

Genome size and ploidy levels in highly fragmented habitats: the case of western Mediterranean *Juniperus* (Cupressaceae) with special emphasis on *J. thurifera* L.

Angel Romo · Oriane Hidalgo · Adam Boratyński ·
Karolina Sobierajska · Anna Katarzyna Jasińska ·
Joan Vallès · Teresa Garnatje

Received: 11 May 2012 / Revised: 15 October 2012 / Accepted: 26 October 2012 / Published online: 30 November 2012
© Springer-Verlag Berlin Heidelberg 2012

Abstract Mediterranean junipers are of special ecological importance as key components of resource islands in semi-arid mountain ecosystems of the Mediterranean basin. The fragmentation of their habitat, which was primarily natural and driven by climatic drought conditions, is currently being aggravated by anthropogenic pressure. In the framework of this concern, the present work aims to contribute establishing a genomic profile of *Juniperus* in its western Mediterranean range, with a special emphasis placed on *J. thurifera*. DNA contents were assessed by flow cytometry in 43 populations of nine taxa within their Mediterranean range (first reports for *J. navicularis*, *J. thurifera* subsp. *africana* and *J. thurifera* subsp. *thurifera*). Chromosome numbers were determined by orcein staining in eight taxa (first counts for *J. oxycedrus* subsp. *badia*, *J. phoenicea* subsp. *phoenicea*, *J. phoenicea* subsp. *turbinata*, of $2n=2x=22$, and for *J. thurifera* subsp. *thurifera*, of $2n=4x=44$). Tetraploid cytotypes have been the only ones found in the 19 populations of *J. thurifera* studied, this being the first report of a *Juniperus* species exclusively polyploid. In *J. thurifera*, *C*-value does not respond to habitat fragmentation, in the same way that genetic diversity within populations was previously shown to be unaltered, suggesting that this factor has not had, at least to date, a significant impact

on populations at genomic and genetic levels. Habitat fragmentation leads to deeply age-biased populations with a male-biased imbalanced sex ratio (lack of females), indicating an urgent need to improve regeneration within the populations of this species.

Keywords Flow cytometry · Gymnosperms · Habitat fragmentation · Juniper · Karyology · Polyploidy

Introduction

The genus *Juniperus* L., consisting of 101 taxa (infraspecific incl.; Adams 2011), is the second largest of living conifers (Miller 1977; Farjon 2010), surpassed only by *Pinus* L.. Predominantly distributed in the Northern Hemisphere (the only exception is *J. procera* Hochst. ex Endl., in Africa), it presents 15 taxa exclusive to the western Mediterranean region (Gauquelin 2006). The phylogeny of the genus shows the Mediterranean taxa scattered into several groups in derived position within Asian lineages (Mao et al. 2010), suggesting their diversification through five to six independent colonization events from Asia. Junipers are xerophytes of a special ecological importance since they are among the few—sometimes the only—trees able to grow in arid and semi-arid climates (Ciesla 2002). This is the case in the Mediterranean basin of *J. excelsa* M. Bieb. (Douaihy et al. 2011) and *J. thurifera* L. (Montes et al. 2002; Romo and Boratyński 2005). These *Juniperus* species are key components of “resource islands” (Reynolds et al. 1999), i.e. patches of remnant woodlands of locally improved soil and microclimatic conditions, plant facilitative interactions and plant dispersal (Maestre and Cortina 2005, and references therein). The positive impact of trees and shrubs on community structure and dynamics of semi-arid areas promoted the formation of small biodiversity hot-spots (Pugnaire and

Communicated by G. G. Vendramin

A. Romo · T. Garnatje
Institut Botànic de Barcelona (IBB-CSIC-ICUB),
Barcelona, Catalonia, Spain

O. Hidalgo (✉) · J. Vallès
Laboratori de Botànica, Facultat de Farmàcia,
Universitat de Barcelona, Barcelona, Catalonia, Spain
e-mail: orianehidalgo@ub.edu

A. Boratyński · K. Sobierajska · A. K. Jasińska
Polska Akademia Nauk, Instytut Dendrologii, Kórnik, Poland

Lázaro 2000). Additionally, junipers represent an essential wood resource for human populations living in semi-arid Mediterranean area (Gauquelin et al. 1999; Montes et al. 2002; Romo and Boratyński 2005), and are used in local pharmacopeia (González-Tejero et al. 2008). Considering the ecological and economic importance of Mediterranean junipers, the increasing fragmentation of their habitats is becoming a concern. The fact is that the fragmentation of semi-arid mountain woodlands was primarily natural and driven by an increased aridity and warmer climates during the Holocene (Quézel and Médail 2003; Carrión et al. 2004, 2010; Fady and Conord 2010). Nevertheless, it is currently being worryingly increased by human impact, such as wood removal and intensive pasture activity (Gauquelin et al. 1999; authors' field observations). The distribution of *Juniperus* across the semi-arid Mediterranean regions results from a compromise between altitude, where competition is avoided, and resources, which are scarcer as altitude increases and limits the reproductive efficiency (Montesinos et al. 2010). Global warming is increasing summer aridity, which is likely to increment pressure acting on junipers (Gauquelin et al. 1999, and references therein, for *J. excelsa* and *J. procera*; De Soto Suárez 2010, for *J. thurifera*).

The impact of habitat loss and fragmentation on species genetics and survival has mainly been observed over a short timescale since most cases available for direct observations, driven by anthropic pressure, are rather recent (e.g. *Araucaria nemorosa* De Laub., since 3,000 years ago; Kettle et al. 2011). The lack of temporal distance is making it difficult to draw inferences about future consequences of these processes. There is a current awareness of the need to widen the knowledge of biodiversity-shaping processes to the evolutionary timescale (e.g. Stigall 2010; Escarguel et al. 2011). In this sense, organisms allowing a larger time perspective could provide valuable data (e.g. Stigall 2010), which may be the case for *Juniperus*. The genus, which arose during the Eocene, experienced major diversification rates during the climatic changes of the Miocene onwards (Mao et al. 2010). Although preferring cold environments, their wide temperature tolerance (Gauquelin et al. 1999) and longevity (up to several hundred years; García and Zamora 2003) permitted juniper taxa to persist through time, and especially to overcome the Holocene warming. During this period, distribution ranges alternated contractions and expansions in function of climatic fluctuations and land usage, which have at some point favoured juniper steppe against pine forest (Riera Mora 2006) before becoming a threat (Gauquelin et al. 1999).

The present work focuses on Mediterranean junipers, with a special emphasis laid on *J. thurifera*, which belongs to *Juniperus* sect. *Sabina*, group IV (Mao et al. 2010). For comparative purposes, representatives of each section and infrasectional group reaching the western Mediterranean area were also considered. These are *J. sabina* L. (sect.

Sabina, group III), *J. phoenicea* L. (sect. *Sabina*, group V), *J. communis* L. (sect. *Juniperus*, 'blue seed cone' group) and *J. navicularis* Gand. along with *J. oxycedrus* L. (sect. *Juniperus*, 'red seed cone' group) (Mao et al. 2010). *Juniperus thurifera* is a medium-sized, 8–15 (20)m tall, dioecious tree (Farjon 2005; Adams 2011), whose range covers western Mediterranean regions, mostly the Iberian Peninsula and Northwest Africa (Jalas and Suominen 1973; Quézel and Pesson 1980; Quézel and Barbero 1981; Greuter et al. 1984; Amaral Franco 1986; Charco 2001; Farjon 2005; Romo and Boratyński 2005; Adams 2011). The species is found from the Western Alps, Corsica and the Aurès mountains in Algeria, to the East, to the Pyrenees, Cantabrian range, Central Iberian range and the Moroccan Atlas Mountains to the West. These areas represent Pleistocene refugia of Tertiary floras (Carrión 2002; Benito Garzón et al. 2007; Thompson 2005; Médail and Diadema 2009), mostly as referred to the Ibero-Moroccan region, and to a lesser extent Corsica (Comes 2004; Médail and Diadema 2009).

Juniperus thurifera is a polymorphic species for which two subspecific entities are recognized: subsp. *thurifera* and subsp. *africana* (Maire) Gauquelin, Idr. Hass. & P. Lebreton ex Romo & Borat., as well as several chemovarieties of doubtful taxonomic value (Romo and Boratyński 2007; Adams 2011). The existence of the two subspecies has been confirmed through biometrical examinations (Gauquelin et al. 1988; Romo and Boratyński 2007), the correlation between biochemical diversity and number of seeds per cone (Adams et al. 2003) and genetic markers (Jiménez et al. 2003; Terrab et al. 2008).

While a pool of genetic data is being constituted for Mediterranean *Juniperus* (Jiménez et al. 2003; Meloni et al. 2006; Michalczyk et al. 2006; Michalczyk 2008; Boratyński et al. 2009; Douaihy et al. 2011; Dzialuk et al. 2011), comparatively very limited data are available regarding genomic aspects. These are mostly restricted to chromosome counts, all taxa being determined as diploids based on $x=11$, with the exception of *J. sabina*, which presents both diploid and tetraploid cytotypes (Muratović et al. 2004; Siljak-Yakovlev et al. 2010). Genome size has been addressed in five Mediterranean species (*J. excelsa*, *J. intermedia* Schur, *J. oxycedrus*, *J. phoenicea*, *J. sabina*; Loureiro et al. 2007; Siljak-Yakovlev et al. 2010), and three other junipers (*J. conferta* Parl., *J. rigida* Siebold & Zucc. and *J. virginiana* L.; Hizume et al. 2001). Characterizing species from the genome size point of view is becoming essential as DNA content relates to morphology, ecology and phylogeny (Doležel et al. 2007), and its variation is thought to trigger taxonomic divergence (Kraaijeveld 2010). Additionally, genome size variants at intraspecific level have been shown to reflect, to a certain degree, the palaeovegetation history (e.g. Slovák et al. 2008) and also the more recent history (e.g. Pellicer et al. 2009).

In the present paper, we have established the karyological and genome size profiles of *J. thurifera* and other Mediterranean congeners, with the aims of investigating whether: (1) interspecific genome size variation may respond to biological factors; (2) the genomic characteristics reflect the subspecific differentiation defined on the bases of genetic, biochemical and morphological studies; and (3) the genome size patterns may respond to habitat fragmentation within *J. thurifera*.

Material and methods

Species studied and collection of material

Efforts to germinate seeds collected in the field were mostly unfruitful, which led us to use root tips from cultivated plants of known origin from the *Proyecto Forestal Ibérico* (a forest tree nursery; www.proyectoforestaliberico.es) for chromosome number determination. Eight taxa were considered (Table 1). For genome size assessments, localities of the 43 studied populations—corresponding to six species and eight infraspecific entities—are indicated in Table 2. *Juniperus thurifera* populations were sampled across its currently fragmented distribution range. The species has not been extensively planted, and its seeds do not have a high dispersion potential (Santos et al. 1999), which guarantees a high preservation of the natural geographic pattern.

Chromosome counts

Root tips were pretreated with 0.05 % aqueous colchicine for 3 h at room temperature or with 0.02 M 8-hydroxyquinoline for 7 h at 16 °C or with a mixture of both for 4–5 h at 17 °C. The root tips were fixed into absolute ethanol/chloroform/glacial acetic acid (6:3:1) for 48 h at 4 °C and transferred to 70 % ethanol and stored at 4 °C. Samples were hydrolyzed in 1 N HCl for 20 min at

60 °C and subsequently stained with Schiff's reagent during 30 min. Meristems were squashed on slides in a drop of 45 % acetic acid/glycerol (9:1) and covered with a coverslip. The best metaphase plates were photographed with a digital camera (AxioCam MRc5 Zeiss) mounted on a Zeiss Axioplan microscope, and images were analysed with Axio Vision Ac software version 4.2.

Genome size assessments

Five male and five female individuals were analysed for each dioecious population, and five individuals for each monoecious one. Samples were independently processed and measured twice. *Triticum aestivum* L. 'Chinese spring' (2C=30.9 pg; Marie and Brown 1993) was used as internal standard. Fresh leaf tissue was chopped in a Petri dish with a razor blade in 1,200 µl of LB01 lysis buffer (Doležel et al. 1989) supplemented with Triton X-100 (8 %v/v) and 100 µg/ml ribonuclease A (RNase A, Boehringer, Meylan, France), together with the chosen internal standard. The resulting nuclei suspension was filtered through a 70 µm pore-size nylon mesh and subsequently stained with 36 µl of propidium iodide (for a final concentration of 60 µg/ml; Sigma-Aldrich Química, Alcobendas, Spain), kept on ice for 5–10 min and measured in an Epics XL flow cytometer (Coulter Corporation), the instrument set up with the standard configuration described in Garnatje et al. (2004). Measurements were made at the *Centres Científics i Tecnològics de la Universitat de Barcelona*.

Demographic indicators for populations

The two parameters observed were the age distribution and sex ratio within the population, which have been roughly estimated during fieldwork. The health state of the populations was defined on the basis of whether they were (good) or were not (bad) showing plants of different ages, including young seedlings. Sex ratio estimates were based on the observation of at least 30 adult trees for each population.

Table 1 Chromosome numbers reported for *Juniperus* individuals from the *Proyecto Forestal Ibérico* forest tree nursery

Taxon	Provenance	Somatic chromosome number	Ploidy level	First report
<i>J. communis</i> var. <i>saxatilis</i>	Spain	2n=22	2x	–
<i>J. communis</i> var. <i>communis</i>	Spain	2n=22	2x	–
<i>J. oxycedrus</i> subsp. <i>badia</i>	Spain, Sistema Central	2n=22	2x	+
<i>J. oxycedrus</i> subsp. <i>oxycedrus</i>	Spain, Sistema Ibérico meridional	2n=22	2x	–
<i>J. phoenicea</i> subsp. <i>phoenicea</i>	Spain, Sistema Ibérico meridional	2n=22	2x	+
<i>J. phoenicea</i> subsp. <i>turbinata</i>	Spain, Murcia	2n=22	2x	+
<i>J. sabina</i>	Spain	2n=22	2x	–
<i>J. thurifera</i> subsp. <i>thurifera</i>	Spain, Sistema Ibérico meridional	2n=44	4x	+

Table 2 *Juniperus* populations studied

Taxon	Locality	Altitude (m)	Coordinates	2C (pg) ^a	IC (Mbp) ^b	HPCV ^c <i>Juniperus</i>	HPCV ^c standard	Sex ^d	State ^e
<i>J. communis</i> L. var. <i>saxatilis</i> Pall.	Spain, Catalonia: Barcelona, Montseny range, above Coll Formic. Romo, 13-04-2010 (BC)	1400	41°48'18.6"N 02°22'06.4"E	19.66±0.27♀ 19.84±0.36♂	9,613.74 9,701.76	3.58±0.72 3.55±1.09	0.35±0.20 1.05±0.97	MF	G
<i>J. communis</i> L. var. <i>saxatilis</i> Pall.	France, Hautes-Alpes: Col du Lautaret. Romo, 05-03-2010 (BC)	1930	45°02'13.1"N 06°23'59.7"E	19.38±0.18	9,476.82	3.94±0.61	1.20±0.68	MF	G
<i>J. communis</i> L. var. <i>saxatilis</i> Pall.	Andorra, Tristaina. Romo, 10-07-2010 (BC)	2270	42°37'49.4"N 01°29'24.1"E	19.37±0.78♀ 18.93±0.75♂	9,471.93 9,256.77	1.89±1.82 4.27±0.73	0.24±0.14 0.60±0.30	MF	G
<i>J. navicularis</i> Gand.	Portugal, Setúbal: Tróia, Sol Tróia. Hidalgo & Vilandrau, 03-09-2011 (BCN)	0	38°27'52.46"N 08°51'54.15"W	18.8 ⁺ ♀ 19.57±0.75 ⁺ ♂	9,193.20 9,569.73	3.16±0.47	2.79±0.37	M	G
<i>J. oxycedrus</i> L. subsp. <i>badia</i> (H. Gay) Debeaux	Spain, Huesca: Gabasa. Romo, 31-10-2010 (BC)	820	42°01'07"N 00°26'08.8"E	18.77±0.72♀ 17.94 ⁺ ♂	9,178.53 8,772.66	0.62±0.80 1.72±1.01	0.95±1.09 2.43±2.58	MF	G
<i>J. oxycedrus</i> L. subsp. <i>macrocarpa</i> (Sibth. et Sm.) Ball.	Italy, Sardinia: l'Alguer/Alghero. Romo, 10-05-2010 (BC)	20	40°34'26.4"N 08°18'40.1"E	19.36±0.61♀ 19.31±0.13♂	9,467.04 9,442.59	4.19±1.47 3.06±1.15	1.97±1.17 2.56±0.62	MF	B
<i>J. oxycedrus</i> L. subsp. <i>macrocarpa</i> (Sibth. et Sm.) Ball.	France, Corsica: Roccapina. Garmatje & Romo, 26-11-2010 (BC)	12	41°29'46.2"N 08°56'04.6"E	19.08±0.68♀ 18.94±0.46♂	9,330.12 9,261.66	3.35±1.57 3.35±3.16	2.65±0.79 2.72±1.47	MF	G
<i>J. oxycedrus</i> L. subsp. <i>macrocarpa</i> (Sibth. et Sm.) Ball.	France, Corsica: San Cipriani. Garmatje & Romo, 24-11-2010 (BC)	5	41°37'56.2"N 09°20'45.7"E	18.61±0.34♀ 19.31±0.20♂	9,100.29 9,442.59	4.00±1.24 2.87±0.68	2.55±0.96 3.11±1.95	MF	G
<i>J. oxycedrus</i> L. subsp. <i>macrocarpa</i> (Sibth. et Sm.) Ball.	France, Corsica: Ostriconi. Garmatje & Romo, 27-11-2010 (BC)	30	42°39'32.2"N 09°03'33.3"E	19.49±0.43♀♂ 19.84±0.39♀	9,530.61 9,701.76	5.47±1.24 3.76±2.19	3.32±1.91 2.43±1.43	MF	G
<i>J. oxycedrus</i> L. subsp. <i>oxycedrus</i>	Spain, Catalonia: Girona, el Port de la Selva. Romo, 04-03-2010 (BC)	25	42°20'48.6"N 03°13'05.0"E	19.70±0.61♀ 18.68±0.70♂	9,633.30 9,134.52	3.26±1.49 3.69±1.36	1.62±0.69 1.85±1.12	MF	G
<i>J. oxycedrus</i> L. subsp. <i>oxycedrus</i>	France, Corsica: Corsica. Garmatje & Romo, 24-11-2010 (BC)	673	42°20'50.1"N 09°03'05.5"E	20.16±0.28♀ 19.47±0.00♂	9,858.24 9,520.83	3.31±1.54 2.82±1.18	1.79±0.77 2.06±0.90	MF	G
<i>J. oxycedrus</i> L. subsp. <i>oxycedrus</i>	Spain, Catalonia: Girona, el Port de la Selva. Romo, 01-04-2010 (BC)	25	42°20'48.6"N 03°13'05.0"E	19.02±0.90♂	9,300.78	3.90±2.27	1.10±0.82	-	G
<i>J. oxycedrus</i> L. subsp. <i>oxycedrus</i>	Morocco, Middle Atlas: Imouzer des Mamoucha. Romo, 22-07-2010 (BC)	1220	33°50'32.0"N 04°21'09.9"E	20.41±0.51♀ 19.64±0.68♂	9,980.49 9,603.96	4.86±1.43 6.17±1.25	1.75±0.88 2.14±1.35	-	G
<i>J. oxycedrus</i> L. subsp. <i>oxycedrus</i>	Morocco, High Atlas: near Tlougguite. Romo, 20-07-2010 (BC)	914	32°07'00.6"N 06°21'09.9"E	20.19±1.16	9,872.91	3.56±2.69	1.17±1.11	MF	G
<i>J. phoenicea</i> L. subsp. <i>phoenicea</i>	Spain, Huesca: Candanos. Romo, 05-02-2010 (BC)	310	41°27'38.6"N 00°03'02.0"E	21.52±0.41♀ 21.54±0.29♂	10,523.28 10,533.06	3.30±0.88 3.31±0.59	0.85±0.76 0.70±0.41	MN	G
<i>J. phoenicea</i> L. subsp. <i>phoenicea</i>	Andorra: Sant Julià de Lòria. Romo, 06-07-2010 (BC)	1080	42°27'58.8"N 01°29'33.8"E	20.96±0.46♀ 20.98±0.41♂	10,249.44 10,259.22	2.14±0.38 2.25±0.72	0.52±0.39 0.54±0.14	MN	B
<i>J. phoenicea</i> L. subsp. <i>turbinata</i> (Guss.) Nyman	Italy, Sardinia: Caccia. Romo, 09-05-2010 (BC)	60	40°35'05.7"N 08°10'01.7"E	21.97±0.19♀ 21.96±0.29♂	10,743.33 10,738.44	3.42±0.69 3.14±0.59	1.28±0.52 1.70±0.63	MN	B

Table 2 (continued)

Taxon	Locality	Altitude (m)	Coordinates	2C (pg) ^a	IC (Mbp) ^b	HPCV ^c <i>Juniperus</i>	HPCV ^c standard	Sex ^d	State ^e
<i>J. phoenicea</i> L. subsp. <i>turbinata</i> (Guss.) Nyman	Morocco, High Atlas: Barrage Bin Ouidane, near Ait Mazig. Romo, 21-07-2010 (BC)	860	32°06'02.8"N 06°21'38.7"E	23.23±0.34♀ 23.12±0.51♂	11,359.47 11,305.68	3.14±0.37 2.96±0.14	1.17±0.56 1.78±0.42	MN	B
<i>J. phoenicea</i> L. subsp. <i>turbinata</i> (Guss.) Nyman	Spain, Huelva: Matalascañas, southern limit of Doñana National Park. Romo, 14-04-2010 (BC)	18	36°59'12.2"N 06°32'26.8"W	22.93±0.12	11,212.77	2.87±0.31	2.61±0.23	MN	G
<i>J. phoenicea</i> subsp. <i>turbinata</i> (Guss.) Nyman	Italy, Sardinia: Baltza. Romo, 07-05-2010 (BC)	40	40°40'32.1"N 08°13'34.5"E	21.45±0.59♀ 21.40±0.31♂	10,489.05 10,464.60	2.40±1.17 4.53±1.08	2.28±0.91 1.56±0.83	MN	B
<i>J. phoenicea</i> subsp. <i>turbinata</i> (Guss.) Nyman	France, Corsica: la Tour de Fautea. Garnatje & Romo, 25-11-2010 (BC)	3	41°42'58.00"N 09°24'05.7"E	21.32±0.73	10,425.48	4.12±1.66	2.65±1.48	MN	G
<i>J. phoenicea</i> subsp. <i>turbinata</i> (Guss.) Nyman	Portugal, Faro: Odeceixe, Praia da Odeceixe. Hidalgo & Vilandrau, 03-09-2011 (BCN)	0	38°28'12.63"N 08°52'59.95"O	21.39±0.3	10,459.71	3.48±0.52	2.48±0.5	MN	G
<i>J. sabina</i> L.	Spain, Palencia: Velilla de Guardo. Romo, 26-03-2010 (BC)	1190	42°50'29.4"N 04°49'54.2"W	21.11±0.46	10,322.79	3.64±0.46	1.20±0.91	MF	G
<i>J. sabina</i> L.	Spain, Teruel: near Abejuelas. Romo, 19-11-2010 (BC)	1200	39°55'13.7"N 00°53'02.2"W	20.99±0.81♀ 22.12±0.20♂	10,264.11 10,816.68	3.62±1.37 2.85±0.65	0.57±0.58 1.04±1.10	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁)	Spain, Zaragoza: Bujaraloz. Romo, 05-07-2010 (BC)	342	41°29'41.7"N 00°14'51.3"E	41.20±0.86 ⁺ ♀ 41.00±0.89 ⁺ ♂	20,146.80 20,049.00	1.09±0.81 0.90±0.59	0.90±0.43 0.59±0.41	M	B
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₂)	Spain, Palencia: Velilla de Guardo. Romo, 26-03-2010 (BC)	1200	42°50'29.2"N 04°49'54.9"W	41.84±0.69 ⁺ ♀ 41.83±0.41 ⁺ ♂	20,459.76 20,454.87	2.30±0.34 2.31±0.56	2.59±0.92 3.21±0.47	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₃)	Spain, Segovia: Prádena. Romo, 24-03-2010 (BC)	1160	41°07'28.9"N 03°42'42.0"W	41.50±1.10 ⁺ ♀ 41.78±0.38 ⁺ ♂	20,293.50 20,430.42	1.96±0.68 2.07±0.83	2.52±0.67 2.39±0.60	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₄)	Spain, Burgos: Covarubias. Romo, 25-03-2010 (BC)	1020	42°03'11.9"N 03°30'16.6"W	41.88±0.90 ⁺ ♀ 41.76±1.01 ⁺ ♂	20,479.32 20,420.64	2.14±0.66 2.18±0.49	2.39±0.52 1.84±0.65	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₅)	Spain, Catalonia: Lleida, Alins. Romo, 19-05-2010 (BC)	1140	42°33'08.8"N 01°18'47.2"W	41.28±0.89 ⁺ ♀ 41.18±0.40 ⁺ ♂	20,185.92 20,137.02	2.56±0.49 1.96±0.31	2.46±1.04 2.12±0.62	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₆)	France, Haute Garonne: Marignac Montagne de Ritè. Romo, 13-05-2010 (BC)	1130	42°53'17.5"N 00°39'27.9"W	40.80±0.51 ⁺ ♀ 41.00±0.77 ⁺ ♂	19,951.20 20,049.00	2.06±1.02 1.23±0.70	2.28±0.61 2.88±0.67	M	B
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₇)	France, Hautes Alpes: Saint Crépin. Romo, 07-03-2010 (BC)	1160	44°42'28.1"N 06°36'23.7"E	41.16±0.28 ⁺ ♀ 41.15±0.60 ⁺ ♂	20,127.24 20,122.35	1.68±0.68 2.20±0.75	2.58±0.72 2.35±0.67	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₈)	France, Ariège: Quié de Lujat, near Sensat. Romo & Vallès, 26-05-2010 (BC)	1010	42°49'19.5"N 10°40'01.4"W	41.31±0.24 ⁺ ♀ 40.98±0.11 ⁺ ♂	20,200.59 20,039.22	2.51±0.11 2.52±0.42	2.23±0.02 2.29±0.30	M	B
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₉)	Spain, Soria: Abejar. Romo & Vallès, 07-06-2010 (BC)	1180	41°47'39.1"N 02°45'59.7"E	40.26±0.01 ⁺ ♀ 39.90±1.36 ⁺ ♂	19,687.14 19,511.10	0.56±0.28 3.00±1.21	3.24±1.31 4.25±0.90	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁₀)	France, Corsica: near Corscia. Garnatje & Romo, 24-11-2010 (BC)	748	42°20'47.3"N 09°03'10.2"E	41.83±1.71 ⁺ ♀ 40.73±0.37 ⁺ ♂	20,454.87 19,916.97	1.84±1.07 1.91±1.35	1.06±0.83 2.27±0.97	M	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁₁)	Spain, Murcia: El Sabinar, near Nerpio. Romo, 28-10-2010 (BC)	1290	38°11'44.4"N 02°11'03.2"W	41.95±0.09 ⁺ ♀ 41.71±0.44 ⁺ ♂	20,513.55 20,396.19	2.58±1.40 3.29±0.57	2.86±0.61 3.46±0.62	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁₂)	Spain, Albacete: Ossa de Montiel. Romo, 28-10-2010 (BC)	966	38°54'09.7"N 02°39'24.1"W	39.84±0.71 ⁺ ♀ 41.22±0.48 ⁺ ♂	19,481.76 20,156.58	2.75±1.16 3.03±0.56	3.29±0.85 3.18±0.52	MF	G

Table 2 (continued)

Taxon	Locality	Altitude (m)	Coordinates	2C (pg) ^a	IC (Mbp) ^b	HPCV ^c <i>Juniperus</i>	HPCV ^c standard	Sex ^d	State ^e
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁₁₃)	Spain, Cuenca: Puerto del Rocho. Romo, 29-10-2010 (BC)	1091	39°53'36.5"N 01°56'29.7"W	40.02±0.08 ⁺ ♀ 40.64±0.31 ⁺ ♂	19,569.78 19,872.96	2.19±0.78 2.38±1.22	2.73±1.01 2.79±0.74	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁₁₄)	Spain, Valencia: el Hontanar. Romo, 29-10-2010 (BC)	1310	42°01'39.1"N 00°24'42.1"W	39.84±0.47 ⁺ ♀ 40.93±1.50 ⁺ ♂	19,481.76 20,014.77	2.31±1.11 1.64±1.14	2.50±0.37 1.90±0.72	MF	G
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁₁₅)	Spain, Castelló: Penyalgosa, near Vistabella del Maestrat. Romo, 18-11-2010 (BC)	1177	40°18'23.9"N 00°20'13.8"W	41.71±0.55 ⁺ ♀ 42.65±0.40 ⁺ ♂	20,396.19 20,855.85	2.18±1.00 1.74±1.19	1.32±0.68 1.32±0.49	M	B
<i>J. thurifera</i> L. subsp. <i>thurifera</i> (J ₁₁₆)	Spain, Teruel: Javalambre, near la Nava de Torrijos. Romo, 19-11-2010 (BC)	1407	40°00'06.3"N 00°58'36.6"W	41.01±2.12 ⁺ ♀ 41.20±0.71 ⁺ ♂	20,053.89 20,146.80	1.76±0.48 1.97±0.97	0.89±0.60 1.53±0.70	MF	B
<i>J. thurifera</i> L. subsp. <i>africana</i> (Maire) Romo & Borat. (J ₁₁₇)	Morocco, Middle Atlas: near Tizi Bou Zabel. Romo, 24-07-2010 (BC)	2200	33°32'53.0"N 04°08'57.7"W	41.62±0.77 ⁺	20,352.18	1.62±0.83	3.96±0.85	M	B
<i>J. thurifera</i> L. subsp. <i>africana</i> (Maire) Romo & Borat. (J ₁₁₈)	Morocco, Middle Atlas: Bou Iblane range, above Talzent. Romo, 25-07-2010 (BC)	1920	33°38'01.3"N 04°10'55.1"W	42.01±1.08 ⁺	20,542.89	2.61±0.91	2.18±2.05	M	B
<i>J. thurifera</i> L. subsp. <i>africana</i> (Maire) Romo & Borat. (J ₁₁₉)	Morocco, High Atlas: above Zaouïa Ahansal. Romo, 26-07-2010 (BC)	1900	31°57'28.9"N 06°07'13.0"W	41.29±0.66 ⁺	20,190.81	1.89±1.24	0.46±0.23	M	B

Localities, nuclear genome sizes and coefficients of variation

^a Amount of nuclear DNA (2C value±standard deviation); the first reports are marked with a cross (†)

^b Amount of nuclear DNA; 1 pg=978 Mbp (Doležel et al. 2003)

^c Half peak coefficient variation for the studied plant and standard

^d Sex: dioecy with sex ratio 1/1 (MF), dioecy with male-biased sex ratio (M), monoecy (MN)

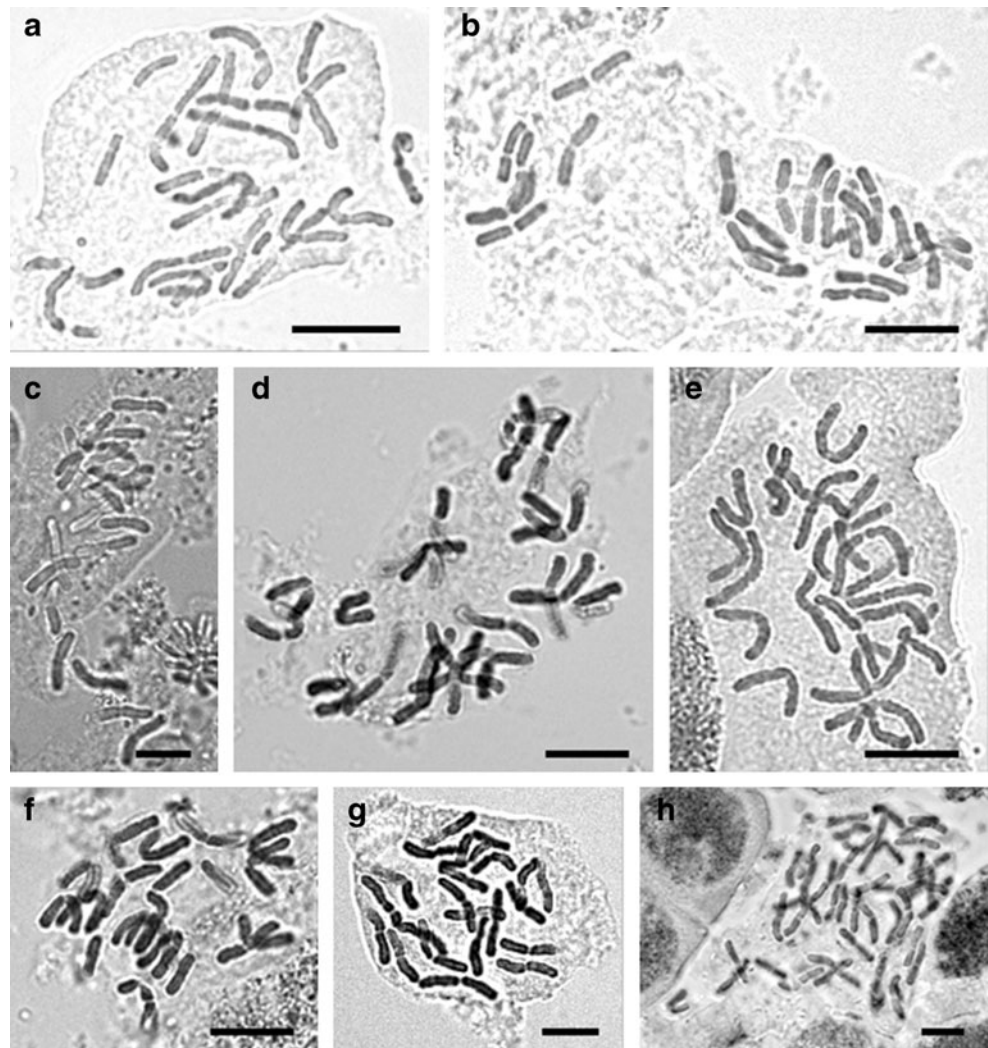
^e State: good (G), bad (B)

^f Only one individual was measured

Statistical analyses

Shapiro–Wilk was used to test the data normality. One-way ANOVA was carried out to test the genome size variation with sex (male and female) between taxa. The monoecious populations were excluded from the analysis. Because both subspecies and varieties are represented in the *Juniperus* studied, comparison of genome size at infraspecific level was restricted to the species whose infraspecific entities were within the same taxonomic category. However, for *J. thurifera*, there were too few populations of subsp. *africana* to allow comparison with subsp. *thurifera*. Differences in genome size among both specific and infraspecific taxa were tested and illustrated with box and whisker plots. A least significant difference (LSD) test was carried out to compare the means. A dendrogram, based on the Euclidean distance and the Ward agglomeration method, including all *J. thurifera* populations, was constructed. Statistical analyses and graphics were elaborated with STATA 10.0 (Stata Corp. Texas, USA) and XLSTAT (Addinsoft NY, USA).

Fig. 1 Somatic metaphase plates. **a** *J. communis* var. *communis*, $2n=2x=22$. **b** *J. communis* var. *saxatilis*, $2n=2x=22$. **c** *J. oxycedrus* subsp. *badia*, $2n=2x=22$. **d** *J. oxycedrus* subsp. *oxycedrus*, $2n=2x=22$. **e** *J. phoenicea* subsp. *phoenicea*, $2n=2x=22$. **f** *J. phoenicea* subsp. *turbinata*, $2n=2x=22$. **g** *J. sabina*, $2n=2x=22$. **h** *J. thurifera* subsp. *thurifera*, $2n=4x=44$. Scale bar=10 μm



Results

Chromosome numbers and ploidy level determination

Chromosome counts indicate diploid cytotypes with $2n=22$ for all taxa, with the exception of *J. thurifera* subsp. *thurifera*, which presents a tetraploid cytotype ($2n=44$, Table 1, Fig. 1). These results are the first counts for *J. oxycedrus* subsp. *badia* (H. Gay) Debeaux, *J. phoenicea* subsp. *phoenicea*, *J. phoenicea* subsp. *turbinata* (Guss.) Nyman ($2n=2x=22$) and for *J. thurifera* subsp. *thurifera* ($2n=4x=44$). All *J. thurifera* populations considered for genome size measurements, including subsp. *africana*, have approximately twice as much DNA amount as analysed congeneric species (Table 2, Fig. 2), this suggesting that tetraploidy probably extends to the whole species.

Genome size quality and data distribution

The global mean in percentage of HPCV was 2.76 ± 1.05 for the target plant and 1.95 ± 0.92 for the standard, indicating

methodological reliability. The Shapiro–Wilk test applied to diploid and tetraploid taxa separately showed that 2C values follow a normal distribution, allowing the use of parametric tests in statistical analyses. There were no significant differences in 1Cx between sexes, which led us to group the genome size measurements of male and female individuals from each population in further analyses.

Genome size and taxa

One-way ANOVA revealed a significant difference in 1Cx amongst the whole set of taxa, although no significant difference was found between *J. communis* and *J. oxycedrus*, and between *J. phoenicea* and *J. sabina*.

Within *J. oxycedrus*, one-way ANOVA shows significant 1Cx differences ($P=0.015$), which discriminates after the LSD test *J. oxycedrus* subsp. *badia* from the other subspecies: subsp. *macrocarpa* (Sibth. et Sm.) Ball. ($P=0.046$) and subsp. *oxycedrus* ($P=0.007$). Within *J. phoenicea*, no significant differences were found between 1Cx of subsp. *phoenicea* and subsp. *turbinata* ($P=0.077$). Except for *J. thurifera*, population sampling was not representative of the species' whole distributions, and the results should be considered as preliminary.

Comparison of *J. thurifera* genome size throughout its geographical range

Genome size values assessed for the populations of *J. thurifera* are very homogenous, from 2C of 40.81 pg (J_{t15}) to 43.2 pg (J_{t13}), representing a 1.06-fold variation. However, significant differences ($P<0.0001$) were found amongst them. Comparison of genome size and genetic structure patterns for populations represented in both Terrab et al. (2008) and the present study do not evidence overlap (Fig. 3a). However, in both cases, the maximum variability is found in Spain (Fig. 3). Genome size of *J. thurifera* populations does not follow a health-related ($P=0.155$) or a sex ratio-related distribution ($P=0.442$; Table 2). In turn, 87.5 % of age-biased populations (lacking young individuals) show an imbalanced sex ratio (lacking females), which is around ten times less frequent in populations of a normal age structure (9.09 %; Table 2).

Discussion

Genome size serves to adequately estimate ploidy level in *Juniperus*

In all the juniper species in which we determined the chromosome number, direct count was consistent with the one expected given the genome size value (Fig. 2). This suggests

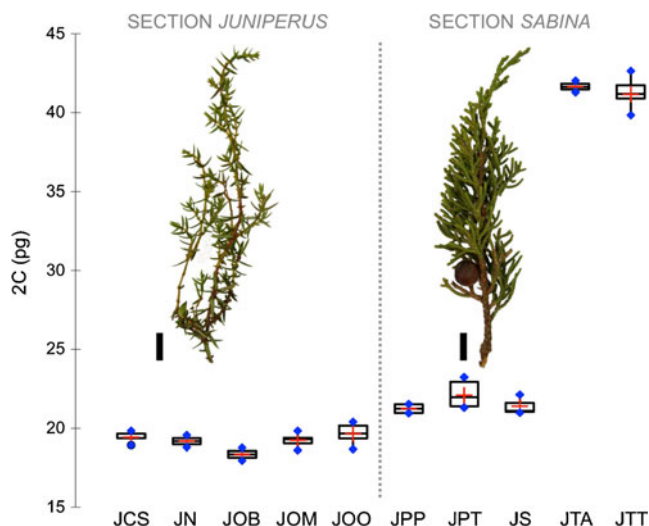


Fig. 2 Box and whisker plots of the genome size in studied juniper species and subspecies, with indication of sectional assignment. *JCS* *J. communis* var. *saxatilis*, *JN* *J. navicularis*, *JOB* *J. oxycedrus* subsp. *badia*, *JOM* *J. oxycedrus* subsp. *macrocarpa*, *JOO* *J. oxycedrus* subsp. *oxycedrus*, *JPP* *J. phoenicea* subsp. *phoenicea*, *JPT* *J. phoenicea* subsp. *turbinata*, *JS* *J. sabina*, *JTA* *J. thurifera* subsp. *africana*, *JTT* *J. thurifera* subsp. *thurifera*. At left, a branch of *J. navicularis* illustrates the acicular-leaved section *Juniperus*, at right, *J. phoenicea* for the scale-leaved section *Sabina*. Scale bars=1 cm

that estimation of the ploidy level through the flow cytometry technique could be an alternative to direct counting within the genus, which is limited by the availability of cultivated material of known wild origin, and the overall low germination rate of junipers.

Adding one more case of polyploidy in conifers

Our result concerning *J. thurifera* represents the first report of an exclusively polyploid species in the genus. Natural tetraploid cytotypes were previously found in rare occurrences in the mostly diploid species *J. chinensis* L. (*J. chinensis* ‘Pfitzeriana’; Sax and Sax 1933) and *J. sabina* (Muratović et al. 2004; Siljak-Yakovlev et al. 2010). Sporadically-occurring triploid and tetraploid cytotypes of *J. chinensis*, *J. sabina*, *J. squamata* Buch.-Ham. (*J. squamata* var. *meyeri* Rehder; Jensen and Levan 1941) and *J. virginiana* were preserved in nursery culture (Hall et al. 1973). Polyploidy, which is frequent in ferns, allied plants (95 % of species; Grant 1981) and angiosperms (70–80 % of species; Masterson 1994; Soltis et al. 2009, and references therein), is in turn rare or even absent from gymnosperm lineages (around 5 % of species; Khoshoo 1959; reviewed by Ahuja 2005). Apart from the three junipers mentioned above, natural polyploids are indeed only known otherwise in gymnosperms from *Encephalartos hildebrandtii* A.Br. & Bouché, triploid (Abraham and Mathew 1966), *Ephedra* L. (in 50 % of the species), *Fitzroya cupressoides* I.M.Johnst.,

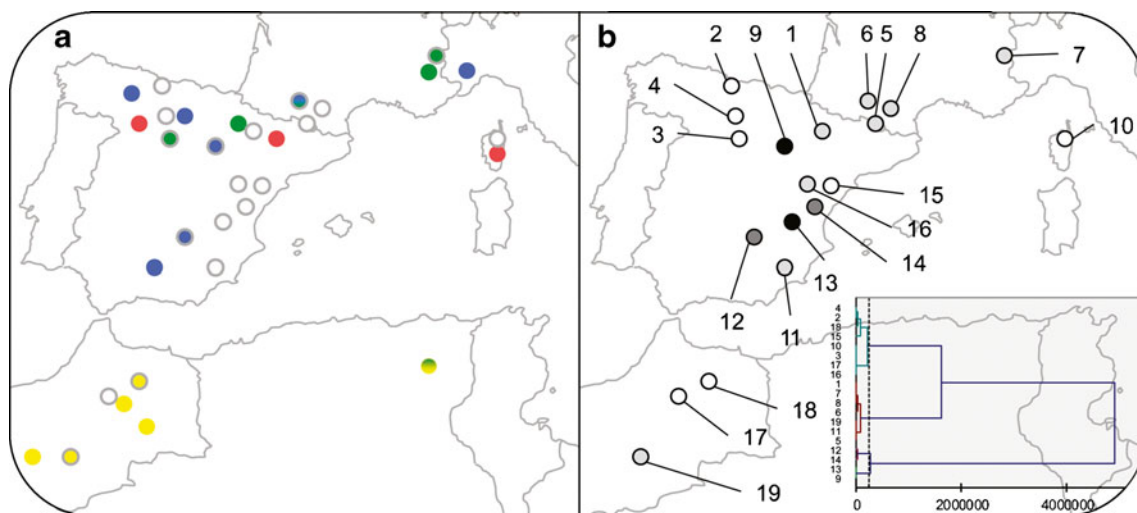


Fig. 3 Distribution of populations of *J. thurifera* studied. **a** Populations from the present study (circles with grey line) and those of Terrab et al. (2008) (colour-filled circles) corresponding to genetic clusters: I (yellow), II (blue), III (green) and IV (red). **b** Populations from the present study. Genome size clusters resulting from Ward agglomeration

method (see dendrogram) are represented by different grey intensities on the map. White: $2C > 41,343.88$ Mbp, light grey: $41,343.88 \geq 2C > 40,625.600$ Mbp, dark grey: $40,625.600 \geq 2C > 40,079.200$ Mbp, black: $2C \leq 40,079.200$ Mbp

tetraploid, and *Sequoia sempervirens* Endl., hexaploid (Ahuja 2005; Ahuja and Neale 2005; and references therein). Therefore, *Juniperus* stands out for grouping three cases of natural polyploids, the most in any gymnosperm genus after *Ephedra*.

Due to the extreme rarity of polyploidy in gymnosperms, and the phylogenetic relevance of this plant group as the most ancient seed plants (Ohri and Khoshoo 1986), much attention has been paid to deciphering the origin of genome duplication in the few species concerned (Ahuja 2005, and references therein). In this sense, the *J. chinensis* tetraploid cytotype was shown to arise from the hybridization between *J. chinensis* and *J. sabina* (De Luc et al. 1999). No such data are available for *J. sabina* and *J. thurifera*. The only statement that may be made so far is the independent occurrence of the polyploidization events in junipers. *Juniperus chinensis* and *J. sabina* comprise both tetraploid and diploid cytotypes, this meaning that the genome duplication events happened within both species. Since the 19 populations of *J. thurifera*, measured throughout its total distribution, are tetraploid, polyploidy certainly happened early in the history of the species. The presence of diploid cytotypes in early- and late-diverging members of sect. *Sabina* group IV, to which *J. thurifera* belongs (respectively in *J. procera*, and *J. chinensis* plus *J. procumbens* Siebold), indicates that diploid cytotypes should be inferred at inner branches of this group (Mehra and Khoshoo 1956; Hall et al. 1973; Nagano et al. 2000 for chromosome counts; Mao et al. 2010, for phylogenetic framework). Taken together, this locates the genome duplication as concomitant with *J. thurifera* speciation, making the species the only one known in the genus to have undergone speciation through

polyploidisation. Karyotype homogeneity—common in gymnosperms—impedes to determinate whether autopolyploidy or hybridization is involved in the formation of this tetraploid. The use of molecular cytogenetic techniques could shed light on this subject as they did in genera *Cedrus* Mill. and *Pinus* (Bou Dagher-Kharrat et al. 2001; Bogunic et al. 2011).

Strong homogeneity of genome size within *Juniperus*

This study contributes genome size values for six *Juniperus* species representing eight subspecific entities, and providing the first data for *J. navicularis*, *J. thurifera* subsp. *africana* and *J. thurifera* subsp. *thurifera*. Along with the previous reports (Hizume et al. 2001; Loureiro et al. 2007; Siljak-Yakovlev et al. 2010), our data account for a 1.7-fold variation of $2C$ within *Juniperus*, with the lowest value found in *J. navicularis* ($2C=19.18$ pg) and the highest in *J. thurifera* ($2C=41.23$ pg), and a 1.27-fold variation of $1Cx$, with the lowest value found in *J. navicularis* ($1Cx=9.59$ pg) and the highest in *J. rigida* ($1Cx=12.15$ pg).

Although our results extend the range of $2C$ values of *Juniperus* at both lower and upper limits, *Juniperus* still fits well with the overall homogeneity of gymnosperms' genome size (Ohri and Khoshoo 1986; Leitch et al. 2001), as its range is still below the 2.17-fold variation found between *Pinus* species, all of them being diploid (Grotkopp et al. 2004). Adaptive response to habitats has been evoked to explain high genome size values and its variation in *Pinus* (Wakamiya et al. 1993). Bogunic et al. (2007) reported a very narrow C -value range amongst five subspecies of *Pinus nigra* Arnold s.l., and concluded that infraspecific

diversification occurred without change in genome size. Beside differences in habitats, *Juniperus* displays a reduced genome size range suggesting that environmental factors such as temperature, precipitation, etc. did not impact *C*-value in the taxa considered.

Previous analyses of gymnosperm chromosomes have revealed a number of unusual features that differentiate them from the remaining seed plants (reviewed by Murray 1998). Amongst them, large and relatively few-in-number chromosomes (producing high linkage disequilibrium, and, as a consequence, genetic hitchhiking), karyotypes of remarkably uniform structure, and, with few exceptions, no polyploidy, and a constant basic chromosome number for families. It has been argued that this low karyological dynamism could in part explain why gymnosperms were unable to diversify as much as angiosperms did (Gorelick and Olson 2011).

Genome size and leaf type

Mediterranean species with squamiform (scaled) leaves from our study were found to have significantly higher *1Cx* values than species with needle-like (acicular) leaves ($P=0.006$; Fig. 2). However, this result should be taken with caution because only six species were considered.

Deciphering whether genome size difference might be reflecting phylogeny rather than leaf shape is necessary since *Juniperus* sections are based on leaf form character. Accordingly, acicular leaves are found in sect. *Juniperus* and its sister group sect. *Caryocedrus*, whereas sect. *Sabina* representatives are scale-leaved, with very few exceptions (*J. coxii* A.B.Jacks., *J. morrisonicola* Hayata, *J. recurva* Buch.-Ham. ex D.Don, *J. saxicola* Britton & P.Wilson, *J. squamata*, and sporadic individuals of *J. barbadensis* L. and other juniper species; Adams et al. 2008; Adams 2011). Acicular-leaved juvenile configuration is the rule in Cupressaceae, while its persistence in the adult plant, restricted to some junipers, is exceptional within the family (Little et al. 2004) and may illustrate cases of neoteny (e.g. *J. saxicola*; Adams 2011). In animals, neoteny has been related to increased genome size (Gregory 2002), but no such research has been done for plants, although it is well recognized that neoteny paid an important role in their evolution (Li and Johnston 2000, and references therein).

The fact that tetraploids—natural or not—exclusively occur in section *Sabina* (*J. chinensis*, *J. sabina*, *J. squamata*, *J. thurifera*, *J. virginiana*; see above), that is to say, are restricted to scale-leaved species, would also deserve more in-depth research.

Genome size and minimum seed-bearing age

A positive correlation between age of sexual maturity and genome size has been observed in *Pinus* (Wakamiya et al.

1993). The very few data available for *Juniperus* species show the minimum seed-bearing age not related to genome size, leaf type/section, but more likely to climatic conditions. The seed-bearing age of the Mediterranean species with acicular leaves, *J. communis*, is 17–25 years (Iszkulo and Boratyński 2011) and that of the also acicular *J. oxycedrus* is 17–21 years (Baldoni et al. 2004), whereas scale-leaved *J. thurifera* reaches sexual maturity at a mean age of 31, according to Montesinos et al. (2006). Since non-Mediterranean members of section *Sabina* reach sexual maturity much earlier, from the age of 10 (*J. virginiana*) or from the age of 12 years (*J. monosperma* Sarg., *J. pinchotii* Sudw., *J. scopulorum* Sarg.; Coder 2008), a possible interpretation could be that late offspring might be climate-constrained (delayed in unfavourable xeric conditions). Following this assumption, late offspring would have been reached independently by *J. thurifera* (together with polyploidy and increased genome size?) and by *J. communis* and *J. oxycedrus* (through a slower developmental rate that has, inter alia, given rise to the neotenic acicular leaves?). The pattern of low genome sizes found in Mediterranean *J. communis* and *J. oxycedrus* compared to non-Mediterranean species reminds the negative correlation between aridity and genome size previously established for pines, all diploids (Wakamiya et al. 1993).

Genome size and sex allocation

Contrariwise to those dioecious species in which small differences in DNA amount were found between males and females [*Manoao colensoi* (Hook) Molloy; Davies et al. 1997; possibly *Ginkgo biloba* L.; Murray 1998], no such trend is detected for dioecious junipers ($P=0.840$). Genome size difference between sexes may be indicative of sex chromosomes (e.g. in *Ginkgo biloba*) that have been reported in a number of gymnosperms (Murray 1998 and references therein), but not in *Juniperus*.

Genome size and subspecific differentiation

No difference of genome size has been found between the well-differentiated *J. thurifera* subsp. *africana* and subsp. *thurifera*, from which the former underwent a morphological (Maire 1926; Gauquelin 1988; Romo and Boratyński 2007), chemical (Adams et al. 2003) and genetic (Jiménez et al. 2003; Terrab et al. 2008) divergence. Subspecific differentiation within *J. thurifera* resulted from the last opening of the Gibraltar Strait c. 5.33 Mya ago (Terrab et al. 2008). The Gibraltar Strait acted as an effective barrier against gene flow between Iberian and Moroccan populations for many species, allowing new species and even new lineages to arise in a number of plant groups (reviewed by Rodríguez-Sánchez et al. 2008; Jaramillo-Correa et al. 2010). Only

subspecies were formed in the case of *J. thurifera*, although vicariant populations were genetically isolated since the last deglaciation started (Terrab et al. 2008), stating for a comparatively lower taxonomic divergence in *J. thurifera* than for other plant groups. Furthermore, a certain degree of genome size heterogeneity indicative of ongoing incipient speciation would have been expectable, but it was not found. This recalls the above-discussed low diversification rate of the gymnosperms, associated to their low karyological dynamism. A gene flow increase has been proposed to counterbalance the effects of population fragmentation in wind-pollinated plants with limited seed dispersal such as *Juniperus*, but testing this hypothesis has shown controversial results (Provan et al. 2008).

However, it has been evidenced that plants having experienced recent polyploidy diversify at a lower rate (Mayrose et al. 2011), and the fact that *J. thurifera* experienced a genome duplication concomitant to its arisal might also account for the comparatively low taxonomic divergence tendency observed within this species.

Genome size and habitat fragmentation

The knowledge of ecological traits and population dynamics of a species is considered to be crucial for predicting the effects of habitat fragmentation (Lindenmayer and Fischer 2006, and references therein). This first study of genome size in a species affected by habitat fragmentation, *J. thurifera*, reveals that *C*-values are not influenced by this factor, at least for the moment, in the same way that genetic diversity within populations remains high (Terrab et al. 2008). *Juniperus thurifera* populations are unfortunately good candidates to pay an extinction “debt” (Lindenmayer and Fischer 2006, and references therein), and their response to the landscape changes, although having possibly been delayed (due to the huge longevity of *Juniperus* trees), is already well visible through a very low regeneration rate and a deeply imbalanced sex ratio. Habitat fragmentation was shown to have altered the genetic range-wide structure of *J. thurifera*, with inter-population divergence higher than many other conifers, indicating a low connectivity between surveyed populations (Terrab et al. 2008). Equally worrying are the effects on population demography, especially with regard to the age and sex structure. Many populations are lacking seedlings and young individuals, pointing to an extremely low fertility (Montesinos et al. 2010). Furthermore, since most age-biased populations also show an imbalanced sex ratio and the scarcity of females reduces the overall seed production, the two factors probably aggravate each other. A critical point for regeneration might be reached, a situation that adult longevity may buffer temporarily (Montesinos et al. 2010). However, the fact that populations are not yet affected at genetic and genomic levels gives hope that recovery might

be possible if measures for encouraging a successful reproduction are undertaken soon.

Acknowledgments The authors thank MÁ Canela for his advice on statistics, SC Brown and O Catrice for supplying *Petunia hybrida* and *Pisum sativum* used as internal standards; J Comas, R Álvarez and R López for technical support in flow cytometry; M Veny for keeping the collections of living plants and S Vilandrau for help in collecting material in the field. We thank S Pyke for improving the English of the text and three anonymous reviewers for their valuable comments and suggestions. The collaboration of the *Proyecto Forestal Ibérico* forest tree nursery is also acknowledged. This work was subsidized by the *Dirección General de Enseñanza Superior*, Spain (project PB 97/1134), *Ministerio de Ciencia e Innovación*, Spain (projects CGL2010-22234-C02-01 and 02 / BOS) and the *Generalitat de Catalunya*, Catalonia (*Ajuts a grups de recerca consolidats* 2005/SGR/00344 and 2009/SGR/00439). AR was funded by “Proyectos Intramurales de Incorporación del Consejo Superior de Investigaciones Científicas: project 20099301161”. OH received a *Juan de la Cierva* postdoctoral grant from the *Ministerio de Ciencia e Innovación*.

References

- Abraham A, Mathew PM (1966) Cytology of *Encephalartos hildebrandtii* A.Br. & Bouche. *Ann Bot* 30:239–241
- Adams RP (2011) *Junipers of the world: the genus Juniperus*, 3rd edn. Trafford Publishing, Bloomington
- Adams RP, Mumba LE, James SA, Pandey RN, Gauquelin T, Badri W (2003) Geographic variation in the leaf essential oils and DNA fingerprints (RAPDs) of *Juniperus thurifera* L. from Morocco and Europe. *J Ess Oil Res* 15:148–154
- Adams RP, Morris J, Schwarzbach AE (2008) The evolution of Caribbean *Juniperus* (Cupressaceae): terpenoids, RAPDs and DNA SNPs data. *Phytologia* 90:103–120
- Ahuja MR (2005) Polyploidy in gymnosperms: revisited. *Silvae Genet* 54:59–69
- Ahuja MR, Neale DB (2005) Evolution of genome size in conifers. *Silvae Genet* 54:126–137
- Amaral Franco J do (1986) *Juniperus* L. In: Castroviejo S, Lainz M, López González G, Montserrat P, Muñoz Garmendia F, Paiva J, Villar L (eds) *Flora Iberica*, vol 1. Real Jardín Botánico, CSIC, Madrid, p 181
- Baldoni M, Biondi E, Ferrante L (2004) Demographic and spatial analysis of a population of *Juniperus oxycedrus* L. in an abandoned grassland. *Plant Biosyst* 138:89–100
- Benito Garzón M, Sánchez de Dios R, Sáinz Ollero H (2007) Predictive modelling of tree species distribution on the Iberian Peninsula during Last Glacial Maximum and Mid-Holocene. *Ecography* 30:120–134
- Bogunic F, Muratovic E, Ballian D, Siljak-Yakovlev S, Brown S (2007) Genome size stability among five subspecies of *Pinus nigra* Arnold s.l. *Environm Exper Bot* 59:354–360
- Bogunic F, Siljak-Yakovlev S, Muratovic E, Ballian D (2011) Different karyotype patterns among allopatric *Pinus nigra* (Pinaceae) populations revealed by molecular cytogenetics. *Plant Biol* 13:194–200
- Boratyński A, Lewandowski A, Boratyńska K, Montserrat JM, Romo A (2009) High level of genetic differentiation of *Juniperus phoenicea* (Cupressaceae) in the Mediterranean region: geographic implications. *Plant Syst Evol* 277:163–172
- Bou Dagher-Kharrat M, Grenier G, Bariteau M, Brown S, Siljak-Yakovlev S, Saviouré A (2001) Karyotype analysis reveals

- interspecific differentiation in the genus *Cedrus* despite genome size and base composition constancy. *Theor Appl Genet* 103:846–854
- Carrion JS (2002) Patterns and processes of Late Quaternary environmental change in a montane region of southwestern Europe. *Quaternary Sci Rev* 21:2047–2066
- Carrion JS, Yll EI, Willis KJ, Sánchez P (2004) Holocene forest history of the eastern plateaux in the Segura Mountains (Murcia, south-eastern Spain). *Rev Paleobot Palynol* 132:219–236
- Carrion JS, Fernández S, Jiménez-Moreno G, Fauquette S, Gil-Romera G, González-Sampériz G, Finlayson C (2010) The historical origins of aridity and vegetation degradation in southeastern Spain. *J Arid Environ* 74:731–736
- Charco J (2001) Guía de los árboles y arbustos del Norte de África. Agencia Española de Cooperación Internacional, Madrid
- Ciesla WM (2002) Juniper forests—a special challenge for sustainable industry. *For Trees Livel* 12:195–207
- Coder KD (2008) Tree sex: gender and reproductive strategies. *Tree Selection Series*. Warnell School of Forestry and Natural Resources, University of Georgia, Athens
- Comes HP (2004) The Mediterranean region—a hotspot for plant biogeographic research. *New Phytol* 164:11–14
- Davies BJ, O'Brien IEW, Murray BG (1997) Karyotypes, chromosome bands and genome size variation in New Zealand endemic gymnosperms. *Plant Syst Evol* 208:169–185
- De Luc A, Adams RA, Zhang M (1999) Using random amplification of polymorphic DNA for taxonomic evaluation of Pfister *Juniperus*. *Hort Sci* 34:1123–1125
- De Soto Suárez L (2010) Efecto del cambio global sobre la especie dioica *Juniperus thurifera* en la Península Ibérica. Dissertation, Universidad de Valladolid-INIA
- Doležel J, Binarová P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. *Biol Plantarum* 31:113–120
- Doležel J, Bartos J, Voglmayr H, Greilhuber J (2003) Nuclear DNA content and genome size of trout and human. *Cytometry* 51:127–128
- Doležel J, Greilhuber J, Suda J (2007) Flow cytometry with plant cells. Analysis of genes, chromosomes and genomes. Wiley, Weinheim
- Douaihy B, Vendramin GG, Boratyński A, Machon N, Bou Dagher-Kharrat M (2011) High genetic diversity with moderate differentiation in *Juniperus excelsa* from Lebanon and the eastern Mediterranean region. *AoB PLANTS* plr003. doi:10.1093/aobpla/plr003
- Dzialuk A, Mazur M, Boratyńska K, Montserrat JM, Romo A, Boratyński A (2011) Population genetic structure of *Juniperus phoenicea* (Cupressaceae) in the western Mediterranean Basin: gradient of diversity on a broad geographical scale. *Ann For Sci* 68:1341–1350
- Escarguel G, Fara E, Brayard A, Legendre S (2011) Biodiversity is not (and never has been) a bed of roses! *C R Biol* 334:351–359
- Fady B, Conord C (2010) Macroecological patterns of species and genetic diversity in vascular plants of the Mediterranean basin. *Divers Distrib* 16:53–64
- Farjon A (2005) A monograph of Cupressaceae and Sciadopitys. Royal Botanic Gardens, Kew
- Farjon A (2010) A handbook of the world's conifers, vol 1–2. Brill, Leiden-Boston
- García D, Zamora R (2003) Persistence, multiple demographic strategies and conservation in long-lived Mediterranean plants. *J Veg Sci* 14:921–926
- Garnatje T, Vallès J, García S, Hidalgo O, Sanz M, Canela MÁ, Siljak-Yakovlev S (2004) Genome size in *Echinops* L. and related genera (Asteraceae, Cardueae): karyological, ecological and phylogenetic implications. *Biol Cell* 96:117–124
- Gauquelin T (1988) Dynamique de la végétation et des formations superficielles dans les montagnes du bassin occidental de la Méditerranée : étude des formations à genévrier thurifère et à xérophytes épineuses en coussinet des atlas marocains. Dissertation, Université Paul Sabatier, Toulouse
- Gauquelin T (2006) Les Genévriers du monde et du bassin occidental de la Méditerranée : diversité, stabilité, expansion, régression. I; Actas del III° coloquio internacional sobre sabinas y enebrales, Soria 24–26 de Mayo de 2006; Junta de Castilla y León; Ponencia inaugural, 25–32
- Gauquelin T, Idrissi Hassani M, Lebreton P (1988) Le genévrier thurifère, *Juniperus thurifera* L. (Cupressaceae): analyse biométrique et biochimique; propositions systématiques. *Ecol Med* 14:31–42
- Gauquelin T, Bertaudière V, Montes N, Badri W, Asmode J-F (1999) Endangered stands of thuriferous juniper in the western Mediterranean basin: ecological status, conservation and management. *Biodiver Conserv* 8:1479–1498
- González-Tejero MR, Casares-Porcel M, Sánchez-Rojas CP, Ramiro-Gutiérrez JM, Molero-Mesa J, Pieroni A, Giusti ME, Censori E, de Pasquale C, Della A, Paraskeva-Hadjichambi D, Hadjichambis A, Houmani Z, El-Demerdash M, El-Zayat M, Hmamouchi M, ElJohrig S (2008) Medicinal plants in the Mediterranean area: synthesis of the results of the project Rubia. *J Ethnopharmacol* 116:341–357
- Gorelick R, Olson K (2011) Is lack of cycad (Cycadales) diversity a result of a lack of polyploidy? *Bot J Linn Soc* 165:156–167
- Grant V (1981) Plant speciation, 2nd edn. Columbia University Press, New York
- Gregory TR (2002) Genome size and developmental complexity. *Genetica* 115:13–146
- Greuter W, Burdet HM, Long G (1984) Med-Checklist, vol 1. Botanischer Garten and Botanisches Museum, Berlin-Dahlem
- Grotkopp E, Rejmanek M, Sanderson MJ, Rost TL (2004) Evolution of genome size in pines (*Pinus*) and its life-history correlates: super-tree analysis. *Evolution* 58:1705–1729
- Hall MT, Mukherjee A, Crowley WR (1973) Chromosome counts in cultivated junipers. *J Arnold Arbor* 54:369–376
- Hizume M, Kondo T, Shibata F, Ishizuka R (2001) Flow cytometric determination of genome size in the Taxodiaceae, Cupressaceae sensu stricto and Sciadopityaceae. *Cytologia* 66:307–311
- Iszkulo G, Boratyński A (2011) Initial period of sexual maturity determines the greater growth rate of male over female in the dioecious tree *Juniperus communis* subsp. *communis*. *Acta Oecol* 37:99–102
- Jalas J, Suominen J (1973) Atlas Florae Europaeae. The Committee for Mapping the Flora of Europe and Societatis Biologica Fennica Vanamo, Helsinki
- Jaramillo-Correa JP, Grivet D, Terrab A, Kurt Y, de Lucas AI, Wahid N, Vendramin GG, González-Martínez SC (2010) The Strait of Gibraltar as a major biogeographic barrier in Mediterranean conifers: a comparative phylogeographic survey. *Mol Ecol* 19:5452–5468
- Jensen H, Levan A (1941) Colchicine-induced tetraploidy in *Sequoia gigantea*. *Hereditas* 27:220–224
- Jiménez JF, Werner O, Sánchez-Gómez P, Fernández S, Guerra J (2003) Genetic variations and migration pathway of *Juniperus thurifera* L. (Cupressaceae) in the western Mediterranean region. *Israel J Plant Sci* 51:11–22
- Kraaijeveld K (2010) Genome size and species diversification. *Evol Biol* 37:227–233
- Kettle CJ, Ennos RA, Jaffré T, McCoy S, Le Borgne T, Gardner M, Hollingsworth PM (2011) Importance of demography and dispersal for the resilience and restoration of a critically endangered tropical conifer *Araucaria nemorosa*. *Divers Distrib*. doi:10.1111/j.1472-4642.2011.00835.x
- Khoshoo TN (1959) Polyploidy in gymnosperms. *Evolution* 13:24–39
- Leitch IJ, Hanson L, Winfield M, Parker J, Bennett MD (2001) Nuclear DNA C-values complete familial representation in gymnosperms. *Ann Bot* 88:843–849

- Li P, Johnston MO (2000) Heterochrony in plant evolutionary studies through the twentieth century. *Bot Rev* 66:57–88
- Lindenmayer DB, Fischer J (2006) Habitat fragmentation and landscape change: an ecological and conservation synthesis. Island Press, Washington, DC
- Little DP, Schwarzbach AE, Adams RP, Hsieh C-F (2004) The circumscription and phylogenetic relationships of *Callitropsis* and the newly described genus *Xanthocyparis* (Cupressaceae). *Am J Bot* 91:1872–1881
- Loureiro J, Capelo A, Brito G, Rodriguez E, Silva S, Pinto G, Santos C (2007) Micropropagation of *Juniperus phoenicea* from adult plant explants and analysis of ploidy stability using flow cytometry. *Biol Plantarum* 51:7–14
- Maestre FT, Cortina J (2005) Remnant shrubs in Mediterranean semi-arid steppes: effects on shrub size, abiotic factors and species identity on understory richness and occurrence. *Acta Oecol* 27:161–169
- Maire R (1926) Contribution à l'étude de la flore de l'Afrique du Nord, Fascicule 10. *Bull Soc Hist Nat Afrique N* 17:125
- Mao K, Hao G, Liu J, Adams RP, Milne RI (2010) Diversification and biogeography of *Juniperus* (Cupressaceae): variable diversification rates and multiple intercontinental dispersals. *New Phytol* 188:254–272
- Marie D, Brown SC (1993) A cytometric exercise in plant DNA histograms with 2C values for 70 species. *Biol Cell* 78:41–51
- Masterson J (1994) Stomatal size in fossil plants—evidence for polyploidy in majority of angiosperms. *Science* 264:421–424
- Mayrose I, Zhan SH, Rothfels CJ, Magnuson-Ford K, Barker MS, Rieseberg LH, Otto SP (2011) Recently formed polyploid plants diversify at lower rates. *Science* 333:1257
- Médail F, Diadema K (2009) Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *J Biogeogr* 36:1333–1345
- Mehra PN, Khoshoo TN (1956) Cytology of conifers. I *J Genet* 54:165–180
- Meloni M, Perini D, Filigheddu R, Binelli G (2006) Genetic variation in five Mediterranean populations of *Juniperus phoenicea* as revealed by inter-simple sequence repeat (ISSR) markers. *Ann Bot* 97:299–304
- Michalczyk IM, Sebastiani F, Buonamici A, Cremer E, Mengel C, Ziegenhagen B, Vendramin GG (2006) Characterization of highly polymorphic nuclear microsatellite loci in *Juniperus communis* L. *Mol Ecol Notes* 6:346–348
- Michalczyk IM (2008) Application of DNA marker systems to test for genetic imprints of habitat fragmentation in *Juniperus communis* L. on different spatial and temporal scales: Integration of scientific knowledge into conservation measures. Dissertation, Philipps-Universität Marburg, Germany
- Miller CN (1977) Mesozoic conifers. *Bot Rev* 43:217–280
- Montes N, Bertaudière-Montes V, Badri N, Zaoui El H, Gauquelin T (2002) Biomass and nutrient content of a semi-arid mountain ecosystem: the *Juniperus thurifera* L. woodland of Azzaden Valley (Morocco). *Forest Ecol Manag* 166:35–43
- Montesinos D, De Luís M, Verdú M, Raventós J, García-Fayos P (2006) When, how and how much: gender-specific resource-use strategies in the dioecious tree *Juniperus thurifera*. *Ann Bot* 98:885–889
- Montesinos D, García-Fayos P, Verdú M (2010) Relictual distribution reaches the top: elevation constraints fertility and leaf longevity in *Juniperus thurifera*. *Acta Oecol* 36:120–125
- Muratović E, Bogunić F, Šolić EM, Soljan D, Siljak-Yakovlev S (2004) 2C DNA values in genus *Juniperus*: genome size revealed the rare case of polyploidy in Conifers. Poster. XI OPTIMA Meeting, Organization for Phyto Taxonomic Investigation of the Mediterranean Area. Belgrad, Serbia
- Murray B (1998) Nuclear DNA amounts in gymnosperms. *Ann Bot* 82:3–15, Supplement A
- Nagano K, Umeda T, Toda Y (2000) Karyomorphological study of *Juniperus*. In: Guttenberger H, Borzan Ž, Schlarbaum SE, Hartman TPV (eds) Cytogenetic studies of forest trees and shrubs review, present status, and outlook on the future, Arbora Publishers, Zvolen, pp. 143–159
- Ohri D, Khoshoo TN (1986) Genome size in gymnosperms. *Plant Syst Evol* 153:119–132
- Pellicer J, Garcia S, Garnatje T, Vallès J (2009) Changes in genome size in a fragmented distribution area: the case of *Artemisia crithmifolia* L. (Asteraceae, Anthemideae). *Caryologia* 62:152–160
- Provan J, Beatty GE, Hunter AM, McDonald RA, McLaughlin E, Preston SJ, Wilson S (2008) Restricted gene flow in fragmented populations of a wind-pollinated tree. *Conserv Genet* 9:1521–1532
- Pugnaire F, Lázaro R (2000) Seed bank and understorey species composition in a semi-arid environment: the effect of shrub age and rainfall. *Ann Bot* 86:807–813
- Quézel P, Barbero M (1981) Contribution à l'étude des formations pre-steppiques à Genévriers au Maroc. *Bol Soc Brot ser* 2(53):1137–1160
- Quézel P, Médail F (2003) Ecologie et biogéographie des forêts du bassin méditerranéen. Elsevier, Paris
- Quézel P, Pesson P (1980) Biogeography and ecology of conifers in the Mediterranean area. Gauthier, Paris
- Reynolds JF, Virginia RA, Kemp PR, de Soya AG, Tremmel DC (1999) Impact of drought on desert shrubs: effects of seasonality and degree of resource island development. *Ecol Monogr* 69:69–106
- Riera Mora S (2006) Cambios vegetales holocenos en la región Mediterránea de la Península Ibérica: ensayos de síntesis. *Ecosistemas* 15:17–30
- Rodríguez-Sánchez F, Pérez-Barrales R, Ojeda F, Vargas P, Arroyo J (2008) The Strait of Gibraltar as a melting pot for plant biodiversity. *Quaternary Sci Rev* 27:2100–2117
- Romo A, Boratyński A (2005) Chorology of *Juniperus thurifera* (Cupressaceae) in Morocco. *Dendrobiology* 54:41–50
- Romo A, Boratyński A (2007) Nomenclatural note on *Juniperus thurifera* subsp. *africana* (Cupressaceae). *An Bot Fenn* 44:72–75
- Santos T, Tellería JL, Virgós E (1999) Dispersal of Spanish juniper *Juniperus thurifera* by birds and mammals in a fragmented landscape. *Ecography* 22:193–204
- Sax K, Sax HJ (1933) Chromosome number and morphology in the conifers. *J Arnold Arboretum* 14:356–375
- Siljak-Yakovlev S, Pustahija F, Šolić EM, Bogunić F, Muratović E, Bašić N, Catrice O, Brown SC (2010) Towards a genome size and chromosome number database of Balkan Flora: C-values in 343 taxa with novel values for 242. *Adv Sci Lett* 3:190–213
- Slovák M, Vít P, Urfus T, Suda J (2008) Complex pattern of genome size variation in a polymorphic member of the Asteraceae. *J Biogeogr* 36:372–384
- Soltis DE, Albert VA, Leebens-Mack J, Bell CD, Paterson AH, Zheng C, Sankoff D, Depamphilis CW, Wall PK, Soltis PS (2009) Polyploidy and angiosperm diversification. *Am J Bot* 96:336–348
- Stigall AL (2010) Invasive species and biodiversity crises: testing the link in the late Devonian. *PLoS One* 5(12):e15584. doi:10.1371/journal.pone.0015584
- Terrab A, Schönswetter P, Talavera S, Vela E, Stuessy TF (2008) Range-wide phylogeography of *Juniperus thurifera* L., a presumptive keystone species of western Mediterranean vegetation during cold stages of the Pleistocene. *Molec Phylogenet Evol* 48:94–102
- Thompson JD (2005) Plant evolution in the Mediterranean. Oxford University Press, Oxford
- Wakamiya I, Newton RJ, Johnston SJ, Price JH (1993) Genome size and environmental factors in the genus *Pinus*. *Am J Bot* 80:1235–1241