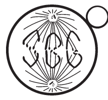

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Research Article

Intra- and interspecific chromosome polymorphisms in cultivated *Cichorium* L. species (Asteraceae)

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Abstract

Endive (*Cichorium endivia* L.) and chicory (*C. intybus* L.) both have $2n = 18$, but until now, there has been no detailed karyomorphological characterization. The present work evaluated five accessions of each species using FISH with rDNA probes and fluorochrome staining with CMA and DAPI. Both species presented distinct banding patterns after fluorochrome staining: while endive had proximal $CMA^{++}/DAPI^{-}$ bands in the short arms of pairs 1, 2 and 3, chicory had proximal CMA-positive bands in chromosomes 1 and 3 and interstitial in the short arm of chromosome 8. Among endive accessions, FISH procedures revealed conserved position and number of 5S and 45S rDNA sites (two and three pairs, respectively), associated with the CMA-positive bands. Notwithstanding, polymorphisms were detected within chicory accessions regarding the number and the distribution of rDNA sites in relation to the most frequent karyotype (two pairs with 45S and one with 5S rDNA). The karyological markers developed allowed karyotypic differentiation between both species, uncovering peculiarities in the number and position of rDNA sites, which suggest chromosome rearrangements, such as translocations in chicory cultivars. The interspecific and intraspecific polymorphisms observed emphasize the potential of karyomorphological evaluations, helping our understanding of the relationships and evolution of the group.

Keywords: compositae, Cichorieae, CMA/DAPI, rDNA sites, chromosome rearrangements.

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Introduction

The Cichorieae tribe (subfamily Cichorioideae, family Asteraceae) comprises about 70 genera and 1,500 species. The group is morphologically well characterized by the presence of latex and perfect flowers within the capitulum. Among the economically most important members of the tribe, lettuce (*Lactuca sativa* L.), chicory (*Cichorium intybus* L.) and endive (*C. endivia* L.) stand out due to their worldwide use as green salad vegetables (Bremer, 1994; Kilian *et al.*, 2009). While being less used than lettuce, the economic importance of chicory and endive is increasing, with growing incorporation in cooking recipes due to their nutritional value (Lucchin *et al.*, 2008), which impacts the size and distribution of the areas cultivated worldwide.

Controversies regarding the taxonomy of *Cichorium* have been raised since the 18th century, especially regarding the total number of species and their delimitation (Lucchin *et al.*, 2008). Some authors suggested the existence of three (Tutin *et al.*, 1976), four (Pignatti, 1982) or seven (Wagenitz and Bedarff, 1989) valid species. However, approaches based on AFLP markers (Kiers *et al.*, 2000) and ITS sequences (Kilian and Gemeinholzer, 2007) recognized six species subdivided into three distinct groups: (1) *C. bottae* A. Deflers., as sister to all the other species, (2) *C. intybus* and *C. spinosum* L., and (3) *C. endivia*, *C. pumilum* Jacq. and *C. calvum* Sch. Bip.

Chromosome studies using FISH (fluorescent *in situ* hybridization) and double fluorochrome staining with CMA (chromomycin A_3 - for GC-rich heterochromatic regions) and DAPI (4',6-diamidino-2-phenylindole - for AT-rich heterochromatic regions) revealed important karyotypic features in Asteraceae, including cultivated (*e.g.* sunflower, Vanzela *et al.*, 2002; lettuce, Matoba *et al.*, 2007) and wild species (Fregonezi *et al.*, 2004; Garcia *et al.*, 2010). Despite their informativeness, few previous reports are available which have employed these methods, especially considering the size and diversity of the family, as

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well as their relevance in an evolutionary context within the angiosperms (Watanabe *et al.*, 2007).

Previous cytogenetic evaluations for *C. endivia* and *C. intybus* using conventional staining reported the diploid number $2n = 18$ for both species and similar chromosome morphologies (*e.g.* Rick, 1953), thus were unable to effectively differentiate between species as has also been observed for other genera of Cichoriodeae (see Matoba *et al.*, 2007). Therefore, aiming to identify chromosomal differences between these species, 10 *Cichorium* accessions were analysed - five from *C. endivia* and five from *C. intybus* - using FISH with 5S and 45S rDNA probes and CMA/DAPI staining. The results uncovered interesting chromosome polymorphisms, especially in the number of 45S rDNA sites between both species, also revealing chromosome variations within *C. intybus* accessions, bringing interesting aspects to light for the understanding of their relationships and evolution.

Materials and Methods

Seeds of five accessions of *C. endivia* and five of *C. intybus* from different provenances (Table 1) were germinated in Petri dishes. Root tips were collected, pre-treated with 8-hydroxyquinoline (2 mM) at 8 °C for 24 h, fixed in 3:1 ethanol:acetic acid (v/v) at room temperature (ca. 25 °C) for 4-24 h and stored at -20 °C as described by Benko-Iseppon and Morawetz (2000). Fixed root tips were digested for 3 h at 37 °C in an enzymatic solution containing 2% (w/v) cellulase (`Onozuka R-10’, Serva) and 20% (v/v) pectinase (Sigma-Aldrich), incubated in 60% acetic acid for 20 min at 37 °C and squashed in a drop of 60% acetic acid. Coverslips were removed after freezing in liquid nitrogen and the slides were air-dried. Slides were aged for three days at room temperature and then stained with CMA (0.5 mg/mL, 1 h) and DAPI (1 μg/mL, 30 min), mounted in McIlvaine’s buffer (pH 7.0):glycerol (1:1, v/v) and stored for three days, according to Schweizer and Ambros (1994). Cell images were acquired using a Leica DMLB epifluorescence microscope and a Leica DFC 340FX camera with the Leica CW4000 software.

The R2 clone, with an 18S-5.8S-25S rDNA repeat unit (6.5 kb) isolated from *Arabidopsis thaliana* (L.) Heynh. (Wanzenböck *et al.*, 1997), and the D2 clone, which consists of two 5S rRNA repeats (~ 400 bp) isolated from *Lotus japonicus* (Regel) K. Larsen (Pedrosa *et al.*, 2002), were used as probes and were labelled by nick translation (Invitrogen) with digoxigenin-11-dUTP (Roche) and biotin-11-dUTP (Sigma), respectively. The FISH pre-treatment and post-hybridization washes were based on Pedrosa *et al.* (2002), in which the stringency wash (77%) was performed with 0.1x SSC at 42 °C. Chromosome and probe denaturation and detection were performed according to Heslop-Harrison *et al.* (1991) and Jiang *et al.* (1996), respectively, with minor modifications. The slides were denatured in 70% formamide at 90 °C for 10 min. The hybridization mixture, containing 50% formamide (v/v), 2x SSC, 10% dextran sulphate (w/v) and 2.5-5 ng/μL of probe, was denatured at 75 °C for 10 min. Each slide received 10 μL of the hybridization mixture and was hybridized at 37 °C for at least 18 h. Digoxigenin-labelled probes were detected using sheep anti-digoxigenin-FITC (Roche) and amplified with donkey anti-sheep-FITC (Sigma), in 1% (w/v) BSA. Biotin-labelled probes were detected with mouse anti-biotin (Dako) and the signal was visualized with rabbit anti-mouse TRITC conjugate (Dako), in 1% (w/v) BSA. Preparations were counterstained and mounted with 2 μg/mL DAPI in Vectashield (Vector) (1:1; v/v).

Cell images were acquired and optimized for contrast and brightness, while DAPI pictures were pseudo-coloured in grey with the Adobe Photoshop CS4 (Adobe Systems Incorporated) software. Metaphase chromosomes of three cells stained with DAPI from each accession were measured by using the MicroMeasure 3.3 (Reeves, 2001) software and the idiograms were constructed using Adobe

Table 1 - Analysed *Cichorium endivia* and *C. intybus* accessions with their provenances.

Accession	Provenance[A]	Additional information
Cichorium endivia L.	Flora-Frey[B]	winter endive
C. endivia subsp. *endivia* var. *endivia*	IPK[C] (CICH 23 Italy)	-
C. endivia subsp. *endivia* var. *crispum* Lam.	IPK (CICH 709 China)	Hua Yie Sheng Tsai
C. endivia subsp. *endivia* var. *latifolium* Lam.	IPK (CICH 388 Netherlands)	Bubikopf
C. endivia subsp. *divaricatum* (Schousb.) P. D. Sell	IPK (CICH 66 Italy)	-
C. intybus L.	Flora-Frey	-
C. intybus var. *intybus* Hegi	IPK (CICH 499 Germany)	Wild chicory
C. intybus var. *foliosum* Hegi	IPK (CICH 615 Netherlands)	Liber vo
C. intybus var. *foliosum* cv. Zoom Hegi	Kiepenkerlb[C]	-
C. intybus var. *sativum* Lam. & DC.	IPK (CICH 75 Czechoslovakia)	Slezka

[A]In parenthesis: registration number and place of origin, if informed. [B]Commercially available seeds. [C]Institut für Pflanzengenetik und Kulturpflanzenforschung (Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany).

Flash CS4 Professional (Adobe Systems Incorporated). Chromosomes were ordered in decreasing order according to their size. The chromosome arm ratio (AR = length of the long arm/length of the short arm) was used to classify chromosome morphologies as metacentric (AR = 1.00-1.49) or submetacentric (AR = 1.50-2.99), according to Guerra (1986).

Results

The chromosome numbers of all *C. endivia* (Figure 1A and 1B) and *C. intybus* (Figure 1C-G) accessions were stable (2*n* = 18), with gradually decreasing chromosome sizes. Size and morphology of the chromosomes, in combination with the cytogenetic markers allowed for the easily identification of all chromosome pairs in *C. endivia* and most of them in *C. intybus* (except for the pairs 5, 6 and 7). Chromosome complements showed averages of 2.84 μm per chromosome for *C. endivia* (ranging from 1.67 μm to 4.01 μm) and 3.20 μm for *C. intybus* (ranging 2.30 μm from to 4.29 μm). The average size of the whole diploid complement for *C. endivia* was 51.13 μm, and 57.58 μm for *C. intybus*.

Nucleolus organizing regions (NORs) were observed (usually not distended) in the three larger chromosome pairs (1, 2 and 3) of *C. endivia* (Figure 2A), while only pairs 1 and 3 of *C. intybus* presented satellites (Figures 2B-D). Chromosome morphology was metacentric, except for *C. intybus* var. *foliosum* cv. Zoom that displayed a submeta-

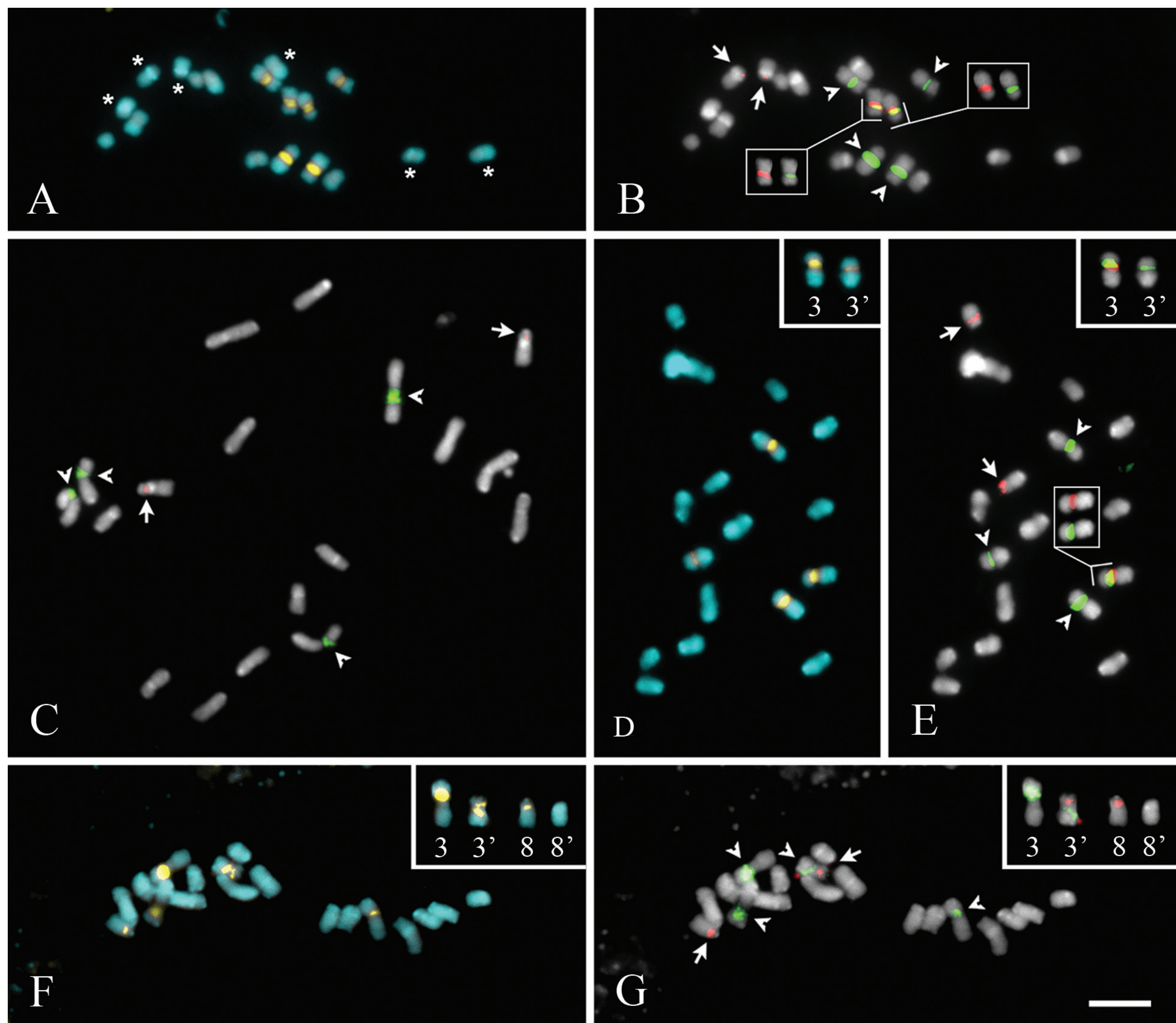

Figure 1 - Metaphase chromosomes of *Cichorium endivia* subsp. *endivia* (A and B), *C. intybus* var. *foliosum* (C), *C. intybus* var. *intybus* (D and E), and *C. intybus* var. *foliosum* cv. Zoom. (F and G). Superposition of chromosomes stained with DAPI (blue) and CMA (yellow) (A, D and F). Chromosomes hybridized with 45S (green) and 5S (red) rDNA probes, counterstained with DAPI (grey) (B, C, E and G). Asterisks indicate DAPI$^+$ bands; arrows and arrowheads indicate 5S and 45S rDNA sites, respectively; inside inserts in B and E indicate chromosomes with both 5S and 45S rDNA sites; and right corner inserts in D, E, F and G indicate heteromorphic chromosome pairs. Scale bar in G corresponds to 5 μm.

centric pair (chromosome 7) and two heteromorphic pairs (3 and 8), each with a submetacentric and a metacentric chromosome (Figure 2D). Considering the similarity regarding the chromosome measurements for *C. endivia* (Figure 2A) and *C. intybus* (Figure 2B) accessions, the sampled mean values for each species were grouped into single idiograms, except for *C. intybus* var. *intybus* Hegi (Figure 2C) and *C. intybus* var. *foliosum* cv. Zoom (Figure 2D) that were processed in exclusive idiograms.

The CMA/DAPI staining revealed the prevalence of $CMA^{++}/DAPI^{-}$ heterochromatin for both species. In *C. endivia,* bands were visible in the short arm of pairs 1, 2 and 3 (Figures 1A and 2A), while in *C. intybus* the same distribution was observed in pairs 1 and 3 (Figures 1D and 2B), with an additional faint $CMA^{+}/DAPI^{-}$ band in the intercalary region of the short arm of pair 8 (data not shown; Figure 2B). Additionally, two *C. intybus* accessions showed heteromorphic chromosomes, regarding both size and distribution of CMA bands. In *C. intybus* var. *intybus*, a larger $CMA^{++}/DAPI^{-}$ band was observed in one chromosome of pair 3 (Figures 1D and 2C). On the other hand, *C. intybus* var. *foliosum* cv. Zoom displayed two heteromorphic chromosome pairs. In pair 3, one of the homologues exhibited a proximal band in the short arm, while the other chromosome had two $CMA^{++}/DAPI^{-}$ bands (one intercalary in the short arm and the other in the proximal region of the long arm) (Figures 1F and 2D). Additionally, in pair 8, besides a small difference between chromosome sizes, one homologue displayed an intercalary $CMA^{++}/DAPI^{-}$ band in the short arm, while the other presented no bands (Figures 1F and 2D).

Additional proximal $CMA^{0}/DAPI^{+}$ bands were visible in the short arms of chromosome pairs 5, 6 and 8 in *C. endivia* (Figures 1A and 2A). Nevertheless, small terminal $CMA^{0}/DAPI^{+}$ bands were observed in *C. intybus*, which were not always visible, especially when the chromosomes were fully condensed (Figure 1D,F). Therefore, these small marks were not represented in the idiogram. In both cases, these additional bands were enhanced after FISH procedures, as well as other marks that became visible in terminal and proximal positions (noticeable when comparing the DAPI staining in Figures 1A and D - before FISH, with C, E, F - after FISH).

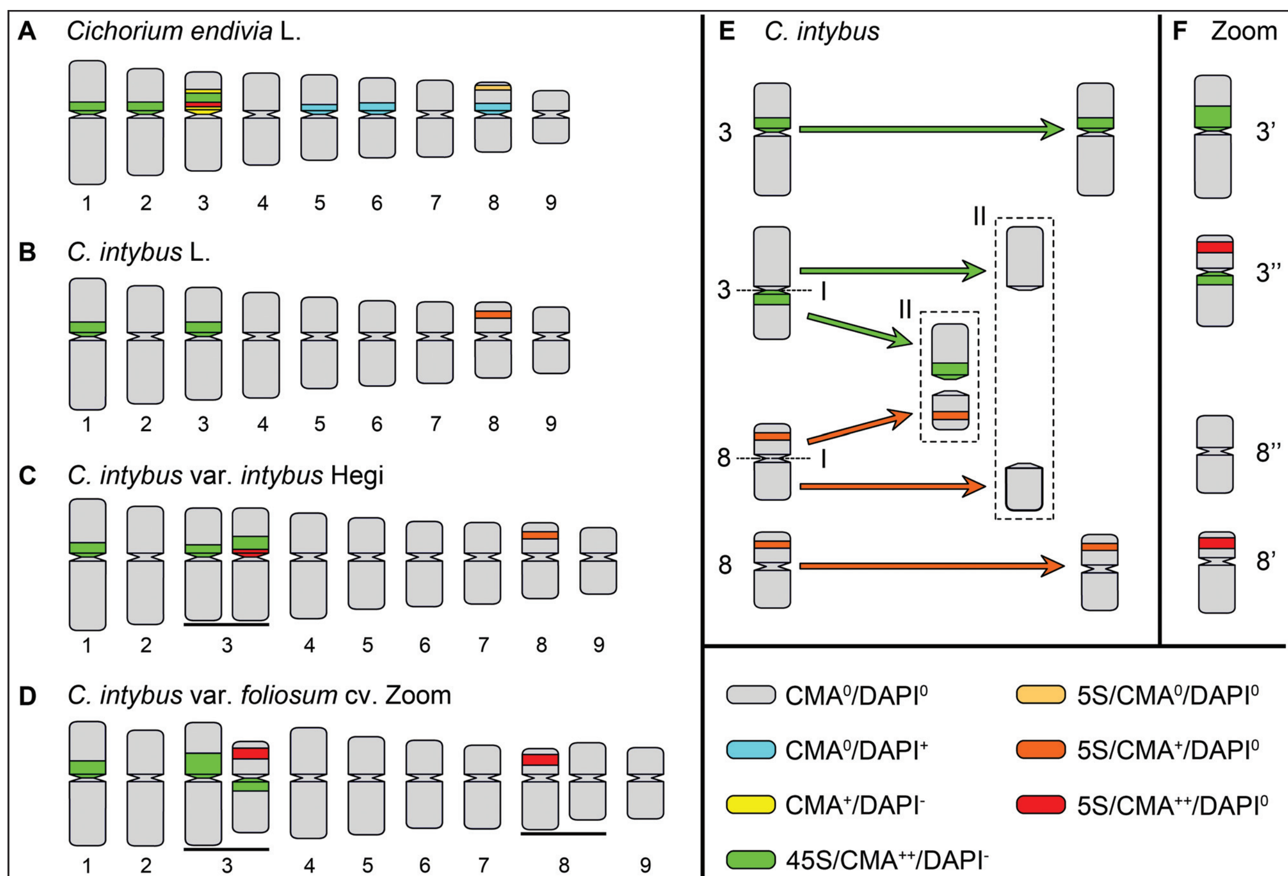

Figure 2 - Schematic representation of *Cichorium endivia* and *C. intybus* chromosomes: idiograms of *C. endivia* (A), *C. intybus* (B), *C. intybus* var. *intybus* (C) and *C. intybus* var. *foliosum* cv. Zoom (D). Heteromorphic pairs in *C. intybus* var. *intybus* and *C. intybus* var. *foliosum* cv. Zoom accessions are underlined (C and D). Representation of possible reciprocal translocation with breakages in the centromeres (centric fission - I, and fusion - II), involving a homologue of pairs 3 and 8 of *C. intybus* (E), in the genesis of the heteromorphic chromosomes pairs of *C. intybus* var. *foliosum* cv. Zoom cytotype (F).

The FISH assay using the 45S rDNA probe uncovered signals in the proximal region of the short arms of the three larger chromosome pairs of *C. endivia*, which were co-localized with the CMA^{++}/DAPI^{-} bands (Figures 1B and 2A). Only in pair 3, did the 45S rDNA site occupied a part of the CMA^{++}/DAPI^{-} band (Figure 2A). Regarding the distribution of the 5S rDNA, two chromosome pairs had hybridization signals: one site was observed in a proximal position of the short arm in pair 3 (co-localized with a CMA^{++}/DAPI^{-} band), adjacent to the distal 45S rDNA site, while the other was visible in the subterminal region of the short arm in pair 8, with no associated fluorochrome band (Figures 1B and 2A).

The FISH assay using the 45S rDNA probe in *C. intybus* revealed two pairs of hybridization sites in the proximal region of the short arms in pairs 1 and 3, and, as observed for *C. endivia*, those sites were co-localized with CMA^{++}/DAPI^{-} bands (Figures 1C and 2B). The FISH procedures with the 5S rDNA probe showed evidence for a single site in the intercalary position of the short arm in pair 8, co-localized with a CMA^{+}/DAPI^{-} band (Figures 1C and 2B). In *C. intybus* var. *intybus*, an extra 5S rDNA site was observed in one chromosome of pair 3 (Figures 1D and 2C).

In *C. intybus* var. *foliosum* cv. Zoom, a heteromorphism related to the distribution of the rDNA sites could be detected in pairs 3 and 8 (Figures 1G and 2D). Regarding the 45S rDNA site of pair 3, one of the homologues displayed a proximal site in the short arm, similar to the other accessions, but slightly larger. However, the other site was in a proximal region of the long arm at the homologue of this chromosome pair. For 5S rDNA, an intercalary site was observed in the short arm of a homologue of pair 3 (carrier of 45S rDNA) while the other was intercalary in the short arm of a homologue of pair 8. The second homologue of pair 8 exhibited no marks. In this accession, all rDNA sites co-localized with the CMA^{++}/DAPI^{-} bands (Figures 1F-G and 2D).

Discussion

Previous karyotypic analyses of *Cichorium* species were restricted to chromosome counts regarding the species *C. endivia* (Rick, 1953), *C. intybus* (Dobes *et al.*, 1997), *C. intybus* var. *intybus* (Lövkvist and Hultgård, 1999) and *C. spinosum* (Montmollin, 1986), all of them with $2n = 2x = 18$. This chromosome number is very frequent within the Cichorioidae, as observed in *Lactuca*, *Sonchus* and *Tolpis*, being considered conserved throughout the subfamily (Bremer, 1994).

Contrary to the classic cytogenetic methods, which could not elucidate the chromosome evolution within other Cichorioideae genera, the distribution of heterochromatic blocks has been shown to be a very informative approach (*e.g.* Matoba *et al.*, 2007). Thus, the presence of repetitive DNA clusters (rDNA sites and fluorochrome bands) observed in both *C. endivia* and *C. intybus*, as well as differences in their distribution patterns, suggest the great importance of such DNA elements during the karyotype evolution within the genus.

In general, 45S rDNA sites occur either in terminal or sub-terminal position of chromosomes within subfamilies of Asteraceae, as observed in *Chaptalianutans* (subfamily Mutisioideae) (Fregonezi *et al.*, 2004) and in the genera *Lactuca* (Matoba *et al.*, 2007), *Tragopogon* (Garcia *et al.*, 2010) and *Vernonia* (Salles-de-Melo *et al.*, 2010) from the subfamily Cichorioideae. However, despite the prevalence of terminal NORs in karyotypes of angiosperms as a whole (Roa and Guerra, 2012), both *C. endivia* and *C. intybus* had only proximal NORs. Similarly, proximal NORs have been reported in karyotypes of *Achyrocline* spp. (subfamily Asteroideae) *Hypochaeris* spp. (subfamily Cichorioideae), which may also be evidences of structural changes (such as paracentric inversions) throughout chromosome evolution within these plant groups (Cerbah *et al.*, 1998; Ruas *et al.*, 2005; Mazzella *et al.*, 2010).

The co-localization of CMA/DAPI bands and 45S rDNA sites in the analysed species revealed the presence of CMA^{++} heterochromatin associated with NORs. This association has also been previously described for Asteraceae (Fregonezi *et al.*, 2004; Mazzella *et al.*, 2010; Salles-de-Melo *et al.*, 2010) and has been shown to be quite frequent within angiosperms (Guerra, 2000). On the other hand, the occurrence of CMA^{+} bands in association with 5S rDNA sites in the family has been mainly associated with the co-localization of 45S and 5S rDNA sites, as observed for species of *Achyrocline* (Mazzella *et al.*, 2010) and *Artemisia* (Pellicer *et al.*, 2008; Konowalik *et al.*, 2010). Moreover, as occurred for both species herein analysed, *Hypochaeris catharinensis* Cabrera also showed co-localization of CMA^{+} bands and 5S rDNA (Reck *et al.*, 2011), a feature described for other higher plants (*e.g.* Cabral *et al.*, 2006; Vasconcelos *et al.*, 2010).

In *C. intybus* var. *foliosum* cv. Zoom, an additional, more intense band (CMA^{++}/DAPI^{-}) associated with the 5S rDNA site was observed, suggesting an amplification of this site, a fact confirmed by a higher intensity of both CMA staining and FISH signals. On the other hand, the lack of CMA-positive bands in the 5S rDNA site of the chromosome 8 of *C. endivia* may probably be related to either a considerably lower number of rDNA repetitions or a distinct GC content in the intergenic sequences Additionally, epigenetic changes in chromatin conformation due to cytosine methylation or post-translational histone changes could affect the CMA association (see Cabral *et al.*, 2006, and references therein).

In contrast to the published results for *Lactuca* species in which the number of rDNA sites were very stable (two pairs with 45S and one with 5S rDNA; Matoba *et al.*, 2007), we observed a slight difference regarding the number of chromosome pairs bearing 45S and 5S rDNA sites between the analysed species (three pairs with 45S and two

with 5S rDNA for *C. endivia*, and two pairs with 45S and one with 5S rDNA for *C. intybus*). Similarly to the *Cichorium* species, *Tragopogon* species (Cichorieae; with $2n = 2x = 12$) also exhibited variation regarding the number of 45S rDNA sites among *T. dubius* Scop. and *T. pratensis* L. (both with one pair of sites) and *T. porrifolius* L. (two pairs) (Pires *et al.*, 2004).

Contrary to the similarity among different *C. endivia* accessions, the two distinct karyotypes noticed among *C. intybus* accessions indicate the occurrence of recent chromosome changes involving rDNA sites in cultivated varieties. The occurrence of an extra 5S rDNA site in one chromosome of pair 3 of *C. intybus* var. *intybus* may be an indication of non-homologous recombination between chromosome pairs 3 and 8, followed by amplification of 5S rDNA repetitive motifs at the new location. On the other hand, the presence of two heteromorphic pairs (3 and 8) in the karyotype of *C. intybus* var. *foliosum* cv. Zoom seem to be the result of a reciprocal translocation with breakages in the centromeres (centric fission/fusion) involving a homologue of each pair (Figure 2E,F). Besides the detected changes, other rearrangements may have occurred, including the amplification of the heterochromatin that was co-localized with the 45S rDNA in the first homologue of the pair 3 and the 5S rDNA sites, and the reduction of part of the second homologue chromosome of pair 8, possibly by means of deletion related or not to the translocation process. Additionally, a different morphology was observed in the submetacentric pair 7 and the first homologue of pair 8 (both with a larger long arm).

The present results uncovered interspecific differences between *C. endivia* and *C. intybus*, besides the occurrence of some unexpected intraspecific karyotypic variations among *C. intybus* accessions. The identified features represent the first pieces of evidence regarding the distribution of the main chromosome markers within this genus, revealing high diversity in the apparently homogeneous karyotypes previously described after standard staining.

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