Extreme environmental conditions and phylogenetic inheritance: systematics of *Myopordon* and *Oligochaeta* (Asteraceae, Cardueae-Centaureinae)

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Running heading: Systematics of *Myopordon* and *Oligochaeta*

Number of Figures: 2

Number of Tables: 2

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ABSTRACT:

The genus *Myopordon*, with six perennial species from Iran, Lebanon, Syria and Turkey, had until recently been placed in the Carduinae subtribe. *Oligochaeta*, composed of four annual species from Afghanistan, Caucasus, India, and the Near East, was considered related to the genus *Rhaponticum* in the Centaureinae subtribe. The two genera have apparently not much in common; however, evidence that both are closely related within the *Rhaponticum* group was provided by recent molecular phylogenetic reconstructions.

New and previously published cytogenetical [fluorescent *in situ* hybridization of rDNA genes, fluorochrome banding of AT- and GC-rich regions, silver nitrate staining of active ribosomal genes, genome size assessment], karyological, molecular phylogenetic, and palynological data are evaluated in the light of the recent evidence concerning the relationships of *Myopordon* and *Oligochaeta*.

Myopordon presents a constant chromosome number and a homogeneous pattern in physical mapping of rDNA genes, contrasting with a strong variability in chromosome banding, genome size, and pollen-type. Such tendencies could be related to the environmental pressures in the high mountains where species of *Myopordon* occur. This also accords well with the phylogeny and the geographic distribution of the genus. Phylogenetic reconstructions resolve *Oligochaeta* as being nested in the clade of *Myopordon* species. The exceptional cytogenetical, karyological, as well as palynological characters of *Oligochaeta* are apparently derived from a *Myopordon*-like ancestor.

KEYWORDS: fluorescent *in situ* hybridization, fluorochrome banding, genome size, high altitude, *Myopordon*, *Oligochaeta*

INTRODUCTION

The genus *Myopordon* Boiss. comprises six species, four from Iran, one from Lebanon, and one from Syria and Turkey. All are perennial, and occur at very high elevations. The genus *Oligochaeta* (DC.) K. Koch comprises four species, three from the Near East and Caucasus, and one from East Pakistan and India. Its representatives are annual, autogamous and grow in dry steppes and mountain areas.

Taxonomic and nomenclatural history - The complicated taxonomic and nomenclatural histories of the genera *Myopordon* and *Oligochaeta*, as well as the species which constitute them, reflect the difficult systematics of these taxa. Boissier (1845) described the genus *Myopordon* based on two new species from Iran, *M. aucheri* Boiss. and *M. persicum* Boiss. (type of the genus). Subsequently, three futher species were added by Wagenitz (1958): *M. hyrcanum* (Bornm.) Wagenitz from Iran, previously described as a species of *Jurinea* Cass. and subsequently as constituing the *Centaurea* section *Erinacella* (Wagentiz, 1955); *M. pulchellum* (C. Winkl. & Barbey) Wagenitz from Lebanon, which constituted the genus *Autrania* C. Winkl.; and *M. thiebautii* (Genty) Wagenitz from Syria and Turkey, which was first described as a *Jurinea* species and later on represented the genus *Haradjania* Rech. f. The most recent addition to the genus *Myopordon* is *M. damavandicum* ["*damavandica*"] Mozaff. from Iran (Mozaffarian, 1991).

Koch (1843) described the genus *Oligochaeta* based on *Serratula* sect. *Oligochaeta*, with *O. divaricata* (Fisch. & C.A. Mey.) K. Koch from Central Asia, Caucasus, and Iran as type and only representative. The genus *Oligochaeta* was not commonly accepted and *O. divaricata* was successively recombined as

Volutarella Cass. (Bentham, 1873), *Microlonchus* Cass. (Boissier, 1875), *Cousinia* Cass. (Winkler, 1889) and *Centaurea* (Urban, 1916). When Boissier (1875) transferred *O. divaricata* to *Microlonchus*, he described the closely related new species, *M. minimus* Boiss. from Central Asia and Iran. Briquet (1930), when redefining *Mantisalca* Cass. (= *Microlonchus*), restored the genus *Oligochaeta* and included *O. albispina* (Bunge) Boiss., *O. divaricata* and *O. minima* (Boiss.) Briq. *Oligochaeta albispina* is now considered in the monotypic genus *Schischkinia* Iljin (Wagenitz, 1962). Wagenitz (1962) added to *Oligochaeta* a species from India and Pakistan, *O. ramosa* (Roxb.) Wagenitz, which was first described as *Carduus ramosus* Roxb. (Roxburgh, 1832). Within the numerous taxonomic combinations of this species, several of them were under the genus *Tricholepis* DC. (*T. candolleana* Wight & Arn., *T. procumbens* Wight, *T. divaricata* (DC.) Iljin.). This may be due to the fact that *Oligochaeta ramosa* and *Tricholepis* share an uncommon, Indian biogeographic distribution in the Centaureinae, only three genera of the subtribe reaching the Indian peninsula: *Goniocaulon* Cass., *Oligochaeta* and *Tricholepis* (Dittrich, 1977). Finally, Czerepanov (1959) added a new species from Caucasus, *O. tomentosa* Czerep.

Subtribal assignment - Traditional classifications placed *Myopordon* in the subtribe Carduinae (Wagenitz, 1958; Dittrich, 1977; Bremer, 1994). This placement was based on the absence of receptacle setae, a character shared with *Onopordon* L., and the insertion areole of the achene described as straight, which is typical of the subtribe Carduinae (Dittrich, 1968). However, Mouterde (1983) reported the insertion areole as oblique, which points toward Centaureinae. The placement of *Oligochaeta* within the Centaureinae was not

questioned, its position in the subtribe, however, remained unclear due to the occurrence of convergent morphological characters in this and other annual taxa. On the basis of pollen types, *Oligochaeta* was considered related to the genus *Centaurea* (Wagenitz, 1955, 1962).

Phylogenetic reconstructions based on molecular sequencing resolved *Oligochaeta* and *Myopordon* within the *Rhaponticum* group (Garcia-Jacas & al., 2001, for *Oligochaeta*; Susanna & al., 2006, for *Myopordon*), among the early branched genera of the subtribe Centaureinae, and lead to a new subtribal placement for *Myopordon*. The molecular data showed that *Myopordon* and *Oligochaeta* constitute a monophyletic clade closely related to the genus *Rhaponticum* Vaill. (Hidalgo & al., 2006; Susanna & al., 2006). In the light of these findings, we have carried out palynological and karyological studies on this group (Hidalgo & al., 2007; Hidalgo & al., in press).

Pollen-type features illustrate an interesting case of convergence between *Myopordon* and *Oligochaeta*, and with *Centaurea* section *Jacea*. They suggest a possible problem in the generic delineation of *Myopordon* and *Oligochaeta*. *Myopordon* exhibits a series of pollen types from the *Serratula*-type (the most primitive) to the *Jacea*-type (the most derived). *Oligochaeta* presents only the *Jacea*-type (Hidalgo & al., in press). Keeping the two taxa as separate genera implies the assumption that such a pollen-type evolution, which is exceptional in the Centaureinae, has occurred separately two times in the *Myopordon* plus *Oligochaeta* clade.

As to karyology, *Myopordon* species present the most primitive basic chromosome number in the *Rhaponticum* group ($x = 14$), whereas *Oligochaeta* shows both the most primitive ($x = 14$, *O. ramosa*) and the most derived basic

chromosome number (*x* = 12, *O. divaricata*; Hidalgo & al., 2007). The occurrence of *x* = 12 in *O. divaricata* seems to be the result of a recent event of chromosome fusion, as suggested by the high fragility of particularly long chromosome pairs (Hidalgo & al., 2007). Such a dysploid tendency within a limited group of species makes it an interesting subject for cytogenetical studies, as previously shown in other Cardueae (e.g., in *Xeranthemum* L. and related genera, Garnatje & al., 2004). *Myopordon* and *Oligochaeta* species, as well as the other representatives of the *Rhaponticum* group, are all diploid.

The main goals of our study were: (1) to improve the molecular phylogenetic reconstruction of *Myopordon* and *Oligochaeta* by adding the cpDNA region *rps4-trnT-trnL* to the previously sequenced nr ETS, ITS, and cp *trnL-trnF* markers, and completing the sampling with *Myopordon pulchellum*, and *Oligochaeta ramosa*, along with new populations of *Myopordon aucheri*, *M. hyrcanum* and *M. persicum*, (2) to provide a cytogenetical characterization of *Myopordon* and *Oligochaeta* species using fluorochrome banding and fluorescent *in situ* hybridization (FISH) of rDNA genes, and (3) to address systematic and evolutionary issues in the light of the new data.

MATERIALS AND METHODS

Plant material **-** The sampling for the molecular phylogenetic study included *Myopordon aucheri*, *M. hyrcanum*, *M. persicum*, *M. pulchellum*, *Oligochaeta divaricata*, *O. minima,* and *O. ramosa*. The outgroup was selected according to the previous work of Hidalgo & al. (2006) and includes *Centaurothamnus maximus* (Forssk.) Wagenitz & Dittrich, *Ochrocephala*

imatongensis (Phillipson) Dittrich, *Rhaponticum pulchrum* Fisch. & C.A. Mey., and *R. repens* (L.) Hidalgo. Both previously published and new sequences were used in the analyses. The origin of the samples and GenBank sequence accession numbers are given in the appendix.

The plants for the cytogenetical study were grown from achenes, either collected in the field or obtained from a botanical garden. The species investigated were *Myopordon aucheri*, *M. persicum*, *M. pulchellum,* and *Oligochaeta divaricata*. Two other representatives of the *Rhaponticum* group were added to the study for comparison: the early branched monotypic genus *Callicephallus* C.A. Mey., and *Rhaponticum carthamoides* (Willd.) Iljin. *Callicephallus nitens* (M. Bieb. ex Willd.) C.A. Mey. is annual and autogamous like *Oligochaeta* species, and presents $x = 14$ like *Myopordon*. *Rhaponticum carthamoides* shows $x = 12$, the same basic chromosome number as *Oligochaeta divaricata* (Hidalgo & al., 2007).

***Molecular phylogeny* -** The amplification and sequencing protocols for ITS, ETS and *trnL-trnF* regions were the same as those described in Hidalgo & al. (2006). The cpDNA *rps4-trnT-trnL* region was amplified and sequenced with the forward primers rps4R2 (Shaw & al., 2005) and trnA2 (Cronn & al., 2002), and the reverse primers trnA2R (5'-AGG TTA GAG CAT CGC ATT TG-3') and trnL-b (Taberlet & al., 1991) using the following profile: warm start at 94ºC for 2 min, followed by 5 min at 80ºC for the addition of the Taq polymerase (Ecotaq, Ecogen S.R.L., Barcelona, Spain), whence proceeding with 35 cycles at 94ºC for 1 min, then 55ºC for 1 min 30 s and 72ºC for 2 min, including additionally 72ºC for 10 min.

Procedures to conduct phylogenetic analyses were as described in Hidalgo & al. (2006). To investigate incongruence between the datasets, the ILD test was performed through the “partition homogeneity test” of PAUP* version 4.0b10 (Swofford, 2002), using tree bisection reconnection branch swapping with 1000 replicates of heuristic search. When given DNA datasets appeared to be incongruent (P-values < 0.05), P-values were calculated removing successively the species one by one, by pairs and by clades, with a view to identifying which of them were implicated.

Cytogenetical studies - For fluorochrome banding and FISH the chromosome spreads were obtained using the air-drying technique of Geber & Schweizer (1987) with slight modifications. Meristems were excised and washed in citrate buffer (0.01 M citric acid-sodium citrate, pH = 4.6) for 10 min at room temperature, and incubated at 37ºC for 18--25 min in an enzyme mix solution [4% cellulase Onozuka R10 (Yakult Honsha), 1% pectolyase Y23 (Sigma) and 4% hemicellulase (Sigma)] diluted at 50% in citrate buffer. Meristems were broken with a pipet and centrifuged for 5 min at 4,000 rpm, twice in the buffer, and once in fresh fixative (3:1 absolute ethanol:glacial acetic acid). The pellet was resuspended in 50 µl of fixative, dropped onto clean slides and air-dried.

Chromomycin A_3 banding - This technique was performed according to Schweizer (1976) and Cerbah & al. (1995) to reveal GC-rich DNA. The slides were incubated in McIlvaine buffer pH = 7 with $MgSO_4$ for 15 min, stained with chromomycin A_3 (0.2 mg/ml in McIlvaine buffer pH = 7) for 10 min to 1 h in the dark, rinsed in McIlvaine buffer pH = 7, and counterstained with methyl green (0.1% in McIlvaine buffer pH = 5.5) for 10 min also in the dark. Finally, the slides

were rinsed in McIlvaine buffer pH = 5.5 and mounted in a drop of Citifluor AF2 (Agar Scientific Oxford Instruments).

Fluorescent in situ *hybridization* - The probe used for 18S-5.8S-26S rDNA location was labelled with direct Cyanine Cy3 using the Nick Translation Kit (Amersham), and the probe used for 5S rDNA location was labelled with digoxigenin-11-dUTP (Boehringer Mannheim). DNA:DNA *in situ* hybridization was carried out according to Heslop-Harrison & al. (1991) and Cerbah & al. (1998) with slight modifications as follows. Slides were washed in 100% ethanol during 2--3 min and air-dried. They were incubated in 100 µg/ml DNase-free RNase in 2xSSC (0.03 M sodium citrate and 0.3 M sodium chloride) for 1 h at 37ºC, rinsed in 2xSSC for 5 min, and incubated in HCl 0.01 N for 2--3 min. The slides were incubated in pepsin (0.1 mg/ml in 0.01 N HCl) for 15 min at 37ºC, washed in water 2 min and next three times in 2xSSC for 5 min each with shaking, dehydrated through an ice-cold ethanol series (70, 90, and 100% for 3 min each), and air dried. The probe hybridization mixture contained 25--50 ng/µl rDNA probes, 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) sodium dodecyl sulphate, 250 µg/ml salmon sperm, and 20xSSC. It was denatured at 75ºC for 10 min, briefly centrifuged, transferred immediately in ice for 5 min, and 50 µl were loaded on slides under coverslips. The preparations were denatured at 72ºC for 10 min and transferred at 55ºC for 5 min. Hybridization was carried out overnight at 37ºC in a humidified chamber. Posthybridization washes were performed with agitation. The first wash at room temperature in 2xSSC for 3 min was followed by several washes at 42ºC for 5 min (three in 2xSSC, two in 20% formamide, one in 0.1xSSC, three in 2xSSC and one in 4xSSC with 0.2% Tween 20). The last wash was at room

temperature for 5 min in 4xSSC with 0.2% Tween 20. For 18S-5.8S-26S signal detection, the slides were treated with 5% (w/v) bovine serum albumin (BSA) in 4xSSC with 0.2% Tween 20 for 5 min. They were next incubated for 1 hour at 37ºC in 20 µg/ml antidigoxigenin-fluorescein (Boehringer Mannheim) in the same buffer, and rinsed three times with the buffer for 5 min at room temperature. Samples were counterstained with Vectashield (Vector Laboratories, Inc. Burlingame, California) a mounting medium containing DAPI. The preparations were observed with an epifluorescence Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07, 15). Hybridization signals were analyzed using the highly sensitive CCD camera (Princeton Instruments, Evry, France) and image analyzer (Metavue).

Silver staining - To determine the activity of the 18S-5.8S-26S ribosomal genes in *Oligochaeta divaricata*, rDNA transcriptional activity was assessed by silver staining according to the method of Frehner & Pazza (2004) with the modifications described in Srisuwan & al. (2006). Two drops of 1% aqueous gelatine solution with 0.25% formic acid and four drops of silver nitrate were placed on test slides which were covered and incubated for 15 min at 65ºC. After this step, the coverslips were removed and the slides were washed under tap water, stained with 5% Giemsa for 30 sec and air dried. The preparations were mounted in Euparal (Agar scientific LTD Stansted, Essex, UK) and observed with an optical microscope.

RESULTS

Molecular phylogeny - Numerical results from the phylogenetic analyses and selected models for Bayesian inferences are presented in Table 1.

nrDNA dataset - Separate ITS and ETS analyses do not show topological conflict for supported branches (PP ≥ 95%, BS ≥ 80%), which led us to present only the nrDNA combined results (Fig. 1A). Nevertheless, the ILD P-value of ITS and ETS datasets is of 0.026, which indicates a certain level of incongruence. The P-value calculation removing species does not evidence a species or a group of species as responsible for the incongruence. In fact, incongruence seems to be diffuse and produced by the level of homoplasy of the nuclear dataset, which is relatively high, given the small number of taxa (CI = 0.6667 for ITS and CI = 0.7483 for ETS; Table 1). Nuclear DNA results confirm the close relationship between *Myopordon* and *Oligochaeta* (PP = 100%, BS = 97%; Fig. 1A) previously suggested by Hidalgo & al. (2006) and Susanna & al. (2006), and provide some new insight in phylogenetic relationships within this group of species. *Myopordon aucheri* appears as sister to the remaining *Myopordon* taxa and the genus *Oligochaeta* (PP = 100%, BS = 99%; Fig. 1A). *Myopordon pulchellum*, *M. hyrcanum* and *M. persicum* form a monophyletic association (PP = 100%; BS = 84%, Fig. 1A), but the relationships within this clade are unsupported. The genus *Oligochaeta* is also monophyletic (PP = 100%; BS = 84%, Fig. 1A), with *O. ramosa* sister to *O. divaricata* plus *O. minima* (PP = 100%; BS = 100%, Fig. 1A).

cpDNA dataset - As the *trnL-trnF* and *rps4-trnT-trnL* tree topologies are congruent, and likewise their corresponding datasets (ILD P-value = 1), only the combined analyses are presented (Fig. 1B). The cpDNA phylogenetic

reconstructions differ from the nrDNA ones in two main points: (1) the *Myopordon pulchellum*, *M. hyrcanum* and *M. persicum* clade is unresolved; (2) the placement of *Oligochaeta divaricata* and *O. minima* is more basal than suggested by nrDNA dataset, the two species being set apart from the clade including all *Myopordon* species plus *Oligochaeta ramosa* (PP = 100%; BS = 100%, Fig. 1B). With such a discrepancy, the ILD P-value of 0.001 signifying a strong incompatibility between nrDNA and cpDNA datasets was expected.

***Cytogenetics* -** The karyology and cytogenetical data are presented in Table 2. and Fig. 2. Fluorescent *in situ* hybridization reveals a homogeneous pattern in the physical mapping of rDNA within the *Myopordon* species (Fig. 2A--L). All of them present two 18S *loci*, which are located in satellites of different chromosome pairs, and one 5S *locus* situated in an intercalary position near the centromere. FISH signals were always collocated with CMA+ bands. However, these species exhibit differentiated patterns of CMA+ and DAPI+ signals. *Myopordon aucheri* presents 44 CMA+ signals (Fig. 2A, D), *M. persicum* 36 (Fig. 2E, G, H), and *M. pulchellum* 58 (Fig. 2I, L). All of them are located in the telomeric region, except those corresponding to the 5S *locus* which are situated in the pericentromeric region. As regards DAPI banding, *M. aucheri* shows 10 positive signals, eight near to the centromere, and two in an intercalary position (Fig. 2C, D). *Myopordon persicum* exhibits only two pericentromeric DAPI+ signals (Fig. 2H). All telomeric regions of the chromosomes of *M. pulchellum* present both DAPI and CMA positive signals except those corresponding to the 18S *loci*, which do not show DAPI signals (Fig. 2L). Furthermore, the 5S *locus* in this species is collocated with DAPI and CMA bands (Fig. 2L).

Oligochaeta divaricata presents twelve 18S sites and two 5S sites as FISH pattern, 24 CMA+ and 20 DAPI signals as banding pattern (Fig. 2M--P). All 18S *loci* are situated in an intercalary position.

DISCUSSION

Incongruence between nuclear and chloroplastic topologies - Among the different factors which could have caused the inconsistency between the nrDNA and cpDNA trees (e.g., hybridization, long branch attraction, lineage sorting), ancestral polymorphism seems to us the most likely one. The earlier branching of *Oligochaeta divaricata* and *O. minima* in the cpDNA tree cannot be attributed to long branch attraction because their branches in the cpDNA phylogeny are very short (Fig. 1B). Hybridization cannot be discarded but there is no morphological evidence supporting it. Moreover if hybridization has occurred, the uncertain placement of *O. divaricata* and *O. minima* in the cpDNA phylogenetic reconstruction (PP = 98% with the HKY+I model, PP = 90% with the F81+G model; BS = 59%, Fig. 1B) does not permit to determine their putative maternal origin. In any case, in all nuclear and plastid reconstructions *Myopordon* and *Oligochaeta* species are intermixed (Fig. 1A--B).

***The* Myopordon *species*: *cytogenetical traits related to extreme environmental conditions* -** The CMA and DAPI banding pattern of the *Myopordon* species strongly differs from that of other representatives of the *Rhaponticum* group studied. In *Callicephalus nitens*, the sister species of the clade including *Myopordon*, *Oligochaeta* and *Rhaponticum* (Hidalgo & al., 2006), there is only a pair of CMA+ signals (Fig. 2Q, T) corresponding to the

18S *locus* (Fig. 2T). Furthermore, *Rhaponticum*, sister to the *Myopordon* plus *Oligochaeta* clade, does not show telomeric marked regions as in *Myopordon* (*Rhaponticum carthamoides*, Fig. 2U; Hidalgo & al., in prep.). This evolutionary tendency seems to appear with *Myopordon* and to increase within the genus. *Myopordon aucheri*, sister to the remaining species of the genus plus *Oligochaeta*, presents 44 CMA+ telomeric signals (Fig. 2D) while in *M. pulchellum*, the most derived species (on the basis of geographic distribution and pollen-types, Hidalgo & al., in press), all telomeric regions are marked both by CMA and DAPI (except the 18S *loci*, which do not present a DAPI signal; Fig. 2L).

An explanation for the telomeric banding pattern within *Myopordon* could be the extreme, high mountain environmental conditions where the species occur (as suggested in *Artemisia* L., Garcia & al., 2007). Such high mountain environments (described in Noroozi *et al.*, 2007) have been related to genomic adaptations, e.g. genome size. Large genome sizes, enhance the capacity for growth at low temperatures and frost resistance (Albach & Greilhuber, 2004, and references therein). Assessments of genome size in *Myopordon* are strongly consistent with this tendency. With DNA amounts from 2C = 3.26(±0.06) pg for *M. pulchellum* to 2C = 4.32(±0.05) pg for *M. hyrcanum*, *Myopordon* shows 2C values much higher than other representatives of the *Rhaponticum* group (Hidalgo & al., in prep.; Table 2). Genome sizes in *Myopordon* species are larger than in *Rhaponticum* [2C 1.53(±0.03) pg to 2.57(±0.06) pg], and the early branched genus *Callicephalus* [2C of 1.28(±0.05) pg]. Other authors (Sparrow & Miksche, 1961) have suggested that ultraviolet (UV) damage increases in larger genomes. UV radiation, which is one of the

major genome-damaging agents, is particularly severe in high mountain environments where *Myopordon* occurs. In contrast to animals, plants are constantly exposed to solar UV radiation, which has led them to develop very efficient strategies to maintain their integrity (Bray & West, 2005). It has been shown that plants growing in high UV radiation environments are less sensitive to high UV intensity than their relatives growing in low UV radiation environments (Sullivan & al., 1992, and references therein). Consequently, genomic adaptation to extreme environments could proceed through a compromise between two opposite tendencies, namely, high DNA values, which optimize growth in extreme conditions (e.g., improving cold-stress tolerance), and limitation of these values due to UV-damage sensitivity, which increases with genome size. This might have caused development of GC and AT-rich heterochromatin in telomeric regions in *Myopordon*. Telomeric regions, which stabilize chromosome ends, enable replication and possess essential protective and repair functions of the chromosomes (Zakian, 1995), are particularly sensitive to UV radiation (Lansdorp, 2005). The pattern of telomeric AT- and GC-rich regions in *Myopordon* could result from an additional adaptation to preserve telomere functions conserving a high DNA-value. Beside its protective function, heterochromatin expansion in telomeric regions could also enhance chromosome pairing during cell division (Siljak-Yakovlev & Cartier, 1986).

***Cytogenetical characteristics of* Oligochaeta divaricata*: additive effects of a shift in life cycle and phylogenetic inheritance* -** The FISH pattern of *Oligochaeta divaricata* (Fig. 2M--P) contrasts with that of *Myopordon* species, by a much higher number of 18S sites. Within *Rhaponticum*, higher numbers of 18S sites have been encountered in species in which recent events

of chromosome rearrangements have occurred, such as chromosome fusion (six 18S sites in *R. carthamoides*, Fig. 2S, U) or rearrangements following introgression events (Hidalgo & al., in prep.). Another peculiarity is that 18S signals in *Oligochaeta* are located in an intercalary position, whereas in *Myopordon* species the 18S signals are always situated in a telomeric position. Intercalary position of a 18S *locus* is not uncommon in the genus *Rhaponticum* (Hidalgo & al., in prep.), but *R. carthamoides* is the only representative of this genus with three intercalary 18S *loci* (Fig. 2U). Besides the high number of 18S sites and their intercalary position, *O.divaricata* and *R. carthamoides* share another unique characteristic in the group, underlining a convergent evolution. With $x = 12$, these two species constitute the end of the dysploid series of basic chromosome numbers, beginning with $x = 14$ (*Callicephalus*, *Myopordon*) and followed by $x = 13$ (most of the *Rhaponticum* species; Hidalgo & al., 2007). Both *O. divaricata* and *R.carthamoides* present fragility of some chromosomes (Fig. 2P, U), related to recent events of chromosome fusion (Hidalgo & al., 2007). These cytogenetical and karyological features indicate that considerable chromosome rearrangement has recently occurred within *O. divaricata*.

Why has *Oligochaeta divaricata* developed such perplexing characteristics? The phylogenetic reconstructions and inference of ancestral character states of pollen-types clearly situate the species of *Oligochaeta* as derived from an ancestor that exhibits the characteristics of *Myopordon*, such as those corresponding to adaptation to an extreme environment (Fig. 2A). In the evolutionary history of the group, some species have evolved from the perennial state and extreme habitats (*Myopordon*), to the annual, autogamous state and moderate habitats (*Oligochaeta*). Optimal requirements in karyological and

cytogenetical aspects in each case are very different, particularly with respect to genome size. As mentioned earlier, *Myopordon* species have particularly high DNA-values, a characteristic which could result from adaptation to extreme environmental conditions. Autogamy as well as an annual life cycle, on the other hand, are related to low genome sizes (Bancheva & Greilhuber, 2006, and references therein). Indeed, *Callicephalus*, the other annual and autogamous genus of the group, presents one of the lowest genome size within the Cardueae tribe, of 2C = 1.28(±0.05) pg (Hidalgo & al., in prep.; Table 2). The DNA-value of *Oligochaeta divaricata* (2C = 1.99(±0.04) pg) is somewhat higher than expected for an annual and autogamous species. The switch in life cycle, breeding system, and environmental conditions correlated with smaller genome sizes has induced, within *Oligochaeta*, a series of chromosomal rearrangements in order to lighten its genome. This may explain why *O. divaricata* presents numerous “residual” CMA+ and DAPI signals in telomeric regions, as in *Myopordon* species. It also explains the particularly high number of 18S *loci* in *O. divaricata*. The high incidence of chromosomal restructurings that have occurred in this taxon could have generated the fragmentation of 18S regions and their scattered occurence in the genome. The silver staining technique in this species yielded 12 positive signals, implying that all 18S *loci* are active.

Concluding remarks - The molecular phylogenetic and cytogenetical data resolve *Oligochaeta* species as being derived from *Myopordon*, and coincide with palynological findings (Hidalgo & al., in press). *Myopordon* and *Oligochaeta* are quite different in morphological, karyological, cytogenetical and palynological aspects, which should be due largely to their very different life

cycle (perennial vs annual) and habitats (high vs low elevation). The bracts with a spiny appendix found in *Myopordon* and *Oligochaeta* differentiates the two taxa from other representatives of the *Rhaponticum* group.

ACKNOWLEDGEMENTS

The comments by two anonymous reviewers are greatly acknowledged. We thank O. Robin and S. Srisuwan (Ecologie, Systématique, Evolution, Université de Paris-Sud, Orsay Cedex, France) for technical support, S. Pyke (Institut Botànic de Barcelona, Spain) and the editors of TAXON for improvement of the English, and S. Garcia (Facultat de Farmàcia, Universitat de Barcelona, Spain) for fruitful discussion. We also thank M. Bou Dagher-Kharrat (Université Saint Joseph, Beyrouth, Lebanon), C.L. Kinter (University of Wyoming, USA), and V. Mozaffarian (Research Institute of Forests and Rangelands, Tehran, Iran) for their assistance with field collecting. This work was subsidized by the Ministerio de Educación y Ciencia, Spain (Project MEC CGL2004-04563-C02-01/BOS) and Generalitat de Catalunya ("Ajuts a Grups de Recerca Consolidats" 2005SGR00344). O. Hidalgo benefited from a pre-doctoral grant from the Ministerio de Educación y Ciencia (Spanish Government).

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Table 1. Numerical results from the phylogenetic analyses. The consistency and retention indexes were calculated excluding uninformative characters.

Data set	ITS	ETS	*trnL-trnF*	*rps4-trnT-trnL*	Combined nr data	Combined cp data
Total characters	**660**	**894**	**848**	**1138**	**1554**	**1986**
Informative substitutions	**44**	**97**	**10**	**12**	**141**	**22**
Autapomorphies	**64**	**129**	**8**	**22**	**193**	**30**
Number of MPTs	**9**	**2**	**2**	**3**	**2**	**3**
Number of steps (tree length)	**69**	**147**	**11**	**13**	**219**	**24**
Consistency Index (CI)	**0.6667**	**0.7483**	**1.0000**	**0.9231**	**0.7123**	**0.9583**
Retention Index (RI)	**0.6761**	**0.7466**	**1.0000**	**0.9600**	**0.7097**	**0.9360**
Rescaled CI	**0.4507**	**0.5587**	**1.0000**	**0.8862**	**0.5055**	**0.9767**
Selected models for Bayesian inference AIC	**SYM+G**	**GTR+I GTR+G**	**F81**	**GTR+G F81**	**GTR+I GTR+G**	**HKY+I F81+G**
hLRT						

Table 2. Karyology, cytogenetics and other characteristics of the studied species. [a] Life cycle: A annual, P perennial; [b] chromosome numbers from Hidalgo & al. 2007, except for *Myopordon aucheri* for which this is the first chromosome report; [c] 2C nuclear DNA content [means ± (SD) of 10 samples of 5 individuals] from Hidalgo & al. (in prep.); [d] palynological data are from Wagenitz (1955) for *Callicephalus nitens*, Martín & Garcia-Jacas (2000) for *Oligochaeta divaricata*, and Hidalgo & al. (in press) for the remaining species. P: polar axis, E: equatorial axis.

Taxon			Cytogenetical data					Palynological data[d]			
	Life cycle[a]	2n[b]	2C [pg][c]	18S sites	5S sites	CMA+ signals	DAPI+ signals	P (µm)	P/E	Spine (µm)	Pollen type
Callicephalus nitens	**A**	**28**	**1.28(±0.05)**	**2**	**2**	**2**	**0**	**34.5**	**0.95**	**-**	***Serratula***
Myopordon aucheri	**P**	**28**	**3.76(±0.03)**	**4**	**2**	**44**	**10**	**32.82**	**1.02**	**1.89**	***Serratula***
Myopordon hyrcanum	**P**	**-**	**4.32(±0.05)**	**-**	**-**	**-**	**-**	**30.46**	**1.03**	**1.04**	***Jacea***
Myopordon persicum	**P**	**28**	**3.38(±0.03)**	**4**	**2**	**36**	**2**	**31.92**	**1.16**	**1.40**	***Jacea***
Myopordon pulchellum	**P**	**28**	**3.26(±0.06)**	**4**	**2**	**38**	**54**	**23.84**	**1.05**	**0.40**	***Jacea***
Oligochaeta divaricata	**A**	**24**	**1.99(±0.04)**	**12**	**2**	**54**	**20**	**16.91**	**1.19**	**0.39**	***Jacea***
Rhaponticum carthamoides	**P**	**24**	**1.82(±0.05)**	**6**	**2**	**10**	**-**	**39.54**	**1.02**	**1.29**	***Serratula***

Figure 1. A- Phylogram from Bayesian phylogenetic analysis of combined ITS and ETS data. Numbers above branches indicate Bayesian clade-credibility values (posterior probability) and numbers below branches the parsimony bootstrap percentages. **B-** Phylogram from Bayesian phylogenetic analysis of combined *trnL-trnF* and *rps4-trnT-trnL* data. Supports of the branches are indicated as in A. Asterisk (*) points out a difference in support obtained with the two selected Bayesian models, the first being F81+G and the second one HKY+I.

Figures 2. A--D. *Myopordon aucheri*, CMA banding (A), FISH (B), DAPI banding (C) and haploid idiogram (D); **E--H.** *M. persicum*, CMA banding (E), FISH (F), prometaphase and metaphase CMA banding showing numerous telomeric marked regions (G), haploid idiogram (H); **I--L.** *M. pulchellum*, CMA banding (I), FISH (J), FISH of interphases nuclei, the arrows evidencing an 18S *locus* with particularly weak intensity (K), haploid idiogram (L); **M--P.** *Oligochaeta divaricata*, CMA banding (M), FISH (N), DAPI banding (O) and haploid idiogram with arrows indicating chromosome fragments (P); **Q, R, T.** *Callicephalus nitens*, CMA banding (Q), FISH (R), haploid idiogram (T); **S, U.** *Rhaponticum carthamoides*, FISH (S), haploid idiogram, the arrows indicating chromosome with centromeric fragility (U).

Scale bars = 10 µm. 18S, 5S, chromomycin, DAPI.

Appendix. Collection data. Origin of the materials, herbarium vouchers, and ITS, ETS, *trnL-trnF* and *rps4-trnT-trnF* GenBank accession numbers of the molecular study; previously published sequences [1] from Susanna & al. (2006), and [2] from Hidalgo & al. (2006)

Centaurothamnus maximus (Forssk.) Wagenitz & Dittrich, YEMEN, *Molero s.n.* (BC), AY826259[1], DQ310971[2], AY772301[1], EU409932; ***Myopordon aucheri*** Boiss., IRAN, *Romashchenko & Susanna 2613* (BC), EU409918, EU409924, EU409930, EU409940; ***Myopordon hyrcanum*** (Bornm.) Wagenitz, IRAN, *Romashchenko & Susanna 2623* (BC), EU409916, EU409922, EU409928, EU409938; ***Myopordon persicum*** Boiss., IRAN, *Romashchenko & Susanna 2610* (BC), EU409917, EU409923, EU409929, EU409939; ***Myopordon pulchellum*** (C. Winkl. & Barbey) Wagenitz, LEBANON, *Bou Dagher-Kharrat, Hidalgo & Romashchenko 407* (BC), EU409915, EU409921, EU409927, EU409937; ***Ochrocephala imatongensis*** (Phillipson) Dittrich, ETHIOPIA, *L. Friis, S. Bidgood, Malaku Wondefrash, Amsalu Ayana & Fantahun Simon 9163* (K), DQ310931[2], DQ310970[2], DQ310897[2], EU409933; ***Oligochaeta divaricata*** (Fisch. & C.A. Mey.) K. Koch, ARMENIA, *Fajvush, Gabrielyan, Garcia-Jacas, Guara, Hovhannisyan, Susanna 1583, Tamanyan & Vallès* (BC), AY826306[1], DQ310973[2], AY772344[1], EU409934; ***Oligochaeta minima*** (Boiss.) Briq., UZBEKISTAN, *Botanical Garden of Tashkent* (BC), AY826307[1], DQ310974[2], AY772345[1], EU409935; ***Oligochaeta ramosa*** (Roxb.) Wagenitz, PAKISTAN, *Rechinger 27481* (MA), EU409914, EU409920, EU409926, EU409936; ***Rhaponticum pulchrum*** Fisch. & C.A. Mey., GEORGIA, *Popova 326, Menitskiy, Medvedeva, Shults & Serov* (LE), DQ310943[2], DQ310991[2], DQ310910[2], EU409941; ***Rhaponticum repens*** (L.) Hidalgo, USA, *Kinter s.n.* (BC), EU409919, EU409925, EU409931, EU409942.