Nuclear ribosomal DNA and karyotypes indicate a NW African origin of South American Hypochaeris (Asteraceae, Cichorieae)

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Abstract

Hypochaeris has a disjunct distribution, with more than 15 species in the Mediterranean region, the Canary Islands, Europe, and Asia, and more than 40 species in South America. Previous studies have suggested that the New World taxa have evolved from ancestors similar to the central European H. maculata. Based on internal transcribed spacer (ITS) sequences and fluorescence in situ hybridization (FISH) with 5S and 18S–25S rDNA of the previously overlooked Hypochaeris angustifolia from Moyen Atlas, Morocco, we show that it is sister to the entire South American group. A biogeographic analysis supports the hypothesis of long-distance dispersal from NW Africa across the Atlantic Ocean for the origin of the South American taxa rather than migration from North America, through the Panamian land bridge, followed by subsequent extinction in North America. With the assumption of a molecular clock, the trans-Atlantic dispersal from NW Africa to South America is roughly estimated to have taken place during Pliocene or Pleistocene.

Keywords: Compositae; FISH; ITS; Hypochaeris angustifolia; Long-distance dispersal; rps16 intron

1. Introduction

The genus Hypochaeris (Asteraceae, Cichorieae) is distributed in North Africa, the Canary Islands, Europe, Asia, and South America. The co-occurrence with its sister genus Leontodon in the Mediterranean region (North Africa and S Europe) and the abundance of Old World species in this region suggest a Mediterranean origin of Hypochaeris (Samuel et al., 2003; Stebbins, 1971). The Hypochaeridinae are distributed in Eurasia and North Africa (Bremer, 1994). There are no other Hypochaeridinae in South America (Bremer, 1994). Old World Hypochaeris (c. 15 species) also contain greater karyotypic (chromosome numbers x = 3, 4, 5, and 6 and all symmet-
been included within the otherwise Eurasian sect. *Achyrophorus* containing *H. grandiflora*, *H. maculata*, and *H. uniflora* based on the presence of just one row of pappus hairs (Hoffmann, 1893). The Eurasian sect. *Achyrophorus* has *n* = 5 and similar karyotypes, including also localization of rDNA loci (Cerbah, 1997; Weiss-Schneeweiss et al., unpubl.).

The biogeographic origin of the South American group is still problematic. Two conflicting hypotheses exist to explain how *Hypochaeris* arrived in South America given its absence from North America. (1) Stebbins' (1971) hypothesis is based primarily on the assumption that the asymmetrical, bimodal karyotype is generally a derived genomic character in flowering plants. It is found in the South American group, in contrast to Old World *Hypochaeris*, which have rather symmetrical karyotypes. Stebbins (1971) suggested that *Hypochaeris* may have existed in North America during the Tertiary as small populations in pioneer habitats, a situation which might have favored evolution of asymmetrical, bimodal karyotypes (two large and two small chromosome pairs). After entering South America during the Pliocene, perhaps by migration through the Panamanian land bridge, *Hypochaeris* might have diversified into different environments, and the North American populations gone extinct. (2) Samuel et al. (2003) suggested that there might be a simpler and hence more attractive explanation, namely long-distance dispersal directly to South America. DNA sequence evidence of Samuel et al. (2003) showed that two Old World sections (*Achyrophorus* and *Metabasis*) are closely related with the South American group. Based on karyotypic similarity of *H. maculata* and relatives of the Eurasian sect. *Achyrophorus* and the South American species (both possess two 18S–25S rDNA loci, and the intrachromosomal location of the single 5S rDNA locus is the same), Weiss-Schneeweiss et al. (2003) presented a model of chromosomal changes during evolution from a *H. maculata*-like ancestor, i.e., *H. maculata* or *H. uniflora*, which share similar karyotypes, or their unknown/extinct relative, to the South American species. These detailed karyotypic analyses, including particularly the number and localization...
of 5S and 45S rRNA genes by FISH, allowed the hypothesis that one species of this group (or a related ancestral taxon of sect. Achyrophanos) gave rise to the South American group, although sect. Achyrophanos differs from the South American group by its chromosome number (n = 5 vs. n = 4).

Determining the closest relative of the South American group could give a clue to migration or dispersal as an agent of evolution. Oberprieler and Vogt (2002) investigated the basic karyotype morphology of the previously poorly studied Moroccan endemic Hypochaeris angustifolia (Litard & Maire) Maire and showed it to have n = 4 with three pairs of chromosomes carrying satellites (rRNA genes). H. angustifolia also has a single row of pappus hairs like other members of sect. Achyrophanos (Galán de Mera and Vicente Orellana, 1998), but it has not yet been included in any phylogenetic analysis. The basic chromosome number and asymmetric karyotype of H. angustifolia suggest that this species could be a closer relative of the South American group than H. maculata.

We therefore extended the previous molecular phylogenetic study of Hypochaeris (Samuel et al., 2003) by including H. angustifolia and other missing taxa using a nuclear (ITS) and a chloroplast (rps16 intron) sequence. A detailed study of the karyotype of H. angustifolia was also carried out using FISH. The specific purposes of this study were to: (1) determine the relationship of H. angustifolia to other Old and New World taxa; and (2) interpret its relationships in context of the biogeographic origin (time and place) of the South American group.
2. Materials and methods

2.1. Plant material

We sampled leaves of *H. angustifolia* and other Western Mediterranean species not previously included in a phylogenetic analysis (Samuel et al., 2003; *H. arachnoidea*, *H. leontodontoides*, *H. rutea*, and *H. salzmanniana*; Table 1) in the field. Because this study focuses on *H. angustifolia*, we sequenced five individuals in Morocco collected from throughout the geographical range of the species. Species classification of Moroccan and Spanish taxa follows Talavera (1987), Oberprieler (2002), and Galán de Mera et al. (1999; for *H. leontodontoides*). A subset of South American taxa was sequenced including representatives of the entire geographical distribution and from all major phylogenetic groups determined by the fingerprinting technique amplified fragment length polymorphism (Tremetsberger et al., unpubl.).

2.2. DNA sequences

Total DNA was extracted from silica-gel dried leaves with the hexadeoxytrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). ITS of 18S–5.8S–28S nuclear ribosomal DNA was amplified by using primers ITS4 and ITS5 of White et al. (1990) and 17SE of Sun et al. (1994). The PCR mix for amplification contained: 45 µl 1.1 x Reddy Mix PCR Master Mix (2.5 mM MgCl2; ABgene), 20 pmol forward and reverse primer each, 2 µl dimethyl sulfoxide (DMSO), and 1 µl template DNA (approx. 30–100 ng/µl). Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research) with the following reaction conditions: 95 °C/4.5 min, 45 °C/1 min, 72 °C/1 min (1 cycle); 95 °C/1 min, 48 °C/1 min, 72 °C/1 min (36 cycles); and 72 °C/7 min, 4 °C/thereafter. Amplification products were purified with the QIAquick Gel Extraction Kit (QIAGEN) and sequenced on a GeneAmp PCR System 9700 by following the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Protocol (Applied Biosystems). As a modification, the total volume of the cycle sequencing reaction was 10 µl (2 µl Terminator Ready Reaction Mix, 2.5 pmol primer, purified amplification product). Cycle sequencing products were run on an ABI Prism 377 DNA Sequencer (PE Applied Biosystems). The chloroplast *rps16* intron was amplified and sequenced by using primers *rpsF* and *rpsR2* of Oxelman et al. (1997) with the same conditions as for ITS except that bovine serum albumin (BSA) was used in addition to DMSO (1:1) in the PCR mix. In some species, a newly designed internal primer (5'-TTTTCAAC GAGACAAAAAGGAGG-3') was used in an additional cycle sequencing reaction to smooth out illegibility of part of the sequence caused by a G-stretch of variable length. Sequences were deposited in the EMBL Database (Stoesser et al., 2003; Table 1).

2.3. Phylogenetic analysis

The forward and reverse sequences were assembled with AutoAssembler ver. 1.4.0 (Applied Biosystems). Alignment of the complete ITS1–5.8S–ITS2 sequences was done manually and guided by the consensus alignment for Asteraceae of Goertzen et al. (2003) using BioEdit ver. 5.0.9 (Hall, 1999). The following ITS sequences from Samuel et al. (2003) were also included (with GenBank Accession Nos.): *H. acaulis*, AF528433; *H. achyrophorus*, AF528434; *H. apargioides*, AF528435; *H. clarionoides*, AF528445; *H. gayana*, AF528450; *H. grandiflora*, AF528448; *H. maculata*, AF528454; *H. meyeniana*, AF528455; *H. palustris*, AF528456; *H. sessiliflora*, AF528482; *H. spathulata*, AF528464; *H. tenuifolia*, AF528469; *H. thrinocioides*, AF528467; and *H. uniflora*, AF528481. Percentage G+C content was determined for ITS1 and ITS2 for each sample (BioEdit ver. 5.0.9; Hall, 1999). Presence of RNA secondary structure motifs (ITS1 and ITS2) described by Goertzen et al. (2003) was tested for each sample with the mfold web server ver. 3.1 (Zuker, 2003) using thermodynamic rules (Zuker et al., 1999). The best-fitting model of DNA substitution determined with PAUP* ver. 4.0b10 (Swofford, 2003) and Modeltest ver. 3.06 (Posada and Crandall, 1998) using the Akaike Information Criterion is the symmetrical model (Zharkikh, 1994) with gamma distributed site-to-site rate variation (SYM+G). A heuristic search was carried out under the maximum likelihood (ML) criterion with 100 random addition sequence replicates and other settings kept as default with PAUP* ver. 4.0b10 (Swofford, 2003). Bootstrap proportion (BP) for each node was estimated with 500 bootstrap replicates (Felstenstein, 1985) with PAUP* ver. 4.0b10 (Swofford, 2003; ML criterion, heuristic searches with simple addition sequence, TBR swapping with no more than 10 trees ≥ score 10 saved at each replicate, and other settings kept as default). Outgroup choice was guided by Samuel et al. (2003), which showed *Leontodon* as the sister group, and by an initial analysis of *Hypochaeris* with all Cichorieae sequenced by Goertzen et al. (2003), which placed *Cicerbita*, *Lactuca*, *Ragadilocus*, and *Taraxacum* next to *Hypochaeris* and *Leontodon*. The following taxa were used as outgroups (with GenBank Accession Nos.: Kim and Jansen, 1994; Kim et al., 1996; Koopman et al., 1998; Samuel et al., 2003; Cicerbita alpina, AJ228652; Lactuca sativa, L13957; Leontodon saxatilis, AF528489; Ragadilocus edulis, AF528495; and Taraxacum officinale, L48337, L48338. After alignment with Clustal X ver. 1.83 (Thompson et al., 1997), ML analysis of the *rps16* intron was carried out in the same manner as that of ITS. The transversion model plus estimated proportion of invariable sites (TVM + I) was chosen as appropriate
<table>
<thead>
<tr>
<th>Taxon</th>
<th>Distribution</th>
<th>Locality</th>
<th>Collector(s) and number</th>
<th>Location of voucher</th>
<th>EMBL Accession number</th>
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<tbody>
<tr>
<td><strong>Ingroup</strong> Achyrophorus</td>
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<td><em>H. acaulis</em> (J. Rémy) Britton</td>
<td>S Am.</td>
<td>Argentina, Neuquén: Volcán Copahue</td>
<td>Tremetsberger, Jiménez &amp; Gómez 1038</td>
<td>WU</td>
<td>AJ627447</td>
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<td></td>
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<td>Morocco, Moyen Atlas, Meknés: 5 km S of Boumia along dirt path to Tounifie</td>
<td>Talavera, Stuessy et al. 27003M</td>
<td>SEV, WU</td>
<td>AJ627450</td>
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<td>Morocco, Moyen Atlas, Meknés: on dirt path to Bekrit, 8 km from junction with road between Timahdite and Col du Zad</td>
<td>Talavera et al. 69303M</td>
<td>SEV</td>
<td>AJ627451</td>
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<td>Morocco, Moyen Atlas, Meknés: near Timahdite</td>
<td>Talavera et al. 67603M</td>
<td>SEV</td>
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<td>Morocco, Taza: Jebel Tazzea between Bab-Azhar and Bab-Bou-Idir</td>
<td>Talavera et al. 63303M</td>
<td>SEV</td>
<td>AJ627453</td>
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<td><em>H. apargioides</em> Hook. &amp; Arn.</td>
<td>S Am.</td>
<td>Chile, Región IX: dirt path from Cordillera de los Raices to Volcán Lonquimay</td>
<td>Stuessy, Baeza &amp; Tremetsberger 18099</td>
<td>CONC, WU</td>
<td>AJ627454</td>
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<td><em>H. chilensis</em> (Kunth) Hieron.</td>
<td>S Am.</td>
<td>Argentina, Jujuy: between Yalda and Leon</td>
<td>Stuessy, Turbey &amp; Tremetsberger 18060</td>
<td>LP, WU</td>
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<td><em>H. chondrillooides</em> (A. Gray) Cabrera</td>
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<td>Argentina, Jujuy: Iturbe</td>
<td>Stuessy, Turbey &amp; Tremetsberger 18072</td>
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<td><em>H. clarionoides</em> (J. Rémy) Reiche</td>
<td>S Am.</td>
<td>Chile, Región Metropolitana: between Farellones and La Parva</td>
<td>Tremetsberger &amp; Hössinger 137</td>
<td>WU</td>
<td>AJ627459</td>
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<td><em>H. elata</em> (Wedd.) Griseb.</td>
<td>S Am.</td>
<td>Argentina, Jujuy: between Humahuaca and El Aguilar</td>
<td>Stuessy, Turbey &amp; Tremetsberger 18080</td>
<td>LP, WU</td>
<td>AJ627461</td>
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<td><em>H. eremophila</em> Cabrera</td>
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<td>Argentina, Jujuy: near Purmamare</td>
<td>Stuessy, Turbey &amp; Tremetsberger 18064</td>
<td>LP, WU</td>
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<td><em>H. gayana</em> (DC.) Cabrera</td>
<td>S Am.</td>
<td>Chile, Región VIII: Cordillera de Nahuelbuta</td>
<td>Tremetsberger &amp; Parra 42</td>
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<tr>
<td><em>H. incana</em> (Hook. &amp; Arn.) Macloskie</td>
<td>S Am.</td>
<td>Chile, Región XII: Punta Arenas</td>
<td>Schönswetter &amp; Tribsch 5640</td>
<td>WU</td>
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<td><em>H. maculata</em> L.</td>
<td>C + N Eur., As.</td>
<td>Austria, Niederösterreich: Wachau, hill 1 km E of village Dürnstein</td>
<td>Dimitrova &amp; M. A. Fischer s. n. (13.05.1999)</td>
<td>WU</td>
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<td><em>H. megapotamica</em> Cabrera</td>
<td>S Am.</td>
<td>Argentina, Buenos Aires: La Plata</td>
<td>Stuessy &amp; Turbey s. n. (27.10.2001)</td>
<td>LP, WU</td>
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<td><em>H. meyeniana</em> (Walp.) Griseb.</td>
<td>S Am.</td>
<td>Bolivia, La Paz: just below Represa</td>
<td>Stuessy, Tremetsberger &amp; Hössinger 18502</td>
<td>LPB, WU</td>
<td>AJ627670</td>
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<tr>
<td><em>H. microcephala</em> (Sch. Bip.) Cabrera</td>
<td>S Am.</td>
<td>Ecuador, Cotopaxi: Pujili</td>
<td>Stuessy et al. 18555</td>
<td>QUSF, WU</td>
<td>AJ627676</td>
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<td><em>H. palastris</em> (Phil.) De Wild.</td>
<td>S Am.</td>
<td>Argentina, Río Negro: Cerro Tronador</td>
<td>Stuessy, Turbey &amp; Tremetsberger 18048</td>
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<tr>
<td><em>H. pampasica</em> Cabrera</td>
<td>S Am.</td>
<td>Argentina, Buenos Aires: Sierra de la Ventana</td>
<td>Turbey &amp; Tremetsberger 128</td>
<td>LP, WU</td>
<td>AJ627473</td>
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<tr>
<td><em>H. sessiliflora</em> Kunth</td>
<td>S Am.</td>
<td>Peru, Junin, Huáncayo</td>
<td>Weigend 5816 (2001-2167)</td>
<td>USM, WU</td>
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<td><em>H. spatulata</em> (J. Rémy) Reiche</td>
<td>S Am.</td>
<td>Chile, Región VIII: Quidico</td>
<td>Tremetsberger &amp; Hössinger 52</td>
<td>WU</td>
<td>AJ627477</td>
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</tbody>
</table>
### Distributions indicated are those used for the biogeographic analysis. If not indicated otherwise, the Old World species are widespread in their distributional areas. Only the native distributions are indicated for *H. glabra* and *H. radicata*, which are also introduced worldwide. Although *H. uniniflora* grows only in the mountains of central Europe, it was assigned the same general distributional area as *H. maculata* (C + N Eur.) for the biogeographic analyses. Exact distributions within South America are not indicated. Sectional classification follows Hoehn (1893). Abbreviations: NW Afr., north-western Africa; SW Eur., south-western Europe; SC + SE Eur., south-central and south-eastern Europe; C + N Eur., central and northern Europe; As., Asia; Can., Canary Islands; S Am., South America. Location of vouchers are indicated by acronyms of Index Herbariorum.

### Sect. Hypochaeris

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<th>Distribution</th>
<th>Vouchers</th>
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<td><em>H. tenutifolia</em> (Hook. &amp; Arn.) Griseb.</td>
<td>S Am.</td>
<td>Argentina, Río Negro: Cerro Catedral</td>
<td>Stuessy, Urbey &amp; Tremetsberger 18025 LP, WU — AJ627479</td>
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<td><em>H. thrinicoside (J. Rêmy) Reiche</em></td>
<td>S Am.</td>
<td>Chile, Región VIII: Talcahuano</td>
<td>Stuessy, Baeza &amp; Tremetsberger 18092 CONC, WU — AJ627480</td>
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<td><em>H. arachnoidea</em> Poir.</td>
<td>NW Afr.</td>
<td>Morocco, Anti-Atlas, Agadir: 23 km from Tafraoute on road to Agadir</td>
<td>Talavera, Stuessy et al. 162/03M SEV, WU AJ627262 AJ627455</td>
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<td><em>H. salzmanniana</em> DC.</td>
<td>NW Afr., SW Eur.</td>
<td>Spain, Cádiz: Conil de la Frontera, Playa de El Palmar</td>
<td>Talavera, Stuessy et al. TS5-1 SEV, WU AJ627272 AJ627475</td>
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<td><em>H. cretensis</em> (L.) Bory &amp; Chaub.</td>
<td>SC + SE Eur.</td>
<td>Italy, Sicilia, Monti di Palermo</td>
<td>Grown from seeds from specimen MA 646061 SEV AJ627263 —</td>
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### Sect. Metabasis

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<tr>
<td><em>H. rutea</em> Talavera</td>
<td>SW Eur. (endemic to Spain)</td>
<td>Spain, Córdoba, Sierra Subbética: Sierra Gallinera near Carcabuey</td>
<td>Talavera et al. s. n. (08.05.2002) SEV AJ627271 AJ627474</td>
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### Outgroup

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<th>Vouchers</th>
</tr>
</thead>
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<td><em>Crepis alpestris</em> Reichb.</td>
<td>Austria, Niederösterreich: mountain Gahns N of Payerbach</td>
<td>G. &amp; M. A. Fischer s. n. (22.06.2002) WU — AJ627482</td>
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<tr>
<td><em>Urospermum dallechampii</em> F. W. Schmidt</td>
<td>Spain, Castellón: Desierto de las Palmas, Benicasim</td>
<td>Rico &amp; Sánchez SALA 32145 SALA — AJ627483</td>
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</tbody>
</table>

Distributions indicated are those used for the biogeographic analysis. If not indicated otherwise, the Old World species are widespread in their distributional areas. Only the native distributions are indicated for *H. glabra* and *H. radicata*, which are also introduced worldwide. Although *H. uniniflora* grows only in the mountains of central Europe, it was assigned the same general distributional area as *H. maculata* (C + N Eur.) for the biogeographic analyses. Exact distributions within South America are not indicated. Sectional classification follows Hoehn (1893). Abbreviations: NW Afr., north-western Africa; SW Eur., south-western Europe; SC + SE Eur., south-central and south-eastern Europe; C + N Eur., central and northern Europe; As., Asia; Can., Canary Islands; S Am., South America. Location of vouchers are indicated by acronyms of Index Herbariorum.
model of DNA substitution. Alignments are published in the EMBL alignment database (ITS: ALIGN_000666; rps16 intron: ALIGN_000665).

2.4. Biogeographic analysis

Occurrence of ingroup taxa in seven distributional areas is indicated in Table 1. Two approaches were used to assign ancestral areas to the nodes of a fully resolved phylogenetic tree derived from the results of the DNA sequence analysis (with the South American group represented on a single terminal node): (1) a most-parsimonious reconstruction (MPR) under the Fitch parsimony criterion (Fitch, 1971; Swofford et al., 1996) was carried out with MacClade ver. 4.0 ( Maddison and Maddison, 2000). (2) A dispersal-vicariance analysis (Ronquist, 1997) was carried out using DIVA ver. 1.1 (Ronquist, 1996) with default settings.

2.5. Age estimation

The age of groups was estimated in two steps. (1) The basal node for calibration is the age of the vernonioid group ( Bremer, 1996) estimated at 25 million years ago (mya) from ML analysis of atpB, rbcL, and 18S rDNA genes (Wikström et al., 2001). This date was fixed on a ML analysis of the 3′-portion of the ndhf gene of selected members of the vernonioid group ( Kim and Jansen, 1995; Park et al., 2001) to estimate the age of the most recent common ancestor (MRCA) of Hypochaeris uniflora and Leontodon saxatilis. (2) This estimate was subsequently fixed on the ML analysis of ITS sequences with Hypochaeris and Leontodon saxatilis.

The likelihood ratio (LR) test ( Felsenstein, 1981, 2004, p. 323) was used to assess clock-like evolution of the 3′-portion of the ndhf gene as defined by Kim and Jansen (1995) in the following taxa (with GenBank Accession Nos.; Kim and Jansen, 1995; Park et al., 2001): Cichorium intybus, L39390; Helianthus annuus, L39383; Hypochaeris uniflora, AF218333; Koelpinia linearis, AF218341; Leontodon saxatilis, AF218330; Schlechtendalia luzulaefolia, L39395; and Tagetes erecta, L39466. The transversion model with gamma distributed site-to-site rate variation (TVM+G) was selected as appropriate model of DNA substitution with PAUP* ver. 4.0b10 ( Swofford, 2003) and Modeltest ver. 3.06 (Posada and Crandell, 1998). Support for nodes was tested with 500 bootstrap replicates (PAUP* ver. 4.0b10; Swofford, 2003). Divergence time between Hypochaeris uniflora and Leontodon saxatilis was estimated in a global molecular clock framework with r8s ver. 1.60 (Sanderson, 2003; with Powell’s optimization method). Magnitude of error due to uncertainty of branch lengths (sampling from a finite number of characters) was approximately estimated through bootstrapping (Sanderson and Doyle, 2001). One hundred bootstrap data matrices obtained with SEQBOOT ver. 3.5c in PHYLIP (Felsenstein, 1995) were used to estimate branch lengths of the original tree with original likelihood settings (PAUP* ver. 4.0b10; Swofford, 2003). Age estimates for the 100 trees were obtained with r8s ver. 1.60 (Sanderson, 2003). Removal of five points from the tails of the resulting histogram (distribution of 100 age estimates) provided a rough approximation of the 95% confidence interval (Sanderson and Doyle, 2001).

The LR test was used to assess that ITS does not evolve clock-like (LR = 71.955 with 30 degrees of freedom; p < 0.000). Therefore, penalized likelihood (PL; Sanderson, 2002), which attempts to simultaneously estimate unknown divergence times and smooth the rate differences across lineages, was used to estimate divergence times with the age of the node connecting Hypochaeris uniflora and Leontodon saxatilis fixed to the point estimate obtained in the previous step using r8s ver. 1.60 (Sanderson, 2003) with the truncated Newton optimization method. The optimal level of smoothing (4.5) was estimated from the data using the cross-validation criterion (Sanderson, 2002) of r8s ver. 1.60 (Sanderson, 2003). Magnitude of error due to uncertainty of branch lengths was estimated in the same way as for 3′-ndhf.

2.6. Classical and molecular cytogenetics

Surface-sterilized seeds of H. angustifolia ( Talavera, Stuessy et al. 270/03M; Table 1) were germinated on wet filter paper on Petri dishes. Seedlings were pre-treated with 0.1% colchicine for 2 h at room temperature and 2 h at 4°C, fixed in 3 ethanol:1 acetic acid for 12 h at room temperature, and stored at −20°C until use. Feulgen staining with Schiff’s reagent was done in accordance with standard protocol (Weiss et al., 2003). Preparations with a least 15 well-spread chromosome plates were chosen for analysis. Asymmetry index (AsI; ratio length of all long arms in haploid chromosome set/total haploid karyotype length × 100; Arano and Saito, 1980) and ratio longest chromosome/shortest chromosome (R; Barghi et al., 1989) were calculated.

Chromosome spreads for FISH were obtained by enzymatic digestion/squashing as described by Weiss-Schneeweiss et al. (2003). The quality of spreads was checked by phase-contrast microscopy and only preparations with adequate numbers of well-spread metaphases (10–15) were selected for FISH. Slides were frozen at −80°C and stored at −20°C after coverslip removal. FISH was carried out in accordance with Weiss-Schneeweiss et al. (2003). Probes used for FISH were: 18S–25S rDNA from Arabidopsis thaliana in plasmid pSK+, labeled with digoxigenin (Roche), and 5S rDNA from Beta vulgaris in plasmid pBx1-2, labeled with biotin (Roche). Both probes were labeled by nick translation in accordance with manufacturer’s instruction (Roche). Chromosome preparations, together with the hybridiza-
tion mix, were denatured on a P2x2 Thermal Cycler (Thermo Hybaid) at 78°C for 3 min and hybridization was carried out overnight. Stringent washes and detection followed the method of Schwartzacher and Heslop-Harrison (2000) and Weiss-Schneeweiss et al. (2003). Analyses of preparations were made with a ZEISS Axioskop epifluorescent microscope. Images were acquired with a CCD camera (Zeiss), and files were processed using Adobe Photoshop ver. 6.0 (Adobe Systems) with only those functions that applied equally to all pixels in the image. For rDNA localization, a minimum of 30 well-spread metaphases and prometaphases was analysed.

3. Results

3.1. DNA sequences

PCR-amplified ITS fragments showed a single band when examined on agarose gels. The aligned ITS1 is 263 nucleotides (nt) long (length of individual sequences: 250–254 nt). The 5.8S rDNA gene is 164 nt long, has no gaps and few mutations (five sites in Hypochaeris plus four sites in outgroup taxa). The aligned ITS2 is 231 nt long (length of individual sequences: 202–226 nt). Length variation is mainly in the loop of helix 2A (see below). Eleven sites in all Hypochaeris sequences are polymorphic within the same individual. The five accessions of H. angustifolia have almost identical ITS sequences. Only the easternmost accession (Taza: Jbel Tazzeza) has two positions with two states in ITS1 (pos. 27: C/T; pos. 182: A/T). Mean G + C content for all taxa analysed (without outgroup) is 51.6% (SD = 1.9) in ITS1 and 53.3% (SD = 1.2) in ITS2.

All ITS1 and ITS2 sequences (ingroup) are able to form the helices conserved among Asteraceae (Goertzen et al., 2003). The stem of helix 1A has a minimum length of six base pairs (bp). The stem of helix 1B has a minimum length of 14 bp. Helix 1C has the 5 bp stem with a completely conserved sequence and 5 nt compound stem including two consecutive pyrimidine–pyrimidine juxtapositions. Helix 2C has two stems of 9–10 and 4 bp separated by an internal loop (Goertzen et al., 2003). The stem of helix 2D is eight (instead of seven; Goertzen et al., 2003) bp long.

The aligned rps16 intron data matrix (with outgroups) is 946 bp long (5′ end not complete and including the first four bases of exon 2 of the rps16 gene). Individual Hypochaeris sequences range from 819 to 850 bp. Informative indels are depicted in Fig. 2. The five accessions of H. angustifolia have identical rps16 intron sequences except for an A-stretch and a G-stretch of variable lengths (9, 10, or 11 A’s; 10, 11, or 12 G’s).

3.2. Phylogeny

ML analysis of ITS sequences produced a single most likely phylogram (−ln L = 3899.732; Fig. 1). Section Hypochaeris is monophyletic (100% BP) with H. glabra sister to H. arachnoidea, H. radicata, and H. salzmanni ana. Hypochaeris arachnoidea is endemic to NW Africa (Morocco and Algeria), and H. salzmanniana is endemic to the coasts of SW Spain and NW Morocco (both sides of the Strait of Gibraltar). Section Seriola is also monophyletic (100% BP). Hypochaeris rutea (Talavera, 1980), endemic to the Sierra Subbética in Córdoba, Spain, is confirmed as a close relative of H. laevigata (80% BP). Hypochaeris leontodontoides, endemic to the Moyen Atlas in Morocco, is confirmed as a member of sect. Seriola, but is more distantly related. Sections Hypochaeris and Seriola are sister groups (90% BP). As in Samuel et al. (2003), sect. Achyrophorus sensu Hoffmann (1893) is paraphyletic. One group comprises H. grandiflora, H. maculata, and H. uniflora (81% BP; sect. Achyrophorus s. str. in the following). Another group comprises H. angustifolia and all South American species analysed (100% BP). Within the South American group (100% BP), H. apargioides, H. clarionoides, H. gayana, H. spatulata, and H. thrincioidea group together (68% BP), and H. meyniana and H. taraxacoides group together (76% BP). These groups are also recovered in an amplified fragment length polymorphism (AFLP) study (Tremetsberger et al., unpubl.). Section Metabasis is monophyletic (91% BP), and sect. Achyrophorus s. str. and Metabasis are sister clades (100% BP).

ML analysis of rps16 intron sequences also produced a single most likely phylogram (−ln L = 1891.621; Fig. 2), which is congruent, but less resolved than that obtained with ITS. The topology described for ITS is also retrieved by rps16 intron in sect. Hypochaeris (99% BP; with H. glabra sister to H. arachnoidea, H. radicata, and H. salzmanniana) and Seriola (64% BP; with H. leontodontoides sister to H. achyrophorus, H. laevigata, and H. rutea). The South American species form a monophyletic clade (87% BP; as with ITS sequences), but sect. Achyrophorus and Metabasis are not resolved.

3.3. Biogeography

Most-parsimonious reconstruction of ancestral distributional areas suggests that the most recent common ancestor (MRCA) of H. angustifolia and the South American species lived in NW Africa (Fig. 3). NW Africa is also reconstructed as area occupied by the MRCA of the whole genus. Dispersal–vicariance analysis resulted in an exact solution, which suggests that the MRCA of H. angustifolia
and the South American species was distributed in NW Africa and South America (Fig. 3). The MRCA of the whole genus is reconstructed to have lived in all unit areas occupied by extant species or in all these areas except SW Europe. Generally, the state of the root node (in our case the MRCA of the whole genus) is less reliably inferred by dispersal–vicariance analysis (Ronquist, 1996). This uncertainty is manifested as a tendency for the root node distribution to include most or all of the areas occupied by the terminals (Ronquist, 1996). Constraining the maximum number of unit areas occupied by an ancestral taxon to two, dispersal–vicariance analysis infers the most likely distribution of the MRCA of the genus *Hypochaeris* as NW Africa or a combination of NW Africa with any one of the other unit areas except SW Europe.

### 3.4. Age estimation

ML analysis of *3'-ndhF* yields a negative log likelihood (− ln L) of 2242.827 (with clock; Fig. 4) and 2241.774 (without clock). The null hypothesis of equal rates of *3'-ndhF* evolution among branches cannot be rejected (LR < 2.106 with 5 degrees of freedom; \( \alpha = 0.834 \)). Bootstrap support ranges from 75% (vernonioid group; Bremer, 1996) to 100% (Cichorieae). Calibration of the chronogram with 25 mya for the vernonioid group yields a rate of nucleotide substitution of 0.0016 substitutions/site/my. The age of the MRCA of *Tagetes* and *Helianthus* is thus estimated at 15.1 mya and the 95% confidence interval due to uncertainty of branch lengths in the *3'-ndhF* tree lies between \( \sim 11.25 \) and \( \sim 20.00 \) mya.

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**Fig. 3.** Phylogenetic tree of the genus *Hypochaeris* with ancestral distributions inferred by most-parsimonious reconstruction [black lettering and line style (different grey tones represent different states; hatching represents equivocal state)] and dispersal-vicariance analysis (grey lettering). Distributional areas of extant taxa are also indicated in Table 1. Coding: A, north-western Africa; B, south-western Europe; C, south-central and south-eastern Europe; D, central and northern Europe; E, Asia; F, Canary Islands; and G, South America.

**Fig. 4.** Single best maximum likelihood phylogram of *3'-ndhF* sequences retrieved from GenBank (Kim and Jansen, 1995; Park et al., 2001) with molecular clock. Model of DNA substitution: TVM + G. Values above branches: substitutions/site; below branches: bootstrap proportions (500 replicates). Node for calibration: 25 mya for vernonioid group (Wikström et al., 2001). Subfamilial and tribal designations according to Panero and Funk (2002).
The age of the MRCA of Hypochaeris uniflora and Leontodon saxatilis is estimated at 6.58 mya and the 95% confidence interval lies between ~4.00 and ~9.25 mya.

Smoothing the rate differences across lineages of the ITS phylogram (Fig. 1) with the calibration point (MRCA of Hypochaeris uniflora and Leontodon saxatilis) fixed at 6.58 mya using the penalized likelihood method (Sanderson, 2002) reveals a mean rate of 0.0157 substitutions/site/my (SD = 0.0004). The age of the MRCA of H. angustifolia and the South American group is estimated at 2.37 mya and the 95% confidence interval due to uncertainty of branch lengths in the ITS tree lies between ~1.75 and ~3.50 mya (Fig. 5A). The age of the MRCA of the South American group is estimated at 0.50 mya and the 95% confidence interval lies between ~0.25 and ~1.00 mya (Fig. 5B). The length of the branch connecting the South American group with its MRCA with H. angustifolia thus corresponds to a time period of ~1.9 my (minimum = ~0.75 my; maximum = ~3.25 my).

3.5. Karyotype

The karyotype of H. angustifolia consists of two pairs of larger chromosomes, one submetacentric (pair 1) and one acrocentric (pair 2), and two pairs of smaller chromosomes, one submetacentric (pair 3) and one acrocentric (pair 4; Figs. 6A–C). Total haploid karyotype length (HKL) is 15.1 μm (SD = 0.9). Individual chromosome lengths are (relative lengths in parentheses): 5.5 μm (SD = 0.5; 36.5%; chromosome 1), 4.7 μm (SD = 1.2; 31.0%; chromosome 2), 2.6 μm (SD = 0.5; 17.1%; chromosome 3), and 2.3 μm (SD = 0.4; 15.4%; chromosome 4). Asymmetry index (AsI) is 67.6% and ratio of the longest chromosome/shortest chromosome (R) is 2.6. Chromosome pairs 2 and 3 carry satellites on their short arms contrary to the observation of Oberprieler and Vogt (2002), who noted satellites on chromosome pairs 2, 3, and 4. FISH revealed one 5S rDNA and two 18S–25S rDNA loci (Fig. 6D). The 5S rDNA locus is localized in an interstitial position within the short arm of chromosome 2. 18S–25S rDNA loci co-localize with satellites (short arms of chromosomes 2 and 3). The number of 5S and 18S–25S rDNA loci is the same as in most South American species (Weiss-Schneeweiss et al., 2003), but the position of the 18S–25S rDNA locus on chromosome 2 differs (short vs. long arm).

4. Discussion

4.1. Usefulness of ITS sequences for phylogenetic inference in Hypochaeris

A number of molecular genetic processes impact ITS sequences in ways that may mislead phylogenetic inference (Álvarez and Wendel, 2003). The main problem is the existence in many plant genomes of extensive sequence variation and presence of paralogous loci, which are derived from a duplication event (Álvarez and Wendel, 2003; Bailey et al., 2003). Hypochaeris species have one or two major 18S–5.8S–28S rDNA loci located in the nucleolar organizer regions of different chromosomes (Cerbah et al., 1998a; Weiss-Schneeweiss et al., 2003). Low proportion of intra-individual polymorphism (a total of 11 sites in all Hypochaeris sequences) stands for uniformity of ITS copies within and between these loci and is attributed to homogenization of ITS.
copies (concerted evolution; Baldwin et al., 1995). However, we did not apply cloning that would allow detection of eventually present rare ITS types (Álvarez and Wendel, 2003). Furthermore, even when concerted evolution is complete or nearly complete, it cannot be assumed that strict orthology has been maintained among sequences amplified among a set of taxa (Álvarez and Wendel, 2003). An associated problem is genomic harboring of pseudogenes (i.e., non-functional copies) in various states of decay (Álvarez and Wendel, 2003). In Hypochaeris, functionality of ITS sequences is supported by the presence of a highly conserved 5.8S rDNA gene, G + C contents (51.6% in ITS1 and 53.3% in ITS2) that compare well with values inferred for Asteraceae (50.0% in ITS1 and 51.8% in ITS2; Goertzen et al., 2003), and the presence of RNA secondary structure motifs conserved among Asteraceae (Goertzen et al., 2003). However, orthology or paralogy of loci in different species cannot be concluded on functionality alone (Bailey et al., 2003).

4.2. Biogeographic origin of the South American group

Analysis of ITS sequences shows with great confidence (100% BP) that H. angustifolia is the closest known relative of the South American group. It thereby replaces the H. maculata group, which was previously thought to hold this position (Samuel et al., 2003). Our biogeographic analysis suggests that the MRCA of H. angustifolia and the South American group was distributed in NW Africa (MPR), where its only known Old World descendent H. angustifolia is growing in the Moyen Atlas, or in NW Africa and South America (DIVA). This strengthens the hypothesis of long-distance dispersal directly from NW Africa to South America and disagrees with Stebbins’ (1971) hypothesis. If an earlier connection of the Old World and South America existed through North America as suggested by Stebbins (1971), either by migration through the Beringian land bridge or by direct dispersal from Europe to North America across the Atlantic ocean, a closer relationship of South American species with Eurasian species would be anticipated. However, the three Eurasian species, H. grandiflora, H. maculata, and H. uniflora (i.e., sect. Achyrophorus s. str.; all with n = 5), group together in a clade and are not closely related with the South American taxa. There are no other Hypochaeris species in Asia and temperate Europe.

Long-distance dispersal from NW Africa is thus the most parsimonious hypothesis for the arrival of Hypochaeris in South America. Means of conveyance cannot be determined with certainty, but wind and birds are the two likely possibilities. Evidence for long-distance dispersal within the family over several thousands of kilometers across open ocean has already been documented in several cases, e.g., in Senecio sect. Senecio (two inferred dispersals from the Old to the New World; Coleman et al., 2003), in the Hawaiian silversword alliance, which has a North American (Californian) origin (Baldwin et al., 1991; Baldwin, 2003), and in Microseris, which dispersed from western North America to Australia and New Zealand (Vijverberg et al., 1999). Long-distance dispersal must also be hypothesized for the arrival of plants on oceanic islands of volcanic origin, which have never been connected to any continental land mass, e.g., St. Helena in the southern Atlantic ocean [where Carlquist (2001) studied the woody Asteraceae], the Juan Fernández Islands in the southern Pacific ocean [where Sang et al. (1994) studied the endemic genus Dendroseris], and Macaronesia [where Moore et al. (2002) studied Tolpis and Kim et al. (1996, 1999) studied
the woody Sonchus alliance]. Long-distance dispersal between continental land masses has less often been documented. Wendel and Albert (1992) hypothesized several intercontinental dispersals in the evolution of cotton (Malvaceae), among which are two dispersals from Africa to the New World, first of D-genome diploids and later of A-genome diploids leading to the formation of AD-allopolyploids (Wendel et al., 1995). Käss and Wink (1997) hypothesized long-distance dispersal of Old World (mainly African) lupins (Fabaceae) to the E parts of South America (“Atlantic region”).

Cichorieae are predominant in the Northern Hemisphere with concentration of genera and species in the Mediterranean region, central Asia and SW North America (Bremer, 1994). Many Cichorieae are widespread, successful and noxious weeds (Bremer, 1994), e.g., Hypochaeris glabra, H. radicata, and Leontodon saxatilis, which colonized South America with the European people several hundred years ago. Some Cichorieae are believed to have undergone adaptive radiation in the Southern Hemisphere, e.g., Microseris, which evolved four ecotypes in New Zealand and Australia after long-distance dispersal from western North America (Vijverberg et al., 1999, 2000), Sonchus, which has a high diversity of species in Africa, Macaronesia (“the woody Sonchus alliance”; Kim et al., 1996, 1999) and the Mediterranean region (Bremer, 1994), and the also woody Dendroseris, which is closely related to the woody Sonchus alliance and radiated into 11 species on the Juan Fernández Islands in the southern Pacific ocean (Sang et al., 1994), Hypochaeris in South America has diversified into c. 40 species, which have adapted to a variety of environments (from subantarctic to subtropical and from sea-level to over 5000 m elevation).

4.3. Dating the dispersal

Age estimation from DNA sequence data can contain several sources of error (Graur and Martin, 2004; Sanderson and Doyle, 2001). These include problems associated with the primary calibration point (e.g., dating the fossil, placing the fossil on the branch), phylogenetic uncertainty and uncertainty of branch lengths due to sampling from a finite number of characters, lineage effects (i.e., differences in substitution rates across lineages), and variance introduced by methods used to smooth these rate differences, transfer of age estimates obtained by molecular clock calculations on independent trees (generated from different, often faster evolving sequences) and their use as secondary calibration points, and uncertainties associated with the molecular evolutionary process itself (e.g., neutrality vs. non-neutrality, deviations from rate constancy through time). The single absolute calibration point, on which our analysis rests, is the split between Fagales and Cucurbitales at 84 mya selected by Wikström et al. (2001). This analysis dates the Asteraceae at 40–44 mya (SE obtained from 100 bootstrap replicates of the original data matrix = 3 mya). This age estimate is not in conflict with fossil evidence (summarized in Graham, 1996; Romero, 1993) and biogeographic considerations (Bremer and Gustafsson, 1997; DeVore and Stuessy, 1995; Stuessy et al., 1994), which suggest a late Eocene appearance of Asteraceae and thus provide an independent confirmation of Wikström et al.’s (2001) estimate. Using the bootstrap approach of Sanderson and Doyle (2001) we have accounted for uncertainty of branch lengths due to sampling from a finite number of characters. We also tested for topological uncertainty using the same approach and found that the variance due to topological uncertainty is smaller than the variance due to uncertainty of branch lengths (data not shown). Wikström et al. (2001) used nonparametric rate smoothing (Sanderson, 1997) and we used penalized likelihood (Sanderson, 2002) to smooth rate differences across lineages. These methods add another layer of assumptions and manipulations, of which the statistical basis is unknown. An unquantified proportion of variance is also introduced in our age estimation by two successive transfers of age estimates on independent trees and their use as secondary calibration points. First, Wikström et al.’s (2001) age estimate of the non-nectoid group (24–28 mya; SE = 3 mya) was used as a calibration point on the 3′-ndhF tree. Second, our age estimate of the MRCA of Hypochaeris and Leontodon (6.58 mya; 95% confidence interval lies between ~4.00 and ~9.25 mya) was used as a calibration point on the ITS tree. Through their use as secondary calibration points these age estimates have been denuded of error and it is not clear how this procedure affects the ranges of our age estimates. For these reasons, we must keep in mind that the range of uncertainty is broader than the confidence intervals that we report (which account only for uncertainty of branch lengths due to sampling from a finite number of characters) would imply.

In the absence of a more recent fossil or geological calibration point, however, 3′-ndhF provides the best estimate of the age of the MRCA of Hypochaeris uniflora and Leontodon saxatilis (6.58 mya; 95% confidence interval lies between ~4.00 and ~9.25 mya). Trans-Atlantic dispersal of Hypochaeris from NW Africa to South America could have occurred at any time along the branch connecting the South American group with its MRCA with H. angustifolia, i.e., between ~3.5 mya (upper limit of the 95% confidence interval of the age estimate of the MRCA of H. angustifolia and the South American group) and ~0.25 mya (lower limit of the 95% confidence interval of the age estimate of the MRCA of the South American group), i.e., during Pliocene or Pleistocene. The start of the diversification in South America is estimated to have occurred between ~0.25 and ~1.00 mya, i.e., during Pleistocene.
4.4. Karyotype evolution

Previous karyotype analysis of South American Hypochaeris (Weiss-Schneeweiss et al., 2003) allowed insight into karyotype evolution within this group, but only weakly resolved possible relationships between karyotypes of South American and Old World taxa. Study of interchromosomal asymmetry (see Romero Zarco, 1986), which estimates variation in chromosome lengths, positioned H. angustifolia between the Old World and South American taxa (this was not the case for intrachromosomal asymmetry, however, which estimates relationships between the chromosome arms; Oberprieler and Vogt, 2002). FISH analysis of H. angustifolia allows re-evaluation of possible pathways for the origin of the karyotypes of South American taxa (Fig. 7). The basic chromosome number of the MRCA of sect. Achyrophorus s. str. (n = 5), sect. Metabasis (n = 3) and the clade of H. angustifolia and South American species (n = 4) is either n = 4 or 5 based on MPR (not shown; see also Cebab et al., 1998b). Starting with n = 5 [an earlier suggestion of Stebbins et al. (1953)], we first have to assume a diploid chromosome number change, leading to n = 4 (hypothetical ancestor of H. angustifolia and the South American species). Next we assume translocation of 18S–25S rDNA from the short arm of chromosome 4 to the short arm of chromosome 2, resulting in the karyotype of H. angustifolia.

Among all South American species, the H. sessiliflora group in the northern Andes has the karyotype most similar to H. angustifolia (Weiss-Schneeweiss et al., unpubl.; Fig. 7) with the 18S–25S rDNA locus on chromosome 3 located distally. It differs only in the position of the 18S–25S rDNA locus on chromosome 2. The karyotype of the H. sessiliflora group could have originated via two alternative pathways (Fig. 7): (1) starting from the karyotype of the hypothetical ancestor with n = 4, translocation of 18S–25S rDNA from the short arm of chromosome 4 to the long arm of chromosome 2; and (2) starting from the karyotype of H. angustifolia, translocation of 18S–25S rDNA from the short arm of chromosome 2 to the long arm of chromosome 2. The second pathway is associated with a direct ancestor–progenitor relationship between H. angustifolia and the South American group, whereas the first pathway assumes a sister group relationship between the two.

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