

1 **Physical mapping of ribosomal DNA and genome size in diploid**
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4 **and polyploid North African *Calligonum* species (Polygonaceae)**
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51 Running title: Cytogenetics in *Calligonum*
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Abstract

Most *Calligonum* species are desert plants, characteristic of the Saharan bioclimatic region. All species karyologically analysed until present have the basic chromosome number $x = 9$ and comprise diploids, triploids and tetraploids. The Tunisian flora comprises diploid *C. arich* and *C. azel*, of restricted distribution, and the tetraploid *C. comosum* with wider distribution. Analyses of their karyotypes and polyploidisation-linked rDNA changes by orcein staining, fluorochrome banding with chromomycin A₃ and fluorescent *in situ* hybridisation with 5S and 26S ribosomal DNA probes have been performed. We report the chromosome number for *C. arich* ($2n = 18$) as well as the diploid level for *C. comosum* for the first time. Chromosome counts have also verified the earlier described tetraploid cytotype ($2n = 36$) of *C. comosum*. A general pattern of six GC-rich bands as well as two 35S sites and four 5S sites is described for *Calligonum* species at the diploid level although there is intraspecific variation regarding the site number in a second type of *A. comosum*, with one pair of 35S rDNA sites and two pairs of 5S rDNA sites. The tetraploid cytotype of *C. comosum* has undergone locus loss and genome downsizing. Genome size assessments confirmed previous data. Nonetheless, statistically significant differences were found depending on the type of tissue used for estimation. Measurements from seeds had always larger values than from leaves. The presence of cytosolic compounds in leaves, interfering with DNA staining, is discussed as a possible cause of the differences.

Key words: 5S rDNA, 35S rDNA, cytogenetics, FISH, fluorochrome banding, genome organisation

Introduction

Calligonum L. (Polygonaceae) is one of the dominant genera in active sand dunes, distributed in the North African desert, the desert sands of the Middle East, south Europe and in West and Central Asia (Mao and Pan 1986). China represents most of the distribution range of the genus with 23 species out of 60–80 species in the world (Bao and Grabovskaya-Borodina 2003; Singh 2004). Many facts have caused the decline of the natural resources of *Calligonum* species (Liu et al. 2001) and some of them are considered even critically endangered, threatened with extinction, according to the criteria of the International Union for Conservation of Nature (IUCN 1997). The threats include overgrazing by the camel herds, use as firewood, and several other uses by Bedouins and local nomadic populations. Local healers have reported the chewed stems and leaves to cure toothache (Liu et al. 2001). Pharmacological and toxicological studies on *Calligonum* aerial parts showed anti-gastric, anti-hypertensive, high antimicrobial potential (Radhakrishnan et al. 1999; Zaki et al. 1984), anti-nociceptive and anti-asthmatic activities (Islam et al. 1999; Liu et al. 2001). Extracts of *Calligonum* species are also described to reduce acute gastric ulcers (Liu et al. 2002) and protecting against toxic agents (Islam et al. 1999; Liu et al. 2001). They have also a great potential as forage and for halting desert encroachment (Badria et al. 2007).

Only three species of this genus have been found in the Tunisian desert, which play an important role in the Saharan bioclimatic region (Le Houérou 1959; Pottier-Alapetite 1979; Gouja et al. 2014). *Calligonum comosum* L'Hér. is a shrub of 1 to 2 m height with

1 the widest geographic distribution (Le Houérou 1959). This plant is abundant in the
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3 North African desert, the desert sands of the Middle East and Pakistan, as well as the
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5 sand dunes in both Central and Eastern Arabia (Lipscombe 1984). *Calligonum azel* Maire
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7 is an endemic tree or tall shrub (up to 4-5 m) of Great Eastern Erg, with a prominent
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9 branching (Le Houérou 1959; Dhief et al. 2012). *Calligonum arich* Le Houér. is a tree up
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11 to 7-9 m tall, considered as a Tunisian endemic of the Great Erg Oriental (Le Houérou
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13 1959; Pottier-Alapetite 1979; Gouja et al. 2014), with a prominent, non-thorned
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15 branching. The three Tunisian species are sometimes found in the same localities,
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17 although not intermixed as they occupy different dune slope positions (Le Houérou
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19 1959).
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29 In systematic and evolutionary studies of plants, karyological data are important and
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31 widely used to determine species relationships (Stebbins 1971; Hong 1990; Stuessy
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33 2011; Garbari et al. 2012). Several karyological studies have been based on family
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35 Polygonaceae, despite its difficulty due to the usually small chromosome size (Mao et al.
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37 1983; Wang and Yang 1985). Most chromosomal researches on this family were centred
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39 on *Polygonum*, *Fagopyrum*, *Rheum* and *Rumex* (Baltisberger and Widmer 2006; Hu et al.
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41 2007). Different basic chromosome numbers have been recorded: $x = 8, 9$ and 10 were
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43 detected for *Rumex* (Bolkhovskikh et al. 1969), $x = 11$ for *Rheum* (Darlington and Wylie
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45 1955) and $x = 8$ for *Fagopyrum* (Zhang 1998). Only $x = 9$ has been reported for genus
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47 *Calligonum*, and chromosome counts have been already performed for 16 species to our
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49 knowledge (Mao et al. 1983; Wang and Yang 1985; Wang and Guan 1986; Ferchichi
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51 1997) including diploid, triploid and tetraploid levels. Regarding Tunisian *Calligonum*,
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1 chromosome counts establish diploidy for *C. azel* and tetraploidy for *C. comosum*
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4 (Ferchichi 1997), while no data are available for *C. arich*. Genome size measurements
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6 are consistent with these results and suggest *C. arich* to be diploid (Gouja et al. 2014).
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9 Fluorescent in situ hybridisation (FISH) with ribosomal DNA (rDNA) probes provides
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11 useful markers for chromosome identification (Singh et al. 2001). In plants, FISH with
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13 ribosomal RNA (rRNA) genes as probes has been widely used for karyotyping (Raina et
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15 al. 2001), for studying genome organisation and chromosome evolution (Leitch and
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17 Heslop-Harrison 1993) and for understanding the systematic and evolutionary
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19 relationships between closely related species (Ali et al. 2000; Ran et al. 2001; Li and
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21 Zhang 2002). In family Polygonaceae few researches have been performed, which
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23 describe the numbers and locations of 5S and 35S (18S-5.8S-25S) rRNA genes for genera
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25 *Fagopyrum*, *Persicaria* and *Rumex* (Kim et al. 2006; Choi et al. 2008; Kikuchi et al. 2008;
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27 Sheng et al. 2013). However these genera are relatively phylogenetically distant from
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29 *Calligonum* (Schuster et al. 2013).
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40 In this study, we investigate for the first time the abundance and distribution pattern of
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42 5S and 35S rDNA loci on the chromosomes of three *Calligonum* species by FISH. The
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44 specific aims of this research are: a) to characterise the numbers and locations of 5S and
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46 35S rDNA sites and b) to study the evolution of rDNA loci with polyploidy. Additionally,
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48 we have complemented a previous genome size study on this group (Gouja et al. 2014)
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50 by undertaking new assessments on these three Tunisian *Calligonum* species both in
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52 seed and in seedlings, in order to study the reach of intraspecific or interpopulation
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1 variability, if any, as well as to detect any significant difference on genome size
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4 estimation with respect to the tissue used.
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10 **Materials and methods**

11 **Plant materials**

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22 Seeds of three *Calligonum* species (*C. arich*, *C. azel* and *C. comosum*) for cytogenetics
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24 and genome size assessments were collected from the wild part of the desert in the
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26 Eastern Great Erg of Southern Tunisia. In this study, seeds were selected at three sites
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28 (El Borma, Tiaret and Kamour, places with the typical arid-type climate of dry and hot
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30 summers and cold winters) between years 1999 and 2012 (Table 1 and Fig. 1). These
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32 were washed and stored (20°C, 30% H) in the seed bank at the Pastoral Ecology
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34 Laboratory (PLE) of the Arid Regions Institute (Médénine, Tunisia) until further use. For
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36 flow cytometric measurements on leaf tissue, seedlings were obtained from seeds from
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38 the corresponding sites. Specimen vouchers of the studied material have been
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40 deposited in the herbarium of the University of Barcelona (BCN).
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50 **Chromosome preparations**

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56 Seeds were germinated on wet filter paper in Petri dishes in the dark at room
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58 temperature. Chemical scarification with sulfuric acid (96%) for 30 min was applied to *C.*
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1 *azel* and *C. arich* seeds, before germination. In order to obtain mitotic chromosomes,
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4 0.5-1 cm long root tips were collected from seedlings and the meristems pretreated in
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6 aqueous colchicine (0.05% w/v) for 3-4 h and then fixed in Farmer's fixative fluid
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8 (absolute ethanol : glacial acetic acid, 3 : 1) at 4°C for at least 4 h and later transferred to
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10 70% ethanol. For chromosome counts, the fixed roots were hydrolyzed in 0.1 M
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12 hydrochloric acid at 60°C for 1 min, washed with water and then stained with acetic
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14 orceine (1%) for 2-12 hours and squashed for observations. Each sampled population
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16 included 10 to 30 individuals. A minimum of 10 well spread metaphases were counted
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18 for each population. Photographs were taken with a digital camera (AxioCamHRm Zeiss)
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20 coupled on a Zeiss Axioplan microscope and images were analyzed with Axio Vision Ac
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22 software version 4.2.
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32 Chromosome preparations for fluorochrome banding and FISH were done using the air-
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34 drying technique of Geber and Schweizer (1987), with some modifications: root tips
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36 were washed with soft shaking in citrate buffer (0.01 M citric acid – sodium citrate, pH =
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38 4.6) for 15 minutes, excised, and incubated in an enzyme solution [4% cellulase Onozuka
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40 R10 (Yakult Honsha), 1% pectolyase Y23 (Sigma) and 4% hemicellulase (Sigma)] at 37°C
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42 for 20 to 25 minutes, depending on the species and meristematic thickness. Protoplasts
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44 were prepared applying gentle pressure (squash) to spread the chromosomes onto
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46 microscope slides, frozen at -80°C overnight and after fast coverslip removal they were
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48 desiccated using absolute ethanol and drying for two days.
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58 Fluorochrome banding
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4 In order to reveal GC-rich DNA bands, chromomycin A₃ (CMA) was used, following the
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6 protocols of Schweizer (1976) and Cerbah et al. (1995) with minor modifications: the
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8 slides were incubated in McIlvaine buffer, pH = 7 (0.1 g/L in McIlvaine buffer, pH = 7) for
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10 15 min; stained with CMA (5 mmol/L MgSO₄) for 60 to 90 min; rinsed in the latter buffer;
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12 counterstained with methyl green (0.1 % in McIlvaine buffer, pH = 5.5) for 10 min;
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14 rinsed in McIlvaine buffer, pH = 5.5; and mounted in Citifluor AF1 (glycerol/PBS
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16 solution).
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26 27 Labelling of rDNA probes and FISH

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31 For hybridisation experiments we have mostly used the same slides than for
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33 fluorochrome banding with CMA after destaining with fixative, dehydration through an
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35 ethanol series (70%, 90% and 100%), and drying for two days. The probe used for 35S
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37 rDNA localization was a plasmid carrying a 2.5 kb insert of 26S rRNA gene from
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39 *Lycopersicon esculentum* Mill. labeled with Cy3 (Jena Biosciences) using the Nick
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41 Translation Mix (Roche). The 5S rDNA probe was an approximately 0.7 kb-long trimer of
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43 5S rRNA genes from *Artemisia tridentata* Nutt., labeled with Green dUTP (Abbot
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45 Molecular) using the Nick Translation Mix (Abbot Molecular). This probe contained
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47 three units of the 5S rRNA gene (120 bp) and the noncoding intergenic spacers (about
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49 290 bp). DNA in situ hybridisation was carried out according to Heslop-Harrison et al.
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51 (1991) and Cerbah et al. (1998) with slight modifications as follows. Slides were
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1 incubated in 100 µg/ml DNase-free RNase in 2×SSC (0.03 M sodium citrate and 0.3 M
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4 sodium chloride) for 1 h at 37°C in a humidified chamber, rinsed in 2×SSC for 5 min with
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6 slow shaking, and incubated in pepsin (0.1 mg/ml in 0.01 N HCl) for 15 min at 37°C,
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8 washed three times in 2×SSC for 5 min each with slow shaking, dehydrated through an
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10 ice-cold ethanol series (70%, 90%, and 100% for 3 min each), and air dried. The probe
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12 hybridisation mixture contained 25–100 ng/µl rDNA probes, formamide, 50% (w/v)
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14 dextran sulphate, and 20×SSC. This was denatured at 75°C for 10 min and immediately
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16 transferred to ice for 5 min. Approximately 30 µl of the probe mixture were loaded on
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18 slides and covered with plastic coverslips. The preparations were denatured at 75°C for
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20 10 min and transferred at 55°C for 5 min. Hybridisation was carried out overnight at
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22 37°C in a humidified chamber. Post-hybridisation washes were performed with shaking,
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24 the first at room temperature in 2×SSC for 3 min, followed by several washes at 42°C for
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26 5 min (three in 2×SSC, one in 0.1×SSC, three in 2×SSC and one in 4×SSC with 0.2% Tween
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28 20). A last wash was done at room temperature for 7 min in 4×SSC with 0.2% Tween 20.
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30 Samples were counterstained with Vectashield (Vector Laboratories, Inc. Burlingame,
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32 California) a mounting medium containing DAPI. FISH preparations were observed with
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34 an epifluorescence Zeiss Axioplan microscope with different combinations of Zeiss
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36 excitation and emission filter sets (01, 07 and 15). Hybridisation signals were analyzed
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38 and photographed using the highly sensitive CCD camera (Princeton Instruments),
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40 images were processed for color balance, contrast and brightness uniformity with
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42 Adobe Photoshop Axiovision HR Rev3, 4.8. Graphics (idiograms) were performed with
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44 PowerPoint 2010, considering three standard chromosome sizes (small, medium and
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46 large).
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4 Flow cytometric measurements
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9 Seven populations of the different *Calligonum* species were studied, and of these, five
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11 individuals per population were selected to estimate the genome size by flow cytometry
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13 (Table 2). We used both seeds and leaves from seedlings for these analyses and each
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15 individual was measured twice. *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg; Marie and
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17 Brown 1993) and *Lycopersicon esculentum* Mill. 'Montfavet 63-5' (2C = 1.99 pg; Catrice
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19 et al. 2006) were used as the internal standards, which were first analyzed separately to
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21 locate its peak position at the fluorescence histogram. Fresh leaf tissue of the standard
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23 and the target species were chopped together in 600 µl of LB01 buffer (8% Triton X-100;
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25 Doležel et al. 1989) supplemented with 100 µg/ml ribonuclease A (RNase A, Boehringer,
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27 Meylan, France) and stained with 36 µl of 1 mg/ml propidium iodide (Sigma-Aldrich,
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29 Alcobendas, Madrid) to a final concentration of 60 µg/ml, and kept on ice for 20 min.
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31 The fluorescence measurements were performed using an Epics XL flow cytometer
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33 (Coulter Corporation, Miami, FL, USA) at the Centres Científics i Tecnològics, Universitat
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35 de Barcelona. More details about the method followed are described in Gouja et al.
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37 (2014). As in Garcia et al. (2008), to ensure that the instrument shows a linear response
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39 across the range of genome sizes studied and that results obtained with either one
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41 internal standard or the other are fully comparable, we performed cytometric assays
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43 with both internal standards and the specimen with the highest genome size at the
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45 same time. The difference between the obtained results with respect to each standard
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47 was negligible (about 1.5-2% of deviation), and we can therefore ascertain the linearity
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1 of the flow cytometer in this interval and the convenience of the use of the chosen
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4 internal standards in any case.
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8 9 Statistical analyses

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14 In order to detect significant difference between genome size estimations from seeds
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16 and leaves from seedlings, the non-parametric Wilcoxon test for paired data and the
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18 parametric paired t-test were performed with XLSTAT (Addinsoft) and the R commander
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20 package (Version 2.0-3) of R.
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27 28 Results

29 30 31 Basic karyology

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34 The metaphase chromosome morphology of the studied species is shown in Fig. 2. Basic
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36 chromosome number of all species was $x = 9$. All observations showed that *C. comosum*
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38 (Kamour), *C. azel* (Tiarert and El Borma) and *C. arich* (Kamour) have a somatic number of
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40 $2n = 18$ (diploid), whereas, for *C. comosum* (El Borma) we have detected diploid and
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42 tetraploid cytotypes (Fig. 2: g, m): $2n = 18$ and $2n = 36$ (Table 1). We find metacentric,
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44 submetacentric and acrocentric chromosomes in *Calligonum* metaphases.
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1 rDNA FISH and fluorochrome banding
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6 The cytogenetic results of the taxa studied are presented in Fig. 2. In all the studied
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8 *Calligonum* species, 5S and 35S signals were always in different chromosomes,
9 separated from each other (S-type arrangement). *Calligonum arich*, *C. azel* and *C.*
10 *comosum* (Kamour) have one locus of 35S rDNA and two loci of 5S rDNA (Fig. 2: b and c,
11 e and f, and k and l, respectively) whereas the diploid *C. comosum* (El Borma) has two
12 loci of 35S rDNA and one locus of 5S rDNA (Fig. 2: h, i). The tetraploid *C. comosum* (El
13 Borma) presents two loci of both the 35S and 5S rDNAs (Fig. 2:n, o). GC-rich regions
14 were detected in all the studied species with CMA staining, which showed six terminal
15 bands in all diploids and eight terminal bands in the tetraploid *C. comosum* from El
16 Borma, in all cases in different chromosomes and coincident with rDNA sites (data not
17 shown).

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35 Genome size
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40 Although genome size measurements for *Calligonum* species were apparently similar in
41 seeds and in leaves, the paired t-student test and the Wilcoxon test for paired data
42 supported significant differences between both datasets ($p < 0.05$ for both). In all cases,
43 values obtained with seeds were slightly higher than those obtained with leaves. The 2C
44 DNA mean values ranged from 2.58 to 2.63 pg (for seeds) and from 2.27 to 2.56 pg (for
45 leaves), and, at the tetraploid level, the values were 4.89 pg (for seeds) and 4.77 pg (for
46 leaves) (Table 2). Half-peak coefficients of variation (HPCV) values were lower than 5%
47 in all cases, which indicates the good quality of the analyses.
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Discussion

Chromosome number

Cytogenetic studies by Hong et al. (1992), Navajas-Pérez et al. (2005), and Baltisberger and Widmer (2006) have revealed highly variable basic chromosome numbers in Polygonaceae ($x = 7$ to 13), in particular in the genera *Rumex* and *Polygonum* (Simmonds 1945; Baltisberger and Widmer 2006). Chromosomal studies are particularly difficult to perform in *Calligonum* due to the small chromosome size. The chromosome numbers of genus *Calligonum* had been previously investigated and our results confirm the basic chromosome number of $x = 9$ and the existence of two cytotypes, $2n = 18$ and $2n = 36$. The chromosome number for *C. arich* is reported here for the first time. Although these diploid and tetraploid cytotypes are the most commonly found in the genus (Sabirhazi and Pan 2009; and references therein), recently, Shi et al. (2013) reported a triploid population ($2n = 3x = 27$) in *C. mongolicum* Turcz., supporting what Mao et al. (1983) had already stated that polyploidy was present in the genus, including triploid and tetraploid forms. They had also detected an unusual count ($2n = 24$), which would point to aneuploidy, in a population of *C. klementzii* Losinsk.

General banding and rDNA pattern in *Calligonum* species

1 In the Polygonaceae, the independent organization of 5S and 35S rDNA loci on
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4 chromosomes, i.e. that both rRNA genes are separated, not linked in a single operon as
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6 found in genera such as *Artemisia*, some other Asteraceae or *Ginkgo*, among others
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8 (Garcia et al. 2009, 2010; Galián et al. 2012; Garcia and Kovařík 2013), had been
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10 previously observed (Sheng et al. 2013). FISH studies on family Polygonaceae are rare
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12 and this is the first molecular cytogenetic investigation in *Calligonum*. Our FISH results
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14 have shown that all 5S and 35S rDNA sites were also separated and always located in
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16 terminal positions of different chromosomes. Actually, rRNA genes (particularly 35S)
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18 occur preferentially in terminal positions in angiosperms (Roa and Guerra 2012). These
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20 regions were always positively stained with CMA, which is also a common finding as
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22 rDNA is typically GC-rich (Garcia et al. 2009). In diploids, the general pattern is that two
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24 terminal 5S rDNA sites and four terminal 35S rDNA sites are observable. However, there
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26 is an exception in the diploid *C. comosum* (El Borma), where we find the inverse result.
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29 Plenty of studies on variation in number of rDNA loci in many plant, insect and
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31 vertebrate groups have described such changes in their number and chromosomal
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33 location, suggesting that rDNA sites are highly dynamic components of the genome
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35 (Britton-Davidian et al. 2012). The tetraploid cytotype of *C. comosum* shows
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37 equal numbers of sites (four) for both 5S and 35S rDNA. Chromosomes with 35S rDNA
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39 genes are called NOR-bearing (nucleolar organizer regions) chromosomes, these are the
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41 regions around which the nucleolus forms after mitosis. When these genes are
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43 transcriptionally active (competent NORs, Kalmarová et al. 2007) they give rise to
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45 decondensed chromatin structures during previous interphase and prophase and to
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47 secondary constrictions at metaphase stage. When these are inactive (i. e. silent) they
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1 are often highly compacted. Due to the reduced size of *Calligonum* chromosomes, the
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3 presence of secondary constrictions (in other words, the number of 35S sites that are
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5 transcriptionally active) is difficult or impossible to see. However, in the slightly
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7 prophasic chromosomes shown for *C. azel* (Fig. 2e) we can see two 35S chromatin knobs
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9 (i. e. satellites) apparently detached (indicated with arrows) but which may be
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11 connected to their respective chromosomes by faint fibers of decondensed
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13 heterochromatin. We assume that these are competent NORs, and therefore one 35S
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15 rDNA locus would be active for this species, while the other remains inactive, a
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17 phenomenon known as amphiplasty or nucleolar competence and which could be
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19 related to past hybrid origin of the species / population (Lacadena et al. 1984).
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26 Altogether, rather than a specific cytogenetic pattern, capable of discerning between
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28 these three taxa, we find a typical karyotype common to *C. arich*, *C. azel* and *C.*
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30 *comosum*. Conversely, the clade formed by these tree taxa in a recent phylogenetic
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32 reconstruction based on nuclear (ITS) and plastid (*trnL-trnF* and *rbcl*) regions (Gouja et
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34 al. 2014) did clearly discern between the three species (see Figure 3), supporting their
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36 separated specific status. Similar findings were reported in the *Tridentatae* subgenus of
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38 *Artemisia* (Garcia et al. 2009). In this species complex, a quite homogeneous cytogenetic
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40 pattern was shared among its members, although phylogenetic approaches clearly
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42 supported the specific status of these species (Garcia et al. 2011).
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50 Previous research determining the number and location of 5S and 35S ribosomal DNA
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52 loci distribution in Polygonaceae does not show a clear tendency of 5S or 35S loci to be
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54 in higher or lower abundance than the other; in *Fagopyrum* and *Persicaria* most species
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56 present more 35S than 5S loci (Kikuchi et al. 2008; Sheng et al. 2013; Choi et al. 2008),
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1 while in *Rumex* it is the other way round (Koo et al. 2004; Kim et al. 2006). Nevertheless,
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3 the number of rDNA sites detected for *Calligonum* is consistent with the most
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5 commonly found numbers in angiosperms. In this line, the typical angiosperm karyotype
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7 at the diploid level, according to data available at the Plant rDNA database
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9 (www.plantrdnadatabase.com, Garcia et al. 2012) has 18 chromosomes with two 5S
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11 sites and two to four 35S sites (being four the most usual), both kinds located at
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13 terminal position (Garcia et al. unpublished research).
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22 Polyploidy, rDNA loci downsizing and intraspecific rDNA loci number variation

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27 Polyploidy is a common phenomenon in plants and has contributed to the evolution of
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29 about 70% of angiosperms species (Cui et al. 2006; Leitch and Leitch 2008; Soltis et al.
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31 2009; Garcia et al. 2009; Ramsey and Schemske 1998). Among many other features,
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33 polyploidy is said to improve fitness in arid habitats (Grant 1981). In our sample, *C.*
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35 *comosum* (El Borma) has the largest geographical distribution, spread across the Sahara
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37 with the exclusion of the Great Erg Oriental to the high Southern plains, with an
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39 extension to the East and North of Tunisian desert. On the other side, *C. arich* is an
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41 endemic restricted, with a low frequency of distribution (one to five plants per ha), to
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43 the top of the large dunes of the Great Erg Oriental. *Calligonum azel* is also localized at
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45 the Great Erg Oriental (Le Houérou 1959) occupying the slopes of large Saharan dunes
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47 (Dhief et al. 2009) with a poor average density of one to 10 plants per ha. It is likely that
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49 the tetraploid cytotype of *C. comosum* has achieved a larger geographical distribution
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51 (Ferchichi 1997) than the others, only known at diploid level.
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4 Regarding the number of rDNA sites, we would expect that the tetraploid *C. comosum*
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6 would have twice the number of rDNA signals than the diploid ones. However, neither
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8 from “doubling” the number of signals from the diploid *C. comosum* individuals from El
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10 Borma nor from the population of Kamour, the observed number of signals, that is four
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12 sites both for 5S and 35S rDNA, is not consistent with a recent autopolyploid origin of
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14 any of them (and this is also consistent with regards to the number of CMA positive
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16 signals: we would expect 12 but we only detect eight GC-rich bands). According to our
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18 results, there has been loss in any case. Loci loss has been previously detected after
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20 allopolyploid and autopolyploid formation (Garcia et al. 2009), although rDNA loci
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22 additivity (i.e. that the tetraploids have twice the number of loci than the diploids, for
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24 example) is also a common finding, particularly in polyploids of recent origin (Soltis and
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26 Soltis 1999).
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37 As stated previously, there is intraspecific variability in *C. comosum*, which may be a
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39 reflection of the higher ecological plasticity and colonising ability of this species with
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41 respect to *C. azel* and *C. arich*. A variable number of rDNA sites occur between different
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43 populations of *C. comosum*, a case of intraspecific variability, quite commonly found in
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45 many plant groups. Two authors have determined the number and location of the 35S
46
47 and 5S rRNA loci in Polygonaceae, also finding a variation in the number of the rDNA
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49 sites in *Fagopyrum esculentum*: Kikuchi et al. (2008) reported the presence of six signals
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51 of both 35S and 5S rDNA, but Sheng et al. (2013) detected eight 35S rDNA and four 5S
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53 rDNA sites. A considerable variation in rDNA loci number was also identified in the
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1 species *Rumex acetosa*: according to Kim et al. (2006), it presented four 35S and 5S
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4 rDNA sites, while Koo et al. (2004) showed two 5S rDNA sites and three to four 35S
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6 rDNA sites. Many other examples outside family Polygonaceae can be found showing
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8 that intraspecific variation in the number of rDNA loci is a common finding (Garcia et al.
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11 2012).

12 13 14 15 16 17 Genome size analysis

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22 This is the first time that the amount of nuclear DNA was assessed in *Calligonum* from
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24 seed tissue. The results confirm the chromosomal counts, indicating that *C. comosum* (El
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26 Borma) has two cytotypes (diploid and tetraploid). Among the diploid taxa, genome
27
28 sizes are fairly similar, i.e. genome size alone would not help distinguishing between
29
30 these species. The flow cytometry profiles of nuclear DNA content obtained both for
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32 seeds and leaf tissue show that 2C-values in seeds were slightly but significantly higher
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34 ($p < 0.05$) than in the leaves in all cases. A similar situation had been observed previously
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36 in *Brassica napus* L. and *Helianthus annuus* L. (Sliwinska et al. 2005) and in *Eucalyptus*
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38 *globulus* Labill. (Pinto et al. 2004), in which a higher genome size was found in seeds
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40 than in leaves. Many angiosperms contain a storage tissue, named endosperm, full of
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42 reserve for the embryo and which, in diploid species, it is triploid. This could interfere
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44 with genome size estimations from seeds. However, our results indicate that the
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46 measurements clearly correspond to the embryo cells, since values from seeds are only
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48 1.5% to 15.8% larger than values from leaf tissue, depending on the species. If we were
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50 measuring mostly endospermic cells we would expect values about 50% larger in seeds
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1 than in leaves. This was also the case of *Zea mays* L. (Sliwinska et al. 2005) in which no
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4 endosperm nuclei were observed in the histograms as a clear fluorescence peak when
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6 the whole seed was analyzed, because the large storage cells of endosperm do not
7
8 contain nuclei. This is probably what explains the relatively similar genome sizes
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11 between seeds and leaves in *Calligonum*.
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14 Additionally, results were overall consistent with previous genome size determination in
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16 these species from leaves (2.30 pg for *C. azel*, 2.45 pg for *C. arich* and 4.68 pg for
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18 tetraploid *C. comosum*, from Gouja et al. 2014). According to several authors (Bino et al.
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20 1993; Sliwinska et al. 2009), species that contain phenolic compounds, among others,
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22 may present technical problems for measurements with flow cytometry. Many species,
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24 particularly woody and aromatic plants, produce numerous secondary metabolites that
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26 can interfere with DNA staining causing stoichiometric error in measurements.
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28 *Calligonum* is rich in polysaccharides, alkaloids and other secondary metabolites (Liu et
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30 al. 2001). Thus, the lower DNA content in leaves could be explained by the presence of
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32 interfering staining inhibitors (Sliwinska et al. 2005). Other authors (Baluska 1990;
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34 Biradar and Rayburn 1994) suggested that the difference between the 2C values in
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36 leaves and seeds could be due to the different structure and condensation of chromatin
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38 in these different tissues.
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50 Genome size in the tetraploid *C. comosum* (2C = 4.89 pg) is 6% lower than expected
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52 from doubling nuclear DNA amount from its closest diploid relative (2C = 2.59 pg).
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54 Genome downsizing is a widespread biological phenomenon in response to
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56 polyploidisation, leading to diploidisation of the polyploid genome (Leitch and Bennett
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1 2004). There may be a relationship between the detected genome downsizing in the
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3 tetraploid and its lower than expected number of rDNA loci previously described.
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5 Although the whole genome downsizing cannot be directly linked (neither totally
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7 attributed) to a decrease in the number of rDNA loci (or rDNA genes copy number) it is
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9 conceivable that rDNAs, as a highly repetitive part of the genome, bear some
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11 relationship with overall genome size (Prokopovich et al. 2003).
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22 **Conclusions**

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27 This study reports for the first time data on ribosomal DNAsite number and distribution
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29 in a representative sample of *Calligonum* species in the desert areas of Tunisia. This
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31 supports a typical signal pattern at the diploid level with mostly two terminal 5S rDNA
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33 and four terminal 35S rDNA sites. Nevertheless, more species and populations should be
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35 analysed in order to confirm this model, since we found some intraspecific variation for
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37 one species. In the tetraploid studied, rDNA loci loss and genome downsizing have been
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39 detected, both findings frequently associated with polyploidy. Finally, the study
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41 demonstrated that genome size can be estimated from seeds in these taxa, and that this
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43 estimate may be more accurate than from leaves since seeds do not contain staining
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45 inhibitors, while leaves may present cytosolic compounds that interfere with the
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47 genome size assessment. As the slight difference in genome size estimations from seeds
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49 and leaves is significant, we recommend the use of seeds for nuclear DNA assessments
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58 in this genus and probably as well in closely related taxa, whenever possible.
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Table 1 Provenance and collection data of the studied *Calligonum* populations, all from Tataouine, Tunisia. All signals appear in terminal or subterminal positions. (*) Chromomycin A₃, (**) detected with 26S rDNA probe.

Taxon	Voucher specimens	Collection year	Chromosome number (2n)	Ploidy level	CMA*	5S rDNA sites	35S rDNA sites**
<i>C. arich</i>	Tunisia, Tataouine: Kamour, Gouja H. BCN 106466	2005	18	2x	6	2	4
<i>C. azel</i>	Tunisia, Tataouine: Tieret, Gouja H. BCN 106463	2012	18	2x	6	2	4
<i>C. azel</i>	Tunisia, Tataouine: El Borma, Gouja H. BCN 106465	2010	18	2x	6	2	4
<i>C. comosum</i>	Tunisia, Tataouine: Kamour, Gouja H. BCN 106456	1999	18	2x	6	2	4
<i>C. comosum</i>	Tunisia, Tataouine: El Borma, Gouja H. BCN 106457	2007	18, 36	2x, 4x	6, 8	4, 4	2, 4

Table 2 Genome size data of the studied *Calligonum* populations, all from Tataouine, Tunisia. SD= standard deviation. *Lycopersicon esculentum** (2C=1.99 pg) and *Petunia hybrida*** (2C=2.85 pg) were used as internal standards.

Taxon	Voucher specimens	Collection year	Poidy level	2C (pg)± SD seeds	HPCV plant (%)	2C (pg)± SD seedlings	HPCV plant (%)
<i>C. comosum</i>	Tunisia, Tataouine: Kamour, Gouja H. BCN-S-1887	2013	2x	2.59±0.07*	3.45	2.36±0.07**	3.16
	Tunisia, Tataouine: El Borma, Gouja H. BCN-S-1877	2012	4x	4.89±0.13*	3.28	4.77±0.10**	3.94
<i>C. azel</i>	Tunisia, Tataouine: Kamour, Gouja H. BCN-S-1888	2013	2x	2.63±0.20*	3.70	2.27±0.06**	3.63
	Tunisia, Tataouine: El Borma, Gouja H. BCN-S-1876	2012	2x	2.60±0.13*	3.93	2.45±0.05**	1.71
	Tunisia, Tataouine: Tieret, Gouja H. BCN-S-1874	2012	2x	2.58±0.08*	3.04	2.54±0.02**	3.18
<i>C. arich</i>	Tunisia, Tataouine: Kamour, Gouja H. BCN-S-1889	2013	2x	2.60±0.10*	2.37	2.45±0.07*	2.39
	Tunisia, Tataouine: El Borma, Gouja H. BCN-S-1878	2012	2x	2.60±0.13*	1.34	2.56±0.01*	4.97

1 **Fig. 1** Map of Tunisia indicating the geographical distribution of the *Calligonum*
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4 populations analysed.
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10 **Fig. 2** Orcein, FISH pictures and idiograms in which chromomycin A₃ positive bands are
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12 marked yellow, 26S rDNA sites, green and 5S rDNA sites, red, for diploid *Calligonum*
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14 *arich* from Kamour (a-c), *C. azel* from El Borma (d-f), *C. comosum* from El Borma (g-i), *C.*
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16 *comosum* from Kamour (j-l) and tetraploid *C. comosum* from El Borma (m-o). Since
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18 information on chromosome morphology is not included in this work, idiograms depict
19
20 them as all metacentric, although submetacentric and acrocentric chromosomes are
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22 also present. In picture e arrows indicate detached 35S rDNA satellites. Scale bars = 10
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36 **Fig. 3** Neighbour-net diagram based on sequences from the merged dataset of three
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38 DNA regions (ITS, *trnL-F*, and *rbcl*), adapted from Gouja et al. (2014). Populations of
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40 the same species are grouped in the same cluster.
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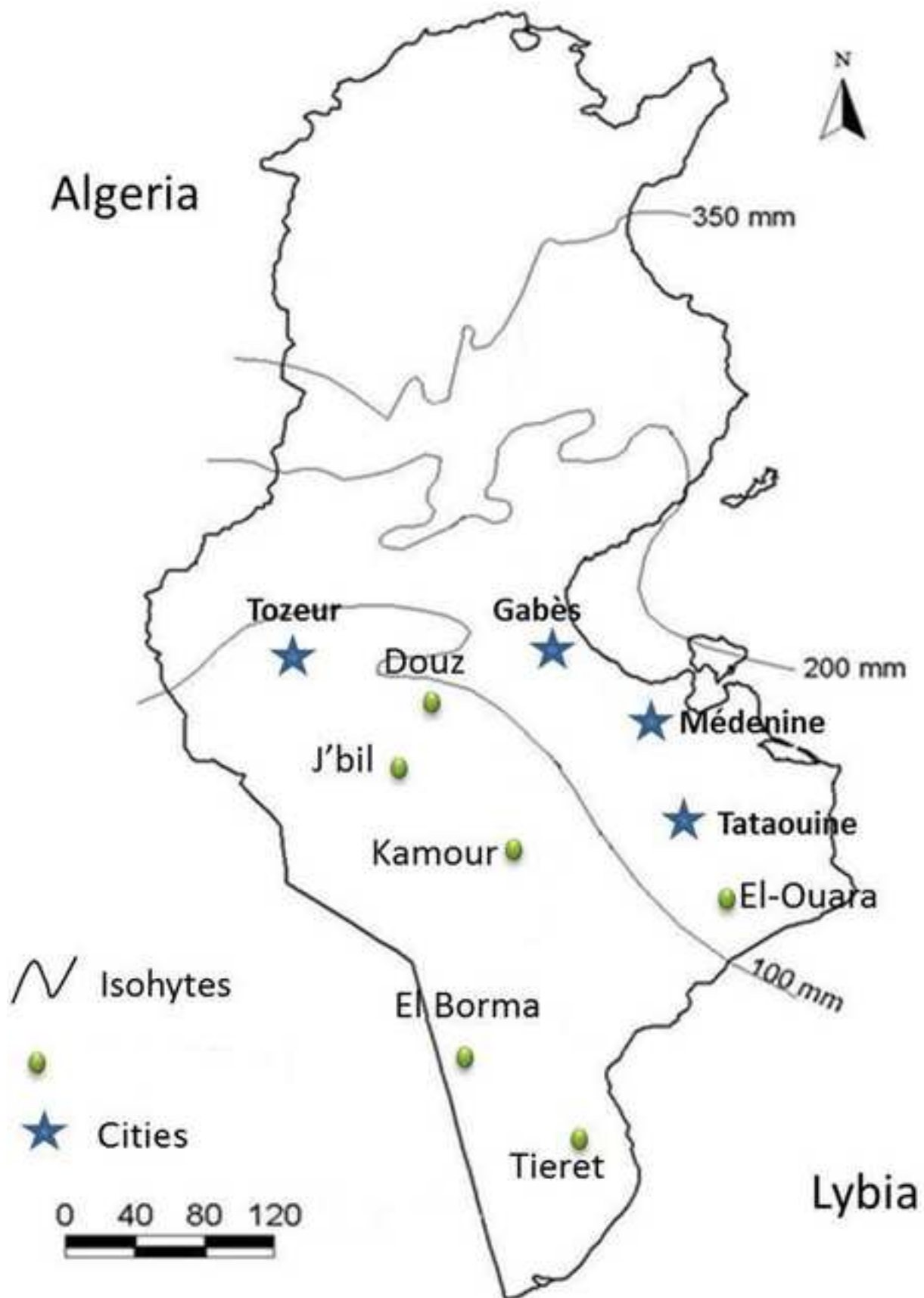
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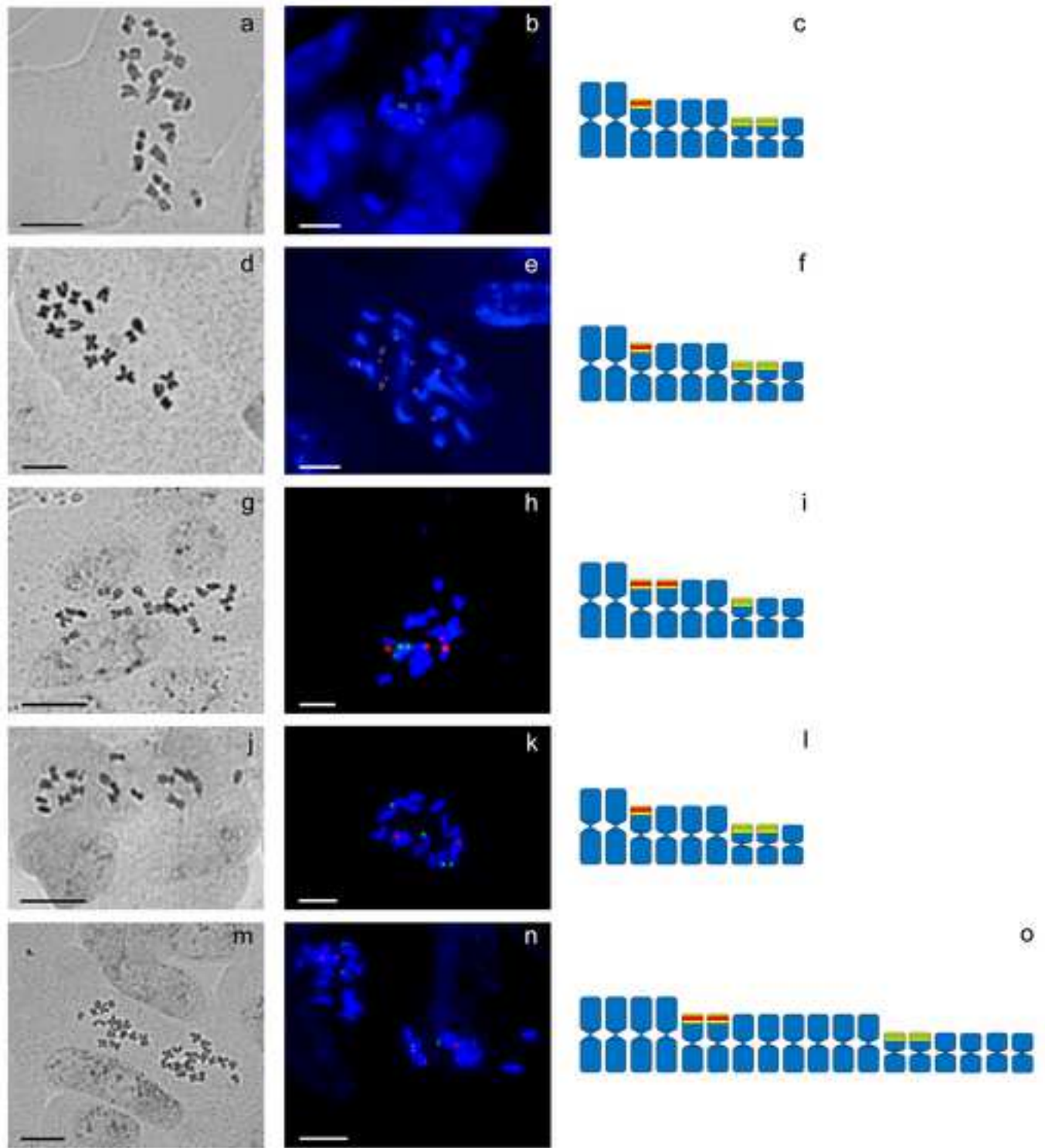


Figure 3

