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# Taxonomy of prickly juniper (*Juniperus oxycedrus* group): A phytochemical–morphometric combined approach at the contact zone of two cryptospecies

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ABSTRACT

Based on different essential oil composition paralleling different genotypes, *Juniperus deltoides* was recently segregated from *Juniperus oxycedrus*. Despite a clear phytochemical and molecular differentiation, *J. deltoides* resulted not clearly morphologically discernible from *J. oxycedrus*, so that it was defined as a cryptospecies. Italy represents the contact zone of their distribution, but the ranges of the two species are not sufficiently known, due to unsatisfactory morphological characterisation. To further complicate the picture, a third closely related species (ecotype), *J. macrocarpa*, occurs all across the Mediterranean coasts. After a preliminary phytochemical analysis to ascertain the (chemo-)identities of the studied populations, we performed a morphometric investigation to test the degree of morphological distinctiveness among the taxa. According to our analysis, some character (e.g. leaf mucro length, leaf width, seed-cone size and seed size) resulted useful to discriminate these cryptic taxa. Finally, based on these characters, an extensive revision of herbarium specimens allowed us to redefine the distribution pattern of the investigated species in the Central Mediterranean area.



## 1. Introduction

It is difficult to provide a general and unambiguous definition of cryptospecies, due to the absence of a general definition of species itself. Cryptospecies are morphologically indistinguishable, but genetically differentiated, reproductively isolated (Paris et al., 1989; Whittall et al., 2004; Bickford et al., 2007), and allopatric (Arrigoni, 1988). On the contrary, cryptic species living in sympatry are more appropriately defined as 'sibling species' (Grant, 1971).

Cryptospecies are frequently recognized and described based on DNA sequence data (Bickford et al., 2007), but phytochemical investigations can also provide valuable information, helping to disentangle taxonomically critical groups (Harborne, 2000; Hadacek, 2002; Stuessy, 2009; Carta et al., 2015; Tundis et al., 2016; Passalacqua et al., 2017).

A paradigmatic case where both DNA and chemosystematics have been jointly used to recognise a cryptospecies is the so-called *Juniperus oxycedrus* group (prickly juniper), i.e. *J. deltoides* R.P.Adams, *J. macrocarpa* Sm., and *J. oxycedrus* L. s.str. (Adams et al., 2005).

The genus *Juniperus* L. (Cupressaceae, gymnosperms) consists of about 60 dioecious woody species, and it is divided into three sections: *J.* sect. *Caryocedrus* Endl., *J.* sect. *Juniperus*, and *J.* sect. *Sabina* Spach (Adams, 2012). The *Juniperus oxycedrus* group is included within *J.* sect. *Juniperus* (Adams, 2014a, 2014b).

*Juniperus oxycedrus* was described by Linnaeus (1753) based on plants from the western part of the Mediterranean basin ("*Habitat in Hispania, G. Narbonensi*"), while *Juniperus deltoides* was segregated from the former species based on plants from the eastern Mediterranean basin (Greece) (Adams, 2004). The description of *J. deltoides* as a distinct species relied mostly on the different composition of essential oil (EO): the leaf oil of east Mediterranean

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populations was poor in α-pinene and rich in limonene, compared to west Mediterranean populations (Adams et al., 2003, 2005; Rajčević et al., 2013, 2015; Adams, 2014b). Interestingly, this phytochemical differentiation was supported and paralleled by DNA molecular markers: nrITS (Adams et al., 2003, 2005, 2015; Mao et al., 2010; Adams and Schwarzbach, 2012); ISSR (Adams et al., 2003); RAPD (Adams et al., 2003, 2005); cpDNA (Mao et al., 2010; Rumeu et al., 2011; Adams and Schwarzbach, 2012; Adams et al., 2015); SSR (Boratyński et al., 2014). Despite this, *Juniperus deltoides* is hardly morphologically discernible from *J. oxycedrus* (Adams et al., 2005; Adams, 2014b; Boratyński et al., 2014), although the lineages including the two taxa have been estimated to diverge since the late Miocene (8–10 Ma) (Mao et al., 2010; Boratyński et al., 2014).

The occurrence of both *J. oxycedrus* and *J. deltoides* was reported in Italy: the first one in the west portion of Liguria (NW Italy) and Sardinia, the second one supposed to occur in the whole peninsular Italy, from Tuscany to Calabria (Adams, 2014a, 2014b). The recent record of *J. oxycedrus* s.str. for peninsular Italy (Lazzeri et al., 2015; Bartolucci et al., 2017), based on some putatively discriminant morphological characters (Adams, 2014b), was the occasion to deeper investigate the morphological distinction and distribution of this species pair. Tuscany, in particular, seems to represent the main contact zone between the ranges of *J. deltoides* and *J. oxycedrus* (Roma-Marzio et al., 2016). All across the Mediterranean coasts, also *J. macrocarpa* Sm. occurs. The latter species represents an ecotype, often treated as an ecologically vicariant subspecies of *J. oxycedrus* (Farjon and Filer, 2013), chemotaxonomically and phylogenetically much closer to *J. oxycedrus* s.str. than to *J. deltoides* (Adams et al., 2003, 2005, 2015; Mao et al., 2010; Adams and Schwarzbach, 2012; Boratyński et al., 2014).

The present study aims to clarify the taxonomy and distribution of the *Juniperus oxycedrus* group in a critical geographical area, where the ranges of the three cited above taxa are coming in contact, i.e. the Central Mediterranean area, and peninsular Italy in particular. A phytochemical approach was followed to detect the (chemo-)identity of the sampled populations and, based on the obtained results, a morphometric analysis was performed on selected *Juniperus* populations, to test the degree of morphological distinctiveness among the taxa, and to highlight the most useful and significant diagnostic morphological characters. Finally, based on these characters, a critical review of herbarium specimens was performed, in order to clarify the distribution of the involved taxa.

## 2. Materials and methods

### 2.1. Phytochemical investigation

Populations of *Juniperus deltoides* R.P.Adams, *J. macrocarpa* Sm., and *J. oxycedrus* L. (Cupressaceae) were collected during 2016 from thirteen localities: ten from Italy, two from Tunisia and one from Croatia (Table 1). For each locality, a herbarium voucher was prepared and deposited at PI (herbarium acronym follows Thiers, 2017). All the EOs were obtained by hydrodistillation from dry aerial parts, using a Clevenger-type apparatus according to the Italian Pharmacopoeia (AOAC, 1990).

The GC/MS analyses were performed with a Varian CP-3800 apparatus, equipped with a DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm) and a Varian Saturn 2000 ion-trap mass detector. The oven temperature was programmed rising from 60 °C to 240 °C at 3 °C/min; injector temperature, 220 °C; transfer-line temperature, 240 °C; carrier gas, He (1 ml/min).

The identification of the EO compounds was based on the comparison of their retention times (Rt) with those of pure reference samples, and their linear retention indices (LRIs) determined

**Table 1**
Sampled localities and source of data used for the phytochemical investigation. For the populations sampled in the present study, decimal degrees coordinates (WGS84) and altitude (m a.s.l.) are provided.

| ID Locality | Locality | Country | Source of data | Coordinates | Altitude |
|---|---|---|---|---|---|
| EL | Elba Island (Livorno, Tuscany) | Italy | Present study | 43.431740, 10.430017 | 80 |
| SI | Castiglione d'Orcia (Siena, Tuscany) | Italy | Present study | 42.998589, 11.605017 | 600 |
| PE | Monte Pelato (Livorno, Tuscany) | Italy | Present study | 43.435018, 10.610169 | 295 |
| VE | Marina di Vecchiano (Pisa, Tuscany) | Italy | Present study | 43.797410, 10.266640 | 2 |
| FL | Finale Ligure (Savona, Liguria) | Italy | Present study | 44.177760, 08.343000 | 187 |
| MV | Monte Vaso (Pisa, Tuscany) | Italy | Present study | 43.435018, 10.610169 | 560 |
| MP | Monte Pisano (Pisa, Tuscany) | Italy | Present study | 43.736920, 10.582490 | 314 |
| LI | Calignaia (Livorno, Tuscany) | Italy | Present study | 43.426810, 10.398950 | 10 |
| OR | Oriolo (Cosenza, Calabria) | Italy | Present study | 40.010620, 16.499150 | 315 |
| SB | Rosignano Solvay (Livorno, Tuscany) | Italy | Present study | 43.376270, 10.438326 | 0 |
| SA | Sidi Ameur | Tunisia | Present study | 35.879110, 9.479410 | 800 |
| BH | Bir Hannun | Tunisia | Present study | 36.083330, 9.250000 | 800 |
| KR | Miljevački Bogatići (Šibenik) | Croatia | Present study | 43.904543, 15.989145 | 230 |
| 1 | Lemo | Greece | Adams 1999 | | |
| 2 | Crysoritsi | Greece | Adams 1999 | | |
| 3 | El Penon | Spain | Adams 1999 | | |
| 4 | Tarifa | Spain | Adams 1999 | | |
| 5 | Raiano (Abruzzo) | Italy | Adams et al., 2005 | | |
| 6 | Marrakech | Morocco | Adams et al., 2005 | | |
| 7 | Lemos | Greece | Adams et al., 2005 | | |
| 8 | Archova | Greece | Adams et al., 2005 | | |
| 9 | Vila Nova de Foz Coa | Portugal | Adams et al., 2005 | | |
| 10 | El Penon | Spain | Adams et al., 2005 | | |
| 11 | Eskisehir | Turkey | Adams et al., 2005 | | |
| 12 | Peyruis | France | Adams et al., 2005 | | |
| 13 | Devin region | Bulgaria | Adams and Tashev 2012 | | |
| 14 | Raiano (Abruzzo) | Italy | Adams and Tashev 2012 | | |
| 15 | Archova | Greece | Adams and Tashev 2012 | | |
| 16 | Benkovac | Croatia | Rajčević et al., 2013 | | |
| 17 | Bursa | Turkey | Hayta and Bagci 2014 | | |