

# Pleistocene refugia and polytopic replacement of diploids by tetraploids in the Patagonian and Subantarctic plant *Hypochaeris incana* (Asteraceae, Cichorieae)

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## Abstract

We report the phylogeographic pattern of the Patagonian and Subantarctic plant *Hypochaeris incana* endemic to southeastern South America. We applied amplified fragment length polymorphism (AFLP) and chloroplast DNA (cpDNA) analysis to 28 and 32 populations, respectively, throughout its distributional range and assessed ploidy levels using flow cytometry. While cpDNA data suggest repeated or simultaneous parallel colonization of Patagonia and Tierra del Fuego by several haplotypes and/or hybridization, AFLPs reveal three clusters corresponding to geographic regions. The central and northern Patagonian clusters (~38–51° S), which are closer to the outgroup, contain mainly tetraploid, isolated and highly differentiated populations with low genetic diversity. To the contrary, the southern Patagonian and Fuegian cluster (~51–55° S) contains mainly diploid populations with high genetic diversity and connected by high levels of gene flow. The data suggest that *H. incana* originated at the diploid level in central or northern Patagonia, from where it migrated south. All three areas, northern, central and southern, have similar levels of rare and private AFLP bands, suggesting that all three served as refugia for *H. incana* during glacial times. In southern Patagonia and Tierra del Fuego, the species seems to have expanded its populational system in postglacial times, when the climate became warmer and more humid. In central and northern Patagonia, the populations seem to have become restricted to favourable sites with increasing temperature and decreasing moisture and there was a parallel replacement of diploids by tetraploids in local populations.

**Keywords:** amplified fragment length polymorphism, chloroplast intergenic spacer regions, flow cytometry, phylogeography, South America, Tierra del Fuego

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## Introduction

Ice age palaeogeography of southern South America is rather well understood, but much less its impact on the resident biota. The history of glaciation in Andean

southern South America started as early as at the Miocene–Pliocene boundary (Rabassa 2008). Widespread ice accumulation took place in the southern Andes during Pliocene and Pleistocene glacial periods. In southernmost Patagonia, even as early as the Middle Pliocene, glaciers expanded from the ice caps close to the extent of the outlet glaciers of the maximum Pleistocene expansion [Greatest Patagonian Glaciation at

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c. 1.1–0.9 Ma BP; Rabassa (2008)]. During the Late Pliocene and Pleistocene, a series of several glacial advances and retreats characterized the Patagonian landscape. During the Early Pleistocene, a single, continuous mountain ice sheet was for the first time fully developed. It extended almost 2500 km, at least between 36° and 56° S, covering almost completely the Patagonian Andean ranges and extending over the piedmont areas to the east (and to the present submarine platform south of the Río Gallegos) and to sea level on the Pacific side (Rabassa 2008). The Greatest Patagonian Glaciation represents the maximum expansion of the ice in extra-Andean Patagonia (Singer *et al.* 2004). Subsequent Pleistocene glaciations also formed a continuous ice sheet, but were less extensive. Glacial advances of the Last Glaciation started at c. 85 ka BP and the last glacial maximum was attained at c. 18–20 ka BP (Rabassa 2008).

South of 40° S, prevailing westerlies resulting in intense winds throughout the year and a strong west to east precipitation gradient generated by the rain shadow effect of the Andes are the main factors influencing plant distributions today. Two types of present vegetational cover developed in the southern continental part and Tierra del Fuego island of South America comprise the phytogeographic provinces 'Patagónica' and 'Subantártica' (Cabrera 1971). The dominant Patagonian shrub steppe extends over the Patagonian plateau, which receives as little as 100–270 mm precipitation annually. In the more humid westernmost parts, Patagonian grass steppe and Subantarctic forests extend in a narrow strip along the Andes to the southernmost part of South America. No continuous pollen records exist from the Patagonian region that expand back considerably in time (Mancini *et al.* 2008). Similarly, there are very scarce palynological data older than Latest Pleistocene for Tierra del Fuego (Borromei & Quattrocchio 2008), thus the biotic response of Patagonian and Fuegian organisms to Pleistocene climatic fluctuations is not well understood.

Polyploidy is an important route for diversification in plants and knowing more about the origins of plants

containing polyploid lineages would contribute to the field of speciation. Despite considerable efforts in polyploidy research, the occurrence and distribution of autopolyploids in relation to their diploid progenitors have seldom been addressed. For example, questions regarding the establishment and persistence of new polyploids include to what extent are new polyploids adapted to novel ecological niches (Ramsey & Schemske 1998) and how do newly formed polyploids spread to colonize habitats not occupied by their diploid progenitors (Thompson & Lumaret 1992).

*Hypochaeris incana* (Hook. & Arn.) Macloskie (Cichorieae, Asteraceae) is a typical component of the Patagonian province and the edge of the Subantarctic province of southern South America. This rosulate herb with conspicuous white flower heads crowned by dark purple styles (Fig. 1) is one of ~45 species of *Hypochaeris* in South America (nested within the *Tenuifolia* group; Tremetsberger *et al.* 2006) and occupies a widespread area from ~38° S to the end of the American continent (Tierra del Fuego, ~55° S), along the eastern side of the Andean mountain range growing from sea level to 2000 m in Argentina and southernmost Chile. Diploid and tetraploid cytotypes have been described in *H. incana* (Moore 1981; Weiss-Schneeweiss *et al.* 2007). It is a common species with in general large population sizes (mostly between 500 and several thousand individuals per population). The low fecundity in the field (mean fruit/flower ratio = 0.25; range = 0.01–0.56; M.Á. Ortiz, M. Talaveia & S. Talaveia, unpublished data) is typical of self-incompatible species with few inter-compatible individuals and found in the majority of self-incompatible South American *Hypochaeris* species. Radiation of the genus *Hypochaeris* in South America is supposed to have started in the Pleistocene (Tremetsberger *et al.* 2005), thus range expansions/contractions and speciation must have been triggered or at least affected by Pleistocene climatic fluctuations.

The aim of this study was to investigate the origin and phylogeographic history shaped by Pleistocene

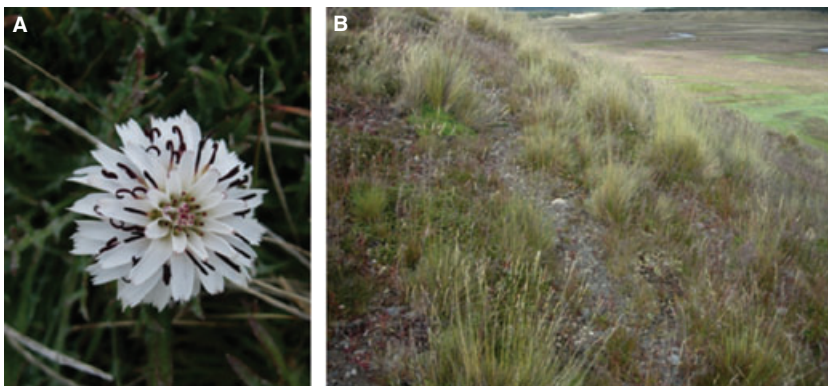


Fig. 1 *Hypochaeris incana* and its habitat in Tierra del Fuego (A, TF-2 and B, TF-6). Photographs by E. Urtubey.

climatic fluctuations of the Patagonian and Subantarctic plant *H. incana* as well as to elucidate the importance of polyploidy in its history. The specific questions to be answered are: (i) Did *H. incana* originate in the northern, central or southern part of its distributional range? Two species, *Hypochaeris hookeri* and *Hypochaeris tenuifolia*, are most closely related with *H. incana* (Tremetsberger *et al.* 2006) and were used as outgroup to answer this question. *Hypochaeris hookeri* is a steppe species, which occurs along the eastern side of the Andes, but with a more northward extension than *H. incana*. The two species co-occur in the Argentinean provinces Neuquén, Río Negro, Chubut and Santa Cruz, but *H. hookeri* does not reach Tierra del Fuego. *Hypochaeris tenuifolia* is a mountain species, which grows at higher altitude in the Andes from 34° to 40° S. (ii) Are polyploids widespread in *H. incana*? And if yes, where are they distributed? (iii) Where did *H. incana* outlive Pleistocene glaciations, in its entire distributional range or in parts of it? (iv) What is the actual genetic diversity in populations? We employed nuclear and chloroplast markers [amplified fragment length polymorphisms (AFLPs) and chloroplast DNA (cpDNA) intergenic spacer sequences] that are supposed to be selectively neutral in order to answer the question of the place of origin, evaluate phylogeographic hypotheses and estimate population diversity. For determination of ploidy levels, we used flow cytometry.

## Materials and methods

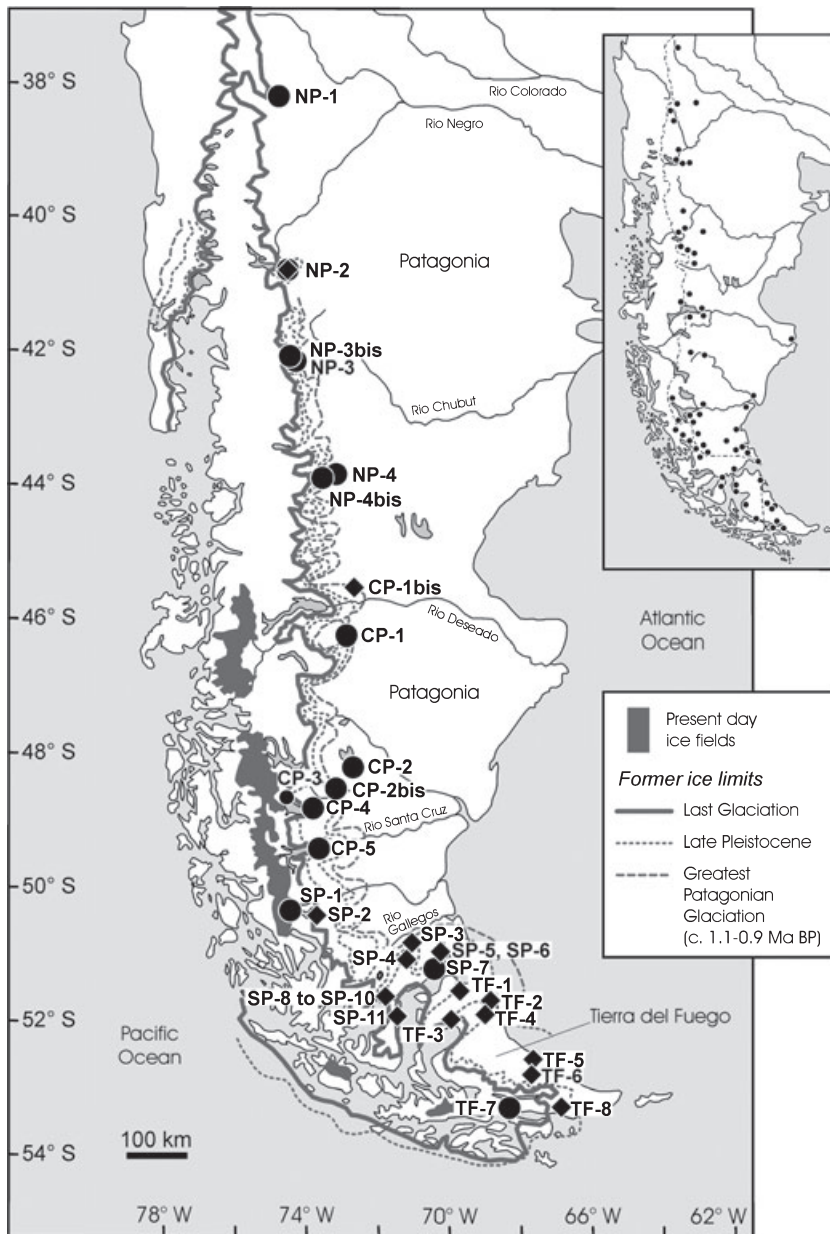
### Populational sampling

In the field, we placed leaves of 20–30 individuals per population in silica-gel from throughout the geographical range of *Hypochaeris incana* (Table S1, Fig. 2). Sampled individuals were separated by 1 m or more depending on the population size. Population sizes were estimated in three categories (S, small population with <200 plants; M, medium-sized population with ~500–1000 plants; and L, large population with more than 1000 plants). Vouchers of each population sampled are kept at the herbaria CONC, LP, SEV and/or WU. The total distribution of the species was inferred from herbarium collections at BM, CONC, LP, NY, OS, P, SEV, UC and WU.

### AFLP fingerprinting

We scored 580 individuals from 28 populations of *H. incana* for three AFLP primer combinations. Nineteen individuals (i.e. ~3%) were replicated from the extraction and used to calculate the error rate after scoring as the ratio between observed number of phenotypic

differences and total number of phenotypic comparisons between replicates (Bonin *et al.* 2004). Two individuals of *Hypochaeris hookeri* (Argentina, Prov. Río Negro, Stuessy *et al.* 18044) and *Hypochaeris tenuifolia* (Chile, Región VIII, Stuessy *et al.* 15555) each were used as outgroup. Genomic DNA was extracted from silica-gel dried leaf material following the CTAB method (Doyle & Doyle 1987) with minor modifications (Tremetsberger *et al.* 2003b). The AFLP protocol followed Vos *et al.* (1995) with modifications. Genomic DNA was digested with two restriction endonucleases *EcoRI* and *MseI* and ligated to double-stranded *EcoRI* and *MseI* adapters in one step at 37 °C for 2 h. For each sample, we combined 1.1 µL 10× T4 DNA ligase buffer (Promega), 1.1 µL 0.5 M NaCl, 0.55 µL BSA (1 mg/mL; New England Biolabs), 1 µL 50 µM *MseI* adaptors (genXpress), 1 µL 5 µM *EcoRI* adaptors (genXpress), 1 U *MseI* restriction endonuclease (New England Biolabs), 5 U *EcoRI* restriction endonuclease (New England Biolabs), 1 unit T4 DNA ligase (Promega), ~0.5 µg DNA, and added up with water to a total volume of 11 µL. Ligated DNA fragments were diluted 10-fold with TE<sub>0.1</sub> buffer. Preselective and selective polymerase chain reaction (PCR) protocols following Vos *et al.* (1995) were performed in a thermal cycler (GeneAmp PCR System 9700; PE Applied Biosystems). For preselective amplification, we combined 1.14 µL 10× RedTaq PCR reaction buffer (Sigma), 0.2 U RedTaq DNA polymerase (Sigma), 0.22 µL dNTPs (10 mM; Applied Biosystems), 0.58 µL preselective primer pairs [*EcoRI*-A and *MseI*-C, each 5 µM; genXpress], 2 µL diluted restriction–ligation product, and added up with water to a total volume of 10 µL. The preselective PCR products were diluted 10-fold with TE<sub>0.1</sub> buffer. The same selective primer combinations already used in other species of the *Tenuifolia* group (Tremetsberger *et al.* 2003a, b; Muellner *et al.* 2005) were applied to *H. incana* [*EcoRI*(Fam)-ACT/*MseI*-CAG, *EcoRI*(Vic)-ACG/*MseI*-CTC and *EcoRI*(Ned)-AGC/*MseI*-CAG]. For selective amplification, we combined 1 µL 10× RedTaq PCR reaction buffer (Sigma), 0.2 U RedTaq DNA polymerase (Sigma), 0.22 µL dNTPs (10 mM; Applied Biosystems), 0.54 µL *MseI*-primer (5 µM; genXpress), 0.54 µL *EcoRI*-primer (1 µM; Applied Biosystems), 2 µL diluted preselective amplification product, and added up with water to a total volume of 10 µL. The selective PCR products were purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences) applied to a MultiScreen-HV 96-Well Plate (Millipore) in three steps of 200 µL each and packed at 600 g (1, 1 and 5 min respectively). The same rotation was used for centrifugation of the samples (5 µL of each selective PCR product), again for 5 min. Three microlitres of the eluate were combined with 10 µL formamide and 0.1 µL GeneScan 500 ROX (Applied Biosystems) and



**Fig. 2** Sampling localities of *Hypochaeris incana* for the AFLP and cpDNA study in Argentina and Chile in relation to former ice limits [redrawn from Singer *et al.* (2004)]. Note that the coastline was extended during glacial times due to lowering of the sea level providing additional refugial areas for Patagonian steppe vegetation in the east of the continent [not shown; see Coronato *et al.* (1999)]. Rhombi represent diploids and large dots tetraploids (the small dot is for population CP-3 of unknown ploidy level). The small map shows the total distributional range of *H. incana*.

run on a capillary sequencer (3130xl Genetic Analyzer; Applied Biosystems). Raw data were aligned with the internal size standard using ABI PRISM GeneScan ver. 3.71 (Applied Biosystems). Presence/absence of bands in all individuals and replicates was scored with Genographer ver. 1.6.0 (Benham 2001; available from [http://www.umanitoba.ca/afs/plant\\_science/psgen/db/doc/genographer/](http://www.umanitoba.ca/afs/plant_science/psgen/db/doc/genographer/)) after normalizing on total signal in one file. Only bands present in *H. incana* were scored in the outgroup. The number of different AFLP phenotypes present in a population was counted with Arlequin ver. 3.1 (Excoffier *et al.* 2005) and only eight different AFLP phenotypes (limited by the minimum number of different AFLP phenotypes found in a popu-

lation) were kept in each population for all further statistical analysis, for which we employed all scored bands in the range of 70–500 bp.

#### *Among-population analysis of AFLPs*

We used distance and parsimony methods to determine population clusters in *H. incana* and their relationship to the outgroup (used to infer the place of origin of *H. incana*). Phylogenetic networks should be preferred over phylogenetic trees, especially when reticulate events are to be expected as is the case at the population level (Huson & Bryant 2006). Split decomposition (Bandelt & Dress 1992) was used to show relationships



among populations based on average genetic distances. Genetic distances were obtained as [1—Dice (1945) similarity] for all pairs of individuals with FAMD ver. 1.108 (Schlüter & Harris 2006). Genetic distance values of individuals belonging to two different populations were averaged to obtain the average genetic distance among individuals between populations. The 'Neighbor Net' produced with SPLITTREE ver. 4.8 (Huson & Bryant 2006) visualizes potentially conflicting signals in the data, such as caused by methodological artefacts, homoplasy or hybridization. Maximum parsimony analysis was carried out as an alternative method to estimate common ancestry of populations. A heuristic search was carried out with PAUP\* ver. 4.0b10 (Swofford 2003) with no more than 10 trees of score  $\geq 10$  retained in each of 1000 random-addition-sequence replicates. Among-population differentiation [analysis of molecular variance (AMOVA)-derived  $F_{ST}$ ] was quantified with Arlequin ver. 3.1 (Excoffier *et al.* 2005).

#### *Divergence of populations and clusters (AFLPs)*

Population structure in conjunction with estimates of divergence was used to interpret population history (Bonin *et al.* 2007). Divergence of populations resp. clusters was estimated via the occurrence of rare bands and private bands, i.e. those bands confined to only one population or cluster. The Rarity Index or DW (frequency-down-weighted marker values) was first applied by Schönswetter & Tribsch (2005) for AFLP data, but is equivalent to range-down-weighted species values in historical biogeographical research (Crisp *et al.* 2001). It was calculated with the R-script AFLPdat (Ehrich 2006; last modified 23 January 2008) in R ver. 2.6.0 (© The R Foundation for Statistical Computing; available from <http://www.r-project.org/>). For each individual, each AFLP band was divided by the total number of occurrences of this band in the data set. These relative values were then summed to the rarity index for this particular individual. Population values were estimated as the average of the individual values. The number of private bands in each population resp. cluster was counted using FAMD ver. 1.108 (Schlüter & Harris 2006). Because the indices of rare and private bands are dependent on the geographic proximity of sampled populations, we calculated these indices using only four populations in each cluster, thus strongly reducing the sampling of populations in the southern cluster, for which we have a very dense populational sampling.

#### *Within-population diversity (AFLPs)*

Population diversity was estimated to interpret the recent status of populations. The percentage of polymorphic

bands was obtained by dividing the number of polymorphic bands by the total number of bands in the data set. Genetic distances were obtained as [1—Dice (1945) similarity] for all pairs of the eight randomly selected AFLP phenotypes within a population with FAMD ver. 1.108. These values were averaged for populations to obtain the average genetic distance among individuals within populations. For correlation of these genetic diversity estimates with population size, we used Spearman's rank correlation implemented in SPSS ver. 15.0 (© SPSS Inc.).

#### *Chloroplast sequences*

Four cpDNA intergenic spacer regions (*psbA-trnH*, *ndhF-rpl32*, *rpl32-trnL* and *3'rps16-5'trnK*) were amplified for two to three individuals from 32 populations of *H. incana* using primers of Sang *et al.* (1997; *psbA-trnH*) and Shaw *et al.* (2007; other three regions). As outgroup, we included individuals of *H. hookeri* and *H. tenuifolia* (see Table S1). The PCR mix for amplification contained 14.4  $\mu$ L ReddyMix™ PCR Master Mix (ABgene), 0.9  $\mu$ L 0.4% bovine serum albumin, 0.9  $\mu$ L forward and reverse primer each (10 pmol/ $\mu$ L) and 0.9  $\mu$ L template DNA. Amplifications were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems) with the following conditions (those for *psbA-trnH* in parentheses): 5 min at 80 °C followed by 36 cycles each of 30 s denaturation at 95 °C (94 °C), 30 s annealing at 50 °C (52 °C) and 4 min (1 min) extension at 65 °C (72 °C), followed by a final elongation phase of 5 min (9 min) at 65 °C (72 °C). PCR products were purified using 0.5  $\mu$ L Exonuclease I, *Escherichia coli*, and 1  $\mu$ L FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) for 45 min at 37 °C followed by enzyme inactivation for 15 min at 85 °C. Cycle sequencing was performed for the forward and the reverse strand with 0.7  $\mu$ L BigDye® Terminator v3.1 Ready Reaction Mix (Applied Biosystems), 1  $\mu$ L forward or reverse primer (3.2 pmol/ $\mu$ L), 1.5  $\mu$ L 5 $\times$  Sequencing Buffer (Applied Biosystems) and 6.8  $\mu$ L PCR product with the following conditions: 1 min at 96 °C followed by 35 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. The samples were run on a capillary sequencer (3730 DNA Analyzer; Applied Biosystems). Sequences were deposited in the EMBL Database (Stoesser *et al.* 2003; see Table S1).

#### *Analysis of chloroplast sequences*

Sequences were assembled using SeqMan II (DNA-STAR) and manually aligned using BIOEDIT ver. 7.0.5.3 (Hall 1999). For all subsequent analyses, the four chloroplast sequences were concatenated for each individual. Insertion/deletions (indels) were recoded using Simmons

& Ochoterena's (2000) simple coding method implemented in SEQSTATE ver. 1.32 (Müller 2005). Presence of a gap ('1' according to the simple coding method) was manually recoded as 'G' and absence of a gap (0) was recoded as 'A'. Length variation at a mononucleotide repeat (A/T) in the *rps16-trnK* sequence was not recoded. The coded indels were included in the subsequent TCS and Arlequin analyses and the original gaps were treated as missing data. A parsimony network was constructed using TCS ver. 1.21 (Clement *et al.* 2000) with a 95% connection limit. AMOVA was used for hierarchical analysis of population structure using Arlequin ver. 3.1 (Excoffier *et al.* 2005). For comparison of haplotype diversities within populations of *H. incana*, we estimated nucleotide diversity (Nei 1987) with Arlequin ver. 3.1. To assess the occurrence of sudden population expansion in regional clusters of *H. incana*, we compared mismatch distributions of pairwise differences among haplotypes with the expected distributions under a model of sudden population expansion (Rogers 1995; Schneider & Excoffier 1999). The validity of the expansion model was tested using a parametric bootstrap approach (500 replicates) as implemented in Arlequin ver. 3.1. Statistical significance of population expansion was further assessed using Fu's (1997)  $F_S$ -statistics in Arlequin ver. 3.1, whereby large negative values of the  $F_S$ -statistics indicate population expansion. The  $P$ -value of the  $F_S$ -statistic was obtained as the proportion of 1000 random  $F_S$ -statistics less or equal to the observation.

#### Flow cytometry

We used Otto *et al.*'s (1981) buffer with propidium iodide to measure ploidy levels from fresh or silica-gel dried leaves of individual plants with a CyFlow<sup>®</sup> ML flow cytometer (Partec) equipped with a green laser (Cobolt Samba; Cobols AB) following the chopping method of Galbraith *et al.* (1983). Fresh leaves of *Pisum sativum* 'Kleine Rheinländerin' (4.42 pg/1C; Greilhuber & Ebert 1994) or *Solanum pseudocapsicum* (1.295 pg/1C; Tensch *et al.* in press) were used for internal or external standardization (the latter was used when dry material yielded only very weak peaks). Flow cytometry and chromosome counts (Baeza *et al.* 2007a, b; Weiss-Schneeweiss *et al.* 2007) are available jointly in three populations (NP-2, SP-5 and TF-1; see Table 3).

## Results

#### AFLP analysis

Scoring of three AFLP primer combinations in 580 individuals of *Hypochaeris incana* resulted in a total of 258 bands, of which 28 (11%) were monomorphic.

The total number of different scorings was 41 of 4921 phenotypic comparisons, thus the error rate after scoring is 0.83% (range for individual phenotypic comparisons = 0–4.25%). Thirty-three bands differed in one phenotypic comparison and four bands differed in two comparisons.

#### Genetic structure and outgroup relationships (AFLPs)

Three clusters were recognized in the Neighbor Net (Fig. 3; a very similar network was also obtained when all scored individuals were subjected to the analysis instead of eight different AFLP phenotypes per population). The first cluster contains northern Patagonian populations from the Argentinean provinces Neuquén, Río Negro and Chubut (NP-1 to NP-4; referred to as northern cluster in the following). The second cluster contains central Patagonian populations from the Argentinean province Santa Cruz (CP-1 to CP-5; referred to as central cluster) and the third cluster contains southern Patagonian and Fuegian populations from the Argentinean provinces Santa Cruz and Tierra del Fuego and the Chilean Región XII (SP-1 to SP-11

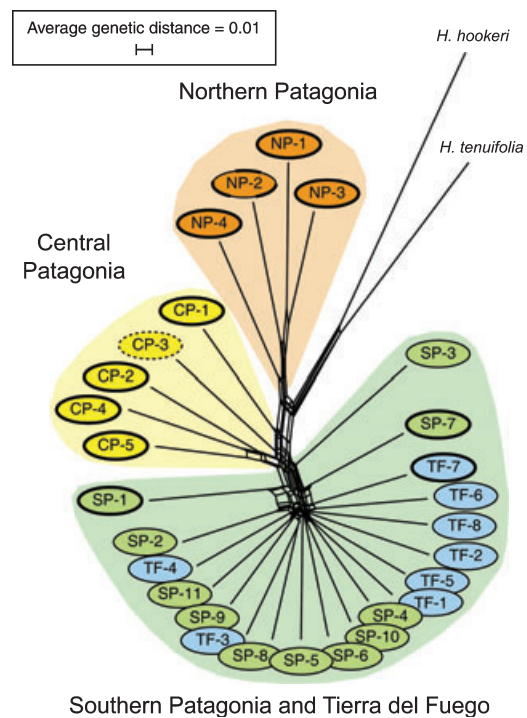


Fig. 3 Neighbor Net showing relationships among sampled populations of *Hypochaeris incana* and the two outgroup species, *Hypochaeris hookeri* and *Hypochaeris tenuifolia*. Thickness of lines of the ellipses refers to ploidy level: thin line, diploid; thick line, tetraploid; and dotted line, unknown. The three distinct clusters northern Patagonia, central Patagonia and southern Patagonia and Tierra del Fuego are seen.

and TF-1 to TF-8; referred to as southern cluster). The central cluster is in intermediate position between the northern and the southern clusters. Maximum parsimony analysis of AFLP data (Fig. S1) revealed the same groups and relationships among them as the Neighbor Net analysis. In both distance and parsimony analyses, the root represented by *Hypochaeris hookeri* and *Hypochaeris tenuifolia* attaches to the base of the northern and central clusters with populations NP-4 and CP-1 being closest to the root. Hierarchical analysis of molecular variance carried out by subjecting four populations in each of the three regional clusters to the analysis (the same as those selected for calculation of Rarity Index and private bands; see Table 1) attributes a proportion of 0.48 (95% CI = 0.42–0.54) to variation among populations across the entire study area ( $F_{ST}$ ), 0.25 (95% CI = 0.18–0.31) to variation among the three clusters ( $F_{CT}$ ), and 0.31 (95% CI = 0.27–0.36) to variation among populations within these clusters ( $F_{SC}$ ). When comparing among-population differentiation within clusters, the highest level is found in the northern cluster, an intermediate level in the central cluster and the lowest level in the southern cluster (Table 1).

#### Divergence of populations and clusters (AFLPs)

The Rarity Index shows similar levels of rare fragments in all three clusters (means = 1.7–1.8; range for individual populations = 1.4–2.2; Table 1, Fig. 4A). Whereas populations of the northern and central clusters have 1.8 private bands on average, populations of the southern cluster have 3.0 private bands on average (Table 1, Fig. 4A). When calculated for clusters instead of populations (again using the same four populations in each cluster as for population level analysis), the northern cluster has 28 private bands (one fixed), the central cluster 25 and the southern cluster 29.

#### Within-population diversity (AFLPs)

The values for total number of bands, percentage of polymorphic bands and average genetic distance among individuals within populations increase from north to south (Table 1, Fig. 4B). For example, populations of the northern cluster have 14.2% polymorphic bands, populations of the central cluster 16.7% and populations of the southern cluster 20.1% (Table 1). Correlations between genetic diversity estimates and population size ( $n = 27$ ) are not significantly different from zero [ $r_S = -0.139$  (sig. = 0.490) for total number of bands,  $r_S = 0.026$  (sig. = 0.899) for percentage of polymorphic bands and  $r_S = 0.028$  (sig. = 0.891) for average genetic distance within populations].

#### Chloroplast haplotypes

Characteristics of the sequence alignments of the four chloroplast intergenic spacer regions *psbA-trnH*, *ndhF-rpl32*, *rpl32-trnL* and *3'rps16-5'trnK* are shown in Table S2. The network produced by tcs (Fig. 5) revealed three cpDNA lineages (A–C). Lineage A comprises only population NP-1. Lineage B comprises both populations of *H. hookeri*, six of seven individuals of *H. tenuifolia*, the remaining northern Patagonian populations NP-2 to NP-4bis, the central Patagonian populations CP-1, CP-1bis, CP-4 (two of three individuals) and CP-4bis and individuals SP-2A and TF-8A. Finally, lineage C comprises one individual of *H. tenuifolia*, the central Patagonian populations CP-2, CP-3, CP-4 (one of three individuals) and CP-5 and all southern Patagonian and Fuegian individuals except SP-2A and TF-8A. The haplotypes that are most closely related to haplotypes of the outgroup (separated by one mutational step) are from populations NP-3bis, CP-1 and CP-1bis (related to population 2 of *H. hookeri*) and from population NP-2 (related to population 1 of *H. tenuifolia*). In lineage B, haplotypes of populations CP-1 and CP-1bis are in a central position and in lineage C, haplotypes of populations CP-3, CP-4 and CP-5 are in a central position. Hierarchical analysis of molecular variance attributes a proportion of 0.67 (95% CI = 0.60–0.72) to variation among populations across the entire study area ( $F_{ST}$ ), 0.29 (95% CI = 0.20–0.36) to variation among the three clusters ( $F_{CT}$ ), and 0.54 (95% CI = 0.48–0.59) to variation among populations within these clusters ( $F_{SC}$ ). Genetic differentiation ( $F_{ST}$ ) among populations within each of the three AFLP clusters, within-population nucleotide diversities and Fu's (1997)  $F_S$ -statistics are shown in Table 2. The mismatch distributions (Fig. S2) and Fu's  $F_S$ -statistics both indicate rapid population expansion in southern Patagonia and Tierra del Fuego in contrast to northern and central Patagonia.

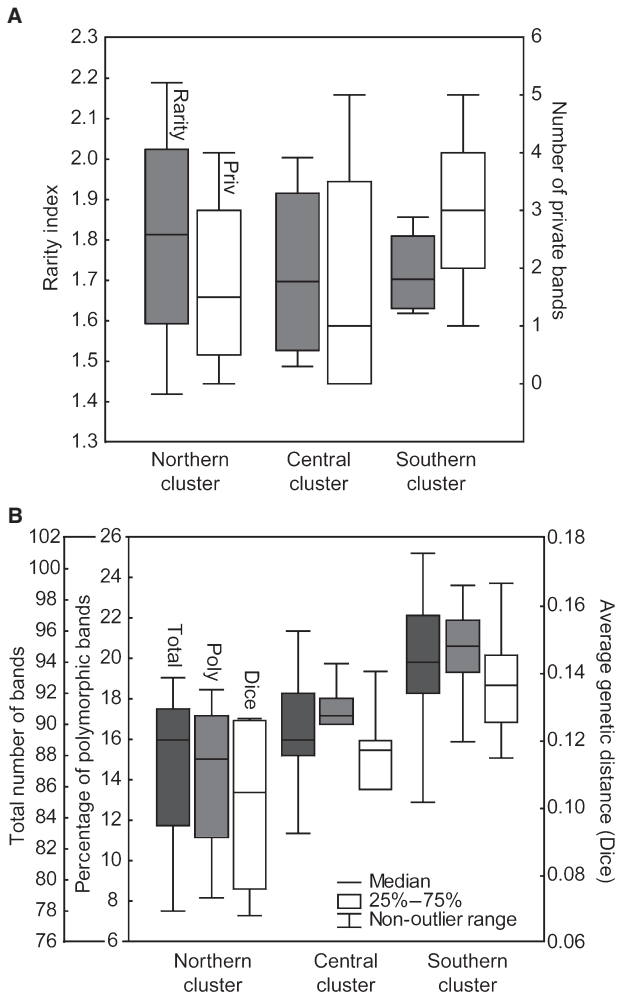
#### Distribution of ploidy levels

We found diploid as well as tetraploid populations occupying different distributional areas (Table 3, Fig. 2). Diploid populations are found in southern Patagonia and Tierra del Fuego and only once in central Patagonia (CP-1bis). Tetraploid populations are found in northern and central Patagonia and more rarely in southern Patagonia and Tierra del Fuego [SP-1, SP-7 and TF-7; also at Ea. José Menéndez close to Río Grande and Ea. Harberton east of Ushuaia in Tierra del Fuego (Moore 1981)]. One mixed diploid and tetraploid population (NP-2) has also been described (Weiss-Schneeweiss *et al.* 2007). In this population, three of the investigated seedlings were revealed to be diploid and

**Table 1** Population characteristics and among-population differentiation in three regional clusters estimated from AFLP analysis of 28 populations of *Hypochaeris incana* (diploids in regular; tetraploids in bold; populations with undetermined ploidy level in italics; \*population NP-2 contains both diploids and tetraploids). Among-population differentiation (AMOVA-derived  $F_{ST}$ ) was determined by subjecting four populations in each cluster to the analysis (the same as those selected for calculation of Rarity Index and private bands)

Population	No. AFLP phenotypes/no. individuals analysed	Average no. bands per AFLP phenotype	Rarity Index	No. private bands	Total no. bands	Percentage of polymorphic bands	Average genetic distance within populations
<b>Northern Patagonian cluster</b>							
<b>NP-1</b>	18/18	69.0	2.2	4	93	18.5	0.13
<b>NP-2*</b>	8/28	68.3	1.9	2	89	15.9	0.13
<b>NP-3</b>	15/20	68.0	1.8	1	89	14.2	0.08
<b>NP-4</b>	8/11	68.4	1.4	0	78	8.2	0.07
Mean (SD)	12/19	68.4 (0.4)	1.8 (0.3)	1.8 (1.7)	87.3 (6.4)	14.2 (4.4)	0.10 (0.03)
AMOVA-derived $F_{ST} = 0.49$ (95% CI = 0.41–0.55)							
<b>Central Patagonian cluster</b>							
<b>CP-1</b>	14/14	62.1	1.5	0	88	17.2	0.12
<b>CP-2</b>	12/21	69.4	1.8	0	96	19.7	0.14
<b>CP-3</b>	21/21	65.5	1.6	5	89	16.7	0.12
<b>CP-4</b>	14/16	67.0			83	11.6	0.07
<b>CP-5</b>	17/18	66.9	2.0	2	92	18.0	0.11
Mean (SD)	16/18	66.2 (2.7)	1.7 (0.2)	1.8 (2.4)	89.6 (4.8)	16.7 (3.1)	0.11 (0.03)
AMOVA-derived $F_{ST} = 0.29$ (95% CI = 0.24–0.34)							
<b>Southern Patagonian plus Tierra del Fuego cluster</b>							
<b>SP-1</b>	8/26	69.5			93	17.2	0.12
<b>SP-2</b>	14/19	63.9	1.8	3	91	17.6	0.12
<b>SP-3</b>	20/20	63.8			98	23.6	0.17
<b>SP-4</b>	23/29	63.3			84	15.9	0.12
<b>SP-5</b>	18/20	64.6	1.9	3	99	23.6	0.16
<b>SP-6</b>	22/23	65.0			97	21.9	0.15
<b>SP-7</b>	12/19	68.5			101	21.0	0.14
<b>SP-8</b>	29/29	64.5			95	19.7	0.13
<b>SP-9</b>	20/21	63.8			92	20.2	0.13
<b>SP-10</b>	20/21	65.4			96	21.5	0.14
<b>SP-11</b>	19/19	64.1			91	19.3	0.13
<b>TF-1</b>	21/24	63.1			92	20.6	0.14
<b>TF-2</b>	20/20	63.0			94	21.5	0.15
<b>TF-3</b>	23/23	65.0			99	21.9	0.15
<b>TF-4</b>	20/21	61.9			93	20.6	0.13
<b>TF-5</b>	17/17	62.8	1.6	1	92	19.7	0.13
<b>TF-6</b>	23/23	63.9			96	22.3	0.14
<b>TF-7</b>	13/19	67.1			85	13.3	0.09
<b>TF-8</b>	20/20	66.0	1.6	5	94	20.6	0.14
Mean (SD)	20/22	64.7 (2.0)	1.7 (0.1)	3.0 (1.6)	93.8 (4.4)	20.1 (2.6)	0.13 (0.02)
AMOVA-derived $F_{ST} = 0.10$ (95% CI = 0.04–0.16)							





**Fig. 4** Box plots showing (A) Rarity Index and number of private bands and (B) total number of bands, percentage of polymorphic bands and average genetic distance among individuals within populations for each of the three AFLP clusters. Similar levels of rare and private bands in all three clusters (A) as well as increasing genetic diversity estimates from the northern to the central to the southern cluster (B) are seen.

three seedlings were revealed to be tetraploid (*H. Weiss-Schneeweiss*, personal communication). The average number of bands per AFLP phenotype is dependent on the ploidy level. Diploid populations have a lower average number of bands per AFLP phenotype (mean = 64.1, SD = 0.7,  $n = 9$ ) than tetraploid populations [mean = 67.6, SD = 2.1,  $n = 10$ ; probability of a one-tailed Student's *t*-test (equal variance) = 0.0001].

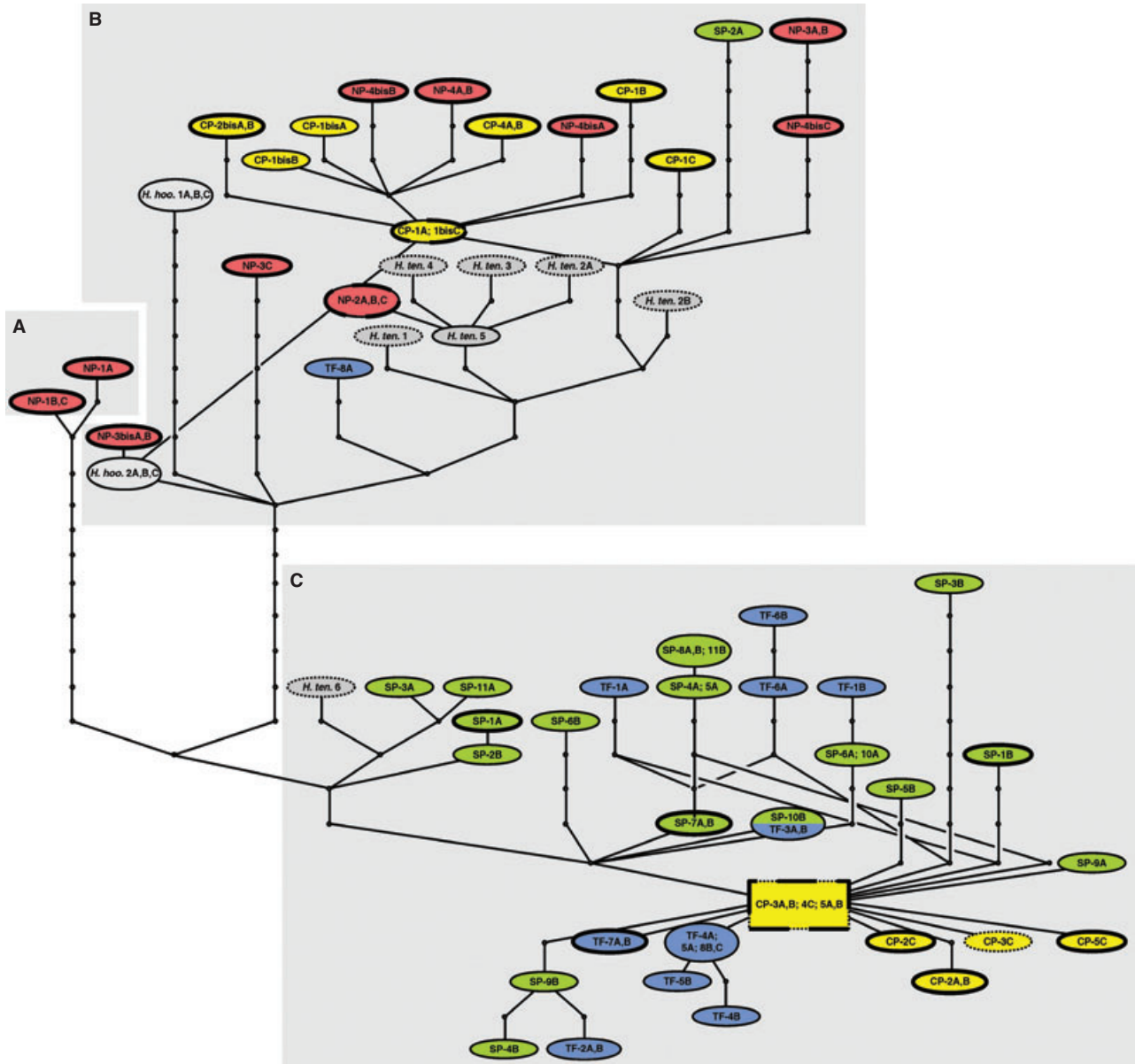
## Discussion

### Central or northern Patagonian origin of *H. incana*

The answer to the question of the place of origin of *Hypochoeris incana* is not straightforward. In AFLPs, the

placement of the root in the Neighbor Net and maximum parsimony analyses suggest that it originated in central or northern Patagonia. Out of these central and northern Patagonian populations, populations CP-1 and NP-4 located at 47° and 44° S have the closest affinities to the outgroup taxa. However, we suppose that the ancestor of *H. incana* was diploid and that tetraploidy is the derived condition, a supposition that somehow contradicts the hypothesis of a central or northern origin, because diploids are mainly found in the south and tetraploids mainly in the centre and north. As an alternative hypothesis for the origin of *H. incana*, we could imagine a more southern origin followed by subsequent northward migration accompanied by polyploidization. Nevertheless, the central and northern populations CP-1bis and NP-2 contain diploid individuals (mixed with tetraploids in NP-2). If we exclude polyhaploidy, i.e. the production of occasional haploid progeny by polyploids, which is supposed to be an unusual event (Ramsey & Schemske 2002), this fact is supportive of the first hypothesis, i.e. a central or northern origin, southward migration and only subsequent doubling of chromosome number in central and northern populations. The scattered occurrence of some tetraploid populations in the otherwise diploid southern cluster also indicates that polyploidization is readily accomplished in *H. incana*. Similarly, the belonging of the single fixed private band to the northern cluster and the high genetic divergence of central and northern populations also suggest that this area has been occupied by *H. incana* for a long time and has not been colonized recently.

Variation in cpDNA should relate to processes dating back longer in time than AFLPs, which in their majority are supposedly derived from the nuclear genome with a higher mutation rate than the chloroplast genome. Thus, cpDNA variation should indicate similarity/divergence probably stemming from before the last glaciation. The pattern of relationships revealed by AFLP analyses is largely upheld in the cpDNA network, though it is less clear. The grouping of haplotypes in three cpDNA lineages correlates somehow with their species affiliations (*H. incana*, *Hypochoeris hookeri* or *Hypochoeris tenuifolia*), the geographical origins or ploidy levels of populations. Northern Patagonian populations are found in lineages A and B and central Patagonian as well as southern Patagonian and Fuegian populations in lineages B and C. This finding could indicate that colonization of Patagonian and Subantarctic regions of South America by *H. incana* occurred repeatedly or simultaneously in parallel by several ancestral haplotypes. Haplotypes of the outgroup are found in two lineages [B (all haplotypes of *H. hookeri* and six of seven haplotypes of *H. tenuifolia*) and C (one haplotype of *H. tenuifolia*)], so that hybridization and



**Fig. 5** Parsimony network generated by TCS among 58 chloroplast haplotypes (*psbA-trnH*, *ndhF-rpl32*, *rpl32-trnL* and *3'rps16-5'trnK*) of *Hypochaeris incana* and outgroup (*Hypochaeris hookeri* and *Hypochaeris tenuifolia*) revealing three lineages (A–C) and partly reticulate relationships. Colour coding refers to geographical origin of populations: red, northern Patagonia; yellow, central Patagonia; green, southern Patagonia; and blue, Tierra del Fuego. Thickness of lines refers to ploidy level: thin line, diploid; thick line, tetraploid; and dotted line, unknown [for ploidy levels of outgroup populations see Weiss *et al.* (2003) and Weiss-Schneeweiss *et al.* (2007)]. Some individuals of populations CP-3, CP-4 and CP-5 have the highest outgroup weight and are inferred to be ancestral to at least some populations of the southern AFLP cluster.

chloroplast capture events between the closely related species can also not be ruled out.

Despite possible repeated and/or simultaneous parallel expansions of *H. incana* and hybridization/introgression events, higher nucleotide diversity of cpDNA haplotypes in central and northern Patagonia and the fact that haplotypes of the outgroup tie mainly to central and northern Patagonian haplotypes in the cpDNA

lineage B suggest an origin of *H. incana* in central or northern Patagonia, consistent with AFLP results. The central haplotypes of lineages B and C (haplotypes of populations CP-1 and CP-1bis for lineage B and haplotypes of populations CP-3, CP-4 and CP-5 for lineage C) suggest expansions from these central Patagonian populations. Polyploidization in populations CP-3, CP-4 and CP-5, therefore, must have occurred after colonization

**Table 2** Among-population differentiation and mean within-population diversity in the three AFLP clusters derived from cpDNA sequence analysis

	<i>n</i>	$F_{ST}$ (95% CI)	Mean within-population nucleotide diversity (SD)	Fu's $F_S$ ( <i>P</i> -value)
Northern Patagonian cluster	6	0.74 (0.63–0.82)	0.0010 (0.0014)	1.294 (0.744)
Central Patagonian cluster	7	0.70 (0.60–0.77)	0.0010 (0.0012)	–0.024 (0.550)
Southern Patagonian plus Tierra del Fuego cluster	19	0.25 (0.15–0.34)	0.0017 (0.0016)	–11.925 (0.000)

**Table 3** Ploidy levels of populations of *Hypochaeris incana* analysed for AFLPs and cpDNA sequences. Ploidy levels were determined by flow cytometry or retrieved from literature [\*Weiss-Schneeweiss *et al.* (2007); \*\*Baeza *et al.* (2007a); \*\*\*Baeza *et al.* (2007b)]. The diploid chromosome number is eight. The 1C value of diploid individuals measured from fresh material is ~2.9 pg (SD = 0.04). The ploidy level of population CP-3 was not measured

Population	Ploidy level (2 <i>n</i> )	No. ind. measured by flow cytometry	No. ind. used for chromosome counts
Northern Patagonia			
NP-1	4 <i>x</i>	5	0
NP-2	2 <i>x</i> , 4 <i>x</i>	1	6*
NP-3	4 <i>x</i>	6	0
NP-3bis	4 <i>x</i>	5	0
NP-4	4 <i>x</i>	8	0
NP-4bis	4 <i>x</i>	5	0
Central Patagonia			
CP-1	4 <i>x</i>	5	0
CP-1bis	2 <i>x</i>	7	0
CP-2	4 <i>x</i>	6	0
CP-2bis	4 <i>x</i>	2	0
CP-4	4 <i>x</i>	5	0
CP-5	4 <i>x</i>	5	0
Southern Patagonia			
SP-1	4 <i>x</i>	6	0
SP-2	2 <i>x</i>	6	0
SP-3	2 <i>x</i>	5	0
SP-4	2 <i>x</i>	5	0
SP-5	2 <i>x</i>	6	7**
SP-6	2 <i>x</i>	5	0
SP-7	4 <i>x</i>	7	0
SP-8	2 <i>x</i>	5	0
SP-9	2 <i>x</i>	5	0
SP-10	2 <i>x</i>	5	0
SP-11	2 <i>x</i>	0	3*
Tierra del Fuego			
TF-1	2 <i>x</i>	7	5***
TF-2	2 <i>x</i>	5	0
TF-3	2 <i>x</i>	5	0
TF-4	2 <i>x</i>	6	0
TF-5	2 <i>x</i>	6	0
TF-6	2 <i>x</i>	5	0
TF-7	4 <i>x</i>	4	0
TF-8	2 <i>x</i>	5	0

of southern Patagonia and Tierra del Fuego from these populations. Thus, the cpDNA network seems to corroborate the AFLP results in suggesting recent polyploidizations in parallel in different locations.

#### *Multiple, polytopic replacement of diploids by tetraploids in central Patagonia*

The AFLP and cpDNA data agree in suggesting that after *H. incana* had become established in the central and southern parts of its distributional area, diploid central Patagonian populations have become replaced *in situ* by tetraploid populations. The northern Patagonian populations could also be the result of multiple, polytopic replacements of diploids by tetraploids or of an expansion of tetraploid central Patagonian populations at a later stage. On an evolutionary timescale, the process of polytopic replacement of diploids by tetraploids can be thought of as a preferential survival of tetraploids over diploids in local populations after a stage of mixed cytotype composition. Thus, this study suggests multiple origins of polyploid populations. It challenges the traditional view that polyploids spread by extending the range of their diploid progenitors (Levin 2002; p. 113) and highlights polytopic replacement of diploids by tetraploids as an alternative mode of establishment. The most common mechanism of polyploid evolution is via unreduced gamete formation, which can be affected by environmental conditions, and such continuous sexual polyploidization may take place repeatedly in different locations (Thompson & Lumaret 1992). Factors that can contribute to polyploid establishment despite an initial minority cytotype mating disadvantage in local populations include a substantial competitive advantage of the polyploid, divergent ecological requirements of the two cytotypes, stochastic processes in small populations and assortative mating (Levin 2002; pp. 108–113).

#### *Persistence in Pleistocene refugia*

In phylogeographic analysis, the occurrence of genetically distinct groups probably represents lineages

descending from different ancestral populations suggesting survival in different refugia (Avice 2000; Ehrich *et al.* 2008). The presence of rare and private bands is characteristic of populations with a long *in situ* history, most probably going back to the last glaciation (Schönswetter & Tribsch 2005; Ehrich *et al.* 2008). Thus, similar levels of rare and private bands in each of the three AFLP clusters (~10% of all bands are private to one cluster respectively) indicate that all three can be considered sites that have been inhabited by *H. incana* for a similarly long time, most probably dating back to the last glaciation, although it is very difficult to infer a time frame for molecular markers lacking an explicit mutation model such as AFLPs (Bonin *et al.* 2007). Similarly, phylogeographic analyses and modelling of the palaeodistributions of *Hordeum* species in Patagonia have suggested that climatic conditions were sufficient for the species to survive Pleistocene cold cycles in Patagonia without significant geographic restrictions, even as far as south of 51° S in southernmost Patagonia and Tierra del Fuego (Jakob *et al.* 2009).

#### *Historical vs. recent status of populations in northern and central Patagonia*

Palaeoclimatic conditions in Patagonia are not well understood (Schäbitz 1999; Mancini *et al.* 2008), but it seems that the climate was cooler and moister than today during glacial periods most probably due to a more northern location of the west wind zone. The major part of Patagonia could have been a bogland dissected by outwash streams and lakes from glacial meltwaters during glacial periods (Auer 1960; Simpson Vuilleumier 1971). Pollen evidence suggests that at 40° S east of the Andes in a present-day *Nothofagus dombeyi*–*Austrocedrus chilensis* woodland, a herb–shrub steppe characterized the full-glacial environments (Markgraf & Bianchi 1999). Markgraf *et al.* (1995) suggest that steppe vegetation has occupied Patagonia east of the Andes throughout glacial–interglacial cycles. The response of Patagonian plants to glacial conditions, therefore, could have been in persistence and dominance shifts rather than in latitudinal range shifts.

Amplified fragment length polymorphism analysis of *H. incana* is in accordance with the hypothesis of cooler and moister glacial conditions in northern and central Patagonia than today. The isolated northern and central Patagonian populations seem to be remnants of a once more continuous distribution in this region, which might have existed during glacial times. Population sizes are not correlated with genetic diversity estimates in *H. incana*, possibly because populations are rather large in general, and cannot be used to explain differences in genetic diversity estimates between popula-

tions of the northern, central and southern clusters. Low genetic diversity within and high differentiation among northern and central Patagonian populations evidenced by AFLPs as well as cpDNA sequences could be explained by geographical isolation of these remnant populations and lack of inter-population gene flow allowing populations to diverge through genetic drift. Similarly, flat, multimodal mismatch distributions as well as nonsignificant values for Fu's (1997)  $F_S$ -statistics indicate no departures from population equilibrium. The fact that geographical provenances of populations correlate with the AFLP groups, but less so with the cpDNA lineages suggests homogenization of nuclear genomes through pollen-mediated gene flow among populations belonging to the same geographical areas after initial repeated and/or simultaneous parallel expansions of haplotypes. We hypothesize that these major episodes of pollen-mediated gene flow were confined to glacial times in northern and central Patagonia, when these regions offered more favourable environmental conditions for *H. incana*.

#### *Historical vs. recent status of populations in southern Patagonia and Tierra del Fuego*

Vast areas were exposed during glacial times due to lowering of the sea level in the east of Tierra del Fuego, thus offering refugial areas for steppe plants (Coronato *et al.* 1999). Pollen evidence supports the interpretation of a much colder and drier palaeoenvironmental setting than today at high latitudes indicative of steppe or tundra environments in eastern Tierra del Fuego during glacial times (Bujalesky *et al.* 1997; Borromei & Quattrocchio 2008). High genetic diversity within and low differentiation among southern Patagonian and Fuegian populations evidenced by AFLPs as well as cpDNA sequences, a rather unimodal mismatch distribution and a significantly negative value of Fu's (1997)  $F_S$ -statistic seems to be associated with rapid post-Pleistocene population growth, when the climate became warmer and more humid again, and high levels of gene flow. Populations seem to be rather continuously distributed across suitable habitat throughout the whole southern Patagonian and Fuegian area facilitating gene flow via pollen and seeds and preventing population divergence. The species possesses fruits with pappus, which are well suited for wind-dispersal in these windy regions, suggesting a high dispersal capability. Dispersal being not a limiting factor for *H. incana*, we can hypothesize rapid postglacial spread into newly available habitats. Placement of the haplotypes of individuals SP-2A and TF-8A in lineage B could represent remnants of an early expansion of central or northern haplotypes (i.e. before polyploidization of these central and northern individuals occurred).



## Conclusions

Combined analysis of AFLPs, cpDNA sequences and ploidy levels revealed a complex populational history of *Hypochoeris incana*. AFLP and cpDNA data seem to agree upon an origin of *H. incana* in central or northern Patagonia as well as multiple recent polyploidizations in local populations and polytopic replacement of diploids by tetraploids in central and possibly also northern Patagonia. The study demonstrates how combination of different markers helps in obtaining a more integrated notion of past and present population processes and highlights polytopic replacement of diploids by tetraploids in local plant populations as mode of polyploid establishment.

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The research groups around T.F.S. (University of Vienna, Austria) and S.T. (University of Seville, Spain) collaborate with E.U. (Instituto de Botánica Darwinion, Argentina) and C.M.B. (University of Concepción, Chile) in research on evolutionary aspects of the genus *Hypochaeris*, in the Old World (mainly Mediterranean region) as well as in the New World (South America), addressing questions of genetic diversity and reproductive biology.

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### Supporting information

Additional Supporting Information may be found in the online version of this article.

**Fig. S1** Maximum parsimony analysis of AFLP data revealing the same population groups as distance analysis (Neighbor Net).

**Fig. S2** Observed and expected mismatch distributions under a model of sudden population expansion in three regional

clusters of *Hypochaeris incana* based on the four chloroplast intergenic spacer regions.

**Table S1** Collection details of populations of *Hypochaeris incana* analysed

**Table S2** Characteristics of the sequence alignments of the four chloroplast intergenic spacer regions *psbA-trnH*, *ndhF-*

*rpl32*, *rpl32-trnL* and *3'rps16-5'trnK* in *Hypochaeris incana* and the outgroup (*Hypochaeris hookeri* and *Hypochaeris tenuifolia*)

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