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Morphological and molecular diversity of the wild carrot *Daucus pusillus*: implications for classification and *ex-situ* conservation

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Abstract: *Daucus pusillus* and *D. montevidensis* are wild carrots from the Americas with unresolved taxonomic status. An investigation was carried out with accessions of *D. pusillus*/*D. montevidensis* from Argentina for a) morphological and molecular (AFLP and ISSR) characterization, b) analysis of congruence of morphological and molecular variation, and c) comparison of diversity for the ITS region with that reported for a North American accession of *D. pusillus*. Twelve accessions of *D. pusillus/montevidensis* -representing their geographical distribution in Argentina-, and one accession of each wild *D. carota* and *D. montanus* -as outgroups- were included. In the multivariate analysis of morphological diversity, two accessions were clearly differentiated; this result is not sustained by multivariate analysis of molecular diversity. Based on multivariate and AMOVA analyses, *D. pusillus/montevidensis* accessions were separated at the molecular level into two groups, associated with geographical origin. Since this result is not supported by morphology, the segregation into two taxa seems unjustified. In all accessions, ITS and 5.8S rDNA regions had identical sequences, which differ in one nucleotide from the corresponding sequence of the North American accession. According to the combined results, *D. pusillus* would be a single taxon distributed from North to South America, and *D. montevidensis* a nomenclatural synonym. Autogamy of *D. pusillus* and its highly structured genetic diversity (F_{st}=0.86) allows the application of a geographically targeted approach for germplasm exploration, conservation and eventual use in pre-breeding.

Key words: wild carrots, genetic resources, molecular diversity, morphological diversity, ITS sequence.

Introduction

Wild and cultivated carrots are included in genus *Daucus* L. (Apiaceae), which comprises approximately 20 (Sáenz Laín 1981) to 60 (Thellung 1926*a*; Zohari 1987) taxonomic species. The center of diversity of the genus is the Mediterranean Region, particularly North Africa, where strong speciation has taken place (Sáenz Laín 1981). In fact, there are four species that have been reported as occurring only outside this region: *D. glochidiatus* (Labill.) Fisch., C.A.Mey. & Avé-Lall. in Australia, *D. montanus* Humb. et Bonpl. ex Schult, *D. pusillus* Michx. and *D. montevidensis* Link ex Sprengel in America (Sáenz Laín 1981).

According to Saénz Laín (1981), *Daucus pusillus* is an annual species distributed in USA, Mexico and Chile, whereas *Daucus montevidensis* is a biennial species distributed in Uruguay, Chile and Argentina. Sáenz Laín (1981) considered that both species could be recognized on the basis of several attributes such as bract morphology, fruit features (especially, number of spines) and flower color. Nevertheless, the taxonomic status of *D. montevidensis* remains in dispute. Heywood and Dakshini (1971) differentiated *D. pusillus* from *D. montevidensis* on the basis of geographical distribution (North and South America, respectively) and overlapping of the primary hair with the spine base in the fruit. Okeke (1978) considered that both species were merely synonyms, and did not accept Heywood and Dakshini´s (1971) classification arguing that hair length could be influenced by the developmental stage of the fruits. Heywood (1982) recognized *D. montevidensis* as a synonym of *D. pusillus*.

Interestingly, both taxa have been cited for the Argentinean Flora, but there is no formal taxonomic revision of genus *Daucus* for this country. According to various regional floristic works, *D. montevidensis* is common in sandy soils of Buenos Aires province (Cabrera 1953; Stutz and Prieto 2003), and also in the provinces of Córdoba, Entre Ríos, Corrientes (Marzocca 1957) and Mendoza (Méndez 2011). *D. pusillus*, on the other hand, has been reported as growing in the same type of soil in the provinces of Buenos Aires (Cabrera and Zardini 1978) and Entre Ríos (Burkart and Bacigalupo 2005) as well, and also in Patagonia (Lincoln Constance, in Correa Maevia 1988). However, in several regional floristic works carried out by Cabrera (1965), Constance (in Correa Maevia 1988), Zuloaga and Morrone (1999) and Burkart and Bacigalupo (2005) *D. pusillus* has

been listed as a synonym of *D. montevidensis*, although no evidence has been presented to support this claim. The loss of the *D. montevidensis* type specimen has added to the taxonomic confusion (Hiepko 1987).

More recently, Camadro et al. (2007) sampled 30 *Daucus* populations in the pampas grasslands of two Argentinean provinces, Buenos Aires and Entre Ríos. These populations were distinguishable by their morphological characters, chromosome numbers and adaptation to characteristic habitats. Thus, accessions (population samples) were assigned to one of two groups according to chromosome number and morphological phenotypes: the first, with 2n = 2x = 18, were classified as wild *D. carota* whereas the second, with 2n = 2x = 22 and 2n = 2x = 22 and 20 due to aneusomaty, were tentatively classified as *D. pusillus* until molecular studies could be carried out (Camadro et al. 2007).

During the last decade, there has been much progress regarding the infra-familiar systematics of Apiaceae. Recently, Downie et al. (2010) summarized the current state of knowledge and compiled the literature on Apiaceae systematics based on phylogenetic analysis of DNA data, including chloroplast gene and intron sequences, cpDNA restriction site variation, and ITS sequences. Notwithstanding, these authors noted that there are still uncertainties regarding *Daucus* taxonomy. The main papers dealing with the taxonomy of *Daucus* at a general level are those of Calestani (1905), Thellung (1926*a* and 1926*b*), Onno (1936), Nehou (1961), Heywood (1968) and Saénz Laín (1981), the latter being the latest taxonomic revision of the genus based on morphology and anatomy. According to Hand (2011), the phylogeny of genus *Daucus* is not completely understood, and changes are to be expected in the near future. In fact, additional data from both molecular and morphological markers are necessary before any workable classification system of *Daucus* can be proposed (Grzebelus 2011). Considering the infra-generic classification of *Daucus*, the current status of *D. montevidensis* as a taxonomic species different from *D. pusillus* is one of the issues to be solved. Due to its wide adaptation, *D. pusillus* could be a valuable source of genes of interest for carrot breeding, including resistance/tolerance to adverse biotic and abiotic factors as well as male sterility for hybrid seed production (Camadro et al. 2008). As *D. carota* and *D. pusillus* appear to be separated mainly by incomplete pre-zygotic barriers, it would be apparently feasible to obtain hybrid seed in controlled crosses between compatible genotypes (Camadro et al 2008; Ibañez MS and Camadro EL unpublished results).

For all the above, the objectives of this work in accessions of Argentinean populations of *D. pusillus*/*D. montevidensis* were to a) carry out their characterization at the morphological and molecular levels, b) analyse the structure and congruence of the diversity evaluated at both levels, and c) compare the diversity of the ITS region with data of a North America population of *D. pusillus* available in Genbank.

Material and methods

Plant material

Twelve accessions (*ex situ* conserved population samples) of *D. pusillus/montevidensis* were included in this study (Table 1). Accessions were chosen as representative samples of natural populations growing in different —some of them contrasting— macroenvironments, including locations where *D. pusillus* and *D. montevidensis* had been reported to occur together (Fig. 1). One accession of *D. montanus* and one of wild *D. carota* were used as outgroups. Seeds of all accessions were deposited at the Laboratory of Genetics, EEA Balcarce, INTA, Argentina.

Ten to 20 mericarps per accession were germinated in Petri dishes under controlled conditions (12 h photoperiod at 21º C). At the 2-4 leaves stage, seedlings of *D. pusillus/montevidensis* were transplanted into 0.5 l pots and those of *D. montanus* and *D. carota* seedlings into 3 l pots containing a sand:soil (2:1) mixture. Plants were grown in a screenhouse without supplementary light.

Chromosome numbers

Chromosome counts were carried out in three plants per accession following the standard technique of pre-treatment with 8-hydroxiquinoline solution (0.29 g/l) for 2 h, fixation in 3 ethanol: 1 glacial acetic acid (v/v), hydrolysis in 1 N HCl at 60º C for 12 min and staining with leucobasic fuchsin (Coleman 1938).

Morphological characterization

Twenty-one quantitative and nine qualitative characters were recorded (Table 2). Measurements were performed at three different phenological stages: *stage 1* - closed flowers in primary umbel, *stage 2* - mostly

opened flowers in primary umbel, and *stage 3* - mature fruits (Fig. 2). Stem and leaf measurements were taken, respectively, at the first internode counting from the base of the plant and on the leaf above that internode. Width of petiole insertion was measured at the union of the petiole with the stem. Spines of vallecular ribs were not considered in measuring length and width of mericarps. Length and number of spines were measured in the dorsal right vallecular ribs. Voucher specimens were deposited in the Herbarium BAL.

Molecular characterization

Young leaves of individual plants were frozen at -20º C. Genomic DNA was extracted using a slightly modified CTAB method (Haymes 1996). DNA concentration was quantified by using a fluorometer (BIO-RAD SmartSpect TM 3000).

Multipoint markers, AFLP and ISSR, were used. AFLP analysis was performed as described by Vos et al. (1995). A total of 250 ng of genomic DNA was double-digested with two restriction enzymes (*Mse*I and *Eco*RI). DNA fragments were ligated to *Eco*RI and *Mse*I adaptors with T4 ligase. Preamplification reactions were performed with a primer mix (+1 primers, M-A and E-C), and subject to 20 cycles of 94º C for 30 s, 56º C for 60 s, and 72º C for 60 s using Mastercycle Gradient (Eppendorf®) thermocycler. Selective AFLP amplification was performed with three primer pairs (Table 3), with the following polymerase chain reaction (PCR) conditions: one cycle at 94º C for 30 s, 65º C for 30 s, and 72º C for 60 s; then the annealing temperature was lowered 0.7º C for each of 12 cycles, that were followed by 23 cycles at 94º C for 30 s, 56º C for 30 s, and 72º C for 60 s.

For ISSR analysis, three primers were used (Table 3). PCR amplifications were performed in a Multigene gradient (Labnet) thermocycler, in 25 µl volumes containing 30 ng template DNA, 2.5 mM $MgCl_2$, 1X buffer (50 mM KCL, 20 mM Tris-HCL, ph 8.4), 125 µM of each dNTP, 1µM of a single primer, 1 mg/ml BSA and 2.5 U of *Taq* polymerase. PCR programmed conditions were 95º C for 1 min, 35 cycles at 94º C for 30 s, 45º C for 45 s, and 72º C for 90 s, and final extension at 72º C for 5 min. Amplification products were electrophoresed in 6% polyacrylamide gels and silver stained, following the protocol described by Bassam et

al. (1991). Individual molecular marker fragments (bands) were scored for each genotype as either present or absent.

Data analyses

For the morphological analysis, two types of matrices were generated, one considering each individual and the other using population means. Euclidean distance was used to generate distance matrices for cluster analysis. For the molecular analysis based on each individual, the Dice coefficient was used to calculate a similarity matrix, which was visualized by principal coordinate analysis (PCO). Dendrograms were generated using the Unweighted Pair Group Method Arithmetic mean (UPGMA) method. Morphological matrices were subjected to principal components analysis (PCA). Correlation between the morphological and molecular distance matrices was determined using the Mantel test (1967). The molecular distance matrix was obtained from the corresponding Dice similarity matrix by transforming each similarity value into a distance value ($d = 1–s$). All analyses were performed using the NTSYS-pc version 2.10t program (Rohlf 1992). For bootstrapping analysis, the FreeTree software, version 0.9.1.50 was used (Pavlicek et al. 1999) (250 bootstraps involving random fragment sampling with replacement).

Combined analysis of morphological and molecular diversity was carried out with *D. pusillus/montevidensis* accessions and, as outgroups, *D. montanus* and wild *D. carota* accessions. Euclidean coefficient was used to obtain similarity matrices for cluster analysis, and correlation matrices were obtained and subjected to PCA. Dendrogram of cluster analysis was generated using the UPGMA method. Data analyses were carried out using NTSYS-c version 2.10t program (Rohlf 1992).

To analyse the distribution of the genetic variation, an AMOVA was carried out with a hierarchical structure [geographical regions, accessions within geographical regions and individuals within accessions]. For assessment of population genetic diversity, the Fixation index of Wright (Fst) was estimated. All analyses were performed using Arlequin 3.1 software package (Excoffier et al. 2005).

Sequence diversity of internal transcribed spacer (ITS) regions

ITS regions of six *D. pusillus/montevidenis* accessions from different geographic regions and macroenvironments were PCR-amplified using ITS5 and ITS4 primers (White et al. 1990) in an equimolar ratio. PCR amplification conditions were modified from Downie et al. (1996). Amplifications were carried out in 25 µl reaction mixture containing 30 ng template DNA, 1.5 mM $MgCl_2$, 1X buffer (50 mM KCL, 20mM Tris-HCL, ph 8.4), 0.2 mM of each dNTP, 1µM of forward and reverse primers, and 1.5 U of *Taq* polymerase, in a Multigene gradient (Labnet) thermocycler as followed: 94º C for 60 s, 35 cycles of 94º C for 60 s, 53º C for 60 s, and 72º C for 60 s; and a final extension at 72º C for 5 m. Successful PCR amplifications resulted in a single DNA band corresponding to approximately 700 bp in size. Each amplified DNA fragment was electrophoresed in a 1% agarose gel, visualized with Sybr® Safe DNA Gel Stain (Invitrogen) under Safe Imager™ 2.0 Blue Light Transilluminator (Invitrogen, Life Technologies), and then excised with a sterilized scalpel. PCR fragments were isolated from agarose using Illustra™ GFX™ DNA and a gel Band Purification Kit (GE). DNA fragments were submitted for direct sequencing (Macrogen, USA). Sequences were assembled and edited using Contig Express (Invitrogen). Boundaries of coding and spacer regions were determined by sequence comparison with the respective boundaries in *Daucus carota* (Yokota et al. 1989). DNA sequences were aligned using Clustal W conducted in MEGA 5 (Tamura et al. 2011) and compared among them and with sequences AF077788.2 and AF077103.2, available in GenBank, from a North America *D. pusillus* population. A combined sequence of the three fragments of each accession was deposited in Genbank as contiguous data: KF467154 (ECpus1), KF467155 (ECpus2), KF467156 (ECpus11), KF467157 (ECMCpus2), KF467158 (ECMCpus11), KF467159 (SIMacpus1).

Results

Chromosome numbers

Plants of the *D. carota* accession were 2n = 2x = 18, whereas those of the *D. montanus* accession were polyploid, with 2n = 6x = 66. Accessions classified as *D. pusillus/montevidensis* were 2n = 2x = 22, although cells with 2n = 2x = 20 were also observed at a low frequency (less than 10%).

Characterization of wild *Daucus* accessions

The three taxonomic species were clearly distinguished by the morphology of leaves, inflorescences and mericarps (Table 4, Fig. 3). Cluster and PCO analysis of morphological and molecular data revealed three groups corresponding to *Daucus pusillus/montevidensis*, *D. montanus* and *D. carota* (Fig. 4). In the dendogram, *D. pusillus/montevidensis* accessions were clustered together in a large group that was close to the *D. montanus* accession, whereas the *D. carota* accession was separated from the other accessions at a larger dissimilarity distance. The first two components of the PCA analysis (PC1 and PC2) accounted for 72% of the variation, which was mainly explained by the molecular characters. Nevertheless, one morphological character, involucral bract petiole membrane, contributed to PC1 (Table 4, Fig. 3: *g*, *h*) and three other morphological characters, umbel area, largest ray of umbel, and stem pubescence (Table 4, Fig.3: *c*, *d*, *e*, *f*), contributed to PC2.

Morphological characterization of Daucus pusillus/montevidensis

Daucus pusillus/montevidensis accessions presented high morphological diversity. The characters which exhibited more variability were plant height, branching (Fig. 3: *a*, *b*); umbel arrangement, loosely to strongly compacted (Fig. 3: *i*, *j*, *k*, *l*, *m*); and involucral bract number (Table 4). Stems and leaves were always pubescent but in different degree (Table 4, Fig. 3: *d*, *e*, *f*). Involucral bracts were 2-3 pinnatisect and shorter than -or as long as- the umbel. Mericarp appearance and endosperm median transverse section were also very variable (Fig. 3: *p*, *q*, *r*, *s*, *v*, *w*, *x*, *y*). When mericarps with more than one shape were present in the same inflorescence, the character was considered as *variable* (1). Spine length in relation to fruit width was also variable: from slightly short to slightly long. On the other hand, some characters were monomorphic: all plants were annuals, with alternate and 2-3 pinnatisect leaves, umbels with straight rays, fruits with glochidiate spines dilated and slightly confluent at the base, and *vittae* triangular in transverse section.

Cluster analysis for both type of matrices and PCA of morphological characters separated accessions into two groups. Those from Patagonia, ECpus1 and ECpus2, were grouped together and separated from the other accessions (Fig. 5). In PCA, the first two components accounted for 60% of the variation. PC1 contributed about 40%, and had the highest contribution from the following characters: involucral bract number, stem length 2, secondary branches and umbel arrangement. PC2 represented 20% of the variation, with length of

largest and smallest involucral bracts and primary branches contributing the most. Patagonian accessions could be differentiated from the others on the basis of PC1.

Molecular diversity

For individual plants of *Daucus pusillus/montevidensis* accessions, 77 polymorphic bands were produced by AFLP and ISSR (Table 3). Cluster analysis and PCO showed a clear grouping of accessions according to geographical origin, with one exception (Fig. 6). Two groups were differentiated in the dendrogram: one (I) with accessions from Patagonia and Pampa regions and one accession from Mesopotamia (ECpus11), and the second (II) with accessions from Mesopotamia (Entre Ríos and Isla Martín García). This grouping was sustained by bootstrap value of 100%. Clustering of individuals in the principal coordinate analysis was similar to the dendrogram grouping. Individuals of each accessions and geographic origin tended to group together, except for genotypes of ECpus11 from Mesopotamia, which was closer to those from Patagonia and Pampa.

An AMOVA was performed to analyse the partitioning of the genetic variance among accessions which had been previously clustered according to the results of the morphological and the molecular studies. Two comparisons were carried out: 1) accessions from Patagonia vs. the rest, and 2) accessions from Patagonia and Pampa vs. accessions from Mesopotamia. In both comparisons, the largest variation was detected among accessions from the same geographical region (α= 0.05). In the first comparison, accessions from Patagonia were not significantly different from the rest whereas both groups of accessions differed significantly in the second (Table 5). Additionally, the Wright's fixation index was significantly high (Fst= 0.86) in the second comparison.

Mantel test of the correlation between the distance matrices for morphological and molecular characters based on individual plant values was low (r = 0.2270, p = 0.001). These results indicate that the morphological variability was weakly associated with the molecular diversity observed among plants of *Daucus pusillus/montevidensis*.

Internal transcribed Spacer

Complete fragments of ITS1 spacer region, 5.8S rDNA coding region, and ITS2 spacer region of six accessions of *D. pusillus/montevidensis* from different geographical origins were sequenced and examined. Sequences were identical for all accessions, with a size of 602 bp: ITS1 contributed with 216 bp, the coding 5.8S rDNA region with 164 bp and ITS2 with 222 bp. This sequence of ITS1 and ITS2 was aligned and compared to a sequence of an accession of *D. pusillus* from North America (California, USA). Both sequences differed in just one nucleotide changing a T for an A in the position 391 corresponding to the ITS2 fragment.

Discussion

There are discrepancies among authors about the distinctive characters and geographical dispersion of populations that allow the differentiation of *Daucus pusillus* from *D. montevidensis*. At the present, in Argentina, these two taxonomic species have been cited both as distinct species and synonyms.

The somatic chromosome numbers determined in the studied *D. pusillus/montevidensis* accessions was coincident with the reported in previous works, n = x = 11 (Constance et al. 1976; Iovene et al. 2008) and also aneusomaty, n = x and x-1 = 11, 10 (Correa Maevia 1988; Camadro et al. 2007).

Individual plants of the evaluated accessions exhibited a wide and continuous range of variation for morphological characters (Table 1S). Some characters such as life cycle, presence of 2-3 pinnatisect leaves, and glochidiate spine in the fruit were constant and matched the description of *D. pusillus* by Sáenz Laín (1981). However, following the same author, the studied accessions should be recognized as D. *montevidensis* because of the length of the mericarps and their geographical distribution. On the other hand, the range of variation observed for some characters such as length of stem, umbel peduncle, bract length in relation to umbels, and spines, exceeded the description given by Sáenz Laín (1981) for both *D. pusillus* and *D. montevidensis.* In her key to *Daucus species*, Sáenz Laín (1981) used the shape of the blade bract to differentiate *D. montevidensis* from *D. pusillus* and *D. carota* but, on describing them separately, she reported the number of spines as eight in *D. pusillus* and 12 in *D. montevidensis*, and the flower color as white in the first and yellowish in the second taxonomic species. She also reported differences in life cycle and stem length, among other characters. This author worked on a few herbarium specimens, which were not representative of

the morphological variation of natural populations as our results indicate. Camadro et al. (2007), who worked with accessions from the Argentinean pampas, reported differences in plant height and number of branches in natural populations, but did not carry out a detailed morphological evaluation of individual plants, as was done in the present study. The main difference, then, between previous works and the present is that we worked with live materials (143 individual plants) that were cultivated in a screenhouse under similar conditions to minimize environmental variation.

Multivariate analysis of morphological diversity of *D. pusillus/montevidensis* accessions showed a clear differentiation of Patagonian accessions from the other populations studied. However, these results were not sustained by the genetic diversity analysis. In fact, at the molecular level, cluster analysis and PCO revealed that *D. pusillus* accessions could be separated into two groups associated with geographical origin: one conformed by those from Patagonia and Pampa and the other by those from Mesopotamia. In this context, also the Mantel test clearly indicated that the results obtained with morphological and molecular tools were not congruent. Since the molecular markers that were used in the characterization are neutral, it can then be hypothesized that morphological differences between accessions from Patagonia versus accessions from Pampa and Mesopotamia are controlled by few major genes whose polymorphism was undetectable by the type of markers used. Phenotypic variation alone, without congruent molecular diversity, precludes the segregation of two *taxa* from the diversity observed among *D. pusillus/D. montevidensis* accessions.

Baldwin et al. (1995) reported that ITS data play a useful role in angiosperm systematics, offering independent assessment of lower-level phylogenetic hypotheses based on morphological evidence. Numerous phylogenetic works in Apiaceae were carried out with ITS regions (Lee and Downie 1999, 2000; Lee et al. 2001; Spalik and Downie 2007). The ITS and 5.8S rDNA regions analysis in this study showed identical sequences for all evaluated accessions. This observation, in addition to the comparison of ITS sequences with the sequence of a North American accession which only differs in one nucleotide, does not allow us to reject the hypothesis that *D. pusillus* is a single taxon distributed from North to South America and that *D. montevidensis* is just a nomenclatural synonym.

Camadro et al. (2008) and Ibañez MS and Camadro EL (unpublished results) reported that *D. pusillus* is an autogamous species, a fact that explains why the largest variation was detected among populations and the lowest within populations (Table 1S). Moreover, AMOVA corroborated the existence of a highly structured molecular diversity. Furthermore, and considering Wright´s (1978) scale, the high Fst value obtained (0.86) is indicative of substantial genetic differentiation among accessions. This evidence supports the existence of a genetic structure among the *D. pusillus* populations under study. In carrot germplasm, on the other hand, and by using various biochemical and molecular markers such as isozymes (St. Pierre et al. 1990; St. Pierre and Bayer 1991), RAPD (Grzebelus et al. 2002), AFLP and ISSR (Bradeen et al. 2002), population genetic structures could not be detected. Recent results of Clotault et al. (2010), based on carotenoid biosynthesis gene sequences and SSR, demonstrated a genetic structure according to geographical origin or root color within the cultivated carrot germplasm. Moreover, Baranski et al. (2012) by using SSR and supported by morphological characters, provided evidence for the divergence of Eastern and Western genetic pools. Recently, Iorizzo et al. (2013) observed a clear separation between wild and cultivated accessions as well as between eastern and western cultivated carrots using 3326 SNP markers. The autogamous condition of *D. pusillus* and its highly structured genetic diversity allows the use of a geographically targeted approach for germplasm exploration, conservation, evaluation and eventual use of this species in pre-breeding.

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Tables captions

Table 1. Argentinean accessions of wild *Daucus* species, geographic location of the originally sampled populations, and number of plants evaluated per accession.

Table 2. Morphological characters used to evaluate wild *Daucus* accessions from Argentina, including phenological stage at measurement, complete character name, abbreviations and character state description (units and scales).

Table 3. Primers sequences and number of polymorphic AFLP and ISSR bands.

Table 4. Quantitative and qualitative morphological characters evaluated in 14 accessions of wild *Daucus* from Argentina, grouped according to species, geographical origin and morphological differences. Accessions grouping: *D. montanus* (ECmon1), *D. carota* (ECMCcar16), *D. pusillus/montevidensis* from Patagonia (ECpus1 and ECpus2), and from Pampa and Mesopotamia regions together (ECpus7, ECpus9, ECpus11, ECpus12, ECMCpus2, ECMCpus3, ECMCpus11, ECMCpus12, SIMacpus1 and SIMacpus2).

Table 5. Results of AMOVA based on AFLP and ISSR data of 12 accessions of *Daucus pusillus/montevidensis.*

Figure captions

Fig. 1. Geographical distribution of wild *Daucus* accessions from Argentina used in this study.

Fig. 2. Phenological stages for morphological characterization: A. *stage 1*- closed flowers in primary umbel, B. *stage 2*- mostly opened flowers in primary umbel, and C. *stage 3*- mature fruits. *i*) complete plant, detail of primary umbel in *ii*) upper and *iii*) lateral view. Scale bars: 10 cm (*i*), 1 cm (*ii*, *iii*).

Fig. 3. Distinctive morphological qualitative characters evaluated in accessions of three wild *Daucus* species from Argentina. Plant aspect: (*a*) short and branched and (*b*) tall and little branched, and scales of characters described in Table 2: stem and leaf pubescence (*c*) 0, (*d*) 1, (*e*) 2, (*f*) 3, involucral bract petiole membrane (*g*) 0, (*h*) 1, umbel arrangement (*i*) 1, (*j*) 2, (*k*) 3, (*l*) 4, (*m*) 5, (*n*) 6, (*o*) 7, endosperm median transverse section (*p-s*) 1, (*t*) 2, (*u*) 3, and mericarp appearance (*v-x*) 1, (*y*)2 , (*z*) 3, (*α*) 4. Specific characters of each species: *D. pusillus* (*a*-g, *i-m*, *p-s*, *v-y*), *D. carota* (*h*, *o*, *t*, *z*), and *D. montanus* (*n*, *u*, *α*). Scale bars: 10 cm (*a*, *b*), 4 cm (*n*), 1 cm (*o*), 0.5 cm (*g-m*), 0.3 cm (*c-f*), 0.1 cm (*v- α*), 0.05 cm (*p-u*).

Fig. 4. Combined analysis based on individual values for 30 morphological characters and 339 polymorphic bands of molecular markers (AFLP, ISSR), in 14 accessions of wild *Daucus* from Argentina. A. Dendrogram based on Euclidean similarity coefficient by the UPGMA method. B. Principal component analysis accounting for 72% of the variation.

Fig. 5. Analysis of 30 morphological characters based on mean values in 12 *Daucus pusillus/montevidensis* accessions from Argentina. A. Phenogram based on Euclidean similarity coefficient by the UPGMA method. B. Principal component analysis accounting for 60% of variation. Accessions origin: Patagonia (black), Pampa (grey) and Mesopotamia (white).

Fig. 6. Molecular analysis of 12 *Daucus pusillus/montevidensis* accessions from Argentina based on individual observations of 77 polymorphic bands of AFLP and ISSR markers using Dice coefficient. A. Dendrogram by UPGMA method, (I) accessions from Patagonia, Pampa, and one from Mesopotamia, ECpus11; and (II)

accessions from Mesopotamia (Entre Ríos and Isla Martín García). The numbers above the branches are bootstrap support values (%). B. Principal coordinate analysis. Accessions origin: Patagonia (black), Pampa (grey) and Mesopotamia (white).

Species	Accession	Region	Province/ Locality	Latitude/ Longitude	Altitude (m.a.s.l.)	Nº evaluated plants
Daucus pusillus/ montevidensis	ECpus1	Patagonia	Río Negro/ Choele Choel	39º 16' 36.73" S/ 65º 37' 08.70" W	196	4
	ECpus2	Patagonia	Chubut / Península de Valdes	42º 26' 53.76" S/ 64º 32' 27.86 "W	50	6
	ECpus7	Pampa	Buenos Aires / San Bernardo	36º 42' 27.12" S/ 56º 41' 14.20" W	7	13
	ECpus9	Mesopotamia	Entre Ríos/ Médanos - Route 11	33º 06' 01.46" S/ 59º 22' 19.97" W	15	12
	ECpus11	Mesopotamia	Entre Ríos/ Victoria	32º 37' 35.24" S/ 60º 11' 54.07" W	5	12
	ECpus12	Mesopotamia	Entre Ríos/ Concepción del Uruguay	32º 25' 01.32" S/ 58º 14' 20.14" W	18	13
	ECMCpus2	Pampa	Buenos Aires/ Abra El Pantanoso	38º 07' 20.47" S/ 61º 45' 33.68" W	261	14
	ECMCpus3	Pampa	Buenos Aires/ Cerro Bahía Blanca	38º 09' 35.13" S/ 61º 54' 44.81" W	660	15
	ECMCpus11	Mesopotamia	Other/ Isla Martín García	34º 10' 55.92" S/ 58º 15' 02.88" W	16	11
	ECMCpus12	Mesopotamia	Entre Ríos/ Médanos	33º 25' 37.91" S/ 59º 04' 46.66" W	6	11
	SIMacpus1	Pampa	Buenos Aires/ Mar Azul	37º 19' 51.39" S/ 57º 03' 36.77" W	9	14
	SIMacpus2	Pampa	Buenos Aires/ Punta Rasa	36º 18' 03.60" S/ 56º 46' 11.36" W	1	14
Daucus montanus	ECmon1	Patagonia	Neuquén/ Villa Traful	40º 39' 43.80" S/ 71º 24' 05.84" W	944	8
Daucus carota	ECMCcar16	Pampa	Buenos Aires/ General Lavalle	36° 24' 09.18" S/ 56° 57' 05.00" W	2	6

Stage	Morphological Character	Abbrev.	Units and scales
1	Stem length	Sl	cm
1	Leaf length	Ll	cm
1	Leaf blade width basal segment	Lbwb	mm
1	Leaf blade width prebasal segment	Lbwp	mm
1	Rachis percentage	Rp	% rachis between basal and prebasal segment/ leaf length
1	Petiole insertion width	Piw	mm
1	Stem pubescence	Sp	(0) glabrous, (1) slightly, (2) densely, (3) very dense
1	Leaf pubescence	Lp	(0) glabrous, (1) slightly, (2) densely, (3) very dense
1	Stem hair length	Shl	(1) short, (2) medium, (3) long
1	Leaf terminal foliole	Ltf	(1) asymmetric trifoliolate, (2) symmetric trifoliolate, (3) asymmetric bifoliolate.
1	Leaf terminal foliole shape	Ltfs	(1) short-thin, (2) medium-thin, (3) long-thin, (4) wide
2	Involucral bracts	Ib	number
2	Largest involucral bract length	Libl	mm
2	Largest involucral bract width	Libw	mm
2	Smallest involucral bract length	Sibl	mm
2	Smallest involucral bract width	Sibw	mm
2	Umbel area	Ua	$cm^2 = \pi$. major radius. minor radius
2	Involucral bract petiole membrane	Ibpm	(0) absence, (1) presence
3	Stem length 2	Sl2	cm
3	Primary branches	Pb	number
3	Secondary branches	Sb	number
3	Largest ray of umbel	Lru	mm
3	Terminal umbel peduncle	Tup	cm
3	Spines	S	number
3	Mericap width	Mw	cm
3	Mericarp length	Ml	cm
3	Spine length	Spl	cm
3	Umbel arrangement	Uar	(1) strongly compacted, (2) compacted, (3) intermediate, (4) half loose, (5) loose, (6) lax, (7) bird's nest
3	Mericarp appearance (based on spine morphology)	Ma	(1) variable, (2) short triangle spines (3) long thin spines, (4) short thin spines
3	Endosperm median transverse section	Emts	(1) trapezoid, (2) crescent, (3) arcuate

1

Molecular marker	Primer code	Sequence 5'-3'	Polymorphic bands (nº)	
			D. pusillus/ montevidensis accessions	All *Daucus* accessions
AFLP	PP1	M-CAT E-AAG	15	74
	PP2	M-CTG E-ACA	6	41
	PP3	M-CAA E-ACA	5	42
ISSR	MM8	(CA)6 GT	16	70
	MM14	(CGT)4 T	17	63
	MM31	CAA (CT)6	18	49
Total polymorphic bands			77	339

1

	D. montanus (ECmon1)		*D. carota* (ECMCcar16)		*D. pusillus/montevidensis* from Patagonia		*D. pusillus/montevidensis* from Pampa and Mesopotamia	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Quantitative characters								
Sl	14.04	± 2.57	101.58	± 17.79	32.25	± 11.36	51.69	± 11.05
Ll	26.64	± 1.67	34.10	± 2.60	11.33	± 1.58	15.60	± 2.97
Lbwb	84.03	± 10.44	98.50	± 12.88	34.03	± 8.40	53.99	± 10.48
Lbwp	73.01	± 8.16	98.75	± 11.38	30.78	± 5.00	47.82	± 10.06
Piw	9.39	± 1.14	13.70	± 1.35	6.06	± 1.23	7.97	± 1.01
Ib	7.00	± 0.53	10.67	± 1.97	6.30	± 1.25	9.54	± 1.96
Libl	34.42	± 4.69	32.78	± 1.95	36.41	± 7.12	30.74	± 5.96
Libw	23.33	± 3.25	29.60	± 6.01	20.47	± 4.88	20.97	± 5.27
Sibl	24.72	± 3.14	22.57	± 2.44	24.63	± 5.08	21.95	± 4.66
Sibw	14.55	± 2.04	14.32	± 5.11	11.37	± 3.41	11.70	± 3.64
Sl2	42.83	± 5.15	113.00	± 13.40	47.00	± 14.77	73.82	± 11.79
Pb	2.14	± 0.69	4.00	± 0.63	2.50	± 0.53	2.26	± 0.96
Sb	0.57	± 0.79	4.50	± 0.55	3.40	± 1.43	0.55	± 0.95
Lru	103.33	± 34.45	46.60	± 8.74	21.67	± 3.18	23.16	± 3.50
Tup	26.67	± 3.78	17.25	± 3.76	22.33	± 6.82	32.57	± 5.12
S	10.60	± 0.55	15.33	± 2.94	13.30	± 1.16	12.45	± 1.23
Mw	0.16	± 0.01	0.18	± 0.02	0.18	± 0.01	0.18	± 0.02
Ml	0.47	± 0.04	0.38	± 0.05	0.35	± 0.03	0.35	± 0.03
Spl	0.09	± 0.01	0.11	± 0.03	0.10	± 0.03	0.19	± 0.03
Ua	21781	± 8398	6259	± 3017	555	± 162	1250	± 312
Rp	16.70	± 2.38	13.63	± 2.17	16.05	± 2.68	16.60	± 2.00
Qualitative characters								
Sp	0		0, 1, 2		2, 3		2, 3	
Shl	0		0, 1, 2, 3		1, 2		1, 2, 3	
Lp	0		0, 1, 2		1, 2, 3		1, 2, 3	
Ltf	1		1		1, 2		1, 2, 3	
Ltfs	3		3, 4		1, 2, 3		1, 2, 3	
Ibpm	0		1		0		0	
Uar	6		7		4, 5		1, 2, 3, 4	
Emts	3		2		1		1	
Ma	4		3		1, 2		1	

Note: Abbrev.: Sl, Stem length; Ll, Leaf length; Lbwb, Leaf blade width basal segment; Lbwp, Leaf blade width prebasal segment; Piw, Petiole insertion width; Ib, Involucral bracts; Libl, Largest involucral bract length; Libw, Largest involucral bract width; Sibl, Smallest involucral bract length; Sibw, Smallest involucral bract width; Sl2, Stem length 2; Pb, Primary branches; Sb, Secondary branches; Lru, Largest ray of umbel; Tup, Terminal umbel peduncle; S, Spines; Mw, Mericap width; Ml, Mericarp length; Spl, Spine length; Ua, Umbel area; Rp, Rachis percentage; Sp, Stem pubescence; Shl, Stem hair length; Lp, Leaf pubescence; Ltf, Leaf terminal foliole; Ltfs, Leaf terminal foliole shape; Ibpm, Involucral bract petiole membrane; Uar, Umbel arrangement; Emts, Endosperm median transverse section; Ma, Mericarp appearance.

Source of variation	df	Sum of squares	Variance components	% variation	*p*
Patagonian vs. the rest of accessions					
Among geographical regions	1	64.099	-0.05471	-0.42	0.43891
Among accessions within geographical regions	10	1379.094	10.92489	84.71	0.00000
Among individuals within accessions	131	265.506	2.02676	15.72	0.00000
Patagonian + Pampean vs. Mesopotamian accessions					
Among geographical regions	1	485.652	5.40754	35.06	0.00000
Among accessions within geographical regions	10	957.541	7.98843	51.80	0.00000
Among individuals within accessions	131	265.506	2.02676	13.14	0.00000

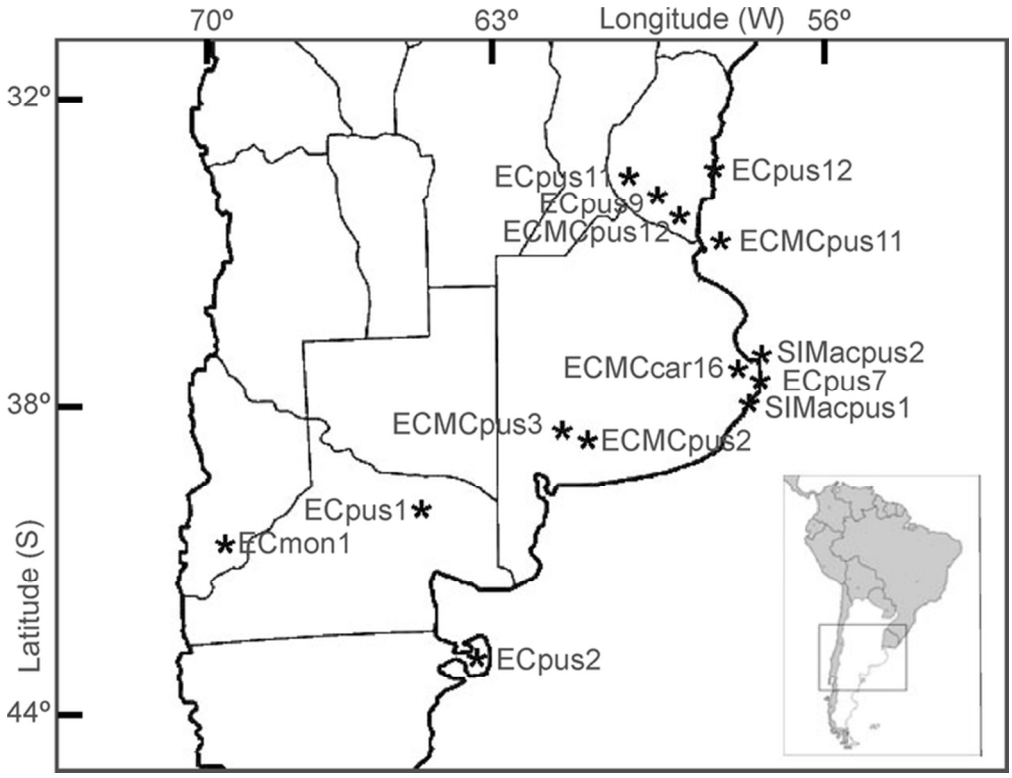

Fig. 1. Geographical distribution of wild *Daucus* accessions from Argentina used in this study.
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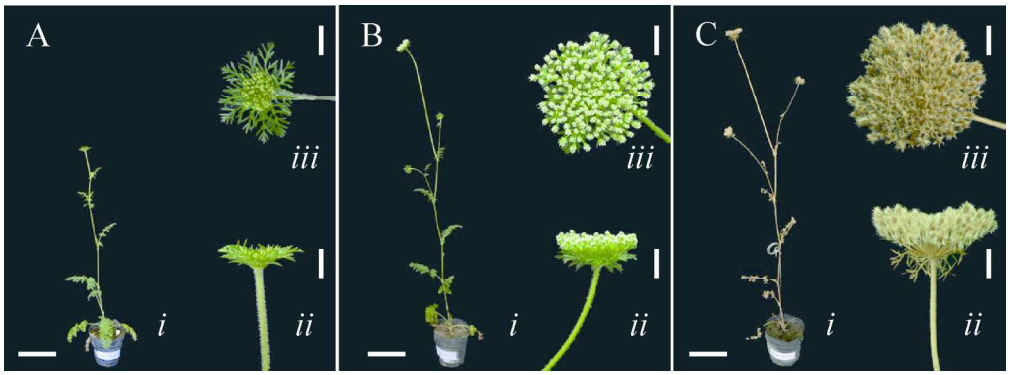

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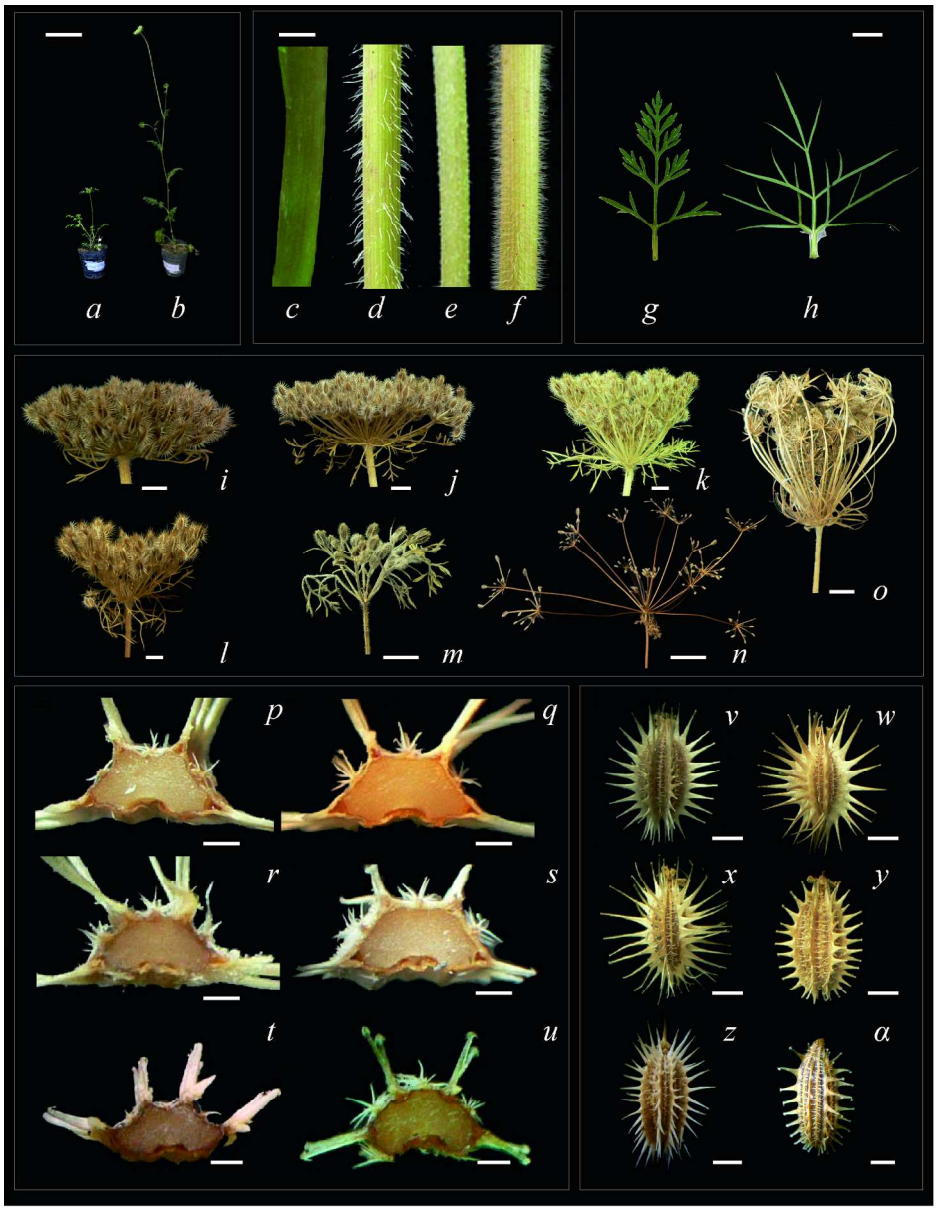
182x66mm (300 x 300 DPI)



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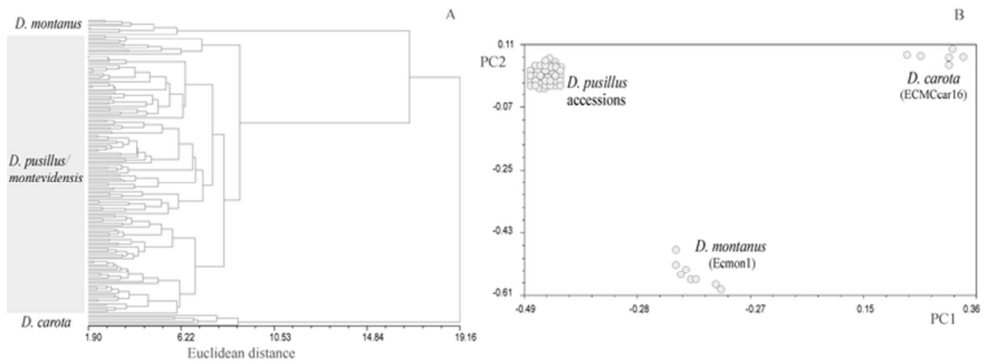
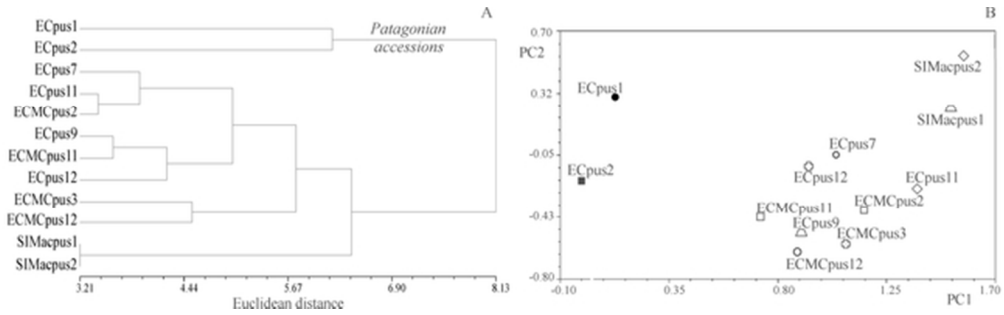


Fig. 4. Combined analysis based on individual values for 30 morphological characters and 339 polymorphic bands of molecular markers (AFLP, ISSR), in 14 accessions of wild *Daucus* from Argentina. A. Dendrogram based on Euclidean similarity coefficient by the UPGMA method. B. Principal component analysis accounting for 72% of the variation.
65x23mm (300 x 300 DPI)



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54x16mm (300 x 300 DPI)

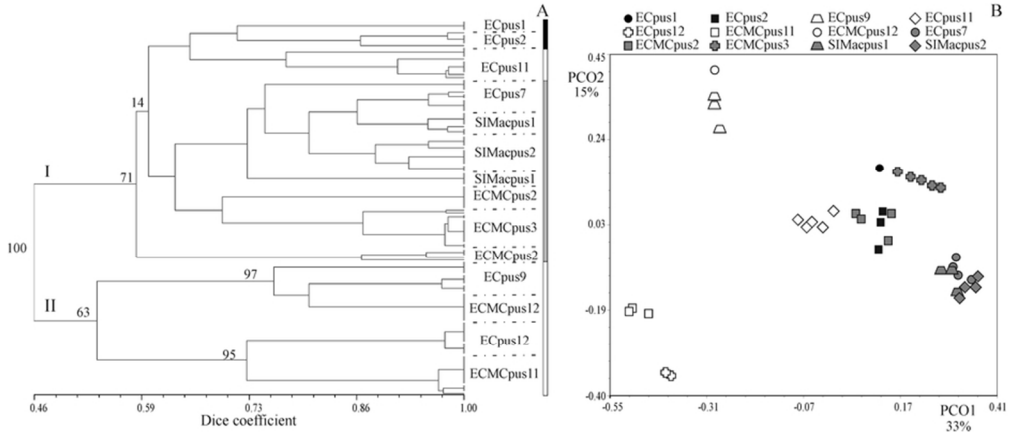

Fig. 6. Molecular analysis of 12 *Daucus pusillus/montevidensis* accessions from Argentina based on individual observations of 77 polymorphic bands of AFLP and ISSR markers using Dice coefficient. A. Dendrogram by UPGMA method, (I) accessions from Patagonia, Pampa, and one from Mesopotamia, ECpus11; and (II) accessions from Mesopotamia (Entre Ríos and Isla Martín García). The numbers above the branches are bootstrap support values (%). B. Principal coordinate analysis. Accessions origin: Patagonia (black), Pampa (grey) and Mesopotamia (white).
79x34mm (300 x 300 DPI)

Table 1S. Range of quantitative morphological characters used to evaluate wild *Daucus* accessions from Argentina.

	ECmon1		ECMCcar16		ECpus1		ECpus2		ECpus7		ECpus9		ECpus11		ECpus12		ECMCpus2		ECMCpus3		ECMCpus11		ECMCpus12		SIMacpus1		SIMacpus2	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Sl	11.00	19.00	81.50	129.50	38.00	46.50	17.00	39.00	37.00	77.00	34.50	62.50	40.50	65.00	47.50	57.50	30.50	58.50	50.00	63.00	30.50	49.50	30.50	49.50	42.00	85.50	44.00	80.00
Ll	24.50	29.00	29.00	36.00	10.65	12.95	7.97	13.15	11.80	16.20	10.90	16.30	12.80	19.80	12.60	17.60	12.23	20.10	16.40	20.50	9.62	14.00	9.62	14.00	16.20	25.00	13.40	20.60
Lbwb	65.90	94.80	87.00	120.30	21.65	35.30	24.00	44.00	34.00	53.90	35.70	61.60	37.30	74.00	45.00	76.30	45.00	69.50	40.20	69.20	29.45	65.60	29.45	65.60	48.20	95.00	41.80	65.00
Lbwp	62.25	87.85	84.80	114.80	23.50	29.20	26.60	39.00	35.00	49.60	30.50	51.20	36.30	62.70	39.00	64.10	43.10	68.70	38.20	69.30	30.20	50.40	30.20	50.40	40.00	73.40	31.00	59.00
Piw	8.00	11.40	11.60	15.00	4.40	5.90	5.40	8.70	6.50	10.00	5.50	9.70	6.80	10.30	6.20	9.00	6.00	9.80	6.90	9.10	5.70	8.30	5.70	8.30	7.40	10.40	7.60	9.70
Ib	6.00	8.00	9.00	14.00	6.00	7.00	5.00	9.00	8.00	12.00	6.00	9.00	9.00	13.00	7.00	9.00	7.00	11.00	9.00	12.00	5.00	8.00	5.00	8.00	10.00	15.00	11.00	14.00
Libl	26.30	40.60	30.00	34.90	28.50	36.80	31.90	48.00	25.00	45.50	28.80	40.60	25.00	34.90	25.20	39.60	24.30	39.00	27.00	46.40	24.80	39.50	24.80	39.50	21.40	30.70	17.60	31.60
Libw	18.25	27.45	23.60	37.80	17.20	24.00	11.60	30.00	14.00	29.30	21.90	30.80	14.60	26.80	16.20	29.20	15.50	32.20	15.50	28.60	15.50	32.30	15.50	32.30	12.80	22.20	10.20	19.60
Sibl	20.20	29.40	20.40	26.10	19.10	20.80	22.80	33.60	17.20	34.00	18.00	31.30	16.20	25.20	17.10	28.80	15.70	30.00	19.20	32.50	19.00	33.60	19.00	33.60	15.50	22.80	10.50	20.70
Sibw	11.80	17.10	6.70	21.20	8.50	11.30	8.70	20.00	5.20	20.00	8.00	17.40	7.90	19.80	6.20	16.00	9.00	18.70	8.80	18.90	7.40	24.50	7.40	24.50	7.80	12.40	5.00	13.10
Sl2	37.00	49.00	94.00	135.00	55.50	62.50	25.00	58.00	50.50	87.50	54.50	86.50	62.00	89.50	62.00	78.00	48.00	93.00	65.00	89.00	50.50	85.00	50.50	85.00	67.00	105.00	60.00	99.50
Pb	1.00	3.00	3.00	5.00	2.00	3.00	2.00	3.00	1.00	3.00	1.00	3.00	1.00	4.00	1.00	3.00	2.00	6.00	1.00	3.00	1.00	3.00	1.00	3.00	1.00	4.00	2.00	6.00
Sb	0.00	2.00	4.00	5.00	2.00	4.00	1.00	6.00	0.00	1.00	0.00	2.00	0.00	0.00	0.00	4.00	0.00	4.00	0.00	1.00	0.00	2.00	0.00	2.00	0.00	0.00	0.00	1.00
Lru	70.00	160.00	38.00	58.00	21.80	27.60	16.70	22.40	18.40	29.80	19.90	25.90	21.50	29.20	20.00	30.30	21.20	31.20	23.60	30.80	17.30	25.00	17.30	25.00	16.30	22.90	18.30	23.40
Tup	22.00	30.00	13.00	23.00	23.00	30.00	12.00	30.00	25.00	40.50	27.50	47.00	30.00	39.00	19.50	36.50	26.50	41.50	29.00	40.50	23.00	42.50	23.00	42.50	23.00	38.00	22.00	41.00
S	10.00	11.00	12.00	20.00	13.00	14.00	11.00	15.00	11.00	15.00	11.00	13.00	11.00	12.00	12.00	15.00	11.00	13.00	13.00	15.00	10.00	12.00	10.00	12.00	11.00	13.00	11.00	16.00
Mw	0.15	0.18	0.15	0.20	0.16	0.19	0.17	0.19	0.13	0.19	0.16	0.19	0.14	0.18	0.18	0.21	0.15	0.18	0.16	0.20	0.16	0.22	0.16	0.22	0.15	0.19	0.16	0.19
Ml	0.40	0.50	0.31	0.41	0.30	0.38	0.32	0.38	0.28	0.38	0.33	0.37	0.30	0.39	0.34	0.38	0.26	0.38	0.35	0.39	0.32	0.39	0.32	0.39	0.32	0.39	0.33	0.38
Spl	0.09	0.10	0.08	0.16	0.10	0.15	0.07	0.12	0.14	0.21	0.18	0.25	0.14	0.24	0.19	0.21	0.15	0.19	0.12	0.17	0.16	0.22	0.16	0.22	0.16	0.22	0.20	0.24
Ua	11902	33938	2671	9807	435	592	367	917	665	1713	911	2075	899	1928	919	1847	857	2304	1102	1691	517	1444	517	1444	757	1630	704	1728
Rp	12.41	19.71	11.44	16.84	12.33	14.44	16.03	20.70	14.44	22.17	11.73	17.56	13.83	20.12	13.02	18.26	16.63	18.57	15.78	21.07	14.38	19.21	14.38	19.21	14.46	18.21	13.64	21.28

Note: Abbrev.: Sl, Stem length; Ll, Leaf length; Lbwb, Leaf blade width basal segment; Lbwp, Leaf blade width prebasal segment; Piw, Petiole insertion width; Ib, Involucral bracts; Libl, Largest involucral bract length; Libw, Largest involucral bract width; Sibl, Smallest involucral bract length; Sibw, Smallest involucral bract width; Sl2, Stem length 2; Pb, Primary branches; Sb, Number of secondary branches; Lru, Largest ray of umbel; Tup, Terminal umbel peduncle; S, Spines; Mw, Mericap width; Ml, Mericarp length; Spl, Spine length; Ua, Umbel area; Rp, Rachis percentage.