rDNA Organization and Evolutionary Relationships in the Genus *Hypochaeris* (Asteraceae)

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The pattern of rDNA sequence distribution in 11 *Hypochaeris* species from South America and Europe has been determined by in situ hybridization using probes of 18S-5.8S-28S and 5S ribosomal RNA genes. Activity of nucleolar organizer regions (NORs) was tested by silver nitrate staining and chromomycin A_3 banding of European species was carried out. In European species the 18S-5.8S-28S rRNA genes were located in the terminal secondary constrictions and the adjacent satellites, except for one locus in *H. achyrophorus.* In South American species, one or two 18S-5.8S-28S rDNA loci were observed in the intercalary secondary constrictions. All the rDNA sites appeared active, except one locus in *H. chillensis.* With the exception of *H. cretensis,* 5S rRNA genes were located on a single chromosome pair. In all the species analyzed, GC-rich heterochromatin has been located around the NORs. The rDNA localization indicated that chromosome rearrangements as centric shifts, centric fusions, and translocations of NORs involve in a decrease of rDNA loci number during the evolution of the complex of *Hypochaeris* species probably from an ancestral genome with two loci of 18S-5.8S-28S rDNA and one locus of 5S rDNA.

The genus *Hypochaeris* (Asteraceae) is distributed principally in two geographical areas, the Mediterranean region and South America. The South American species are numerous (about 40 species), and those in which karyotypes have been studied have the same basic chromosome number ($x = 4$) and very similar karyotype morphologies (Ruas et al. 1995; Saez 1949; Siljak-Yakovlev et al. 1994; Stebbins et al. 1953). The European group (about 12 species) is karyotypically more diverse, having basic chromosome numbers of $x = 3, 4, 5, 6$. Several hypotheses for karyotype evolution in the genus have been discussed (Cerbah et al. 1993, 1995) based on details of chromosome morphology, fluorochrome banding and DNA amounts. In the European group, it has been assumed that a decrease in basic chromosome number and DNA content is associated with a decrease in the number of nucleolar organizer regions (NORs). An exception is *H. robertia,* for which different phylogenetic positions have been proposed. This species, which possesses karyological features similar to South American species, was expected to be directly related to the latter (Barghi et al. 1989; Siljak-Yakovlev et al. 1994). The analysis of the genome size and the pattern of fluorochrome banding suggest that *H. robertia* is the sole representative of a specific group (Cerbah et al. 1995).

NORs are, in general, located at the secondary constrictions of chromosomes and represent the sites of genes coding for 18S-5.8S-28S ribosomal RNA. These genes are present as tandem repeats, form NORs when they are transcribed, and their number varies greatly between species (Sumner 1990). In eukaryotes, 5S rRNA genes (referred as 5S rDNA) also consist of tandemly repeated sequences (Flavell 1986), but are often of lower copy number than in 18S-5.8S-28S rDNA. However, in barley for example, 5S rRNA genes represent many thousands of tandem repeats (Leitch and Heslop-Harrison 1993). These two families of genes are generally not at the same chromosomal location, although there exist certain exceptions (Leitch and Heslop-Harrison 1993).

Fluorescent in situ hybridization is a powerful technique for physical mapping of DNA sequences, allowing elucidation of genome organization and chromosome structure (Heslop-Harrison 1991). It therefore provides information on the relationships between related species. Several studies have been published on the pattern of rDNA distribution in karyotypes of

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 89:312–318

Table 1. Origins of population samples studied

Species	2*n*	Source of samples	Reference number
H. maculata L.	10	Bosnia, Mount Trebevic	SSY 15/80
H. glabra L.	10	Sicily, Mount Quacella	SSY 11/83
H. radicata L.	8	Sicily, Mount Quacella	SSY 12/83
H. robertia Fiori	8	Sicily, Mount Etna	SSY 13/83
H. cretensis L. Chaub. & Bory	6	Sicily, Mount Portella Mandarine	SSY 14/83
H. achyrophorus L.	12	Deiva Marina, Liguria, Italy	Cult. Hanbury Botanical Garden, Gênes, Italy
H. laevigata (L.) Ces., P. & G.	12	Sicily, Mount Quacella	SSY 10/83
H. chillensis (H.B.K.) Hieron	8	Argentina, Buenos Aires	BAA 22 589
H. microcephala (Sch. Bip.) Cabr. var. *albiflora*	8	Argentina, Buenos Aires	BAA 22 600
H. megapotamica Cabr.	8	Argentina, Buenos Aires	BAA 22 590
H. pampasica Cabr.	8	Argentina, Buenos Aires	BAA 10/90

cultivated species, mainly cereals (Fukui et al. 1994; Kamisuki et al. 1994; Leitch and Heslop-Harrison 1992), on rDNA sequence organization in relation to ancestral species (Abbo et al. 1994) or in relation to the activity of these genes (Hizume 1992). The location of 18S-28S genes has proved to be an excellent chromosomal marker in studying the evolution of polyploid wheats (Jiang and Gill 1994) and was considered by Porter et al. (1994) to be a useful derived character in the evolutionary study of squamate reptiles. Few data have been collected concerning rDNA localization in the family Asteraceae (Hall and Parker 1995; Maluszynska and Schweizer 1989). In the genus *Hypochaeris,* the number of NORs and the location and characterization of tandemly repeated sequences by fluorochrome banding (with chromomycin A_3) are useful markers for the investigation of karyotype evolution. The objective of this work was to research other chromosomal markers that could provide additional information on genome differentiation and organization as well as species relationships in *Hypochaeris.* For this purpose we have determined the pattern of rDNA sequence distribution in several species of the genus using in situ hybridization of rRNA genes, tested NOR activity by silver nitrate staining and characterize the heterochromatin by chromomycin banding.

Material and Methods

We studied four South American and seven European species. The sources from which the species were obtained are listed in Table 1. Most of the European species were collected by Dr. S. Siljak-Yakovlev (SSY). The South American species were collected by Dr. A. Bartoli (BAA). Voucher specimens are conserved in the Botanical Garden of the University Paris XI.

Chromosome Preparations

Root tips of *Hypochaeris* species were pretreated with 0.05% colchicine for 2 h 15 min and fixed in 3:1 ethanol and acetic acid. Chromosome spreads were obtained using the air-drying technique of Geber and Schweizer (1987) with slight modifications. Meristems were excised, washed in citrate buffer (0.01 M citric acid-sodium citrate, pH 4.6) and incubated at 37°C for 3 h in enzyme solution [3% cellulase Onozuka R10 (Yakult Honsha Co.), 30% liquid pectinase (Sigma Chemical Co.) in citrate buffer]. The cellular mass obtained was centrifuged for 5 min at 4,000 rpm, twice in buffer, once in fresh fixative (3:1 ethanol and acetic acid). The pellet was resuspended in 50 μl of fixative, dropped onto clean slides, and air dried. Before in situ hybridization, and according to Fukui et al. (1994), the slide preparations were washed in 45% acetic acid for 5 min, dehydrated through an ethanol series, and air dried again in order to reduce background.

In situ Hybridization

For 18S-5.8S-28S rDNA localization, the probe used was a clone of a 0.8 kb *Taq*I fragment specific for the 18S ribosomal gene obtained from the pTa71 (Gerlach and Bedbrook 1979) and cloned in pUC18. The probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by the polymerase chain reaction (PCR). For 5S rDNA localization the probe used was a clone of pTa794, a 410 bp *Bam*HI fragment of 5S rDNA isolated from wheat and cloned in pBR322 (Gerlach and Dyer 1980). It contains the 5S rRNA gene (120 bp) and the noncoding intergenic spacer (290 bp). This probe was labeled by PCR with Fluoro-Red-dUTP (Amersham).

DNA:DNA in situ hybridization was carried out following the method of Heslop-Harrison et al. (1991) with slight modifications. Slide preparations were first incubated in 100 μg/ml DNase-free RNase in 2× SSC (0.03 M sodium citrate and 0.3 M sodium chloride) for 1 h at 37°C, washed 3 times in 2× SSC for 5 min, dehydrated through an ethanol series, and air dried. The rDNA probes were mixed each at a concentration of about 1.5 μg/ml for simultaneous hybridization with 50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1% (w/v) sodium dodecyl sulfate, 250 μg/ml salmon sperm, and 2× SSC. The probe mixture was denatured at 70°C for 10 min and immediately chilled in ice. Forty to 50 μl were loaded on slides under coverslips. The preparations were then denatured for 10 min at 80°C, transferred at 55°C for 5 min, and hybridization was carried out overnight at 37°C.

Posthybridization washes were done at 42°C for 5 min and consisted of three washes in 2× SSC, two stringent washes in 20% formamide in 0.1× SSC, a wash in 0.1× SSC, three washes in 2× SSC, and a wash in 4× SSC, 0.2% Tween 20.

For 18S-5.8S-28S signal detection, the slides were treated with 5% (w/v) bovine serum albumine (BSA) in 4× SSC, 0.2% Tween 20 for 5 min, incubated for 1 h at 37°C in 20 μg/ml antidigoxigenin-fluorescein (Boehringer Mannheim) in the same buffer, and rinsed with 4× SSC, 0.2% Tween 20, three times for 5 min at room temperature. Chromosomes were counterstained for 10 min with 2 μg/ml 4′,6-diamino-2-phenylindole (DAPI) in McIlvaine buffer, pH 7. Preparations were mounted in antifade solution (AF1, Citifluor), examined with a Zeiss Axiophot microscope with different filter sets, and photographed on Fujichrome Super HG 1600 or Ektar 1000. Fluorescence signals were observed in about 50 metaphase plates or interphase nuclei for each species.

Silver Staining

It was assumed that when a single pair of rDNA sites was present, they were active. Thus rDNA transcriptional activity was checked in species with at least two pairs of 18S-5.8S-28S rDNA sites by silver staining, following the method of Lacadena et al. (1984). Squashes were made in 45% acetic acid after maceration in enzyme mixture [3% cellulase Onozuka R10 (Yakult Honsha), 30% liquid pectinase (Sigma) in citrate buffer] for 30 min and air dried. The slides were incubated for 30 s in 2× SSC at 60°C, rinsed in sodium citrate solution (0.04 g/L trisodium citrate adjusted to pH 3 with formic acid), and stained

Table 2. Number and localization of rDNA sequences

Species	Number of 18S rDNA loci per haploid set	Number of 5S rDNA loci per haploid set
H. maculata	2 (sat. of pairs 2 and 3)	1 (centro., SA of pair 2)
H. glabra	1 (sat. of pair 2)	1 (inter., SA of pair 4)
H. radicata	1 (sat. of pair 3)	1 (inter., LA of pair 2)
H. robertia	3 (sat. of pairs 1, 2, and 4)	1 (centro., SA of pair 3)
H. cretensis	1 (sat. of pair 3)	2 (centro., LA of pair 1; term., SA of pair 1) plus 1 (term. on one chromosome of pair 2)
H. achyrophorus	2 (sat. of pair 3 and centro., SA of pair 4)	1 (centro., SA of pair 6)
H. laevigata	2 (sat. of pairs 2 and 3)	1 (centro., SA of pair 6)
H. megapotamica	1 (SC of pair 3)	1 (term., SA of pair 2)
H. chillensis	2 (inter., pair 2 and SC of pair 3)	1 (term., SA of pair 2)
H. microcephala	2 (SC of pairs 2 and 3)	1 (term., SA of pair 2)
H. pampasica	1 (SC of pair 3)	1 (term., SA of pair 2)

SC: secondary constriction; sat.: satellite; term.: terminal; centro.: paracentromeric; inter.: intercalary; SA: short arm; LA: long arm.

with 50% silver nitrate in the same buffer for 5 min in a humid chamber at 60°C.

Chromomycin A_3 (CMA_3) Banding

This technique was carried out according to Schweizer (1976) with modifications described in Cerbah et al. (1995).

Results

Localization of 18S-5.8S-28S rDNA Sequences

In European species the 18S-5.8S-28S rRNA genes were located at the secondary constrictions and the adjacent satellites (Table 2). The number of sites varied between species. Two loci were observed in *H. maculata* (Figure 1a), *H. achyrophorus* (Figure 1j), *H. laevigata* (Figure 1k), and one locus in *H. cretensis* (Figure 1b,c), *H. radicata* (Figure 1f), and *H. glabra* (Figure 1g). In *H. robertia* (Figure 1d), three loci of 18S-5.8S-28S rDNA were seen. It was often difficult to observe all sites together in a single metaphase plate because the satellites were sometimes removed from the chromosomes during the preparation of slides and the signals were smaller than in the other species studied. Nevertheless, in interphase nuclei, six signals were easily observed (Figure 1e).

In all four South American species, one locus of 18S-5.8S-28S rDNA was observed at the secondary constriction of pair 3 (Figure 1h). *H. microcephala* had a further hybridization signal at the subtelomeric secondary constriction of pair 2, while *H. chillensis* had a hybridization signal in the same region on pair 2 but where no secondary constriction was observed. Sizes of signals were similar in the four species.

Localization of 5S rDNA Sequences

With the exception of *H. cretensis,* the 5S rDNA probe detected one locus on a single chromosome pair in all the species. In *H. cretensis,* two loci were observed on pair 1 and one site on pair 2 (Figure 1b). Pair 2 was heterozygous. In interphase nuclei, five signals were always observed (Figure 1c). In the South American species, 5S rDNA loci were paracentromeric or intercalary (Figure 1h), while in European species, 5S rDNA sites might be near the centromere (*H. maculata, H. robertia, H. cretensis, H. achyrophorus, H. laevigata*), intercalary (*H. glabra, H. radicata*), or subtelomeric (*H. cretensis*). The number of interphase signals corresponded to the number of metaphase signals.

To compare the physical mapping of 5S rDNA and CMA_3 bands, chromomycin banding (after in situ hybridization) was carried out in some species. In *H. cretensis, H. maculata, H. glabra,* and *H. radicata,* locations of CMA_3 bands were difficult to determine precisely. They were very close to 5S rDNA sites except in *H. radicata.* CMA_3 bands were thinner than 5S rDNA signals and sometimes reduced to small dots. However, these results must be viewed with caution in species in which chromosome pairs have similar lengths and morphologies. In interphase nuclei, 18S rDNA spots were located around the nucleolus while 5S rDNA spots had no visible preferential position (Figure 1c,e,i).

Activity of 18S-5.8S-28S rDNA Genes

The maximum number of nucleoli observed was assumed to correspond to the number of active sites. Silver staining revealed that all rDNA sites appeared active, with the exception of one locus in *H. chillensis* (Table 3). In *H. robertia,* one nucleolus per cell was most frequently observed, but up to five nucleoli have been seen. In *H. achyrophorus,* one or two nucleoli were most often observed, but one or two additional micronucleoli were also visible in a relatively high percentage of cells (Table 4).

Chromomycin A_3 Banding

Five European species (*H. cretensis, H. glabra, H. radicata, H. achyrophorus,* and *H. laevigata*) were analyzed in this study for chromomycin A_3 banding. The remaining species were analyzed in Cerbah et al. (1995). All these species showed positive bands, which characterize GC-rich repeated sequences, in the satellite region (Figure 2). In addition, *H. glabra* had an intercalary band on the short arm of pair 4, while *H. cretensis* had two bands on pair 1, one paracentromeric on the short arm, and one subterminal on the long arm. Moreover, *H. achyrophorus* showed a thin paracentromeric positive band on the short arm of pairs 4 and 6 and *H. laevigata* had also a thin paracentromeric CMA_3-positive band on the short arm of pair 6. The number of CMA_3 signals in interphase nuclei corresponded to the number of metaphase bands.

Discussion

Organization of rDNA Sequences in the Genus *Hypochaeris*

The South American species showed less variation in the location of rDNA loci than the European species. This finding parallels that for other karyotypic features (Cerbah et al. 1995). Because fluorescent in situ hybridization is a semiquantitative

Table 3. Percentage of cells observed with different numbers of silver-stained nucleoli in some *Hypochaeris* species

Species	Number of silver-stained nucleoli				
	1	2	3	4	5
H. chillensis	86.89	13.1			
H. microcephala	34.15	35.75	21.65	8.43	
H. maculata	50	36.6	11.6	1.78	
H. laevigata	66.72	23.74	8.45	1.07	
H. robertia	85.38	12.08	2.04	0.38	0.09

Table 4. Percentage of cells observed with various numbers of silver-stained nucleoli (n) and micronucleoli (μn) in *H. achyrophorus*

	1n	1n + 1μn	1n + 2μn	2n	2n + 1μn	2 + 2μn	3n
% of cells	72.90	10.34	0.70	13.5	1.97	0.07	0.49

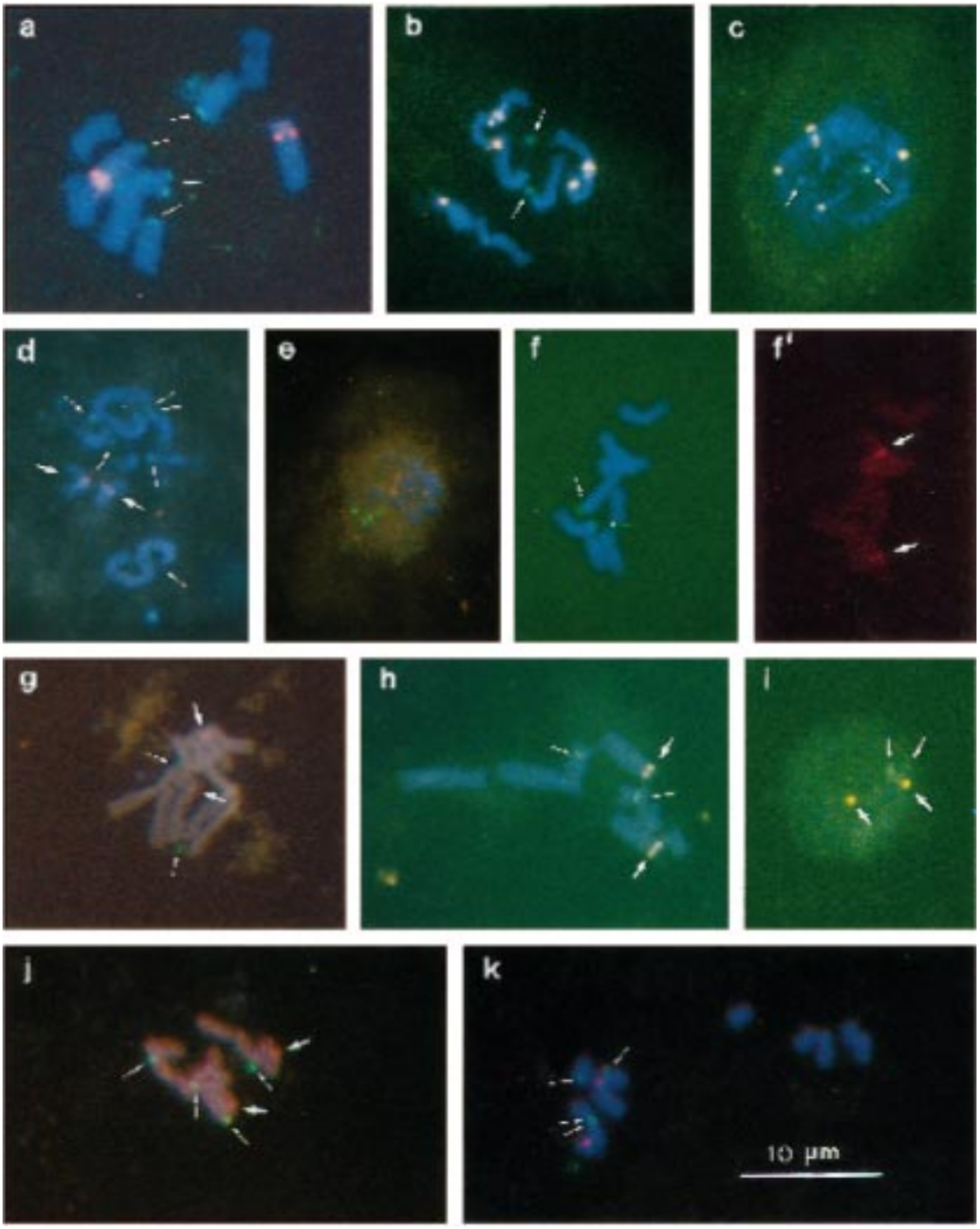

Figure 1. Sites of in situ hybridization with 18S rDNA (green fluorescence) and 5S rDNA (red fluorescence) probes. **(a)** *H. maculata* metaphase plate; **(b)** *H. cretensis* metaphase plate and **(c)** interphase nucleus; **(d)** *H. robertia* metaphase plate and **(e)** interphase nucleus; **(f, f′)** *H. radicata* metaphase plate; **(g)** *H. glabra* metaphase plate; **(h)** *H. megapotamica* metaphase plate and **(i)** interphase nucleus; **(j)** *H. achyrophorus* metaphase plate; **(k)** *H. laevigata* metaphase plate.

technique, the intensity of signals reflects the copy number of genes (Maluszynska and Heslop-Harrison 1991). In general the larger the genome the higher the number of ribosomal repeats (Macgregor 1993). Thus *H. robertia,* which has a low DNA content (Cerbah et al. 1995), might be expected to have a small number of rDNA copies**.** The small size of signals in *H. robertia* corroborates this hypothesis, despite this species possessing the greatest number of 18S-5.8S-28S rDNA loci.

There was a little variability between species in the number of 5S rDNA loci. All the species studied, except *H. cretensis,* had a single chromosome pair with 5S rDNA signals. In the four South American species, the 5S rDNA locus was on the chromosome pair 2, while in the European species, location of 5S rDNA was variable. *H. cretensis* had three 5S rDNA loci, two being on pair 1. This result supports the hypothesis that this pair has derived by Robertsonian fusion of two chromosome pairs, each bearing a 5S rDNA locus. The additional signal observed on one chromosome of pair 2 needs more investigation to determine its origin; it could be population specific or due to stringency conditions.

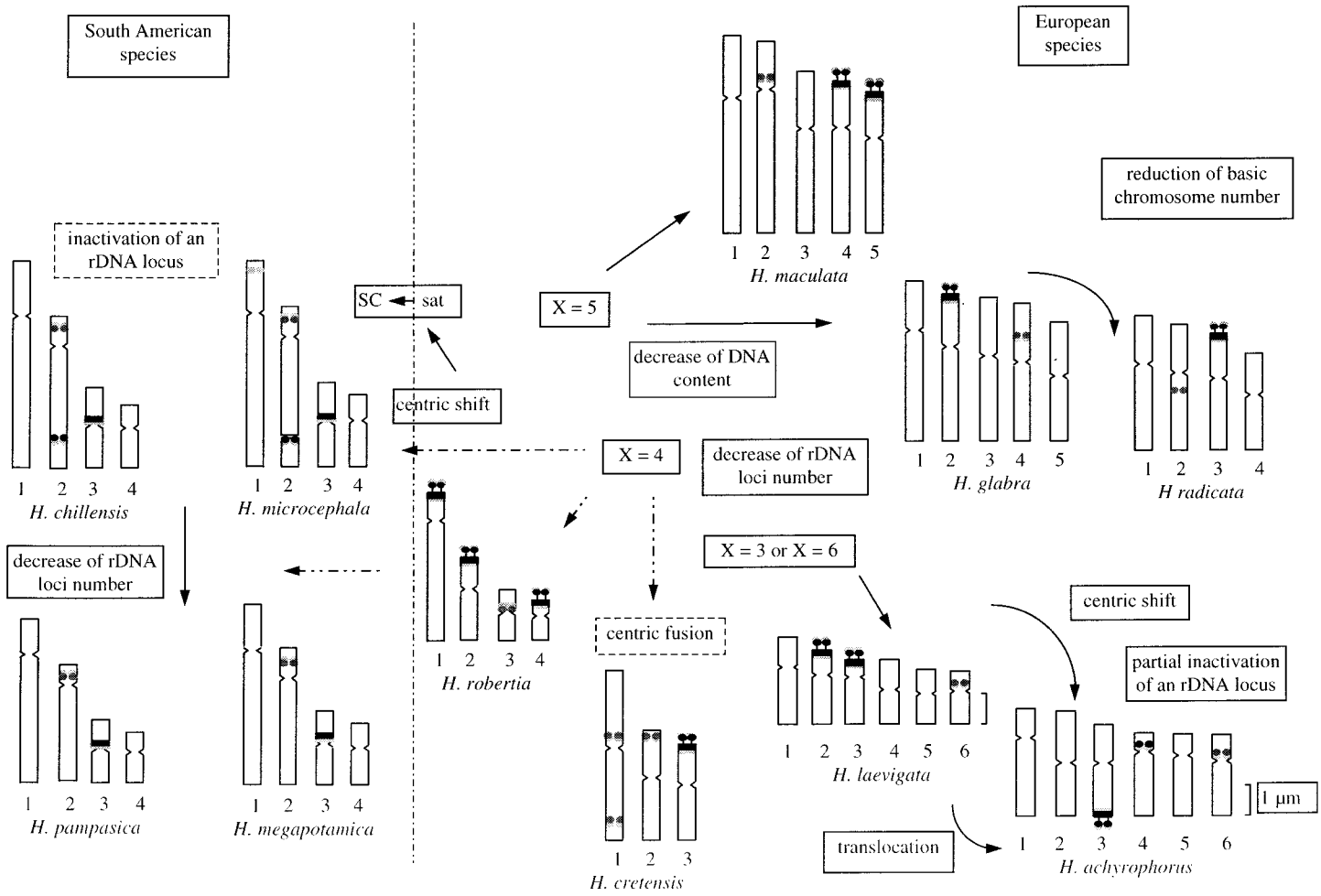

Figure 2. Hypothetical scheme of karyotype evolution in *Hypochaeris* genus. ■ 18S rDNA sites, ▨ 5S rDNA sites, □ CMA_3^+ bands.

The CMA_3-positive bands were present around the NORs in all the species studied, indicating that GC-rich heterochromatin accompanied ribosomal genes coding for 18S-5.8S-28S rRNA. The same has already been reported in many species (Doudrick et al. 1995; Hizume 1992; Schweizer 1976). In our case, colocation of 5S rDNA and CMA_3 bands was also observed in all the species, except in *H. radicata,* for which further experiments are needed. In slash pine (Doudrick et al. 1995) 5S rDNA and CMA_3 bands are not found at the same chromosomal location. However, more data about genome organization of 5S rDNA in relation with heterochromatin are lacking.

18S-5.8S-28S rDNA Activity

In *H. microcephala* and *H. maculata,* the maximum number of nucleoli per nucleus was four, indicating that both rDNA loci are transcriptionally active. In *H. robertia,* the percentage of cells with one or two nucleoli visible after silver staining was particularly high, although previous observations of meiosis (Cerbah et al. 1995) revealed three NOR-bearing bivalents. This result could be explained by the presence of few rDNA copies at each site. In such instances NORs often fuse and form a reduced number of nucleoli.

H. chillensis had two pairs of 18S rDNA sites, but the maximum number of nucleoli in interphase nuclei was only two. Consequently one locus appeared to be inactive. Because the presence of a secondary constriction generally indicates activity of the rRNA genes, the inactive rDNA locus probably is located on pair 2, which did not show a secondary constriction. In *Vicia faba* (Hizume 1992) in situ hybridization of an 18S rDNA probe has shown signals at the secondary constriction and on each side of it, but silver nitrate has produced deposits only in the interstitial region. The author concluded that a large proportion of rRNA genes located on each side of the secondary constriction remained inactive during the cell cycle in the root meristematic tissue. In the case of *H. chillensis a* whole locus appeared methylated, while in *H. achyrophorus* the locus observed on pair 4 would be partially inactive, considering the micronucleoli observed (Linde-Laursen 1984) and the absence of a secondary constriction. In all the species, heterochromatin was present at all rDNA loci, either active or not. This result supports the hypothesis that the presence of heterochromatin facilitates structural rearrangements (Miklos et al. 1980), especially those involving NORs, which are known for their mobility in the genome (Schubert 1984).

Evolutionary Relationships Between Species

The primary center of diversification of the genus *Hypochaeris* is the Mediterranean region, the relatively few species possessing various basic chromosome numbers and karyotypes, as well as variable rDNA localization. South America, which contains the largest number of species, is a secondary center of diversification of the genus. The American species studied show only one basic chromosome

number of $x = 4$, one karyotype (bimodal and asymmetric) considered as derived (Stebbins 1971), and have very similar patterns of rDNA distribution.

According to obtained results, we tried to trace the evolutionary history of rDNA location and to develop hypotheses of the chromosomal evolution in the genus *Hypochaeris* (Figure 2). The ancestral karyotype of *Hypochaeris* may be symmetrical with a basic chromosome number of $x = 5$, following Stebbins et al. (1953). Alternatively, the $x = 4$ karyotype might be ancestral, considering that this is the most common basic chromosome number of the genus.

Positions of the rDNA loci indicate some phylogenetic relations. Based on the number and intercalary position of 5S rDNA signals, *H. radicata* is closely related to *H. glabra.* Moreover, Parker (1975) reported complete homology of the NOR-bearing chromosomes in these two species after study of meiosis in their hybrids. In *H. cretensis,* the two 5S rDNA loci observed on the metacentric chromosome pair 1 and the CMA_3-positive bands in the same region, suggest that this species derived from a $2n = 8$ ancestor by a Robertsonian fusion.

In European species, all 18S rDNA sites were located at the secondary constrictions of the satellite regions of chromosomes with the exception of *H. achyrophorus.* This species seems to be very close to *H. laevigata.* These are the only species that have $2n = 12$ chromosomes. They displayed a single locus of 5S rDNA at similar positions on pair 6, and two loci of 18S rDNA, but at a different chromosomal location. Therefore we suggest that the paracentromeric locus of 18S rDNA on pair 4 in *H. achyrophorus* is the result of a centric shift of the short arm of a chromosome bearing a satellite. The locus observed in the satellite region of pair 3 could be the result of a translocation on the long arm of a chromosome. Translocation of rDNA sequences has already been reported in *Hypochaeris.* Hall and Parker (1995) have observed such a translocation in an individual of *H. radicata* after a Robertsonian fission. Translocation could be also suggested for *H. robertia.* According to the small size of the signal in *H. robertia,* partial translocations of a major site could explain the increased rDNA loci number.

The South American species are related to the group of *H. maculata* group, since they belong to the same primitive taxonomic section, *Achyrophorus,* as proposed by Stebbins et al. (1953). Thus their karyotype evolution would be the result of chromosomal rearrangements involving translocations of rDNA genes from satellite regions to intercalary sites, with the secondary constriction of chromosome pair 3 probably bearing the major locus of 18S rDNA. Moreover, the same paracentromeric position of 5S rDNA signals found in *H. maculata* and in the South American species supports this hypothesis. *H. microcephala* has conserved two 18S-28S rDNA loci like *H. chillensis,* but in this latter species an inactivation of one locus (on pair 2) occurred during evolution. The same locus was probably eliminated in species like *H. megapotamica* and *H. pampasica.*

These results suggest that chromosome rearrangements, such as centric shifts and centric fusions, and inactivation of rDNA genes occurred during the course of evolution of *Hypochaeris* species from the two geographical areas. These rearrangements have led to a decrease of rDNA loci number from an ancestral genome probably possessing two loci of 18S-5.8S-28S rDNA and one locus of 5S rDNA. Nevertheless, more South American species must be studied to establish the behavior of rDNA loci during evolution of this group.

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Received December 20, 1996
Accepted December 13, 1997

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