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Auto- and allopolyploidy in *Centaurea* sect. *Acrocentron s. l.* (Asteraceae, Cardueae): karyotype and fluorochrome banding pattern analyses

M. Font[1], J. Vallès[2], A. Susanna[1] & N. Garcia-Jacas[1]

[1]Institut Botànic de Barcelona (CSIC-Ajuntament de Barcelona), Psg. Migdia s/n, 08038 Barcelona, Catalonia, Spain.
[2]Laboratori de Botànica, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII s/n, 08028 Barcelona, Catalonia, Spain.

Author for correspondence: N. Garcia-Jacas (ngarciajacas@ibb.csic.es)

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Abstract
Dysploidy and polyploidy are well documented in the large genus *Centaurea*, especially in sect. *Acrocentron* and in a small group of species from the Iberian Peninsula described as sect. *Chamaecyanus*, closely related to *Acrocentron*. We have explored two interesting cases of polyploid series in both sections: the polyploid series of *Centaurea toletana* in sect. *Chamaecyanus* and the series of *C. ornata* group in sect. *Acrocentron*. We have carried out a karyological study using both classic karyotype analyses and chromosome banding with fluorochromes.

The *C. toletana* complex exhibits three different ploidy levels, diploid, tetraploid and hexaploid, the latter usually considered a different species, *C. argecillensis*. Our results do not contradict the original hypothesis of tetraploid *C. toletana* being an autopolyploid, but they suggest that this case of autopolyploidy, as many others, is the result of multiple events of polyploidization.

Regarding *C. argecillensis*, our results confirm that it is an allopolyploid originated by hybridization of *C. toletana* and an unknown species. The colonizing habit of *C. argecillensis*, as it was the case of tetraploid *C. toletana*, suggests that the allopolyploid has multiple origins.

The *C. ornata* complex exhibits four ploidy levels, diploid, tetraploid, hexaploid and endecaploid. In this section, however, the banding patterns have not helped us to determine the relation between the polyploid series and neither have allowed us to confirm the hypothesis of the possible progenitors of *C. kunkelii*.

Key words: *Acrocentron*, *Centaurea*, *Chamaecyanus*, cytogenetics, fluorochrome banding, hybridization, karyology, polyploidy.

Resum
La disploïdia i la poliploïdia són fenòmens que estan ben documentats dins del gènere *Centaurea*, especialment en la sect. *Acrocentron* i en un petit grup d'espècies de la Península Ibèrica descrit com a sect. *Chamaecyanus*, estretament relacionada amb *Acrocentron*. Hem estudiat dos casos interessants de sèries poliploides d'ambdues seccions: la sèrie poliploide de *Centaurea toletana* de la sect. *Chamaecyanus* i la sèrie de *C. ornata* de la sect. *Acrocentron*. Hem realitzat un estudi cariològic utilitzant les anàlisis de cariotip clàssiques i el bandeig de cromosomes amb fluorocroms.

El complex de *C. toletana* presenta tres nivells diferents de ploïdia, diploide, tetraploide i hexaploide, aquest últim ha estat normalment considerat com una espècie diferent, *C. argecillensis.* Els resultats obtinguts no contradiuen la hipòtesi original que l'espècie tetraploide *C. toletana* sigui un autopoliploide, però suggereixen que aquest cas d'autopoliploïdia, com molts altres, és el resultat de múltiples esdeveniments de poliploïdització. Pel que fa a *C. argecillensis,* els nostres resultats confirmen que és un al·lopoliploide que es va originar per hibridació de *C. toletana* i una espècie desconeguda. A més, tractant-se d'una espècie colonitzadora, igual que l'espècie tetraploide *C. toletana*, es suggereix que l'al·lopoliploïdia té orígens múltiples.

El complex de *C. ornata* presenta quatre nivells de ploïdia, diploide, tetraploide, hexaploide i hendecaploide. En aquesta secció, però, els patrons de les bandes no ens han ajudat a determinar la relació entre la sèrie poliploide i tampoc no ens han permès confirmar la hipòtesi dels possibles progenitors de *C. kunkelii.*

Paraules clau: *Acrocentron*, bandeig de cromosomes, cariologia, *Centaurea*, *Chamaecyanus*, citogenètica, fluorocroms, hibridació, poliploïdia.

INTRODUCTION

Until recently, polyploidy has been considered as an uncommon and isolated fact and polyploids have been thought genetically uniform, with low adaptive capacity and tending to disappear (STEBBINS, 1971). Currently, polyploidy is regarded as a prominent force in plant evolution (SOLTIS & SOLTIS, 2000; WENDEL, 2000; SOLTIS *et al.*, 2003, 2004). In the last 20 years, molecular studies in plants have changed the understanding of polyploidy evolution. The estimated frequency of polyploidy has increased, and it is now recognised that multiple origins are the rule of most polyploids (SOLTIS *et al.*, 2003).

Traditionally, polyploidy has been thought to result from either duplication of a single genome (autopolyploidy) or from the combination of two or more differentiated genomes (allopolyploidy) (STEBBINS, 1947, 1950; GRANT, 1981). However, GRANT (1981) also noted that both concepts are the extremes of a graded series. Autopolyploidy has historically been considered maladaptive and relatively uncommon compared to allopolyploidy (WENDEL & DOYLE, 2005). Whereas it is clear now that allopolyploidy is probably more common than autopolyploidy in nature, the latter is far more prevalent than was once thought. In addition, it is necessary to say that the autopolyploids never are maladaptive (RAMSEY & SCHEMSKE, 1998; WENDEL & DOYLE, 2005; SOLTIS *et al.*, 2003, 2004).

An interesting case of study of polyploids is found in the genus *Centaurea.* Karyological features of *Centaurea* include both dysploidy and polyploidy. As to dysploidy, chromosome numbers in *Centaurea* range from $x = 12$ to $x = 7$ in a series well-correlated to phylogeny (GARCIA-JACAS *et al.*, 2001). As to polyploidy, it is especially frequent in sects. *Acrocentron* (Cass.) DC. and *Chamaecyanus* Willk. (GARCIA-JACAS, 1998).

Centaurea sect. *Chamaecyanus* was described on the basis of a small group of species from the Iberian Peninsula (WILLKOMM & LANGE, 1870). *Chamaecyanus* is related to sect. *Acrocentron*, one of the largest of *Centaurea* (FONT *et al.*, 2002), with about 100 species and a high percentage of endemics (GARDOU, 1975; WAGENITZ, 1975). The Ibero-North African region stands as the second most important centre of diversification after Turkey and the Balkans (GARCIA-JACAS & SUSANNA, 1992). Differences between two sections rely only on the combination of acaulescent habit and reduced pappus in *Chamaecyanus*. Even though the boundaries are clear-cut, both sections are extremely close as demonstrated by the many fertile hybrids described between species of both sections (FERNÁNDEZ CASAS & SUSANNA, 1986) and subsectional rank looks certainly more appropriate for *Chamaecyanus*. Anyway, no matter the rank adopted (section or subsection), species of *Chamaecyanus* are probably a natural, monophyletic group, however unconfirmed by DNA sequence analyses (FONT *et al.*, 2002).

The most extreme and interesting case of polyploidy in *Chamaecyanus* is *C. toletana* Boiss. & Reuter, which exhibits three different ploidy levels ($2n = 2x = 20$, $2n = 4x = 40$, and $2n = 6x = 60$). Tetraploid *C. toletana* was described as a different species, *C. cavanillesiana* Graells. However, careful examination of the type and new collections from the type localities in central Spain demonstrated that morphological differences between *C. toletana* and *C. cavanillesiana* were inexistent (FERNÁNDEZ CASAS & SUSANNA, 1986). In contrast, it is possible to characterize morphologically the hexaploid, which was also described as a different species, *C. argecillensis* Gredilla, later merged into *C. toletana* as a variety because many intermediate forms between the diploid type variety and the hexaploid exist (FERNÁNDEZ CASAS & SUSANNA, 1986). These authors concluded that the tetraploid was a true autopolyploid because it was impossible to differentiate the diploid from the tetraploid without carefully measuring pollen size or stigmas (LEWIS, 1980; RAMSEY & SCHENKE, 1998). However, FERNÁNDEZ CASAS & SUSANNA (1986) did not reach any conclusion on the hexaploid.

There is another case of similar polyploid series in sect. *Acrocentron* formed by three species *C. gabrielis-blancae* Fern. Casas (*C. ornata* var. *microcephala* Willk.), a diploid species, *C. ornata* Willd. a tetraploid and *C. saxicola* Lag. a hexaploid. DOSTÁL (1976) subordinated *C. saxicola* to *C. ornata* but a phylogenetic analysis of sect. *Acrocentron* (FONT *et al.*, 2002) supports its status as a different species. In addition *C. kunkelii*, described recently by GARCIA-JACAS (1998) on the basis of a plant from south Spain that had been much confused with *C. ornata*, is also member of this group. Curiously GARCIA-JACAS (1998) suspected a hybridogenic origin for

this endecaploid species, and one of the hypothesized parental species was *C. saxicola*, the other putative progenitor belonging to sect. *Chamaecyanus*, *C. haenseleri* a tetraploid species (Fernández Casas & Susanna, 1986).

Interest for polyploidy has increased lately with the emergence of new molecular techniques (extensively revised in Soltis *et al.*, 2004). Traditional views on meiotic pairing cannot be considered a valid criterion to distinguish between auto- and allopolyploids (Qu *et al.*, 1998). Instead, karyotype analysis may be very helpful in establishing systematic and evolutionary relationships within polyploid complexes, particularly those data which constitute what Schweizer & Ehrendorfer (1983) named banding style, i.e. the distribution pattern of different chromosome regions, such as constitutive heterochromatin (revealed with Giemsa C-banding method) or AT- and GC-rich DNA portions (revealed with fluorochrome banding methods). We have used banding methodology a) to verify whether autopolyploidy of *C. toletana* is reflected in the banding pattern, b) to explore the relationships of hexaploid *C. argecillensis* to the diploid and tetraploid levels of *C. toletana*, c) to establish the relationship between *C. gabrielis-blancae*, *C. ornata* and *C. saxicola* and verify if they constitute a polyploid series and d) to look after a confirmation of the hypothesis that *C. saxicola* and *C. haenseleri* are the progenitors of *C. kunkelii*.

Materials and Methods

Adult plants were collected from wild populations of each taxon. The species studied are listed in Table 1, with indication of their origins.

Chromosome counts were made on somatic metaphases using the squash technique. Root meristems from germinating seeds collected in the wild or from wild plants cultivated in pots were used.

Samples were pretreated with 0.05% aqueous colchicine at room temperature for 3 h as a minimum. The material was fixed with Carnoy for 24 h

Table 1. Origin of the studied populations.

Taxon	Collection data
Centaurea argecillensis Gredilla	Spain, Guadalajara: Argecilla, mountain slopes on the road to Ledanca, *M. Font & Susanna 1811* (BC).
Centaurea gabrielis-blancae Fern. Casas	Spain, Navarra: Lumbier, Foz de Lumbier, *Garcia-Jacas & Susanna 1592* (BC).
Centaurea haenseleri Boiss.	Spain, Málaga: 10 km to Jubrique, *Garcia-Jacas & Susanna 1888* (BC).
Centaurea kunkelii N. Garcia	Spain, Almería: road AL-411 between Roquetas and Canjáyar, *Garcia-Jacas, Susanna 1612 & Vilatersana* (BC).
Centaurea ornata Willd.	Spain, Soria: near San Esteban de Gormaz, *Garcia-Jacas & Susanna 1823* (BC). Spain, Huesca: between Salinas and de la Peña dam (A-132), *Vilatersana 58* (BC).
Centaurea saxicola Lag.	Spain, Murcia: La Azohía, near the watchtower, *Garcia-Jacas, Susanna 1616 & Vilatersana* (BC).
Centaurea toletana Boiss. & Reut. (2*x*)	Spain, Toledo: Risco de las Paradas, *M. Font & Susanna 1817* (BC). Spain, Toledo: mountains above San Pablo on the track to Baños del Robledillo, *M. Font & Susanna 1818* (BC).
Centaurea toletana Boiss. & Reut. (4*x*)	Spain, Madrid: Redueña, road N-320, 4 km to Torrelaguna, *M. Font & Susanna 1819* (BC).

at -20ºC. Before staining, the material was hydrolyzed with 5N HCl for 1 h at room temperature. It was stained with 1% acetic orcein and mounted in 45% acetic acid. For all the counts, a minimum of ten plates (diploid and tetraploid taxa) or five plates (hexaploid taxa) from different individuals was examined. Preparations were made permanent by CO_2 freezing, dehydrating in ethanol and mounting in Canada balsam. The majority of photographs were taken using a Zeiss Standard microscope, and digital photographs were also taken using an Olympus 3030 camera mounted on an Olympus microscope U-TV1 X. The preparations, the negatives and the herbarium vouchers are preserved in the Botanical Institute of Barcelona (BC).

Numerical parameters of the karyotypes were calculated and the idiograms were obtained with software running on Lotus 1-2-3 prepared by J. Simon (Universitat de Barcelona).

For fluorochrome banding, root tips were hydrolysed with 45% acetic acid for 5-10 min at 60ºC, and squashed in a drop of 45% acetic acid. After metaphase plate selection and cover slip removal by CO_2 freezing, slides were rinsed with absolute ethanol and dried at room temperature in covered containers until use. Two different fluorochrome banding protocols were applied to reveal GC-rich (with chromomycin A3, abridged CMA) and AT-rich (with bisbenzimide Hoechst 33258, abridged Hoechst) regions. For chromomycin A3 staining the techniques of SCHWEIZER (1976), KONDO & HIZUME (1982), COULAUD *et al.* (1995), CERBAH *et al.* (1995) and VALLÈS & SILJAK-YAKOVLEV (1997) were used with minor modifications as follows: the slides were incubated in McIlvaine buffer pH 7; treated with distamycin A (0.1 g/l in McIlvaine buffer pH 7) for 10 min; stained with chromomycin A3 (0.1 g/l in McIlvaine buffer pH 7 + 0.005 M $MgSO_4$) for 10 min; rinsed in the same buffer; counterstained with methyl green (0.1% in McIlvaine buffer pH 5.5) for 10 min; rinsed in McIlvaine buffer pH 5.5; and mounted in antifade glycerol solution (Citifluor AF1) - McIlvaine buffer pH 7 1:1. Bisbenzimide staining was carried out according to the techniques of MARTIN & HESEMANN (1988), COULAUD *et al.* (1995), CERBAH *et al.* (1995) and VALLÈS & SILJAK-YAKOVLEV (1997) with minor modifications as follows: the slides were successively rehydrated in 70, 50, and 30% ethanol and distilled water, each for 5 min; incubated in McIlvaine buffer pH 5.5 for 10 min; stained with bisbenzimide (2 x 10-3 g/l in McIlvaine buffer pH 5.5) for 1 min; and mounted in 60% sucrose. The observations were made with an epifluorescence Zeiss Axioplan microscope with filter sets 07 (excitation 457, emission 530 nm long pass) for chromomycin stained slides and 01 (excitation 365, emission 480 nm long pass) for bisbenzimide stained slides. ISO 400 Fujichrome, ISO 200 Kodachrome and ISO 100 TMax films were used to photograph the chromosomes and the interphase nuclei.

RESULTS AND DISCUSSION

A summary of cytogenetic results, including chromosome number, chromosomal formula, karyotype symmetry data and the number of GC- and AT-rich regions, is presented in Table 2, except for the endecaploid species *C. kunkelii*, for which it was only possible to obtain a single metaphasic plate and we have not been able to make the calculations. In this table we can appreciate the different chromosome sizes between the species belonging to sect. *Acrocentron* and those from sect. *Chamaecyanus*.

Figure 1 (*C. toletana* series) and Fig. 2 (*C. ornata* series) show orcein-stained metaphase plates and chromosome morphology of the taxa studied. Karyotypes are very symmetrical in all the populations, and most chromosomes are metacentric, with some submetacentric ones present. All the species have satellites, not always in the same position.

In all the taxa, GC- and AT-rich zones were detected. Fluorochrome banded metaphase plates are shown in Fig. 3. The haploid idiograms of each population with the location of GC- and AT-rich regions are presented in Fig. 4. For the hexaploid population (*C. argecillensis)*, the exact assignation of chromomycin and bisbenzimide bands in the idiogram was not performed (Fig. 4C), because of the extreme similarity in length and symmetry of all the chromosomes. We have not included either the idiograms of *C. saxicola* and *C. kunkelii*, since it was impossible to assign a banding patterns adjusted to the reality in these species.

Table 2. Summary of cytogenetic results. The superscripts indicate: [1]chromosomal formula according to LEVAN *et al*. (1964); [2]mean chromosome length; [3]chromosome length range; [4]total karyotype length; [5]centromeric index (I or index in LEVAN *et al*., 1964); [6]length ratio of long and short chromosome arms (LEVAN *et al.,* 1964); [7] intrachromosomal asymmetry index (ROMERO, 1986); [8]interchromosomal asymmetry index (ROMERO, 1986); [9]symmetry class according to STEBBINS (1971). CMA= chromomycin positive/ negative marks; BB= bisbenzimide positive/negative marks. A= *Acrocentron*; Ch= *Chamaecyanus*.

Taxon	2*n*	PL	S	Chromosomal formula[1]	MCL[2]	CLR[3]
Centaurea toletana complex						
C. toletana	20	2x	Ch	6m + 2m^{sat} + 2sm	5.89	3.93-8.68
C. toletana	40	4x	Ch	16m + 4m^{sat}	11	7.02-16.09
C. argecillensis	60	6x	Ch	3M + 22m + 1m^{sat} + 3sm + 1sm^{sat}	7.28	4.93-11.25
Centaurea ornata complex						
C. gabrielis-blancae	20	2x	A	7m + 1m^{sat} + 1sm + 1sm^{sat}	2.82	1.99-4.28
C. ornata	40	4x	A	1M + 15m + 3sm + 1sm^{sat}	2.15	1.39-3.50
C. haenseleri	40	4x	Ch	1M + 18m + 1m^{sat}	4.20	3.96-15.70
C. saxicola	60	6x	A	25m + 3sm + 2sm^{sat}	2.54	1.51-4.11
C. kunkelii	110	11x	A			

Taxon	TKL[4]	CI[5]	R[6]	A1[7]	A2[8]	Sc[9]	CMA+/-	BB+/-
Centaurea toletana complex								
C. toletana	58.85	41.75	1.41	0.29	0.24	2B	3/0	1/2
C. toletana	220.07	42.76	1.35	0.25	0.22	2A	5/0	2/1
C. argecillensis	218.49	42.67	1.37	0.24	0.22	2A	5/0	2/2
Centaurea ornata complex								
C. gabrielis-blancae	28.16	42.01	1.41	0.27	0.22	2B	2/2	1/0
C. ornata	42.92	41.29	1.44	0.29	0.25	2A	4/1	3/0
C. haenseleri	339.6	41.85	1.43	0.26	0.21	2A	4/1	3/0
C. saxicola	76.17	42.05	1.41	0.26	0.24	2A	10/0	10/1
C. kunkelii							16-18	6

Centaurea toletana complex

In *C. toletana* series, GC- and AT-rich regions are predominantly terminal. This banding pattern agrees with those of other Cardueae, such as that of the *Xeranthemum* group (GARNATJE *et al.*, 2004), relatively close to the genus considered here. Chromomycin-positive bands are often coincidental with bisbenzimide-negative zones.

In diploid *C. toletana* three telomeric bands are present in the chromomycin staining, while there is only one in the bisbenzimide staining, together with two negative satellites (Figs. 3A, 3D, 4A).

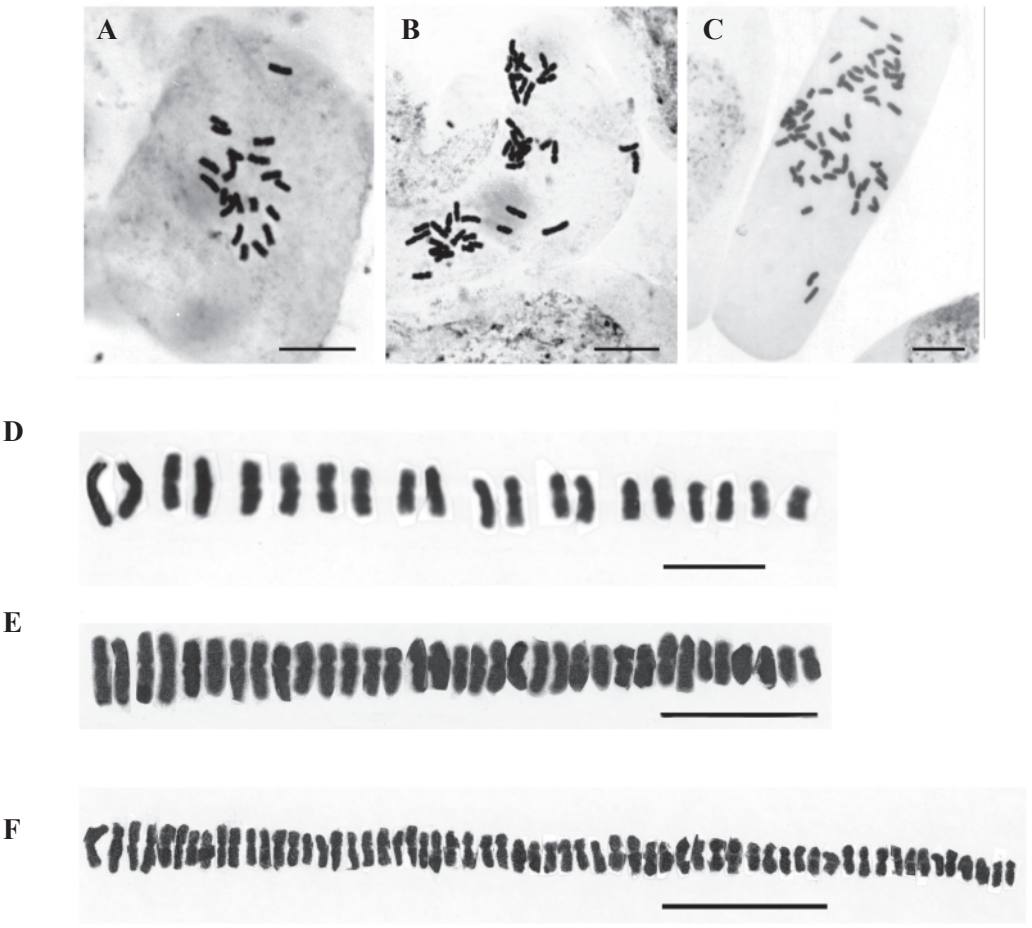

Figure 1. Orcein-stained metaphase plates (A-C) and karyograms (D-F) of the studied populations. (A) and (D), *Centaurea toletana* (2*x*); (B) and (E), *Centaurea toletana* (4*x*); (C) and (F), *Centaurea argecillensis*. Scale bars: 10 μm.

In the tetraploid population, there are also three telomeric bands in the chromomycin staining with two more signals in two satellites (Figs. 3B, 4B). In the bisbenzimide staining, however, there are two positive signals, one telomeric and another one in a satellite, together with a negative satellite (Figs. 3E, 4B). Regarding *C. argecillensis*, we have already pointed out the impossibility of determining exactly the chromosomes where the signals are located, but there are five chromomycin-positive signals, three of them telomeric, one centromeric and the last one in a satellite (Fig. 3C). As for bisbenzimide, there are two telomeric bands and two negative satellites (Fig. 3F).

According to these results, the fluorescent banding pattern of the tetraploid population is not a perfect duplicate of the diploid one: even though diploid and tetraploid *C. toletana* studied populations share at least three of the signals, there are obvious differences between an ideal duplication of the 2*x* pattern and our results.

In our opinion, this does not contradict the hypothesis of autopolyploidy, which, as we have seen, has very sound basis. In fact, we could not expect the tetraploid to be a perfect duplication of the genome of the diploid, which would be possible only in an ideal case that we would seldom find in nature. Many factors are responsible for these differences, but we can stress upon two obvious ones.

First factor is relative age of polyploidy. If tetraploid populations have long evolved without ge-

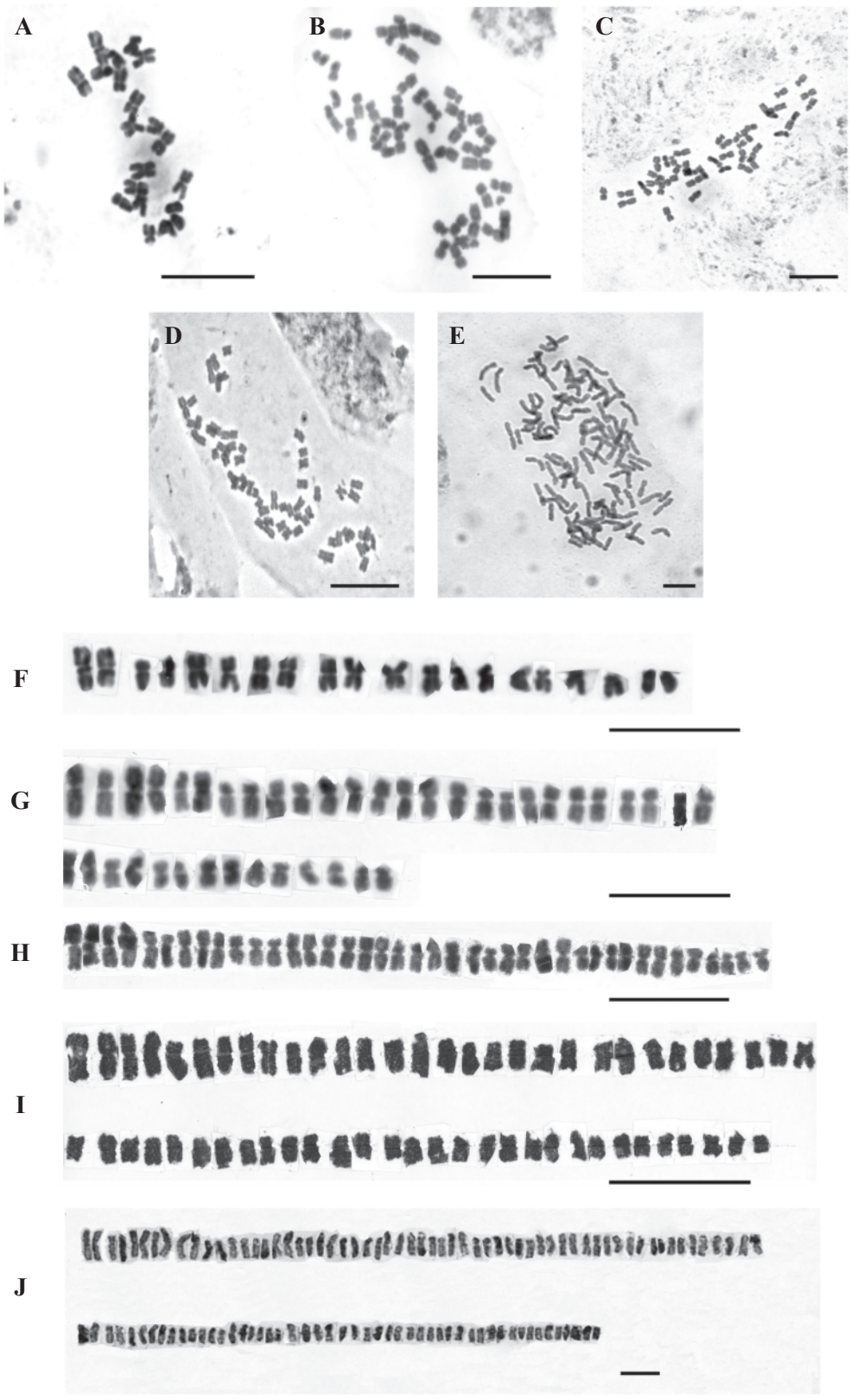

Figure 2. Orcein-stained metaphase plates (A-E) and karyograms (F-J) of the studied populations. (A) and (F), *Centaurea gabrielis-blancae*; (B) and (G), *Centaurea ornata*; (C) and (H), *Centaurea haenseleri*; (D) and (I), *Centaurea saxicola*; (E) and (J), *Centaurea kunkelii*. Scale bars: 10 μm.

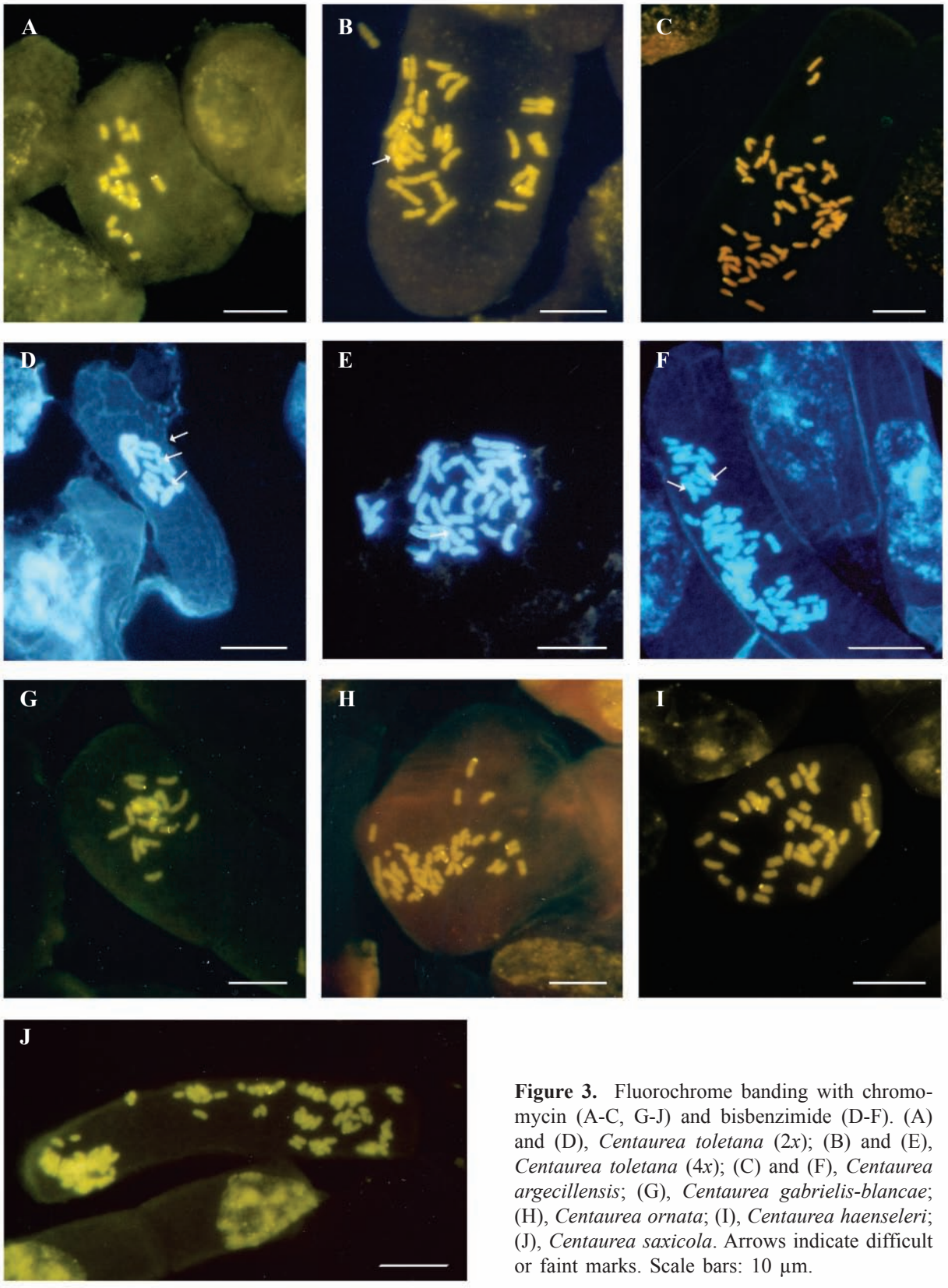

Figure 3. Fluorochrome banding with chromomycin (A-C, G-J) and bisbenzimide (D-F). (A) and (D), *Centaurea toletana* (2*x*); (B) and (E), *Centaurea toletana* (4*x*); (C) and (F), *Centaurea argecillensis*; (G), *Centaurea gabrielis-blancae*; (H), *Centaurea ornata*; (I), *Centaurea haenseleri*; (J), *Centaurea saxicola*. Arrows indicate difficult or faint marks. Scale bars: 10 μm.

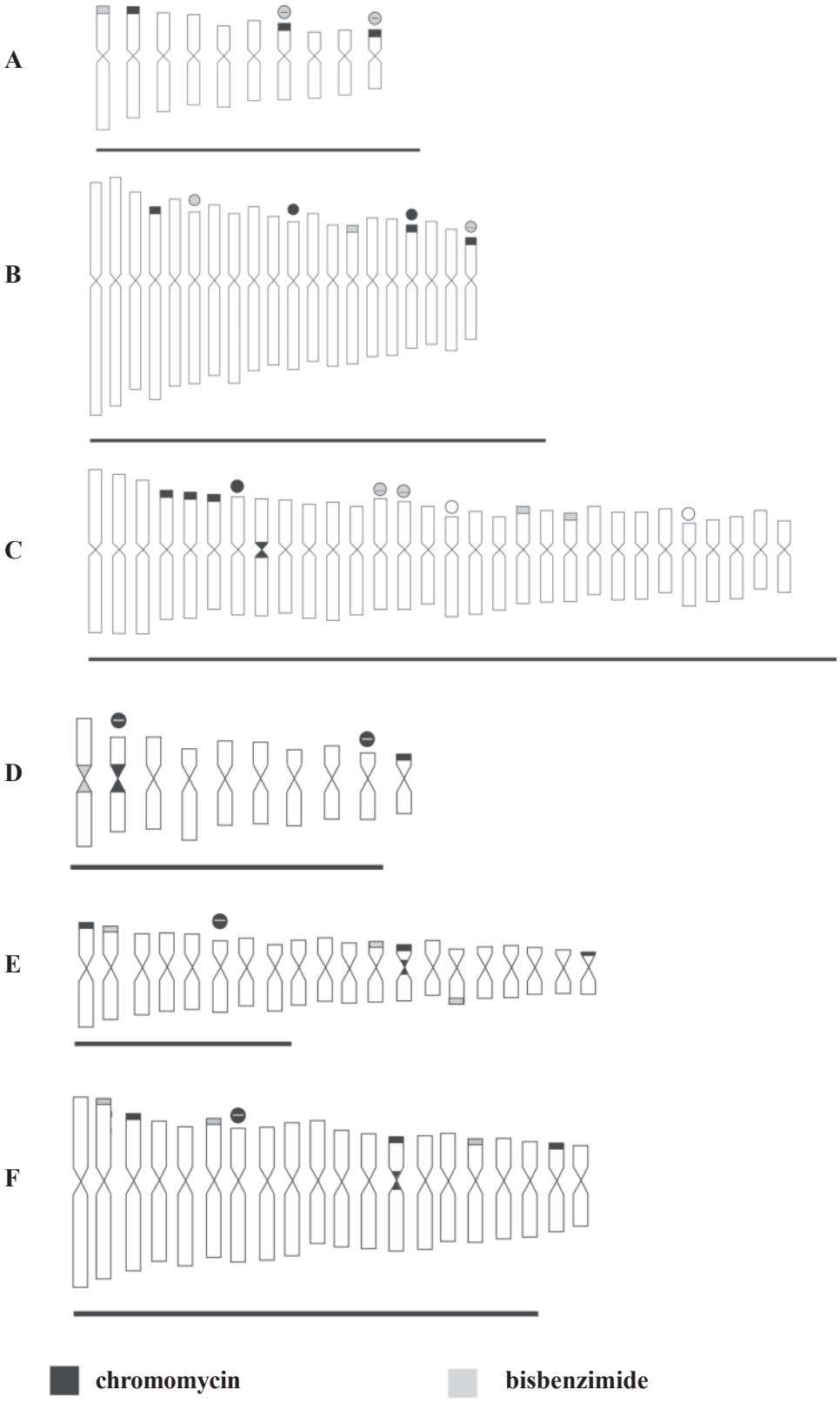

Figure 4. Haploid idiograms of the studied populations with the location of GC-rich and AT-rich regions. Round dots indicate satellite markers. Chromomycin positive: solid black; bisbenzimide positive: grey. (A), *Centaurea toletana* (2*x*); (B), *Centaurea toletana* (4*x*); (C), *Centaurea argecillensis* (estimated position of the satellites); (D), *Centaurea gabrielis-blancae*; (E), *Centaurea ornata*; (F), *Centaurea haenseleri.* Scale bars: 10 μ.

netic exchange with the diploid ones, we can expect some differences, and diploid and tetraploid *C. toletana* do not coexist because their soil requirements are not compatible (FERNÁNDEZ CASAS & SUSANNA, 1986).

Second, and probably the most important factor, tetraploid *C. toletana* is a typical colonizing polyploid (STEBBINS, 1950; LEVIN, 1983). While diploid populations of *C. toletana* grow only on siliceous soils, tetraploid populations have a wider ecological tolerance and have colonized a very large extension in central Spain on clay soils, occupying frequently somewhat disturbed habitats (FERNÁNDEZ CASAS & SUSANNA, 1986). Multiple origins of these successful, colonizing polyploids are now accepted as a rule (SOLTIS & SOLTIS, 1993 and 1995; VAN DIJK & BAKX-SCHOTMAN, 1997). The model of distribution of diploid *C. toletana* also supports a multiple origin of the tetraploid race: the diploid cytotype grows in small and disjunct populations in which genetic drift could easily lead to partial reproductive isolation, favouring the occurrence of polyploidy, as was suggested for *Centaurea* and related genera by VILATERSANA *et al.* (2000) and GARNATJE *et al.* (2001). Our preliminary tests using RAPDs (FONT, unpublished data) suggest that there are differences between diploid populations of *C. toletana*. Thus, the origin of the tetraploid would be in multiple events of intraspecific hybridization between partly genetically isolated diploid individuals, rather than a single duplication in a population (intraspecific hybridization has been suggested for many other autoployploids by SOLTIS *et al.* (2004). This would justify the differences found between a simple duplication of the 2*x* genome and our results.

Regarding hexaploid *C. argecillensis*, we have found no cytogenetic connections to the two other cytotypes of the polyploid series. An outstanding difference is the presence in the hexaploid species of a centromeric chromomycin-positive region, absent from both ploidy levels (diploid and tetraploid) of *C. toletana*, making it difficult to point out autopolyploidy as the mechanism of origin for the 6*x* taxon from the 2*x* and 4x one. Morphological relationships to *C. toletana* are so obvious that cannot be disregarded, and populations of *C. toletana* with tetraploid cytotype are common in the area of distribution of *C. argecillensis*. However, *C. argecillensis* is very probably an allopolyploid: the extreme morphological variability pointed out by FERNÁNDEZ CASAS & SUSANNA (1986) also suggests a hybrid origin. As was the case for the tetraploid, a multiple origin of the hexaploid is very probable, not only because of its variability, but also because the species is a very active colonizer (FERNÁNDEZ CASAS & SUSANNA, 1986). The question that remains open is the identity of the other species involved in the origin of *C. argecillensis*. Achene morphology is very reliable here: the wanting parental species must be probably a species of sect. *Chamaecyanus* because hybridization with species of sect. *Acrocentron* is reflected in the length of the pappus and in the frequent loss of the caulescent habit (revised in depth by GARCIA-JACAS, 1998). The identity of the second species that has originated *C. argecillensis* is a question that further studies using molecular markers and fluorescent and genomic *in situ* hybridization will try to make clear.

Centaurea ornata complex

Regarding the species belonging to the *C. ornata* series, we can observe in the diploid *C. gabrielis-blancae* the presence of four markings with the chromomycin staining, a positive centromeric band and another telomeric, and the presence of two negative satellites. However, we have found a single centromeric marking in the larger chromosome in the case of the bisbenzimide staining (Figs. 3G, 4D). In the two tetraploid species we observe four positive markings, three telomeric bands and a centromeric one, and a negative satellite marking with CMA in *C. ornata*, while with Hoechst staining we can see three telomeric markings. In *C. haenseleri* we also observe four positive CMA bands, three telomeric and one centromeric, together with a negative satellite. We must recall that the exact position of marks in two tetraploid species is different (Figs. 3H, 3I, 4E, 4F).

With respect to the hexaploid and endecaploid species, we can only point out the number of signals observed, since the high similarity in symmetry of the whole karyotype does not allow us to check which chromosomes bear the marks. In *C. saxicola* we can see with CMA staining a minimum of 10 markings, and also 10 with Hoechst, of which some are centromeric, and the presence of some negative satellites is also shown.

In *C. kunkelii* we observe 16 to 18 CMA markings and a minimum of six Hoechst bands, with two positive satellites.

In accordance with the results obtained, species from the *C. ornata* complex do not present a banding pattern allowing us to determine which has been the origin of the polyploid series. The bands present in the diploid species do not coincide with those revealed in the tetraploid and hexaploid species. A possible explanation to this fact could be the intra- and intergenomic reorganization of polyploid genomes, which can be extensive and may occur rapidly (Soltis *et al.*, 2003). In addition, tetraploid *C. ornata* does not share the same habitat that *C. gabrielis-blancae* (a diploid species) and *C. saxicola* (hexaploid). We must hypothesize that the formation of *C. saxicola* is an ancient event, and the long time ellapsed allowed extensive genomic reorganization.

The banding patterns obtained make it difficult to point out any relationship with *C. kunkelii* (endecaploid species); since it is not possible to determine to which chromosomes the bands belong. Anyway, the number of bands found in *C. kunkelii* does not agree with the number obtained in the other species. *Centaurea kunkelii* is known from a single population, which probably implies a long isolation. This isolation could also be the reason for a deep restructuring of the genome that has evolved in a different way.

In later analyses using nuclear-ribosomal DNA sequences of the ETS region (Font *et al.*, unpublished data) we have verified that the diploid and tetraploid species are grouped in the Iberian clade, whereas *C. saxicola* and *C. kunkelii* belong to a clade with the African species and some Iberian ones like *C. haenseleri*, *C. prolongi* Boiss. and *C. lainzii* Fern. Casas with which they share the area of distribution in South Spain. These results do not contradict our starting hypothesis that the possible progenitors of *C. kunkelii* are *C. saxicola* and *C. haenseleri*. Conversely, the ETS-based phylogeny groups *C. gabrielis-blancae* and *C. ornata*, a relationship that does not agree with the very different banding patterns obtained in the present work. In the case of *C. ornata*, this lack of correlation probably is caused by the multiple origins of polyploid species as previously stated.

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