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ORIGINAL ARTICLE

Evolution of *Iris* subgenus *Xiphium* based on chromosome numbers, FISH of nrDNA (5S, 45S) and *trnL–trnF* sequence analysis

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Abstract The subgenus *Xiphium* is one of the six infrageneric divisions of the genus *Iris*. Chromosome numbers of six of the seven Xiphium species are known. Here the aim was to infer genetic and phylogenetic relationships based on chromosome numbers, chromosome markers and plastid sequences. Chromosomal locations of 5S and 45S rDNA loci were determined in 19 populations of the 7 species by fluorescence in situ hybridization (FISH). Additionally, the *trnL–trnF* plastid spacer was sequenced and a phylogenetic analysis performed. Based on chromosome markers, subgenus *Xiphium* species were classified into four groups that differed in the number and locations of both types of nrDNA: (1) *I. tingitana* ($2n = 28$), *I. filifolia* ($2n = 30$, 34) and *I. xiphium* ($2n = 34$), (2) *I. juncea* ($2n = 32$) and *I. boissieri* ($2n = 36$), (3) *I. serotina* ($2n = 34$) and (4) *I. latifolia* ($2n = 42$). Although the *trnL–trnF* phylogeny was not fully resolved, the sequence analysis showed a well-supported subgroup of *I. filifolia*, *I. tingitana* and *I. xiphium*, as well as *I. juncea*. FISH physical maps of the *Iris* subgenus *Xiphium* taxa are species dependent. *I. filifolia*, *I. tingitana* and *I. xiphium* are very closely related species and share cytogenetic characteristics. Disploidy appears to have been central in the evolution of this subgenus, given a series of chromosome numbers ($2n = 28$, 30, 32, 34, 36, 42) and our phylogenetic results. Clear differences were found among European and African populations of *I. filifolia*. A different taxonomic treatment of *I. filifolia* is supported for populations on both sides of the Strait of Gibraltar.

Keywords 45S rDNA · 5S rDNA · cpDNA sequences · Disploidy · FISH · Iridaceae · *Iris* subgenus *Xiphium* · Strait of Gibraltar

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Introduction

The genus *Iris* L., with c. 210 species, is one of the two largest and most diverse genera of the family *Iridaceae* Juss. It is currently divided into six subgenera. One of them [*Iris* subgen. *Xiphium* (Mill.) Spach.] consists of the only species with bulbs, non-persistent roots and canaliculated leaves. The geographical cohesion of this group is observed since it is distributed exclusively in SW Europe and North Africa (Mathew 1981; Christiansen 1997). The worldwide revision of *Iris* by Mathew (1981) recognizes the subgenus *Xiphium* (hereafter *Xiphium*) as having seven species. Within the western Mediterranean, *I. filifolia*, *I. xiphium* and *I. serotina* are distributed in northern Morocco and the Iberian Peninsula, *I. boissieri* and *I. latifolia* are endemic to the Iberian Peninsula, *I. tingitana* is endemic to northwestern Morocco, and *I. juncea* is distributed in Algeria and Tunisia (Fig. 1). In addition, some local flora provide alternative classifications, including *I. fontanesii* from Morocco and Algeria (Colasante et al. 2002). A new species (*I. rutherfordii*), endemic to Morocco, has also been recently described (Martínez et al. 2009).

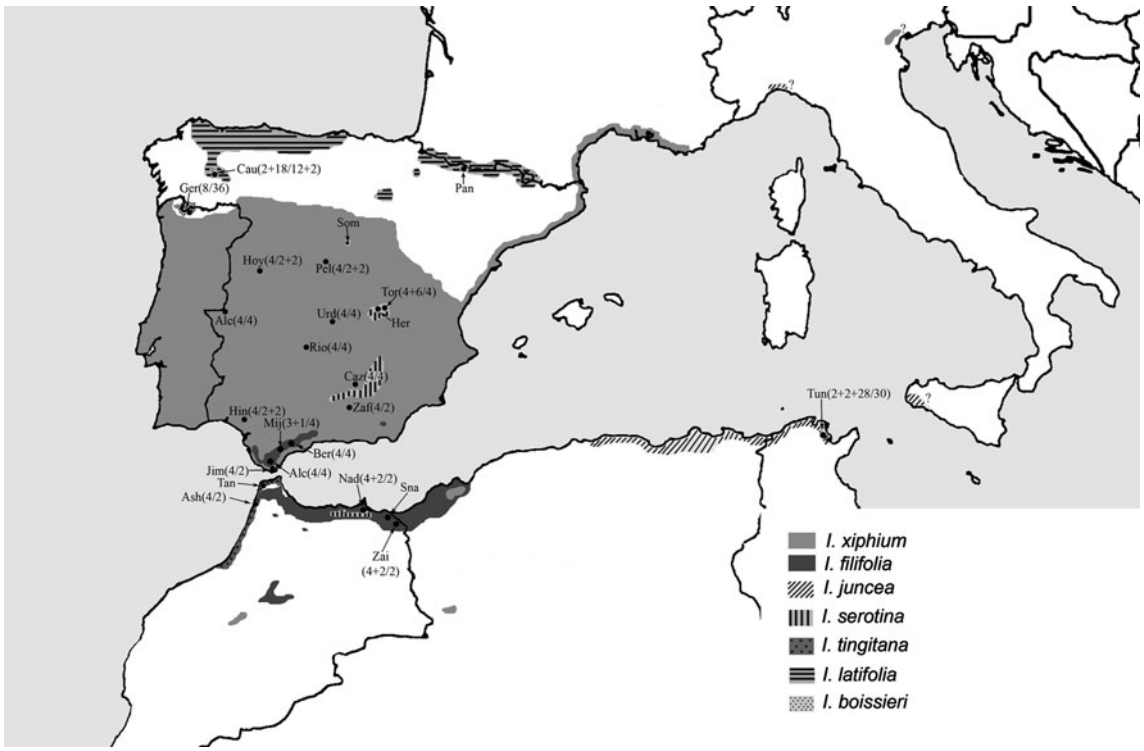

Fig. 1 Distributional map of the seven species of the *Iris* subgenus *Xiphium* used in this study, based on bibliography and herbarium material (Martinez et al., unpublished). Question marks indicate bibliographic doubtful locations. Population codes as in Table 1. *Numbers* indicate cytotypes (as explained in Table 1) found in this study and geographical location of the populations. *First number* indicates 45S rDNA signals, *second number* 5S rDNA signals

The Strait of Gibraltar (SG) has been proposed as the most important barrier to gene flow (both by seed and pollen dispersal) in the western Mediterranean since its formation around 5 million years ago (Rodríguez-Sanchez et al. 2008). Accordingly, geographical isolation on both sides of the SG is expected not only to have generated different taxa, but also to be reflected in cytogenetic differentiation at the populational level.

Apart from chromosome number, the use of chromosome markers is rather limited in wild plants of no economic importance, knowledge of which is fundamental to achieving a classification that reflects evolution (Stebbins 1971; Stace 2000). This is the case of *Xiphium*, where only chromosome numbers (six of the seven *Xiphium* species) are known (Christiansen 1997). Since the widespread adoption of fluorescence in situ hybridization (FISH) in plants, the physical mapping of tandemly repeated DNA sequences has become a new source of chromosomal marking for identifying chromosome regions (Adams et al. 2000; Ruas et al. 2005) and provides "valuable information on homologies between chromosomal segments, mainly between closely related species" (Dobigny et al. 2004). This set of markers may provide a phylogenetic tool for inferring genome evolution during speciation (de Bustos et al. 1996; Cuadrado and Jouve 1997, 1999; Stace 2000). In plants, where technical difficulties still remain in the physical mapping of low-copy genes (Jiang and Gill 2006), the most frequently mapped DNA sequences are ribosomal multigene families. In higher eukaryotes, the ribosomal rRNA 5S genes and the genes encoding the large preribosomal RNA (45S) are both present in multiple copies organized in arrays of tandem repeat sequences. Large preribosomal RNA genes (45S) are arranged in the nucleolus organizer region (NOR) and consist of clusters (18S, 5.8S, 26S and ITS regions) separated by an intergenic spacer (IGS) (Rogers and Bendich 1987; Hillis and Dixon 1991). The 5S genes are mostly independent of the nucleolus organizer region (NOR). Despite the fact that in some species, such as *Arabidopsis thaliana* (Murata et al. 1997), these two classes of rRNA occur in close proximity on the chromosomes bearing the NOR, both multigene families are physically separate and map independently in most cases. Arrangement of 45S and 5S genes is used to identify specific chromosomes. Indeed, simultaneous fluorescence in situ hybridization of 45S rDNA and 5S rDNA probes enabled the identification of all of the chromosomes of species such as those of *Hordeum vulgare* (Leitch and Heslop-Harrison 1993) and *A. thaliana* (Murata et al. 1997). Another reason to investigate the number and location of rDNA in plants is to elucidate the evolutionary

dynamics of these genes. Although the potential utility of plant rDNA variation for inferring phylogenies has been questioned (Thomas et al. 1997; Zhang and Sang 1999; Adams et al. 2000; Mishima et al. 2002), the chromosomal organization of rDNA loci has been successfully used to complement molecular-based phylogenetic analysis and subsequently to elucidate patterns of chromosome evolution and understand the evolution in of rDNA in plants (Ran et al. 2001; de Melo and Guerra 2003).

DNA molecular data applied to plant phylogenetics are basically obtained from two sources: plastid DNA (cpDNA) and nuclear ribosomal DNA (rDNA) (Alvarez and Wendel 2003; Nieto Feliner and Rosselló 2007). Plastid molecular phylogenetic studies have established major lineages of *Iris* (Tillie et al. 2000; Wilson 2004), although most sectional and species level relationships still remain unsolved. Molecular inference of plant relationships requires markers that are informative at the level being investigated. The properties of cpDNA regions that provide useful markers for molecular plant systematics has been reviewed extensively (Alvarez and Wendel 2003; Shaw et al. 2007). Maternal inherited cpDNA sequences would be suitable for inferring phylogenetic relationships of subgenus *Xiphium*, avoiding any reticulation effect. Indeed, they have been proven to be useful to elucidate evolutionary relationships in Siberian *Iris* species (Makarevitch et al. 2003).

The aims of this study were to investigate changes in chromosome number and physical organization (FISH) of both nrDNA (5S and 45S) loci. Chromosome numbers and FISH markers were further analyzed in the context of a *trnL–F* phylogeny to infer the evolutionary change in chromosomes of *Xiphium*. Correlation between karyotype variation and plant distribution was also investigated in nine Iberian populations of *I. xiphium* and five population of *I. filifolia* from both sides of the Strait of Gibraltar. Finally, geographical isolation related to chromosome evolution is discussed.

Materials and methods

Plant material and chromosome preparation for in situ hybridization

Thirty-one individuals from 19 populations of 7 species of *Iris* subgen. *Xiphium* were collected from the wild in Spain, Portugal, Morocco and Tunisia (Fig. 1). Unfortunately, *Iris rutherfordii* was not considered given that it was described after the analysis presented here had been finished. Dry plants are deposited in herbarium (MA), and the living collection is maintained by the authors at the Royal Botanic Garden of Madrid (Spain). Chromosome numbers, plant origin, voucher numbers, number and position of the major 45S rDNA and 5S rDNA loci on metaphases, sequence population origin and GenBank accessions are summarized in Table 1.

Chromosomes were prepared from the meristem of root tips, up to 2 cm in length, after pre-treatment with 2 mM 8-hydroxyquinoline for 4 h at 18°C and followed by fixation for at least 24 h in a freshly prepared 3:1 ethanol-acetic acid solution. Chromosome preparations were made as described by Schwarzacher et al. (1989), using enzymatically digested root tips prior to squashing the meristematic cells in a drop of 45% acetic acid onto clean microscope slides. After removal of the cover slips by freezing, the slides were air-dried. The slides were then pre-treated with RNase, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in an ethanol series and air-dried again, as described by Cuadrado and Jouve (1994).

When possible the samples selected for sequencing were those already used for in situ hybridization, or at least taken from the same population (see Table 1). The three taxa included as the outgroup (*Iris pseudacorus*, *I. nigricans* and *I. planifolia*) were selected to represent taxonomic series of the genus *Iris* distantly related to *Xiphium* (sect. *Limniris*, sect. *Oncocyclus* and subgen. *Scorpiris*, respectively) (Tillie et al. 2000; Wilson 2004).

DNA probes and labeling

Two probes isolated from *Triticum aestivum* were hybridized: pTa71, containing the repeat unit of 18S-5.8S-26S rDNA and the intergenic spacer (IGS) (hereafter called 45S) (Gerlach and Bedbrook 1979) and pTa794, containing the complete 5S gene unit (Gerlach and Dyer 1980). Probes were labeled either with digoxigenin-11-dUTP or biotin 16-dUTP. The entire plasmid pTa71 was labeled using nick translation kit from Gibco BRL (Bionick labelling system). The polymerase chain reaction (PCR) was used to amplify and label the sequence of pTa794.

In situ hybridization, signal detection and microscopy

In situ hybridization was performed as described by Cuadrado and Jouve (1994). Briefly, the chromosomes were denatured at 75°C for 10 min in 30 µl of previously denatured hybridization solution containing 50% (v/v) formamide and 50–100 ng of each labeled probe, using a programmable thermocycler (PT-100, M. J. Research). After hybridization the slides were washed, essentially following the post-hybridization process as described by Heslop-Harrison et al. (1991). The highest stringent wash was in 20% formamide/0.1× SSC at 42°C, allowing only target sequences of more than 80–85% homology to remain hybridized.

Table 1 Chromosome number and location of FISH signals of *Iris* subgenus *Xiphium* species. Chromosome numbers from bibliography are in normal typing

Taxon	*2n*	Valid name [as in Mathew (1981)]	Plant provenance Country: locality (Province) cultivated (C)/wild (W)/uncertain origin (U) (references)	Population code	Voucher number (Acc. number)	No. and position of 45S rDNA	No. and position of 5S rDNA	*trnL–trnF* GenBank
I. boissieri	**36**	***I. boissieri***	**Portugal: Serra do Gerés (Tras o Montes)**	**Ger**	**169JM03 (86)**	**8 (st)**	**36 (pr)**	–
I. boissieri	–	***I. boissieri***	**Portugal: Serra do Gerés (Tras o Montes)**	**Ger**	**169JM03 (84)**	–	–	HQ180212
I. boissieri	–	***I. boissieri***	**Portugal: Serra do Gerés (Tras o Montes)**	**Ger**	**169JM03 (85)**	–	–	HQ180213
I. boissieri	36	*I. boissieri*	W (Fernández 1950)	–	–	–	–	–
I. filifolia	**34**	***I. filifolia***	**Spain. Alcaornocales (Cádiz)**	**Alc**	**246JM04 (418)**	**4 (st)**	**4 (int)**	–
I. filifolia	**34**	***I. filifolia***	**Spain: Sierra Bermeja (Málaga)**	**Ber**	**251JM04 (420)**	**4 (st)**	**4 (int)**	–
I. filifolia	**34**	***I. filifolia***	**Spain: Mijas (Málaga)**	**Mij**	**146JM03 (41)**	**3 + 1 (st)**	**4 (int)**	HQ180220
I. filifolia	32	*I. filifolia*	W (Pérez and Pastor 1994); U (Simonet 1952)	–	–	–	–	–
I. fontanesii	28	*I. filifolia*	C (Simonet 1932)	–	–	–	–	–
I. fontanesii	56[a]	*I. filifolia*	C (Simonet 1932)	–	–	–	–	–
I. filifolia var. La France (*hort*)	24	*I. filifolia*	C (Simonet 1928b)	–	–	–	–	–
I. filifolia	**30**	***I. filifolia***	**Morocco: Nador (Nador)**	**Nad**	**233JM04 (319)**	**4 + 2 (st)**	**2 (int)**	–
I. filifolia	–	***I. filifolia***	**Morocco: Beni Snassen**	**Sna**	–	–	–	HQ180221
I. filifolia	**30**	***I. filifolia***	**Morocco: Zaïo (Oujda)**	**Zai**	**235JM04 (341)**	**4 + 2 (st)**	**2 (int)**	–
I. juncea	**32**	***I. juncea***	**Tunisia: Carthago (Tunis)**	**Tun**	**Cult. (695)**	**2 + 2 + 28 (pr)**	**30 (pr)**	HQ180223
I. juncea	32	*I. juncea*	C (Simonet 1928a, 1932)	–	–	–	–	–
I. latifolia	**42**	***I. latifolia***	**Spain: Seoane do Caurel (Lugo)**	**Cau**	**172JM03 (88)**	**2 + 18 (st, int, pr)**	**12 (st) + 2 (int)**	HQ180210
I. latifolia	–	***I. latifolia***	**Spain: Somosierra (Madrid)**	**Som**	**165JM03 (73)**	–	–	HQ180209
I. latifolia	–	***I. latifolia***	**Spain: Panticosa (Huesca)**	**Pan**	**66JFA03 (149)**	–	–	HQ180211
I. latifolia	42	*I. latifolia*	U (Simonet 1928a, 1932)	–	–	–	–	–
I. serotina	**34**	***I. serotina***	**Spain: las Torcas (Cuenca)**	**Tor**	**174JM03 (109)**	**4 + 6 (st, int)**	**4 (int)**	HQ190214
I. serotina	–	***I. serotina***	**Spain: Hermita San Miguel (Cuenca)**	**Her**	**173JM03 (99)**	–	–	HQ180215
I. tingitana	**28**	***I. tingitana***	**Morocco: Asilah (Tanger)**	**Ash**	**207JM04 (299)**	**4 (st)**	**2 (int)**	–
I. tingitana	–	***I. tingitana***	**Morocco: Tanger (Tánger)**	**Tan**	**211JM04 (309)**	–	–	HQ180222
I. tingitana	42	*I. tingitana*	C (Simonet 1928b, 1932), U (Simonet 1930)	–	–	–	–	–
I. tingitana	56[a]	*I. tingitana*	U (Simonet 1930)	–	–	–	–	–
I. tingitana var. *fontanesii* G.G.	28	*I. tingitana*	C (Simonet 1932)	–	–	–	–	–
I. xiphium	**34**	***I. xiphium***	**Spain: Hoyocasero (Avila)**	**Hoy**	**162JM03 (68)**	**4 (st)**	**2 + 2 (int)**	HQ180218
I. xiphium	**34**	***I. xiphium***	**Spain: Ventas de Zafarraya (Granada)**	**Zaf**	**156JM03 (50)**	**4 (st)**	**2 (int)**	–
I. xiphium	**34**	***I. xiphium***	**Spain: Jimena de la Frontera (Cádiz)**	**Jim**	**249JM04 (656)**	**4 (st)**	**2 (int)**	–
I. xiphium	**34**	***I. xiphium***	**Spain:Rio Frío (Ciudad Real)**	**Rio**	**140JM03 (32)**	**4 (st)**	**4 (int)**	–

Table 1 continued

Taxon	*2n*	Valid name [as in Mathew (1981)]	Plant provenanceCountry: locality (Province) cultivated (C)/wild (W)/uncertain origin (U) (references)	Population code	Voucher number (Acc. number)	No. and position of 45S rDNA	No. and position of 5S rDNA	*trnL–trnF* GenBank
I. xiphium	**34**	***I. xiphium***	**Spain: Cazorla (Jaén)**	**Caz**	**13BGA03 (23)**	**4 (st)**	**4 (int)**	HQ180217
I. xiphium	**34**	***I. xiphium***	**Spain: Hinojos (Huelva)**	**Hin**	**134JM03 (10)**	**4 (st)**	**2 + 2 (int)**	HQ180219
I. xiphium	**34**	***I. xiphium***	**Spain: Pelayos de la Presa (Madrid)**	**Pel**	**161JM03 (63)**	**4 (st)**	**2 + 2 (int)**	–
I. xiphium	**34**	***I. xiphium***	**Spain: Urda (Toledo)**	**Urd**	**138JM03 (14)**	**4 (st)**	**4 (int)**	HQ180216
I. xiphium	**34**	***I. xiphium***	**Spain: Alcántara (Cáceres)**	**Alc**	**259JM04 (461)**	**4 (st)**	**4 (int)**	–
I. lusitanica	34	*I. xiphium*	C (Simonet 1932)		–	–	–	–
I. taitii	34	*I. xiphium*	W (Fernandes and Queirós 1970–1971)		–	–	–	–
I. xiphium	34	*I. xiphium*	W (Löve 1973; Ruiz Rejon 1976; Fernández Casas et al. 1978; Pérez and Pastor 1994); C (Simonet 1932)	–	–	–	–	–
I. xiphium var. *battandieri* Fost.	36	*I. xiphium*	C (Simonet 1932)	–	–	–	–	–
I. xiphium var. *praecox* Hort	34	*I. xiphium*	C (Simonet 1932)	–	–	–	–	–
I. subgen. *Xiphion*	30	Prob. *tingitana*[b]	W (Simonet 1952)	–	–	–	–	–
I. subgen. *Xiphion*	30	Prob. *tingitana*[b]	W (Simonet 1952)	–	–	–	–	–

Accessions in bold letters are data from this study indicating origin. The number of nrDNA signals is indicated as the first number being the strongest FISH signals plus the weak ones. In the case of *I. juncea*, the first two numbers indicate the number of SAT chromosomes, with clear differences between them and with the rest of the chromosomes

int internal, *pr* proximal, *st* satellite

[a] Observed number simultaneously with $2n = 28$

[b] "Environs de Tanger"

To identify signal-bearing chromosomes, two-color FISH was performed. Detection of biotin and digoxigenin was undertaken by incubating the slides in streptavidin-Cy3 (Sigma) and fluorescein anti-digoxigenin, respectively, in 5% (w/v) bovine serum albumin (BSA) for 1 h at 37°C before staining the DNA with DAPI (4′, 6-diamino-2-phenylindole). Slides were examined with a Zeiss Axiophot epifluorescence microscope. The separate images from each filter set were captured by a cooled CCD camera and analyzed by the computer program Adobe Photoshop using only functions that affect the whole image equally.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from silica-dried material collected in the field (Table 1) using the Dneasy Plant Mini Kit (Quiagen Laboratories, Germany). DNA was amplified using primers *trne* and *trnf* for the *trnL*(UAA)–*trnF*(GAA) intergenic spacer (Taberlet et al. 1991). PCR conditions were performed following those of Taberlet et al. (1991) with some variations: 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 51°C for 1.30 min and 72°C for 1 min, and to finish 72°C for 8 min, using a Perkin-Elmer PCR System 9700 thermal cycler and MJ Research MiniCycler^{TM} thermal cycler. Amplified products were cleaned using spin filter columns (PCR Clean-up Kit, MoBio Laboratories, California) according to the manufacturer's protocol. Cleaned products were directly sequenced using dye terminators (Big Dye Terminator v2.0, Applied Biosystems, California) and run on polyacrylamide electrophoresis gels (7%) in a Perkin-Elmer/Applied Biosystems model 377 automatic sequencer. The primers *trne* and *trnf* were also used for cycling sequencing of *trnL–trnF* spacer under the following conditions: 95°C for 2 min followed by 25 cycles of 95°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequence data were assembled in a contig file and edited using the program Sequed (Applied Biosystems). IUPAC symbols were used to represent nucleotide ambiguities for DNA stretches not properly sequenced.

Sequence alignment and phylogenetic analysis

Sequences were aligned using the program Clustal X 1.62b (http://evolution.genetics.washington.edu/phylip/software.etc1.html) with further manual adjustments minimizing the number of informative characters following the logic of Kelchner (2000) and Simmons and Ochotorena (2000). Indels were not coded.

Phylogenetic analyses were conducted using Fitch parsimony as implemented in PAUP* (Swofford 2002) with equal weighting of all characters (Kelchner 2000). Branch and bound searches were implemented with the option MULPARS in effect. Relative support for clades identified by parsimony analysis was assessed by bootstrapping (BS) (100 resamplings each) using the heuristic search replicated 100 times with random taxon-addition sequences, tree bisection-reconnection (TBR) branch swapping and MULPARS option in effect. Nucleotide substitutions within indel regions were not considered in parsimony analysis because mutations within these regions are influenced by surrounding sequence structure (Kelchner 2000). In addition, phylogenetic analysis of *trnL–F* sequences was also performed by using Bayesian inference. To determine the simplest model of sequence evolution that best fits the sequence data, the hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC) as implemented in MrModeltest v.2 (Nylander 2004) were used. Among the 24 models of nucleotide substitution, F81 + G was selected. A Bayesian inference of the *trnL–F* matrix was conducted using MrBayes 3.0b4 (Ronquist and Huelsenbeck 2003). MrBayes was performed sampling for 1 million generations (with 4 MC chains, chain temperature 0.2, sampling every 100 tree, burn-in 1,000). A 50% majority rule tree was constructed and posterior probability (pp) used as branch support.

Results

Chromosome numbers and physical mapping of ribosomal genes

In agreement with previous chromosome counts of *Xiphium* (Table 1), the widest variation in chromosome numbers has been found between *I. tingitana* ($2n = 28$) and *I. latifolia* ($2n = 42$). Intermediate numbers were displayed by *I. juncea* ($2n = 32$), *I. serotina* and *I. xiphium* ($2n = 34$) and *I. boissieri* ($2n = 36$). The Iberian populations of *I. filifolia* showed $2n = 34$, whereas those from northern Morocco had $2n = 30$ (Table 1).

Figure 2a–m shows the physical localization after two-color FISH of 45S and of 5S rDNA probes in the somatic metaphase chromosomes of the seven *Xiphium* species. Chromosome number as well as the number and localization of both ribosomal DNA repeat families was consistently observed in all metaphases analyzed (Table 1). Secondary constrictions were observed in all species. In most cases the nucleolar organizer regions (NORs) are in the subterminal position, so they delimit a short piece of the well-stained chromosomes separated from the rest of the chromosome arm. When a chromosome satellite is significantly separated from the chromosome body, the connecting strands of DNA labeled with the 45S rDNA probe permit its precise localization (Fig. 2e, g, i) and avoids confusions with extra chromosomes as the satellites

are as large as in *I. latifolia* (Fig. 2i). Although not all secondary constrictions were always observed, we defined the chromosome-bearing satellites as those chromosomes with major 45S rDNA sites revealed by FISH.

Different intensity observed in fluorescence signals revealed that both the 45S or 5S rDNA probes had a variable number of repeats between loci. In situ hybridization analysis in *I. tingitana* ($2n = 28$, Fig. 2a) indicated the presence of one pair of metacentric chromosomes with strong 5S rDNA signal. Also, up to eight weakly signaled sites could be observed with the 5S rDNA probe, depending of the quality of the metaphase. Four sites of major 45S rDNA gene clusters were found in two pairs of chromosomes showing the secondary constriction and satellite associated with the NOR. We consistently observed faint signals in the pair of chromosomes carrying a stronger 5S rDNA locus (Fig. 2, arrows).

Differences were observed in chromosome numbers and the physical mapping of both rDNA probes between the northern African and the Iberian populations of *I. filifolia*. The African populations displayed $2n = 30$ chromosomes (Fig. 2b), whereas the Iberian populations had $2n = 34$ (Fig. 2c). The most important difference in chromosomal localization of ribosomal genes between the individuals from both sides of the Strait of Gibraltar is that the African populations showed three pairs of SAT chromosomes, whereas the Iberian populations showed only two pairs. Additionally, the Iberian populations displayed two pairs of

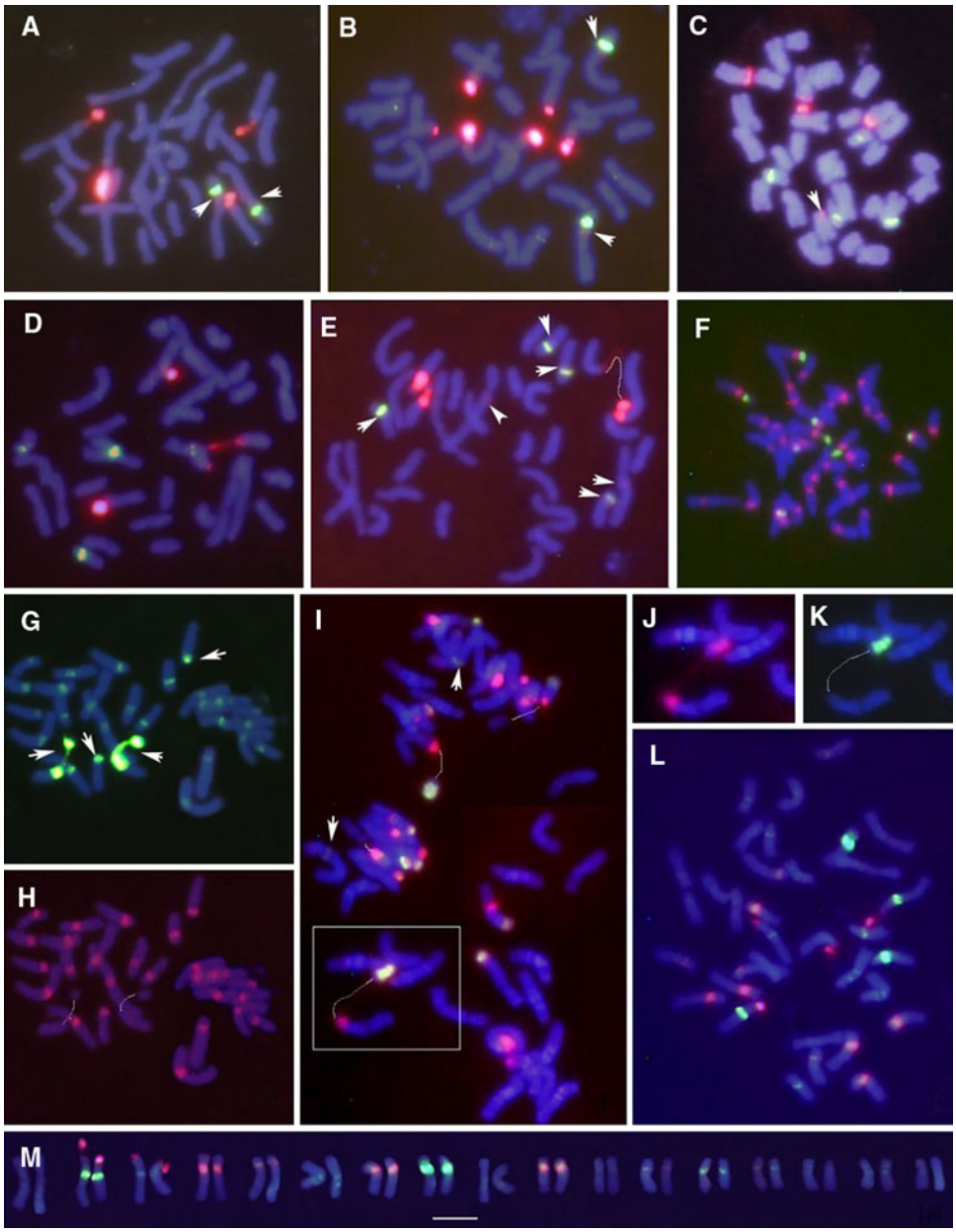

Fig. 2 **a–l** Comparative 45S and 5S loci patterns of rDNA fluorescence in situ hybridization (FISH) analysis in different species of *Iris* subgenus *Xiphium* after DAPI (blue) staining. **a–e** "Xiphium" group species showing double visualization of 45S rDNA (red) signals and 5S rDNA (green) signals, **a** *I. tingitana* showing $2n = 28$ chromosomes, **b** *I. filifolia* from northern Africa showing $2n = 30$ chromosomes, **c** *I. filifolia* from Iberia showing $2n = 34$ chromosomes, **d**, **e** two different populations of *I. xiphium* both showing $2n = 34$ chromosomes. **f–h** Double visualization of 45S (green) signals and 5S (red) signals of **f** *I. boissieri* showing $2n = 36$ chromosomes, **g–h** *I. juncea* showing $2n = 30$ chromosomes, **g** 45S rDNA and **h** 5S rDNA. **i–m** Double visualization of 45S rDNA (red) signals and 5S rDNA (green) signals of **i–k** *I. latifolia* showing $2n = 42$ chromosomes, **j**, **k** *inset* in **i** satellized chromosome showing **j** 45S rDNA and **k** 5S rDNA, and **l–m** *I. serotina* showing $2n = 34$ chromosomes. *Lines* connect satellites to their chromosome of origin. *Scale bar* 10 μm, valid for every photograph. *Arrows* showing the positions of weakly marked 45S rDNAs

medium-size chromosomes with bright 5S rDNA signals, whereas the African populations had only one pair of large metacentric chromosomes with bright 5S rDNA and weakly marked 45S rDNA signals. Interestingly, the morphology and physical map of both ribosomal genes in the later pair of chromosomes of the African populations of *I. filifolia* are similar to the pair of chromosomes carrying 5S rDNA and a minor 45S rDNA signal in the African *I. tingitana* (compare arrowed chromosomes in Fig. 2a, b).

All nine populations of *I. xiphium* showed $2n = 34$ chromosomes. Consistency in FISH patterns is manifested by the presence of four satellized chromosomes with the NOR at a subterminal position. Besides these two pairs of SAT chromosomes, up to six weak signals with the 45S rDNA probe were observed in another three pairs of chromosomes: one pair of large submetacentric and two pairs of medium-size metacentric chromosomes (Fig. 2d,e). All accessions displayed minor 45S rDNA signals in similar morphological chromosomes. The *I. xiphium* populations can be grouped in three classes depending on the number and intensity of the 5S rDNA signals: (1) populations containing two pairs of chromosomes with strong 5S rDNA signals, such as those from Alcántara, Rio Frío, Urda and Cazorla (Fig. 2e), (2) populations of two pairs of chromosomes with bright and weak intensities of 5S rDNA signals (2 + 2 in Table 1), such as those from Hoyocasero, Hinojos and Pelayos de la Presa (Fig. 2d), and (3) two populations from Jimena de la Frontera and Venta de Zafarraya with only one pair of chromosomes with strong 5S rDNA signals. Despite interpopulational differences in the number and intensity of the 5S rDNA signals, as well as in the number of minor 45S rDNA signals (see Fig. 2d, e), intrapopulation polymorphisms were not observed in the five populations represented by various individuals (data not shown).

Iris boissieri ($2n = 36$) displayed four pairs of satellized chromosomes with similar intensity of 45S rDNA in situ signals (8) at a subterminal position. Unexpectedly, one 5S rDNA signal of similar intensity was localized near the centromere of every single chromosome (Fig. 2f). We are not aware of any other report for flowering plants with 5S rDNA sites on all chromosomes.

All chromosomes of *I. juncea* ($2n = 32$) showed a signal with the 45S rDNA probe; however, only two pairs of SAT chromosomes are highlighted (Fig. 2g). One pair of SAT chromosomes showed a small satellite with the NOR in a very subterminal position (arrowed in Fig. 2g). The other pair had a large satellite separated from the rest of chromosome arm. Fluorescent signals were stronger in the last pair of chromosomes, indicating a higher number of 45S rDNA repeats. The rest of the chromosomes showed a similar intensity signal, but a weaker 45S rDNA fluorescent signals at a proximal position (Fig. 2g). With the exception of the NOR bearing chromosome containing the largest satellite, the other chromosomes showed similar intensity 5S rDNA signals co-localized with the 45S rDNA signals, apparently near the centromeres (Fig. 2h).

The species with the highest number of chromosomes ($2n = 42$) and satellites was *I. latifolia*. The limited material available of this species to be analyzed by FISH did not allow us to draw a complete and detailed physical map. Microphotographs were however obtained to establish a general pattern of distribution with both ribosomal probes, which were compared with those of the other *Xiphium* species. *Iris latifolia* has at least nine pairs of satellized chromosomes, of which one pair displayed two 45S rDNA signals. Secondary constrictions were found at different localizations, and then satellites have very different sizes (Fig. 2i). In total, 20 45S rDNA signals were observed. Beside the two sites co-localized with the 45S rDNA all over the length of the largest satellites (Figs. 1, 2j–k), 12 other stronger 5S rDNA signals were observed at a very subterminal position in other NOR-bearing chromosomes. Ten of them also co-localized with the 45S rDNA signals. Interstitial minor 5S and 45S rDNA signals were also observed in some pairs of chromosomes; however, the quality of the metaphases obtained do no allow a more detailed description. An interesting feature found in this species was the clear pattern of DAPI-positive bands observed, consisting of a high number of bright bands in almost all the chromosomes.

Although details affecting the 45S rDNA and 5S rDNA bearing chromosomes are described for almost all *Xiphium* species, the karyotype is only presented for *Iris serotina*, since this species has been investigated at the chromosomal level for the first time (Fig. 2l). Using the 45S rDNA and 5S rDNA probes in combination, we were able to discriminate nearly all the 17 chromosomes pairs of *I. serotina*. There are two pairs of chromosomes with stronger interstitial 5S rDNA signals (chromosome 2 and 8) and five pairs of satellized chromosomes. Two pairs of chromosomes with the NORs at the terminal position are distinguished by the presence of interstitial 5S rDNA signals of very different intensity (chromosome 2 and 3). The other three pairs of satellized chromosomes (4, 7, 10) are clearly distinguished by satellites sizes. In addition to the five pairs of SAT chromosomes with major 45S rDNA signals, there are two other weaker pairs marked with the 45S rDNA probe (in chromosomes 5 and 12), the smallest also with weak 5S rDNA signals. A pair of metacentric chromosomes showed two weakly marked 5S rDNA loci in the same arm (chromosome 6). The remaining eight pairs of chromosomes displayed weakly marked 5S rDNA loci in most cases and could be identified by differences in the morphology and intensity of the 5S rDNA signals (Fig. 2m).

Phylogenetic analyses of *trnL–trnF* sequences

The aligned matrix of the *trnL–F* spacer was 510 base pairs (bp) long, ranging from 394 bp (*I. latifolia*) to 426 bp (*I. planifolia*). This matrix yielded 64 variable and 31 phylogenetically informative characters including 15 accessions of *Xiphium*. Nine indels longer than five bp were detected (four in *Xiphium*). The transitions/transversions were 1.46.

The strict consensus tree (Fig. 3) ($L = 74$, CI = 0.905, RI = 0.892) revealed that *Xiphium* is a well–defined monophyletic group (BS = 100%). Within this clade, the tree showed four subclades in a polytomy. Three of them were formed by the same species accessions, with high bootstrap support (*I. latifolia* BS = 98%, *I. boissieri* BS = 99% and *I. serotina* BS = 83%). The fourth subclade displayed lower support (BS = 69%) and was formed by *I. xiphium*, *I. fililfolia* (both sides of the Strait of Gibraltar), *I. tingitana* and *I. juncea* accessions.

The tree obtained by Bayesian inference (results not shown) supported the monophyly of *Xiphium* with high posterior probability values. Within *Xiphium*, two clusters of species are shown, but with low support values. The first clade (0.51 pp) is that containing *I. latifolia* and *I. serotina* accessions. The second clade (0.76 pp) was made up by one subcluster (1.00 pp) containing *I. xiphium*, *I. filifolia*, *I. tingitana* and *I. juncea* accessions and another subcluster (1.00 pp) with all *I. boissieri* accessions.

Congruence between cytogenetic and molecular results encouraged us to define a group (Xiphium group) including three species (*I. xiphium*, *I. filifolia* and *I. tingitana*), which is separated from the well-defined clades of *I. latifolia*, *I. boissieri* and *I. serotina*. The only species displaying an

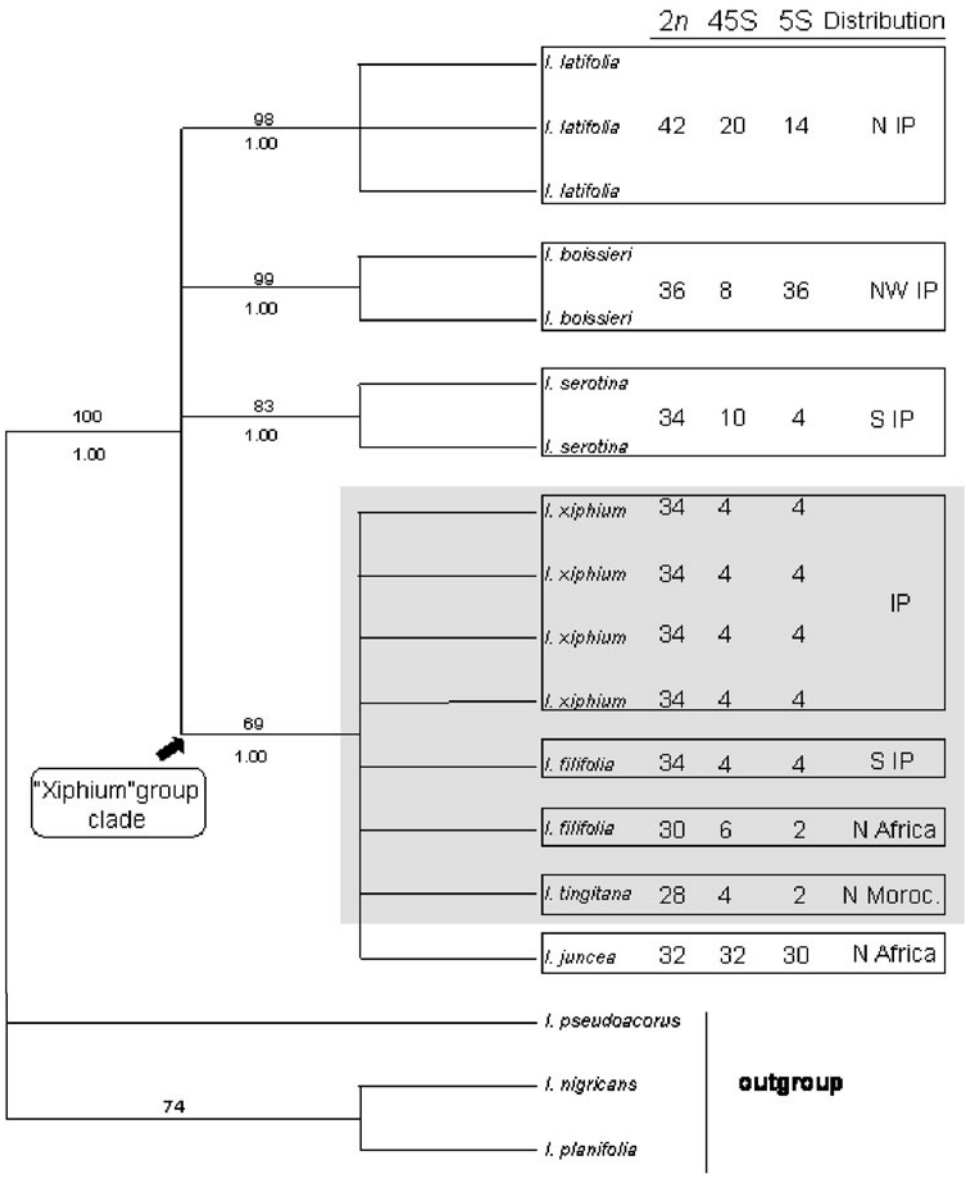

Fig. 3 Strict consensus tree from *trn*L–F analysis of 17,201 most parsimonious trees, CI = 0.905, RI = 0.892. *Numbers* above branches indicate >50% bootstrap values; numbers below the branches indicate posterior probability values. Diploid numbers from this study are indicated. The 45S and 5S nrDNA numbers are the sum of the bright and weak signals (see Table 1). Distribution makes reference to the geographical area of the sampled individuals. The Xiphium group is in a grey square (see text). *IP* Iberian Peninsula, *N* north, *S* south, *W* west

intermediate situation is *I. juncea*. It has a complex FISH pattern as *I. boissieri* does, but the phylogenetic reconstructions include *I. juncea* in the Xiphium group.

Discussion

Chromosome number variation

Polyploidy was evidently important in the early diversification of *Iridaceae* (Goldblatt and Takei 1997). In *Iris*, where changes in basic chromosome numbers are frequent, the ancestral base number, however, remains uncertain (Goldblatt and Takei 1997). Chromosome numbers from the literature in *Xiphium* basically agree with those we found here (Table 1). The cultivated origin of most of the individuals used in some studies implies an additional source of variation as including artificial hybrids or polyploids. Plant misidentification may also be contemplated, particularly with species not clearly morphologically differentiated such as *I. filifolia* and *I. tingitana*.

Taking only into account our original counts (Table 1), the seven species of *Xiphium* studied here displayed a series of chromosome numbers of $2n = 28$, 30, 32, 34, 36 and 42. Considering the results obtained by other authors, our counts of $2n = 32$ for *I. juncea*, $2n = 36$ for *I. boissieri* and $2n = 42$ for *I. latifolia* fit into the chromosome numbers found in the literature for these species (Table 1). Furthermore, we herein provide for the first time the chromosome number for *Iris serotina* ($2n = 34$). Two cytotypes have been reported for *I. xiphium,* with $2n = 34$ for a plant from the Iberian Peninsula and $2n = 36$ for plants from Algeria (*I. xiphium* var. *battandieri*). Our results of $2n = 34$ in the nine analyzed populations of *I. xiphium* are congruent with no variation in the chromosome number for the Iberian populations previously investigated. Unfortunately, we had no access to any living population of *I. xiphium* from North Africa. Without a voucher specimen we cannot rule out that the counts of $2n = 36$ for the populations of Algeria might have been taken from misidentified plants (Simonet, 1932), as differences in chromosome numbers may be related to two geographical areas (northern Africa and the Iberian Peninsula) of *I. xiphium*. Two cytotypes of *I. filifolia* were found in northern Africa and the Iberian Peninsula. Although many counts are available in the literature ($2n = 28$, 32, 34, 56, Table 1), we only counted $2n = 34$ for the three Iberian populations and $2n = 30$ chromosomes for the two populations collected in northern Africa. Our count of $2n = 28$ for *I. tingitana* coincides with the lower number reported for subgenus *Xiphium*. Chromosome numbers of $2n = 42$ and 56 previously reported for *I. tingitana* clearly indicate polyploid or misidentification. The origin of these polyploid plants could be questioned, as these counts might have been taken from cultivated material. Alternatively, polyploidy can occur in *Xiphium* at the population level.

Polyploidy versus disploidy

In addition to a series of chromosome numbers revealed by the cytogenetic data (see above), an increase in chromosome complements can be also inferred. A basic number $x = 7$ would explain the counts of $2n$ ($4x$) = 28 as a tetraploid level in *I. tingitana* and $2n$ ($6x$) = 42 as a hexaploid level in *I. latifolia.* The pattern of chromosomal variation found in the remaining species may be interpreted as stepwise changes from $n = 14$ to $n = 15$ in northern Africa populations of *I. filifolia*, $n = 16$ in *I. juncea*, $n = 17$ in *I. xiphium*, *I. serotina* and the Iberian populations of *I. filifolia* and $n = 18$ in *I. boissieri*.

The phylogenetic reconstructions do not render sufficient resolution to interpret either an ascendant or descendant disploidy. Although few studies assess disploid variation of chromosome number in a phylogenetic framework, a decrease in chromosome numbers appears not to be unusual (Martel et al. 2004; Hidalgo et al. 2007). In addition, descendant disploidy has been suggested for several genera of the Iridaceae based on chromosome number variation (Goldblatt and Takei 1997). If the ancestral base number of *Xiphium* was $x = 9$, and *I. boissieri* ($n = 18$) represented a polyploidy event, descending disploidy may explain the remaining chromosome numbers ($n = 17$, 16, 15, 14). Taking into account the distribution of the species of the subgenus, a pattern of descendent disploidy might coincide with that found for *Nonea* (Boraginaceae) (Selvi and Bigazzi 2002), for which a correlation between descending chromosome numbers and adaptations to a more arid environment is observed.

Variation in the number and localization of nrDNA loci

Variation in the number of FISH markers reveals a higher number of 45S than that of 5S in *Xiphium*. Every subgenus *Xiphium* species is unique in the chromosomal distribution of the 45S and 5S rDNA repeat sequences. The distribution pattern of both ribosomal regions is, however, well maintained in *I. tingitana*, *I. filifolia* and *I. xiphium,* with 45S rDNA loci in the subterminal position in two or three pairs of NOR-bearing chromosomes and one or two other pairs of chromosomes with stronger interstitial 5S rDNA signals. In short, less than one-fourth of the chromosomes display rDNA loci, which are 45S and 5S rDNA probes mapped in six to eight different chromosomes. *Iris serotina* showed a similar but more complex FISH pattern, in which two pairs of chromosomes have stronger interstitial 5S rDNA sites

and two pairs of SAT chromosomes with 45S rDNA at a terminal position. However, the 45S rDNA family is also found at an interstitial position in other pairs of chromosomes in some cases (in *I. tingitana*, northern Morocco *I. filifolia* populations and populations of *I. xiphium*) mapping together with the 5S rDNA probe. The likelihood of insertion and amplification of new element copies is much more common than removal by unequal recombination of current copies, with a subsequent trend of repetitive sequence increase (Bennetzen and Kellogg 1997). Amplification events that increase the number of rDNA loci and/or translocation of chromosomes bearing 45S and 5S rDNA loci may explain the differences in the distribution pattern of rDNA between *I. serotina* and the group comprising *I. filifolia, I. tingitana and I. xiphium.* Accordingly, we interpreted that *I. serotina* may be related to the above-mentioned group of three species.

Iris boissieri and *I. juncea* share a similar distribution pattern of the 5S rDNA loci. The recurrent distribution pattern of 5S rDNA in both species (on 36 and 30 chromosomes, respectively) could also be explained by multiplication events. Irrespective of the fact that most of the loci could correspond to pseudogenes or to degenerated or non-functional 5S rDNA sequences that were poorly revealed (Fig. 2), we are not aware of any other report of plant species with 5S rDNA sites on all chromosomes. Both species are, however, different in the 45S rDNA in situ pattern. In *I. boissieri* the two ribosomal families are physically separate in the same chromosome arms in all satellized chromosomes, whereas in *I. juncea* both families physically colocate, including one 45S rDNA loci on almost every chromosome. If we assumed a close phylogenetic relationship between both species, the 45S rDNA sequences might have been amplified during the divergence process. The number and physical distribution of both ribosomal loci vary widely among plants, from the occurrence of only one 45S and one 5Sr rDNA loci in species of the genus *Lobelia* ($2n = 28$) (Vanzela et al. 1999), maize ($2n = 20$) (Li and Arumuganathan 2001) and *Hypochaeris* ($2n = 8$) (Tremetsberger et al. 2004) to one 45S rDNA locus on every chromosome as reported for species of *Peonia* ($2n = 10$) (Zhang and Sang 1999). Nonetheless, usually less than one-third of chromosomes display either 45S rDNA or 5S rDNA sites (Castilho and Heslop-Harrison 1995; Linares et al. 1996, Tagashira and Kondo 2001, Mishima et al. 2002, Fregonezi et al. 2004, Muravenko et al. 2004). It is therefore notable that *I. juncea*, with $2n = 32$, presents one 45S rDNA locus on every chromosome pair that colocates with 5S rDNA sites on 30 chromosomes.

The highest chromosomal differences found between *I. latifolia* and the other species of *Xiphium*, may be explained either by ancient polyploidization with extensive and rapid genome re-structuring (Soltis and Soltis 1995) or by being the consequence of different phenomena, such as chromosome rearrangement, unequal crossing-over and transpositional events commonly reported in other plant species (Leith and Heslop-Harrison 1993; Hall and Parker 1995). *I. latifolia* differs from the other *Xiphium* species by the amount and distribution of ribosomal DNA families (2 bright plus 18 weak 45S signals either satellite, internal or proximal positions and 12 bright satellite plus 2 weak internal 5S signals) (Table 1).

Phylogenetic relationships

The *trnL–F* consensus tree showed certain evolutionary patterns within *Xiphium* (Fig. 3). The subgenus *Xiphium* is clearly a monophyletic group, which has already been suggested by previous studies (Tillie et al. 2000, Wilson 2004). Taking into account new sources of data (FISH and sequence data combined), we might conclude that: (1) *I. latifolia*, *I. serotina* and *I. boissieri* constitute three independent lineages, (2) *I. xiphium*, *I. filifolia* and *I. tingitana* are closely related (Xiphium group), and (3) *I. juncea* displays an intermediate position between the Xiphium group and *I. boissieri* based on the *trnL–F* tree (both MP and BI) and the FISH pattern (i.e., four 45S large signals and extensive 5S signals in similar chromosome positions), respectively.

Although cohesiveness of the Xiphium group is well supported (i.e., the group has no more than four 45S bright signals, no more than four 5S bright signals and is retrieved as monophyletic in the *trnL–F* phylogeny), there are differences between populations on both sides of the Strait of Gibraltar. *I. filifolia* from Spain is similar to *I. xiphium*, whereas *I. filifolia* from Morocco resembles *I. tingitana*. The first relationship is supported by the same chromosome number ($2n = 34$), whereas the second is supported by the number and location of the 45S and 5S signals (two bright 5S signals and weak 45S signals colocate in the northern African *I. filifolia* and *I. tingitana*).

A constant genomic organization has been suggested for those groups of recent origin and with limited cytogenetic differentiation (Heslop-Harrison 2000; Torrell et al. 2003). Taking into account the FISH pattern, the most homogeneous group within the subgenus is the Xiphium group. The inclusion of *I. juncea* in the Xiphium group, as suggested by the *trnL–F* reconstructions, might mean that shifting of the number of 45S and 5S sites has been relatively easy. Variation of the chromosome number also appears to have occurred relatively easily ($2n = 28$, 30, 32 and 34) within the group. Our results are congruent with a chromosome variation line in which a most common ancestor gave rise to multiple cytogenetic features.

In summary this study reveals a great variation in the rDNA FISH patterns in a small group within a limited distribution area (the western Mediterranean). In addition, our analysis indicates a species-dependent pattern of DNA FISH markers. A close relationship between *I. juncea* and the Xiphium group (*I. filifolia*, *I. tingitana* and *I. xiphium*) is suggested by our *trnL–F* phylogeny, a result to be further investigated given that the former displays a very distinct nrDNA FISH pattern. Although polyploidy cannot be ruled out, a widespread disploidy appears to have been predominant. The separation of the Strait of Gibraltar appears not only to have influenced the establishment of exclusive distributional patterns of *Iris* species (*I. xiphium* in Iberia, *I. tingitana* in northern-Africa), but also the formation of different patterns of chromosome numbers and FISH markers in disjunct populations of *I. filifolia* on the two sides of the Strait of Gibraltar.

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