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Research Article

Inter- and intraspecific morphometric variability in *Juniperus* L. seeds (Cupressaceae)

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In this study, a statistical classifier for Mediterranean taxa of *Juniperus*, based on 98 seed morphometric parameters, was tested at interspecific, specific and intraspecific levels. Ripe cones of 10 taxa were collected in different regions of the Mediterranean Basin to compare and discuss their taxonomic treatments according to two different sources. High percentages of correct identification were reached for both taxonomic treatments at the specific and intraspecific level and from the comparison among taxa of the *J. oxycedrus*, *J. communis* and *J. phoenicea* complexes. Moreover, ripe cones of *J. macrocarpa* were collected from four Sardinian populations, in two seasons, and from plant and soil, in order to analyse inter-population, seasonal and source variability in seed morphology. This statistical classifier discriminated *J. macrocarpa* seeds collected in spring more accurately than those collected in autumn, but it failed to distinguish between the seeds collected from plants and soil, or between those collected from different populations of the same geographic region.

Key words: EFDs, image analysis, LDA, Mediterranean vascular flora, ripe cones, taxonomic treatment

Introduction

The family Cupressaceae shows great ecological diversity among its species (Farjon, 1999). They are more scattered in southern temperate regions and in northern and eastern Africa, with single genera present from either hemispheres (Stevens, 2001). The genus *Juniperus* L. includes about 75 species (Adams & Schwarzbach, 2013), most of them growing in the northern hemisphere, except *Juniperus procera* Hochst. ex Endl., which inhabits the Great Rift Valley and East African Mountains (Adams *et al.*, 1993). This genus can be divided into three monophyletic sections (Adams, 2011; Adams & Schwarzbach, 2013): *Caryocedrus* Endlicher, with only one species for the Mediterranean region (*J. drupacea* Labill.); *Juniperus* L. (syn: *Oxycedrus* Spach), consisting of 14 species (12 only in the eastern hemisphere, one endemic to North America and plus the circumboreal *J. communis* L.); and *Sabina* (Mill.) Spach, consisting of approximately 60 species distributed in south-western regions of North America, Asia and the Mediterranean Basin (e.g. Adams & Turuspekov, 1998; Mao *et al.*, 2010; Silva *et al.*, 2011; Adams & Schwarzbach, 2013).

The entire genus was genetically investigated at the interspecific (Mao *et al.*, 2010; Adams, 2011) and intraspecific (Opgenoorth *et al.*, 2010; Douaihy *et al.*, 2011; Adams *et al.*, 2013) level, revealing high genetic diversity. Moreover, numerous genetic studies analysed specific taxa or groups. In particular, Jiménez *et al.* (2003) analysed genetic diversity and differentiation in Moroccan and Spanish *J. thurifera* L.; Douaihy *et al.* (2011) revealed a high level of genetic diversity within *J. excelsa* M. Bieb. subsp. *excelsa*; Adams *et al.* (2005), using DNA sequencing and leaf terpenoids and morphology, proved that *J. oxycedrus* L. var. *oxycedrus* and *J. deltoids* R.P. Adams, are about as different from each other as *J. navicularis* and *J. macrocarpa* are from *J.*

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oxycedrus var. *oxycedrus*. High levels of inter-population (Meloni *et al.*, 2006) and genetic variability (Boratyński *et al.*, 2009) were also detected for *J. phoenicea* L. populations, as well as for *J. communis* sampled in Britain (Van Der Merwe *et al.*, 2000) and throughout Europe (Michalczyk *et al.*, 2010). Inter-population differences within the various species of this genus have been studied. Thus, Mazur *et al.* (2004) biometrically analysed (number, length, width of cones and seeds, and features of shoots and leaves) the inter-population variation on *J. excelsa* from Crimea and from Balkan Peninsula, which was lower than that of *J. phoenicea* from the Iberian Peninsula (Mazur *et al.*, 2003) and more in general from the western Mediterranean region (Mazur *et al.*, 2010), analysed on the same characters. On the basis of length and width of cones, seeds and needles, and seed number per cone, Klimko *et al.* (2007) found intra- and inter-population variation between *J. oxycedrus* subsp. *oxycedrus* western Mediterranean populations and eastern ones.

The potential of biometric indices for seed studies is well known and demonstrated by many authors, particularly regarding morpho-colorimetric evaluations (e.g. Granitto *et al.*, 2003; Shahin & Symons, 2003; Kiliç *et al.*, 2007; Venora *et al.*, 2007, 2009*a*; Wiesnerová & Wiesner, 2008; Grillo *et al.*, 2011; Smykalova *et al.*, 2013). In particular, Bacchetta *et al.* (2008) characterized seeds of wild vascular plants of the Mediterranean Basin, using digital images and implementing statistical classifiers able to discriminate seeds belonging to different genera and species. Grillo *et al.* (2010) developed 10 specific statistical classifiers at the family level for Angiosperms and tested the system on the genus *Juniperus*, demonstrating that the method is also reliable for Gymnosperms. Recently, Orrù *et al.* (2012*b*) confirmed the effectiveness of this identification method, studying seeds of *Vitis vinifera* L. varieties. Afterwards, many authors have successfully used elliptic Fourier descriptors (EFDs) in seed studies (e.g. Terral *et al.*, 2010; Mebatsion *et al.*, 2012; Orrù *et al.*, 2012*a*).

Given the taxonomic controversies and different systematic treatments on *Juniperus* genus such as those proposed by *Flora Europaea* (Amaral Franco do, 1980) and The Plant List (2012) (FE and PL, hereafter), the aims of this study were: (1) to validate and improve the statistical classifier, based on seed morphometric parameters, at the specific and intraspecific level, previously implemented by Grillo *et al.* (2010) for the Mediterranean *Juniperus* taxa; (2) to compare the results with the taxonomic treatments proposed by FE and PL; and (3) to test the capability of the classification system in the discrimination of seed lots of the same species (*J. macrocarpa* Sm.) collected in different populations, seasons and sources (plants or soil).

Materials and methods

Seed-lot details

Ripe cones of 10 selected *Juniperus* taxa were collected from natural populations in Algeria (Ag), Balearic Islands (Bl), Corsica (Co), Italy (It), Sardinia (Sa) and Spain (Hs), for a total of 43 seed lots and then stored at the Sardinian Germplasm Bank (BG-SAR) in Cagliari (Table 1). Seeds were manually removed out from the cones and washed by stirring them in water for 90 min. Among these seed lots, 18 accessions of *J. macrocarpa* were collected in Sardinia in 2010 in order to analyse inter-population and seasonal variability in seed morphology (Table 1).

Seed-size and -shape analysis

Digital images of seed samples were acquired using a flatbed scanner (Epson Perfection V600 Photo), with a digital resolution of 400 dpi and a scanning area not exceeding 2048×2048 pixels. Image acquisition was performed before drying the seeds at 15 °C to 15% of RH to avoid spurious variation in dimension and shape. Sub-samples consisting of 100 seeds were randomly chosen from the original seed lots and arrayed on the flatbed tray for scanning. When the original accession was numerically lower than 100 units, the analysis was executed on the whole seed lot. The images were processed and analysed using the software package KS-400 V.3.0 (Carl Zeiss, Vision, Oberkochen, Germany). A macro specifically developed for characterizing wild seeds (Bacchetta *et al.*, 2008) was later modified to further measure 20 seed features (Mattana *et al.*, 2008) and afterwards was improved to automatically perform all the analysis procedures, simultaneously reducing the execution time and mistakes in the analysis process (Grillo *et al.*, 2010). This macro, used to analyse seed images, was further enhanced adding algorithms able to compute the EFDs for each analysed seed, increasing the number of discriminant parameters (Orrù *et al.*, 2012*a*).

A total of 98 morphometric features (Table 2) were measured on 2343 seeds (Table 1).

Statistical analysis

Morphometric and EFDs data were analysed applying the stepwise linear discriminant analysis (LDA) method, in order to compare the *Juniperus* seeds at the section level (Adams, 2011), as well as at the specific and intraspecific level, according to the taxonomic treatments proposed by PL and FE (Table 1). In particular, intraspecific analyses were performed for three species complexes (*J. communis*, *J. oxycedrus*, *J. phoenicea*). LDA was also used to assess seed morphological variability of *J. macrocarpa* collected in different populations, seasons and sources (plants and soil). To avoid the influence of the production

Table 1. Location of the *Juniperus* taxa and populations studied (1 = cones collected in spring; 2 = cones collected in autumn; * = cones collected from plant; ** = cones collected from soil). Ag: Algeria; Bl: Balearic Islands; Co: Corsica; It: Italy; Sa: Sardinia; Hs: Spain.

Section (Adams, 2011)	*Taxon* according to: The Plant List (http://www.theplantlist.org/)	*Taxon* according to: Flora Europaea (Amaral Franco do, 1980)	Locality	Region	Number of sampled seeds
Juniperus	*J. communis* var. *saxatilis* Pall.	*J. communis* subsp. *nana* Syme	Desulo	Sa	1813
			Albertacce-Evisa	Co	412
			Desulo	Sa	760
	J. communis L.	*J. communis* L. subsp. *communis*	Laconi	Sa	1221
		J. communis L. subsp. *hemisphaerica* (J.Presl & C. Presl) Nyman	Santiago de la Espada, Andalusia	Hs	1728
			Cabañas, Andalusia	Hs	273
	J. oxycedrus var. *badia* H.Gay	not reported	Buggerru	Sa	836
			Huescar, Andalusia	Hs	244
			Domus de Maria	Sa	266
	J. oxycedrus L.	*J. oxycedrus* L. subsp. *oxycedrus*	Cuesta Carrascal, Andalusia	Hs	1129
			Capoterra	Sa	380
	J. macrocarpa Sm.	*J. oxycedrus* L. subsp. *macrocarpa* (Sibth. & Sm.) Neilr.	Domus de Maria	Sa	3522
			Narbolia	Sa	1409
			Cecina, Tuscany	It	147
			Domus de Maria	Sa	445
			Arbus	Sa	46 [1*]
			Arbus	Sa	137 [1**]
			Arbus	Sa	2477 [1**]
			Arbus	Sa	220 [2*]
			Arbus	Sa	1010 [2**]
			Buggerru	Sa	213 [1*]
			Buggerru	Sa	273 [1**]
			Buggerru	Sa	2414 [1**]
			Buggerru	Sa	2465 [2*]
			Buggerru	Sa	1984 [2**]
			Domus de Maria	Sa	100 [1*]
			Domus de Maria	Sa	430 [1**]
			Domus de Maria	Sa	3527 [2*]
			Domus de Maria	Sa	2087 [2**]
			Villasimius	Sa	543 [1*]
			Villasimius	Sa	269 [1**]
			Villasimius	Sa	2210 [2*]
			Villasimius	Sa	1763 [2**]
	J. phoenicea L.	*J. phoenicea* L.	Lula	Sa	1200
	J. phoenicea var. *turbinata* (Guss.) Parl.		Aïn Sefra, wilaya de Naâma	Ag	392
			Montagne des Lions, Oran	Ag	317
			Villasimius	Sa	897
			Almerimar, Andalusia	Hs	338
			Mallorca, Balearic Islands	Bl	ND
Sabina	*J. sabina* L.	*J. sabina* L.	Comunidad Valenciana	Hs	1023
			Jerez del Marquesado, Andalusia	Hs	843
	J. thurifera L.	*J. thurifera* L.	Comunidad Valenciana	Hs	1005
			Pedro Martinez, Andalusia	Hs	554
Total amount of measured seeds					2343

Table 2. List of 18 morphometric features measured on seeds, excluding the 80 Elliptic Fourier Descriptors (EFDs) calculated according to Hâruta (2011).

Feature		Description
$\boldsymbol{A}$	Area	Seed area (mm^2)
$\boldsymbol{P}$	Perimeter	Seed perimeter (mm)
$\boldsymbol{P_{conv}}$	Convex Perimeter	Convex perimeter of the seed (mm)
$\boldsymbol{P_{Crof}}$	Crofton Perimeter	Crofton perimeter of the seed (mm)
$\boldsymbol{P_{conv}/P_{Crof}}$	Perimeter ratio	Ratio between $\boldsymbol{P_{conv}}$ and $\boldsymbol{P_{Crof}}$
$\boldsymbol{D_{max}}$	Max diameter	Maximum diameter of the seed (mm)
$\boldsymbol{D_{min}}$	Min diameter	Minimum diameter of the seed (mm)
$\boldsymbol{D_{min}/D_{max}}$	Feret ratio	Ratio between $\boldsymbol{D_{min}}$ and $\boldsymbol{D_{max}}$
$\boldsymbol{EA_{max}}$	Maximum ellipse axis	Maximum axis of an ellipse with equivalent area (mm)
$\boldsymbol{EA_{min}}$	Minimum ellipse axis	Minimum axis of an ellipse with equivalent area (mm)
Sf	Shape Factor	Seed shape descriptor = $(4\pi \boldsymbol{A})/\boldsymbol{P}^2$ (normalized value)
Rf	Roundness Factor	Seed roundness descriptor = $(4\boldsymbol{A})/(\pi \boldsymbol{D_{max}}^2)$ (normalized value)
Ecd	Eq. circular diameter	Diameter of a circle with equivalent area (mm)
F	Fiberlength	Seed length along the fibre axis
C	Curl degree	Ratio between $\boldsymbol{D_{max}}$ and $\boldsymbol{F}$
Conv	Convessity degree	Ratio between $\boldsymbol{P_{Crof}}$ and $\boldsymbol{P}$
Sol	Solidity degree	Ratio between $\boldsymbol{A}$ and convex area
Com	Compactness degree	Seed compactness descriptor = $[\sqrt{(4/\pi)\boldsymbol{A}}]/\boldsymbol{D_{max}}$

year, only *J. macrocarpa* seed lots collected in 2010 were considered.

LDA is commonly used to classify/identify unknown groups characterized by quantitative and qualitative variables (Fisher, 1936, 1940), finding the combination of variables able to minimize the within-class distance while simultaneously maximizing the between-class distance, thus achieving maximum class discrimination (Hastie *et al*., 2001; Holden *et al*., 2011). The stepwise method identifies and selects the most statistically significant features among the 98 measured on each seed, using three statistical variables: Tolerance, *F*-to-enter and *F*-to-remove. The Tolerance value indicates the proportion of a variable variance not accounted for by other independent variables in the equation. *F*-to-enter and *F*-to-remove values define the power of each variable in the model and are useful to describe what happens if a variable is inserted and removed, respectively, from the current model. This method starts with a model that does not include any of the variables. At each step, the variable with the largest *F*-to-enter value that exceeds the entry criterion chosen ($F \geq 3.84$) is added to the model. The variables left out of the analysis at the last step have *F*-to-enter values smaller than 3.84, and therefore no more are added. The process was automatically stopped when no remaining variables increased the discrimination ability (Venora *et al*., 2009*b*; Grillo *et al*., 2012). Finally, a cross-validation procedure was applied to verify the performance of the identification system, testing individual unknown cases and classifying them on the basis of all others (SPSS, 2007). Analyses were performed using the SPSS software package release 16.0 (SPSS, 2007).

All the raw data were standardized before starting any statistical elaboration. Moreover, in order to evaluate the quality of the discriminant functions achieved for each statistical comparison, the Wilks' Lambda, the percentage of explained variance and the canonical correlation between the discriminant functions and the group membership, were computed. The Box's *M* tests was executed to assess the homogeneity of covariance matrices of the features chosen by the stepwise LDA; while the analysis of the standardized residuals was performed to verify the homoscedasticity of the variance of the dependent variables used to discriminate among the groups' membership.

The differences among groups (species and populations) were graphically highlighted by drawing box plots using the Mahalanobis' square distance values. This measure of distance, defined by two or more discriminant functions, ranges from 0 to infinity. Samples are increasingly similar at values closer to zero. Higher values indicate that a particular case includes extreme values for one or more independent variables, and can be considered significantly different from other cases of the same group (Bacchetta *et al*., 2008).

Results

Juniperus genus

In comparisons of the seed lots belonging to two of the three *Juniperus* sections proposed by Adams (2011), an overall cross-validation percentage of correct identification of 86.8% was reached, with performance values of 81.6% and 73.4% for *Juniperus* and *Sabina* sections, respectively.

Following the PL taxonomic treatment at the species level, an overall performance of correct identification of 73.8% was found, ranging between 63.6% (*J. thurifera*)

Table 3. Percentage of correct identification at species level according to 'The Plant List' (PL; http://www.theplantlist.org/, accessed 20 Dec 2013) and 'Flora Europaea'(FE; Amaral Franco do, 1980) where *J. macrocarpa* is included in *J. oxycedrus*. The number of analysed seeds is in parentheses.

***Taxon* according to PL**	*J. communis*	*J. oxycedrus*	*J. macrocarpa*	*J. phoenicea*	*J. sabina*	*J. thurifera*	**Total**
J. communis	**77.2 (447)**	1.2 (7)	0.0 (0)	14.2 (82)	4.3 (25)	3.1 (18)	100 (579)
J. oxycedrus	1.0 (5)	**66.7 (323)**	15.9 (77)	10.7 (52)	0.0 (0)	6.0 (27)	100 (484)
J. macrocarpa	0.0 (0)	29.8 (89)	**66.9 (200)**	2.0 (6)	0.0 (0)	1.3 (4)	100 (299)
J. phoenicea	10.6 (62)	7.0 (41)	0.5 (3)	**80.4 (471)**	0.2 (1)	1.4 (8)	100 (586)
J. sabina	13.0 (26)	0.0 (0)	0.0 (0)	0.0 (0)	**81.5 (163)**	5.5 (11)	100 (200)
J. thurifera	11.8 (23)	4.1 (8)	0.0 (0)	15.4 (30)	5.1 (10)	**63.6 (124)**	100 (195)
Overall							**73.8 (2343)**

***Taxon* according to FE**	*J. communis*	*J. oxycedrus*	*J. phoenicea*	*J. sabina*	*J. thurifera*	**Total**
J. communis	**76.9 (445)**	1.2 (7)	14.5 (84)	4.3 (25)	3.1 (18)	100 (579)
J. oxycedrus	0.8 (6)	**88.0 (689)**	7.0 (55)	0.0 (0)	4.2 (33)	100 (783)
J. phoenicea	9.6 (56)	7.3 (43)	**81.4 (477)**	0.2 (1)	1.5 (9)	100 (586)
J. sabina	12.5 (25)	0.0 (0)	0.0 (0)	**82.0 (164)**	5.5 (11)	100 (200)
J. thurifera	12.3 (24)	3.6 (7)	14.9 (29)	5.6 (11)	**63.6 (124)**	100 (195)
Overall						**81.0 (2343)**

and 81.5% (*J. sabina* L.) (Table 3). Table 3 also summarizes the performance of correct identification for the comparison according to the FE taxonomic treatment. An overall percentage of correct identification of 81.0% resulted, ranging between 63.6% (*J. thurifera*) and 88.0% (*J. oxycedrus*).

At the intraspecific level, on the basis of the PL taxonomic treatment, percentages of correct identification ranged between 13.5% (*J. phoenicea* var. *phoenicea*) and 81.5% (*J. sabina*), with an overall performance of 60.6% (Table 4). Regarding the performance according to FE (where *J. oxycedrus* subsp. *oxycedrus* includes also *J. oxycedrus* var. *badia* H. Gay seed lots), the overall percentage of correct identification was 67.6%, ranging between 33.0% (*J. communis* subsp. *communis*) and 83.5% (*J. sabina*).

Juniperus communis, J. oxycedrus and *J. phoenicea* complexes

According to PL, the two varieties belonging to the *J. communis* complex were compared, giving percentages of correct identification of 76.2% and 86.5% for *J. communis* var. *saxatilis* and *J. communis* var. *communis*, respectively, with an overall performance of 80.0%. In Fig. 1, the scores of the only discriminant function implemented are reported as box plots for both *J. communis* varieties. *Juniperus communis* complex was also analysed on the basis of the FE taxonomic treatment, registering an overall identification performance of 71.8%, but correctly identifying only 33.0% of *J. communis* subsp. *communis*. That is, 51.0% of the cases were misattributed to *J. communis* subsp. *nana* Syme, which reached 81.2% of correct identification, and in 16.0% of the cases were misattributed to *J. communis* subsp. *hemisphaerica* (J.Presl & C.Presl) Nyman, which reached 78.2% of correct identification.

According to PL, the two varieties belonging to the *J. oxycedrus* complex (*J. oxycedrus* var. *badia*, *J. oxycedrus* var. *oxycedrus*) were also compared with *J. macrocarpa* (Fig. 2A), achieving an overall percentage of correct identification of 69.2%, with misattributions evenly distributed among the three taxa. The histogram of the standardized residuals (Fig. 2B), the normal probability plot (Fig. 2C) and the dispersion plot of the standardized residuals (Fig. 2D) were also included to better understand the normal distribution of the data.

Discriminant analysis between the two varieties belonging to *J. phoenicea*, according to PL, showed an overall performance of 70.8%, with percentages of correct identification of 25.0% and 93.1% for *J. phoenicea* var. *phoenicea* and *J. phoenicea* var. *turbinata* (Guss.) Parl., respectively (Fig. 3).

Juniperus macrocarpa seed lots

The four *J. macrocarpa* populations showed percentages of correct identification between 22.3% (Domus de Maria) and 54.5% (Buggerru), with an overall performance of 37.9% (Table 5). From the comparison between the *J. macrocarpa* seed lots collected in spring and autumn 2010, overall percentages for the population ranged between 66.6% (Villasimius) and 70.1% (Domus de Maria), with a global identification performance of 63.5%. Similarly, the discriminant analyses conducted between the seed collected from plants and those collected from the soil, showed overall percentages of correct identification, distinguished by population, ranging from 61.5% (Buggerru) to 70.2% (Villasimius), with a global identification performance of 59.2%.

Table 4. Percentage of correct identification among taxa (specific, subspecific and variety level), according to 'The Plant List' (PL; http://www.theplantlist.org/, accessed 20 Dec 2013) and 'Flora Europaea'(FE; Amaral Franco do, 1980; FE where *J. oxycedrus* var. *badia* is included in *J. oxycedrus* subsp. *oxycedrus*. The number of analysed seeds is in parentheses.

***Taxon* according to PL**	*J. communis* var. *saxatilis*	*J. communis* var. *communis*	*J. oxycedrus* var. *badia*	*J. oxycedrus* var. *oxycedrus*	*J. macrocarpa*	*J. phoenicea* var. *phoenicea*	*J phoenicea* var. *turbinata*	*J. sabina*	*J. thurifera*	**Total**
J. communis var. *saxatilis*	**52.5 (148)**	20.6 (58)	0.0 (0)	5.0 (14)	0.0 (0)	4.3 (12)	9.6 (27)	3.2 (9)	5.0 (14)	100 (282)
J. communis var. *communis*	16.8 (50)	**71.7 (213)**	0.0 (0)	0.0 (0)	0.0 (0)	1.3 (4)	3.4 (10)	6.7 (20)	0.0 (0)	100 (297)
J. oxycedrus var. *badia*	0.0 (0)	0.0 (0)	**59.4 (171)**	15.3 (44)	21.5 (62)	0.0 (0)	3.8 (11)	0.0 (0)	0.0 (0)	100 (288)
J. oxycedrus var. *oxycedrus*	3.6 (7)	0.0 (0)	19.4 (38)	**36.7 (72)**	11.2 (22)	0.5 (1)	13.8 (27)	0.0 (0)	14.8 (29)	100 (196)
J. macrocarpa	0.0 (0)	0.0 (0)	16.1 (48)	11.0 (33)	**69.9 (209)**	0.0 (0)	2.0 (6)	0.0 (0)	1.0 (3)	100 (299)
J. phoenicea var. *phoenicea*	14.1 (27)	6.3 (12)	0.0 (0)	3.6 (7)	0.0 (0)	**13.5 (26)**	59.9 (115)	1.0 (2)	1.6 (3)	100 (192)
J. phoenicea var. *turbinata*	5.3 (21)	4.1 (16)	3.0 (12)	6.9 (27)	0.8 (3)	3.8 (15)	**74.6 (294)**	0.0 (0)	1.5 (6)	100 (394)
J. sabina	3.0 (6)	10.5 (21)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	**81.5 (163)**	5.0 (10)	100 (200)
J. thurifera	12.8 (25)	1.0 (2)	0.0 (0)	7.7 (15)	0.0 (0)	2.1 (4)	8.2 (16)	5.1 (10)	**63.1 (123)**	100 (195)
Overall										**60.6 (2343)**

***Taxon* according to FE**	*J. communis* subsp. *nana*	*J. communis* subsp. *communis*	*J. communis* subsp. *hemisphaerica*	*J. oxycedrus* subsp. *oxycedrus*	*J. oxycedrus* subsp. *macrocarpa*	*J. phoenicea*	*J. sabina*	*J. thurifera*	**Total**
J. communis subsp. *nana*	**48.6 (137)**	7.4 (21)	11.3 (32)	2.5 (7)	0.0 (0)	20.9 (59)	2.5 (7)	6.7 (19)	100 (282)
J. communis subsp. *communis*	31.0 (31)	**33.0 (33)**	20.0 (20)	0.0 (0)	0.0 (0)	8.0 (8)	8.0 (8)	0.0 (0)	100 (100)
J. communis subsp. *hemisphaerica*	17.3 (34)	9.1 (18)	**62.9 (124)**	0.0 (0)	0.0 (0)	4.6 (9)	5.6 (11)	0.5 (1)	100 (197)
J. oxycedrus subsp. *oxycedrus*	1.0 (5)	0.0 (0)	0.0 (0)	**67.8 (328)**	15.1 (73)	10.1 (49)	0.0 (0)	6.0 (29)	100 (484)
J. oxycedrus subsp. *macrocarpa*	0.0 (0)	0.0 (0)	0.0 (0)	33.1 (99)	**64.5 (193)**	1.3 (4)	0.0 (0)	1.0 (3)	100 (299)
J. phoenicea	6.3 (37)	0.7 (4)	1.9 (11)	7.0 (41)	0.5 (3)	**81.4 (477)**	0.3 (2)	1.9 (11)	100 (586)
J. sabina	3.5 (7)	4.0 (8)	4.0 (8)	0.0 (0)	0.0 (0)	0.0 (0)	**83.5 (167)**	5.0 (10)	100 (200)
J. thurifera	10.3 (20)	0.0 (0)	1.0 (2)	4.1 (8)	0.0 (0)	14.4 (28)	5.6 (11)	**64.6 (126)**	100 (195)
Overall									**67.6 (2343)**

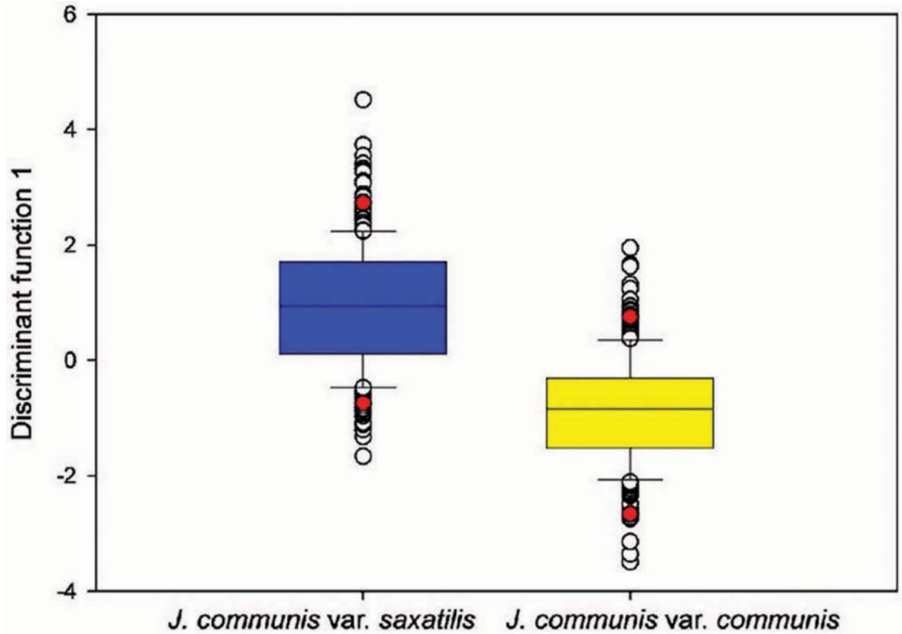

Fig. 1. Graphic representation of the discriminant function scores for both the *J. communis* varieties, according to 'The Plant List' (PL; http://www.theplantlist.org/, accessed 20 Dec 2013).

The best five key parameters

In the evaluation of the parameters influencing the discrimination process in the comparison between the two *Juniperus* sections, the shape-descriptive features proved more powerful than the dimensional ones, showing high F-to-remove values, although many steps were necessary in the discrimination process. At the specific and intraspecific level, both according to the PL and to the FE

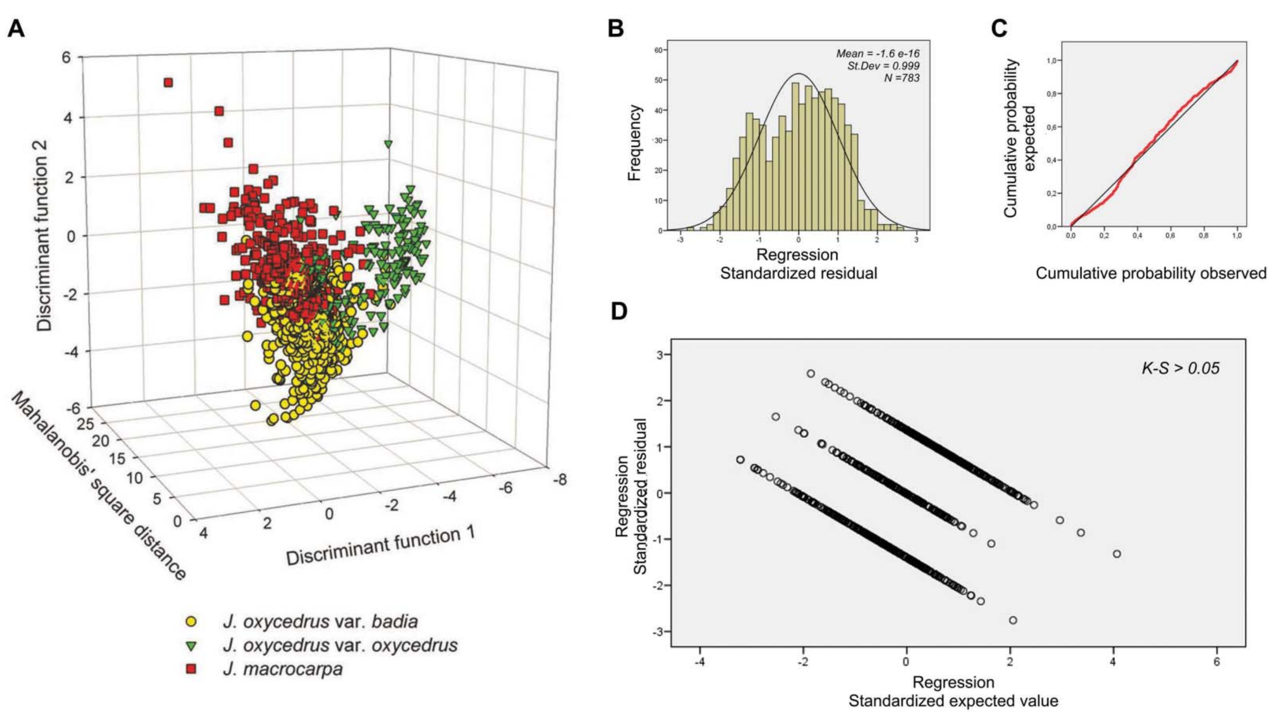

Fig. 2. (A) Discriminating analysis of the varieties belonging to the *J. oxycedrus* complex, according to 'The Plant List' (PL; http://www.theplantlist.org/, accessed 20 Dec 2013), with *J. macrocarpa*; (B) histogram of the standardized residuals; (C) Normal Probability Plot (P-P) tested with the Kolmogorov-Smirnov test (K-S); (D) dispersion plot of the standardized residuals.

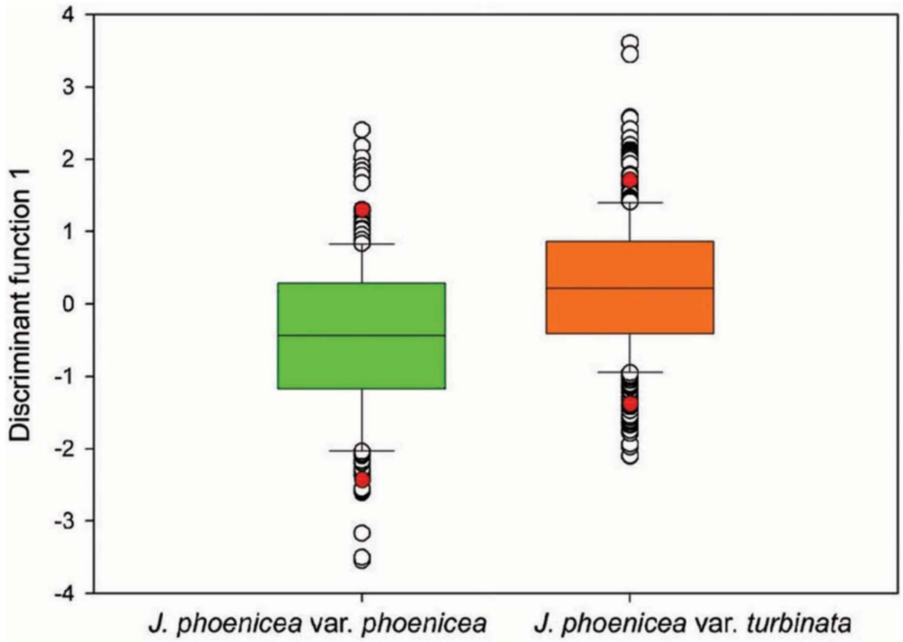

Fig. 3. Graphic representation of the discriminant function scores for the two varieties belonging to *J. phoenicea*, according to 'The Plant List' (PL; http://www.theplantlist.org/, accessed 20 Dec 2013).

taxonomic treatments, parameters related to seed size proved to be more discriminant than the shape-descriptive ones; in particular, mainly seed area (A) and convex perimeter (P_{conv}) were powerful. Also in these cases, among 19 and 26 steps were necessary for the taxon identification. The four comparisons among species aggregates at the subspecies and varietal level showed various useful size- and shape-descriptive features, with emphasis on the seed-perimeter features (P, P_{conv}, P_{conv}/P_{Croft}). Finally, regarding the comparison among the populations of *J. macrocarpa*, mainly size-descriptive features were used. In all these discriminant analyses, the EFDs were found to be particularly powerful among the best five key parameters despite the reduced relative F-to-remove values both at section level and the *J. communis* and *J. phoenicea* aggregates, according to PL (Table 6).

Discussion

Section level: the consistency with current taxonomy

The satisfactory discrimination achieved by the comparison between the seed morphometric data belonging to the *Juniperus* and *Sabina* sections agrees with the results reported by Mao *et al.* (2010) and Adams (2011) on the basis of cpDNA, nrITS and nrITS/cpDNA analysis, confirming the current taxonomic treatment at the section level. These results illustrate that this method is effective also when the morphometric variability within each group is high.

Species level: *J. oxycedrus* and *J. macrocarpa*

The results at the species level reached good percentages of correct identification for both of the taxonomic

Table 5. Percentage of correct identification among Sardinian populations of *J. macrocarpa*. The number of seeds analysed is in parentheses.

Locality	Arbus	Buggerru	Domus de Maria	Villasimius	**Total**
Arbus	**40.2 (194)**	30.2 (146)	12.8 (62)	16.8 (81)	100 (483)
Buggerru	21.8 (106)	**54.5 (265)**	14.8 (72)	8.8 (43)	100 (486)
Domus de Maria	29.9 (117)	38.1 (149)	**22.3 (87)**	9.7 (38)	100 (391)
Villasimius	36.5 (142)	23.4 (91)	10.3 (40)	**29.8 (116)**	100 (389)
Overall					**37.9 (1749)**

Table 6. The best five key parameters of correct classifications. The number of steps, the tolerance, and F-to-remove values are reported in parentheses.

Classifier	1	2	3	4	5
Sections	$\boldsymbol{Com}$ (24; 0.074; 242.355)	$\boldsymbol{EFD_{14}}$ (24; 0.473; 137.652)	$\boldsymbol{P_{conv}/P_{Crof}}$ (24; 0.482; 53.325)	$\boldsymbol{EFD_{22}}$ (24; 0.675; 31.940)	$\boldsymbol{F}$ (24; 0.760; 26.951)
Species according to PL	$\boldsymbol{A}$ (23; 0.004; 45.394)	$\boldsymbol{P_{conv}}$ (23; 0.002; 30.524)	$\boldsymbol{D_{max}}$ (23; 0.004; 25.934)	$\boldsymbol{Ecd}$ (23; 0.001; 24.011)	$\boldsymbol{Sf}$ (23; 0.070; 19.493)
FE species *J. badia* in *oxycedrus*	$\boldsymbol{A}$ (26; 0.003; 47.401)	$\boldsymbol{Com}$ (26; 0.011; 24.077)	$\boldsymbol{Ecd}$ (26; 0.001; 23.879)	$\boldsymbol{D_{max}}$ (26; 0.004; 22.983)	$\boldsymbol{Rf}$ (26; 0.008; 18.341)
PL intraspecific	$\boldsymbol{A}$ (19; 0.004; 41.734)	$\boldsymbol{P_{conv}}$ (19; 0.002; 25.568)	$\boldsymbol{Sf}$ (19; 0.069; 24.239)	$\boldsymbol{EFD_{6}}$ (19; 0.017; 22.642)	$\boldsymbol{Ecd}$ (19; 0.001; 21.050)
FE intraspecific	$\boldsymbol{A}$ (22; 0.004; 43.030)	$\boldsymbol{P_{conv}}$ (22; 0.002; 31.697)	$\boldsymbol{D_{max}}$ (22; 0.004; 17.652)	$\boldsymbol{Com}$ (22; 0.013; 15.094)	$\boldsymbol{Rf}$ (22; 0.009; 14.626)
PL *taxa J. communis* vs. *J. saxatile*	$\boldsymbol{P_{conv}}$ (12; 0.007; 112.470)	$\boldsymbol{P}$ (12; 0.007; 99.101)	$\boldsymbol{D_{min}}$ (12; 0.244; 27.435)	$\boldsymbol{EFD_{14}}$ (12; 0.390; 25.428)	$\boldsymbol{EFD_{11}}$ (12; 0.717; 13.685)
FE *taxa J. communis* vs. *J. nana* vs. *J. hemisphaerica*	$\boldsymbol{EA_{max}}$ (13; 0.572; 183.525)	$\boldsymbol{Conv}$ (13; 0.555; 58.720)	$\boldsymbol{Sf}$ (13; 0.345; 49.828)	$\boldsymbol{Sol}$ (13; 0.359; 17.433)	$\boldsymbol{EFD_{12}}$ (13; 0.945; 15.604)
PL *taxa J. oxycedrus* complex	$\boldsymbol{P}$ (18; 0.009; 35.181)	$\boldsymbol{P_{conv}}$ (18; 0.002; 28.510)	$\boldsymbol{A}$ (18; 0.009; 27.808)	$\boldsymbol{EFD_{11}}$ (18; 0.563; 22.905)	$\boldsymbol{Rf}$ (18; 0.029; 18.992)
PL *taxa J. phoenicea* vs. *J. turbinata*	$\boldsymbol{P}$ (8; 0.715; 11.558)	$\boldsymbol{EFD_{50}}$ (8; 0.933; 6.407)	$\boldsymbol{EFD_{18}}$ (8; 0.863; 5.105)	$\boldsymbol{P_{conv}/P_{Crof}}$ (8; 0.834; 4.981)	$\boldsymbol{EFD_{12}}$ (8; 0.940; 4.981)
Sardinian populations of *J. macrocarpa*	$\boldsymbol{A}$ (5; 0.006; 15.832)	$\boldsymbol{Ecd}$ (5; 0.004; 8.972)	$\boldsymbol{Sol}$ (5; 0.475; 7.899)	$\boldsymbol{P_{conv}}$ (5; 0.025; 6.279)	$\boldsymbol{D_{min}}$ (5; 0.084; 5.946)

treatments (PL and FE). *Juniperus macrocarpa* reached almost 70% correct identification according to PL, thereby demonstrating a clear differentiation with respect to *J. oxycedrus*, which received almost all the misattributions, according to FE, in which *J. macrocarpa* does not appear as a species. However, the performance of *J. oxycedrus* reaches 88.0% following the FE classification, indicating a certain similarity between the two species as reported by Adams (2000).

Intraspecific level: *J. oxycedrus* and *J. macrocarpa*

The two varieties of *J. oxycedrus* proposed by the PL registered lower identification percentages than the two subspecies proposed by FE. Nevertheless, it is important to note that FE does not report *J. oxycedrus* var. *badia*, and does not list *J. macrocarpa* as an independent species but as a subspecies of *J. oxycedrus*. *Juniperus macrocarpa* seems to be fairly well identifiable in both cases, although in view of the misidentifications, a certain similarity to the *J. oxycedrus* taxa is doubtless (Farjon, 1998; Adams, 2000).

Regarding the *J. oxycedrus* species complex according to the PL taxonomic treatment, also considering *J. macrocarpa*, a clear correlation among the three entities is evident, casting legitimate doubt on the most appropriate taxonomic treatment. In any case, the result from the comparison between *J. oxycedrus* var. *badia* and *J. oxycedrus* var. *oxycedrus* points to a relationship at the varietal level between these two taxa, as confirmed by several authors (e.g. Pignatti, 1982; Amaral Franco do, 1986; Farjon, 1998; Adams, 2000; Jeanmonod & Gamisans, 2013).

Intraspecific level: the *J. communis* complex

According to our results, the *J. communis* taxa are more distinguishable following the taxonomic treatment proposed by the PL rather than the one by FE, although in both cases higher percentages of misattributions have been detected in relation to *J. phoenicea* species.

The results of the interactions between the taxa of the *J. communis* complex, according to the PL, confirmed the taxonomic distance between these taxa, although a varietal taxonomic rank is proposed (Adams & Pandey, 2003). The performance following FE showed that the three subspecies considered (*J. communis* subsp. *communis*, *J. communis* subsp. *nana* and *J. communis* subsp. *hemisphaerica*) are not consistent on the basis of seed morphometric data. In a recent work, Grillo *et al.* (2010), registering rather high percentages of correct identification, confirmed the taxonomic distance between *J. communis* subsp. *communis* and *J. communis* subsp. *nana*, identified by several authors as two distinct subspecies (Amaral Franco do, 1980, 1986; Jeanmonod & Gamisans, 2013) or species (Pignatti, 1982; Lebreton *et al.*, 2000), but recently considered to be a single taxon by Farjon (2001) and Adams (2011). The different percentages of correct identification and the distribution of the misclassified cases reached in this work, in respect to the achievements reported by Grillo *et al*. (2010), are certainly to be attributed to the greater amount of seeds analysed and compared, to the larger number of measured parameters and to the effect of *J. communis* subsp. *hemisphaerica*, as third taxon of the complex, that was not considered by Grillo *et al*. (2010).

Intraspecific level: the *J. phoenicea* complex

The results of correct classification for the *J. phoenicea* complex indicate that, according to the PL classification, the two taxa *J. phoenicea* var. *phoenicea* and *J. phoenicea* var. *turbinata* are sufficiently distinguished, considering the taxonomic rank of variety as proposed by Adams *et al*. (1996, 2002, 2013), Farjon (2005) and Adams (2010). However, this result disagrees with the findings of Grillo *et al.* (2010) who, considering these taxa to be two different subspecies, instead reached very high percentages of correct classification, according to many other authors (e.g. Lebreton, 1983; Amaral Franco do, 1986; Valdés *et al*., 1987; Mazur *et al.*, 2003; Conti *et al*., 2005; Farjon, 2005; Jeanmonod & Gamisans, 2013). In this case, considering the similar amount of studied seeds and the analogous numerical proportion between the two compared taxa, it is evident that the non-compliance with the results reached by Grillo *et al.* (2010) derives from the increase in the morphometric features measured on each seed.

The Sardinian *J. macrocarpa* meta-population

The comparison among the four Sardinian populations of *J. macrocarpa* gave low performance of correct identification with misattributions evenly distributed, suggesting that seed morphometry is not able to discriminate among different populations from the same geographical region due to the low intra-population variability. These achievements are consistent with the results reported by Juan *et al.* (2012), who investigated the genetic structure of *J. macrocarpa* in three regions of Spain and found only one meta-population without geographical structure. Klimko *et al*. (2004) also found low genetic differentiation of Italian *J. macrocarpa* populations for most of the morphological features studied. However, some morphological variability for *J. macrocarpa* was found in south-western Spanish populations (Juan *et al.*, 2003). Furthermore, the absence of geographic structure was also observed by

Brus *et al.* (2011) in *J. oxycedrus* subsp. *oxycedrus* from the Balkan Peninsula. According to the results found in this study and the literature, the presence of a single population of *J. macrocarpa*, lacking geographic differences, is feasible in the southern sector of Sardinia. Other works on different species of the genus *Juniperus* showed contradictory and different results (e.g. Jiménez *et al*., 2003; Meloni *et al*., 2006; Boratyński *et al*., 2009; Michalczyk *et al*., 2010; Douaihy *et al*., 2011, 2012; Sertse *et al*., 2011; Yücedağ & Gailing, 2013). In addition, the classification system identified *J. macrocarpa* seeds collected in spring but did not discriminate between collection sources (plant and soil).

The predominance of seed-size features

At the specific and intraspecific level, parameters related to the seed size (i.e. morphometric) proved to be more discriminant than the shape-descriptive ones. Grillo *et al.* (2010) found that for the families Apiaceae, Brassicaceae and Fabaceae, morphometric features were the first discriminant parameters. Also in Bacchetta *et al.* (2011*a*), regarding the *Lavatera triloba* aggregate, the first three parameters with the highest discriminatory power were of morphological type, although colour evaluation was very important in this work for correct seed identification. By contrast, in a previous study regarding *Astragalus* sect. *Melanocercis*, the only morphometric parameters taken into account were related to seed length (Bacchetta *et al*., 2011*b*).

Conclusions

The present results confirmed the validity of the proposed method for the taxonomic differentiation of *Juniperus*, both at specific and intraspecific levels, and its identification capability after adding the EFDs among the features measured, increasing the accession number of the database implemented by Grillo *et al*. (2010). Moreover, morphometric seed analysis did not discriminate among different populations, which could mean the presence of a single meta-population in southern Sardinia. Finally, the classification system was able to discriminate seeds of *J. macrocarpa* collected in different seasons, while those collected in spring being more identifiable, but could not identify seeds collected from different sources (plants and soil). The latter results represent the first application of statistical classifier based on seed morphometric parameters to discriminate seed lots of the same species at the season and source level.

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