Research Article

Chromosomal organization and phylogenetic relationships in *Hypochaeris* species (Asteraceae) from Brazil

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Abstract

The association of cytogenetic and molecular techniques has contributed to the analysis of chromosome organization and phylogeny in plants. The fluorochrome GC-specific CMA_3, fluorescent in situ hybridization (FISH) and RAPD (Random Amplified Polymorphic DNA) markers were used to investigate chromosome structure and genetic relationships in *Hypochaeris* (Asteraceae). Seven species native to South America, and two species introduced from Europe (*H. glabra* and *Hypochaeris* sp) were studied. FISH with rDNA probes identified one or two loci of 18S-5.8S-25S rDNA in the South American *Hypochaeris* species and one locus in the European species. Only one 5S rDNA locus was seen in all species studied. Blocks of GC-rich heterochromatin (CMA-positive bands) associated to 18S-5.8S-25SrDNA loci were detected in all species investigated. Co-location of 5S rDNA and CMA bands was also observed, except for three South American species and *Hypochaeris* sp. In two South American species, additional CMA bands not related to rDNA were observed on the long arm of chromosome 2, near to the centromere. *Hypochaeris glabra* exhibited additional CMA-positive signals distributed at pericentromeric regions, on the short arms of all chromosomes. A total of 122 RAPD markers were used to determine the genetic relationships among species. The level of polymorphism was very high, revealing two genetic groups comprising the South American and the European species, thus supporting a previous hypothesis of monophyly of the South American *Hypochaeris* species. The coefficients of genetic similarity between European and South American species were 0.35, on average. Polymorphism was also high within the two groups. The genetic associations observed with RAPD markers were consistent with chromosome characteristics. Species carrying similar distribution of 45S rDNA loci and CMA-positive signals were included in the same group revealed by RAPDs. Cytogenetic and molecular data support the view that not only chromosome rearrangements, but also changes in DNA sequence took place during the diversification of the South American *Hypochaeris* species.

Key words: chromosome banding, FISH, *Hypochaeris*, phylogenetic relationship, RAPD.

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Introduction

The genus *Hypochaeris* (Asteraceae) is represented by around 60 species occurring in two major areas of distribution, comprising the Mediterranean region and South America. While the Mediterranean group includes about 13 species, the South American species are more numerous with around 50 species (Stuessy, 2001). Species relationships within the genus have been investigated in several studies. Stebbins (1971) suggested, on the basis of distinct chromosome characterists, that the Mediterranean was the primary center of origin and that South America represents the center of diversification of the genus. Cytogenetic stud-

ies have shown a basic chromosome number of x = 3, 4, 5 and 6 associated with symmetric karyotypes that characterizes the European Hypochaeris species (Stebbins, 1971; Cerbah *et al*., 1995, 1998a; Ruas *et al*., 1995). On the other hand, the South American species have a basic chromosome number of x = 4 and highly asymmetric and bimodal karyotypes (Stebbins, 1971; Barghi *et al*., 1989; Ruas *et al*., 1995). According to Stebbins (1971), asymmetric karyotypes would have originated through changes occurred in less complex chromosome sets, thus, the South American *Hypochaeris* species would have derived from European species (Siljak-Yakovlev, 1994; Cerbah *et al*., 1995, 1998a; Ruas *et al*., 1995).

Differences on chromosome size and satellite distribution have been observed among *Hypochaeris* species

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(Ruas *et al.*, 1995; Cerbah *et al.*, 1995, Cerbah *et al.*, 1998a). Analyses of DNA content revealed a C-value varying from 1.68 to 8.10 pg and, with few exceptions, there was no significant correlation between AT or GC content, suggesting that variability in genome sizes is a consequence of differences in the content of interspersed repetitive DNA sequences (Cerbah *et al.*, 1995, 1999).

Recently, fluorescent in situ hybridization (FISH) has been the method of choice for assigning DNA sequences to chromosomes. The technique is specially appropriated to understand the chromosomal organization and to determine the physical distribution of specific DNA sequences on the chromosomes (Heslop-Harrison, 2000). Differences in number and distribution of 45S and 5S rDNA loci among related species have proven to be suitable to study genome evolution and species relationships in plants such as *Calycadenia* (Baldwin, 1993), *Hordeum* (Leitch and Heslop-Harrison, 1993), *Aegilops* (Castilho and Heslop-Harrison, 1995), *Vigna* (Galasso *et al.*, 1998) and *Clivia* (Ran *et al.*, 2001). Application of FISH to determine the distribution of 45S and 5S rDNA loci on *Hypochaeris* chromosomes, combined with fluorochrome banding, suggested that chromosome rearrangments such centric shifts and centric fusions, transposition, and inactivation of rDNA have occurred during the evolution of European and South American species of *Hypochaeris* (Cerbah *et al.*, 1998a). These rearrangements were also concluded to be implicated in the reduction of rDNA loci in the genus.

Major advances on molecular techniques have permitted a better understanding of genome structure in Angiosperms. Recently, molecular systematic analysis has been used for phylogenetic studies within the genus *Hypochaeris* (Cerbah *et al.*, 1999; Samuel *et al.*, 1999). Sequence analyses of nuclear ITS regions and chloroplast trnL-F intron, trnL/trnF spacer and matK sequences, carried out to assess phylogenetic relationships among European and South American species of *Hypochaeris*, revealed that, except for *H. Robertia*, the clades formed by the ITS and the combined trees agree with sections previously stablished for the genus on the basis of morphological and cytological studies (Cerbah *et al.*, 1999; Samuel *et al.*, 1999, 2003). These observations provided evidence for monophyly of the South American species, which would have evolved from a single introduction of European progenitors rather than from *H. robertia*, as previously suggested (Barghi *et al.*, 1989).

Among the different classes of molecular markers applied to assess genetic variation and taxonomic relatedness between plant groups, random amplified polymorphic DNA (RAPD) (Wesh and McClelland, 1990; Williams *et al.*, 1990) has proved to be particularly appropriated. The technique is based on DNA amplification using 10-mer oligonucleotides in a PCR (Polymerase Chain Reaction). It produces a large number of easily generated markers without the need of previous knowledge of the genome. The

success of RAPD markers for taxonomic and evolutionary studies has been observed in several plant species such as *Stylosanthes* (Kazan *et al.*, 1993), Malus (Dunemann *et al.*, 1994), *Actinidia* (Cipriani *et al.*, 1996), *Pisum* (Hoey *et al.*, 1996), *Vigna* (Santala *et al.*, 1998) and *Ananas* (Ruas *et al.*, 2001).

Considering the need for more studies on the Brazilian group of South American *Hypochaeris* species, the present work associated chromosome analysis, using FISH with 45S and 5S rDNA probes and fluorochrome banding, with RAPD makers to evaluate the interspecific relationships between seven Brazilian and two introduced European species of *Hypochaeris*. The phylogenetic schema was compared with the mechanisms proposed for chromosome evolution in the genus. Moreover, the karyotype of *H. grisebashii* is described here for the first time.

Materials and Methods

The specimens studied include seven species native to the Brazilian flora of the South American *Hypochaeris* and one species (*H. glabra*) introduced from Europe. An additional species (*Hypochaeris* sp) was also studied. Plant material used for the analysis of *Hypochaeris* sp was obtained from cypselae collected from plants of a population of *H. radicata* for which no karyotype analysis was available. Information about the origin of the species is summarized in Table 1. Voucher-specimens are preserved in the Herbarium of the Botanic Department of the Universidade Federal do Rio Grande do Sul (ICN), Porto Alegre, Rio Grande do Sul, Brazil.

Chromosome preparations

Root-tip meristems obtained from potted plants were pre-treated with 0.1% colchicine at room temperature for 1 h

Table 1 - Chromosome numbers and collection sites of *Hypochaeris* species investigated.

Species	2n	Collection sites
H. brasiliensis (Less) Benth et Hook	8	Guaíba, RS[a]; Curitiba, PR; Piracicaba, SP
H. grisebashii Cabr.	8	Guaíba, RS
H. megapotamica Cabr.	8	Guaíba and Capão da Canoa, RS
H. microcephala (Sch.-Bip) Cabr. var. *albiflora* (O.K.) Cabr.	8	Guaíba, RS
H. microcephala (Sch.-Bip) Cabr. var. *microcephala* (O.K.) Cabr.	8	Guaíba, RS
H. pampasica Cabr.	8	Guaíba, RS
H. rosengurttii Cabr.	8	Guaíba, RS
H. variegata Baker	8	Guaíba, RS
H. glabra L.	8	Curitiba and Ponta Grossa, PR
Hypochaeris sp.	10	Capão da Canoa, RS

[a]The abbreviations following collection sites correspond to Brazilian states: RS = Rio Grande do Sul; PR = Paraná; SP = São Paulo.

and 30 min and then fixed in ethanol-acetic acid 3:1 for at least 12 h. Chromosome identification was carried out for *Hypocheris* sp and for H. grisebashii, using the Feulgen staining method, as previously described by Ruas *et al*. (1995).

Chromosome preparations for FISH and chromomycin A_3 banding were obtained as described by Schwarzacher *et al*. (1980). Briefly, pre-treated root-tips were washed twice in citrate buffer (0.01 M citric acid-sodium citrate, pH 4.6) and macerated in enzyme solution (2% cellulase (w/v) and 20% pectinase (v/v) at 37 °C for 2 h. After enzymatic treatment, the roots were washed in citrate buffer and the cells were dissociated in a drop of 45 % acetic acid.

In situ hybridization

The ribosomal probes pTa71 and pTa794, isolated from *T. aestivum* (Gerlach and Bedbrook, 1979; Gerlach and Dyer, 1980) were used to locate the position of the 45S and 5S rDNA sites on the chromosomes. The probes were labeled with biotin-14-dATP by nick translation (Bio Nick, Gibco). FISH was carried out following the methods of Heslop-Harrisson *et al*. (1991) and Cuadrado and Jouve (1994) with minor modifications. The slides were incubated in 100 µg/mL DNase-free RNase in 2x SSC (0.03 M sodium citrate and 0.3 M sodium chloride) for 1 h at 37 °C, washed for 5 min in each 2x SSC plus 4% paraformaldehyde and 2x SSC, dehydrated through an ethanol series, and air- dried. The labeled rDNA probes (100-200 ng/slide) were mixed with 50% formamide, 10% (w/v) polyethylenoglycol, 0.3% (w/v) sodium dodecyl sulfate, 300 mg/mL calf thymus DNA, and 2x SSC. The hybridization mixture was denatured at 70 °C for 10 min and immediately chilled on ice. Then the slides were loaded with 30 µL of probe mixture, denatured at 90 °C for 10 min, transferred to 50 °C for 10 min and to 38 °C for 10 min. Hybridization was performed overnight at 37 °C in a moist chamber. Posthybridization washes were done as described by Cuadrado and Jouve (1994). The hybridization sites were detected with avidin-FITC conjugate (5µL/mg/mL) in 5% bovine serum albumin (BSA) at 37 °C for 1 h. Chromosomes preparations were counterstained with 1 µL (2.5 µL/mL) propidium iodide for 30 min, mounted in 20 µL of antifade solution (Vectashield H1000, Vector Laboratories). Preparations were examined with a Nikon Labophot microscope and photographed on Kodak 400 Proimage film.

Chromomycin A_3 banding

Chromosome preparations aged for three days were incubated in 45% acetic acid at 60 °C for 10 min, washed in double-distilled water and treated with 5% $Ba(OH)_2$ for 5 min at room temperature. Preparations were then incubated in 2x SSC at 60 °C for 1 h and 20 min, washed in double-distilled water, and air-dried. Three-day-old slides were incubated in McIlvaine buffer (0.1 M citric acid plus 0.2 M Na_2 HPO_4 7 H_2O, pH 7.0) for 10 min and stained with CMA_3 (0.5mg/mL in McIlvaine buffer plus 2.5 mM

$MgCl_2$) for 1 h and 30 min in a dark chamber. The preparations were then mounted in 1:1 (v/v) glycerol:McIlvaine buffer and the slides aged for another three days. Metaphases were examined with a Nikon microscope using a CMA_3 selective filter (425-445 nm) and photographed on Kodak Tmax 100 film.

DNA extraction and RAPD amplification

Genomic DNA was isolated from young leaves of at least five individual plants of each species using the CTAB (cetyltrimethyl-ammonium bromide) procedure (Doyle and Doyle, 1989) with modifications, as previously described (Ruas *et al*., 2001).

Amplification reactions were carried out in volumes of 15 µL containing 1X Taq polymerase buffer (10 mM Tris-HCl pH 8.3; 50 mM KCL); 1.5 mM $MgCl_2$; 0.1 mM each of dATP, dTTP, dCTP, and dGTP (Pharmacia), 4 mM of 10-mer arbitrary primers (Operon Technologies); 1 U of Taq DNA polymerase (Pharmacia); and 20 ng of template DNA, using a PTC-200 (MJ Research) thermal cycler with the same cycling parameters as reported by Ruas *et al*. (2001). Amplification products were resolved in 1.2% metaphor agarose (FMC) in 1x TAE buffer (40 mM Trisacetate; 1 mM EDTA, pH 8.0) for 3 h at 120 V. Gels were stained with ethidium bromide, visualized under UV light, photographed with a video camera and stored in a microcomputer for later analysis.

Data analysis

DNA markers were scored for the presence (1) or absence (0) of homologous DNA bands. The genetic similarities were estimated using the Dice coefficient of the NTSYS package (Numerical Taxonomy and Multivariate Analysis for personal computer), version 2.1 (Rohlf, 2000). A dendrogram was constructed using the UPGMA (unweighted pair-group method using arithmetic averages). The values of the bootstrap method, employed to evaluate the reliability of tree topology, were calculated with 1000 replicates using the BOOD software, version 3.0 (Coelho, 2001). The cophenetic coefficient between the genetic similarity matrix and the dendrogram was computed using an appropriate routine of the NTSYS-pc software. The significance of the cophenetic correlation was determined by using the Mantel correspondence test (Mantel, 1967).

Results and Discussion

Karyotype analysis of *H. grisebashii* and *Hypochaeris* sp.

Chromosome analysis of *H. grisebashii* was performed for the first time. The karyotype formula is 2n = 8 = 4m+4st, with the same bimodal and asymmetric pattern that characterizes the South American *Hypochaeris* species analyzed so far (Stebbins, 1971; Ruas *et al*., 1995; Cerbah *et al*., 1995; 1998a). The size of the chromosomes varied from

2.11 to 5.93 μm, with the secondary constriction located on chromosome pair 3 near to the centromere. The total haploid length (15.0 ± 1.52 μm) was considerably smaller than in other South American species reported by Ruas *et al.* (1995). This difference suggests that structural alterations involving decrease of DNA content are still playing a role in the chromosome evolution of South American *Hypochaeris* species.

Chromosome measurements of *Hypochaeris* sp revealed a symmetrical karyotype with 2n = 10 = 8m+2sm-sat, following the pattern of European species. The specimens evaluated displayed morphological characters of *H. radicata*. For instance, all cypselae were rostrated and the trichomes at the involucral bracts exhibited specific morphology and distribution only found in *H. radicata*. However, most investigations reported 2n = 8 chromosomes for *H. radicata* (Ruas *et al.*, 1995; Cerbah *et al.*; 1998a). The only exception was reported by Hall and Parker (1995) who described, in an experimental population of *H. radicata* with 2n = 8, individuals presenting centric fission in one chromosome and then, heterozygous and homozygous individuals with 2n = 9 and 2n = 10 chromosomes, respectively. The findings of the present study

show that the plants analyzed could represent a chromosome race of *H. radicata* and further studies are needed to substantiate this possibility.

Localization of 45S and 5S ribosomal genes

The results of FISH with rDNA 45S and 5S probes are shown in Figures 1 and 2, respectively, and summarized in Figure 5 and Table 2. All South American species showed one 45S rDNA locus, identified around the secondary constriction of chromosome pair 3. This locus was pericentromeric in all species, except for *H. rosengurttii* (Figure 1F), which had the hybridization signal in a terminal position and satellite of chromosome 3, revealing that paracentric inversions are implicated in changes in the position of 45S rDNA loci of South American *Hypochaeris*. Analysis of metaphases and interphase nuclei revealed variation in the size of hybridization signals among species, suggesting differences in the number of gene copies at this locus. The signals were stronger, covering most of the chromosome arm (Figure 1 C, E, G), in H. megapotamica, H. pampasica and H. variegata than in *H. brasiliensis*, *H. grisebashii* and *H. microcephala* (Figure 1 A, B, D). Other hybridization signals were observed in a distal position on
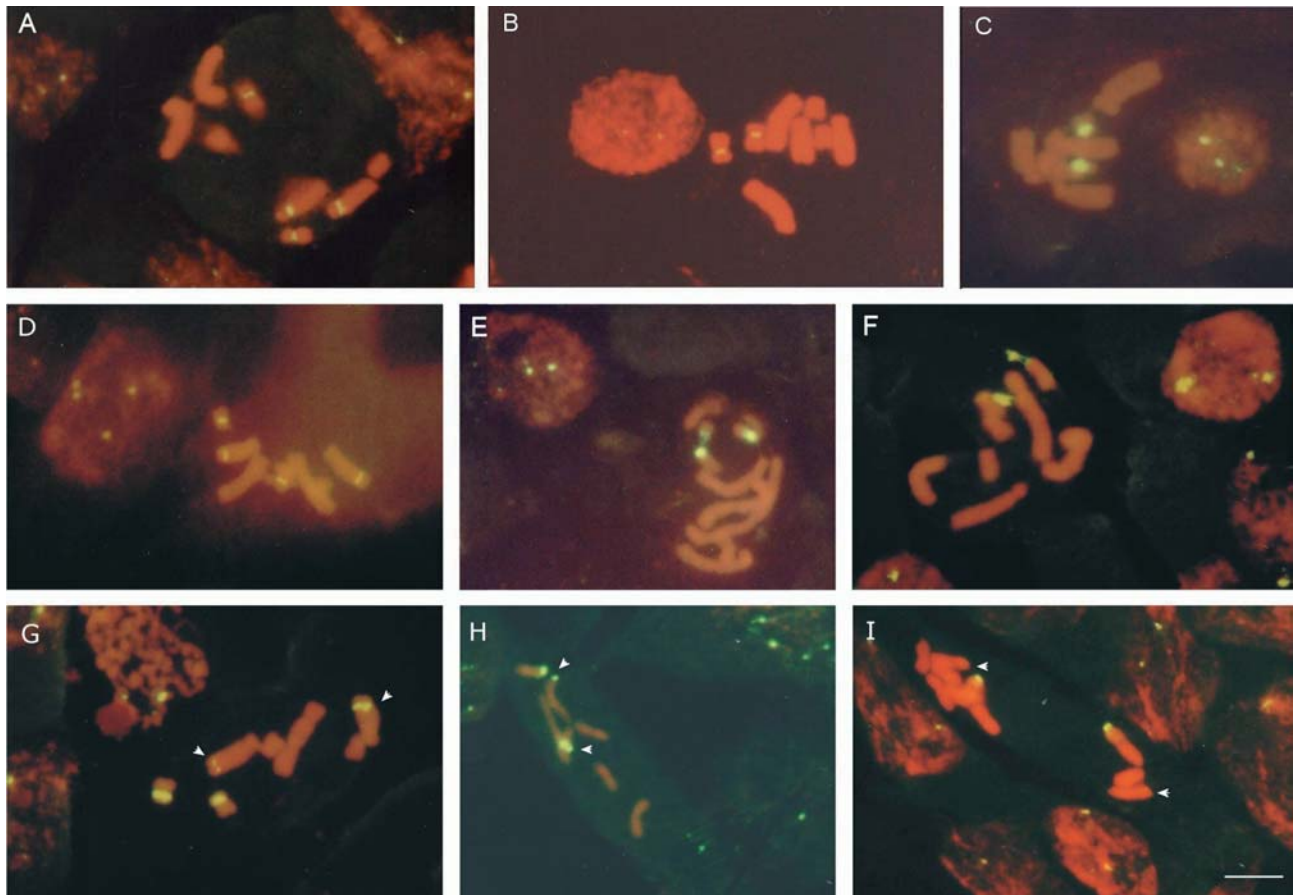

Figure 1 - Hybridization signals of mitotic metaphases probed with 45S rDNA genes in *Hypochaeris* species: (A) *H. brasiliensis*, (B) *H. grisebashii*, (C) *H. megapotamica*, (D) *H. microcephala*, (E) *H. pampasica*, (F) *H. rosengurtii*, (G) *H. variegata* (arrows = size polymorphism of 45S rDNA on chromosome pair 2), (H) *H. glabra*, and (I) *Hypochaeris* sp (arrows = weak 45S hybridization signals on chromosome 4) Bar = 6 μm.
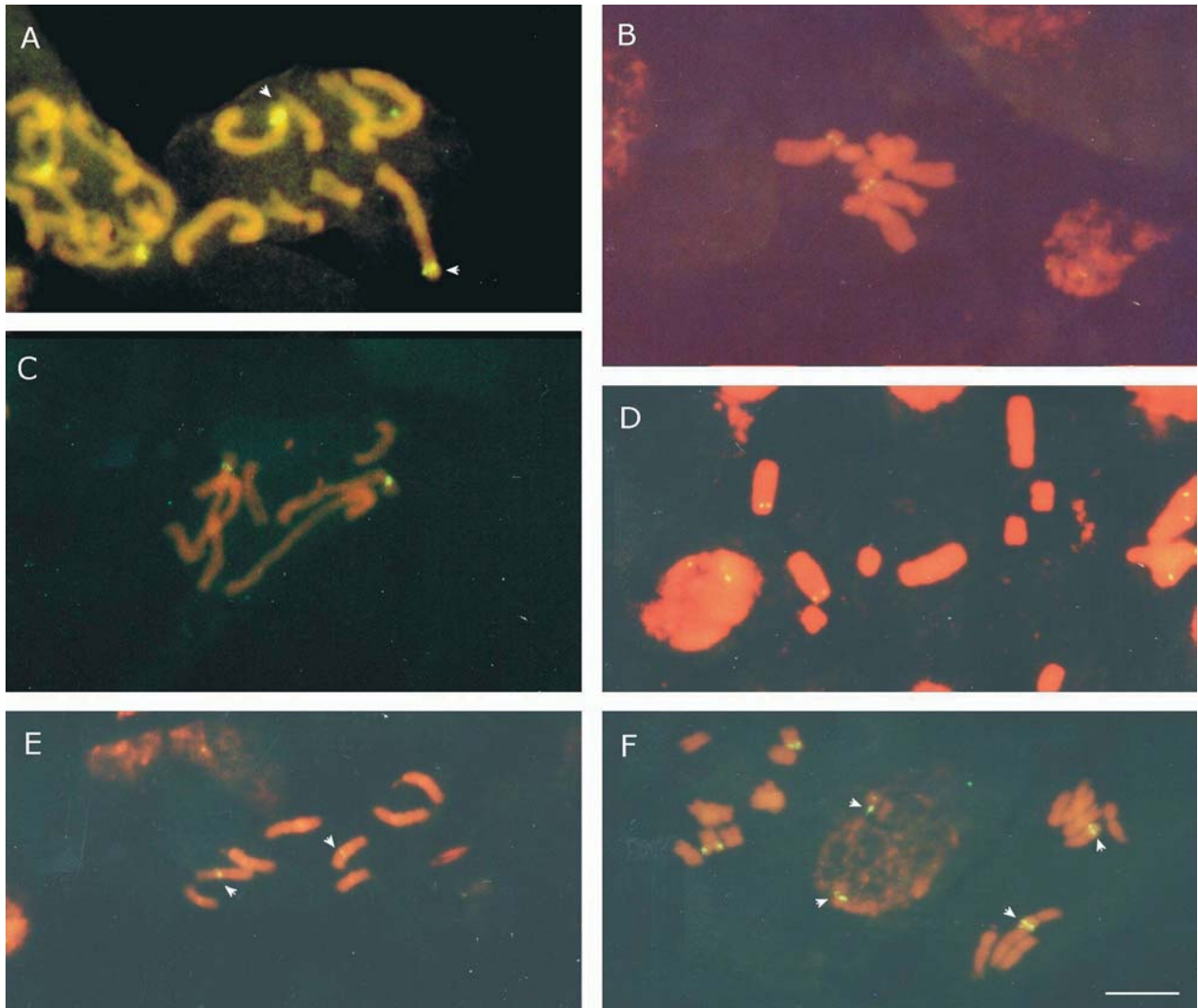
Figure 2 - Hybridization signals on mitotic metaphases probed with 5S rDNA genes in *Hypochaeris* species: (A) *H. grisebashii*, (B) *H. microcephala*, (C) *H. rosengurtii*, (D) *H. variegata*, (E) *H. glabra*, and (F) *Hypochaeris* sp. Bar = 6 μm.

the long arm of chromosome 2 in *H. brasiliensis* and in the two varieties of *H. microcephala* (Figure 1 A, D). Analysis of *H. brasiliensis* using silver impregnation (Ruas *et al.*, 1995) detected transcriptional activities in both chromosome pairs 2 and 3. *Hypochaeris variegata* also displayed an additional 45S rDNA locus in the same position of chromosome 2 (Figure 1 G). However, analysis of several individuals of this species showed that chromosome pair 2 is heteromorphic for the NOR locus, showing a conspicuous difference in the intensity of the FISH signals, which could be related to a reduced number of rDNA copies. The maximum number of nucleoli per nucleus was three in *H. variegata* (data not shown), suggesting that the NOR locus is transcriptionally inactive in one chromosome of pair 2. However, this suggestion needs further investigation. Variation in the expression of 45S rDNA loci was also reported in *H. chilensis* (Cerbah *et al.*, 1998a). In *H. variegata*, the

polymorphism observed in the size of the rDNA site could have originated by unequal crossing-over or by amplification and transposition of single rDNA copies, as suggested for other plant species (Schubert and Wobus, 1985; Garrido *et al.*, 1994).

Cerbah *et al.* (1998a) suggested that the main 45S rDNA site for the South American species is located on chromosome 3. We consistently observed one 45S rDNA locus located on the short arm of chromosome 3 of all South American species investigated. Nevertheless, an additional 45S rDNA site appeared regularly on the long arm of chromosome 2 of *H. brasiliensis*, *H. microcephala*, and *H. variegata*. These data provide additional evidence to the observations of Cerbah *et al.* (1998a) that the 45S rDNA site located on chromosome 2 was conserved in some species and inactivated or eliminated during the karyotype evolution of *Hypochaeris*.

The two populations of the Mediterranean species *H. glabra* showed the same chromosome number (2n = 8) reported by Ruas *el al*. (1995), in contrast with 2n = 10 reported for European populations of this species. FISH analysis revealed a single terminal 45S rDNA locus on the small arm of chromosome 2 of H. glabra Figure 1 H). In *Hypochaeris* sp, the 45S rDNA locus was located at the end of the small arm of chromosome 3, besides an additional signal of weak intensity detected in a terminal position on the short arm of chromosome 4, in which a satellite was absent (Figure 1 I).

Variability in rDNA regions have been explained by translocation, as for instance in humans, in which translocations would have involve the NOR chromosomes 13, 14, 15, 21, and 22 (for review, see Hall and Parker, 1995). In some plant genomes, variability in the location of rDNA loci has been interpreted as a consequence of transposition of rDNA sequences, as suggested for *Allium cepa* (Schubert and Wobus, 1985). The same mechanism was proposed for *H. radicata* by Hall and Parker (1995). The authors suggested that the origin and stabilization of a fission rearrangement in individuals of *H. radicata* with 2n = 8 involved transposition of rDNA from the NOR region on chromosome 3 into the centromeric region of a fission derivative from chromosome 1. In the present study, we could suppose that some rDNA sequences could have transposed from chromosome 3 to chromosome 4 of *Hypochaeris* sp. Considering the chromosome number (2n = 10) of the analyzed plants, we could also hypothesize that the karyotype of *Hypochaeris* sp was derived by chromosome rearrangements from a plant with 2n = 8 (possibly *H. radicata*).

Previous investigations in *Hypochaeris* showed one, two, or a maximum of three 45S rDNA sites per haploid genome (Cerbah *et al*., 1995; Cerbah *et al*., 1998a). The intensity of hybridization signals was associated with the number of rDNA gene copies. In *H. robertia*, the presence of tiny signals was related with a low number of copies in each of the three 45S rDNA loci and with the small genome of this species (Cerbah *et al*., 1995; 1998a). In the present

study, the hybridization signals observed in the two 45S rDNA loci of *H. brasiliensis* and *H. microcephala* (Figure 1 A, D) were brighter than that of the single locus of *H. grisebashii* (Figure 1 B). Indeed, chromosome measurements of *H. grisebashii* revealed a rather smaller haploid genome in this species, suggesting a possible loss of 45S rDNA sequences that may be related to a lower DNA content.

We have confirmed the presence of a single cluster of 5S rDNA genes supporting the results reported by Cerbah *et al*. (1998a). In all South American species, the 5S rDNA clusters were in an intercalary position on the small arm of chromosome 2, as seen in Figures 2 (A-D) and 5. The regular distribution and the uniformity of the hybridization signals observed in the 5S rDNA loci revealed that these clusters are more stable than 45S rDNA clusters in the genome of South American *Hypochaeris*.

The European species *H. glabra* and *Hypocheris* sp showed different distribution of 5S rDNA sites. *Hypochaeris glabra* revealed one 5S rDNA site, identified near to the centromere on the long arm of chromosome 3. *Hypochaeris* sp showed two closely adjacent 5S rDNA sites identified near the terminal region on short arm of chromosome 2 (Figure 2 E-F). These chromosome locations of the 5S rDNA locus also differed from that reported by *Cerbah et al*. (1998a) for *H. glabra* and *H. radicata*, where the 5S rDNA genes were located at an intercalary position, on the short arm of chromosome 4 and on the long arm of chromosome 2, respectively. These results support the hypothesis that structural rearrangements, such as centric shift may have occurred in populations of European species established in South America.

Chromomycin A$_3$ Banding

The chromomycin A3 banding patterns were comparatively analyzed as summarized in Table 2. In all species, CMA-positive bands were found associated with regions identified as 45S rDNA sites by FISH (Figure 3 A-I and Figure 5). The association between CMA-positive bands

Table 2 - Distribution of CMA$_3$ positive bands and 45S and 5S rDNA loci per haploid genome of *Hypochaeris* species.

Species	Number and distribution of CMA$_3$ bands associated to 45S rDNA	Number and distribution of CMA$_3$ bands associated to 5S rDNA	Number and distribution of additional CMA$_3$ bands
H. brasiliensis	2 (SC of LA of pair 2 and SA of pair 3)	1 (SA of pair 2)	-
H. grisebashii	1 (SC/SA of pair 3)		1 (PC/LA of pair 2)
H.megapotamica	1 (SA of pair 3)	1 (SA of pair 2)	-
H. microcephala	2 (SC/LA of pair 2 and SC/SA of pair 3)	1 (SA of pair 2)	-
H pampasica	1 (SA of pair 3)	1 (SA of pair 2)	1 (PC/LA of pair 2)
H. rosengurttii	1 (SA of pair 3)	-	-
H. variegata	2 (SA of pair 3)	-	-
H. glabra	1 (sat of pair 2)	1 (LA of pair 3)	4 (PC/SA of pairs 1-4)
Hypochaeris sp	1 (sat of pair 3)	1 (SA of pair 2)*	-

PC = pericentromeric; SC = secondary constriction; sat = satellite; SA = short arm; LA = long arm.
* 5S rDNA not associated with CMA bands.

and 45S rDNA loci was previously observed by Cerbah *et al*. (1995; 1998a) in European and South American *Hypochaeris* species. This distribution pattern of GC- rich heterochromatin has also been described in many other related or unrelated plant groups (Galasso *et al*., 1995, 1998; Fukui and Nakayama, 1996).

Co-location of 5S rDNA and CMA+ bands was clearly observed in *H. brasiliensis*, *H. megapotamica*, both varieties of *H. microcephala*, *H. pampasica* and *H. glabra* (Figures 3 A, C, D, E, H and Figure 5), but this correspondence was not detected in the other species investigated, including *Hypochaeris* sp. Cerbah *et al*. (1998a) reported the co-location of 5S rDNA loci and CMA bands in South American and European species, except *H. radicata*. These findings need further investigation concerning the organization of 5S rDNA repeat units in *Hypochaeris*, to understand this CMA response.

Additional CMA-positive bands, not related to rDNA, were observed on the long arm of chromosome 2, near to the centromere, in *H. grisebashii* and *H. pampasica* (Figure 3 B, E). In *H. glabra*, additional bands exhibiting equilocal distribution were seen near to the centromere on the short arm of all chromosomes. (Figure 3 H). This pattern of CMA bands is totally different from that described by Cerbah *et al*. (1998a), for a European population of *H. glabra* where only one intercalary band was observed on the short arm of pair 4, besides the satellite-associated band. Cerbah *et al*. (1995) also detected a thin CMA_3 band on the short arm of chromosome 1 in *H. microcephala*, which was absent in the populations analyzed in this study.

Determination of DNA content and DNA base composition showed little differences in GC percentage in *Hypochaeris* species (Cerbah *et al*., 1995), supporting the relatively homogeneous pattern of CMA_3 blocks observed in the present study, especially in the South American species.

Genetic relationships based on RAPD markers

RAPD markers have been considered as suitable characters for genetic analysis since they allow to examine accumulated genetic differences that are important at various taxonomic levels. Molecular markers such as RAPDs are neutral characters with no known phenotypic effects (Aagaard *et al*., 1998) and therefore, they are excellent
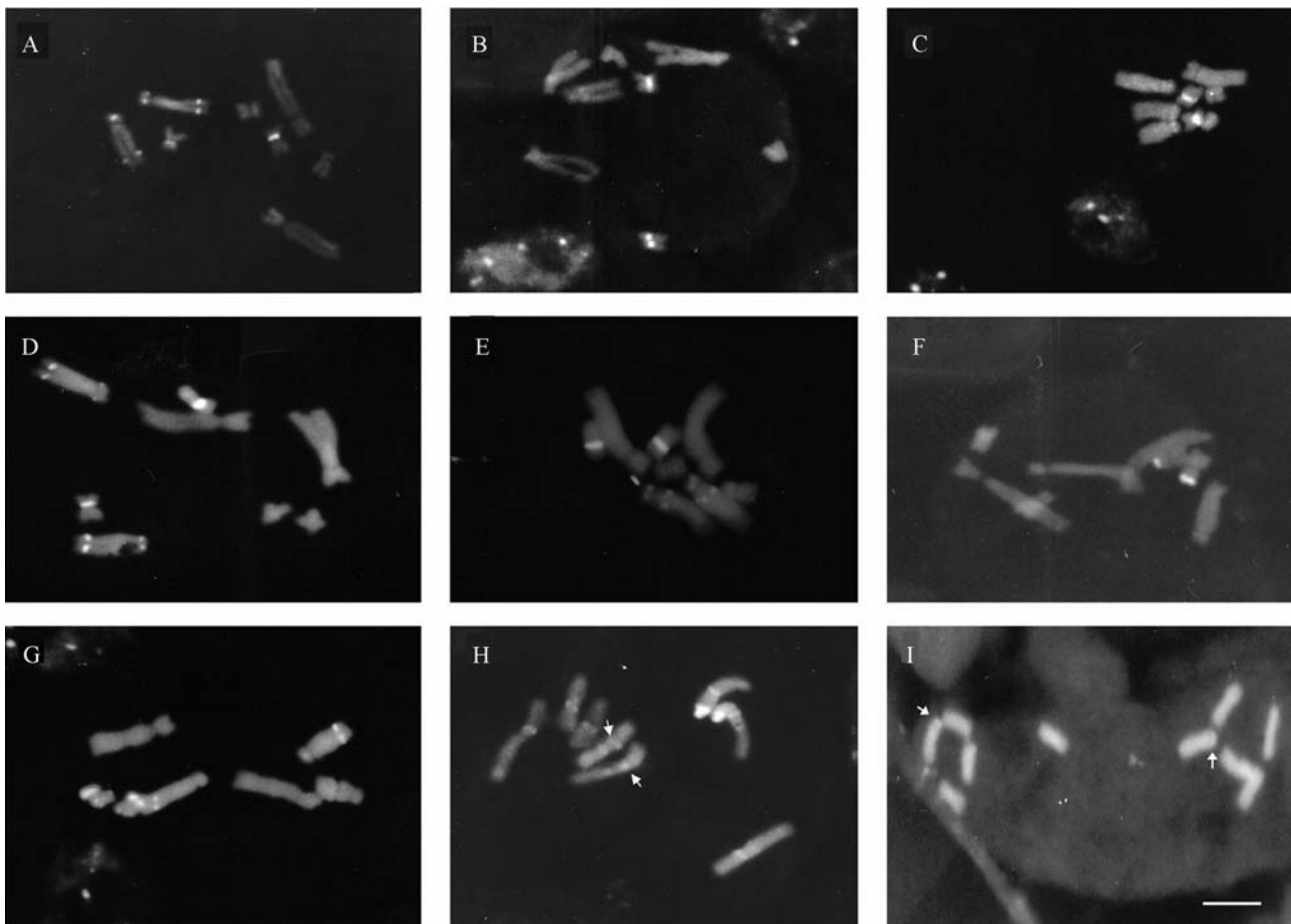

Figure 3 - Mitotic metaphases stained with chromomycin A_3 showing the distribution of GC-rich heterochromatin in *Hypochaeris* species: (A) *H. brasiliensis*, (B) *H. grisebashii*, (C) *H. megapotamica*, (D) *H. microcephala*, (E) *H. pampasica*, (F) *H. rosengurttii*, (G) *H. variegata*, (H) *H. glabra*, and (I) *Hypochaeris* sp (arrows = weak CMA_3 bands)*. Bar = 6 μm.

tools for studying genetic variability of natural populations. Additionally, because RAPDs are randomly distributed over the entire genome, extensive amount of polymorphism can be detected.

RAPD markers were applied to identify intra- and interspecific variation and to evaluate the phylogenetic relationships among nine species of *Hypochaeris*. Ninety-eight RAPD primers were initially tested, to select those that were suitable in generating reproducible and polymorphic bands. Twenty-five primers were then selected and used for DNA amplification in all *Hypochaeris* species, rendering a total of 122 markers, 120 (98%) of which were polymorphic. Figure 4 illustrates the electrophoresis profile obtained with primer OPS-10. The data matrix of genetic similarities and the dendrogram with the respective bootstrap values are illustrated in Table 3 and Figure 5, respectively. The value of cophenetic correlation (r = 0.98) between the similarity matrix and the dendrogram indicates to which extent the clustering of genotypes accurately represents the estimates of genetic similarities among species. Cluster analysis associated the species under study into two major groups, comprising the South American and the European species, with a mean genetic similarity of only 34.7% (Table 3) and a 100% bootstrap support. These results are in agreement with the hypothesis of a monophyletic origin for the South American *Hypochaeris* species (Samuel *et al.* 1999; 2003).

The groups represented by the South American species showed a consistent association between molecular markers and karyotype features (Figure 5), showing substantial levels of polymorphism. The genetic similarity values ranged from 55% between *H. microcephala* var *albiflora* and *H. pampasica* to 79% between *H. grisebashii*
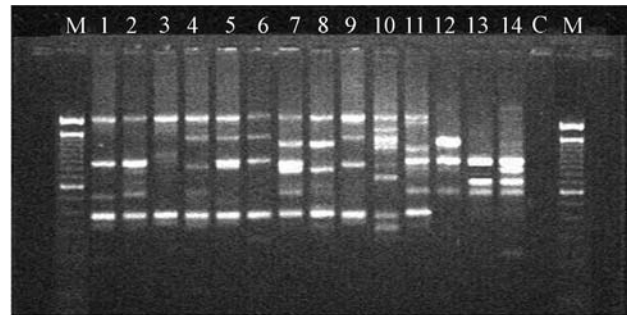

Figure 4 - Electrophoretic pattern of primer OPS-04 in *Hypochaeris* species: 1-3 = *H. brasiliensis* (populations of Guaíba, RS; Curitiba, PR and Piracicaba, SP), 4 = *H. grisebashii*, 5-6 = *H. megapotamica* (populations of Guaíba and Capão da Canoa, RS), 7 = *H. microcephala* var *albiflora*, 8 = *H. microcephala* var *microcephala*, 9 = *H. pampasica*, 10 = *H. rosengurttii*, 11 = *H. variegata*, 12-13 = *H. glabra* (populations of Curitiba and Ponta Grossa, PR) 14 = *Hypochaeris* sp. C = Control, M = ladder (123 bp, Gibco).

and *H. megapotamica*. Based on the RAPDs, the South American species were divided into three subgroups (Figure 5). The first subgroup associated *H. brasiliensis* and *H. microcephala*, which also display similar chromosome characteristics, with an average of 68% genetic similarity. For instance, they both exhibit CMA bands and the 45S rDNA loci at the same position on chromosomes 2 and 3. The second group associates *H. grisebashii*, *H. megapotamica* and *H. pampasica* with a 76% genetic similarity, on average. These species show the same distribution of the 45S rDNA genes, identified in the pericentromeric region on the small arm of chromosome 3 besides an additional CMA band observed on chromosome 2 of *H. megapotamica* and *H. pampasica*. The third subgroup associated *H. rosengurttii* and *H. variegata* with a

Table 3 - Similarity matrix generated from Dice coefficient based on 122 RAPD markers obtained for *Hypochaeris* species.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *H. brasiliensis* (RS)	1.00													
2. *H. brasiliensis* (PR)	0.85	1.00												
3. *H. brasiliensis* (SP)	0.90	0.82	1.00											
4. *H. grisebashii* (RS)	0.68	0.62	0.71	1.00										
5. *H. megapotamica* (RS)	0.67	0.61	0.69	0.79	1.00									
6. *H. megapotamica* (RS)	0.68	0.62	0.67	0.78	0.88	1.00								
7. *H. microcephala var. albiflora* (RS)	0.71	0.64	0.69	0.59	0.61	0.67	1.00							
8. *H. microcephala var. microcephala* (RS)	0.71	0.64	0.69	0.62	0.66	0.70	0.88	1.00						
9. *H. pampasica* (RS)	0.63	0.60	0.60	0.77	0.76	0.76	0.55	0.62	1.00					
10. *H. rosengurttii* (RS)	0.65	0.58	0.68	0.72	0.72	0.69	0.67	0.65	0.67	1.00				
11. *H. variegata* (RS)	0.70	0.65	0.70	0.68	0.67	0.64	0.65	0.68	0.66	0.74	1.00			
12. *H. glabra* (PR)	0.32	0.37	0.32	0.23	0.29	0.24	0.38	0.38	0.25	0.26	0.32	1.00		
13. *H. glabra* (PR)	0.27	0.40	0.37	0.24	0.29	0.24	0.36	0.35	0.23	0.32	0.38	0.81	1.00	
14. *Hypochaeris* sp (RS)	0.33	0.38	0.37	0.28	0.31	0.29	0.41	0.42	0.36	0.40	0.44	0.73	0.76	1.00

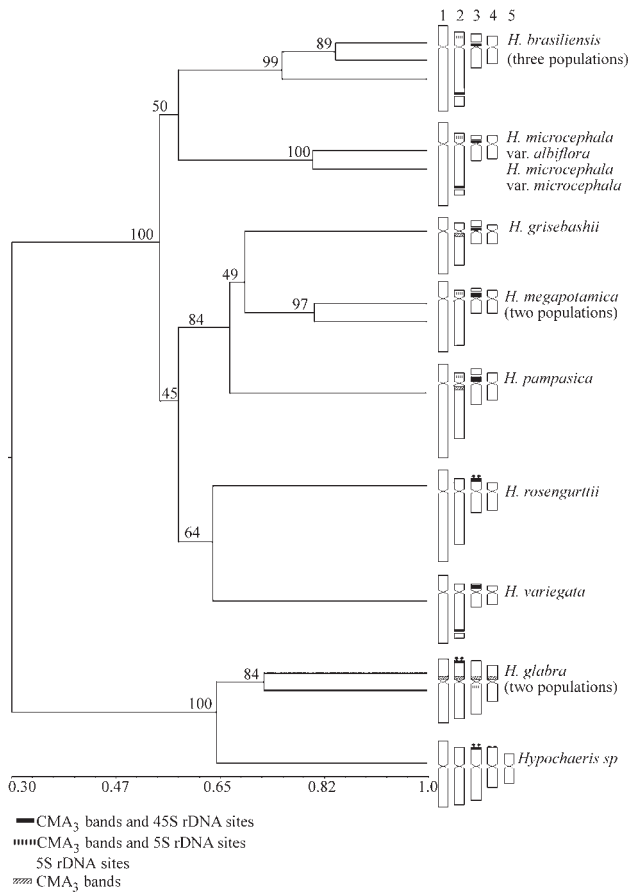The letters in parenthesis represent Brazilian states as described in Table 1.

Figure 5 - Dendrogram showing the association between RAPD markers and chromosome data in *Hypochaeris*. The numbers above the branches are the boostrap values calculated for 1000 replications.

similarity of 74%. However, in *H. rosengurttii*, the single locus of the 45S rDNA clusters was located at the secondary constriction and adjacent satellites of chromosome 3, while *H. variegata* had the 45S rDNA genes on chromosome pairs 2 and 3, like *H. brasiliensis* and *H. microcephala*.

The European species *H. glabra* and *Hypochaeris* sp (possible a cytotype of *H. radicata*), which were isolated from the South American group, showed an average genetic similarity of 74.5%. Whereas these species are known to display symmetrical karyotypes, we detected a distinct distribution of CMA$_3$ bands and 5S rDNA genes (Figures 2 E-F; 3 H-I; and 5).

RAPD markers also identified considerable levels of intraspecific variation. Analysis of three populations of *H. brasiliensis* showed a genetic similarity that ranged from 82% to 90% (Table 3). Similar levels of variation were documented between the two populations of *H. megapotamica* and between the two populations of *H. glabra*, which showed a genetic similarity of 88% and 81%, respectively. The two varieties of H. microcephala had a genetic similarity of 88%. This pattern of intraspecific variation is com-

mon for pioneer species, such as those of the genus *Hypochaeris* (Stuessy, 2001).

The importance of molecular phylogenetic studies to understand changes in plant genome organization and chromosome evolution has been emphasized in many works. Taxonomy and phylogenetic relationships of the genus *Hypochaeris* have been discussed based on a sequencing data set of ITS regions (Cerbah *et al*., 1998b; Samuel *et al*., 1999; 2003) and plastidial intergenic TrnL and mat-K sequences (Samuel *et al*., 1999; 2003). These authors suggested that South American *Hypochaeris* species constitute a monophyletic group that was probably derived from a single progenitor introduced from Europe. They also considered that *H. maculata* rather than *H. robertia*, as previously suggested by Barghi *et al*. (1989), is closer to the South American species than any other European species. Cerbah *et al*. (1995) showed that *H. maculata* has a high DNA content (7.48 pg) when compared with the DNA content of the South American species (4.15 to 4.65 pg), suggesting that, in addition to chromosome rearrangements, which coordinated the changes in position and number of NOR genes, as suggested by Cerbah *et al*. (1998a), loss of DNA accompanied the origin and establishment of the South American karyotypes. Likewise, the pattern of genetic variation detected with RAPD markers is also consistent with changes in DNA repetitive elements, which are known to evolve more rapidly than single sequences (Wu *et al*., 1999).

The evolutionary dynamics of the 45S rDNA sites points to some trends among South American species. It has been noted that while some species have two rDNA sites, the main locus is probably the one located on the small arm of chromosome 3, as suggested by Cerbah *et al*. (1998a). Paracentric inversion may have moved this site from terminal chromosome regions, as in *H. rosengurttii*, to a perhaps more stable pericentromeric position, as observed in all other species. Furthermore, differences detected in the hybridization signals suggest the occurrence of reduction in the number of 45S rDNA copies at this site, implying that deletion of DNA sequences is also implicated in the diversification of genomes in the South American species.

The results obtained in the present work concerning karyotype features, chromosome location of 45S rDNA loci and CMA-positive bands showed that the species diversification in *Hypochaeris* involved chromosome rearrangements, as previously reported (Ruas *et al*., 1995; Cerbah *et al*., 1995; 1998a). The nature of the RAPD markers also suggests that changes in DNA content may have resulted from amplification or deletion of repetitive DNA sequences. Genome diversification has involved considerable alteration of repetitive DNA sequence arrays in several groups of plants (Heslop-Harrison, 2000) and therefore, the variability of RAPD markers may represent a result of variation in different classes of repetitive DNA sequences among *Hypochaeris* species.

The high genetic variability detected using RAPD and the correlation between cytogenetic and molecular markers give additional support to the idea that a major evolutionary pathway operated toward genome organization and karyotype differentiation, leading the separation of European and South American species of *Hypochaeris*.

Acknowledgements

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