

Genome size and cytogenetic characterization of three Algerian *Retama* species

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Abstract Thirty-three populations belonging to the three *Retama* species, *Retama monosperma*, *Retama raetam* and *Retama sphaerocarpa*, were collected to study species differentiation using flow cytometry for 2C DNA assessment and molecular cytogenetics for karyotype organisation. All were $2n=48$. Genome size ranged from 1.76 to 1.97 pg and revealed significant intraspecific variation correlated to the geographic distribution of the populations. The number and position of the two ribosomal gene families 5S and 45S were determined by fluorescent in situ hybridization, revealing chromosome reorganisation between species. In *R. raetam* and *R. monosperma*, the minor 5S loci co-localised with 45S on the satellite chromosome pair. Fluorochrome banding identified GC- and AT-rich DNA regions. In *R. monosperma* a unique chromomycin positive GC-rich band was observed associated with the secondary constriction. In contrast, an original pattern

showing two chromomycin positive bands localised at each side of the extended rDNA locus was observed in *R. sphaerocarpa* and *R. raetam*. The polymorphism revealed in our cytogenetic data allowed us to separate the group of *R. raetam* and *R. monosperma* from *R. sphaerocarpa*.

Keywords FISH · Flow cytometry · Fluorochrome banding · Genome size · rDNA

Introduction

Retama species which belong to the Papilionideae subfamily are distributed in Northern Africa, Canary Island, Southern Europe and Western Asia (Zohary 1959). They correspond to perennial herbaceous plants, 3–4-m high shrubs, which have an important ecological role in the formation of “fertility islands” where the growth of numerous annual and woody species are favoured (Pugnaire et al. 1996). *Retama* shrubs create the natural barriers which prevent the weakening of ecosystems and desertification.

They are included in the vegetation programmes dedicated to the semiarid Mediterranean area in order to improve fixation and soil fertilization (Caravaca et al. 2003). The *Retama* species also contain rare flavones, retamatriosides, with cytotoxic and antimicrobial activities (Louaar et al. 2007; Martín-Cordero et al. 2000).

On the basis of morphological characters, Webb (1843) and Maire (1952–1987) reorganised the genus *Retama* including species *Retama monosperma* (L.) Boiss., *R. raetam* Webb and Berthel., *Retama sphaerocarpa* Boiss., *Retama dasycarpa* Coss. and several subspecies and varieties. *R. monosperma* subsp. *eu-monosperma* var. *webbii* (Spach) Maire is endemic to Morocco and the subsp. *bovei* Webb, endemic to Algeria and Morocco, is

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located in a biodiversity hotspot of the Mediterranean region (Véla and Benhouhou 2007). These species which present taxonomic difficulties were included in the tribe of Genisteae (Käss and Wink 1997; Pardo et al. 2004). They were first classified in *Spartium* L. then in *Retama* Boiss. (Lopez et al. 1998).

Genome size is a useful trait in systematics and evolution (Cerbah et al. 1999a, b, 2001; Godelle et al. 1993; Ohri 1998). Its evaluation by flow cytometry is rapid and precise, allowing to detect inter- and intraspecific variations (Biradar and Rayburn 1993; Doležel and Bartoš 2005). In the three studied *Retama* species: *R. raetam*, *R. sphaerocarpa* and *R. monosperma*, the genome size was assessed by flow cytometry for different populations showing polymorphism.

The *Retama* species have the same chromosome number $2n=48$ (Bhattacharya et al. 1971), showing 24 ring bivalents at meiotic metaphase I (Gallego-Martin et al. 1988; Reese 1957). However, the karyotype has not yet been studied. In the Fabaceae family, karyotyping has been widely based on the chromosome organisation of the ribosomal genes allowing examination of evolutionary relationships between species (e.g. *Phaseolus*, Moscone et al. 1999; *Glycine*, Singh et al. 2001; *Lupinus*, Hajdera et al. 2003; *Medicago*, Cerbah et al. 1999a, b; Abirached-Darmency et al. 2005).

The aims of this study were: (1) to characterise the karyotype of *Retama* species by physical mapping of

ribosomal genes 5S and 18S-5.8S-26S using fluorescent in situ hybridization (FISH); (2) to examine the heterochromatin organisation by chromomycin, Giemsa-C banding and DAPI after FISH; (3) to determine the genome size and its potential variability in several populations by flow cytometry of nuclear DNA content and (4) to understand the cytogenetic mechanisms of the microevolution among three investigated species.

Material and methods

Plant material

Thirty-three populations belonging to the three species of genus *Retama* were included in this study: *R. monosperma* subsp. *bovei* var. *oranensis* (Maire) in Oran and Mostaganem, *R. raetam* var. *rigidula* in High Plateaux and in Saharian Atlas dunes and *R. sphaerocarpa* var. *atlantica* (Pomel) in High Plateaux and in Saharian Atlas. The fourth species, *Retama dasycarpa* Coss. was not found in Algeria. Seeds and leaves have been collected from different localities all over Algeria as shown in Fig. 1 and Table 1. Species identification was assessed following the herbarium at the “Museum d’Histoire Naturelle de Paris” and two flora (Maire 1987; Quezel and Santa 1962) and by personnel surveys (thanks to participation of first author to ecological caravans organised by the Laboratory of

Fig. 1 Geographic distribution of three *Retama* species in Algeria and origin of studied populations (surrounded with circle)

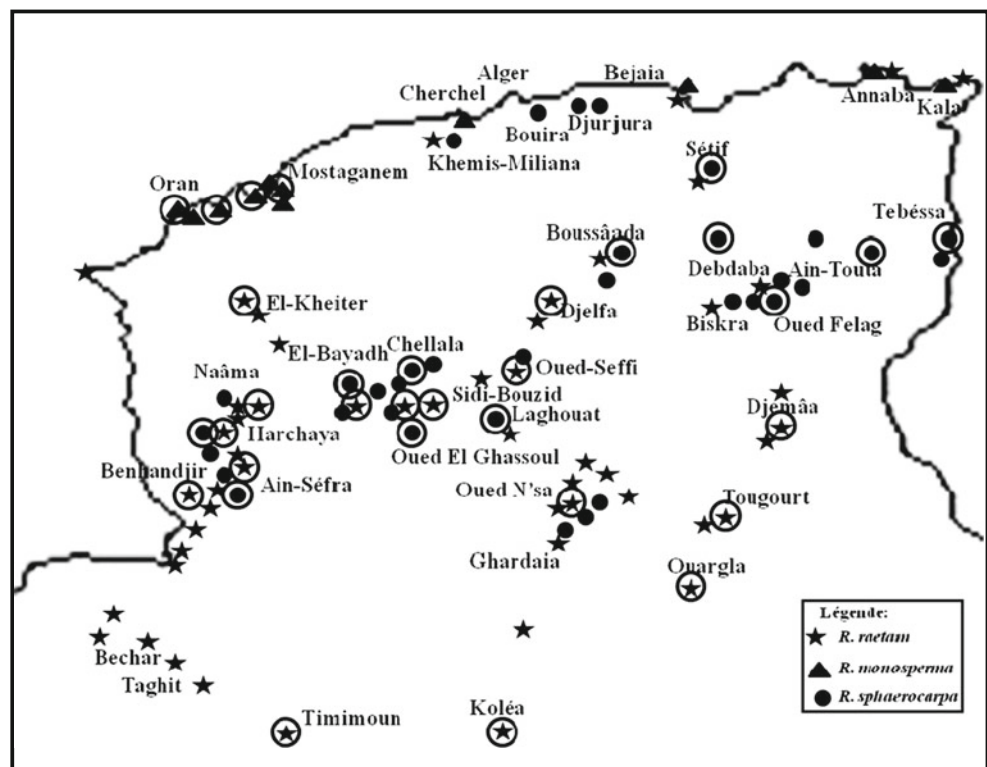


Table 1 Geographic origin of studied populations of three *Retama* species

Species	Localities of populations	Altitude (m)	Latitude Longitude	Collectors
1 <i>R. monosperma</i>	1. NW Algeria, Bousfer near village of Oran in cost	24	35°41'11.36" N 0°56'58.65" O	R. Benmiloud-Mahieddine
2	2. NW Algeria, Cap Falcon near village of Oran in cost	1	35°46'02.21" N 0°48'06.18" O	R. Benmiloud-Mahieddine
3	3. NW Algeria, Marsat El Hadjadj near village of Mostaganem in cost	6	35°47'23.91" N 0°08'48.24" O	R. Benmiloud-Mahieddine
4	4. NW Algeria, Mactaa near village of Mostaganem in cost	7	35°51'55.87" N 0°09'11.20" O	R. Benmiloud-Mahieddine
1 <i>R. raetam</i>	5. SW Algeria, El-Kheiter near village of Saida in "high plateaux"	986	34°09'02.38" N 0°04'03.39" E	R. Benmiloud-Mahieddine
2	6. SW Algeria, Harchaya near village of Naâma, Saharan Atlas	1,184	33°10'55.66" N 21°40'82.0" O	R. Benmiloud-Mahieddine, A. Djabeur
3	7. SW Algeria, Naâma in Saharan Atlas	1,172	33°16'05.71" N 0°19'01.72" O	R. Benmiloud-Mahieddine, A. Djabeur
4	8. SW Algeria, Ain-Séfra, in Saharian Atlas	1,069	32°45'26.05" N 34°35'03.0" O	R. Benmiloud-Mahieddine, A. Djabeur
5	9. SW Algeria, Benhandjir near village of Ain-Séfra, Saharan Atlas	1,148	32°41'29.60" N 0°45'18.22" O	R. Benmiloud-Mahieddine, A. Djabeur
6	10. SW Algeria, Chellala near village of El-Bayadh in "high plateaux"	1,115	33°02'08.65" N 0°03'37.15" E	R. Benmiloud-Mahieddine, A. Djabeur
7	11. SW Algeria, El-Bayadh in "high plateaux"	1,374	33°39'37.45" N 1°02'03.49" E	R. Benmiloud-Mahieddine, A. Djabeur
8	12. SW Algeria, Sidi Bouzid near village of El-Bayadh "high plateaux"	1,226	34°20'45.32" N 2°15'54.93" E	R. Benmiloud-Mahieddine, A. Djabeur
9	13. SW Algeria, Oued Seffi near village of Aflou in "high plateaux"	1,464	33°57'17.74" N 1°54'56.61" E	R. Benmiloud-Mahieddine, H. Benmiloud
10	14. SC Algeria, Djelfa south of City of Algiers in 'high plateaux'	1,140	34°41'16.52" N 3°15'17.77" E	R. Benmiloud-Mahieddine, A. Djabeur
11	15. SC Algeria, Ghardaïa in centre of Sahara	585	32°56'56.46" N 3°46'43.50" E	R. Benmiloud-Mahieddine, H. Benmiloud
12	16. SC Algeria, Oued N'sa near village of Ghardaïa in centre of Sahara	500	32°50'56.55" N 3°54'00.53" E	R. Benmiloud-Mahieddine, H. Benmiloud
13	17. SE Algeria, Ouargla in eastern of Sahara	134	31°57'52.89" N 5°20'35.65" E	R. Benmiloud-Mahieddine, H. Benmiloud
14	18. SE Algeria, Djamâa north of City of Tougourt in eastern of Sahara	37	33°32'10.91" N 5°59'30.55" E	R. Benmiloud-Mahieddine, H. Benmiloud
16	19. SC Algeria, Koléa in centre of Sahara	418	30°36'09.77" N 2°54'02.06" E	R. Benmiloud-Mahieddine
17	20. SE Algeria, Tougourt in eastern of Sahara	77	33°06'26.74" N 6°03'38.72" E	R. Benmiloud-Mahieddine
17	21. SW Algeria, Timimoun in western of Sahara	279	29°15'06.35" N 0°13'40.68" E	R. Benmiloud-Mahieddine
1 <i>R. sphaerocarpa</i>	22. SW Algeria, Harchaya near village of Naâma Saharan Atlas	1,184	33°10'55.66" N 21°40'82.0" O	R. Benmiloud-Mahieddine, A. Djabeur
2	23. SW Algeria, Ain-Séfra in Saharan Atlas	1,069	32°45'26.05" N 34°35'03.0" O	R. Benmiloud-Mahieddine, A. Djabeur
3	24. SW Algeria, El-Bayadh in "high plateaux"	1,374	33°39'37.45" N 1°02'03.49" E	R. Benmiloud-Mahieddine, A. Djabeur
4	25. S Algeria, Laghouat in centre of Saharan Atlas	787	33°47'55.94" N 2°53'04.14" E	R. Benmiloud-Mahieddine, A. Djabeur
5	26. SW Algeria, Oued El Ghassoul near village of El-Bayadh in "high plateaux"	1,222	33°19'10.29" N 1°14'49.81" E	R. Benmiloud-Mahieddine, H. Benmiloud
6	27. SW Algeria, Chellala near village of El-Bayadh in "high plateaux"	1,115	33°02'08.65" N 0°03'37.15" E	R. Benmiloud-Mahieddine, A. Djabeur

Table 1 (continued)

Species	Localities of populations	Altitude (m)	Latitude Longitude	Collectors
7	28. SC Algeria, Boussâada south of City of Algiers in “high plains”	579	35°13'21.37" N 4°11'55.01" E	R. Benmiloud-Mahieddine H. Benmiloud
8	29. SE Algeria, Setif south of City of Algiers in “high plateaux”	1,066	36°10'22.28" N 5°29'40.31" E	R. Benmiloud-Mahieddine, H. Benmiloud
9	30. SE Algeria, Debdaba south of City of Constantine	894	35°20'59.42" N 5°46'27.86" E	R. Benmiloud-Mahieddine, H. Benmiloud
10	31. SE Algeria, Ain-Touta near village of Batna in “high plateaux”	977	44°01'38.1" N 21°57'16.2" E	R. Benmiloud-Mahieddine, H. Benmiloud
11	32. SE Algeria, Tébessa “high plateaux” near of Tunis frontiers	1,199	35°24'51.95" N 8°04'31.09" E	R. Benmiloud-Mahieddine, H. Benmiloud
12	33. SE Algeria, Oued Felag near village of Batna in “high plateaux”	863	35°20'29.67" N 5°50'59.40" E	R. Benmiloud-Mahieddine, H. Benmiloud

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Flow cytometry

For flow cytometry fresh leaves from five individual shrubs of each population were collected. Genome size and GC percentage were estimated following Marie and Brown (1993). *Petunia hybrida*, PxPc6, (2C=2.85 pg) was used as internal standard. The leaves from *Retama* and *Petunia* were chopped together with a razor blade in 600 µl of Galbraith nuclei buffer (Galbraith et al. 1983) containing 0.1 (w/v) Triton X-100 with addition of fresh 10 mM sodium metabisulfite and 1% polyvinylpyrrolidone 10,000. Then, the suspension was filtered through nylon mesh (pore size 30 µm) and kept at 4°C. Total nuclear DNA was estimated using a DNA intercalating dye propidium iodide (25 µg/ml; Sigma) after addition of RNase (2.5 units/ml; Roche). After at least 20 min incubation, the measurements were made on 2,000–3,000 nuclei and repeated two or three times for each of the five individuals per population. Total nuclear DNA was calculated as the ratio between sample and standard peaks (*Retama/Petunia*) multiplied by the DNA content of the standard. All measurements were made using an Elite ESP flow cytometer (Beckman-Coulter, Roissy, France) with an air-cooled argon laser. Statistical analysis was applied to DNA content values using a one-factor ANOVA with XLSTAT 2009.

Karyotype analysis

For karyotype analysis, roots tips were hydrolysed in 1 N HCl at 60°C for 14 min, stained with Schiff reagent for

30 min and squashed in a drop of 1% aceto-carmin. For each population 10–30 good metaphase plates from at least five individuals were examined in order to establish the number and morphology of chromosomes.

For each species, 10–12 metaphase plates from several individuals were used for chromosome measurements. When it was possible, the arm ratio ($r=l/s$) was used to determine the centromere position and chromosome type following Levan et al. (1964).

Chromosome preparation for FISH and fluorochrome banding

Seeds were scarified with 96° sulphuric acid for 6 h to allow germination. Root tip meristems were obtained by germination in petri dishes on wet filter paper in dark at 24°C. Root tips (1–1.5 cm long) were pretreated with 0.002 M 8-hydroxy-quinoleine solution at 16°C for 4 h, and then fixed in freshly prepared mix solution: 96% ethanol/chloroform/glacial acetic acid (6/3/1, v/v/v) for 24 h at 4°C. For storage, the fixed root tips were transferred to 70% ethanol.

Mitotic chromosome preparations were obtained by the air-drying technique (Geber and Schweizer 1987). Before microspreading, the digestion of the cell wall was carried out in an enzymatic mixture with 4% R-10 cellulase (Yakult Honsha Co., Tokyo, Japan), 1% pectolyase Y-23 (Seishin Co., Tokyo, Japan), 4% hemicellulase (Sigma Chemical Co.) in citrate buffer (pH=4.2) for 30 min up to 1 h (depending on root size and species). The cells in suspension were washed three times in the same buffer, centrifuged and finally fixed in ethanol/acetic acid (3/1, v/v). Protoplasts were dropped on a clean slide and kept at room temperature for different staining techniques.

Fluorochrome banding

For detection of GC-rich DNA regions, chromomycin A₃ (CMA; Serva) was used according to Schweizer (1976). The AT-rich DNA regions were detected by Hoechst 33258 (Ho; Serva) according to Siljak-Yakovlev et al. (2002). Slides were stained with 0.2 mg/ml of CMA solution for 10 min, and with 2 µg/ml of Hoechst 33258 for 1 min and 40 s, in the dark. The same slides were used for FISH experiment after discolouration in ethanol/glacial acetic acid (3/1, v/v), and dehydration in a graded ethanol series (70%, 90%, 100%) 3 min for each.

For detection of constitutive heterochromatin, DAPI (4',6-diamidino-2-phenylindole) 2 µl/ml in McIlvaine buffer pH 7 was used after FISH experiment.

Fluorescent in situ hybridization

For the simultaneous FISH experiment, two DNA probes were used: the probe for the 18S-5.8S-26S ribosomal gene was pTa71 (Gerlach and Bedbrook 1979), a clone of a 0.8-kb Taq I fragment obtained from wheat DNA and cloned in pUC18. This probe was labelled with digoxigenin-11-dUTP (Boehringer Mannheim). The probe used for 5S rDNA localization was a clone of pTa794, a 410-kb BamHI fragment of 5S rDNA isolated from wheat and cloned in Pbr322 (Gerlach and Dyer 1980): it contains the 5S rDNA gene (120 bp) and the noncoding intergenic spacer (290 bp). This probe was labelled directly with fluoro-Red-dUTP (Amersham).

Fluorescent in situ hybridization was carried out following Heslop-Harrison et al. (1991) with minor modification (Siljak-Yakovlev et al. 2002). The pepsin treatment (70 µl, 0.1 mg/ml in HCl 0.01 N) was carried out for 30 min at 37°C. Chromosome DNA was denatured with 70% formamide in 2×-SSC for 10 min at 70°C.

Image acquisition and analysis

The slides were observed using a Zeiss Axiophot epifluorescence microscope. The fluorescence signals were analysed in about 30 metaphase plates or interphase nuclei for each population with different Zeiss filters sets: 01 (excitation 365 nm emission >397 nm) for bisbenzimidazole and DAPI, 07 (excitation 436–460 nm, emission >520) for chromomycin, 09 (excitation 490 nm, emission 525 nm) to detect 18S signals and 15 (excitation 540–560 nm, emission 397 nm) to detect 5S signals. Detection and analysis of fluorescent signals were performed using the highly sensitive CCD camera (RETIGA 200R; Princeton Instruments, Evry, France) and an image analyzer (Metavue, Evry, France).

Results

Genome size

The 2C DNA values obtained for 33 populations of three *Retama* species are presented in Table 2. The 2C DNA value ranged from 1.76 to 1.89 pg in *R. sphaerocarpa*, from 1.88 to 1.93 pg in *R. monosperma* and from 1.80 pg to 1.97 pg in *R. raetam*. A single case of tetraploidy was observed for one individual of *R. raetam* from the Djemâa population (2C=3.67 pg). This value was not taken into account for the mean for this population.

The variance analysis (ANOVA) showed significant genome size differences which separate *R. sphaerocarpa* from *R. raetam* and *R. monosperma* ($r^2=0.750$, $P=0.0001$). Intraspecific variation in *R. raetam* ($r^2=0.595$, $P=0.0001$) and in *R. sphaerocarpa* ($r^2=0.576$, $P=0.0001$) was also found among populations from different geographic localities and concerned mainly the desert populations (Fig. 2a–c).

The Ascendant Hierarchic Classification (CHA) from XLSTAT 2009 (expressing distances in sums of squares of deviation Ward's method) for homogeneous group determination was performed as shown in Fig. 2e–g. For all populations, the identified groups showed the separation of *R. sphaerocarpa* from *R. raetam* and *R. monosperma* and revealed three groups for *R. raetam*, and four groups for *R. sphaerocarpa*.

A clear intraspecific separation relative to the geographic localisation of the populations was observed (Fig. 2, Table 1).

In *R. raetam* (Fig. 2e), the group G1 includes three populations from the western High Plateaux and one population from the desert. Group G2 includes eight populations, belonging to the *R. raetam* var. *rigidula*, from the Saharan Atlas and one population from the desert. Group G3 including five populations from the desert corresponds to the lowest C value, 2C=1.80–1.85 pg.

In *R. sphaerocarpa* var. *atlantica* (*Retama atlantica* Pomel, Fig. 2f), the group (G1) includes one population from the east of the Saharan Atlas with the highest 2C value=1.89 pg. Group G2 includes two populations from the western part of the Saharan Atlas. Group G3 includes four populations from the eastern part of the Saharan Atlas and group G4 includes five populations from the centre.

Karyotype feature and chromosome banding

The analysis of mitotic metaphases showed the same chromosome number 2n=48 in the three *Retama* species (Fig. 3a). The metaphase chromosomes were relatively small and morphologically similar, their lengths ranging

Table 2 Chromosome number and 2C DNA values for 33 populations of three *Retama* species

Species and populations	2C DNA in pg ^a (sd)	Min–max	1C DNA (Mbp)	CV %	N
<i>R. monosperma</i>					
Bousfer (Oran)	1.93 ^b (0.02)	1.91–1.96	943	1.11	5
Cap Falcon (Oran)	1.89 (0.07)	1.79–1.97	924	3.42	5
Mactaa (Mostaganem)	1.88 (0.02)	1.86–1.91	919	1.07	5
Marsta el Hadjadj (Mostaganem)	1.87 (0.02)	1.85–1.89	914	0.86	5
Mean value	1.89 (0.03)		924 (13)		
<i>R. raetam</i>					
El-Kheiter	1.87 (0.07)	1.80–1.91	914	3.57	5
Harcahaya	1.89 (0.03)	1.85–1.92	924	1.76	5
Naâma	1.92 (0.04)	1.87–1.96	938	2.15	5
Ain-Séfra	1.93 (0.04)	1.90–2.00	943	2.56	5
Benhandjir	1.89 (0.05)	1.85–1.94	924	2.86	5
Chellala	1.89 (0.05)	1.85–1.96	924	2.56	5
El-Bayadh	1.97 (0.06)	1.92–2.07	963	2.86	5
Sidi Bouzid	1.97 (0.04)	1.91–2.03	963	2.24	5
Oued Seffi	1.91 (0.04)	1.87–1.96	933	0.32	5
Djelfa	1.90 (0.03)	1.87–1.94	929	1.53	5
Ghardaia	1.91 (0.01)	1.91–1.93	933	0.39	5
Oued N'sa	1.86 (0.01)	1.86–1.88	909	0.87	5
Ouargla	1.85 (0.03)	1.82–1.90	904	1.77	5
Djamâa	1.86 (0.02)	1.84–1.89	909	0.69	4 ^c
Timimoun	1.80 (0.01)	1.79–1.82	880	0.47	2
Koléa	1.96 (0.01)	1.95–1.97	958	0.87	5
Tougourt	1.83 (0.01)	1.83–1.84	894	0.18	5
Mean value	1.90 (0.05)		929 (23)		
<i>R. sphaerocarpa</i>					
Harchaya	1.78 (0.02)	1.76–1.80	870	0.94	5
Ain-Séfra	1.77 (0.06)	1.72–1.86	865	3.27	5
El-Bayadh	1.76 (0.06)	1.80–1.91	860	3.22	5
Laghout	1.79 (0.07)	1.79–1.81	875	0.37	5
Oued El-Ghassoul	1.82 (0.03)	1.76–1.87	889	1.05	5
Chellala	1.79 (0.03)	1.77–1.82	875	1.43	5
Boussâada	1.79 (0.04)	1.76–1.85	875	2.21	5
Setif	1.79 (0.02)	1.76–1.81	875	1.22	5
Debdaba	1.81 (0.02)	1.79–1.83	885	0.88	5
Ain-Touta	1.89 (0.04)	1.87–1.94	924	1.97	5
Tébessa	1.81 (0.02)	1.79–1.83	885	1.03	5
Oued Felag	1.81 (0.02)	1.79–1.84	885	1.15	5
Mean value	1.80 (0.03)		880 (16)		

^a 1 pg=978 Mbp (according to Doležel et al. 2003)

^b Mean value followed by the standard deviation in parentheses

^c One tetraploid individual (2C=3.67 pg) was observed, but not included in this mean

from 1 to 3.5 μm . The karyogram follows the bimodal type with one group of 28–32 very small chromosomes ($\sim 1 \mu\text{m}$) and one group of 12–16 larger chromosomes ($\geq 1 \mu\text{m}$).

All *Retama* species showed a chromomycin positive band (CMA⁺) at the secondary constriction of the satellite chromosome pair. A weak terminal CMA⁺ bands was also observed on one small chromosome pair (Fig. 3b, d, e). In *R. sphaerocarpa* and *R. raetam*, two CMA⁺ bands co-localise with the secondary constriction at each side of the extended rDNA locus (Fig. 3d, e). In *R. monosperma* a

unique CMA⁺ band was associated with the secondary constriction (Fig. 3c). Interphase nuclei showed two large and two small signals corresponding to the CMA bands of metaphase chromosomes in all three species (Fig. 3f). One Hoechst AT-rich positive band was detected on the longest chromosome pair at an intercalary position (Fig. 3f). Centromeric DAPI positive bands were observed in all the chromosomes of the karyotype which enabled us to identify metacentric, submetacentric and subtelocentric chromosome types (Fig. 4a, c).

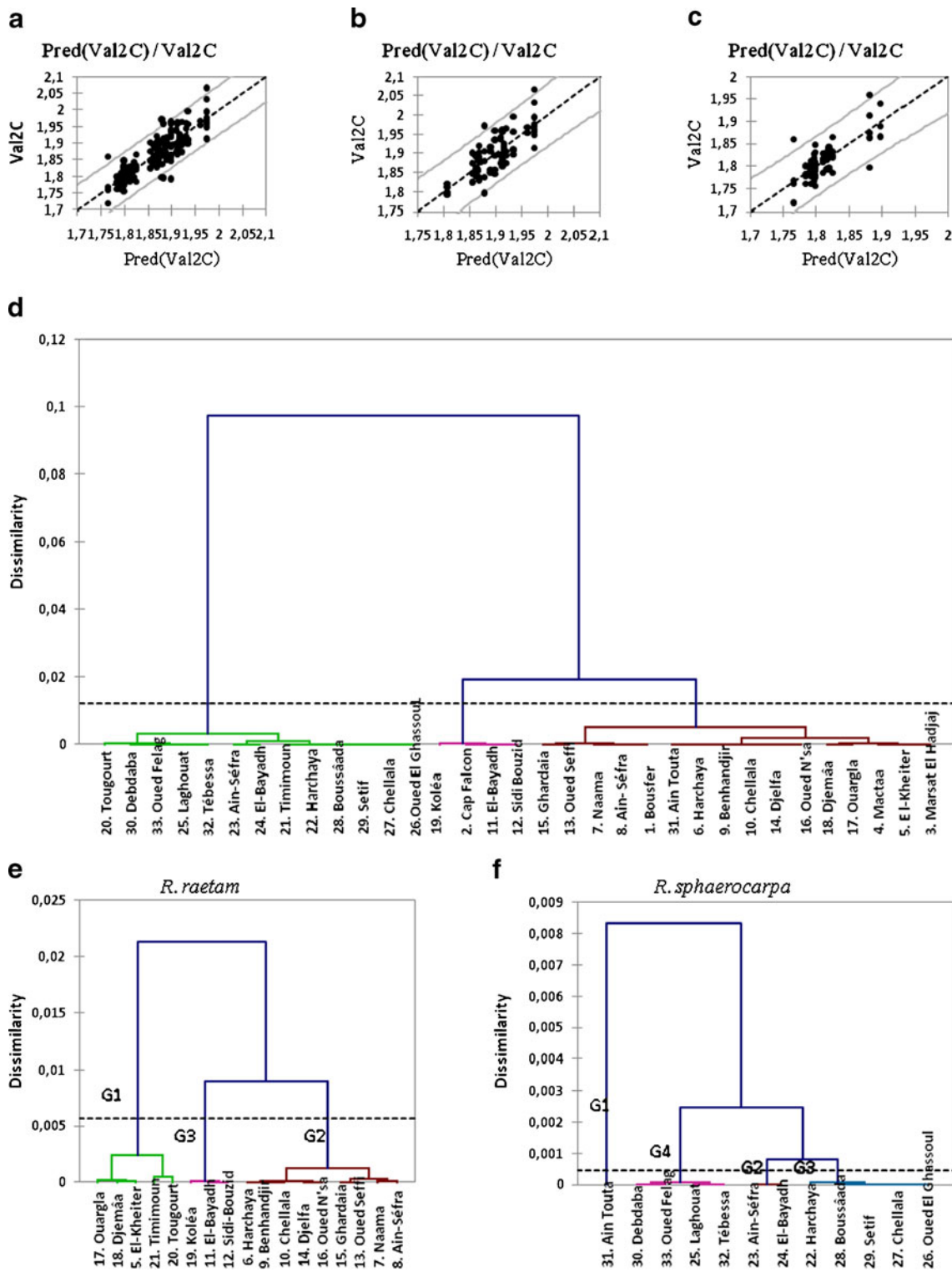
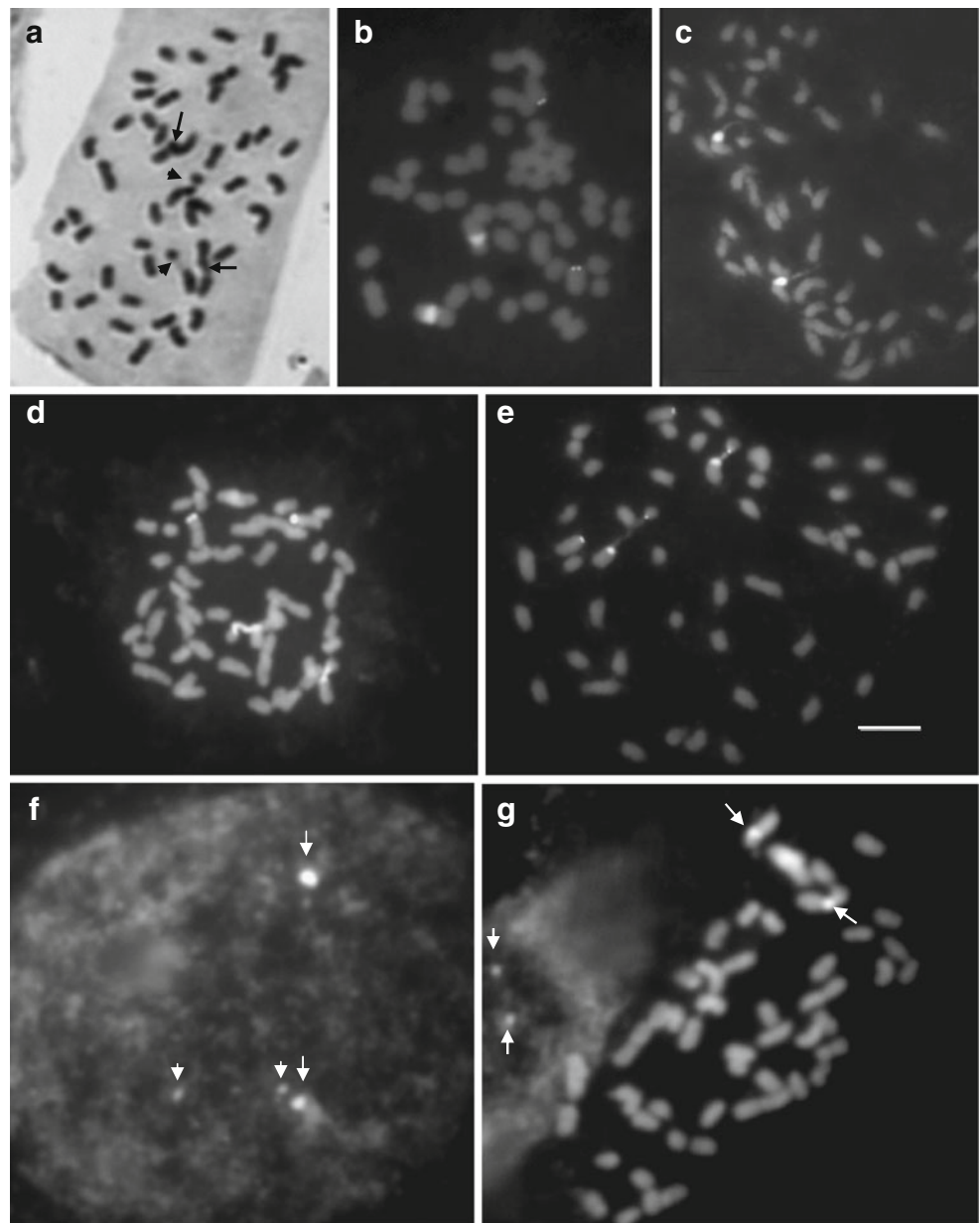


Fig. 2 ANOVA linear regression of variance analysis of 2C DNA values **a** for all *Retama* species, **b** of *R. raetam* populations and **c** of *R. sphaerocarpa* populations. CHA dendrograms of homogenous groups

using 2C DNA **d** for all investigated populations, **e** for *R. raetam* populations and **f** for *R. sphaerocarpa* populations

Fig. 3 **a** Feulgen staining of *R. monosperma* chromosomes. Note the presence of secondary constrictions (SC; *small arrows*) and the longest chromosome pair (*long arrows*). **b**, **c** CMA-banded metaphase of *R. monosperma* with condensed (**b**) and extended (**c**) SC. **d** CMA-banded metaphase of *R. sphaerocarpa*, four strong CMA signals, two in SC and two terminals. **e** *R. raetam* two strong GC-rich bands at SC level, and two weak bands at terminal position on one acrocentric chromosome pair. **f** Chromomycin-stained interphase nucleus in *R. raetam* with two strong and two weak signals (*arrows*). **g** Hoechst-banded metaphase and interphase nucleus of *R. monosperma*, AT-rich bands in intercalary position (*arrows*). Bar=10 μ m



Physical mapping of rRNA genes

In the studied species one 18S-5.8S-26S rDNA locus and up to three 5S loci were identified by FISH. The chromosome pair carrying the 18S-5.8S-26S rRNA genes cluster is one of the largest in the karyotype. The major 5S rDNA site present in all three species was localised on the short arm of one subtelocentric, chromosome pair 5, at telomeric position (Fig. 4a–d). The minor 5S site detected in *R. raetam* and *R. monosperma*, except for two desert populations of *R. raetam*, was localised on the satellite chromosome pair at the extremity of the short arm. In *R. raetam*, an additional 5S locus adjacent to the 18S-5.8S-26S was identified on the satellite chromosome at interca-

lary position (Fig. 4a, d). The extended secondary constriction detected by chromomycin and 18S-5.8S-26S probe was frequently observed in *R. sphaerocarpa* and *R. raetam* populations (Fig. 4a, b). Synthesis of cytogenetic results for three *Retama* species is presented in Table 3.

Discussion

Genome size

The 2C DNA amount found for the three *Retama* species ranges from 1.76 to 1.97 pg. These are the first data on the C value for *Retama* species. The 1C value ranged from 860 to

Fig. 4 a–c Double target FISH with 18S-5.8S-26S (green signal), 5S (red signal) and DAPI (blue signals) in *R. raetam* (a), *R. sphaerocarpa* (b) and *R. monosperma* (c). Note the strongly extended secondary constriction with 18S rRNA genes (green) and two 5S loci of different fluorescent intensity in *R. raetam* (a); two 5S loci were the same intensity in *R. monosperma* (c). Idiograms with position of 18S, 5S, DAPI and Hoechst signals for the three species (d). Bar=10 μm

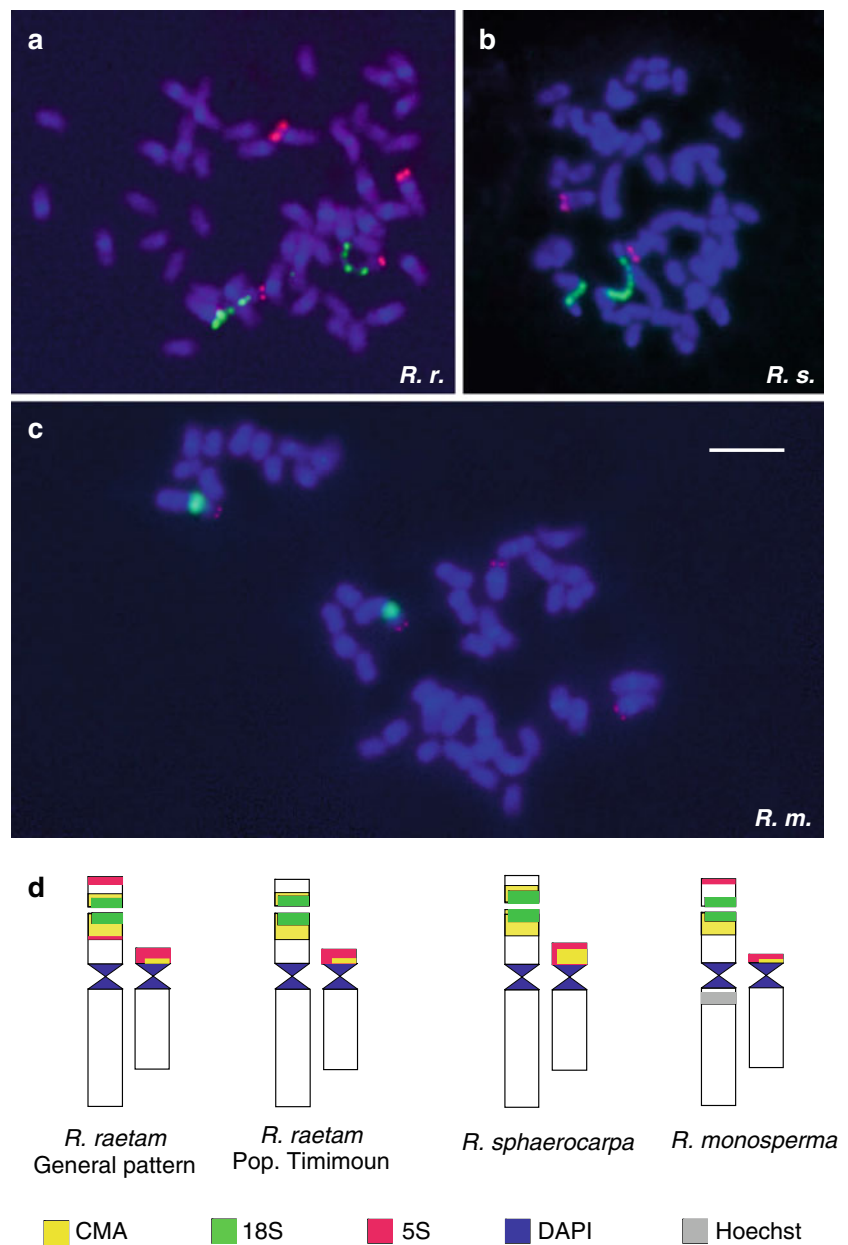


Table 3 Synthesis of cytogenetic results for three *Retama* species

Species	2n	2C DNA (pg)	CMA bands	Hoechst bands	DAPI and C bands	Number of loci 18S and 5S	Number of nucleoli
<i>R. monosperma</i> ssp. <i>bovei</i>	48	1.89 (±0.03) ^a	2 (1 in SC, 1 weak terminal)	Intercalary	Centromeric paracentromeric	1 (ch. 2) 2 (ch. 2, 5)	1
<i>R. raetam</i>	48	1.90 (±0.05) ^a	2 (1 in SC, 1 weak terminal)	Intercalary	Centromeric paracentromeric	1 (ch. 2) 2 (ch. 2, 5) 1 (ch. 2) 1 (ch. 5)	1
<i>R. sphaerocarpa</i>	48	1.80 (±0.03) ^a	2 (1 in SC, 1 weak terminal)	Intercalary	Centromeric paracentromeric	1 (ch. 2) 1 (ch. 2)	1

^a Standard deviation

SC secondary constriction, *ch.* chromosome pair 2 or 5

963 Mbp which represent about twice the genome of the model legume *Medicago truncatula* (1C=466 Mpb) and *Phaseolus* species: *Phaseolus macvaudhii* (1C=444 Mpb), *Phaseolus vulgaris* (588 Mpb, data from <http://www.rbgekew.org.uk/cval/homepage.html>). The *Retama* genus like *Lupinus* species belongs to the Genisteae group. The *Lupinus* species have their 2C nuclear DNA estimated between 0.97 and 2.44 pg. This variation corresponds to different chromosome numbers (Naganowska et al. 2003). *Lupinus mutabilis* with $2n=48$ and $2C=1.90$ pg has the same chromosome number and very similar genome size as *Retama* species. *Lupinus* species were assumed to derive from polyploid ancestors and their evolution was accompanied by changes in DNA amount (Naganowska et al. 2003).

It was thought that interspecific variation of genome size has an adaptive significance correlated to environmental conditions (Bennett 1987). In the investigated *Retama* populations, except for the extreme climatic conditions, no evident correlation can be shown relative to their environmental situation and geographical distribution. The *R. raetam* populations of High Plateaux with the coldest temperatures showed the highest nuclear DNA content $2C=1.97$ pg. In maize, cold tolerant populations also exhibit larger genome size (McMurphy and Rayburn 1992). Genome size variation in *R. raetam* populations was not correlated to altitude variations. The lowest genome size in *R. raetam* ($2C=1.80$ pg) corresponds to the population situated at 312 m in the western desert (Timimoun), the highest nuclear DNA content ($2C=1.97$ pg) being in *Retama* populations of the south-western desert at 165 m (Koléa). The variation between those two populations was estimated to be 10%.

Statistical analyses on the C values have shown a clear separation of *R. sphaerocarpa* from *R. monosperma* and *R. raetam* which was also confirmed by chromosome analysis of the rDNA organisation. *R. monosperma* and *R. raetam* shared also some morphological features, such as flowering periods, seeds and flowers colours in comparison to *R. sphaerocarpa* with different morphological characteristics (Benmiloud-Mahieddine, data not shown). These results are corroborated by the molecular phylogenetic analyses in Papilionideae using the *rbcL* and cpDNA sequences and ITS-1 and ITS-2 (Käss and Wink 1997; Pardo et al. 2004).

The difference in genome size between related species may be the result of several independent evolutionary events. Differences in DNA content among *Retama* species are probably due to changes in the amount of repetitive DNA sequences such transposable elements (Bennetzen and Devos 2005). We noticed that the lowest genome size in *R. raetam* carried only one locus of 5S rDNA.

Small intraspecific genome size variations were shown in both *R. raetam* and *R. sphaerocarpa* species. The data on genome size of all the *Retama* species from Mediterranean

region could be used to better understanding of evolutionary history of the genus.

Karyotype analysis and heterochromatin organisation

The three investigated *Retama* species have the same chromosome number ($2n=48$). In Genisteae, this chromosome number was assumed to correspond to tetraploid species (Cusna Velari and Feoli Chiapella 1991). Two ancestral basic chromosome numbers $x=6$ and $x=8$ have been proposed with the possibility to have a secondary polyploidy or polyploidy series of euploids and aneuploids (Gallego-Martin et al. 1988). It has been postulated that polyploids are better adapted than their diploid progenitors to extreme conditions (Stebbins 1971). We therefore considered that *Retama* species could be an allopolyploid with a disomic inheritance. Indeed, *Retama* species have shown a disomic inheritance with 24 bivalents observed at meiotic metaphase (data not shown), so their basic chromosome number may well be six ($6 \times 4 = 24$) or eight ($8 \times 3 = 24$).

Pericentromeric constitutive heterochromatin was revealed by DAPI banding on the denaturated chromosome preparations dedicated to FISH experiments, which allowed identification of the centromere position. The DAPI positive centromeric bands were specifically revealed in species with small chromosomes (Guerra 2000). In species with higher 2C DNA values, additional bands at interstitial chromosome regions or at telomeres are also observed, such as in *Vicia faba*, *Allium* and *Aveneae* (Fuchs et al. 1998; Do et al. 2001; Winterfeld et al. 2009, respectively)

CMA + fluorochrome banding revealed GC-rich heterochromatin associated with NORs, and occasionally with the 5S rDNA loci. The co-localisation of GC-rich heterochromatin and ribosomal genes has been frequently reported; e.g. *Brassica* (Hasterok and Maluszynska 2000), *Cedrus* (Bou Dagher-Kharrat et al. 2001), *Picea* (Siljak-Yakovlev et al. 2002), *Solanum* (Srebniak et al. 2002). In *R. raetam* and *R. sphaerocarpa*, an original pattern associating two adjacent CMA⁺ bands was observed at each side of the secondary constriction. In *R. monosperma* only one band was present. This banding pattern is probably related to the mode of rDNA compaction. DNA compaction in heterochromatin is associated with the inactivation of the coding sequences (Hennig 1999). In *Quercus* genus there appears to be a relation between the facultative GC-rich heterochromatin and the functional state of rRNA genes (Zoldoš et al. 1999, 2000)—compacted when inactive—which may also apply to *Retama*.

One band of AT-rich DNA was detected by Hoechst banding at an intercalary position on the longest metacentric chromosome pair. This band may correspond

to a chromosome knob associated with highly repetitive AT-rich DNA arrays as reported in *Arabidopsis* (Franz et al. 1998).

In conclusion, only three chromosome pairs can be recognised and used as marker chromosomes in these investigated species (Fig. 4d).

Ribosomal genes organisation

The number and size of the fluorescent signals corresponding to 5S and 18S-5.8S-26S rDNA sites, observed on metaphase chromosomes, were confirmed in the interphase nuclei. The CMA⁺ extended DNA filaments which were also labelled by FISH corresponded to the decondensed secondary constriction during rDNA transcription. Similar observations were reported in *Quercus*, *Medicago* and *Lupinus* (Zoldoš et al. 1999; Cerbah et al. 1999a, b; Naganowska and Zielinska 2004, respectively).

The localisation of the two rDNA families (18S and minor 5S) on the same chromosome pair showed inter- and intraspecific variation. In *R. raetam* the partial superposition of the two fluorescent FISH-signals relative to the 5S and 18S-5.8S-26S rDNA loci was probably of ancestral origin. It has been mainly observed in lower eukaryotes, such as *Saccharomyces cerevisiae*, *Pythium* species, algae and bryophytes (Bell et al. 1977; Belkhiri et al. 1992; Gilson et al. 1995; Sone et al. 1999). The uncommon CMA⁺ band detected at the major 5S rDNA locus may be related to the presence of an ancestral 18S-5.8S-26S rDNA remnant.

Further experiments using Fiber-FISH are needed to characterise the organisation of the rDNA units and to know if 5S intercalates inside the large 18S-5.8S-26S unit as shown for *Capsicum* species (Kwon and Kim 2009).

In many Fabaceae species, the linkage on the same chromosome arm of the two rDNA families was reported: in *Lotus japonicum*, *Phaseolus vulgaris*, *M. truncatula* and *V. faba* where three rDNA loci are linked (Fuchs et al. 1998; Cerbah et al. 1999a, b; Pedrosa et al. 2002, 2003; Abirached-Darmency et al. 2005). This co-localisation was absent in *R. sphaerocarpa* and in two populations of *R. raetam* due to intraspecific variation of the 5S loci number. In *R. raetam*, the minor 5S rDNA locus was absent in two desert populations probably as the consequence of a chromosome deletion. However, the occurrence of a single 5S rDNA locus in *R. sphaerocarpa* populations may reflect an older origin of the species, as suggested in paleopolyploids, such as *Hevea* (Leitch et al. 1998) or rather the lost of one locus during evolutionary process from one common ancestor for three *Retama* species.

In summary, our results demonstrate differentiation among these three species and a clear separation of *R. sphaerocarpa* from *R. raetam* and *R. monosperma* based on both chromosome structure and genome size.

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