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ORIGINAL ARTICLE

High population genetic substructure in *Hypochaeris leontodontoides* (Asteraceae), an endemic rupicolous species of the Atlas Mountains in NW Africa

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Abstract *Hypochaeris leontodontoides* is a rupicolous species endemic to the Atlas Mountains (Morocco), where it occurs in scattered populations. This study aims to understand the biogeographic structure of a high mountain species in a rather small area of NW Africa. We used Amplified Fragment Length Polymorphism (AFLP) to investigate the population structure and phylogeography of *H. leontodontoides* in 19 populations sampled from the entire species distribution range. Multivariate analyses including PCoA, UPGMA analysis, and Bayesian clustering were applied to infer the influence of past biogeographic events. The AFLP differentiation among the populations was high ($F_{ST} = 0.508$). A significant geographical pattern by mountain region was found. The different phylogeographical analyses revealed four main groups corresponding to four well-defined geographic regions: Middle Atlas, Eastern High Atlas, Central High Atlas, and Western High Atlas, and highlighted the Western High Atlas as the most divergent group. Our data also indicate two regions as refuges during the Pleistocene ice ages: the Middle Atlas and the northernmost area of the Western High Atlas.

Keywords AFLP · Asteraceae · Atlas Mountain range · High Mountain · nrITS · Pleistocene refuges · Quaternary climate changes

This research focuses on genetic structure of *Hypochaeris leontodontoides* (Asteraceae), M. A. Ortiz and R. Berjano contributed to data analysis and manuscript writing. F. J. Jiménez-López helped with field sampling molecular data analysis. A. Terrab and S. Talavera substantially contributed to the conception, manuscript writing and design of this work.

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Introduction

Phylogeographical research has been conducted on many organisms worldwide. However, the geographical coverage of such studies is unevenly distributed, with some areas having numerous accounts, whilst others remain largely unexplored. A notable regional contrast exists between much of Europe vs North Africa, with the latter remaining poorly studied. This imbalance is unfortunate because North Africa harbours numerous major "hotspots" of plant biodiversity and endemism (Médail and Quézel 1997), and is also one of the regions of the world that is likely to suffer marked negative effects with the advance of global warming, in particular a significant increase in aridity (IPCC 2001). Due to its climatic history and strikingly montane topography, phylogeographical surveys typically detect a much greater allelic richness and evolutionary divergence of populations in this region compared with those further north (Fineschi et al. 2002; Hampe et al. 2003; Petit et al. 2003), and they may trace range dynamics dating back well into the Tertiary (Lumaret et al. 2002; Caujapé-Castells and Jansen 2003; Magri et al. 2007). For North African taxa, detailed range-wide population surveys of exclusively Moroccan plant species are limited (but see El Mousadik and Petit 1996; Terrab et al. 2006, 2009) and since the fossil record of this region is also fragmentary (Elenga et al. 2000; Magri

and Parra 2002), the past vegetation dynamics in the area and their impacts on gene pools remain largely unknown.

The altiplano of the Atlas mountains of Morocco presents an extensive range that constitutes the biogeographical province known as Atlasica, which contains the highest mountains of North Africa, several of which exceed 4000 m, and which is subdivided into the Middle Atlas, High Atlas and Anti Atlas (Benabid and Fennane 1994; Deil and Galán de Mera 1996). The Atlas Mountains are situated at the Africa–Eurasia plate boundary, formed during the Mesozoic and Cenozoic by the uplift and deformations of Palaeozoic terrains (Hughes et al. 2011). The uppermost mountains comprise Palaeozoic granites and volcanic lavas such as basalt, andesite and rhyolite, whilst elsewhere the mountains are formed by uplifted and deformed Mesozoic carbonate rocks (Dresch 1941; Piqué 1994; Pouclet et al. 2007). The highest peaks are situated at the Western High Atlas and culminate in the Jbel Toubkal massif (4167 m).

In an ongoing study of the phylogeography and genetic structure of populations of species of the genus *Hypochaeris* L. (Tremetsberger et al. 2006, 2009; Ortiz et al. 2007, 2008; Terrab et al. 2009) we here focus on *H. leontodontoides* Ball, a rupicolous species that is endemic to the Atlasica region (NW Africa), where it occurs in isolated populations from 1800 to 3200 m. A molecular phylogenetic study using ITS and chloroplast (*rps16* intron) markers has shown that *H. leontodontoides* is the basal species of the sect. *Seriola* Benth and Hooker (Tremetsberger et al. 2005), and molecular clock estimations indicate that sect. *Seriola* has been separated from sect. *Hypochaeris* since the Miocene, about 6.9 Mya ago. Within sect. *Seriola*, *H. leontodontoides* separated from *H. laevigata* (L.) Ces. and al. in the Pliocene, about 3.2 Mya ago (Tremetsberger et al. 2013).

We used Amplified Fragment Length Polymorphism (AFLP) to investigate the phylogeographic structure of *H. leontodontoides* in order to understand the processes that have shaped its current spatial genetic pattern. Additionally, the nuclear ribosomal internal transcribed spacer (nrITS) was analysed in six populations to potentially further assess genetic differences among populations. Few phylogeographical studies of rupicolous species are known for NW Africa (but see Vargas et al. 1999), contrary to the diverse studies in Europe, especially in the Alps and Pyrenees (e.g. López-Pujol et al. 2001; Jiménez et al. 2002; Segarra-Moragues et al. 2007; Segarra-Moragues and Catalán 2008; Kropf et al. 2012; Winkler et al. 2012, 2013; Christe et al. 2014). Within the context of our AFLP parameters, we aimed at evaluating the influence of geomorphological structures and past climate changes on population differentiation across the whole range of the species. The palaeoclimatologic information available for the studied region was used to infer the history of past changes leading to the present-day distribution of the species. Specifically, we addressed the following questions: (1) Does the current genetic population structure of *H. leontodontoides* show any geographical pattern? (2) Which region appears as the most probable ancestral area of the species, and what are the possible colonisation routes that gave rise to its current distribution? (3) Is the current genetic structure of the species a result of isolation by distance? (4) How did Pleistocene events shape the distribution of this species? (5) Did any areas serve as ice-age refugia for populations of this species?

Materials and methods

Species

Hypochaeris leontodontoides [section *Seriola*] was described by Ball (1873), as a perennial rupicolous plant growing on calcareous, porphyry or granite rocks between 1800 and 3650 m in the Middle and High Atlas mountain regions (Morocco). The plant is woody at the base, with monocephal scapes, and entire and toothed leaves that are glabrous to hairy. Plants with hairy leaves were described as var. *villosa* by Maire (1926), but this feature is very variable and does not warrant taxonomic recognition (Talavera, pers. obs). Galland and Favarger (1985) described *H. leontodontoides* var. *glauca* from the Rif Mountains (N Morocco), and Galán de Mera et al. (1999) described *H. leontodontoides* var. *atlantica* from the Middle Atlas but judging from their descriptions, both are synonyms of *H. laevigata*. The chromosome number of *H. leontodontoides* is $2n = 12$ (Oberprieler and Vogt 2002). *H. leontodontoides* is an allogamous plant pollinated by small and medium-sized solitary bees (Talavera, pers. obs.), and greenhouse experiments (Ortiz et al. unpublished) have shown that it is a self-incompatible species.

Plant material and DNA extraction

We obtained plant material from 19 populations of *H. leontodontoides* (Table 1; Fig. 1a) from four mountain regions: Middle Atlas (five populations), Eastern High Atlas (five populations), Central High Atlas (three populations) and Western High Atlas (six populations). These areas effectively represent the entire distribution range of this species. For each locality we collected fresh leaf samples from 9–19 individuals (mean = 14), with a total of 265 individuals. Samples were dried and stored in silica gel until DNA extraction. Vouchers of all populations sampled were deposited in the Herbarium of the University of Seville (SEV, Spain).

Total genomic DNA was extracted from dry leaf using the unmodified Invisorb® Spin Plant Mini Kit protocol. Quality

Table 1 Localities and their details, and AFLP parameters, for the 19 populations of *H. leontodontoides* collected in the Atlas Mountains: elevation (m a.s.l.), geographical coordinates, number of individuals analysed (N), total number of fragments per population ($Frag_{tot}$), percentage of polymorphic fragments (% $Frag_{poly}$), private fragments ($Frag_{priv}$), average gene diversity (H_D), rarity index (DW), and genetic differentiation among populations (F_{ST}). Population that includes individual analysed by ITS marker are specified by +

Populations	Elevation (m)	Geographical coordinates	N	$Frag_{tot}$/%$Frag_{poly}$	$Frag_{priv}$	AFLP			ITS	
						H_D	DW	F_{ST}	ITS	GenBank accession no.
Middle Atlas								0.331*		
Pop.1. Jbel Bou-Iblan	1960	N 33°39′10″/W 04°08′41″	17	208/70	3	0.053 ± 0.02	28.479		+	KP226625
Pop.2. Ari-Hayane	2167	N 33°06′40″/W 05°09′11″	17	372/85	10	0.119 ± 0.03	78.208			
Pop.3. Tizi-Ali-ou-Mansour	2000	N 33°02′27″/W 05°13′39″	16	398/87	24	0.126 ± 0.03	92.937			
Pop.4. Col Zad 1	2050	N 32°58′46″/W 05°03′55″	12	325/88	12	0.105 ± 0.04	59.688			
Pop.5. Col Zad 2	2148	N 32°59′53″/W 05°04′25″	17	223/84	3	0.065 ± 0.02	29.377		+	KP226626
Mean	2065		16	305/83	10	0.094	57.738			
Eastern High Atlas								0.243*		
Pop.6. Between Midelt & Rich	2218	N 32°34′52″/W 04°34′25″	19	251/89	7	0.076 ± 0.02	37.271			
Pop.7. Jbel Ayachi (near Ait Ouden)	1905	N 32°34′24″/W 04°54′49″	13	215/78	5	0.066 ± 0.02	33.198			
Pop.8. Jbel Ayachi (Cirque de Jaffar)	1967	N 32°33′46″/W 04°55′01″	10	221/82	3	0.072 ± 0.09	29.693		+	KP226627
Pop.9. Near Tounfite	1941	N 32°26′20″/W 05°21′46″	9	210/87	6	0.079 ± 0.03	30.254			
Pop.10. Gorge Arhbalou	1935	N 32°21′46″/W 05°22′19″	15	266/94	9	0.082 ± 0.03	42.651			
Mean	1993		13	232/86	6	0.075	34.613			
Central High Atlas								0.225*		
Pop.11. Imi-n-Ouaqqa	1842	N 31°37′25″/W 06°44′59″	11	227/88	5	0.080 ± 0.03	32.282			
Pop.12. Tarbat-n-Tirsal	2155	N 31°36′23″/W 06°43′01″	15	271/87	8	0.080 ± 0.03	46.577			
Pop.13. Jbel Rat	2458	N 31°36′17″/W 06°41′09″	12	203/81	0	0.064 ± 0.03	23.813			
Mean	2151		13	234/85	4	0.075	34.224			
West High Atlas								0.318*		
Pop.14. Oukaimeden 1	2880	N 31°11′16″/W 07°51′12″	14	283/78	4	0.067 ± 0.02	43.224		+	KP226628
Pop.15. Oukaimeden 2	2763	N 31°12′30″/W 07°51′51″	20	396/84	22	0.105 ± 0.05	89.055		+	KP226629
Pop.16. E of Imlil	2340	N 31°08′49″/W 07°52′56″	11	191/64	1	0.050 ± 0.02	20.255			
Pop.17. W of Aremd	2250	N 31°07′36″/W 07°55′28″	12	220/74	3	0.065 ± 0.02	26.149		+	KP226630
Pop.18. S of Ait Daoud	2400	N 31°04′21″/W 08°23′25″	12	186/68	1	0.053 ± 0.02	20.200			
Pop.19. Jbel Erdouz	2620	N 31°03′37″/W 08°23′40″	13	168/66	1	0.051 ± 0.02	23.690			
Mean	2542		14	241/72	5	0.065	37.095			

* $p < 0.00001$

and quantity of extracted DNA were determined electrophoretically after SYBR green staining using a ladder with known amounts of DNA as standards (HyperLadder™, Bioline) and by using a NanoDrop UV–Vis spectrophotometer (Thermo®).

nrDNA ITS analyses

One randomly chosen individual per population from six populations (pops. 1, 5, 8, 14, 15 and 17) of *H. leontodontoides* was used in the nrDNA ITS analyses (Genbank No. KP226625 through KP226630; see Table 1). In the ITS analysis we also included the only sequence of *H. leontodontoides* available in GenBank (AJ627266.1), and two individuals of *H. achyrophorus* L. (sect. *Seriola*) were used as outgroup (GenBank No. KP226631 and KP226632). ITS of 18S–5.8S–28S nuclear ribosomal DNA was amplified using primers ITS4 and ITS5 published by White et al. (1990). The Polymerase Chain Reaction (PCR) was optimised with the following conditions for each sample: 25 µl reaction contained 12.6 µl H_2O, 2.5 µl of buffer 10×, 1.6 µl MgCl 50 mM, 2 µl dNTPs 10 mM, 1 µl BSA 1×, 1 µl

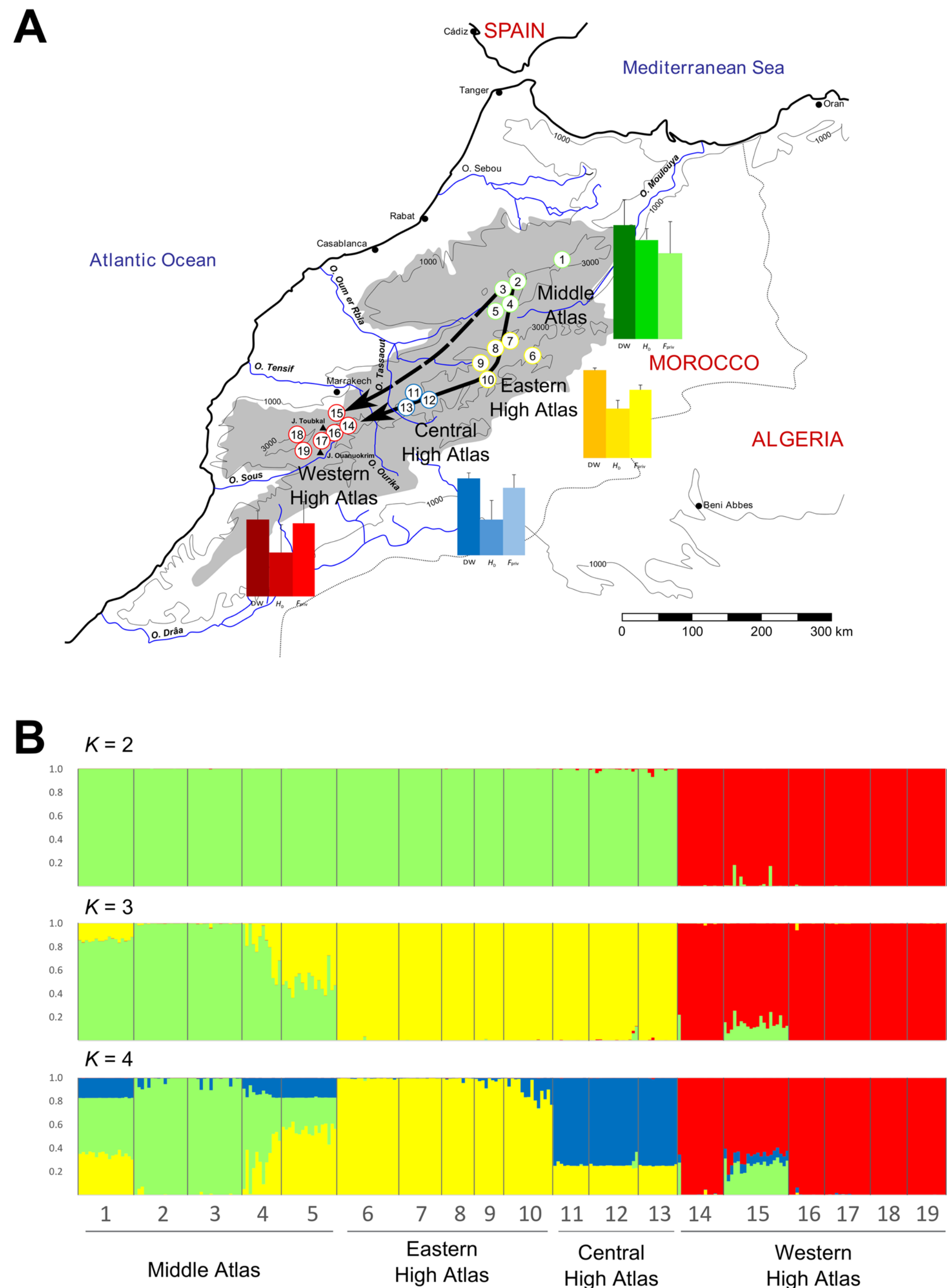

Fig. 1 **a** Localities of the 19 sampled populations of *H. leontodontoides* (for details of numbered *circles* see Table 1). *Grey* area indicates the Atlas Mountain range, in Morocco. Elevations shown at 1000 and 3000 m a. s. l. (Map modified from Deil and Galán de Mera 1996). The *bars* indicate the mean number ±SE of the private fragments (F_{priv}; min–max:4–10), the rare fragments index (DW; min–max:34–58) and the genetic diversity (H_D; min–max: 0.065–0.094). *Colour* coding of populations indicates results of Bayesian clustering at $K = 4$. **b** Population structure at individual level for Bayesian Analysis conducted with STRUCTURE with optimal value, $K = 2$, and suboptimal values $K = 3$ and $K = 4$

DMSO, 1 µl of each primer (forward and reverse), 0.3 µl of Taq DNA polymerase (Bioline) 5 and 2 µl template DNA (approx. 30–100 ng/µl). Amplification was performed in a thermal cycler (Applied Biosystems Thermal Cycler) with the following reaction conditions: 94 °C/5 min (one cycle); 94 °C/1 min, 49 °C/30 s, 72/1 min (30 cycles) and 72 °C/15 min (1 cycle); 4 °C at the end. The amplified fragments were checked with 1.5 % agarose gel and purified using Exosap-IT enzyme (Bioline). The purified fragments were sequenced on an ABI 3730xl sequencer in collaboration with STAB vida laboratory. The sequences were analysed using Geneious software to generate consensus sequences for each sample (Drummond et al. 2011). The alignment of the complete sequences of ITS1, 5.8S and ITS2 were analysed using the MEGA version 6 (Tamura et al. 2013) and Clustal IW software (Thompson et al. 2002). To select the model of nucleotide evolution that better suited to the data we used Modeltest 3.06 (Posada and Crandall 1998), according to the Akaike criterion. The Model used was the Kimura 2 parameter (*K*2). 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 support (BP) for each node was estimated with 500 bootstrap replicates with PAUP* ver. 4.0b10 (Swofford 2003).

AFLP analyses

The AFLP procedure followed established protocols (Vos et al. 1995). An initial screening of selective primers using 36 primer combinations with three selective nucleotides was performed on eight individuals of four populations (2 individuals per population). The six primer combinations selected for the selective PCR were *EcoRI* (Fam)-ATC/*MseI*-CTC, *EcoRI* (Fam)-ATC/*MseI*-CTGA, *EcoRI* (Vic)-ACG/*MseI* CAT, *EcoRI* (Vic)-AGG/*MseI* CTC, *EcoRI* (Ned)-AGC/*MseI*-CAG, and *EcoRI* (Ned)-ACC/*MseI*-CTA. We analysed the 265 collected samples and, additionally, 25 individuals were replicated in order to exclude non-reproducible bands and to calculate the error rate according to Bonin et al. (2004). The fluorescence labelled selective amplification products were separated by capillary gel electrophoresis at the ''Genomic Unit'' (Universidad Complutense, Madrid, Spain), on an automated sequencer (3730 DNA Analyser, PE Applied Biosystems, Foster City, CA, USA) with an internal size standard (GeneScan 500 LIZ, Applied Biosystems). Raw data were exported to GeneMarker 1.8 (SoftGenetics, PA, USA) for automatic scoring of fragments, after normalisation of the profiles. The peaks were considered present when they were stronger than a scoring fluorescence intensity threshold set at 50 relative fluorescent units. The minimum percent of allele peaks to the highest peak in the lane was set at 1 %, and the local region percent, that defines the peak detection threshold based upon the percentage of the highest peak in one locus, was set at 1 %. Amplified fragments from 100 to 500 base pairs were scored. The results of the scoring were exported as a presence/absence matrix.

The presence/absence matrix that originated with the six primer combinations was imported into PAUP* (v. 4.0b10; Sinauer Associates). Within-population genetic diversity was assessed for each population using the total number of AFLP fragments present ($Frag_{tot}$), the percentage of polymorphic fragments ($Frag_{poly}$), the number of private fragments ($Frag_{priv}$), and the average gene diversity (H_D). Additionally, the frequency down-weighed marker value (DW), also called Rarity Index (Schönswetter and Tribsch 2005), was calculated using the function DW of the AFLPdat sourcecode in R (Ehrich 2006). This function calculate the rarity index per population (rarity 2) by making a table with the presence of markers by population; then each marker is divided by the total number of occurrences of this marker in the dataset.

We assessed patterns and levels of population genetic differentiation using analyses of molecular variance (AMOVA), conducted with ARLEQUIN v. 3.01, (Excoffier et al. 2005) that we undertook with four different groupings of the *H. leontodontoides* populations. The first grouping (a) has two hierarchical levels and describes differentiation among all populations of the species. Groupings b, c and d each have three hierarchical levels that additionally describe differentiation among geographical regions: between Middle Atlas populations versus High Atlas populations (grouping b); between Western High Atlas populations versus the remaining populations (grouping c); and between Middle Atlas versus Eastern High Atlas versus Central High Atlas versus Western High Atlas populations (grouping d). In this way, we aimed to test which potential geographical barrier (represented by groupings b, c and d), if any, had the largest effect on genetic differentiation in *H. leontodontoides*. We also calculated the AMOVA-derived fixation index F_{ST} (ARLEQUIN v. 3.01), which describes the reduction in heterozygosity within populations relative to the total population (Wright 1951) and is an indirect approach to estimate gene flow.

We used principal co-ordinate analysis (PCoA) among individuals of the different populations to investigate genetic distances and relationships between populations. The PCoA was calculated and plotted in R software. The matrix of Jaccard similarities among individuals was calculated with the function "vegdist" of the vegan package (Oksanen et al. 2013), and the PCoA was calculated using the resulting matrix and the function "pco" of the ecodist package (Goslee and Urban 2007).

To represent overall genetic relationships among all the analysed individuals of *H. leontodontoides*, we constructed a dendrogram applying the UPGMA algorithm based on Nei and Li (1979) genetic distances. Support for each node was tested by 100,000 bootstrap replicates. The NJ method was also applied to the distance matrix and resulted in a similar dendrogram (data not shown). The UPGMA tree was rooted with *H. glabra* L. and *H. achyrophorus*.

To test for isolation by distance (IBD), we compared the 19 populations pairwise F_{ST} values with their geographical distance (in kilometres) using Mantel test based on Spearman correlations (on 9,999 random permutations). Also, Mantel tests were performed within each of the four Atlas Mountain regions (Middle Atlas, Eastern High Atlas, Central High Atlas and Western High Atlas), with Bonferroni correction. The matrix of geographic distances between population pairs were calculated using the spDistsN1 function of the "sp" package in R, with the Great Circle distance (WGS84 ellipsoid) method. Mantel test was performed using the "mantel" function of the "vegan" package in R (Oksanen et al. 2013).

The overall population genetic structure was explored using model-based Bayesian assignment running STRUCTURE 2.3.4 (Pritchard et al. 2000; Pritchard 2010). Clustering of individuals was conducted without using the geographic origin of the samples as a prior informative. Analyses were based on an admixture ancestry model with correlated allele frequencies, for a range of *K* genetic clusters from one to 11, with 12 replicates for each *K*. The analyses were performed with a burn-in period of 150,000 and a run length of the Monte Carlo Markov Chain (MCMC) of 10^6 iterations, respectively. The most likely number of genetic clusters (*K*) was determined according to the ΔK and Mean LnP(K) (Evanno et al. 2005; Pritchard 2010), using STRUCTURE Harvester (Earl and vonHoldt 2012). Afterwards, CLUMPP 1.1.2 (Jakobson and Rosenberg 2007) was used to align the 12 runs of the most representative *K* values, the average pairwise similarity (*H*) for the 12 replicates, and the average probability of belonging to each cluster (*Q*). For $K = 2$, the Full search method with 1000 replicated was used. For $K = 3$ and $K = 4$, we used the Large K-Greedy algorithm, with 30,000 random input orders.

Results

ITS data

PCR-amplified ITS fragments showed a single band when examined on agarose gels. The complete sequence comprised ITS1, 5.8 rDNA gene and ITS2. The aligned ITS1 was 251 nucleotides (nt) long. The 5.8S rDNA gene was 163 nt long, and had no gaps. The aligned ITS2 was 220 nt long. There was no change in the sequences of ITS1 and ITS2 among individuals of the different populations. The six accessions of *H. leontodontoides* had almost identical ITS sequences.

AFLP data

The six AFLP primer combinations generated 873 unambiguously scorable DNA fragments (*EcoRI*-AGC/*MseI*-CAG: 138; *EcoRI*-ACG/*MseI*-CAT: 131; *EcoRI*- ACC/*MseI*-CTA: 177; *EcoRI*- ATC/*MseI*-CTC: 128; *EcoRI*-AGG/*MseI*-CTC: 155; *EcoRI* -ATC/*MseI*-CTGA: 144) of which 848 were polymorphic. All 265 analysed individuals had unique AFLP profiles. The error rate, based on phenotypic comparisons among the 25 replicated individuals, amounted to 2 %.

Genetic diversity

The Middle Atlas region included three populations with moderate high gene diversity (H_D), namely pop. 3 (H_{D}=0.126), pop. 2 (H_{D}=0.119), and pop. 4 (H_{D}=0.105) with a mean value for the five populations of $H_D = 0.094$ (Table 1). In contrast, all High Atlas populations had low or very low gene diversity (H_D range between 0.050 and 0.082) and mean values of H_D between 0.065 and 0.075 with the exception of the pop. 15 from the Western High Atlas, with $H_D = 0.105$. The highest number of private fragments was found in populations of the Middle Atlas (between 10 and 24 $Priv_{frag}$ in pops. 2–4), and also in the pop. 15 from the Western High Atlas ($Priv_{frag} = 22$). In addition, the highest values of rarity index (DW) were found in the same populations (Table 1).

Phenetic phylogeographical analyses

In the PCoA conducted at the level of individuals (Fig. 2), the first two axes explained 65.5 and 21.7 % of the total variation, respectively, and separated perfectly the Western High Atlas populations (pops. 14–19) from the rest of the data set. Middle Atlas populations formed another group, albeit individuals were more scattered, while populations from Eastern (pops. 6–10) and Central (pops. 11–13) High Atlas were situated in a relatively intermediate position between the Middle Atlas and Western High Atlas populations. The PCoA conducted at the level of populations (Fig. S1) showed similar results, and highlighted the genetic divergence of the population 15 from the other Western High Atlas populations, and of populations 2 and 3 from the other Middle Atlas ones. Otherwise, the Eastern and Central High Atlas population groups appeared more closely related.

The UPGMA tree (Fig. 3) rooted with *H. glabra* and *H. achyrophorus* was highly congruent with the results of the PCoA. All *H. leontodontoides* populations/samples clustered together with high bootstrap support (BS). The Western High Atlas populations (pops. 14–19) clustered with (100 % BS) and formed the sister group to the remaining populations (75 % BS). The latter comprised

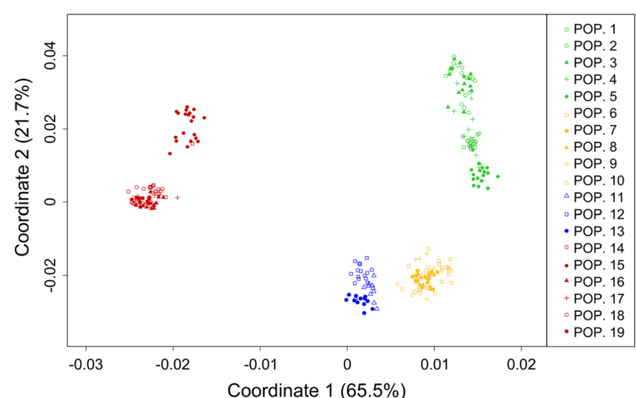

Fig. 2 Principal coordinates analysis (2D-PCoA) based on Nei and Li distances at the individual level

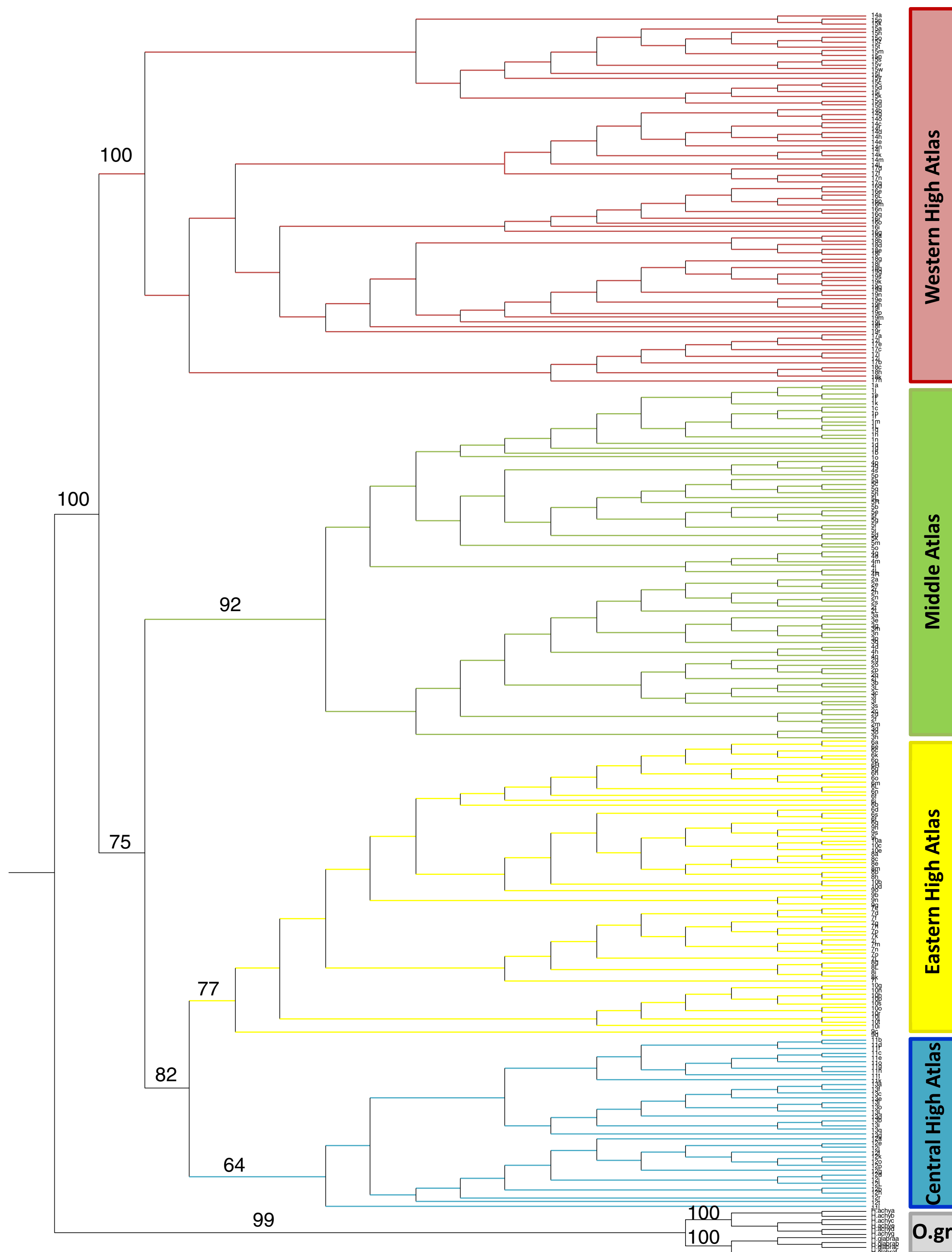

Fig. 3 UPGMA analysis of AFLP phenotypes of *H. leontodontoides*, based on Nei and Li's genetic distance. Bootstrap values higher than 50 % are indicated at each node (based on 10,000 replicates). *O.gr.* Outgroup

three groups: (a) Middle Atlas populations (pops. 1–5; 92 % BS); (b) Eastern High Atlas populations (pops. 6–10; 77 % BS); and (c) Central High Atlas populations (pops. 11–13; 64 % BS).

A non-hierarchical AMOVA (Table 2) revealed that half of the overall genetic variation was explained by the within-population component (49.17 %; $\Phi_{ST} = 0.508$). A nested AMOVA (i.e. grouping Middle Atlas vs. High Atlas)

Table 2 Results of three analyses of molecular variance (AMOVA) of AFLP data (*Squared* Euclidean distance) from 19 populations of *H. leontodontoides* considering 0, 2, 3 or 4 regions

Grouping	N	Source of variation	df	SS	Variance components	Percentage of variance	Fixation index (95 % CI)
a [1–19]	19						$F_{ST} = 0.508$
		Among populations	18	9513	35.52	50.83	
		Within populations	246	8452	34.35	49.17	
b Middle Atlas/High Atlas [1–5] [6–19]	2						$F_{ST} = 0.572$
		Among regions	1	2518	18.61	23.19	$F_{CT} = 0.232$
		Among populations	17	6994	27.27	33.99	$F_{SC} = 0.442$
		Within populations	246	8452	34.35	42.81	
c West High Atlas/remaining pop. [14–19] [1–13]	2						$F_{ST} = 0.595$
		Among regions	1	3487	27.55	32.43	$F_{CT} = 0.324$
		Among populations	17	6025	23.05	27.14	$F_{SC} = 0.401$
		Within populations	246	8452	34.35	40.43	
d Middle Atlas/EHA/CHA/WHA [1–5] [6–10] [11–13] [14–19]	4						$F_{ST} = 0.548$
		Among regions	3	6000	27.18	35.78	$F_{CT} = 0.358$
		Among populations	15	3512	14.44	19.01	$F_{SC} = 0.296$
		Within populations	246	8452	34.35	45.22	

Groupings *b*, *c* and *d* were used to test the effectiveness of geographical barriers in *H. leontodontoides* by maximising the percentage of variation among regions

df degrees of freedom, *SS* mean sum of squares, general fixation index (F_{ST}), fixation index for the region (F_{CT}); and population within region (F_{SC}) level are shown

EHA Eastern High Atlas, *CHA* Central High Atlas, *WHA* Western High Atlas

attributed 57.2 % of the global variation to differences between the different regions, and 33.99 % to within-region population differentiation (both at $p < 0.001$). These variation components were increased when we considered the grouping Western High Atlas vs remaining populations ($\Phi_{ST} = 0.595$), and the among-region component increased even more when we considered the grouping: Middle Atlas vs Eastern High Atlas vs Central High Atlas vs Western High Atlas (35.78 %).

The values of the pairwise fixation index (F_{ST}) between populations (Table S1) indicated, mostly, high values. Populations 18 and 19 ($F_{ST} = 0.119$), 9 and 10 ($F_{ST} = 0.149$), 11 and 13 ($F_{ST} = 0.154$), 6 and 8 ($F_{ST} = 0.163$), 8 and 9 ($F_{ST} = 0.167$), and 2 and 3 ($F_{ST} = 0.175$) were the most genetically similar populations.

The Mantel test, which compared the geographical distances between populations with the respective pairwise F_{ST} values, indicated a very strong positive correlation for these parameters ($r = 0.846, p = 0.0001$. By contrast, the Mantel test indicated no correlation within the four mountain regions ($p > 0.05$).

Bayesian analyses

Bayesian clustering with STRUCTURE was consistent with the results of the ordination and UPGMA analyses. Bayesian clustering showed that the greatest informative representation of overall genetic structure was achieved with $K = 2$ ($\Delta K = 306$, and Mean LnP(K) $= -64402$; Fig. S2). For the 12 replicates of $K = 2$, the average pairwise similarity (H′) was 0.998 for the matrices of populations. The first cluster was very homogeneous and consisted exclusively of Middle Atlas, Eastern and Central High Atlas individuals while the second cluster comprised only individuals of the Western High Atlas populations (Fig. 1b). Bayesian mean F_{ST} values corresponding to the divergence of each inferred K cluster from the hypothetical ancestral population were 0.39 for the first cluster (Middle Atlas, Eastern and Central High Atlas group) and 0.59 for the second cluster (Western High Atlas group), indicating that populations from the first cluster were less diverged from the hypothetical ancestral population (Viruel et al. 2012).

A second maximum $\Delta K = 95$ value, with a Mean LnP(K) = −57203, was obtained for $K = 3$ (Fig. S2), which separated the populations of Middle Atlas (pops. 1–5) from those of Eastern and Central High Atlas (pops. 6–13), and Western High Atlas (pops. 14-19) in three distinct clusters (Fig. 1b). The average pairwise similarity (H') in this case was 0.925 for the matrices of populations. Bayesian mean F_{ST} values oscillated between 0.678 (Western High Atlas populations) and 0.430 (Middle Atlas populations). The lowest Bayesian mean F_{ST} value of this latter cluster points out this group as the least diverged from the hypothetical ancestral population. A further maximum $\Delta K = 55$ value, with a Mean LnP(K) = -54539; was obtained for $K = 4$ (Fig. S2) which separated the 19 analysed populations in four distinct clusters: Middle Atlas, Eastern High Atlas, Central High Atlas, and Western High Atlas (Fig. 1b). The average pairwise similarity (H′) was 0.779 for the matrices of populations. Bayesian mean F_{ST} values oscillated between 0.649 (Western High Atlas) and 0.384 (Middle Atlas), indicating once again that the Middle Atlas population group is the least diverged from the hypothetical ancestral population. The populations 1, 4 and 5 from Middle Atlas appeared as a mixed population in $K = 3$ and even more in $K = 4$ clustering.

Discussion

Strong genetic differentiation among Mountain regions

Our AFLP data showed a highly geographical structuring of the populations of *H. leontodontoides* along the Atlas Mountain range. Two main outcomes deserve to be highlighted. The first one is the marked group differentiation between the Western High Atlas populations from the remainder, which were supported by the UPGMA dendogram and PCoA analyses and also the Bayesian analysis at the optimal peak, $K = 2$. It is possible that the basins that delimit Western High Atlas region, that is, the Oued Tensift basin to the north, and the Oued Tassaout basin to the east, together with the Ourika river valley, act as insurmountable barriers that limit gene flow between the Western High Atlas populations and the other populations. Similarly, the Aosta valley (western Alps) and River Adige valley (eastern Alps) have been identified as efficient barriers against gene flow for many alpine herbaceous plants, such as *Androsace obtusifolia*, *Phyteuma betonicifolium*, *Ranunculus glacialis*, etc. (Schönswetter et al. 2005; Thiel-Egenter et al. 2011). Moreover, it is interesting to note that the populations from the Western High Atlas are at higher altitudes compared with the remainder populations (see Table 1), a fact that also could limit gene flow between the populations in this area and those of other areas.

The second outcome from our study is the appreciable differentiation found between the three remaining geographical regions: Middle Atlas, Eastern High Atlas and Central High Atlas, with the two latter groups being more genetically related (see Bayesian analyses at $K = 3$). Again, geomorphological barriers such as high montane topography or river valleys might explain this genetic structure. Similar patterns of genetic differentiation have been observed in other organisms (Brown et al. 2002; Fritz et al. 2006; Sousa et al. 2011). Likewise, a differential genetic structure among different mountain regions from the Atlas mountain range (Western High Atlas vs Central High Atlas and South Middle Atlas) was observed in *Arabidopsis thaliana* (Brennan et al. 2014).

Despite the genetic differentiation among regions found with AFLP markers, almost identical ITS sequences were found for individuals from the different regions, which suggest that *H. leontodontoides* is a well supported taxa.

Current gene flow patterns

Our AFLP data reveals a sequence of genetically distinct populations of *H. leontodontoides* occurring across the Atlas Mountain range, which is exemplified a high genetic differentiation ($F_{ST} = 0.508$). Although significant correlation between populations pairwise F_{ST} values with their geographical distance (Mantel test) could suggest isolation by distance (IBD), this result should be considered with caution. Indeed, geographically distant populations did show low F_{ST} values, (e.g. Eastern High Atlas vs Central High Atlas populations), while geographically closer populations showed higher F_{ST} values (e.g. Eastern High Atlas vs Middle Atlas populations; see Table 1S and Fig. 1a). Meirmans (2012) argued that IBD tests can lead to a large number of false positives, and suggested that a partial Mantel test can be used to test whether geography contributes to apparent clustering. In our scenario, the Mantel tests performed within each of the four mountain regions (Middle Atlas, Eastern, Central and Western High Atlas) were not significant, which indicate that at regional scale, gene flow between populations was not correlated with geographical distance. Indeed, Mantel tests do not distinguish between patterns resulting from clustering and those resulting from isolation by distance (Meirmans et al. 2011). Our results could be due to two different factors: (1) the scattered distribution populations of *H. leontodontoides,* occurring only rocky outcrops on north slopes, and (2) geographical barriers due to the abrupt topography of this region.

The isolation pattern found among populations of *H. leontodontoides* is also found in several European montane plant species of the Alps, Pyrenees, Apennines, Betic, Balkan and Carpathian systems (Zhang et al. 2001; Despres

et al. 2002; Schönswetter et al. 2004; Schönswetter and Tribsch 2005; Kropf et al. 2006). Also, the congeneric *H. angustifolia*, another species from the Altas Mountain range, showed a similar sequence of isolated populations across the high mountain altiplano (Terrab et al. 2009). However, *H. angustifolia* inhabits high montane marshlands of the Atlas and it seems that its current population structure pattern most likely arose by habitat fragmentation following volcanic activity during the Quaternary of a previously much more extensive population system (i.e. a vicariance origin).

Diversification centre and colonization throughout the Atlas Mountain range

Morocco has been inferred as the ancestral area of Section *Hypochaeris*, which is sister to section *Seriola* that include *H. leontodontoides*. The Sebou valley seems to have played an important role in diversification within sect. *Hypochaeris*. Three species of that section (*H. glabra, H. radicata* and *H. salzmanniana*) evolved in the north-western foothills of the Middle Atlas, and another, *H. arachnoidea*, in the Atlas Mountain range (Ortiz et al. 2009).

Since all species from section Seriola (*H. leontodontoides* and their closest relatives *H. laevigata, H. achyrophorus*; Tremetsberger et al. 2005) inhabits the Middle Atlas, this region seems to be the diversification centre of section Seriola. Our molecular results also imply a possible origin of the species in the Middle Atlas. This view is supported by the Bayesian F_{ST} values, and the highest number of private fragments and rare fragments index (DW), as well as the highest genetic diversity index, all found within this region (see Table 1; Fig. 1a). Although we cannot trace in detail the routes of the colonisation by *H. leontodontoides*, the most likely scenario, based on the Bayesian Analysis, is that populations subsequently dispersed southwards from the Middle Atlas, to the Eastern, Central and Western High Atlas. Moreover, the genetic structure of pop. 15 could suggest another colonisation event by long distance dispersal, from the Middle Atlas to the Western High Atlas (see Fig. 1a, b).

Floristic consequences of Quaternary climate fluctuations in the Atlas Mountain

Pliocene–Pleistocene ice ages are likely to have modelled the distribution and genetic structure of populations of *H. leontodontoides*. In fact, there is evidence that most of the Atlas Mountain range was affected at least by the last glacial maximum (Peulvast et al. 2000; Hughes et al. 2011). The largest glaciers in the whole Atlas mountain range were found in Toubkal massif (Western High Atlas), where valley glaciers emanated from a central ice field which formed between the two highest summits, Toubkal (4167 m a.s.l.) and Ouanuokrim (4067 m a.s.l.) (Hughes et al. 2011; see Fig. 1a). This glaciated area is coincident with the location of the pops. 16 and 17. In fact, these populations exhibit a depauperate genetic structure with low values of gene diversity, private fragment and rare fragment index. The westernmost populations (pops. 18 and 19) of the Western high Atlas, also showed similar impoverished gene diversity (see Table 1), which suggest that this area was also colonized more recently.

Overall, our data suggest that two regions within the Atlas Mountain range may have acted as refuges during the Pleistocene ice ages: The Middle Atlas and, to a lesser extent, the northernmost part of the Western High Atlas. Effectively, the highest values of private and rare fragments, and population gene diversity, were found in most populations of the Middle Atlas (pops. 2-4), and also in the northernmost population (pop. 15) of the Western High Atlas (Table 1). Other authors have proposed the occurrence of glacial refuges in the Middle and High Atlas regions (Médail and Diadema 2009; Hughes et al. 2011; Brennan et al. 2014). Populations of *H. leontodontoides* in the Middle Atlas region, currently located at 1900–2200 m a.s.l., should have been weakly affected by the ice ages, since the glacial snowline, at least of the last glacial period, were situated much higher at 2800 m (Hughes et al. 2011). The High Atlas region, however, showed a more severe impact of the ice ages, especially the Western High Atlas, a region that was extensively covered by ice. Despite this, it seems that in the northernmost part of this region, *H. leontodontoides* may have found a suitable area that permitted its survival, possibly by changing elevation during the coldest periods. Elevational shifts could also assure glacial survival in other regions; however, genetic structuring and diversity patterns found make a colonisation history for the populations from the Eastern and Central High Atlas more likely.

Conclusions

The present study shows a highly structured phylogeographic pattern in *H. leontodontoides*, with notable population differentiation within the Atlas Mountain range, with the population group from the Western High Atlas being most differentiated. Furthermore, a substructuring of the populations from the Middle Atlas, the Eastern High Atlas and the Central High Atlas was also detected. The high genetic differentiation among those groups of populations possibly reflects the impact of geomorphological features in the context of Quaternary climatic fluctuations as dispersal barriers.

The most likely scenario of the colonisation history of *H. leontodontoides* is that populations dispersed southwards from the Middle Atlas, to the Eastern, Central and Western

High Atlas. Two regions could be suggested as refuges during the Pleistocene ice ages: the Middle Atlas and, more strikingly given its glacial history, the northernmost area of the Western High Atlas. Similar patterns of genetic structure of populations have been found in other Atlas Mountain species such as *H. angustifolia* (Terrab et al. 2009), or *Arabidopsis thaliana* (Brennan et al. 2014), although the hypotheses explaining these patterns are in detail not necessarily the same (Terrab et al. 2009).

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