*Biological Journal of the Linnean Society*, 2017, **120**, 626–636. With 2 figures.

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

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*Received 5 August 2016; revised 30 August 2016; accepted for publication 30 August 2016*

The perception that the turnover of 45S rDNA site number in plants is highly dynamic pervades the literature on rDNA evolution. However, most reported evidences come from the study of polyploid systems and from crop species subjected to intense agronomic selection. In sharp contrast with polyploids, the evolutionary patterns of rDNA loci number in predominantly diploid lineages have received less attention. Most studies on rDNA loci changes lack explicit temporal frames, and hence their dynamics could not be assessed. Here, we assess the temporal patterns of rDNA site evolution in *Cistus*, an entirely diploid lineage. We assessed the number and chromosomal position of 45S rDNA loci in *Cistus* species using fluorescence *in situ* hybridization (FISH) and Ag-nucleolus organizing regions (Ag-NOR) staining. Maximum likelihood and maximum parsimony reconstructions of the ancestral state of the 45S rDNA locus number were inferred onto a dated phylogeny. 45S rDNA locus number in *Cistus* ranged from one to four. Maximum likelihood and maximum parsimony reconstructions suggested that the two-loci state was the ancestral condition in Cistaceae, including the sister genera *Tuberaria* and *Cistus*. The most likely basal number of rDNA loci (two) has been maintained from the hypothesized ancient splitting events between *Fumana* and the remaining Cistaceae lineages in the Oligocene to most of the recent clades of *Cistus* diversified in the Middle Pleistocene. Our results support the view that evolutionary stasis regarding the number of 45S rDNA loci have been prevalent in several *Cistus* lineages and close relatives along their evolutionary history. It is suggested that conservation in rDNA site number likely occurred along more than 25 Mya of plant evolution, leading support to hypothesize that rDNA stasis in site number may have been neglected and underestimated in plant evolution at the diploid level.  *Biological Journal of the Linnean Society*, 2017, **120**, 626–636.

KEYWORDS: 45S rDNA – Cistaceae – *Cistus* – diploid lineages – fluorescent *in situ* hybridization – *Fumana* – *Tuberaria*.

## 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 & Dixon, 1991). In contrast to 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 & Schlessinger, 1991).

Although rDNA is the most abundant gene family and occupies a large fraction of the nuclear genome,

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it is also one of the most unstable genomic region (Kobayashi, 2008). The reasons for this instability are not fully understood; however it has been reported that rDNA loci are the predominant sites of repeated recombination (Kobayashi & Ganley, 2005). Thus, illegitimate recombination between loci may trigger both intragenomic fluctuation in rDNA copy number and amplification of new arrays (Cronn *et al.*, 1996; Tsang & 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 at 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 is available for a substantial number of species (García, Garnatje & Kovařík, 2012; Roa & Guerra, 2012; García *et al.*, 2014). However, information concerning the dynamics of rDNA loci among closely related species only becomes biologically relevant when under an explicit phylogenetic framework.

Polyploidy in plants provide an excellent opportunity for studying the dynamic turnover of rDNA loci. The rapid rearrangement of parental genomes after polyploid formation is now a well-established paradigm in plant genome evolution (Wendel, 2015). The available evidence suggests that the fate of 45S rDNA units in recently formed plant polyploids does not follow a consistent evolutionary pattern (Volkov, Komarova & Hemleben, 2007). Because the ancestors of many plant polyploids have been finely identified, and the temporal frame and exact origin of polyploid formation are usually known, the dynamic distribution patterns of rDNA loci have been confidently assessed.

In sharp contrast with polyploids, the evolutionary patterns of rDNA loci number in predominantly diploid lineages has received less attention (e.g. Datson & Murray, 2006). Unfortunately, most previous studies on rDNA loci changes lack explicit temporal frames, and hence their dynamics could not be assessed.

Rock roses, *Cistus* (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 ancestral dated nodes are available (Guzmán & Vargas, 2005, 2009a, b, 2010; Guzmán, Lledó & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010, 2011; Civeyrel *et al.*, 2011). In addition, several important evolutionary patterns and processes have been revealed for *Cistus* 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 (e.g. Guzmán & Vargas, 2009b, 2010; Guzmán *et al.*, 2009).

In this study, the number and chromosomal position of 45S rDNA loci have been determined in *Cistus* species (including the genus *Halimium*, Démoly, 2006) using FISH studies and Ag-NOR staining, and mapped onto dated phylogenies in an attempt to ascertain whether (1) a phylogenetically-associated variation in number of rDNA loci features is present within the genus, (2) rates of rDNA site evolution are equivalent in all lineages, and (3) organismal evolutionary histories show association with the patterns of rDNA site location and number.

## MATERIAL AND METHODS

### Plant materials

Thirty-five taxa of the Cistaceae family belonging to *Cistus*, *Tuberaria* and *Fumana* genera were analysed in this study. Seeds were obtained from field sampling, botanical gardens, plant breeding stations and research centers. The origin of the plant analysed material is provided in Table 1. Representative herbarium vouchers for all populations and species are deposited at the herbarium of the Botanical Garden of Valencia University (VAL).

### Cytogenetic analysis

*Cytological preparations*

For mitotic chromosome preparations, the protocols described in Rosato, Castro & Rosselló (2008) were followed.

*Fluorescence in situ* hybridization

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

*Ag-NOR staining*

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

**Table 1.** List of studied taxa and the sources of material analysed in this study

| Taxon | Origin | Source (accession number) |
|---|---|---|
| ***Cistus*** | | |
| *C. albidus* L. | Portugal, Algarve, Lagoa | BGB-D (460. PT-O.B-0100208) |
| *C. atriplicifolium* Lam. | Spain, Alicante, Villena | CIEF (A26E) |
| *C. calycinus* L. | Spain, Cádiz, Trafalgar | JAR (2015-1) |
| *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-O-B-2400300) |
| subsp. *eriocephalus* (Viv.) Greuter & Burdet | Italy, Grosseto, Marina di Grosseto | BGB-D (469. IT-O-B-1971186) |
| *C. crispus* L. | Spain, Valencia, Torreblanca | CIEF (C67A) |
| *C. grancanariae* Marrero-Rodríguez *et al.* | Spain, Canary Islands, Gran Canaria | 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* Démoly | Spain, Canary Islands, Gran Canaria | JBCVC (3580-B) |
| *C. ladanifer* L. subsp. *ladanifer* | Spain, Valencia, Sinarcas | CIEF (V51A) |
| *C. lasianthus* Lam. subsp. *alyssoides* (Lam.) Démoly | 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) |
| *C. monspeliensis* L. | [1]Spain, Canary Islands, Gran Canaria | JBCVC (2880/B) |
| | [2]France, Montpellier, Saint-Mathieu-de-Tréviers | BGB-D (474. FR-O-B-2042205) |
| *C. ochreatus* Chr. Sm. ex Buch | Spain, Canary Islands, Gran Canaria | JBCVC (1402/B) |
| *C. ocymoides* Lam. | Spain, Huelva, Aracena | CIEF (HulA) |
| *C. osbeckiifolius* Webb ex Christ. | Spain, Canary Islands, Tenerife | JBCVC (3641/B) |
| *C. palmensis* Bañares & Démoly | Spain, Canary Islands, La Palma | JBCVC (3617/B) |
| *C. parviflorus* Lam. | Greek, 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 | JBCVC (3656/B) |
| *C. umbellatus L.* subsp. *micranthus* Démoly | Spain, Valencia, Castielfabib | BGB-D (580. GR-O-B-1901502) |
| subsp. *viscosum* (Willk.) Démoly | Greece, Messenia, Sparta | CIEF (A65A) |
| ***Tuberaria*** | | |
| *T. 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) |

Seed from wild origins accessed from personal recollections (JAR) and research institutions: Botanischer Garten Berlin-Dahlem (BGB-D); Conservatoire Botanique National Méditerranéen de Porquerolles (CBNM); Centro para la Investigación y Experimentación Forestal (CIEF); Jardí Botànic de Barcelona (IJBB); Jardín Botánico de Córdoba (JBC); Jardín Botánico El Castillejo (JBCA); Jardín Botánico Canario Viera y Clavijo (JBCVC); Jardín Botánico Dunas del Odiel (JBDO); Muséum d'Histoire Naturelle (MHN); Real Jardín Botánico de Madrid (RJB-CSIC). The superscripts in *C. monspeliensis* refer to each different accession studied.

### Karyotype analysis

Chromosome measurements were made on digital images using the computer application MicroMeasure version 3.2 (Reeves, 2001). Idiograms were obtained from chromosome measurements of at least five well-spread metaphase plates.

### rDNA site-number evolution

Cytogenetic features were mapped onto a phylogenetic tree following the likelihood reconstruction methods in Mesquite, version 3.04 (Maddison & 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 [Markov k-state one-parameter model (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 our study was the backbone consensus tree obtained by previous authors using several plastidial and nuclear markers, e.g. *rbc*L/*trn*L-*trn*F (Guzmán & Vargas, 2009a), *rbc*L/*trn*K-*mat*K (Guzmán & Vargas, 2009b), *trnL-trnF*/*trnS-trnG*/*trnK-matK*/*rbcL*/*ITS*/*ncpGS* (Guzmán *et al.*, 2009), *trn*L-*trn*F/*mat*K (Fernández-Mazuecos & Vargas, 2010), *trn*S-*trn*G/*trn*K-*mat*K (Guzmán & Vargas, 2010), and *trn*S-*trn*G/*trn*L-*trn*F (Civeyrel *et al.*, 2011). A representative species of the closely related genus *Tuberaria* (*T. lignosa*) was also included in the phylogenetic tree. The genus *Fumana*, hypothesized to be basal in Cistaceae phylogeny, was used as outgroup (Guzmán & Vargas, 2005, 2009a, b, 2010; Guzmán *et al.*, 2009; Fernández-Mazuecos & Vargas, 2010). Estimated node dates obtained by Guzmán *et al.* (2009) and Guzmán & Vargas (2009b) using relaxed clock methods were indicated in the consensus tree.

## 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* ($2n = 18$) 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 (Table 2). In contrast, the *Tuberaria lignosa* karyotype ($2n = 14$) was characterized by the presence of two metacentric and five submetacentric chromosome pairs. In *Fumana* species ($2n = 32$) the degree of chromatin condensation of most chromosomes did not allow a clear recognition of the centromeric region and idiograms could not be constructed.

### Variation in the number and localization of 45S rDNA loci

45S rDNA locus number in *Cistus* ranged from one to four, whereas two loci were present in *Tuberaria*, the sister genus of *Cistus*, and in the four analysed species of *Fumana* (Table 2).

Most species of *Cistus* showed one or two 45S rDNA loci, accounting for 38.89% and 50% of the entire sample analysed, respectively. Higher numbers of loci were rarely present; thus, 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* (Fig. 1).

The rDNA sites were mostly localized at the subterminal 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 labelled chromatin. No interstitial or proximal rDNA signals were observed.

Four chromosome landmarks were defined (Fig. 1G) according to the 45S rDNA site location on the chromosomes (short or long arms) and the presence of a terminal chromosome chromatin portion adjacent to the rDNA sites. In types I and II the chromosomes bears rDNA sites at the short chromosome arms. Type I shows a sub-terminal position of the rDNA site whereas in type II a small terminal chromosome portion is found. Landmarks types III and IV show the rDNA sites located at the long arms of the chromosomes. In type III the rDNA site is sub-terminal and in IV it is attached to a small chromosome portion.

Chromosome type I was present in all *Cistus* accessions, whereas type III co-occurred in seven accessions. The less represented rDNA phenotypes were present 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 single rDNA locus their NOR activity was assessed by silver staining (Supporting Information, Fig. S1) and the maximum number of interphase nucleoli was recorded. In all but one of the analysed taxa all 45S

**Table 2.** Karyological features of the analysed species

| Taxon | 2*n* | Haploid karyotype formula | 45S rDNA loci | Chromosome landmark pairs | | | | Max. no. of nucleoli |
|---|---|---|---|---|---|---|---|---|
| | | | | I | II | III | IV | |
| *Cistus albidus* | 18 | 8 m + 1sm | 1 | 1 | – | – | – | |
| *C. atriplicifolium* | 18 | 7 m + 2sm | 2 | 2 | – | – | – | 3 |
| *C. calycinus* | 18 | 8 m + 1sm | 2 | 2 | – | – | – | 4 |
| *C. chinamadensis* subsp. *gomerae* | 18 | 8 m + 1sm | 1 | 1 | – | – | – | |
| *C. clusii* | 18 | 3 m + 6sm | 2 | 2 | – | – | – | 4 |
| *C. creticus* subsp. *creticus* | 18 | 8 m + 1sm | 1 | 1 | – | – | – | |
| subsp. *eriocephalus* | 18 | 8 m + 1 sm | 1 | 1 | – | – | – | |
| *C. crispus* | 18 | 6 m + 3sm | 2 | 1 | – | 1 | – | 3 |
| *C. grancanariae* | 18 | 8 m + 1sm | 4 | 3 | – | 1 | – | 6 |
| *C. halimifolius L.* subsp. *multiflorus* | 18 | 7 m + 2sm | 2 | 2 | – | – | – | 4 |
| *C. heterophyllus* | 18 | 7 m + 2sm | 1 | 1 | – | – | – | |
| *C. horrens* | 18 | 7 m + 2sm | 1 | 1 | – | – | – | |
| *C. ladanifer* | 18 | 7 m + 2sm | 1 | 1 | – | – | – | |
| *C. lasianthus* subsp. *afyssoides* | 18 | 7 m + 2sm | 2 | 1 | 1 | – | – | 3 |
| *C. laurifolius* subsp. *atlanticus* | 18 | 8 m + 1 sm | 2 | 1 | – | 1 | – | 4 |
| subsp. *laurifolius* | 18 | 7 m + 2sm | 2 | 1 | – | 1 | – | 3 |
| *C. libanotis* | 18 | 8 m + 1sm | 2 | 1 | – | – | 1 | 4 |
| *[1]C. monspeliensis* | 18 | 8 m + 1sm | 3 | 2 | – | 1 | – | 6 |
| *[2]C. monspeliensis* | 18 | 8 m + 1sm | 3 | 2 | – | 1 | – | 5 |
| *C. ochreatus* | 18 | – | 1 | 1 | – | – | – | |
| *C. ocymoides* | 18 | 7 m + 2sm | 2 | 2 | – | – | – | 4 |
| *C. osbeckiifolius* | 18 | 8 m + 1sm | 1 | 1 | – | – | – | |
| *C. palmensis* | 18 | 8 m + 1sm | 1 | 1 | – | – | – | |
| *C. parviflorus* | 18 | 7 m + 2sm | 2 | 1 | – | 1 | – | 3 |
| *C. populifolius* | 18 | 7 m + 2sm | 2 | 2 | – | – | – | 4 |
| *C. pouzolzii* | 18 | 7 m + 2sm | 2 | 2 | – | – | – | 4 |
| *C. psilosepalus* | 18 | 8 m + 1sm | 2 | 1 | 1 | – | – | 3 |
| *C. salviifolius* | 18 | 8 m + 1sm | 2 | 1 | – | – | 1 | 4 |
| *C. symphytifolius* | 18 | 7 m + 2sm | 1 | 1 | – | – | – | |
| *C. umbellatus* subsp. *micranthus* | 18 | 6 m + 3sm | 1 | 1 | – | – | – | |
| subsp. *viscosum* | 18 | 3 m + 6sm | 3 | 3 | – | – | – | 5 |
| *Tuberaria lignosa* | 14 | 2 m + 5sm | 2 | 1 | 1 | – | – | 4 |
| *Fumana clausonis* | 32 | – | 2 | 2 | | | | nd |
| *F. ericifolia* | 32 | – | 2 | 2 | | | | 3 |
| *F. fontanesii* | 32 | – | 2 | 2 | | | | nd |
| *F. thymifolia* | 32 | – | 2 | 2 | | | | nd |

The chromosome number, the haploid karyotype formula, the number of 45S rDNA loci, the number of each rDNA chromosome landmarks, and the maximum number of nucleoli observed after Ag-NOR staining are indicated. Please, note that the number of nucleoli refers to a diploid cell whereas the number of loci is given for a haploid cell.
Nd, not determined. The superscripts in *C. monspeliensis* refer to the accessions indicated in Table 1.

rDNA loci were transcriptionally active, as the number of the FISH signals equated to 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.

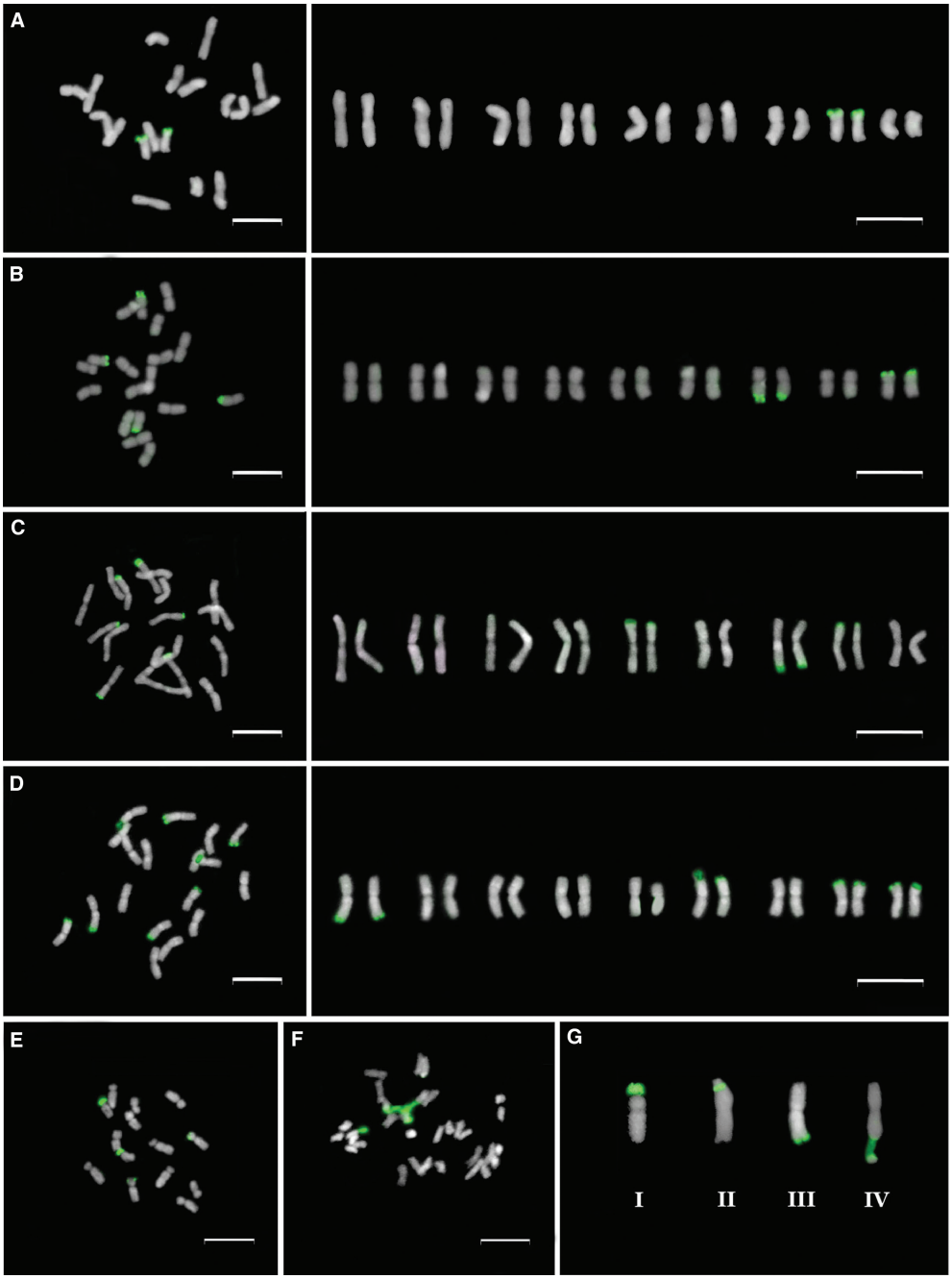


**Figure 1.** 45S rDNA locus on mitotic chromosomes and corresponding ideograms of *Cistus* (A–D), *Tuberaria* (E), and *Fumana* species (F). A, *Cistus creticus*; (B) *C. libanotis*; (C) *C. monspeliensis*; (D) *C. grancanariae*; (E) *Tuberaria lignosa*; (F) *Fumana thymifolia.* The four chromosome landmarks defined on the basis of the location of 45S rDNA loci are depicted (G). Chromosomes are counterstained with DAPI staining. Scale bars represent 10 μm.

### EVOLUTIONARY TRENDS IN rDNA SITE-NUMBER CHANGE

The maximum likelihood and maximum parsimony reconstructions of the ancestral state of the 45S rDNA locus number yielded identical results, suggesting unambiguously that the two-loci state was the ancestral condition in Cistaceae, including the sister genera *Tuberaria* and *Cistus* (Fig. 2). It is inferred that an evolutionary stasis spanning more than 4 Mya (from node 2 to node 8; Fig. 2) resulted in no changes in rDNA loci number. In fact, most of the multiple shifts involving changes in the number

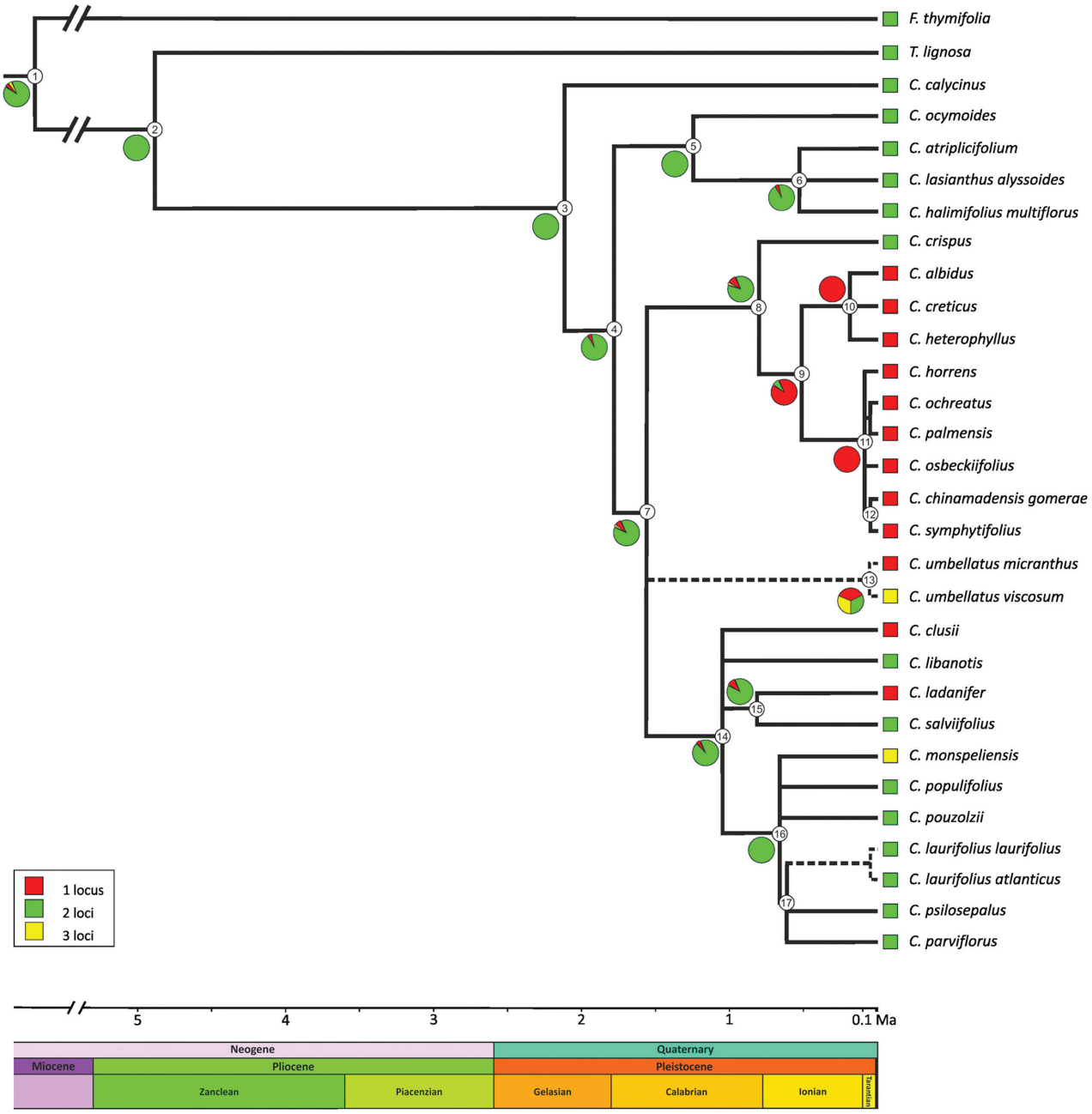


**Figure 2.** Likelihood reconstruction of ancestral 45S rDNA locus number in *Cistus* species, *Tuberaria lignosa* and *Fumana* (outgroup) using Mesquite v. 3.04. The backbone consensus tree and the hypothesized chronogram were built using the phylogenies obtained by several plastidial and nuclear markers (Civeyrel *et al.*, 2011; Fernández-Mazuecos & Vargas, 2010; Guzmán & Vargas, 2009a, b, 2010; Guzmán *et al.*, 2009). Pie charts illustrate the relative likelihood of the 45S rDNA loci number at each node under the specified model of evolution (Mk1 model, in this study). Likelihood scores for each node are indicated in Supporting Information (Table S1). Node age estimates are indicated in Supporting Information (Table S2). Dotted lines indicate the two clades for which a detailed phylogenetic position and age estimates are uncertain.

of the rDNA loci likely occur within the last 1 Mya (Middle Pleistocene). rDNA site losses (from two to a single locus) occurred independently at least twice (the inferred ancestral state in the *C. umbellatus* lineage is ambiguous). Interestingly, in the monophyletic purple-flowered clade the loss of a locus

occurred after the splitting of the basal *C. crispus* lineage, during a relatively short evolutionary frame (Fig. 2). The continental diversification and insular radiation of the purple-flowered lineage was not linked to rDNA changes in the last 500 000 years. In contrast, the white-flowered lineage (as defined by Guzmán *et al.*, 2009) retained for most species the ancestral two-loci state during its radiation, and rDNA changes occurred only in two species, *C. ladanifer* (a rDNA site loss) and *C. monspeliensis* (a rDNA site amplification).

## DISCUSSION

### Evolutionary stasis on rDNA site number in *Cistus*

The perception that the turnover of 45S rDNA site number in plants is highly dynamic pervades the literature on rDNA evolution (Dubcovsky & Dvorák, 1995; Raskina *et al.*, 2004a). Most reported evidences come from the study of polyploid systems which may show greater variation in rDNA locus numbers than their natural counterparts (Pontes *et al.*, 2004; Kovařik *et al.*, 2008), and from the analysis of artificial hybrids subjected to agronomic selection (e.g. Schubert, 1984; Schubert & Wobus, 1985). In addition, rapid genome restructuration and epigenetic control affecting rRNA genes and ribosomal loci behaviour are some of the somaclonal variants influenced by *in vitro* culture (Brettell *et al.*, 1986; Breiman *et al.*, 1987; Lee & Phillips, 1988; Bairu, Aremu & Van Staden, 2011).

Although reports on rDNA loci number are currently available for more than 1700 plant species (García *et al.*, 2014; S. García, pers. comm.), most of them have not been discussed within a dated phylogenetic context, which precludes drawing conclusions about patterns of rDNA site change in vascular plants.

In addition, most diploid organisms where rDNA site dynamism has been documented are crop species. Hence, modifications in their rDNA genome organization may have occurred as a result of intense agronomic selection (Pedrosa-Harand *et al.*, 2006) or from the artificial origin of the accessions used, e.g. in the Jemalong J5 and R-1081 lines obtained in the model species *M. truncatula* (Cerbah *et al.*, 1999; Rosato *et al.*, 2008). Thus, it could be hypothesized that part of the rDNA site dynamism reported so far is not occurring in nature or, alternatively, that natural selection or population bottlenecks may be able to purge much of this rDNA site variation.

It should be stressed that most of the lineages where substantial rDNA dynamism has been documented are not evolutionarily random, but belong to grasses (Poaceae). The genomes from this family have accumulated a high diversity and frequency of mobile elements (retrotransposons; e.g. Bennetzen, 1996; Kalendar *et al.*, 2000; Li *et al.*, 2004; Belyayev *et al.*, 2010), which may be more sensitive to activation than in other plant lineages and may be effective molecular drivers of intragenomic rDNA mobility (Raskina *et al.*, 2004b).

In sharp contrast with the above findings, our results convincingly support the view that evolutionary stasis regarding the number of 45S rDNA loci have been prevalent in several *Cistus* lineages, and close relatives (*Tuberaria*), along their evolutionary history. The most likely basal number of rDNA loci (two) has been maintained from the hypothesized ancient splitting events between *Fumana* and the remaining Cistaceae lineages in the Oligocene to most of the recent clades of *Cistus* diversified in the Middle Pleistocene (Fernández-Mazuecos & Vargas, 2010). Such conservation in rDNA site number likely occurred along more than 25 Mya of plant evolution, leading support to hypothesize that rDNA stasis in site number may have been neglected and underestimated in plant evolution at the homoploid level. This should be further assessed in those groups where neopolyplodization events have not occurred, and where extensive sampling, phylogenetic hypotheses, and temporal evolutionary frames are available.

### rDNA site number variation in *Cistus*: evolutionary and taxonomic implications

The use of karyological features has provided fruitful insights in plant systematics over more than 50 years (Stace, 2000; and references therein). The assessment of the chromosome number and morphology, genome size, chromosome and genome disposition in the cell, chromosome behaviour and homology, and chromosome banding pattern has been of paramount value in taxonomic decision making during the second half of the 20$^{th}$ Century. The new knowledge acquired through the developments of molecular cytogenetic analysis has produced a wealth of data of great potential on taxonomic significance, which to date has had less impact in plant taxonomy than in plant evolutionary studies.

One of the best studied chromosomal landmarks are the ribosomal RNA genes, that usually show considerable variation in number, size and position among closely related species (Appels *et al.*, 1980; Adams *et al.*, 2000; Weiss-Schneeweiss *et al.*, 2007). Yet, variation at the intragenomic level have been deemed a concern in reconstructing the evolution of rDNA loci for taxonomic studies; specifically the occurrence of intragenomic polymorphisms in the location and number of rDNA sites and NORs within individual plants of a few genera, like *Allium*

(Bougourd & Parker, 1976; Schubert, 1984; Schubert & Wobus, 1985).

However, increasing knowledge on rDNA site number variation in plants suggests that these concerns have been overemphasized and should be reassessed. Thus, the judicious use of the rDNA site number as a potential marker should be not discouraged since it may provide molecular cytogenetic evidence on the evolution of plant lineages and support taxonomic decisions.

All phylogenetic hypothesis assessed using nuclear and plastidial DNA markers are at odds with current and past infrageneric classification of *Cistus*, revealing that the morphological characters traditionally used to draw supraspecific taxonomic boundaries are unreliable (Guzmán & Vargas, 2005, 2009a, b, 2010; Guzmán *et al.*, 2009; Fernández-Mazuecos & Vargas, 2010, 2011; Civeyrel *et al.*, 2011). In accordance with these results, our findings on rDNA site number variation also failed to support any subgeneric or sectional classification in *Cistus*, as parallel rDNA site gains and losses have occurred in independent evolutionary lineages (Fig. 2). Even, one of the best supported *Cistus* lineages on morphological and DNA-based phylogenies, the purple-flowered species (Guzmán & Vargas, 2005; Civeyrel *et al.*, 2011), did not show a complete association with rDNA site number, as the basal *C. crispus* shows a divergent number of loci (2) as compared with the remaining species (1).

While the obtained results did not confidentially support the use of rDNA site number as markers in *Cistus* infrageneric classification (and likely in many other plant vascular groups), their use at low taxonomic levels merits discussion. We have recently showed that intraspecifc rDNA site number variation in *Cistus heterophyllus*, is absent (Rosato *et al.*, 2016). This has been corroborated in the present study in additional species where two or more populations have been analysed.

The white-flowered *Cistus grancanariae* was recently described from the Canary Islands (Marrero, Almeida & Ríos, 2008). The species is closely related to *C. monspeliensis*, growing on the same island, and with which it was confused in the past. Both species showed the same plastid *trnS-trnG* and *psbK-trnS* DNA sequences, leading Fernández-Mazuecos & Vargas (2011) to suggest that the former was not worth of taxonomic recognition from *C. monspeliensis*. Our study has revealed that *Cistus grancanariae* and *C. monspeliensis* (accessions sampled from the same Gran Canaria Island) showed a different number of 45S rDNA loci (four and three, respectively; Table 1); in fact, *C. grancanariae* showed the highest number of rDNA loci so far found in *Cistus*.

On the basis of the 45S rDNA results, we suggest that *Cistus grancanariae* and *C. monspeliensis* lineages have underwent a divergent genomic evolution that has resulted in the amplification of new rDNA loci in the former, and a later epigenetic silencing of one locus. We speculate that these genomic changes occurred after the colonization of *C. monspeliensis* to the Canary Islands. These evidences together with morphological differences support the evolutionary distinction of *C. grancanariae* from *C. monspeliensis* that should be recognised at some taxonomic rank. In addition, if the high identity in plastid DNA sequences between the two species mirrors a recent origin of *C. grancanariae* from *C. monspeliensis*-like ancestors, the uniqueness of rDNA features in *C. grancanariae* strongly supports that the occurrence of *C. monspeliensis* in the Canary Islands is not the result of a recent, human-mediated introduction (as previously suggested, Fernández-Mazuecos & Vargas, 2011), but the result of natural long-range dispersal to the archipelago.

## CONCLUDING REMARKS

In this work we have assessed the phylogenetic and temporal frames of 45S rDNA site-number variation in *Cistus*. We have inferred that a stasis in 45S rDNA site-number occurred during most of the evolutionary history of *Cistus* and allied genera. It is suggested that most of the multiple shifts involving changes in the number of the rDNA loci likely occur since the Middle Pleistocene, and rDNA site losses and gains occurred independently in divergent lineages. The continental diversification (excluding *C. crispus*) and insular radiation of the purple-flowered group occurred from an ancestral lineage showing a single rDNA site, and was not associated to rDNA changes in the last 500 000 years. In contrast, the white-flowered lineage retained for most species the ancestral two-loci state during its radiation, and rDNA changes occurred only in two species, *C. ladanifer* (a rDNA site loss) and *C. monspeliensis* (a rDNA site amplification).

## ACKNOWLEDGEMENTS

We thank the curators of the Botanical Gardens listed in Table 1 for kindly providing seeds for this study. Our colleague G. Nieto-Feliner provided insightful comments that improved the manuscript. We thank two anonymous reviewers for their insightful comments and criticisms that improved the manuscript. This work was supported by funds from the Spanish Ministry of Science and Innovation (Project CGL2010-15693/BOS) and the Spanish Ministry of Economy and Competitiveness (Project CGL2013-49097-C2-1-P). The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Ag-NOR staining of interphase cells of *Cistus libanotis* (A) and *C. monspeliensis* (B) showing four and six nucleoli, respectively. Scale bars represent 10 μm.

**Table S1.** Likelihood scores and parsimony estimates for the ancestral states of 45S rDNA loci number at nodes indicated in Figure 2.

**Table S2.** Mean divergence time estimates (Mya) for the nodes depicted in Figure 2 using several plastidial (*rbc*L, *trn*K-*mat*K, *mat*K, *trn*L-*trn*F, *trn*S-*trn*G) and nuclear markers (*ITS*, *ncpGS*).