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ORIGINAL ARTICLE

Karyotype and AFLP data reveal the phylogenetic position of the Brazilian endemic *Hypochaeris catharinensis* (Asteraceae)

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Abstract The genus *Hypochaeris* offers an excellent model for studies of recent adaptive radiation in the South American continent. We used karyotype analysis with chromomycin A_3 (CMA_3)/4',6-diamidino-2-phenylindole (DAPI) banding and fluorescence in situ hybridization (FISH), and amplified fragment length polymorphism (AFLP) fingerprinting to investigate for the first time the Brazilian endemic *H. catharinensis* and define its position within the South American group of species. Strong CMA-positive signals were seen at the end of both arms of chromosome 3 and at the end of the long arm of chromosome 4. DAPI bands were only detected in subterminal position on short arm of chromosome 4. FISH with 5S and 35S ribosomal DNA (rDNA) probes revealed a single 5S rDNA locus on short arm of chromosome 2, typical for all other South American *Hypochaeris* taxa analyzed to date. The 35S rDNA locus was identified at subterminal position on the short arm of chromosome 3, as reported so far for only two of the known species (*H. lutea* and *H. patagonica*). The AFLP study included 55 individuals, comprising nine species of the South American *Hypochaeris* plus their putative ancestor *H. angustifolia*. Eleven AFLP primer combinations generated a total of 401 fragments, of which 388 (96.7%) were polymorphic. High genetic similarities were observed among taxa, with all South American *Hypochaeris* species falling into one main cluster [100% bootstrap (BS)]. *Hypochaeris catharinensis* is closely related to *H. lutea* (82% BS), forming a well-separated subcluster within the South American species. Taken together, the karyological and AFLP data contribute to the placement of *H. catharinensis* within the phylogenetic framework of South American species of *Hypochaeris* and allow the definition of a novel and well-resolved phylogenetic group (the Lutea group).

Keywords AFLP · Asteraceae · Cytogenetics · FISH · *Hypochaeris catharinensis* · Phylogeny

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Introduction

The genus *Hypochaeris* L. (Asteraceae) comprises around 65 species with a disjunct distribution, including 15 species in the Mediterranean region (DeFillips 1976) and possibly

more than 50 in South America (Bortiri 1999). Information based on chromosome numbers and plant morphology suggested that the genus is probably of Mediterranean origin, whereas South America is the secondary center of diversification (Stebbins 1971; Ruas et al. 1995; Cerbah et al. 1998a). Recent evidence from DNA sequence analyses suggested that *H. angustifolia* (Litard. & Maire) Maire, a species endemic to Morocco, is the sister taxon to the entire South American clade of *Hypochaeris*, supporting the view that the New World is a secondary center of genus diversification (Stuessy et al. 2003; Tremetsberger et al. 2005).

Hypochaeris is considered to be a very young genus in South America, where it was apparently derived from a single ancestral species through events of long-distance dispersal, during the Pliocene or Pleistocene (between $\sim$0.25 and $\sim$3.50 mya; Tremetsberger et al. 2005). Single colonization by a common ancestor, followed by rapid radiation, has allowed the genus to diverge into new habitats throughout the South American continent (Stuessy et al. 2003; Tremetsberger et al. 2005), accompanied by considerable morphological diversification. Comparative studies among South American *Hypochaeris* have, however, revealed a low degree of chromosome and genetic divergence. Studies based on chromosome morphology revealed the same pattern of stable chromosome number ($x = 4$), putative autopolyploidy, and a highly asymmetrical and bimodal karyotype for all South American taxa (Stebbins 1971; Cerbah et al. 1995, 1998a; Ruas et al. 1995, 2005; Weiss-Schneeweiss et al. 2003, 2007, 2008). More detailed chromosome analyses were carried out using chromomycin A_3 (CMA_3) staining to investigate the distribution of GC-rich heterochromatin in a group of Mediterranean and South American species of *Hypochaeris*. In most of the investigated species CMA-positive bands co-localized with the 5S and 35S rDNA loci, with less intense CMA_3 signals occurring at similar positions of corresponding chromosomes within the Mediterranean and the South American species (Cerbah et al. 1995, 1998a; Ruas et al. 2005; Fiorin 2008). CMA_3 staining was used into investigate the distribution of GC-rich heterochromatin on the chromosomes of ten populations of *H. catharinensis* (Fiorin 2008). The author described an unusual pattern in the distribution of CMA_3-positive signals, where large GC-rich blocks were found associated with the subtelomeric regions of chromosomes 3 and 4. Additional but less intense signals were distributed at several other chromosomal locations. The patterns of heterochromatin distribution and its role in the evolution of plant chromosomes have been broadly discussed (for revision see Guerra 2000), showing different trends in a variety of plant species. However, the co-location of GC-rich heterochromatin and ribosomal RNA (rRNA) genes has been repeatedly reported in many plant groups, for example, in Asteraceae (Vanzela et al. 2002), Pteridophyta (Marcon et al. 2004), Cyperaceae (Silva et al. 2008), Leguminosae (Nardy et al. 2010), and Solanaceae (Fregonezi et al. 2006; Fernandes et al. 2009).

Despite the overall uniformity of chromosome morphology observed among *Hypochaeris* species analyzed, recent interpretation of the karyotypic information in a phylogenetic context suggests that the early evolution of *Hypochaeris* in South America was characterized by at least some karyotype differentiation. Weiss-Schneeweiss et al. (2003, 2008) suggested that new karyotypic variants had independent origin from the ancestral karyotype, mostly via changes in the position and activity of the 35S rDNA loci. These changes most likely involved inversions and/or transpositions of rDNA within chromosome 3 and inactivation and/or loss of loci on chromosome 2 (Cerbah et al. 1995, 1998a; Ruas et al. 1995, 2005; Weiss-Schneeweiss et al. 2003, 2007, 2008).

Phylogenetic relationships in the genus *Hypochaeris* have been studied using DNA sequence analysis of nuclear internal transcribed spacers (ITS; Cerbah et al. 1998b) and ITS plus chloroplast (*trn*L intron and *trn*L/*trn*F spacer and *mat*K) regions (Samuel et al. 2003); however, the results provided only limited insight into phylogenetic relationships of the South American *Hypochaeris* (Samuel et al. 2003). Among other applications, amplified fragment length polymorphism (AFLP; Vos et al. 1995; Mueller and Wolfenbarger 1999) is currently widely used to create robust phylogenetic inferences, especially within taxa in which interspecific relationships have been difficult to resolve (Barluenga et al. 2006; Pellmyr et al. 2007) or that have diverged or radiated recently (Bussell et al. 2005; Koopman 2005; Koopman et al. 2008). Despite its widespread application, there are restrictions on the use of AFLP markers in phylogenetic analyses resulting from their unknown mutation characteristics and anonymous nature (Felsenstein 2004; Koopman 2005). The AFLP technique detects differences in restriction sites in the genome, combining the reliability of restriction enzymes with the power of the polymerase chain reaction (PCR) technique (Vos et al. 1995). AFLPs have been successfully applied in studies of *Hypochaeris* (Stuessy et al. 2003, 2004; Tremetsberger et al. 2005, 2006; Ortiz et al. 2007, 2008; Terrab et al. 2009) and prove to be a good source of information for understanding relationships among species of the South American continent. Based on AFLP data, Tremetsberger et al. (2005, 2006) suggested that the South American *Hypochaeris* form a monophyletic group. This result is consistent with recent and rapid speciation proposed for the genus.

In Brazil, the genus *Hypochaeris* is represented by eight to ten species that are mostly endemic to the southern region. The exception is *H. chillensis* (Kunth) Britton that is widespread, occurring from Rio Grande do Sul to São

Paulo States of Brazil, as well as in other South American countries such as Argentina, Uruguay, Paraguay, Peru, Bolivia, Ecuador, and Colombia (Bortiri 1999; Cabrera 1937, 1974, 1976; Cabrera et al. 2000; Azevêdo-Gonçalves and Matzenbacher 2007). Among the Brazilian taxa, the endemic *H. catharinensis* Cabrera grows in restricted areas, mostly in cold regions at relatively high altitudes (800–1,400 m). The distribution of this species extends from the north of Rio Grande do Sul to the south-eastern regions of Santa Catarina States (Cabrera 1963; Azevêdo-Gonçalves and Matzenbacher 2007), where the winter temperature can reach −5°C with many episodes of frost and occasional occurrences of snowfall. *Hypochaeris catharinensis* usually grows in grass-dominated areas, slopes, or fields with *Araucaria angustifolia*. The species is adapted only to dry, rocky, and shallow soils resulting from basaltic weathering (Azevêdo-Gonçalves and Matzenbacher 2007), which characterize a nutrient-poor environment caused by regional high rate of bleaching (Almeida 2009). Adaptation to such an environment is facilitated by horizontal rhizomes, a character of taxonomic importance for the species (Azevêdo-Gonçalves and Matzenbacher 2007) that also influences the vegetative spread observed in *H. catharinensis*. Morphologically, *H. catharinensis* has entire oblanceolate leaves, forming a rosette that favors total light incidence, and a solitary stem with or without bracts, capitula with florets of yellow corollas, and beaked achenes with the beak longer than the fertile portion (Azevêdo-Gonçalves and Matzenbacher 2007).

AFLP markers have been used in an attempt to trace the phylogenetic relationships of the South American species of *Hypochaeris* (Tremetsberger et al. 2003, 2006; Weiss-Schneeweiss et al. 2008), whereas FISH has been applied to around 50% of the species to assess the reorganization of rDNA and to provide insights into the karyotype evolution among these closely related taxa (Cerbah et al. 1998a; Ruas et al. 2005; Weiss-Schneeweiss et al. 2003, 2007, 2008). Although a considerable amount of information is now available for the South American species of *Hypochaeris*, detailed studies are still lacking for many species. The present work was undertaken to investigate the endemic species *H. catharinensis*, which has never been included in any detailed chromosome and molecular studies, with the principal aim of determining the phylogenetic relationship of this species within the South American group of species.

Materials and methods

Species and sampling

Hypochaeris catharinensis Cabrera was investigated together with five additional species, including *H. apargioides* Hook. & Arn, *H. argentina* Cabrera, *H. megapotamica* Cabrera, *H. neopinnatifida* C.F. Azevêdo-Gonçalves & N.I. Matzenbacher (synonym *H. pinnatifida* (Speg.) Azevêdo-Gonçalves & Matzenbacher), and *H. pampasica* Cabrera, representing three of the six phylogenetic groups described by Tremetsberger et al. (2006). Three other species, consisting of *H. lutea* (Vell.) Britton, *H. petiolaris* (Hook. et Arn.) Griseb, and *H. variegata* (Lam.) Baker, that were not assigned to any molecular group in the work of Tremetsberger et al. (2006) were also included in this study. The Moroccan endemic *H. angustifolia* (Litard. & Maire) Maire, the putative ancestor of the entire South American group of species (Tremetsberger et al. 2005), was used as outgroup.

Plant material consisted of young leaves sampled from 55 plants, including 17 individuals representing three populations of *H. catharinensis*, plus three to six individuals for each of the eight South American species of *Hypochaeris* (Table 1). Leaves of *H. catharinensis*, *H. lutea*, and *H. megapotamica* were collected in silica gel during fieldwork in 2006 and 2007 in South Brazil. The other species (*H. angustifolia*, *H. apargioides*, *H. argentina*, *H. neopinnatifida*, *H. pampasica*, *H. petiolaris*, and *H. variegata*) were sampled from collections at the University of Sevilla, Spain. *Hypochaeris angustifolia* was represented by five individuals taken from four populations from Morocco. Details of localities, number of individuals, and collection numbers are given in Table 1.

Chromosome staining with CMA_3, DAPI, and FISH

Chromosome preparations of *H. catharinensis* were obtained using pretreated and fixed root tips (Ruas et al. 2005), pooled from at least 10 plants of three populations of *H. catharinensis*. Fluorescent CMA/DAPI banding was performed following the procedure described in Ruas et al. (2005). FISH was carried out according to Vanzela et al. (2002), using probes of 35S and 5S rDNA. The p*Ta*71 clone with a 9-kb fragment from *Triticum aestivum* containing the 18S–5.8S–25S rDNA and intergenic spacers (Gerlach and Bedbrook 1979) was used to detect 35S rDNA. The 5S rDNA (120 pb) genic region was detected using probe isolated from *H. catharinensis* by PCR with primers UP 46 (forward: 5′-GTG CGA TCA TAC CAG CRY TAA TGC ACC GG-3′) and UP 47 (reverse: 5′-GAG GTG CAA CAC GAG GAC TTC CCA GGA GG-3′). The 35S and 5S rDNA probes were labeled with biotin and digoxigenin, respectively, using nick translation kit (Invitrogen). Root tips were digested in mixture of 2% (w/v) cellulase and 20% (v/v) pectinase at 37°C for 1.5 h and squashed in a drop of 60% acetic acid. Chromosome preparations were treated with 100 µg/mL RNase, dehydrated in a 70–100% graded ethanol series, and air-dried.

Table 1 Species, number of individuals (*N*), and collection sites of South American species of *Hypochaeris* and *H. angustifolia* (outgroup) analyzed by AFLP markers

Species	*N*	Collection details
Pampasica group		
H. megapotamica Cabrera	4	Brazil, Rio Grande do Sul: Montenegro and Jaquirana (NM, 2106 and 2606)
H. pampasica Cabrera	6	Argentina, Prov. Buenos Aires: Saladillo (EU, 234); Prov. San Luis: Villa Mercedes (EU, 236 and 237)
H. neopinnatifida C.F. Azevêdo-Gonçalves & N.I. Matzenbacher	4	Argentina: Buenos Aires. Balcarce: Ladera Oeste del cerro "La Brava", Ruta 226, km 41 (EU, MAO, 218). Tandil: Cerro El Centinela, Bajo las aerosillas (EU, MAO, 216, LP.SEV)
Microcephala group		
H. argentina Cabrera	3	Argentina, Prov. Córdoba: Córdoba Calamuchita. A 5 km de la base del Champaquí (EU, EMB, 200 and 208)
Apargioides group		
H. apargioides Hook. & Arn.	4	Chile (KT et al. 42)
Ungrouped species		
H. catharinensis Cabrera	17	Brazil, Rio Grande do Sul: São José dos Ausentes and Cambará do Sul (MAO, NM, 1906 and 2806); Santa Catarina, Rancho Queimado (CR, 0407)
H. lutea (Vell.) Britton	4	Brazil, Rio Grande do Sul: Fazenda Capão Muniz, between São Francisco de Paula and Cambará do Sul (NM, 1306) and Canyon Fortaleza Município of Cambará do Sul (NM 2706)
H. petiolaris (Hook. et Arn.) Griseb.	4	Argentina, Prov. Buenos Aires: Mar del Plata and La Plata (EU, 220 and 223)
H. variegata (Lam.) Baker	4	Argentina, Prov. Buenos Aires: Olavarría and Tandil (EU, 212 and 172)
Outgroup		
H. angustifolia (Litard. & Maire) Maire	5	Morocco, Middle Atlas: Bekrite and Larais (ST, 267, 193, 703/03 and 73/03)

AT, A. Terrab; CR, C.F. Ruas; EMB, E.M. Baztarrica; EU, E. Urtubey; KT, Karin Tremetsberger; MAO, M.A.Ortiz; NM, N.I. Matzenbacher; ST, S. Talavera; TS, T. Stuessy

For FISH, we used a 30 µL volume mixture composed of 100% formamide, 50% polyethylene glycol, 2× saline-sodium citrate (SSC) (0.03 M Na citrate and 0.3 M NaCl), 100 ng sonicated calf thymus DNA, 10% sodium dodecyl sulfate, and 100 ng of each 35S and 5S rDNA probe. The hybridization mixture was denatured at 70°C for 10 min, chilled on ice for 5 min and added to the slide. Chromosome denaturation/hybridization was done at 90°C for 10 min, 48°C for 10 min, and 38°C for 5 min, using a thermal cycler (MJ Research, Inc., USA), and the slides were then incubated at 37°C overnight in a humid chamber. Post-hybridization washes were carried out in 2× SSC, 20% formamide in 0.1× SSC, 0.1× SSC, and 4× SSC/0.2% Tween 20, all at 37°C. Probes were detected with avidin–fluorescein isothiocyanate conjugate or avidin—Alexa Fluor 488 conjugate (green) and with anti-dig-rhodamine (red). Post-detection washes were applied twice in 4×SSC/0.2% Tween 20 at room temperature in the dark. Slides were mounted with 25 µL of solution composed of glycerol (90%), 1,4-diazabicyclo[2.2.2]octane (DABCO, 2.3%), 20 mM Tris–HCl, pH 8.0 (2%), 2.5 mM $MgCl_2$ (4%), and distilled water (1.7%), plus 1 µL 2 µg/mL DAPI. Analyses of chromosome preparations were carried out using an epifluorescent microscope (NIKON, Eclipse E600), and images were acquired using a digital camera (Cool SNAP cf, Photometrics) and processed with Canvas™ (version X build 898), using only those functions that could be applied to the entire image. For localization of the rDNA loci, a minimum of 10 well-spread metaphases were analyzed for each population of *H. catharinensis*.

DNA isolation and AFLP fingerprinting

Total genomic DNA was extracted from dried leaves using a cetyl trimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987) with minor modifications (Tremetsberger et al. 2003) and quality-checked on 1% Tris–acetate–ethylenediamine tetraacetic acid (TAE) agarose gels. For each sample, approximately 800 ng DNA was restricted with 5 U *Eco*RI (Fermentas) and 5 U *Mse*I (NEB), in total volume of 20 µL, at 37°C overnight. *Eco*RI and *Mse*I adaptors were then ligated to the resulting DNA fragments by incubating with T_4 DNA ligase (Fermentas) at 37°C for 3.5 h (Vos et al. 1995). Preselective PCR amplification included GoTaq® Green Master mix (Promega), 0.25 µM of each *Eco*RI + A and *Mse*I + C primers and restriction-ligation solution with volume adjusted to 10 µL with double-distilled water. The pre-amplification PCR program consisted of 1 cycle at 72°C for 2 min followed by 20 cycles at 90°C for 1 s, 56°C for 30 s, and 72°C for 2 min, and 1 final cycle of 60°C for 30 min.

Selective amplification reactions contained GoTaq® Green Master mix (Promega), 5 pmol of each of the *Eco*RI/*Mse*I selective primers, and diluted (5× in sterile water) pre-amplification product, adjusted to final volume of 10 μL with double-distilled water. For selective amplification the primer combinations had an extension of two or three additional nucleotides. Primer selection was performed on four samples using 16 *Eco*RI/*Mse*I primer combinations. Based on the number and quality of polymorphic fragments, 11 informative primer combinations were screened in all 55 samples (Table 2). A reproducibility assay was performed to increase the consistency of the results using two selected samples that were processed independently twice with all 11 primer combinations. Selective PCR conditions consisted of an initial cycle of 94°C for 2 min, 65°C for 30 s, and 72°C for 2 min; 8 cycles of 94°C for 1 s, 64°C for 30 s, and 72°C for 2 min; 23 cycles of 94°C for 1 s, 56°C for 30 s, and 72°C for 2 min, and a final extension of 60°C for 30 min. The products of the selective amplification were run on a 7% acrylamide:bisacrylamide (29:1) gel (200 W for 3:30 h), silver stained, visualized by exposing the gels to white light, and photographed using a digital camera.

Data analysis

Only the highly reproducible AFLP profiles were considered for data analysis. The raw AFLP fragments, ranging between 50 and 1000 bp in length, were scored as present (1) or absent (0). The binary AFLP matrix was imported into dBoot version 1.1 (Coelho 2001) to check the consistency of the data using the coefficient of variation (CV%) for the total number of amplified products. The distance-based neighbor-joining (NJ) method was used to create phylogenetic trees, and the results compared with those obtained with Bayesian methods. The Nei–Li distance (Nei and Li 1979) was implemented in PAUP version 4.0b10 (Swofford 2003), and its robustness was confirmed using the bootstrap (BS) method (Felsenstein 1985) with 10,000 replicates using PAUP version 4.0b10 (Swofford 2003). For Bayesian analysis (data not shown) we used MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with the restriction sites model. As it is designed for restriction site data, this model may oversimplify some more complex evolutionary processes underlying AFLP polymorphisms. We used the option "noabsencesites" to correct the bias of the AFLP data (Ronquist et al. 2005; Koopman et al. 2008).

Results and discussion

Chromosome analysis

Chromosome analysis of *H. catharinensis* confirmed the presence of an asymmetrical and bimodal karyotype (Fig. 1a), as previously reported for 10 populations of this species (Fiorin 2008), following the pattern that characterizes the South American species of *Hypochaeris* analyzed so far (Stebbins 1971; Cerbah et al. 1995, 1998a, b; Ruas et al. 1995, 2005; Weiss et al. 2003; Weiss-Schneeweiss et al. 2007). The fluorochrome banding (CMA_3/DAPI) and double fluorescent in situ hybridization (FISH) with 5S and 35S rDNA probes, applied on the same chromosome plates, are shown in Fig. 1b–j.

The base-specific fluorochrome chromomycin A_3 (CMA_3) was used to investigate the distribution of GC-rich heterochromatin in a group of *Hypochaeris* species (Cerbah et al. 1998a; Weiss-Schneeweiss et al. 2003; Ruas et al. 2005). In all investigated species the authors found strong CMA_3^+ signals co-localized with the 35S rDNA loci, while less intense signals were seen in some species in the vicinity of 5S rDNA loci, and in a few other chromosomal locations (Ruas et al. 2005). CMA_3 staining identified a unique pattern of distribution of the GC-rich heterochromatin on chromosomes of *H. catharinensis* (Fig. 1c) and

Table 2 Selective primer combinations used for AFLP analysis of *Hypochaeris* species with number of markers and levels of polymorphism obtained from each primer pair

Primers combinations	Number of markers	Polymorphism (%)
*Eco*RI-ACT + *Mse*I-CTGA	55	100.0
*Eco*RI-ACT + *Mse*I-CAG	30	93.3
*Eco*RI-AGC + *Mse*I-CAG	53	94.3
*Eco*RI-ACG + *Mse*I-CTC	30	100.0
*Eco*RI-AGC + *Mse*I-CTCG	42	100.0
*Eco*RI-ATC + *Mse*I-CTCG	40	97.5
*Eco*RI-ACG + *Mse*I-CAT	35	100.0
*Eco*RI-ACT + *Mse*I-CAC	24	91.7
*Eco*RI-AGC + *Mse*I-CAT	24	95.8
*Eco*RI-AGC + *Mse*I-CTGA	30	100.0
*Eco*RI-ATC + *Mse*I-CAG	38	92.1

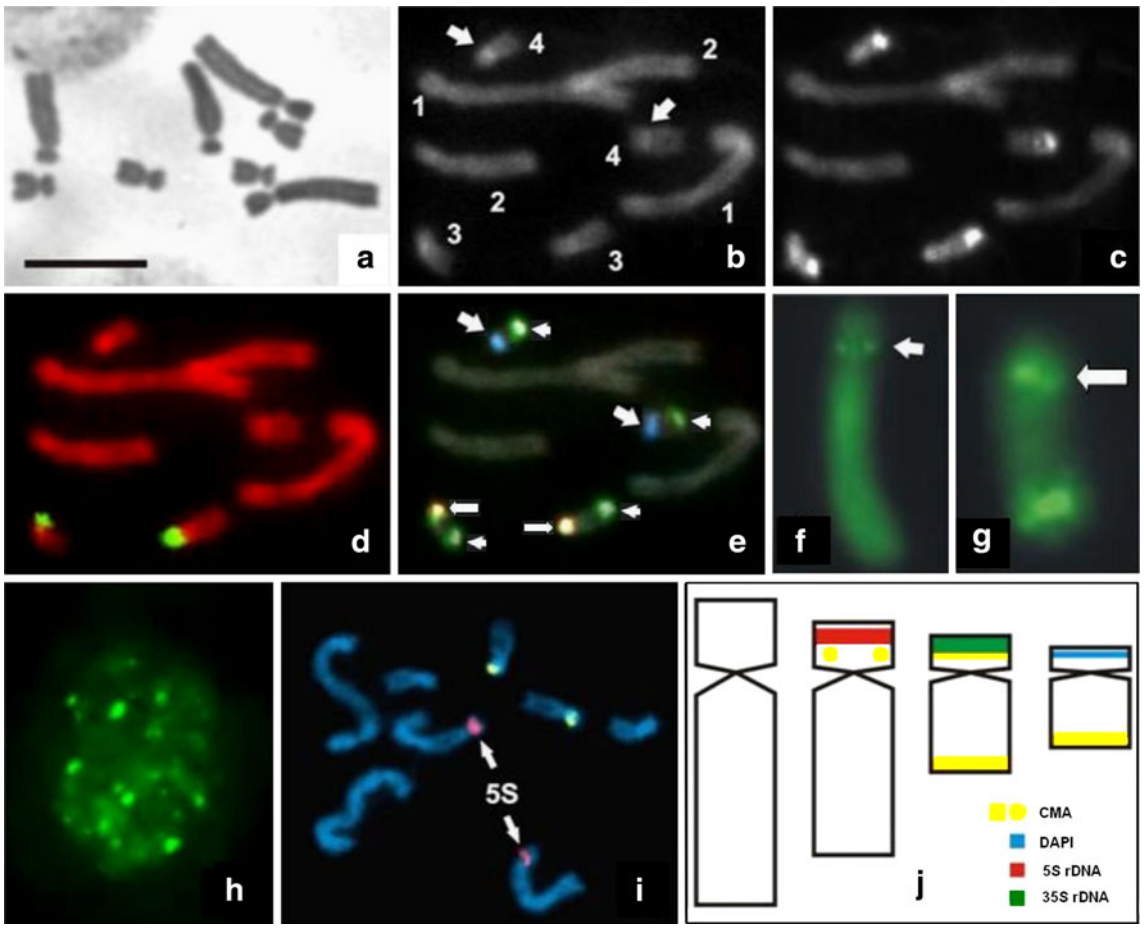

Fig. 1 Conventional staining and physical location of repetitive DNA families on mitotic chromosomes and nuclei of *Hypochaeris catharinensis*. **a** Feulgen staining; **b** tenuous DAPI$^+$ band located near to centromere on short arm of pair 4 (*arrow*); **c** distribution of major CMA signals on long and short arms of pair 3 and long arm of pair 4; **d** FISH signals detected with 35S rDNA probe on short arm of pair 3; **e** overlap of DAPI (*short arrow*), CMA (*arrow head*), and 35S rDNA (long arrow) signals; (**f**–**h**) details of CMA_3^+ signals co-localized with rDNA loci and in the interphase nuclei; **i** 5S rDNA signals on short arm of pair 2; **j** ideogram showing the combined distribution of CMA_3 (*yellow*) and DAPI (*blue*) banding, and the position of the 35S (*green*) and the 5S (*red*) rDNA loci. *Scale bar* 10 μm

confirmed the results of a recent chromosome investigation on 10 populations of this species (Fiorin 2008). *Hypochaeris catharinensis* showed three strong CMA_3^+ signals: at subterminal position of long and short arms of chromosomes 3 and long arms of chromosome 4 (Fig. 1c, f–g, and j). Less intense CMA_3^+ signals (small spots) were observed in a near-centromeric region of short arm of chromosome 2 (Fig. 1f). Some other small blocks of GC-rich heterochromatin, represented by less intense CMA signals that were sometimes undetectable on the chromosomes, were regularly seen in the interphase nuclei (Fig. 1h).

A faint DAPI$^+$ signal, unknown to date in *Hypochaeris*, was found at pericentromeric position on the short arm of chromosome 4 of *H. catharinensis* (Fig. 1b). The presence of AT-rich (DAPI-positive) band within short arm of chromosome 4 might be suggestive of the presence of the unique AT-rich DNA repeats. None of the other species of South American *Hypochaeris* have had so far revealed the presence of any distinct heterochromatic bands that would suggest the presence of tandemly repeated satellites. Therefore, *H. catharinensis* might offer a chance to isolate first tandemly repeated DNA in the New World *Hypochaeris*. Previous analyses of the diversity of *rt* domains of Ty1-*copia* retrotransposons in New World taxa of *Hypochaeris* have revealed the exclusion of these in some chromosomal regions of chromosome pairs 3 and 4 (Ruas et al. 2008). The presence of AT- and GC-rich signals in these chromosome pairs of *H. catharinensis* might support one of the hypotheses presented in the former study that these regions devoid of retroelements might be enriched in tandem repeats of other types.

FISH with the 35S rDNA probe identified a single locus, located at the subterminal position on the short arm of chromosome 3 of *H. catharinensis* (Fig. 1d). The 5S rDNA probe revealed the expected single locus localized

on short arm of chromosome 2 (Fig. 1i) as seen in all New World *Hypochaeris* species investigated to date (Ruas et al. 2005; Weiss-Schneeweiss et al. 2007, 2008). The GC-rich heterochromatin in *H. catharinensis* co-localized with both 35S and 5S rDNA loci (Fig. 1e–g, j) corroborating the results found in other South American species (Cerbah et al. 1998a; Weiss-Schneeweiss et al. 2003, 2008; Ruas et al. 2005). The correlation between heterochromatin and rDNA genes supports the hypothesis that the presence of heterochromatin facilitates structural rearrangements, especially those involving nucleolus organizing regions (NORs) that, as seen in *Hypochaeris*, are known for their mobility in the genome (Schubert 1984).

Chromosome studies concerning localization of rDNA have been interpreted previously in a phylogenetic context in South American *Hypochaeris*. Weiss-Schneeweiss et al. (2003, 2007, 2008) used analyses of basic chromosome morphology and distribution of rDNA loci to separate the South American species into different karyotype groups. In the present work we have focused on determining the phylogenetic position of *H. catharinensis* among the South American species. Distribution of 35S rDNA genes in a unique locus on the short arm of chromosome 3 is less frequent among species of this group. In addition to *H. catharinensis*, this pattern of distribution is known only in two other South American species, *H. lutea* (a synonym of *H. rosengurttii* Cabrera; Ruas et al. 2005) and *H. patagonica* Cabrera (Weiss-Schneeweiss et al. 2008). Based on similar distribution of the 35S rDNA, Weiss-Schneeweiss et al. (2008) included *H. lutea* and *H. patagonica* in the same karyotype group (named the Patagonica group). The authors suggested that this karyotype assemblage might have been derived from the ancestral *H. angustifolia*-like karyotype by a single-step loss of the 35S rDNA locus on chromosome 2. Hence, considering the distribution of the 35S rDNA signal, the Patagonica karyotype group previously composed by *H. lutea* and *H. patagonica* (Weiss-Schneeweiss et al. 2008) now also includes *H. catharinensis*.

Phylogenetic relationships

Eleven AFLP primer combinations yielded 401 reproducible and unambiguously scorable markers, ranging in size from 50 to 1,000 bp, of which 388 (96.75%) were polymorphic. The number of markers over all samples ranged from 24 (for primer combinations *Eco*RI-ACT/*Mse*I-CAC and *Eco*RI-AGC/*Mse*I-CAT) to 55 (for *Eco*RI-ACT/*Mse*I-CTGA; Table 2). The coefficient of variation (CV%) calculated for the total number of markers was 4.32%, demonstrating satisfactory level of confidence in our results.

AFLP markers have already proven useful for delimitation of South American species of *Hypochaeris* (Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2008). We used the NJ distance method and the Bayesian analysis (not shown) to test our AFLP data. Both trees were identical in topology with some differences in branch support, and we show the former (Fig. 2). Phylogenetic relationships in *Hypochaeris* have been inferred using the NJ distance trees (Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2008), and since both the NJ and the Bayesian trees have similar topologies, we focus the discussion on the NJ tree. Our NJ analysis separates the South American species from *H. angustifolia* (outgroup) with 100% bootstrap support. Whereas some phylogenetic relationships remain unresolved (weak BS), the associations among individuals within species are highly supported (BS 88–100%; Fig. 2). Tremetsberger et al. (2006) used AFLPs to separate the South America species of *Hypochaeris* into six phylogenetic groups, the backbone of which, however, was not well resolved because the basal nodes received only low bootstrap support (below 50%). A summary of those results together with chromosome and AFLP data reported in previously published studies is given in Table 3. In the present study, the NJ analysis identified two subclusters among the South America species (Fig. 2). The first subcluster includes three species (*H. megapotamica*, *H. neopinnatifida*, and *H. pampasica*) belonging to the Pampasica group of Tremetsberger et al. (2006). However, these associations received no BS support (BS <50%). Two other species, *H. variegata* and *H. petiolaris*, which were near but not within the Pampasica group in the previous studies (Stuessy et al. 2004; Ruas et al. 2005; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2008), appeared intermixed with *H. megapotamica*, *H. neopinnatifida*, and *H. pampasica* (but without BS support). All five species exhibit similar geographical distribution, growing in the hills and plains of the central-eastern part of the South American continent, from Buenos Aires Province (Argentina) to Santa Catarina State (Brazil). Occurrence in the same broad geographical area has also been observed for the species of the other phylogenetic groups (e.g., Chondrilloides group—Patagonian region; Tenuifolia group—southern Andean region; Sessilifolia group—northern Andean region; Tremetsberger et al. 2006). Taking into account the distributions of the species in addition to the AFLP data, it is therefore reasonable to assume that all five species, *H. megapotamica*, *H. neopinnatifida*, *H. pampasica*, *H. petiolaris*, and *H. variegata*, should be included in the Pampasica group (Table 3; Fig. 2). Similarly to the study of Tremetsberger et al. (2006), *H. apargioides* and *H. argentina*, the only representatives in our study of the Apargioides and the Microcephala groups, respectively, associated with the Pampasica group with 63% BS (Fig. 2).

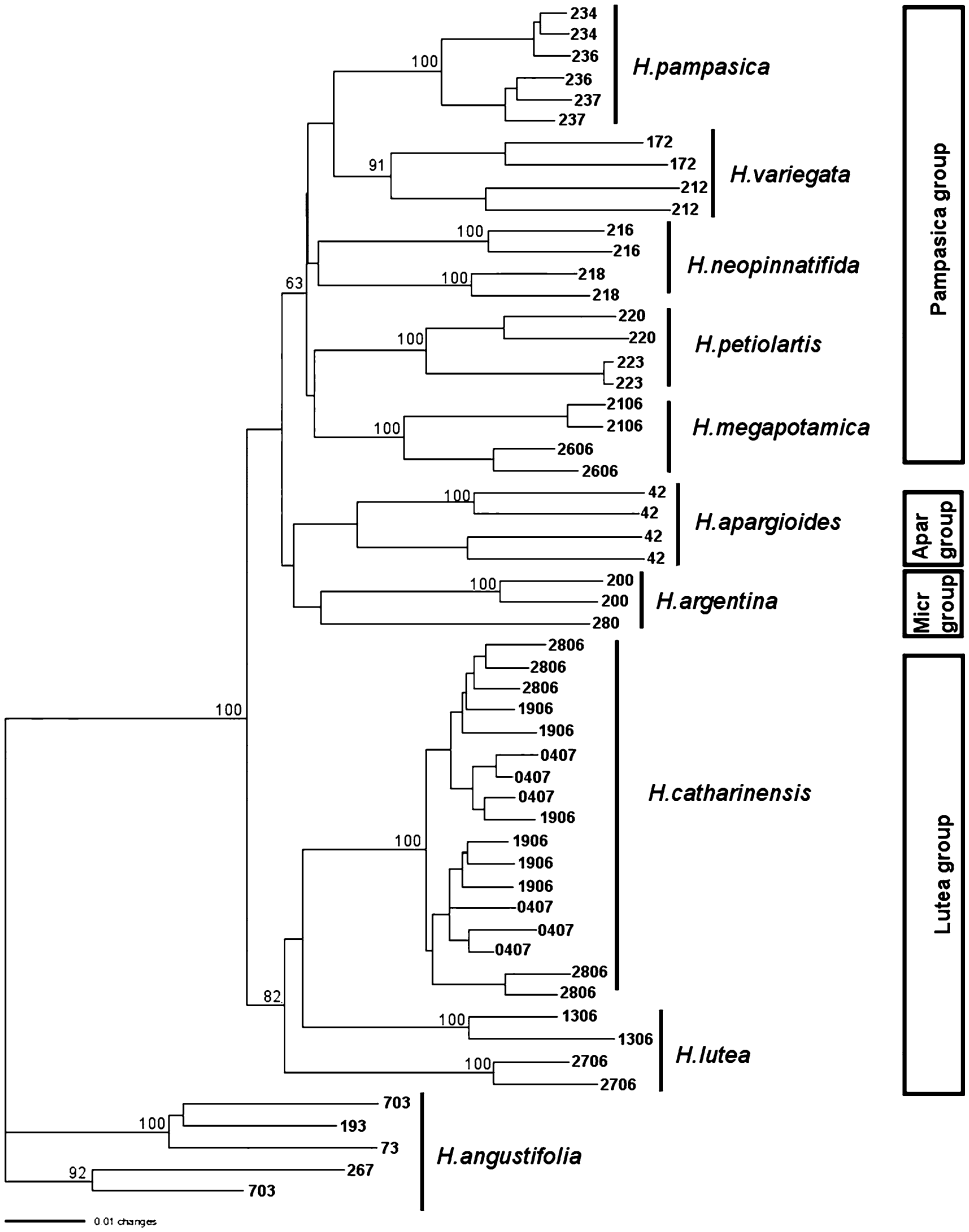

Fig. 2 Consensus tree including groups compatible with 10,000 bootstrap replicates of South American species of *Hypochaeris* plus *H. angustifolia* (outgroup) based on neighbor-joining (NJ) analysis of 401 AFLP fragments. Numbers on branches are bootstrap proportions. Numbers at ends of branches represent individual samples from each species according to their respective collection numbers (for details see Table 1). Bootstrap values below 50% are not shown

The inclusion of the Brazilian endemic *H. catharinensis* in the molecular study was based on the analysis of 17 individuals obtained from three populations from southern Brazil. Whereas individuals of *H. catharinensis* from different populations appeared intermixed in the tree, they represent a monophyletic entity (100% BS) that forms a well-resolved subcluster with *H. lutea* (82% BS). Several studies with South American species of *Hypochaeris*, including *H. lutea* (syn. *H. rosengurttii* var. *rosengurttii*; Azevêdo-Gonçalves and Matzenbacher 2005, 2006, 2007), used molecular markers and cytogenetic data to identify the phylogenetic relationships among species (Ruas et al. 1995, 2005; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008). However, none of the molecular groups, defined by Tremetsberger et al. (2006) on the basis of AFLP markers, included *H. lutea*. The present AFLP data showing the association between *H. lutea* and *H. catharinensis* is, therefore, of relevance for defining a new phylogenetic group within the South American *Hypochaeris* (Fig. 2).

AFLP data obtained in this and previous studies show that relationships among South American species of *Hypochaeris* are partly difficult to resolve, most probably because diversification occurred within a short period of time. According to Stuessy et al. (2004), ecological differentiation may have occurred independently in different lineages of *Hypochaeris* during evolution in the continent, reflecting a basic pattern of adaptive radiation. Even

Table 3 Organization of South American *Hypochaeris* spp. into karyotype and phylogenetic informal groups (Weiss-Schneeweiss et al. 2007; Tremetsberger et al. 2006, respectively) along with the information on geographical distribution of the species

Species	Chromosome/ phylogenetic groups	Distribution areas	References
H. acaulis (J.Rémy) Britton	Acaulis/ Tenuifolia	Argentina, Bolivia, and Chile	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2007, 2008
H. alba Cabrera	Apargioides/ Microcephala	Argentina	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008
H. albiflora (O.K.) Azevedo-Gonçalves & Matzenbacher	Apargioides/ Microcephala	Argentina, Brazil, and Paraguay	Ruas et al. 2005; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008
H. apargioides Hook. & Arn	Apargioides/ Apargioides	Argentina and Chile	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2007, 2008; present study
H. argentina Cabr.	Microcephala/ Microcephala	Argentina and Bolivia	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2008; present study
H. caespitosa Cabrera	Caespitosa/ ungrouped	Argentina	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008; present study
H. catharinensis Cabr.	Lutea/Lutea	Brazil	Fiorin, 2008, present study
H. chillensis (Kunth) Hieron.	Tenuifolia/ Microcephala	Argentina, Bolivia, Brazil, Colombia, Ecuador, Paraguay, Peru, and Uruguay	Cerbah et al. 1995, 1998a, b; Samuel et al. 2003; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2003, 2008
H. chondrilloides (A. Gray) Cabrera	Chondrilloides/ Chondrilloides	Argentina and Chile	Cerbah et al. 1995, 1998a, b; Samuel et al. 2003; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008
H. clarionoides(J. Rémy) Reiche	Apargioides/ Apargioides	Chile	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2008
H. echegarayi Hieron.	Unknown/ Sessiliflora	Argentina, Bolivia, and Peru	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2008
H. elata (Wedd.) Griseb.	Apargioides/ ungrouped	Argentina, Bolivia, Colombia, Ecuador, and Peru	Tremetsberger et al. 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2007, 2008
H. eremophila Cabr.	Sessiliflora/ Sessiliflora	Argentina, Bolivia, and Chile	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2008
H. gayana (DC.) Cabrera	Apargioides/ Apargioides	Argentina and Chile	Tremetsberger et al. 2005, 2006; Weiss-Schneeweiss et al. 2008
H. grisebachii Cabr.	Acaulis group/ unknown	Brazil	Ruas et al. 2005; Weiss-Schneeweiss et al. 2007
H. hookeri Phil.	Apargioides/ Tenuifolia	Argentina	Samuel et al. 2003; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008
H. incana (Hook. & Arn.) Macloskie	Apargioides/ Tenuifolia	Argentina and Chile	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008
H. lutea (Vell.) Britton	Sessilifolia/ Lutea	Argentina, Brazil, and Uruguay	Ruas et al. 1995, 2005 (*H. rosengurtii*); Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2008; Fiorin 2008; present study
H. megapotamica Cabr.	Acaulis/ Pampasica	Argentina, Brazil, and Uruguay	Cerbah et al. 1995, 1998a, b; Ruas et al. 1995, 2005; Samuel et al. 2003; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2003, 2008; present study
H. meyeniana (Walp.) Griseb.	Apargioides/ Sessiliflora	Argentina, Bolivia, Ecuador, and Peru	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2008
H. microcephala (Sch. Bip.) Cabrera	Apargioides/ Microcephala	Argentina, Brazil, Paraguay, and Uruguay	Cerbah et al. 1995, 1998a, b; Ruas et al. 1995, 2005; Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss-Schneeweiss et al. 2003, 2007, 2008; present study
H. neopinnatifida Azevedo-Gonç. & Matzemb.	Apargioides/ Pampasica	Argentina and Brazil	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008; the present study
H. palustris (Phil.) De Wild.	Acaulis/ Tenuifolia	Argentina and Chile	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2008

Table 3 continued

Species	Chromosome/ phylogenetic groups	Distribution areas	References
H. pampasica Cabr.	Acaulis/ Pampasica	Argentina and Brazil	Cerbah et al. 1995, 1998a, b; Ruas et al. 1995, 2005; Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss-Schneeweiss et al. 2003, 2007, 2008; present study
H. parodii Cabrera	Apargioides/ Microcephala	Argentina, Bolivia, and Ecuador	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008
H. patagonica Cabrera	Patagonica/ Chondrilloides	Argentina	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008
H. petiolaris (Hook. & Arn.) Griseb.	Acaulis/ ungrouped	Argentina	Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2007, 2008; present study
H. scorzonerae (DC.) F. Muell.	Ungrouped/ ungrouped	Chile	Samuel et al. 2003; Tremetsberger et al. 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2007, 2008
H. sessiliflora Kunth	Apargioides/ Sessiflora	Bolivia, Colombia, Ecuador, Peru, and Venezuela	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2007, 2008
H. setosa (Wedd.) Rusby	Ungrouped/ ungrouped	Colombia, Ecuador, Peru, and Venezuela	Weiss et al. 2003.
H. sonchoises Kunth	Ungrouped/ ungrouped	Ecuador	Weiss et al. 2003
H. spathulata (J. Rémy) Reiche	Apargioides/ Apargioides	Chile	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2007, 2008;
H. stenocephala (A. Gray ex Wedd.) Kuntze	Ungrouped/ ungrouped	Bolivia and Peru	Weiss et al. 2003
H. taraxacoides (Walp.) Benth. & Hook. F.	Apargioides/ Sessiflora	Argentina, Bolivia, and Chile	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss-Schneeweiss et al. 2007, 2008
H. tenuifolia (Hook. & Arn.) Griseb.	Tenuifolia/ Tenuifolia	Argentina and Chile	Samuel et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2007, 2008
H. thrincioides (J. Rémy) Reiche	Apargioides/ Apargioides	Chile	Weiss et al. 2003; Tremetsberger et al. 2005, 2006; Weiss et al. 2003; Weiss-Schneeweiss et al. 2003, 2007, 2008
H. variegata (Lam.) Baker	Apargioides/ ungrouped	Argentina, Brazil, Paraguay, and Uruguay	Ruas et al. 1995, 2005; Tremetsberger et al. 2006; Weiss-Schneeweiss et al. 2003, 2007, 2008; present study
H. angustifolia (Litard. & Maire) Maire[a]	Ungrouped	Morocco	Tremetsberger et al. 2005, 2006; Weiss-Schneeweiss et al. 2008; present study

[a] *H. angustifolia* is considered the sister species of the South American *Hypochaeris* and was included in the study as the outgroup

though AFLP and karyotype data revealed a close relationship between *H. catharinensis* and *H. lutea*, the genetic similarity is not positively correlated with morphological and ecological characteristics. Morphologically, *H. catharinensis* and *H. lutea* are clearly distinguishable as seen by the simple versus ramified stems, exclusively basal versus basal and stem leaves, corollas that are equal or shorter versus longer than the involucres, ligules that are shorter versus longer than the tube of the corolla, and shorter versus longer achenes (Azevêdo-Gonçalves and Matzenbacher 2007). In addition, the leaves of *H. catharinensis* form rosettes, which favors total light incidence (Azevêdo-Gonçalves and Matzenbacher 2007). Furthermore, the distribution of *H. catharinensis* is limited to dry, shallow, and rocky soils that facilitate the development of a horizontal rhizome system, characteristic of this species. *Hypochaeris catharinensis* occurs in association with herbs or shrubs, in open forests of *Araucaria angustifolia*, and on the edges of roadsides. The ecological requirements of *H. catharinensis* suggest that this species may have passed through environmental constraints motivated by adverse climatic features of the plateau region where it occurs.

In contrast, *H. lutea* is adapted only to wet environments, being a very plastic species and occurring in a variety of physiographic regions (Azevêdo-Gonçalves and Matzenbacher 2007; Matzenbacher 1998). *Hypochaeris lutea* is generally associated with grasses, *Sphagnum* sp., *Baccharis* sp., ferns, and other species that are adapted to deep, wet, and swampy soils. These characteristics have favored in *H. lutea* the development of pivotant roots, a long and thin flower stem, and very narrow long leaves that assist this species in searching for photosynthetic resources (Azevêdo-Gonçalves and Matzenbacher 2007; Matzenbacher 1998). Interestingly, the ecological features of

H. lutea are shared with the Moroccan *H. angustifolia*, the putative ancestor of South American species of *Hypochaeris*.

Delimiting boundaries of species within a group of very young plants, such as *Hypochaeris* in South America, has been difficult because the phylogenetic relationships of species, as well as the genetic characteristics that distinguish them, are still not completely understood (Samuel et al. 2003; Stuessy et al. 2003; Tremetsberger et al. 2005, 2006; Weiss-Schneeweiss et al. 2008). Furthermore, hybridization between *Hypochaeris* species with overlapping distribution (Azevêdo-Gonçalves and Matzenbacher 2007) complicates this scenario. In the present study, chromosome and AFLP data allow determination of the phylogenetic position of *H. catharinensis* among other South American species of *Hypochaeris*. Whereas *H. lutea* and *H. patagonica* have been previously placed in the same karyotype group (Weiss-Schneeweiss et al. 2008), they were not associated genetically in the AFLP study of Tremetsberger et al. (2006), suggesting independent karyotype evolution in these species. Our results, therefore, contribute not only to the positioning of *H. catharinensis* within the South American species, but also permit to suggest a novel and well-resolved phylogenetic group (the Lutea group), containing *H. lutea* and *H. catharinensis*.

Conclusions

Hypochaeris catharinensis is closely related, based on both karyotypic features and DNA-based phylogeny, to *H. lutea*. Hence, the new results strengthen and complete previously proposed chromosome- and phylogenetic-based informal groups within South American *Hypochaeris* species, emphasizing the role of proper sampling for making evolutionary inferences. The present study further demonstrates the predictive value of karyotypic features, and the involvement of chromosome changes in speciation processes in South American species of *Hypochaeris*. Additionally, chromosome analyses revealed the presence of additional and strong GC-rich regions in addition to a novel AT-rich heterochromatin in the *Hypochaeris* genome, which require further and more detailed investigation. Such studies might provide new markers to analyze chromosomal rearrangements in this rapidly speciating plant group.

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