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Assessment of *Daucus carota* L. (Apiaceae) subspecies by chemotaxonomic and DNA content analyses

Ana Cristina Tavares [a,b,1,2], João Loureiro [b,2], Sílvia Castro [b,2], António P. Coutinho [b,2], Jorge Paiva [b,2], Carlos Cavaleiro [c,3], Lígia Salgueiro [c,3], Jorge M. Canhoto [b,*]

[a] Department of Life Sciences/CEF and CNC, University of Coimbra, Ap 3046, 3001-401 Coimbra, Portugal
[b] CFE, Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, PO Box 3046, 3001-401 Coimbra, Portugal
[c] Faculty of Pharmacy/CEF and CNC, Azinhaga de Santa Comba, University of Coimbra, 3000-548 Coimbra, Portugal

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ABSTRACT

For a clearer distinction between the four subspecies of *Daucus carota* native from Portugal (subsp. *carota*, subsp. *maximus*, subsp. *gummifer* and subsp. *halophilus*), morphological features of the fruits, DNA content analyses by flow cytometry, and chemical characterization of the essential oils were undertaken.

We found chemotaxonomic evidences to consider *D. carota* subsp. *maximus* as a separate species rather than a subspecies of *D. carota*. This separation is based on the morphometric analysis of the fruits and in the high levels of asarone present only in the essential oil of the subsp. *maximus*. The remaining subspecies are difficult to distinguish from each other based on the morphology of the fruits and in DNA content. However, based on the essential oils, it was possible to distinguish the subspecies *halophilus* from the other two (subsp. *gummifer* and subsp. *carota*) because of its high content of elemicin, with the other two having high levels of geranyl acetate.

Based on these results, the subspecies *maximus* is proposed as a different species (*Daucus maximus* Desf.) and the taxonomic status of other three subspecies (subsp. *carota*, subsp. *gummifer* and subsp. *halophilus*) is maintained. Still, the latter three *taxa* need to be further studied for a more precise taxonomic characterization.

1. Introduction

Apiaceae is a large family represented by 2500–3700 species widely distributed worldwide. The taxonomy of this family is complex as it is evident, for example, in *Daucus* genus and, in particular, among *Daucus carota* infraspecific *taxa* (Castroviejo et al., 2003). *Daucus carota* is native to temperate regions of Europe and southwest Asia, and the wild ancestors most probably originated in what is today the Afghanistan, the center of diversification of this *taxon* (Bradeen et al., 2002). The species is

* Corresponding author. Tel.: +351 239 855212, +351 239 855210; fax: +351 239 855211.
E-mail addresses: actavar@bot.uc.pt (A.C. Tavares), jloureiro@bot.uc.pt (J. Loureiro), scastro@bot.uc.pt (S. Castro), cafe@bot.uc.pt (A.P. Coutinho), jaropa@bot.uc.pt (J. Paiva), ccavaleiro@ff.uc.pt (C. Cavaleiro), ligia@ff.uc.pt (L. Salgueiro), jorgecan@ci.uc.pt (J.M. Canhoto).
[1] Tel.: +351 239855233.
[2] Tel.: +351 239855210.
[3] Tel.: +351 239488400.

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morphologically highly variable being found in wild and feral forms and being widespread in different ecosystems. As a consequence, a large number of infraspecific *taxa* have been described in Iberian and European Floras (Pereira Coutinho, 1939; Sampaio, 1946; Tutin et al., 1972; Amaral Franco, 1974; Castroviejo et al., 2003).

Daucus carota subsp. *sativus* is the only cultivated form of the species and an important crop worldwide (Rong et al., 2010). This domesticated form has been assumed to have originated by selective breeding over the centuries from an ancestral wild form of *Daucus carota* subsp. *carota* (Just et al., 2007). Molecular characterization of wild and cultivated forms revealed that this group holds high genetic diversity and, thus, it has great potential for improving plant breeding efficiency (Bradeen et al., 2002). Numerous molecular studies are being developed to explore the potential of this crop and its wild relatives (e.g., Shim and Jorgensen, 2000; Grzebelus et al., 2007; Iorizzo et al., 2011, 2013; Alessandro et al., 2013).

Among the nine *D. carota* subspecies described for the Iberian Peninsula (Castroviejo et al., 2003), five are represented in Continental Portugal from which four are native, namely: *D. carota* L. subsp. *carota*; *D. carota* L. subsp. *maximus* (Desf.) Bal; *D. carota* L. subsp. *gummifer* (Syme) Hook. and *D. carota* L. subsp. *halophilus* (Brot.) A. Pujadas. The first three subspecies are distributed throughout Portugal, while the latter *taxon* is a Portuguese endemism, occurring only in three provinces in the centre and southwest regions (Pujadas Salvá, 2003).

Daucus carota subspecies display a high level of morphological variation and, despite of the several descriptions available in different Floras, the distinction of the different subspecies is difficult. Thus, other diagnostic characteristics, such as, DNA content and chemical characterization, could be helpful tools to achieve a better identification of these *taxa*. In fact, secondary metabolites such as essential oils have been reported as biological markers (Nogueira et al., 2008; Sena Filho et al., 2010), as well as the DNA content (Loureiro et al., 2007a, 2007b; Sunnucks, 2000). These methods were shown to be particularly relevant for the study of endemic species, restricted to particular habitats and populations (Qiu et al., 2004). Considering the doubts about the taxonomy of the subspecies of *D. carota* in Portugal and the potential of the above mentioned techniques, the main goal of this study was to clarify the taxonomy of this *taxon* based on the chemistry of its essential oils and DNA content. Considering the high variability of fruits morphology, this trait was also studied in more detail.

2. Materials and methods

2.1. Plant material

All plant material analysed was collected taking into account the phenology and the geographic distribution of the four native *D. carota* subspecies documented in Continental Portugal, following Tavares et al. (2012). Specimens were repeatedly harvested during seven consecutive years, between 2005 and 2011, and used for the analyses of DNA content and essential oil characterization (Table 1), and for fruit morphometric analysis (Table 2). The plant material was collected in 14 localities from five different provinces of the center and south of Portugal (Fig. 1). Voucher specimens from all the localities were collected and deposited at the Herbarium of the University of Coimbra (COI, http://www.uc.pt/herbario_digital).

2.2. Morphometric characterization of the fruits

Fruit samples were collected from a total of 33 specimens of *Daucus carota* subspecies ($n = 10$ for subsp. *carota* and subsp. *gummifer*; $n = 9$ for subsp. halophilus; and $n = 4$ for subsp. *maximus*) in 13 natural populations (Table 2). Three fruits were collected per specimen, observed under a binocular microscope and photographed. The following characters were measured in the photographs using ImageTool (v.3.0 for Windows, University of Texas Health Science Center, San Antonio, TX, USA):

Table 1
Locations where *Daucus carota* subspecies were collected and period of the year in which specimens were harvested. Places or times marked by an asterisk refer to plant material used for genome analyses and essential oil characterization.

Subspecies	Places/provinces	Year
halophilus	Ag: Cabo de S. Vicente*	May/June 2005 and 2006; May 2008*; May 2010
	Ag: Arrifana*	
	BAl: Cabo Sardão*	
	E: Cabo Espichel	June/July 2006 and 2007; June 2008; June 2009; June 2010
	E: Cabo da Roca	
	E: Cabo Carvoeiro	
gumifer	BL: Nazaré*	June/July 2005 and 2007; June 2008*; July 2010*
	BL: Praia do Norte*	
	BL: S. Pedro de Moel*	
	BL: Figueira da Foz	
carota	BL: Póvoa da Lomba*	June 2008*, June/July 2010* and 2011
	BL: Meãs do Campo*	July 2005; July 2010*
	BL: Figueira da Foz	
maximus	Cartuxa/AAL*	June/July 2006 and 2007; June 2008*; July 2010*
	Montemor-o-Novo*	

Provinces: Ag – Algarve, AAl – Alto Alentejo, BAl – Baixo Alentejo, Bl – Beira Litoral and E – Estremadura.

Table 2
Locations where *Daucus carota* subspecies were collected and period of the year in which specimens were harvested for morphometric analyses.

Subspecies	Places/provinces	Year
carota	BL: Vale das Pombas, Figueira da Foz	2005
	BL: Meãs do Campo	2010
	BL: Póvoa da Lomba	2011
	BL: Meãs do Campo	2011
gummifer	BL: Praia do Norte	2005
	BL: Nazaré	2005, 2006
	BL: S. Pedro de Moel	2005, 2006
	BL: Vale das Pombas, Figueira da Foz	2005
halophilus	Ag: Cabo de S. Vicente	2006, 2007, 2008, 2010
	BAl: Cabo Sardão	2006
	Ag: Arrifana	2007
	E: Cabo da Roca	2007
	E: Cabo Espichel	2007
maximus	AAl:Cartuxa	2006, 2007
	AAl: Montemor-o-Novo	2010

Provinces: Ag – Algarve, AAl – Alto Alentejo, BAl – Baixo Alentejo, BL – Beira Litoral and E – Estremadura.

fruit length, fruit width, spine length, width of spine at the base, and number of spines in one secondary ridge. The fruits were also mounted in microscopy slides and observed, with a Motic BA 310 light microscope using an amplification of 100×. In these observations the spine apex morphology was analysed and the width of secondary ridges measured. To increase the reliability of the binocular microscope measurements of the spines, their length and base width were also measured in the light microscope. Scanning electron microscopy (SEM) was used to study morphological details of the fruits and spines. For this purpose, the fruits were air dried, mounted on aluminium stubs and coated with a 30 nm layer of gold-paladium for 8 min at high vacuum in a sputtering chamber (Jeol JFC-1100 Ion Sputter). Fruits were then observed with a JEOL JSM-5400 scanning electron microscope (operating at 10 kV) and micrographs were taken.

Descriptive statistics (mean and standard deviation of the mean) of the quantitative variables were calculated for each subspecies. One-way ANOVA were used to assess differences among subspecies for the fruit variables studied. The analyses of variance were performed using the mean values for each specimen to avoid pseudo-replication. Values for fruit width and secondary ridge width were log transformed to achieve normality and homoscedasticity. All the remaining variables were normally distributed and homoscedastic. Multivariate analyses were performed to investigate the structural organization of the subspecies studied based on fruits characters combined. Principal component analysis (PCA) was performed using the specimen mean values for all the variables measured.

2.3. DNA content

The nuclear DNA content of up to 6 individuals per population of each subspecies of *Daucus carota* was estimated through flow cytometry (Table 1), following the procedure described by Loureiro et al. (2007a, b). Briefly, nuclei were released after co-chopping 0.5 cm^2 of fresh leaf tissue of *Daucus carota* together with 0.5 cm^2 of fresh leaf tissue of *Solanum lycopersicon* cv. Stupické (internal reference standard with 2C = 1.96 pg; Doležel et al., 1992) with a sharp razor blade in a glass 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, and 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 also 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 1300 nuclei per G1 peak was analysed in a Partec CyFlow Space flow cytometer (Partec GmbH., Münster, Germany), equipped with a green solid state laser for PI excitation, using the FloMax software (Partec GmbH). The G_1 peak of the sample 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) x genome size of the standard.

2.4. Chemical characterization of the essential oils

The essential oils of air-dried ripe umbels were isolated by hydrodistillation for 3 h using a *Clevenger*-type apparatus according to the European Pharmacopoeia (Council of Europe 1997). The oils of the four *taxa* were obtained from plants at the same developmental stage. The oils were preserved in a sealed vial at 4 °C. Oil analyses were carried out by 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 described by Cavaleiro et al. (2006).

The volatile compounds were identified by both their retention indices and 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

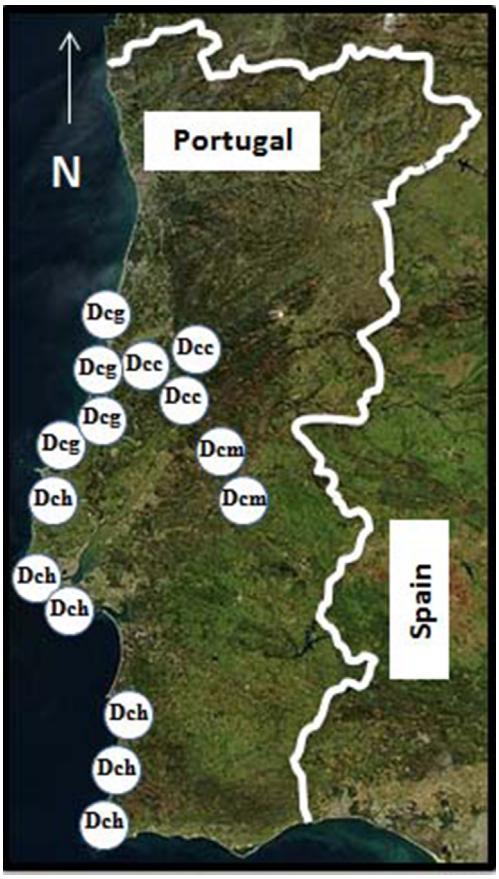

Fig. 1. Location of the individuals collected. Dch – *Daucus carota* subsp. *halophilus*; Dcg – *D. carota* subsp. *gummifer*; Dcc – *D. carota* subsp. *carota*; Dcm – *D. carota* subsp. *maximus* (map adapted from www.google.com/images).

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.

3. Results

3.1. Morphometric characterization of the fruits

The morphometric analysis of the fruits from *D. carota* subspecies enabled to separate the subsp. *maximus* based on the number of spines (having a significantly lower number of spines per secondary ridge in comparison with the other three

Table 3
Morphometric analysis of the fruits of *Daucus carota* subspecies.

Taxon	Fruit length	Fruit width	Secondary ridge width	Spine length	Spine width at the base	N. spines in 1 s ridge	Morphology of spine apex			
							Stelullate	One-pointed	Two-pointed	Simple
D. carota ssp. *carota*	2.5 ± 0.38^{b}	1.5 ± 0.10^{b}	0.4 ± 0.08^{b}	0.9 ± 0.22^{b}	0.2 ± 0.04^{b}	11 ± 1.5^{a}	63.6 ± 25.2%	0.0 ± 0.0%	25.1 ± 24.2%	11.4 ± 14.7%
D. carota ssp. *gummifer*	2.3 ± 0.26^{b}	1.8 ± 0.20^{a}	0.4 ± 0.15^{ab}	0.8 ± 0.21^{b}	0.2 ± 0.04^{b}	11 ± 1.0^{a}	42.1 ± 31.9%	8.3 ± 10.0%	35.4 ± 23.1%	14.2 ± 18.6%
D. carota ssp. *halophilus*	3.1 ± 0.57^{a}	1.8 ± 0.22^{a}	0.5 ± 0.10^{a}	0.9 ± 0.23^{b}	0.3 ± 0.06^{a}	11 ± 1.6^{a}	59.8 ± 30.0%	3.8 ± 6.1%	21.2 ± 18.2%	15.2 ± 19.0%
D. carota ssp. *maximus*	2.8 ± 0.22^{ab}	1.7 ± 0.08^{ab}	0.4 ± 0.12^{ab}	2.1 ± 0.36^{a}	0.3 ± 0.02^{a}	7 ± 0.5^{b}	100.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%
Statistical test	$F = 6.10$, $P = 0.002$	$F = 5.62$, $P = 0.004$	$F = 5.13$, $P = 0.003$	$F = 32.07$, $P < 0.001$	$F = 10.31$, $P < 0.001$	$F = 8.51$, $P < 0.001$	–	–	–	–

Different letters represent significant differences ($P < 0.05$) for a given trait according to a multiple comparison Tukey test.

subspecies, $P < 0.05$), spine length (having significantly longer spines than the other three subspecies, $P < 0.05$) and morphology (always stelullate) of spine apices (Table 3). Concerning the remaining three subspecies, a fairly continuum in the variables studied was observed (Table 3, Fig. 2). Indeed, most of the characters were highly variable within infructescence, plant and population. As a result, it was not possible to completely discriminate subsp. *carota*, subsp. *gummifer* and subsp. *halophilus* based on the fruit characters studied.

A detailed SEM and LM analysis of the fruits revealed similar results as morphometric analyses, i.e., that individuals from populations of subsp. *halophilus*, subsp. *carota* and subsp. *gummifer* presented similar characteristics. Moreover, SEM (Fig. 3) and LM observations of the length and basis of the fruits' spines did not present a pattern and often the fruits from populations that belong to supposed different *taxa* could yet present the same characteristics. In reality, the light and scanning microscopic fruits evaluation revealed that fruits with simple spine, stelullate spine, one-pointed spine or two-pointed spine could be observed in all three subspecies and in populations collected in very different localities.

Consequently, only the subsp. *maximus* could be separated from the other three subspecies based on a few fruit traits, as follows:

1. Stelullate spines, length ≥1.50 mm .....*D. carota* subsp. *maximus*
   - Stelullate, two-pointed, one-pointed or simple spines, length <1.50 mm or absent.....*D. carota* subsp. *carota*, *D. carota* subsp. *gummifer*, *D. carota* subsp. *halophilus*

Nevertheless, some partial differences among the other three subspecies were also found, namely: a) the secondary ridges are always <0.50 mm in *D. carota* subsp. *carota*, and sometimes (ca. 26%) ≥0.50 mm in *D. carota* subsp. *gummifer* and *D. carota* subsp. *halophilus*; b) the spine length is always ≤0.75 mm in *D. carota* subsp. *gummifer*, and sometimes (ca. 50%) > 0.75 mm in *D. carota* subsp. *halophilus*.

3.2. DNA content

The nuclear DNA content of the four subspecies of *D. carota* (Table 1) was estimated using flow cytometry. Overall, mostly due to the release of some cytosolic compounds (see Section 3.3), it was difficult to obtain histograms with CVs of the G_1 peak of sample nuclei below 3% (mean CV value was 5.6%). Despite of this, the variation in genome size values among individuals of the same population was low (CV values of genome size were always below 3%). Significant differences in genome size were detected between the subsp. *maximus* and the subsp. *halophilus* and *gummifer*, with subsp. *maximus* presenting the highest genome size value (2C = 1.3 ± 0.04 pg) and subspecies *halophilus* presented the lowest genome size value with 2C = 1.2 ± 0.04 pg (Table 4). The subsp. *carota* was not distinguishable from any of the other subspecies based on DNA content (Table 4).

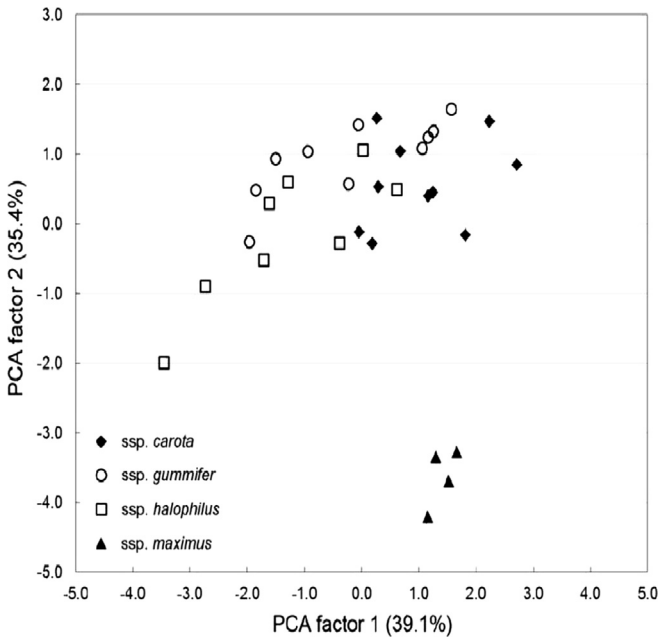

Fig. 2. Principal component analysis (PCA) of the morphometric analysis of the fruits.

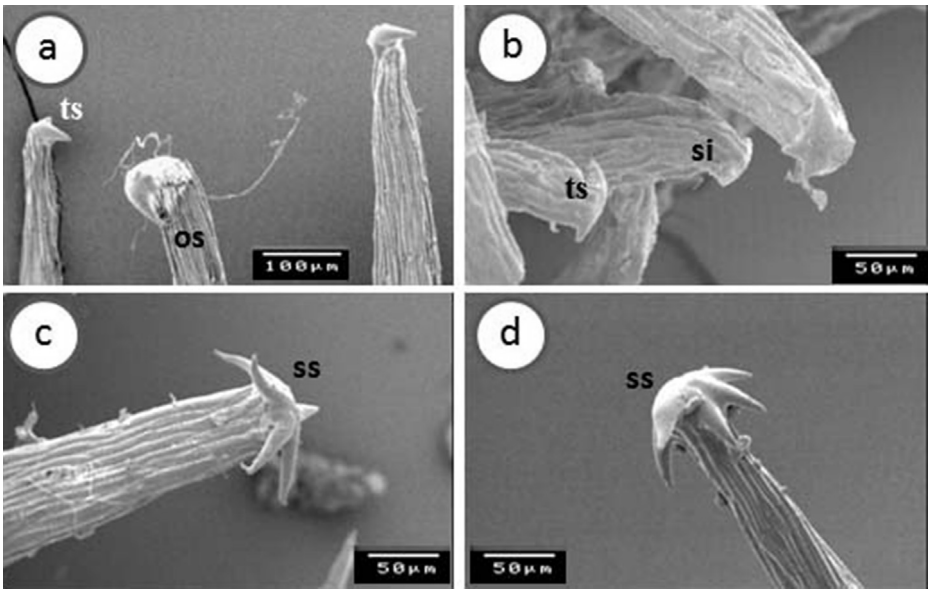

Fig. 3. Details of the fruit's spines: a) *Daucus carota* subsp. *carota*; b) *D. carota* subsp. *gummifer*; c) *D. carota* subsp. *halophilus*; d) *D. carota* subsp. *maximus*. si – simple spine, os – one–pointed spine, *ss* – stelullate spine; ts – two-pointed spine. Bars: a = 100 µm; b–d = 50 µm.

3.3. Chemical characterization of the essential oils

The qualitative and quantitative compositions of the oils are presented in Table 5, where compounds are listed in order of their elution on a polydimethylsiloxane column. Monoterpene hydrocarbons and oxygen containing monoterpenes were the main group of constituents in the oils of the subsp. *carota* and subps. *gummifer*, whereas monoterpene hydrocarbons and phenylpropanoids where the main groups in the oils of the subsp. *maximus* and subsp. *halophilus*.

Based on the essential oils it was not possible to distinguish the subsp. *carota* from the subsp. *gummifer* because the oils of both subspecies are mainly composed by α-pinene (13.0–27.1% and 11.0–31.0%, respectively) and geranyl acetate (28.7–65.0% and 18.0–55.0%, respectively) in similar amounts (Table 5). Some minor quantitative differences were only observed in the amounts of carotol and 11αH-himachal-4-en-1-β-ol, depending on the origin of the plants.

Contrarily to the above, it is possible to distinguish the subsp. *maximus* from the subsp. *halophilus* by the composition of their essential oils, besides their chemical polymorphism. Important differences concerning their major constituents were observed, namely: asarone, one of the major compounds of the subsp. *maximus* (5.8–25.8%), is absent in the subsp. *halophilus* oil; sabinene, one of the major compound of the subsp. *halophilus* (9.0–29.0%), is a minor compound in the oil of the subsp. *maximus* (1.2–1.3%); elemicine is present in higher amounts in the oil of the subsp. *halophilus* (15.0–31.0% *vs* 4.9–13.6%), whereas *E*- methylisoeugenol and β-bisabolene are present in higher amounts in the subsp. *maximus* oil (8.2–15.7% *vs* 0.5–7.4% and 8.3–15.1% *vs* 0.4–3.5%, respectively) (Table 5).

4. Discussion

The results of the present study provide significant clues for the taxonomic delimitation of *D. carota* subspecies, namely to support the delimitation of the subspecies *maximus* in a higher taxonomic rank. Altogether, the morphometric evaluation of

Table 4
Nuclear DNA content of *Daucus carota* subspecies.

Taxon	Nuclear DNA content				No. pop.	No. ind.
	Mean ± SD	Min.	Max.	1Cx (Mbp)		
D. carota subsp. *carota*	1.22 ± 0.02^{ab}	1.18	1.24	594	2	8
D. carota subsp. *gummifer*	1.22 ± 0.04^{b}	1.13	1.29	590	3	15
D. carota ssp. *halophilus*	1.21 ± 0.04^{b}	1.12	1.33	593	6	22
D. carota ssp. *maximus*	1.26 ± 0.02^{a}	1.23	1.28	617	2	6

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, the number of analysed populations (No. pop.) and the total number of analysed individuals (No. ind.) are also provided. Different letters represent significant differences ($P < 0.05$) according to a multiple comparison Tukey test. 1 pg DNA = 978 Mbp.

Table 5
Composition of the essential oils of four subspecies of *Daucus carota*.

RI[a]	RI[b]	Compounds[c]	Amount of the essential oil (%)			
			D. carota subsp. *maximus*	*D. carota* subsp. *gummifer*	*D. carota* subsp. *carota*	*D. carota* subsp. *halophilus*
922	1030	α-Thujene	*t*-0.2	*t*-0.2	*t*	0.2–0.4
930	1030	α-Pinene	10.0–25.9	11.0–31.0	13.0–27.1	12.2–23.0
943	1073	Camphene	0.5–1	0.6–1	0.5–0.6	0.3–05
964	1128	Sabinene	1.2–1.3	2.1–10	0.6–0.9	9.0–29.0
970	1118	β-Pinene	4.0–6.8	3.8–5.2	2.3–4.5	2.2–2.8
980	1161	Myrcene	1.4–3.0	2.1–3.7	1.2–2.5	2.0–3.1
997	1171	α-Phellandrene		t		*t*-0.1
1010	1187	α-Terpinene		0.1–0.7		0.3–1.3
1011	1275	*p*-Cymene		t–0.3		0.1–0.3
1020	1206	Limonene	1.8–3.3	5.8–9.0	1.2–9.0	5.5–12.0
1020	1215	β-Phellandrene	0.3–0.4	*t*-0.5		0.1–05
1025	1235	*Z*-β-Ocimene	*t*-0.1	0.6–2	0.1–0.2	0.1–0.5
		cis-Verbenol	*t*-0.1			
1035	1250	*E*-β-Ocimene	*t*-0.1	0.2–4.1	0.1–0.2	*t*-0.1
1046	1249	γ-Terpinene		0.2–1.7		0.8–2.6
1050	1458	*trans*-Sabinene hydrate		*t*-0.3		0.1–0.4
1162	1624	Myrtenal	0.1–0.2			
1169	1690	α-Terpineol	*t*-0.1			
1076	1288	Terpinolene		*t*-0.3		0.2–0.5
1081	1543	Linalool	0.9–1.3	0.4–1	1.3–1.6	0.5–1.1
1081	1542	*cis*-Sabinene hydrate		*t*-0.2		*t*-0.1
1105	1556	*cis*-p-2-Menthen-1-ol		*t*-0.1		0.1–0.2
1120	1620	*trans*-p-2-Menthen-1-ol		t		*t*-0.2
		trans-Verbenol			*t*-0.1	
1135	1553	Pinocarvone				*t*
1144	1695	Borneol				*t*
1158	1597	Terpinene-4-ol		0.1–2.1	*t*-0.1	2.0–4.7
1169	1692	α-Terpineol		*t*-0.1	*t*-0.1	0.2–0.3
1176	1699	Verbenone			*t*-0.1	
1177	1673	*cis*-Piperitol				0.1-*t*
1187		*trans*-Piperitol	*t*	*t*		*t*
1208		Nerol			*t*-0.1	
1233	1838	Geraniol			0.5–1.2	
1239	1555	Linalyl acetate	0.1–0.3			
1240	1730	Geranial			*t*-0.3	
1264	1574	Bornyl acetate		0.3–0.4		0.1–0.3
1330	1465	δ-Elemene		*t*-0.1		0.1–0.4
1328	1688	α-Terpinyl acetate		0.1–0.3		0.1–0.4
1341	1723	Neryl acetate			*t*-0.1	
1346		α-Longipinene			*t*-1.0	0.1–0.3
1363	2225	Geranyl acetate	3.4–16.0	18.0–55.0	28.7–65.0	*t*-1.0
1369	1487	α-Copaene		*t*-0.1		*t*-0.1
1369	2006	Methyleugenol	0.1–0.2			*t*-0.3
1381	1534	β-Cubebene	*t*	*t*		
1383	1586	β-Elemene		*t*-0.1		*t*-0.1
1405	1563	α-Cedrene				0.1–0.3
1411	1563	Aristolene				*t*-0.2
1411	1590	*E*-β-Caryophyllene	0.1–0.5	0.6–1.1	0.4–1.3	0.5–1.4
1442	1662	α-Humulene		0.3–0.6	*t*-0.1	*t*-0.1
1446	1661	*trans*-β-Farnesol				*t*-0.3
1448	1665	*trans*-β-Farnesene	0.1–0.3	0.2–0.3	*t*-0.1	
1461	2219	*E*- Methylisoeugenol	8.2–15.7		1.0–1.3	0.5–7.4
		Neocalitropsene			0.1–0.5	
1466	1699	Germacrene D		2–5.5	*t*-0.2	0.1–0.3
1473	1708	β-Selinene	*t*		*t*-0.1	
1484	1724	Bicyclogermacrene	*t*-0.1	*t*-0.3	0.1–0.3	0.2–0.3
1489		β-Himachalene		*t*-0.1	*t*-1.3	0.1–0.3
1498	1720	β-Bisabolene	8.3–15.1		0.3–0.5	0.4–3.5
1508	1751	δ-Cadinene				0.2–0.5
1518		Elemicin	4.9–13.6		0.1–1.6	15.0–31.0
1530	1766	*E*-α-Bisabolene	0.5–4.2	0.1–0.2		*t*-0.3
1542	1816	Germacrene B				*t*-0.2
1557	1968	Caryophyllene oxide	*t*		0.1–0.2	0.1–0.3
1581	2001	Carotol	*t*-0.2	5.0–15.0	1.5–6.1	
1618	2174	T-Muurolol		*t*		*t*-0.3
1622	2089	11αH-himachal-4-en-1-β-ol		0.1–1.1	0.5–9.4	
1630	2219	T-Cadinol		*t*-0.1		*t*-0.2

Table 5 (*continued*)

RI[a]	RI[b]	Compounds[c]	Amount of the essential oil (%)			
			D. carota subsp. *maximus*	*D. carota* subsp. *gummifer*	*D. carota* subsp. *carota*	*D. carota* subsp. *halophilus*
1630	2216	α-Cadinol		*t*-0.2		0.1–0.7
1644	1755	α-Asarone	5.8–25.8			
1663		β-Bisabolol		*t*-0.4		*t*-0.1
1668		Juniper camphor				0.2–0.6
1777		Isocalamendiol				0.1–0.3

t = traces (<0.05%).
n.d. = Not determined.
[a] RI = Retention indices on the SPB-1 column relative to C_8 to C_{24} *n*-alkanes.
[b] RI = Retention indices on the SupelcoWax-10 column relative to C_8 to C_{24} *n*-alkanes.
[c] Compounds listed in order to their elution on the SPB-1 column.

the fruits from different populations, the different habit of the plants (tall herbs up to 170 (200) cm, with wide umbels of 12–23 cm in diameter), DNA content and the chemical composition of the essential oils (namely, presence of significant amounts of asarone), clearly distinguish the subspecies *maximus* from the other ones, providing evidences that this *taxon* should be considered as a separate species (*Daucus maximus* Desf.), rather than a *D. carota* subspecies.

As observed in other groups, essential oils were an important tool to characterize and distinguish *D. carota* subspecies. The essential oils of *D. carota* subsp. *maximus* and subsp. *halophilus* are differentiated from the other two ones subspecies (subsp. *carota* and subsp. *gummifer*) by the amount of phenylpropanoids. The subsp. *maximus* can be distinguishable from the subsp. *halophilus* by the high amount of asarone in the former and its absence in the oils of the latter subspecies. After excluding the subsp. *maximus* based on several traits, the other three subspecies (subsp. *halophilus*, subsp. *gummifer* and subsp. *carota*) are with difficulty distinguishable by their fruit morphology, as well as based on their overall plant morphology. Nevertheless, essential oil composition, namely the presence of elemicin could be considered a chemical marker that distinguishes the subsp. *halophilus* from the other two taxa. This trend is also present in DNA content with plants from this subspecies tending to have smaller genome sizes. However, although *D. carota* subsp. *halophilus* can be distinguishable by some morphological characteristics, genome size and a few chemical constituents of the essential oils, the differences are not strong enough to separate this *taxon* in a different taxonomic rank. In reality, the high levels of elemicin in the essential oil, with no other taxonomic evidence is not sufficient to support this separation. Also, there is no cytological, morphological and genomic data to support the separation of subsp. *carota* and subsp. *gummifer* neither between them nor in relation to the subsp. *halophilus*. Consequently, in the absent of sufficient support, we suggest to maintain the three *taxa*, subsp. *halophilus*, subsp. *gummifer* and subsp. *carota*, as subspecies in agreement with Pujadas Salvá (2003). This difficulty in distinguishing the subspecies is in agreement with molecular and ecological descriptions of the species. *D. carota* has been described as an outcrossing species where gene flow is likely to be widespread; this feature together with range overlap, could promote gene flow between populations of different subspecies contributing to the maintenance of high levels of morphological variability observed in all traits of the plants and to the non-structured and extensive genetic diversity observed in wild populations (Bradeen et al., 2002).

In conclusion, and considering the range and variation of the species with a reasonable amount of subspecies (around 10), the *taxon Daucus carota* subsp. *maximus* should be better considered a different species, as René Desfontaine has stated (*D. maximus* Desf.) (Desfontaine, 1798). On the contrary and in agreement with Pujadas Salvá (2003), the others three *taxa* should be maintained in the same rank, as follows:

Daucus maximus Desf.
Daucus carota L. subsp. *carota*
Daucus carota L. subsp. *halophilus* (Brot.) A. Pujadas
Daucus carota L. subsp *gummifer* Syme

Nevertheless, further studies should be developed in order to allow a better characterization of *D. carota* subspecies, particularly the Portuguese endemism, *D. carota* subsp. *halophilus*.

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