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ORIGINAL ARTICLE

Characterization and distinction of two subspecies of *Eryngium duriaei* J. Gay ex Boiss., an Iberian endemic Apiaceae, using flow cytometry and essential oils composition

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Abstract *Eryngium duriaei* (Apiaceae) is an endemic taxa from Iberian Peninsula. Some doubts exist in the literature about the taxa relationships, especially among Iberian populations at different altitudes. Also, as other Apiaceae, this species presents a large potential for essential oil production. Considering all this, a multidisciplinary study comprising taxonomic, cytological (using flow cytometry and chromosome counts) and chemical (essential oils) analyses was performed with the objective to morphologically characterize this species and to evaluate the cytotaxonomical and chemical diversity of *E. duriaei* Portuguese populations. FCM and chromosome counts have shown that every individual presented the same ploidy level, i.e., $2n = 2x = 16$ chromosomes. However, flow cytometric analyses revealed that individuals of *E. duriaei* from higher altitudes (»1,700 m) presented a significantly higher genome size than those belonging to *E. duriaei* populations below 1,700 m ($2C = 6.20 \pm 0.04$ vs. $2C = 5.52 \pm 0.05$ pg). Moreover, the essential oils analyses revealed that most chemical constituents were sesquiterpenes, but relevant differences in the main components were observed: α-neocallitropsene (28–53 %), β-betulenal (8.5–15.8 %) and 14-hydroxy-β-caryophyllene (5.8–13.7 %) were the main compounds of *Eryngium duriaei* oil below 1,700 m, whereas caryophyllene oxide (47 %) and *E*-caryophyllene (6 %) were the major compounds of *E. duriaei* oil of higher altitude populations. The results provide important evidences to support the taxonomic separation of *E. duriaei* in two *taxa*: *E. duriaei* J. Gay ex Boiss. subsp. *duriaei* and *E. duriaei* subsp. *juresianum* (M. Laínz) M. Laínz, as previously considered by this author.

Keywords *Eryngium duriaei* · Chromosome counting · Essential oils · Genome size · Taxonomy

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Introduction

The genus *Eryngium* L. belongs to the Apiaceae and it is distributed practically all over the world. According to Nieto Feliner (2003) it includes about 250 species mainly distributed in temperate regions of North, Central and South America (Calviño et al. 2008, 2010). Two centers of diversity are recognized in each hemisphere: one in central-west Mexico and central-east South America and the other in southwest Asia and western Mediterranean, where *Eryngium* occurs in dry, rocky areas (Calviño et al. 2008).

The wide distribution of the genus and the strong morphological and chemical diversity of *Eryngium* species in terms of height, structure and essential oils composition seem to point out to a very old origin of this *taxon* (Berenbaum 2001; Bylebyl et al. 2008; Fidelis et al. 2008; Perthuy et al. 2010; Paul et al. 2011). *Eryngium* can be easily distinguished from other Apiaceae by their capitate inflorescences surrounded by bracts and by the presence of a single bracteole per flower. Data from dispersal-

vicariance analyses indicated that the most likely candidates for its origin are western Mediterranean subgenera: *Eryngium* subgen. *Eryngium* and *Eryngium* subgen. *Monocotyloidea* (Calviño et al. 2008). The first taxon includes all the species from the Old World (Africa, Europe and Asia), while the latter includes all the species of the New World (North, Central and South America, and Australia). However, as stressed out by Calviño et al. (2008, 2010) the interpretation of the evolutionary history of *Eryngium* remains extremely difficult, as it combines several complex processes, such as rapid radiations, reticulate evolution and long distance dispersals.

Twenty-six species of *Erygium* are referred in Flora Europaea (Tutin et al. 1968) from which sixteen have been described for the Iberian Peninsula (Castroviejo et al. 2003). Seven of the sixteen *Eryngium* Iberian species are present in both countries; eight only in Spain and one species is exclusive to Portugal (Nieto Feliner 2003). Two of these species, *E. galioides* and *E. duriaei*, are Iberian endemisms (Castroviejo et al. 2003; Gimenez et al. 2004). Endemic species have become a main concern for plant conservationists, as these rare species, with restricted geographic distributions are endangered by several factors such as over-exploitation, global warming and habitat destruction (Malcom et al. 2006; Hawkins et al. 2008). Yet, for the establishment of effective conservation practices it is essential to understand the genetic variation within and among populations, as pointed out by the studies of Qiu et al. (2004) on the genetic variation of the endangered and endemic species of *Changium smyrnioides,* an aromatic Apiaceae. Furthermore, within aromatic plants, the chemical characterization and the taxonomic correlations are also important issues to assess (Nogueira et al. 2008; Pála-Paúl et al. 2010; Paul et al. 2011).

Eryngium duriaei is an outcrossing, insect-pollinated, herbaceous species displaying $x = 8$ as the most common basic chromosome number, although lower numbers (i.e., $x = 5$–7) have also been reported (O'Leary et al. 2004). Different ploidy levels are also common within the genus (Perthuy et al. 2010). Several species of *Eryngium* have been previously studied concerning the chemistry, diversity and properties of their essential oils (Ayoub et al. 2003, 2006; Pála-Paúl et al. 2005, 2010; Cavaleiro et al. 2011; Darriet et al. 2012).

Like other Apiaceae, *E. duriaei* is an aromatic species whose essential oils may have some practical applications as reported for other members of this family (Tavares et al. 2008, 2010; Gonçalves et al. 2012a, b). However, the available data concerning the composition and bioactivities of *E. duriaei* essential oils are scarce. Moreover, the information about the different populations that have been reported in Portugal is quite limited and doubts exist about the *taxa* relationships and the taxonomical level. Indeed, Jansen (1998) and Nieto Feliner (2003) pointed out some morphological differences among Iberian populations of *E. duriaei*.

Considering the gaps in the literature about this species, its potential for essential oil production, and its endemic status, a multidisciplinary study comprising taxonomic, cytological and chemical analyses was performed with the objective to morphologically characterize this species and to evaluate the cytotaxonomical and chemical diversity of *E. duriaei* Portuguese populations.

Materials and methods

Plant material

All plant material was sustainably collected from four populations of *E. duriaei* growing in different provinces and altitudes (Figs. 1, 2). Table 1 gives further details about the areas where the plants were collected. Voucher specimens from all the collected material were deposited at the Herbarium of the University of Coimbra (COI) (Table 1).

Plant morphological analysis

To evaluate the taxonomic relationships within four Portuguese populations of *E. duriaei*, some morphological features were selected, such as, plant height, spinescency (soft or hard) and the morphology of the basal leaves.

Cytological characterization

The genome size of up to 13 individuals per population of different altitudes (above and below 1,700 m) was estimated using flow cytometry. The procedure described by Loureiro et al. (2007a, b) was used. Briefly, nuclei were released after co-chopping 0.5 cm^2 of fresh leaf tissue of *E. duriaei* together with 0.5 cm^2 of fresh leaf tissue of *Pisum sativum* (internal reference standard with $2C = 8.76$ pg) with a sharp razor blade in a plastic Petri dish containing 1 ml of WPB (0.5 mM spermine.4HCl, 30 mM sodium citrate.$3H_2O$, 20 mM MOPS, 80 mM KCl, 20 mM NaCl, 0.5 % (w/v) Triton X-100) with the pH adjusted to 7.0. The nuclear suspension was then filtered through a 50 mm nylon filter to remove cell fragments and large debris. Afterwards, nuclei were stained with 50 mg ml^{-1} propidium iodide (PI) (Fluka, Buchs, Switzerland), and 50 mg ml^{-1} RNase (Sigma, St Louis, MO, USA) was added to the nuclear suspension to prevent staining of double-stranded RNA. After a 5 min incubation period, the relative fluorescence intensity (FL) of at least 1,500 nuclei per G_1 peak was analyzed in a Partec CyFlow Space flow cytometer (Partec GmbH., Münster, Germany),

Fig. 1 Areas where *Eryngium duriaei* populations were analyzed: *NPG* (National Park of Gerês); *NPSE* (National Park of Serra da Estrela) and two areas in Serra do Açor (*A* Colcurinho and *B* Margaraça)

equipped with a green solid state laser for PI excitation, using the FloMax software (Partec GmbH). The G_1 peak of the standard was set to channel 200, and then the amplification system was set to a constant voltage and gain throughout the experiment. The resulting histograms were evaluated and the genome size of each sample was determined using the following formula: Mean FL of sample's G_1 peak/Mean FL of standard's G_1 peak) × genome size of the standard.

Only histograms with symmetrical peaks and with a coefficient of variation (CV) of the sample's G1 peak below 5 % were considered. To confirm the reliability of the estimated C-values, simultaneous analyses of the individuals from the populations with more dissimilar genome sizes were performed.

Chromosome counts were carried out in root tips from seedlings germinated in vitro. Mature seeds from plants growing in Serra da Estrela and Mata da Margaraça were sterilized in a calcium hypochlorite solution (7 % w/v) with 2–3 drops of Tween 20, for 20 min under stirring, and washed 3 times with sterilized distilled water. Zygotic embryos isolated from sterilized seeds were cultured on half-strength MS basal medium (Murashige and Skoog 1962). Root tips (5 mm length) of seedlings (20–30 days after germination) were cut and treated with colchicine 0.05 % (w/v) for 2–2.5 h, in the dark and then fixed in 3:1 (v/v) ethanol/acetic acid for approximately 4 h at room temperature. The meristematic zone of the root (apical 1–2 mm) was cut and the Feulgen technique was applied (Darlington and La Cour 1976). Samples were hydrolyzed in 1 N HCl at 60 °C for 6 min in a water bath stained in Schiff reagent for 1–3 h in the dark and squashed in 45 % acetic acid. Microscopic slides were observed using a Nikon Eclipse E400 microscope and images registered with a Nikon Digital Sight DS-U1 camera using the Act-2U software.

Isolation and characterization of essential oils

Air-dried stems from field-growing plants at the stage of fruit ripening were used for essential oil extraction. Oils were isolated by hydrodistillation for 3 h using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe 1997). When necessary, the oils were stored in dark vials at 4 °C for further assays.

Analyses of the volatile compounds were carried out by both gas chromatography (GC) and gas chromatography/mass spectrometry (GC–MS), using fused silica capillary columns with two different stationary phases (SPB-1 and SupelcoWax-10), as previously reported (Tavares et al. 2010).

The volatile compounds were identified by both their retention indices and their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of a series of *n*-alkanes, were compared with those of authenticated samples from the database of the Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Coimbra. Mass spectra were compared with reference spectra from a home-made library or from literature data (Adams 1995; Joulain and Konig 1998). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

Statistical analysis

Genome size data were analyzed using an one-way ANOVA. Differences in mean genome size values among

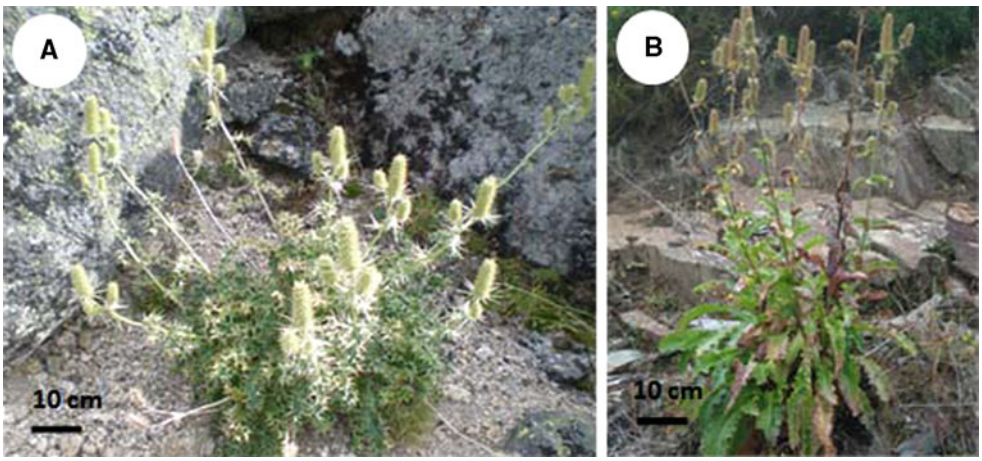

Fig. 2 Plants of *Eryngium duriaei*. **a** Plants growing in the NPSE, **b** plants growing in Colcurinho

Table 1 Places, altitudes and dates for the collected *Eryngium duriaei* populations

Places	Voucher—COI herbarium
Natural Park Serra da Estrela, Cântaro Raso (>1.700 m)	Beira Alta, Serra da Estrela, Cântaro Raso, 1,700 m, 16.09.2008, *A.C. Tavares,* 112 (COI). Beira Alta, Serra da Estrela, Cântaro Raso, 1,700 m, 11.07.2010
Serra do Açor, Colcurinho (800–1,000 m)	Beira Litoral, Serra do Açor, between Colcurinho and Senhora das Necessidades, 30.06.2005, *A.C. Tavares*, 8 (COI); 17.07.2006, *A.C. Tavares,* 54 (COI); 20.09.2008, *A.C. Tavares*, 113 (COI); 19.06.2010, *A.C. Tavares*, 136 (COI)
Serra do Açor, Mata da Margaraça (600–800 m)	Beira Litoral, Arganil, Mata da Margaraça, 08.10.2010, *A. C. Tavares*, 143 (COI)
Natural Park Serra do Gerês, Mata da Albergaria (800–900 m)	Minho, Serra do Gerês, Mata da Albergaria, 26.07.2007, *A. C. Tavares,* 74 (COI). Minho, Serra do Gerês, Mata da Albergaria, edges of Homem river, 04.10.2010

populations of each subspecies were evaluated using the multiple comparison Tukey test (SigmaPlot 12.2, SyStat Software).

Results and discussion

Morphological characterization

Morphological comparisons showed no differences between the three populations of lower altitudes (<1,700 m, Table 1). However, when these populations were compared with populations growing above 1,700 m (Fig. 2a) some morphological differences were found. Thus, plants growing at higher altitudes were usually spinosous herbs, of 30–60 cm tall, with narrower basal leaves, linear-oblanceolate, undulate, pinnatifite, showing a regularly sinuate-dentate margin, whereas the plants growing at lower altitudes were soft spinousous herbs often reaching 100 cm, possessing the basal leaves more or less linear, spathulate, and with a denticulate margin (Fig. 2b).

Based mainly on these morphological differences, Laínz considered two different *taxa* (Laínz 1965, 1967) at the specific level (Laínz 1965): *E. juresianum* (M. Laínz) M. Laínz and *E. duriaei* J. Gay ex Boiss. In a further work the same author established two subspecies for *E. duriaei: E. duriaei* subsp. *juresianum* and *E. duriaei* subsp. *duriaei* (Laínz 1969). This classification has been followed by other authors (Jansen et al. 2000; Pinto da Silva and Teles 1999). Our data seem to indicate that the *Eryngium duriaie* populations of higher altitudes (Serra da Estrela) correspond to *E. duriaei* subsp. *duriaei* whereas the others belong to *E. duriaei* subsp. *juresianum.*

Flow cytometry and chromosome counting

The genome size of the four populations of *E. duriaei* was estimated using flow cytometry (Table 2). The variation in this character among individuals of the same population was very low and not significant; differences in genome size were detected between populations growing at lower altitudes and the population of Serra da Estrela. Plants of this last area showed a significantly larger nuclear DNA content ($2C = 6.21 \pm 0.05$ pg/DNA) than the other populations ($2C = 5.68 \pm 0.07$ pg/DNA). This ≈ 9 % difference was confirmed to be genuine, as two clear distinct peaks were repeatedly observed in simultaneous analysis of both populations (Fig. 3). Despite the observed differences

Table 2 Genome size estimations of *Eryngium duriaei* populations

Population	2C (pg)	Min.	Max.	1Cx (Mbp)	*n*
Colcurinho	5.66 ± 0.08[b]	5.54	5.78	2,768	13
Gerês	5.73 ± 0.05[b]	5.67	5.79	2,802	4
Margaraça	5.68 ± 0.05[b]	5.63	5.79	2,778	8
Estrela	6.21 ± 0.05[a]	6.14	6.32	3,037	9

The values are given as mean and standard deviation of the mean of the holoploid nuclear DNA content (2C in pg) of individuals of each population. The minimum (Min.) and maximum (Max.) values obtained for each population, as well as, the monoploid nuclear DNA content (1Cx) in Mbp, and the number of analyzed individuals (*n*) are also provided for each population. The different letters (a or b) represent significant differences ($P < 0.05$) according to a multiple comparison using the Tukey test. 1 pg DNA = 978 Mbp

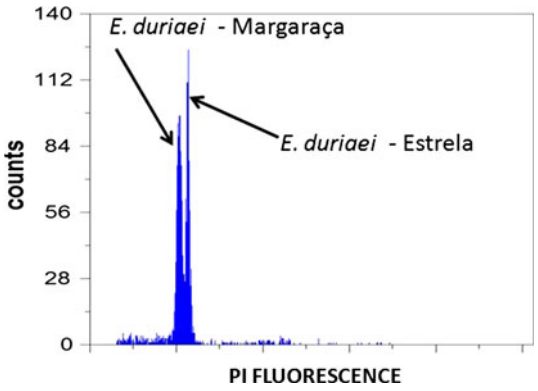

Fig. 3 Histogram of PI fluorescence (in relative units) obtained after simultaneous analysis of nuclei isolated from leaves of *Eryngium duriaei* from an individual collected at Margaraça and other gathered at Serra da Estrela

in genome size, karyological analyses of individuals from two populations of different altitudes showed that both presented the same number of chromosomes, $2n = 2x = 16$ (Fig. 4), which is the most common number within the genus (Nieto Feliner 2003). Considering cytological data and the observed morphological differences, it seems that two distinct homoploid entities occur, i.e., *taxa* with the same number of chromosomes but different amounts of nuclear DNA. Only recently, with the advancement of flow cytometry, it started to be possible to resolve the taxonomy of homoploid groups. One of the most remarkable examples on homoploid groups with some taxonomic relevance is the genus *Helleborus* (Zonneveld 2001). All the species from this genus have 32 chromosomes, but the largest genome is nearly twice as large as that of the smallest, and thus, genome size can be a diagnostic tool to distinguish several species. Furthermore, the observed variation fitted very well with the sectional division. If there are numerous examples at genus level (e.g. Leong-Skornickova et al. 2007; Zonneveld et al. 2003; Zonneveld and van Jaarsveld 2005), below species level as it seems to be this case, fewer cases can be found in the literature. For example, Loureiro et al. (2007a) was able to distinguish *Festuca ampla* subsp. *ampla* from subsp. *transtagana* due to its 5 % smaller genome, despite having the same number of chromosomes. Differences in genome size are known to be largely caused by different amounts of non-coding repetitive DNA, to which transposable elements, satellite DNA, introns and pseudogenes can contribute (Bennett and Leitch 2005).

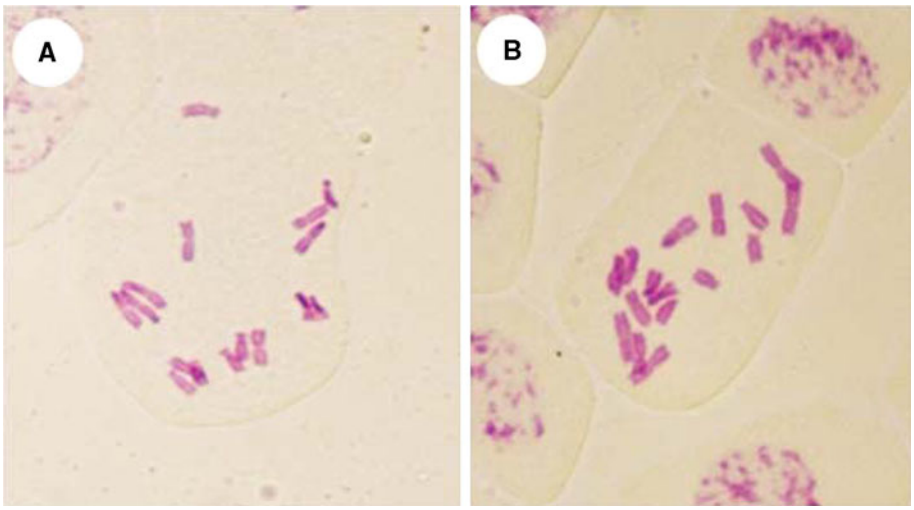

Fig. 4 Karyological analyses of *Eryngium duriaei* showing 16 chromosomes in c-metaphase root seedlings either from Mata da Margaraça (**a**) or from Serra da Estrela (**b**). Chromosomes are amplified ×840

Table 3 Composition of the volatile oil of *Eryngium duriaei*

RI[a]	RI[b]	Compounds	Colcurinho (<1,700 m)	Gerês (<1,700 m)	Margaraça (<1,700 m)	Serra Estrela (»1,700 m)
929	1030	α-Pinene	0.7	0.4	0.2	t
978	1290	*n*-Octanal	0.4	0.6	0.2	1.8
978	1161	Myrcene	t	t	t	t
1019	1206	Limonene	t	t	t	1.2
1024	1237	*Z*-β-Ocimene	0.3	0.6	0.8	–
1034	1255	*E*-β-Ocimene	0.8	0.3	t	–
1079	1393	*n*-Nonanal	t	t	t	–
1181	1495	*n*-Decanal	0.7	t	t	0.3
1235	n.d	2-Decenal	0.1	t	t	0.5
1327	n.d.	Bicycloelemene	t	t	t	t
1369	1493	α-Copaene	–	–	–	1.4
1376	1518	β-Bourbonene	–	–	–	0.4
1386	1585	β-Elemene	0.7	0.3	0.5	2.9
1386	n.d.	*n*-Dodecanal	t	t	0.1	t
1408	1595	*E*-β-Caryophyllene	6.6	12.2	5.8	6.0
1440	1665	α-Humulene	0.3	0.4	0.4	1.4
1444	1665	*E*-β-Farnesene	0.4	0.2	0.4	0.5
1461	1685	α-Neocallitropsene	28.0	32.3	53.0	–
1464	1680	γ-Muurolene	–	–	–	0.4
1468	1676	γ-Curcumene	0.8	0.7	0.4	–
1468	1699	Germacrene D	–	–	–	5.6
1471	1711	β-Selinene	3.1	2.4	1.5	3.0
1481	1727	Bicyclogermacrene	3.4	3.8	2.4	–
1483	1714	α-Selinene	–	–	–	1.2
1491	1752	Germacrene A	–	–	–	0.6
1503	n.d.	Z-Calamelene	–	–	–	0.5
1508	1749	δ-Cadinene	–	–	–	1.8
1521	1904	α-Calacorene	–	–	–	0.5
1556	1969	Caryophyllene oxide	7.4	7.3	4.1	47.3
1556	2112	Spathulenol	1.0	0.2	0.5	–
1561	2063	Globulol	0.5	0.2	0.5	–
1567	1992	Salvial-4(14)-en-1-one	–	–	–	1.2
1583	2022	Humulene oxide	1.1	0.4	0.4	4.1
1615	2145	Isocaryophyllen-14-al (β-Betulenal)	15.8	15.8	8.5	1.1
1631	2214	α-Cadinol	–	–	–	1.7
1637	2344	14-Hydroxy-β-caryophyllene	13.7	6.6	5.8	–
		Total	86.1	85.0	85.9	85.6

Compounds listed in order of elution from the *Supelco SPB-1* column

[a] GC-retention indices relative to C_9–C_{23} *n*-alkanes on the *Supelco SPB-1* column

[b] GC-retention indices relative to C_9–C_{23} *n*-alkanes on the *SupelcoWax-10* column

t traces (≤0.05 %)

Chemical characterization of the essential oils

Essential oils of individuals from the four populations were extracted with yields of 0.2–0.3 % (v/w). Constituents of the essential oils are listed in Table 3 according to their elution on a polydimethylsiloxane column. The oils are predominantly composed by sesquiterpenic hydrocarbons and oxygen-containing sesquiterpenes. In all samples the caryophyllane derivates are important compounds. Nevertheless, significant differences were found in the main constituents of populations growing at different altitudes (Table 3). The oils obtained from plants collected in lower

altitudes were characterized by the presence of high amounts of α-neocallitropsene (28–53 %), β-betulenal (8.5–15.8 %) and 14-hydroxy-β-caryophyllene (5.8–13.7 %) whereas the oil of Serra da Estrela populations (above 1,700 m) was characterized by high levels of caryophyllene oxide (47.3 %) and *E*-caryophyllene (6 %). Moreover, α-neocallitropsene, the major compound of the oil of plants from lower altitudes, is not present in the oil of plants from higher altitudes. This compound can be considered as a taxonomic marker that helps to distinguish these populations. Furthermore, as far as we know, this compound was not previously identified in other *Eryngium* species and in a recent study of *Eryngium aquifolium* from Spain no specific compounds could be used as chemotaxonomic markers for this *taxon* (Pála-Paúl et al. 2010). In the material of *E. duriaei* from Serra da Estrela (above 1,700 m) no variation in the oil composition between the populations of this region it was found, being the oil always characterized by high levels of caryophyllene oxide. Several studies on *Eryngium* species have signaled the presence of this compound in essential oils although in lower amounts (Capetanos et al. 2007; Pála-Paúl et al. 2005, 2006, 2010; Rodriguez et al. 2002; Paul et al. 2011). Some other species of *Eryngium* have also shown compositions dominated by sesquiterpenes, some of them uncommon, like muurol-9-en-15-al, cadina-9-en-15-al and cadina-9-en-15-ol in the oil of *E. maritimum* (Darriet et al. 2012), and eryng-9-en-15-al in the oil of *Eryngium creticum* (Ayoub et al. 2003). High amounts of sesquiterpenes are not very usual in the Apiaceae family (Picman 1986).

Conclusions

The present results confirm the earlier observation of Laínz (1965, 1969) who divided the species *E. duriaei* into the subspecies *duriaei* and *juresianum*. Our data indicate that these subspecies are different not only in morphological characteristics but also in genome size and essential oils composition, and occupy different ecosystems, with *E. duriaei* subsp. *duriaei* thriving at higher altitudes (Serra da Estrela) and *E. duriaei* subsp. *juresianum* growing at lower altitudes. How these differences have emerged during evolution and their ecological significance are questions that need to be clarified by further studies involving crossing and progeny analysis between the two subspecies. These differences, in particular those related with nuclear DNA content, seem to be genetically determined and might have appeared due the geographical isolation of the population at higher altitude. Differences in oil composition may also be genetically determined. However, it is well known that climatic and soil factors can affect essential oil composition. Thus, more studies need to be carried out to determine whether the differences found in oil composition can be ascribed to genetic factors or are more environmental related. In this way, the analysis of populations growing at altitudes between 1,000 and 1,700 m would be useful. Unfortunately, no known locations are available for populations growing at these altitudes.

This work also shows how relevant modern techniques of chemical and genomic analysis are for helping in taxonomic studies.

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