

Karyological evolution in *Rhaponticum* Vaill. (Asteraceae, Cardueae) and related genera

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Abstract: Chromosome counts are reported in 29 populations representing 20 species and one subspecies of the genera *Callicephalus* (1 sp.), *Klasea* (7 sp.), *Myopordon* (2 sp.), *Oligochaeta* (1 sp.) and *Rhaponticum* (9 sp., one subsp.); Eleven reports are new, the others supplement limited previous data. New and published cytological data are summarized and critically evaluated in the light of current taxonomic treatments and evolutionary hypotheses. Basic chromosome numbers are a valuable source of taxonomic information and relate well to the phylogeny. They are quite conserved in *Klasea* (only $x = 15$), the sister group of the remaining genera and in the early branching genera of the *Rhaponticum* group ($x = 14$ for *Centaurothamnus* and *Callicephalus*). However, a more complex pattern of genome evolution is present among the more derived clades, *Oligochaeta* plus *Myopordon* ($x = 12$ and 14) and the genus *Rhaponticum* ($x = 12, 13$). The occurrence of $x = 12$ in *Oligochaeta divaricata* and *Rhaponticum carthamoides* seems to be the result of a recent event of chromosome fusion, as suggested by the high fragility of particularly long pairs of chromosomes. The use of chromomycin staining, including an enzymatic digestion step that facilitates spreading and permits a reduction of potential fragmentation, only partially resolves this problem.

ADDITIONAL KEYWORDS: Centaureinae - chromomycin A₃ banding - chromosome basic number - Compositae - dysploidy - *Klasea* - polyploidy - symploidy.

INTRODUCTION

Rhaponticum Vaill. and the related genera *Callicephalus* C. A. Mey., *Centaurothamnus* Wagenitz & Dittrich, *Myopordon* Boiss., *Ochrocephala* Dittrich and *Oligochaeta* (DC.) C. Koch constitute a natural group of ca. 40 species ranged among the early branching taxa of the Asteraceae, Cardueae-Centaureinae (Hidalgo *et al.*, 2006). Recent phylogenetic reconstructions based on molecular data have considerably modified the generic delimitation of the group (Garcia-Jacas *et al.*, 2001; Hidalgo *et al.*, 2006; Susanna *et al.*, 2006). The *Rhaponticum* group first included the genera *Acroptilon* Cass., *Leuzea* DC. and *Rhaponticum* (Dittrich, 1977), three taxa that are currently considered as synonymous of *Rhaponticum* (Hidalgo *et al.*, in press). Such a delimitation leads to strong character homogeneity, especially from a karyological point of view. Despite the fact that previous chromosome counts of *Acroptilon*, *Leuzea* and *Rhaponticum* representatives are incomplete, always isolated and often doubtful, they show mainly $2n = 26$ chromosomes (Guinochet, 1957). The recent enlargement of the group has included genera of unknown chromosome number, such as *Myopordon* and *Ochrocephala*, together with genera for which previous chromosome counts are already available. *Callicephalus* and *Centaurothamnus* show $2n = 28$ (Wagenitz, Dittrich & Damboldt, 1982 for the latter; Chouksanova, Sveshnikova & Alexandrova, 1968; Hellwig, 1994; Garcia-Jacas *et al.*, 1998 for the former), whereas *Oligochaeta* exhibits $2n = 24$ or 28 chromosomes. All the counts of *O. ramosa* (Roxb.) Wagenitz report $2n = 28$ (Bhandari & Singhir, 1977; Gupta & Gill, 1981, 1983, 1984, 1989; Razaq, Vahidy & Ali, 1994). Those of *O. divaricata* (Fisch. & C. A. Mey.) C. Koch are not uniform: $2n = 24$ (Tonjan, 1968, 1980; Avetisian & Tonjan, 1975; Ghaffari, 1999) or $2n = 28$ (Garcia-Jacas *et al.*, 1998). Furthermore, molecular data have defined the genus *Klasea* Cass. as closely related to the *Rhaponticum* group (Hidalgo *et al.*, 2006). *Klasea* exhibits $x = 15$, with many ploidy levels leading to $2n = 30, 60$ and 90 (Löve & Löve, 1961; Cantó, 1982, 1984, 1985; Garcia-Jacas *et al.*, 1998, Garcia-Jacas, Susanna & Mozaffarian, 1998). This diversity in chromosome counts, as a consequence of the generic re-delimitation of the *Rhaponticum* group, makes the group an interesting subject for evolutionary studies based on karyological data. Cytological data are of great importance in the study of plant evolution and diversification (Levin, 2002), and have been successfully used in studies of several groups of the subtribe Centaureinae (Garcia-Jacas, Susanna & Ilarslan 1996; Vilatersana *et al.*, 2000).

The aims of this first karyological study focused on *Rhaponticum* and its relatives were; (1) to verify or establish the chromosome numbers of several representatives of the

genera belonging to the re-defined *Rhaponticum* group and the genus *Klasea*; (2) to establish the main processes of karyological evolution in our focus of study; (3) to discuss the results on a phylogenetic basis in order to infer where changes in basic chromosome numbers took place in the course of the evolutionary history of *Rhaponticum* and its relatives.

MATERIAL AND METHODS

Plant material

The studied plants came from germinated achenes, either collected in the field or obtained from Botanical Gardens. The sampling was done on the basis of our earlier molecular work (Hidalgo *et al.*, 2006). The origin of the samples and voucher information are given in Table 1.

Chromosome preparation

Root tip meristems were obtained either by germinating achenes on wet filter paper in Petri dishes at room temperature, or from plants cultivated in pots in a greenhouse. They were pretreated with 0.002 M 8-hydroxyquinoline for 3 at 16 °C. The material was fixed in 3:1 v/v absolute ethanol:glacial acetic acid for 24-48 h at -4 °C, and stored in 70% ethanol at -20 °C.

Chromosome number assessment:

Meristems were hydrolyzed in 1N HCl for 12 min at 60 °C, and stained in Schiff's reagent for 30 min in the dark. Squashes were made in acetocarmine. Preparations were made permanent by freezing, ethanol-dehydrating and mounting in Euparal (Agar Scientific Ltd., Stansted, Essex, UK). Metaphase plates were photographed with a Lock Focus JVC color video KY-F50 camera.

Some of the metaphase plates obtained showed telocentric chromosomes, which are not usual. To ensure that they are not a product of chromosome fragmentation, we used chromomycin staining, a technique that includes an enzymatic digestion step, which facilitates the spreading of chromosomes and minimizes fragmentation.

Chromomycin A₃ (CMA₃) banding

Samples were washed in citrate buffer (0.01 M citric acid-sodium citrate, pH = 4.6) for 10 min at room temperature, and incubated at 37 °C for 20-40 min in an enzyme mixture [4%

Table 1. Origin of the material, somatic chromosome number ($2n$), ploidy level, and previous chromosome counts of the corresponding species

Species	Voucher	$2n$, Fig.	Previous counts
<i>Klasea alatanica</i> C. A. Mey.	KAZAKHSTAN: Jambulskaiia Ob., Kurdai mountain pass, <i>Susanna 2151 et al.</i> (BC)	$4x=60$ (Fig. 1)	First count
	KAZAKHSTAN: 120 km W of Almaty, <i>Susanna 2214 et al.</i> (BC)	$4x=60$ (Fig. 2)	
<i>K. algida</i> (Iljin) Hidalgo	TAJIKISTAN: mountains above Kara-Churá, <i>Susanna 2558 & Romashchenko</i> (BC)	$2x=30$ (Fig. 3)	First count
<i>K. centauroides</i> (L.) Cass.	MONGOLIA: Chogno-Chan-Uul, <i>Bot. Gard. Osnabrück 01-163</i>	$2x=30$ (Fig. 4)	New ploidy level
	RUSSIA: Tannu mountains, Tuva, <i>Bot. Gard. of Siberia Centralis, Novosibirsk 2003/2004-1065</i>	$4x=60$ (Fig. 5)	
<i>K. cerintbifolia</i> (Sm.) Greuter & Wagenitz	IRAN: Azarbayjan-e-Gharbi, Orumiyeh, near Boulherha, <i>Susanna 1700 et al.</i> (BC)	$2x=30$ (Fig. 6)	Garcia-Jacas, Susanna & Mozaffarian (1998).
	LEBANON: Aanjar, <i>Hidalgo & Romashchenko 423</i> (BC)	$2x=30$ (Fig. 7)	
<i>K. lyratifolia</i> (Schrenk) L. Martins	KAZAKHSTAN: Shimkientskaya Ob., Aksu Džabagly reservation, Aksu canyon, <i>Susanna 2172 et al.</i> (BC)	$2x=30$ (Fig. 8)	First count
<i>K. cf. sogdiana</i> Bunge	KAZAKHSTAN: Shimkendskaiia Ob., Mayevka, <i>Susanna 2193 et al.</i> (BC)	$2x=30$ (Fig. 9)	First count
<i>Callicephalus nitens</i> (M. Bieb. ex Willd.) C. A. Mey.	ARMENIA: Ararat, Khosrov reservation, <i>Susanna 1578 et al.</i> (BC)	$2x=28$ (Fig. 10)	$2n = 28$ Chouksanova <i>et al.</i> (1968), Hellwig (1994), Garcia-Jacas <i>et al.</i> (1998); $2n = 32$ Tonjan (1968), Avetisian & Tonjan (1975), Tonjan (1980)
	IRAN: 6 km from the cross-road to Baladeh on the road to Chalus, <i>Susanna 1623 et al.</i> (BC)	$2x=28$	
<i>Myopordon persicum</i> Boiss.	IRAN: South of Borujend, Sabzeh Kuh, <i>Romashchenko & Susanna 2610</i> (BC)	$2x=28$ (Fig. 11)	First count
<i>M. pulchellum</i> (Winkler & Barbey) Wagenitz	LEBANON: Jabal el Mekmel, <i>Romashchenko 407 et al.</i> (BC)	$2x=28$ (Fig. 12)	First count
<i>Oligochaeta divaricata</i> (Fisch. & C. A. Mey.) K. Koch	ARMENIA: Ashtarak, between the villages of Aragat and Udzhan, <i>Susanna 1583 et al.</i> (BC)	$2x=24$ (Fig. 13, 14, 15)	$2n = 24$ Tonjan (1968, 1980), Avestian & Tonjan (1975); $n = 12$ Ghaffari (1999); $2n = 28$ Garcia-Jacas <i>et al.</i> (1998)

<i>Rhaponticum australe</i> (Gaudich.) Soskov	AUSTRALIA: <i>Funk</i> 12203 (BC)	2x=26 (Fig. 16)	First count
<i>R. carthamoides</i> (Willd.) Iljin	RUSSIA: Novosibirsk, Altai, <i>Bot. Gard. of Siberia Centralis, Novosibirsk 2003/2004-1062</i> (BC)	2x=24 (Fig. 17, 18)	2n = 24 Fedorov (1974), Krogulevich (1976), Stepanov (1994); n = 16 Rostovtseva (1979); 2n = 26 Rostovtseva (1979)
<i>R. integrifolium</i> C. Winkl.	TAJIKISTAN: mountains above Mazor, <i>Susanna</i> 2568 & <i>Romashchenko</i> (BC)	2x=26 (Fig. 19)	First count
<i>R. acaule</i> (L.) DC.	MOROCCO: Taza, Jbel Tazekka, Dayat Chiker, <i>Romo</i> 13093 <i>et al.</i> (BC) ALGERIA: 16 km N Aflou, <i>Montserrat</i> 2331 <i>et al.</i> (BC)	2n=2x=26 (Fig. 20) 2n=2x=26	2n=26 Guinochet (1957)
<i>R. berardioides</i> (Batt.) Hidalgo	MOROCCO: Grand Atlas Central, Ait Bougmaze valley, <i>Hidalgo & Romo</i> 12749 (BC)	2x=26 (Fig. 21)	2n = 26 Guinochet (1957), Galland (1988)
<i>R. coniferum</i> (L.) Greuter	SPAIN: Girona, Serra d'Altrera, <i>Font s. n.</i> (BC) SPAIN: Guadalajara, near Brihuega, <i>Garcia-Jacas & Susanna</i> 2685 (BC)	2x=26 (Fig. 22) 2x=26	2n = 26 Lorenzo-Andreu & García-Sanz (1950), Guinochet (1957), Dittrich (1968), Hellwig (1994), Van Loon, Gadella & Kliphuis (1971), Verlaque, Reynaud & Vignal (1995); 2n = 18 Lorenzo-Andreu & García-Sanz (1950), Afzal-Rafii <i>et al.</i> (1985); 2n = 22 Colombo, Marceno & Princiotta (1979)
<i>R. cynaroides</i> Less.	SPAIN: Huesca, <i>Hidalgo</i> 504 <i>et al.</i> (BC)	2x=26 (Fig. 23)	2n = 26 Fedorov (1974).
<i>R. fontqueri</i> (Sauvage) Hidalgo	MOROCCO: West Rif, Jbel Lakraa, <i>Hidalgo & Romo</i> 12621 (BC)	2x=26 (Fig. 24)	First count
<i>R. heleniifolium</i> Godr. & Gren.	FRANCE: Alps, mountain pass of Gleize, near Gap, <i>Hidalgo</i> 506 (BC)	2x=26 (Fig. 25)	First count
<i>R. heleniifolium</i> subsp. <i>bicknellii</i> (Briq.) Greuter	ITALY: vallée d'Aoste, la Thuile, <i>Bot. Gard. of Pietra Corva</i> 98-2001 (BC) ITALY: Alps, near Passo della Guardia, <i>Hidalgo</i> 508 & <i>Romashchenko</i> (BC)	2x=26 (Fig. 26) 2x=26	First count
<i>R. scariosum</i> Lam.	FRANCE: Alps, Champsaur valley, path between le Jas de Jartier and le Cellon, <i>Hidalgo</i> 507 (BC) SLOVENIA: Lemež, <i>Bot. Gard. of Universitatis Labacensis Slovenia</i> 2003-863 (BC)	2x=26 (Fig. 27) 2x=26	2n = 26 Guinochet (1957)

cellulase Onozuka R10 (Yakult Honsha), 1% pectolyase Y23 (Sigma) and 4% hemicellulase (Sigma)] diluted to 50% in citrate buffer. For *Oligochaeta divaricata*, meristems were spread in a drop of 45% acetic acid. Coverslips were removed following the technique of Conger & Fairchild (1953), and slides were air-dried for 24 h. In the case of *Rhaponticum carthamoides* (Willd.) Iljin, after enzymatic treatment, meristems were centrifuged for 5 min at 4,000 rpm, twice in buffer, and once in fresh fixative (3:1 absolute ethanol:glacial acetic acid). The pellet was re-suspended in 50 μ l of fixative, dropped onto clean slides and air-dried. The banding was performed following the procedure of Schweizer (1976), with slight modifications according to Cerbah *et al.* (1995). Chromosome plates were observed with an epifluorescence microscope with Zeiss excitation and emission filter set 07. Fluorescent signals were analysed using a highly sensitive CCD camera (Princeton Instruments, Evry, France) and image analyser (Metavue).

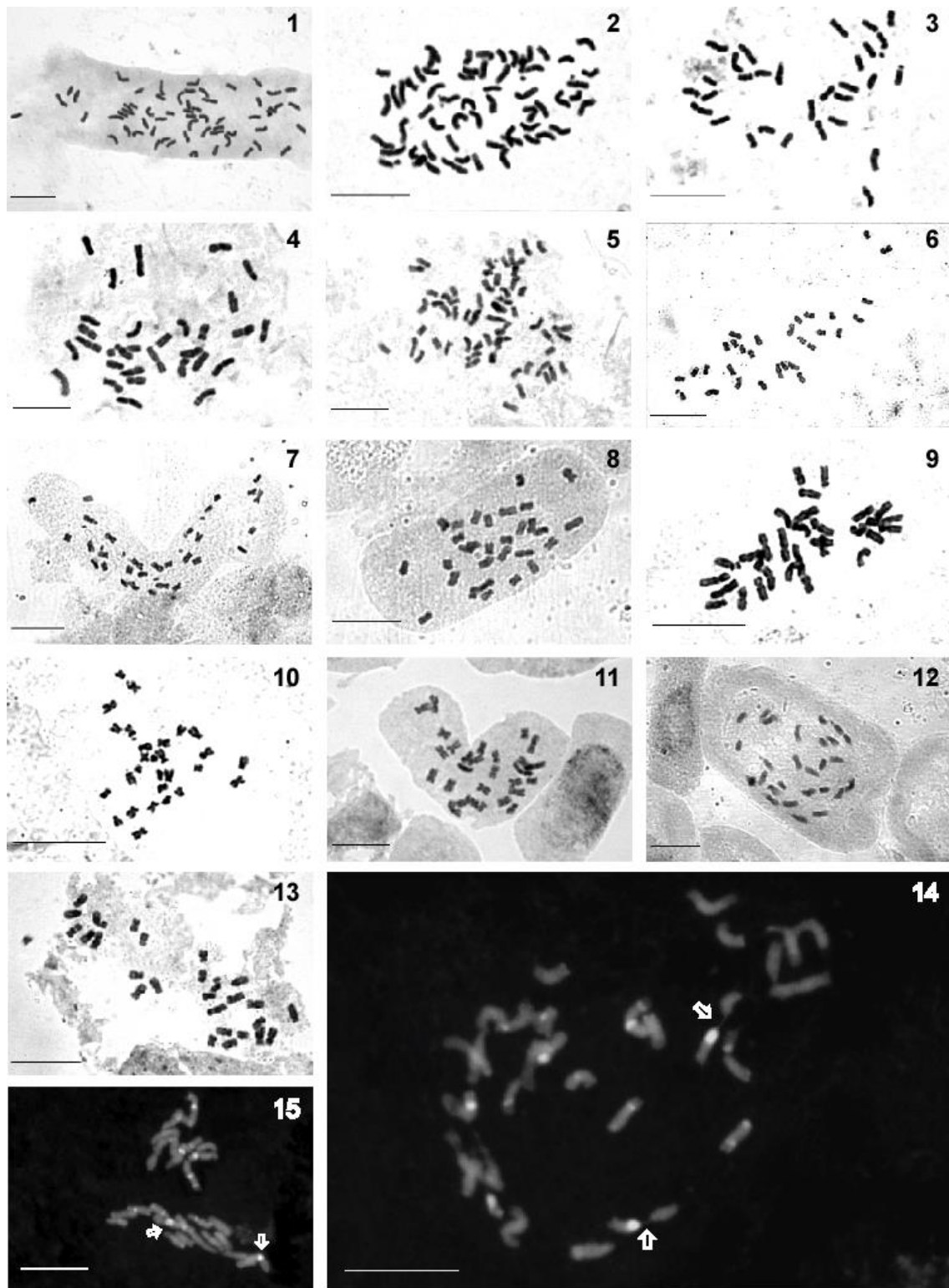
RESULTS AND DISCUSSION

The origin of the material, results of the new chromosome counts, and previous data for the corresponding taxa are shown in Table 1.

Dysploidy versus polyploidy: representation of these two main strategies

One evident outcome of this study is that the representatives of the *Rhaponticum* group show various basic chromosome numbers ($x = 14, 13$ and 12) but all the species are diploid (or more precisely ancient polyploids which have completed diploidization; Stebbins, 1971), whereas species of the genus *Klasea* have a constant basic number of $x = 15$ and exhibit various ploidy levels.

In the case of *Rhaponticum* and related genera, not all the basic numbers of its strong dysploid series are represented equally. While $x = 14$ and 13 are common (*Centaurothamnus*, *Callicephalus*, *Myopordon*, and *Oligochaeta ramosa* have $x = 14$; and most of the *Rhaponticum* species show $x = 13$; only two species, *Oligochaeta divaricata* and *Rhaponticum carthamoides*, have $x = 12$ *Oligochaeta divaricata* presents chromosomes which are considerably larger than the others and displays centromeric fragility, indicating that chromosomal fusions (symploidy) could have taken place (Clark & Wall, 1996). The chromomycin staining has enabled the manifestation of this phenomenon, especially for a chromomycin marked chromosome



Figures 1-15. Somatic metaphase plates. Fig. 1. *Klasea alata* (S-2151), $2n = 60$. Fig. 2. *K. alata* (S-2214), $2n = 60$. Fig. 3. *K. algida*, $2n = 30$. Fig. 4. *K. centauroides* (Mongolia), $2n = 30$. Fig. 5. *K. centauroides* (Russia), $2n = 60$. Fig. 6. *K. cerinthifolia* (Iran), $2n = 30$. Fig. 7. *K. cerinthifolia* (Lebanon), $2n = 30$. Fig. 8. *K. lyratifolia*, $2n = 30$. Fig. 9. *K. sogdiana*, $2n = 30$. Fig. 10. *Callicephalus nitens* (Armenia), $2n = 28$. Fig. 11. *Myopordon persicum*, $2n = 28$. Fig. 12. *M. pulchellum*, $2n = 28$. Figs. 13-15. *Oligochaeta divaricata*. Fig. 13. $2n = 28$ “chromosome fragments”. Figs 14, 15 Fluorochrome banding with chromomycin. Arrows indicate a pair of larger chromosomes showing centromeric fragility. Scale bars = 10 μ m.

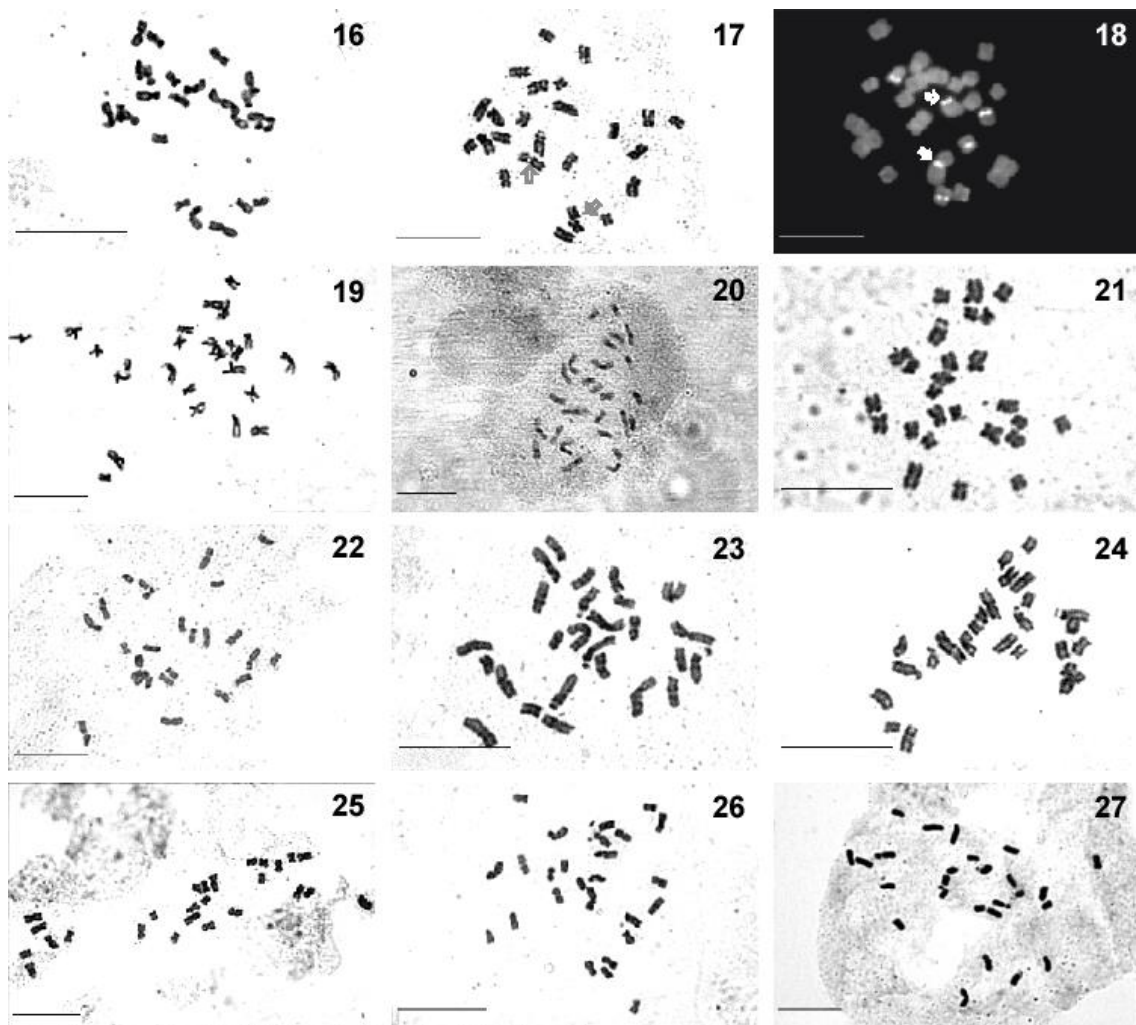
pair (Figs. 14, 15), while orcein or Feulgen staining used for the same material always lead to the observation of 28 “chromosomes” or chromosome fragments (Garcia-Jacas *et al.*, 1998 and Fig. 13). However, the finding of $x = 12$ is consistent with previous counts of *O.divaricata* (Table 1). In the case of *Rhaponticum carthamoides*, both Feulgen and chromomycin staining lead to variable results of $2n = 24$ (Fig. 17), 25 (Fig. 18) and 26. Our result of $x = 12$ agrees with most of the previous reports (Table 1), and with counts in taxa closely related to *R. carthamoides*, such as *R. chamarensis* Peschkova (Krogulevich, 1978) and *R. orientale* Serg. (Murín, 1997). Previous reports of $n = 13$ and $2n = 26$ (Rostovtseva, 1979) could have been a consequence of chromosome breakage. The phenomenon of chromosome fragmentation during the squash constitutes a serious problem concerning the reliability of the counts in *Oligochaeta* and *Rhaponticum*. It is especially true in the case of species showing acrocentric and telocentric chromosomes (in *R. australe* (Gaudich.) Soskov, for example, Fig. 16), which could in fact result from chromosome fragmentation. Nevertheless, it is interesting from an evolutionary perspective, since it indicates, were this the case, that fusion events occurred recently in evolutionary time.

Our results confirm those of Löve & Löve (1961), Cantó (1982, 1984, 1985), Garcia-Jacas *et al.*, (1998) and Garcia-Jacas, Susanna & Mozaffarian (1998) in establishing a constant chromosome base number of $x = 15$ for *Klasea*. Contrary to the *Rhaponticum* group, *Klasea* shows various ploidy levels, mostly diploid and tetraploid (Table 1), although polyploidy exists up to the hexaploid level in some species in the Iberian Peninsula (Cantó, 1982, 1985). The occurrence of polyploidy in *Klasea* but not in the *Rhaponticum* group could be explained in part by a difference in their hybridization potential. *Klasea* species show overlapping areas of distribution where interspecific hybrids are documented (Wagenitz, 1955), whereas *Rhaponticum* and its relatives are mainly endemics with restricted areas and isolated populations, which do not favour hybridization and resulting allopolyploidy events.

Location of changes in chromosome basic numbers during the evolutive history of *Rhaponticum* and its relatives

A hypothesis of the direction of chromosome number evolution, as well as the location of those changes in the evolutionary history of the studied group, can be inferred through the mapping of chromosome reports on the phylogenetic reconstructions. The number of *Rhaponticum* species and its relatives for which both reliable cytological and molecular data are available is at present sufficient for us to reach firm conclusions on the existence of a

decreasing dysploid series from $x = 15$ to 12, coinciding notably with the phylogeny of these taxa (Hidalgo *et al.*, 2006; Fig. 28). *Klasea*, sister to the *Rhaponticum* group, presents the highest basic chromosome number of $x = 15$, while the early-branching genera *Centaurothamnus* and *Callicephalus* show $x = 14$. In the more derived clades, $x=14$ and 12 appear in *Myopordon* and *Oligochaeta* respectively, and $x = 13$ and 12 in *Rhaponticum*. This illustrates another example within the Centaureinae that shows dysploidy as one of the main mechanisms of chromosomal evolution, reported previously in *Centaurea* L. (Fernández Casas & Susanna, 1986; Siljak-Yakovlev, 1986; Garcias-Jacas & Susanna, 1992; Garcia-Jacas *et al.*, 1996) and in *Carthamus* (Vilatersana *et al.*, 2000).



Figures 16-27. Somatic metaphase plates. Fig. 16. *Rhaponticum australe*, $2n = 26$. Figs 17, 18. *R. carthamoides*, $2n = 24$. Fig. 18. Fluorochrome banding with chromomycin. Arrows indicate a pair of chromosomes showing fragility. Fig. 19. *R. integrifolium*, $2n = 26$. Fig. 20. *R. acaule*, $2n = 26$. Fig. 21. *R. berardioides*, $2n = 26$. Fig. 22. *R. coniferum*, $2n = 26$. Fig. 23. *R. cynaroides*, $2n = 26$. Fig. 24. *R. fontqueri*, $2n = 26$. Fig. 25. *R. heleniifolium*, $2n = 26$. Fig. 26. *R. heleniifolium* subsp. *bickenellii* (Aoste), $2n = 26$. Fig. 27. *R. scariosum* (Hautes Alpes) $2n = 26$. Scale bars = 10 μm .

Our results confirm that most of the *Rhaponticum* species show $x = 13$. This number is uncommon within the Centaureinae and, apart from *Rhaponticum*, is known only for *Plectocephalus* D. Don (Hellwig, 1994), *Stizolophus* Cass. (Chouksanova *et al.*, 1968; Tonjan, 1968; Bakhshi Khaniki, 1996; Garcia-Jacas *et al.*, 1998b; Ghaffari, 2001) and *Volutaria crupinoides* (Desf.) Maire (Oberprieler & Vogt, 1993; Hellwig, 1994). The molecular phylogeny established by Hidalgo *et al.* (2006) shows a polytomy consisting of two clades of *Rhaponticum* species and the *Myopordon* plus *Oligochaeta* clade (Fig. 28). Decreasing dysploidy suggests that $x = 14$ is more ancient than $x = 13$. This implies that *Myopordon* and *Oligochaeta*, which exhibit mostly $x = 14$, emerged before *Rhaponticum* in the evolutionary history of the group, and that the two main clades of *Rhaponticum* species, which share mainly $x = 13$, are sisters and constitute a monophyletic genus. As a matter of fact, the karyological data could provide an answer to help resolve the more vexing question of polytomy which molecular reconstruction has failed to solve.

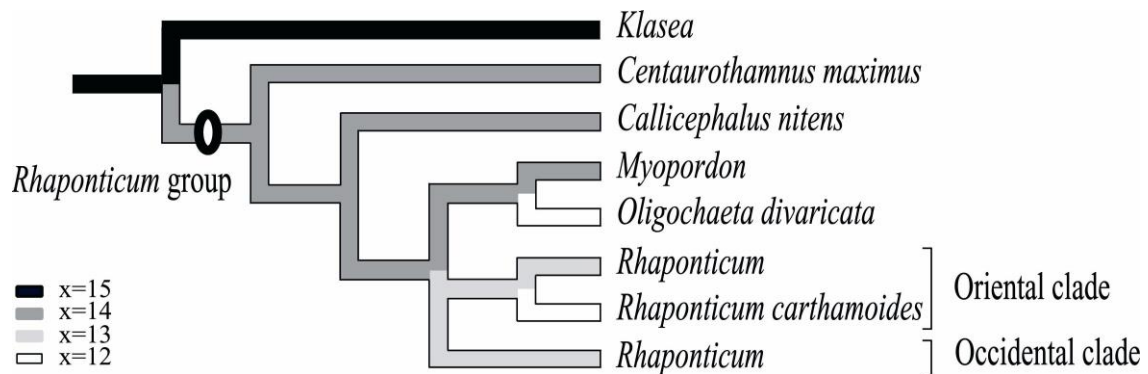


Figure 28. Synoptic phylogeny of the *Rhaponticum* group based on Hidalgo *et al.* (2006), showing the evolution of basic chromosome numbers.

In the case of $x = 12$, which is the most recent basic chromosome number in the decreasing dysploid series, its occurrence in *Oligochaeta divaricata* and *Rhaponticum carthamoides*, two taxa not closely related in the phylogeny (Fig. 28), suggests convergent evolution. Regarding this number, one of the most interesting results of this study is the manifestation of recent chromosome fusions in *Oligochaeta divaricata*. Basic chromosome numbers of $x = 14$ for *O. ramosa* and $x = 12$ for *O. divaricata* imply strong dysploidy within the genus, which exhibits at the same time the highest and the lowest chromosome numbers

of the *Rhaponticum* group. Unfortunately, there is no information about the chromosome number of the three other species of the genus *Oligochaeta*. Furthermore, the two annual genera studied, *Callicephalus* and *Oligochaeta*, show $x = 14$ and $x = 14$ and 12 , respectively. Such difference can not be interpreted in terms of difference their life cycle (both are annual), their breeding system (they are autogamous; Wagenitz & Hellwig, 1996 for *Callicephalus*; Hellwig, 2004 for *Oligochaeta*), neither their environmental conditions (which are not radically different). The explanation for the strong disploidy within *Oligochaeta* should be rather searched for in the reproductive history of the species.

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