

## SPECIAL FEATURE

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# A new species of *Odontarrhena* (Brassicaceae) endemic to Greek ultramafics: From taxonomy to metal accumulation behavior

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## Abstract

A new species of *Odontarrhena* (Brassicaceae) is described from Mount Vourinos in western Macedonia, Greece. The species is restricted to the ridge and the northern slopes of Mt. Vourinos above 1500 m a.s.l. and grows on stony slopes and dry rocky grassland on ultramafic soil with elevated trace metal concentrations. Based on morphological, karyological, and nrDNA sequences, the species is tetraploid with supernumerary B-chromosomes ( $2n = 4 \times = 32 + 2B$ ) and related to the Balkan endemic *Odontarrhena decipiens*. It differs from the latter mainly by the habit of subshrub with woody base and a denser indumentum of stellate trichomes, resulting in a white-silvery color of the leaves and shoots. The presence of four degenerate positions in the ITS1 region was detected only in the new species from Mt. Vourinos. Shoot Ni concentrations determined by x-ray fluorescence analysis and atomic absorption spectroscopy were largely variable ( $6200\text{--}18,700 \mu\text{g g}^{-1} \text{dw}$ ), but always significantly higher than in roots. Hydroponic trials confirmed the typical features of Ni hyperaccumulating plants, such as growth stimulating effect at low metal doses and shoot metal concentration above the hyperaccumulation threshold. The new species of *Odontarrhena* is a Ni hyperaccumulator with substantial aboveground biomass production, making it a promising candidate for phytomining applications.

## KEYWORDS

endemic, Greek flora, nickel-hyperaccumulator, taxonomy, ultramafic

## 1 | INTRODUCTION

Ultramafic ecosystems worldwide represent paradigmatic examples of continental edaphic islands due to their discontinuous, island-like distribution across the landscape and the distinct properties of the soils that originate from the ultramafic rocks (“serpentine soils”; Kruckeberg, 2004). These characteristics are not found in

the “normal” soils of the surrounding environments and include the strong nutrient deficiency, especially in nitrogen and phosphorus, the low Ca:Mg ratio  $<1.0$ , the pH values from basic to ultrabasic, and the phytotoxic concentrations of trace metals such as Ni, Zn, and Cr (Brady et al., 2005; Brooks, 1987; Kazakou et al., 2008). Together with physical anomalies often causing drought and temperature extremes in the Mediterranean region (Coppi

et al., 2021), these factors exert a strong selective pressure on the local biotas and drive the establishment of specialized plant communities with many endemic taxa and distinct ecotypes (Chiarucci et al., 1995; Selvi, 2007). Endemism and ecotypic variation are the results of adaptation and differentiation processes to which plant populations are subject under geographic separation on the ultramafic “islands,” which acts as a barrier to gene flow (O’Dell & Rajakaruna, 2011). After the acquisition of ultramafic stress tolerance by populations of pre-adapted species, these circumstances can promote their gradual divergence and lead to the origin of distinct and reproductively isolated plant species (Rajakaruna, 2004). Hence, ultramafic landscapes provide unique settings to study the factors and mechanisms that determine first the acquisition of “serpentine” tolerance, secondly the generation and maintenance of intraspecific ecotypes and, ultimately, the origin of endemic plant species (O’Dell & Rajakaruna, 2011). Depending on the degree of specialization and the amplitude of their ecological niche, endemic species of a given region can be obligate or facultative, for example, occurring either on or off ultramafic soils (Kruckeberg, 2004; Selvi, 2007). In relation to this, speciation modes can also vary from allopatric to parapatric, peripatric, and sympatric (O’Dell & Rajakaruna, 2011).

In Europe, ultramafic “islands” are particularly widespread in the Balkan peninsula, from Serbia to the Peloponnese in Greece (Brooks, 1987; Stevanović et al., 2003). These areas are major centers of plant speciation and diversity, with c. 335 endemic taxa (species and subspecies) of which 123 are strictly found on ultramafic soils (Stevanović et al., 2003). One of the most diversified plant groups is *Odontarrhena* C.A.Mey. (Brassicaceae), a genus of 87 species primarily distributed in the Euro-Mediterranean and Irano-Turanian regions (Španiel et al., 2015). In Europe, Greece has 15 native species, seven of which are strict serpentine endemics and four are found either on or off ultramafic soils (Cecchi et al., 2020; Hartvig, 2002; Strid, 2016). This high diversity is primarily due to the large extent of ultramafic areas across the country, from the northern parts of the mainland to the southern ones and the islands, including Crete (Brooks, 1987). Additionally, these ultramafic areas extend from sea-level up to over 2600 m a.s.l. on Mt. Smolikas, thus covering a wide variety of bioclimatic and vegetation types.

However, the species taxonomy of *Odontarrhena* is still poorly resolved, mainly due to the phenotypic plasticity and the extensive occurrence of polyploidy, hybridization and introgression in several groups, as recently documented for *Odontarrhena chalcidica* (Janka) Španiel, Al-Shehbaz, D. A. German and Marhold, and other taxa

from Albania (Cecchi et al., 2018; Coppi et al., 2020). During recent geobotanical surveys in Greek ultramafic areas, we collected plant material of an *Odontarrhena* species growing on ultramafic soil on the upper slopes and summit of Mount Vourinos, western Macedonia. Apparently, there was only a single previous collection of this taxon (*E. Stamatiadou* 6972, ATH!) and it was provisionally identified as *Alyssum pichleri* Velen., now *Odontarrhena muralis* (Waldst. & Kit.) Endl. The taxon was regarded as a “hirsute” variety of *O. muralis* (Ball & Dudley, 1993; Nyárády, 1949) and more recently included in the circumscription of the species (Hartvig, 2002; Marhold, 2011). *Odontarrhena muralis* is a morphologically variable species occurring in most of continental Greece, mostly on nonultramafic soils (Hartvig, 2002; Strid, 2023). However, our morphological, karyological, and genetic studies of the *Odontarrhena* species from Mt. Vourinos suggested no close affinity to this species (and hence “*A. pichleri*”). The distinctive traits in the growth form (shrub) and indumentum of the species from Mt. Vourinos, coupled with its geographic isolation, provided enough differentiation to justify recognition of a new species.

In addition to the systematic study, we also analyzed plant tissue metal concentrations, as well as its capacity for Ni-accumulation using an experimental approach. This allowed us to test the hypothesis that this new species belongs to the group of Ni hyperaccumulators in *Odontarrhena*, plants that are able to concentrate Ni in shoots to over 1000  $\mu\text{g g}^{-1}$  dw (Reeves et al., 2017; van der Ent et al., 2013). Based on current knowledge, nearly all the ultramafic endemics in the genus are Ni hyperaccumulators with the only exception of *O. sibirica* (Willd.) Španiel et al. (Bettarini et al., 2020, 2021). The Ni-hyperaccumulator species of *Odontarrhena* have important applications in phytoremediation and phytomining, explaining the growing interest in their phylogeny, ecology, distribution, physiology, and genetics (Cecchi et al., 2010, 2013; Kidd et al., 2018; Mohtadi & Schat, 2024; van der Ent et al., 2021). Studying new populations of *Odontarrhena* growing on ultramafic soils is thus important to advance knowledge of these Ni hyperaccumulators and to identify the best candidates for practical applications in Ni phytomining and agromining.

## 2 | MATERIALS AND METHODS

### 2.1 | Field collection of plant, seed, and soil samples

*Odontarrhena* plants were observed and collected in July 2022 (G. Echevarria) and August 2022 (F. Selvi and

L. Cecchi) at two sites of Mt. Vourinos, one at lower altitude on the northern slopes close to the ridge, at 1530 m a.s.l. (40.1876° N, 21.6574° E) and one at higher altitude on the summit of the mountain, at ca. 1835 m a.s.l. (40.1760° N, 21.6724° E). The study of *Odontarrhena* populations on Mt. Vourinos was conducted within the framework of investigations on the systematics and metal accumulation in this genus (Cecchi et al., 2010, 2013, 2018, 2020; Coppi et al., 2020). A total of 12 plants were collected in the two sites (8 at the low site, of which 3 in July and 5 in August; 4 at the high site in August) for preparation of herbarium specimens and morphological analysis. Herbarium specimens were deposited in FI, P, ATH, G, K (Herbarium codes after *Index Herbariorum*), and in the laboratories of the International Hellenic University, Thessaloniki (IHU).

For the Ni-hydroponics experiment, seeds were collected at the lower site from 10 to 15 randomly selected plants with well-developed fruits. At the same site, five additional plants were collected at a distance of at least 5 m from each other and washed with distilled water for elemental analysis with atomic absorption spectrometry (AAS, see below). Here, five bulk soil samples (ca. 40 g) were collected at ca. 1–10 cm depth, placed in a plastic bag, and mixed to get a consolidated bulk soil sample ~200 g in weight.

## 2.2 | Morphology

Morphological analysis was conducted on the *exsiccata* obtained from the field-collected plants and on various herbarium specimens of related taxa from Greece and other Balkan countries, kept in FI. We examined numerous specimens of *O. muralis* including collections from the type locality of Deva, Romania (coll. Selvi, September 6, 2017; FI050439!) and Bulgarian specimens identified as “*Alyssum pichleri*”; the holotype of this taxon in PRC was examined using on-line digital resources (JStorPlants). Specimens of *Odontarrhena decipiens* (Nyár.) L. Cecchi and Selvi from our own collections in Albania and Greece were also examined. The analysis was conducted with a Nikon stereo microscope connected to a DinoLite digital photographic system. Specimens were placed on a flat surface, preferably with a light-colored background and examined from a corner-to-corner position to obtain measurements in detail. Due to their taxonomic relevance in *Alyssum* s.l. and other Mediterranean groups of Brassicaceae (Hopewell et al., 2021), microcharacters of the trichomes were examined with a scanning electron microscope-energy dispersive x-ray spectrometry (SEM-EDS). Gold-coated samples were analyzed with SEM-EDS (ZEISS EVO MA 40) with W filament, using

the following operative conditions: an acceleration potential of 10 kV, 20 pA beam current, and working distance between 8.5 and 9 mm.

## 2.3 | Karyology

Chromosome observations were performed on mitotic metaphase plates of cells samples collected from meristematic tissue of root tips of germinating seeds from the low- and high-altitude sites. Additionally, we also analyzed *O. decipiens* from north Albania (coll. L. Cecchi et al., July 11, 2016, voucher FI050443!; Cecchi et al., 2018) and *O. muralis* s.l. corresponding to the “*A. pichleri*” morphotype from a Greek locality of the Rhodope region north of Paranesti, Drama (41.3370° N, 24.4485° E; coll. F. Selvi & I. Bettarini, August 10, 2018; FI055807!). Tissue samples were pretreated with 0.002 M 8-hydroxyquinoline for 2.5 h at room temperature, fixed overnight in ethanol/glacial acetic acid (3:1), rinsed in distilled water, hydrolyzed in 1 M HCl at 60°C for 6–7 min and stained in lacto-propionic orcein overnight (Cecchi et al., 2013; Dyer, 1979). The meristems were dissected and squashed in a drop of 45% acetic acid. Metaphase plates were examined with a Zeiss Axioscop light microscope under oil immersion ( $\times 100$ ).

## 2.4 | Molecular phylogenetic analyses

We used the ITS region (ITS1-5.8S-ITS2) of the nrDNA for a first genetic analysis of the species from Mt. Vourinos with respect to the morphologically closer taxa, *O. muralis* and *O. decipiens*. This marker has been widely used in previous analyses of Alyssae for its phylogenetic signal (Cecchi et al., 2010, 2013; Coppi et al., 2021; Li et al., 2015; Melichárková et al., 2019; Rešetnik et al., 2013; Salmerón-Sánchez et al., 2018). Genomic DNA was extracted from silica-gel dried leaf tissue samples collected from four plants (two at the low and two at the high site), using a modified 2  $\times$  CTAB protocol (Doyle & Doyle, 1987). Quality control of extracted DNA, amplification, and sequencing of the nrITS region was performed as described in Coppi et al. (2020). The electropherograms showing overlaps between any IUB (International Union of Biochemists) nucleic acid (except N) were scored as follows: A or C = M; A or G = R; A or T = W; C or G = S; C or T = Y; G or T = K; A or C or G = V; A or C or T = H; A or G or T = D; C or G or T = B; A or C or G or T = X or N. These codes are compatible with the GenBank data libraries and indicate two or more alternatives in the single locus due to paralogous DNA traits (hereafter termed “degenerate bases”).

The four original sequences were deposited in GenBank and aligned with 24 accessions of the most closely related taxa of *Odontarrhena* and three accessions of *Alyssum* as outgroup representatives, based on Coppi et al. (2020). The multiple alignments of the 31 total accessions in our dataset was achieved using the Q-INS-i algorithm with MAFFT version 7 (Kato & Standley, 2013). The phylogenetic analysis was performed using the Bayesian approach as implemented in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). The model of nucleotide substitution used was GTR for ITS-5.8S, with gamma-distributed rate variation across sites. The analysis was performed using four incrementally heated Markov chains, simultaneously starting from random trees, and running for a maximum of 1 million cycles, sampling one tree every 1000 generations. The stationary phase was reached at 900,000 cycles. Trees that preceded the burn-in threshold were discarded, and the remaining trees were used to calculate the consensus phylogram. The phylogenetic tree was displayed and edited with MEGA vs. 11 (Tamura et al., 2021), indicating the internal tree nodes posterior probability values when  $>0.6$ .

## 2.5 | Ni-hydroponics study

After a period of cold stratification in a refrigerator, seeds were sown onto peat soil and 2-week-old seedlings were then transferred to hydroponic culture, in 1-L polyethylene pots containing a modified half-strength Hoagland's solution (Hoagland & Arnon, 1950) in milliQ-water (Millipore, Billerica, MA, USA) buffered with 2 mM 2-morpholinoethanesulphonic acid, pH 5.5, adjusted with KOH. Background Ni concentration in the growing medium was about  $0.1 \mu\text{M}$  and this was considered as the control condition. Plants were grown in a growth chamber (24/16°C day/night; light intensity  $100 \mu\text{mol}^{-2} \text{s}^{-1}$ , 16-h (day) photoperiod; relative humidity 60%–65%). After 10 days of preculture, root and shoot length of each plant was measured and then plants were exposed to 0, 50, 150, 250, 500, 1000, 2000, and 3000  $\mu\text{M}$  of  $\text{NiSO}_4$  concentrations (12 plants per treatment), in a background solution of the same composition as the preculture solution for 7 days. Root and shoot length of all plants were then measured again and harvested. For each plant, the shoots were separated from roots. Shoots were rinsed with deionized water. Roots and shoots were desorbed with  $\text{Pb}(\text{NO}_3)_2$  at 4°C for 30 min as in Bazihizina et al. (2015).

Data analysis was carried out using the four-parameter hormetic model proposed by Brain and Couzens (1989). This was used to fit the experimental data of root and shoot growth response to Ni treatment. The

model allowed us to estimate the half-maximal effective concentration ( $\text{EC}_{50}$ ), the maximum stimulation dose, and the maximum mean response (MAX, necessary for a reliable calculation of the percentage of the hormetic effect as  $[100 * (\text{MAX} - \text{length in control condition}) / \text{length in control condition}]$ , here named hormetic percentage [HP]). The drc package (Ritz et al., 2015) as implemented in R Studio version R 4.3.0 (R Core Team, 2023) was used to fit the concentration–response curve. The significance of differences was analyzed by one-way ANOVA followed by a Mann–Whitney pairwise test using Past 4.03 (Hammer et al., 2009).

## 2.6 | AAS analysis of elemental concentrations in soil and plant samples

Once in the laboratory, the soil samples were air-dried, sieved to 2 mm, and then further dried in an oven at 50°C for 7 days. Plants samples (roots and shoots) collected from the field ( $n = 5$ ) and used for the Ni-hydroponics experiment ( $n = 12$ ) were carefully washed with deionized water, blotted dry with filter paper, oven-dried at 50°C for 48 h and ground to a fine powder with mortar and pestle. Three analytical replicates of each root and shoot samples and five subsamples of the pooled bulk soil were digested using 10 mL of 69%  $\text{HNO}_3$  in a microwave system (Mars 6, CEM); the concentration of K, Ca, Mg, Ni, Cr, and Co was determined by flame AAS using PinAAcle 500 (Perkin Elmer).

## 2.7 | X-ray fluorescence analysis of elemental concentrations in herbarium specimens

In addition to AAS analysis, elemental concentrations (K, Ca, Mg, Zn, Ni, Cr, and Co) were analyzed in the three herbarium specimens kept in P, using monochromatic x-ray fluorescence (XRF) spectroscopy (Z-SPEC JP-500 apparatus). XRF spectroscopy is most suitable for tissue elemental analysis of herbarium specimens since it is less destructive than alternative methods such as acid digestion of a much larger tissue sample (0.5 g) and analysis by inductively coupled plasma-optical emission spectrometry (ICP-OES) or AAS. Samples consisted of 8–12 leaves (range between 0.05 and 0.1 g) removed and organized to completely a 10 mm-diameter disc placed on the sample holder. The sample was then covered with a thin film (12  $\mu\text{m}$ ) of polypropylene. Samples were measured for 30 s with the Z-SPEC JP-500. Three replicate measurements per sample were taken.

### 3 | RESULTS

#### 3.1 | Morphology

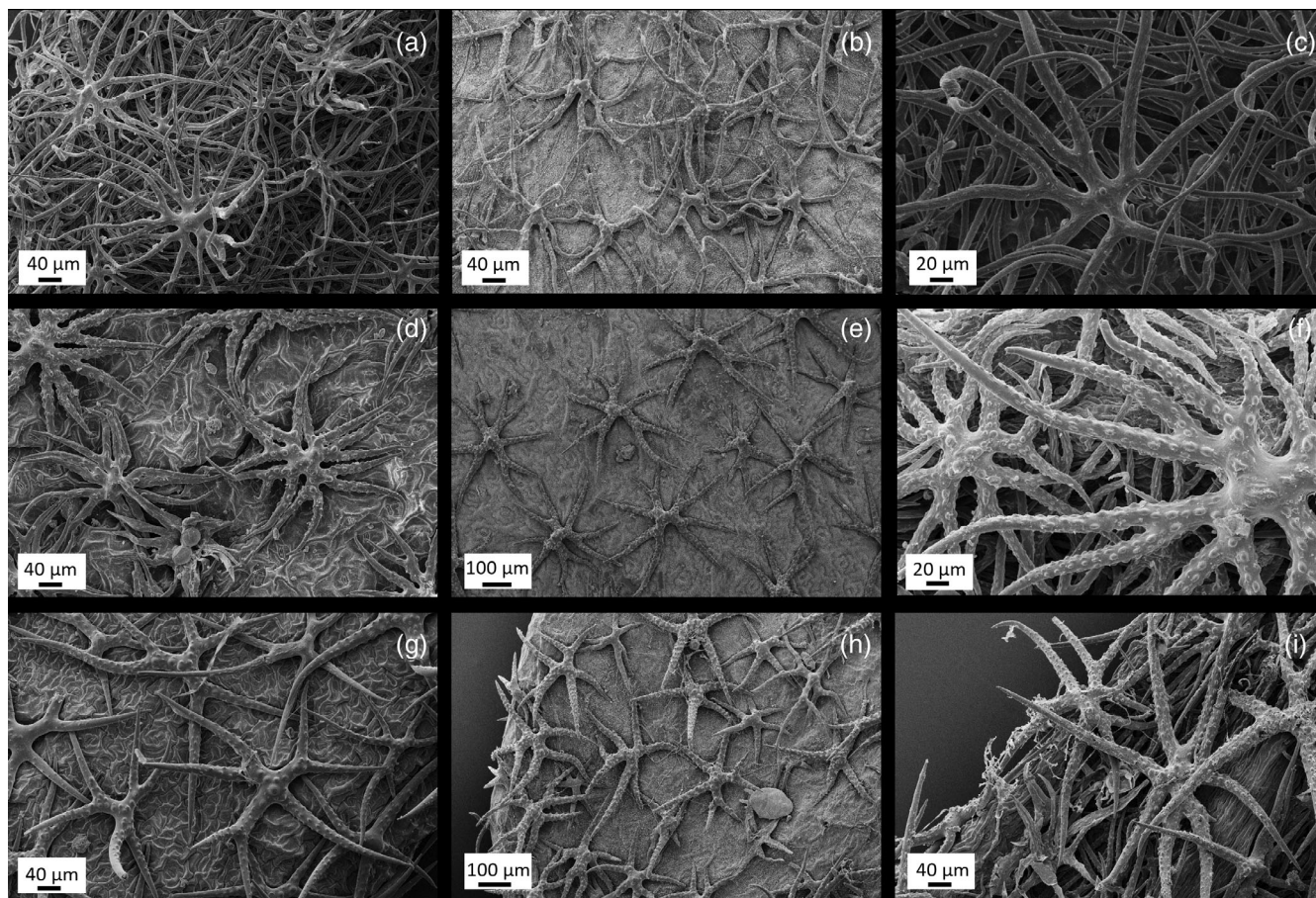
The *Odontarrhena* species from Mt. Vourinos is a subshrub up to 80 cm wide and 70 cm tall. It is highly branched from the base with numerous sterile shoots that are densely leafy (Figure 1a,b); the stems are up to 7 mm in diameter towards the base and woody (Figure 1g). This species approaches *O. decipiens* in growth form, but it is even more densely branched and with thicker stems. In comparison, *O. muralis* (including “*A. pichleri*”) differs in the less robust habit and fewer sterile shoots; the slender stems did not have a similarly thickened base. The leaves of the sterile shoots are obovate-spathulate, 12–20 × 4–6 mm in size (including the petiole), like the Mt. Vourinos species and *O. decipiens* (Figure 1e). The characteristic feature of the Mt. Vourinos species is the dense cover of stellate hairs (indumentum) on the upper part of the stem and on both leaf surfaces, giving to it a white-silvery appearance (Figure 1b,e). SEM observations revealed that these trichomes are arranged in overlapping appressed layers, such that the leaf epidermal surface is completely covered and not visible, even on the upper (adaxial) surface (Figure 2a,c). Trichomes are 0.5–0.9 mm across and formed by 6–8 thin-forked

rays (12–16 rays total) covered with granular thickenings (Figure 2c). The density of the indumentum is diagnostic with respect to *O. decipiens* since the trichomes on stem and adaxial leaf surface of this species are arranged in a single layer and the epidermal cells remained visible, so that these parts appear gray-green rather than white-silvery (Figure 2d,f). The leaf trichomes of *O. decipiens* also differ in the smaller size (0.3–0.6 mm across) and less numerous (10–15), but thicker rays (Figure 2d). Plants of *O. muralis* from the Rhodope region north of Paranesti, corresponding to the concept of “*A. pichleri*”, are not white-silvery due to the lower density of hairs on stem and leaves (Figure 2g,i). The type specimen of *A. pichleri* shows the same type of indumentum and is also different in the shape of the cauline leaves (narrowly oblanceolate).

The Mt. Vourinos species had flowering stems in mid-July, with oblanceolate leaves. By late August, the leaves had senesced and dropped off the plants, resulting in leafless stems at fruiting stage. In *O. decipiens* and *O. muralis* s.l., instead, the cauline leaves usually remain present at the fruiting stage. The inflorescence of the Mt. Vourinos species is broadly corymbose, with branching of third and sometimes even fourth order. Similar to *O. decipiens*, the partial racemes support up to 15 dense flowers (Figure 1c), while in *O. muralis* s.l. these are less



**FIGURE 1** Summit area of Mt. Vourinos with fruiting plants of *Odontarrhena vourinensis* sp. nov. on ultramafic rocks (a), (photo F. Selvi, August 28, 2022); habit of the plant in fruiting stage (b), (F. Selvi, August 28, 2022); portion of inflorescence and flowers (c), (G. Echevarria, July 9, 2022); ovary with dense hairs (bar 0.1 mm; d); upper leaves of sterile shoots (e), (G. Echevarria, July 9, 2022); silicles (f); basal part of the stem (bar 0.2 mm; g), (G. Echevarria, July 9, 2022); silicles and seed (bar 1 mm; h), (L. Cecchi, August 28, 2022).



**FIGURE 2** Scanning electron micrographs of trichomes on adaxial leaf surface and silicle valves. Plant from Mt. Vourinos *Odontarrhena vourinensis* sp. nov. (a–c), *Odontarrhena decipiens* from Lake Aooos (Limni Aooou, Metsovo, Greece FI055822) (d–f) and *O. muralis* variant “*pichleri*” from the Rhodope region (Paranesti, Greece, FI055807) (g–i) adaxial leaf surface (a,d,g); silicle surface (b,e,h); details of leaf trichomes (c,f,i).

numerous (up to 8–10). The flower pedicels of the Mt. Vourinos species are erecto-patent, thin, while these are thicker and more rigid in *O. muralis*. The inflorescence branches are more densely hairy than in *O. decipiens*, as well as the flower pedicels, sepals, petals and, even more, the ovary (Figure 1d). The silicles of the Mt. Vourinos *Odontarrhena* species average  $2.2 \times 4$  mm and are similar to *O. decipiens* in shape and size, being narrowly to broadly elliptical, symmetrical and not undulate (Figure 1f,h), but the hairs on the valves are denser than in the latter (Figure 2b,e). In *O. muralis* s.l. the silicles are usually larger (4–5 mm) and more rounded, nearly orbicular, mostly glaucous and with more or less undulate margins. In the “*A. pichleri*” variant, the valves have a higher density of hairs (Figure 2h), resulting in a more strigose surface. The style is 2 mm long in all three taxa. The main diagnostic morphological characters of the Mt. Vourinos species, *O. decipiens* and *O. muralis* s.l. (incl. “*A. pichleri*”) are summarized in Table 1.

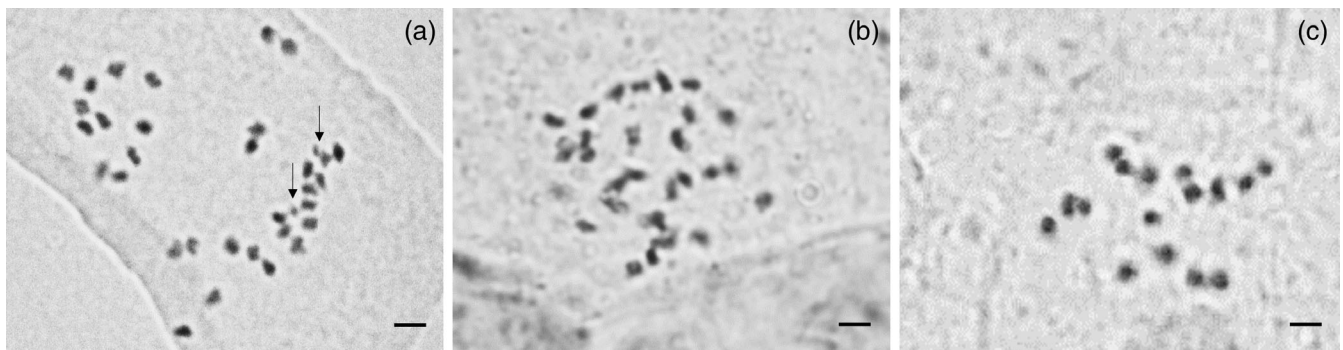
The seeds of the Mt. Vourinos *Odontarrhena* species are 2.0–2.2 mm in diameter and winged, similarly to *O. decipiens* and *O. muralis* s.l. (Figure 1h).

Phenological characters also pointed to differentiation between the plant from Mt. Vourinos and *O. decipiens*. In the former, flowering occurred around mid-July, with most reproductive individuals bearing at least 60%–70% of the flowers in anthesis at the same time. Fruiting was also relatively synchronous between mature individuals, with most of them bearing ripe silicles at the end of August. In *O. decipiens*, the flowering phase was temporally more diluted, even in the same region and within groups populations, starting in late June and extending to mid-August. Hence, the overlapping time of flowering between the two species was around mid of July. Fruit ripening was from the end of July to the first half of October, hence extending more towards the end of the summer and early autumn than in the species from Mt. Vourinos.

An updated key to the species of *Odontarrhena* in Greece is provided as Supporting information S1.

**TABLE 1** Main diagnostic morphological characters of *Odontarrhena vourinensis* sp. nov., *O. decipiens* and *O. muralis* s.l. (incl. “*A. pichleri*”).

	<i>O. vourinensis</i> sp. nov.	<i>O. decipiens</i>	<i>O. muralis</i> s.l.
Growth form	Subshrub up to 80 cm wide and 70 cm tall, densely branched from base, with thick woody base and many nonflowering shoots	Densely caespitose perennial herb, up to 40 cm wide and 90 cm tall, branched from base, with many nonflowering shoots	Caespitose perennial herb, with few to numerous nonflowering shoots
Leaf indumentum	Very dense, white-silvery with overlapping layers of stellate hairs on both surfaces; hairs 0.5–0.9 mm across, with 12–16 rays	Very dense beneath (abaxial surface white-silvery), sparser above (hairs not in overlapping layers, adaxial surface greenish); stellate hairs 0.3–0.6 mm across with 10–15-rays	Dense beneath (abaxial surface gray), sparse above (adaxial surface green with few hairs); stellate hairs 0.5–0.8 mm across, with 8–15 rays
Indumentum of flower pedicels, sepals, petals and ovary	Very dense	Dense	Sparse
Number of flowers on partial racemes	10–15 (18)	10–15	4–8 (10)
Fruiting pedicels	Thin, 3.5–4 mm	Thin, 3.5–4 mm	Stout, rigid, c. 3.5 mm
Silicles	Elliptical, c. 2.2 × 4 mm, valves not undulate, with dense (but not overlapping), 5–10-rayed stellate hairs 0.3–0.5 mm across, appressed	Elliptical, c. 2.2 × 4 mm, valves not undulate, with 6- to 8-rayed stellate hairs 0.3–0.5 mm across, often sparse (not overlapping), appressed	Mostly orbicular, 3 × 4–5 mm, glaucous, valves usually undulate covered with dense, mostly overlapping 5–10-rayed stellate hairs 0.3–0.5 mm across, patent-strigose in “ <i>A. pichleri</i> ”
Chromosome number	2n = 32 + 0-2B	2n = 32 + 0-3B	2n = 16

**FIGURE 3** Metaphase chromosome plates. (a) *Odontarrhena vourinensis* sp. nov. from Mt. Vourinos at 1530 m a.s.l. (voucher FI078193), 2n = 32 + 2B (arrows indicate B-chromosomes); (b) *Odontarrhena decipiens* from north Albania (Pukë, voucher FI050443), 2n = 32; (c) *Odontarrhena muralis* variant “*pichleri*” from the Rhodope region, (Paranesti, Greece; voucher FI055807), 2n = 16; scale bars: 5 µm.

### 3.2 | Karyology

Both populations from Mt. Vourinos resulted tetraploid with  $2n = 4 \times = 32$ . Additionally, we observed the almost consistent presence of two small supernumerary

B-chromosomes (Figure 3a). A very similar chromosome complement was observed in *O. decipiens* ( $2n = 4 \times = 32$ , Figure 3b), while countings on the plants of the “*A. pichleri*” variant of *O. muralis* showed the diploid level  $2n = 2 \times = 16$  (Figure 3c).

**TABLE 2** Number of variable positions in the ITS1 and ITS2 alignment in four accessions of the *Odontarrhena* taxon from Mt. Vourinos, one accession of *O. decipiens* and one of *O. muralis* (see Section 2 for details).

	GenBank accession	ITS1 variable loci						ITS2 variable loci	
		40	70	73	86	111	197	393	513
<i>O. vourinensis</i> 1	OR735473	G	C	Y	Y	Y	R	C	C
<i>O. vourinensis</i> 2	OR735476	G	C	Y	Y	Y	R	C	C
<i>O. vourinensis</i> 3	OR735475	G	C	Y	Y	Y	R	C	C
<i>O. vourinensis</i> 4	OR735474	G	C	Y	Y	Y	R	C	C
<i>O. decipiens</i>	MK775294	G	C	C	T	C	G	C	C
<i>O. muralis</i>	MK775296	A	T	C	T	C	G	T	T

Note: Accessions *Odontarrhena vourinensis* sp. nov. 1–3 were from the summit of Mt. Vourinos at 1835 m (reference collection number FI078194); accession *Odontarrhena vourinensis* sp. nov. 4 was from the lower site at m a.s.l. (reference collection number FI078193, holotype).

### 3.3 | DNA sequences and phylogenetic relationships

The ITS1-5.8s-ITS2 alignment of the 31 examined accessions resulted in 619 base-pair positions. The four accessions from Mt. Vourinos showed identical nucleotide sequences and were very similar to *O. decipiens* (GB: MK775294), from which they differed by four degenerate bases in singleton positions 73, 86, 111 (C or T = Y) and 197 (A or G = R; Table 2). The phylogenetic relationship between these accessions was not resolved in the Bayesian phylogram (Figure 4). The three accessions of *O. muralis* were identical to each other and different from the Mt. Vourinos accessions, showing four substitutions equally distributed in the ITS1 and ITS2 region. This species was resolved as sister to *O. chalcidica* (GQ284867 and GQ284885) in the phylogenetic tree (Figure 4).

### 3.4 | XRF analysis of herbarium specimens

Leaf elemental concentrations in herbarium specimens of *Odontarrhena vourinensis* are reported in Table 3. Potassium varied from 0.6% to 1.1% and Ca varied from 2.0% to 3.2%. Zinc concentrations were relatively elevated for plants growing on ultramafic soils. Nickel concentration was variable among the three specimens and within each specimen, reaching 1.2% in the plants from the summit area (1835 m). Cobalt was also higher in the summit plants and varied with Ni concentration.

### 3.5 | Taxonomic treatment

Based on the morphological, karyological and molecular data, together with geographical isolation on ultramafic

soil, the plant from Mt. Vourinos is described here as a new species.

*Odontarrhena vourinensis* Selvi, Echevarria and Bianchi, sp. nov.—HOLOTYPE: “Greece, W. Macedonia, Mt. Vourinos, stony grassland at the forest edge on north-facing slopes close to the ridge, 40.1876 N, 21.6574 E, 1530 m a.s.l., serpentine.” F. Selvi and L. Cecchi, August 28, 2022 (FI078193; Figure 5); isotypes P (P02168139), ATH, G, K.

### 3.6 | English diagnosis

Differs from *O. decipiens* by the more robust, densely branched growth form (shrub) and white-silvery indumentum, the upper leaf surface and stem with trichomes in overlapping layers completely covering the epidermis, the more densely hairy inflorescence branches, floral pedicels, sepals, petals and ovary, the larger leaf trichomes with thinner rays, the leafless stems at fruiting stage, and the earlier fruiting. Differs from *O. muralis* sensu lato by the more robust, densely branched growth form (shrub) with white-silvery indumentum, the thinner flower pedicels, the smaller elliptical silicles, not glaucous, without undulate margins and without dense, overlapping stellate hairs, the tetraploid chromosome complement.

#### 3.6.1 | Etymology

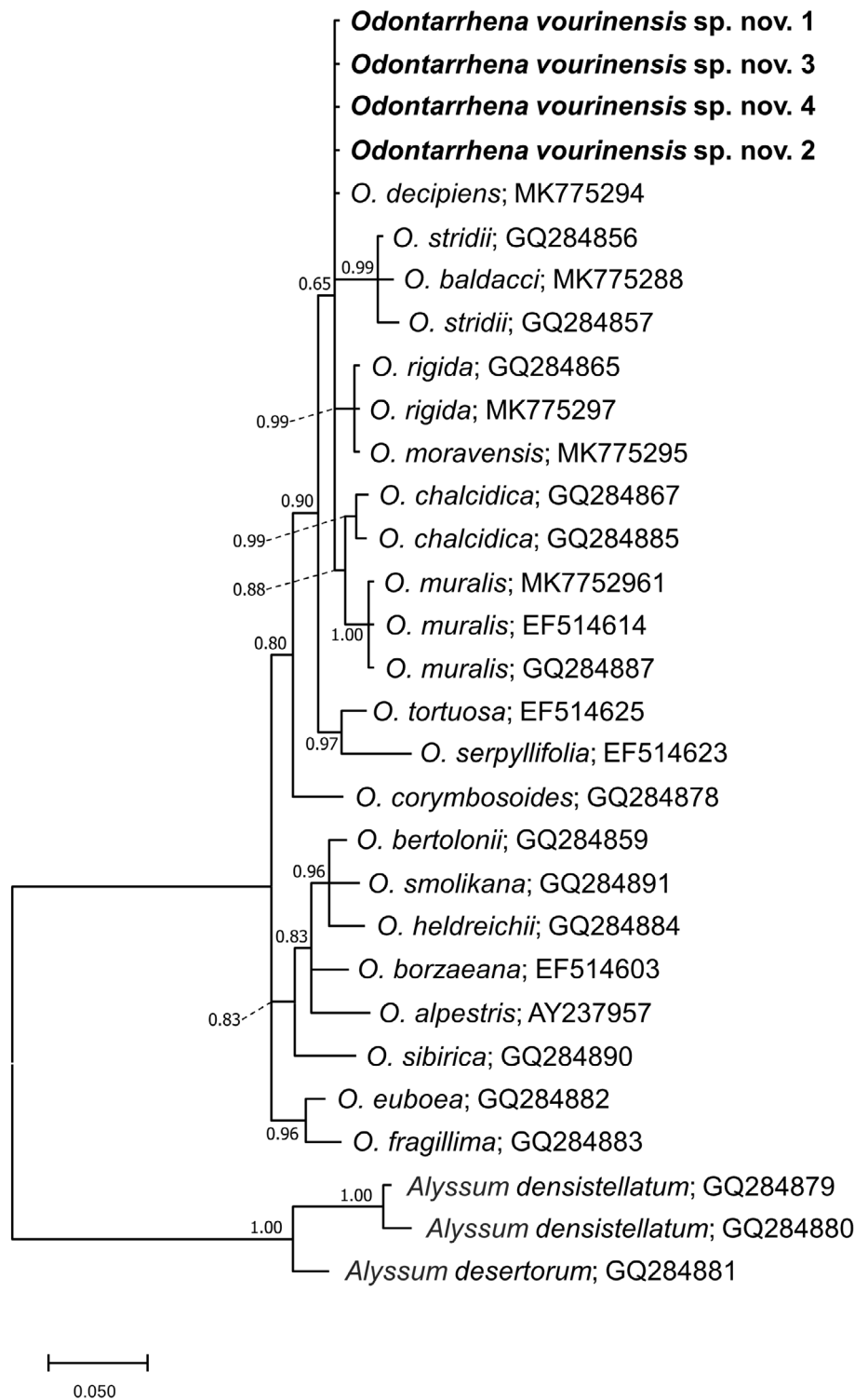
This species takes its name from the mountain where it grows, Mount Vourinos.

#### 3.6.2 | Examined specimens

W. Macedonia: prov. and distr. Kozani. N. side of Mt. Vourinos (SE of Siatista), on the ridge from



**FIGURE 4** Bayesian consensus phylogram from ITS-5.8S sequences of *Odontarrhena*, showing relationships of the species from Mt. Vourinos to other taxa. Posterior Probability values are shown at the nodes.



Mavrovouni to summit, S the place called “Pigi Tsamia.” Alt. 1450–1600 m. Open, dry rocky slopes and stony grassland between the first and second saddle. Serpentine. coll. E. Stamatiadou 6972, July 31, 1969 (sub *A. pichleri* Velen., ATH 3445!). Mt. Vourinos, footpath from Katafigio to the summit, north-facing slope close to the ridge, above forest belt, 1535 m, serpentine. G. Echevarria, July 9, 2022 (P02168140!). Greece, W. Macedonia, Mt. Vourinos, among serpentine rocks

near the summit, 40.1760 N; 21.6724 E, 1835 m, F. Selvi and L. Cecchi, August 28, 2022 (FI078194!; P02168138!).

### 3.7 | Description

Small shrub with woody stems up to 7 mm thick, densely branched from base, up to 80 cm wide, white-silvery. Sterile stems numerous, with densely leafy shoots; leaves

**TABLE 3** Elemental concentrations ( $\mu\text{g g}^{-1}$  DW) in subsamples of leaves from the herbarium specimens of *Odontarrhena vourinensis* sp. nov. (vouchers), analyzed by quantitative monochromatic x-ray fluorescence spectrometer ( $n = 3$  from the same specimen).

Voucher no.	Stage	Altitude m a.s.l.	K	Ca	Zn	Ni	Co
P02168140	Flower	1535	11,100 $\pm$ 5020	31,920 $\pm$ 13,480	56.3 $\pm$ 35.1	10,980 $\pm$ 3180	10.5 $\pm$ 1.2
P02168139 (Isotype)	Fruit	1530	6110 $\pm$ 2230	22,850 $\pm$ 10,100	13.0 $\pm$ 3.0	6506 $\pm$ 1215	3.5 $\pm$ 1.6
P02168138	Fruit	1835	9780 $\pm$ 1680	20,270 $\pm$ 1480	53.4 $\pm$ 23.4	12,440 $\pm$ 1470	13.6 $\pm$ 1.2

Note: Mean  $\pm$  standard error.

obovate-spathulate, 12–20  $\times$  4–6 mm, with both surfaces densely covered by stellate trichomes 0.5–0.9 mm across, with 6–8 forked rays (12–16 rays in total), arranged in overlapping layers. Flowering stems up to 70 cm, stellate-pubescent, with oblanceolate leaves during flowering time; fruiting stems aphyllous. Inflorescence broadly corymbose, with branching of the third and fourth order, branches hairy; partial racemes with up to 10–15 (18) densely spaced flowers. Flower pedicels erecto-patent, thin, straight, hairy. Sepals c. 2.2  $\times$  0.5–0.8 mm, petals c. 1.8–2.8 mm, both with stellate hairs; ovary densely hairy. Silicles 2.2  $\times$  4 mm, narrowly to broadly elliptical, symmetrical, not undulate, with relatively dense hairs c. 0.2 mm across, 5- to 10-rayed; style 2 mm long. Seeds 2.0–2.2 mm across, with a wing 0.4–0.5 mm. Flowering in the first half of July; fruit maturity in late August and early September.

### 3.8 | Distribution and habitat

The species occurs on the ridge and north-facing slopes of Mt. Vourinos (Kozani, western Macedonia), at an altitudinal range of 1540–1860 m a.s.l. It appears to be a strict endemic of ultramafic substrates and a local endemic of Mt. Vourinos, growing within the open vegetation of rocky ultramafic soils. It is locally abundant in dry, rocky grasslands, as well as in rock fissures and crevices (Figure 1a,b).

### 3.9 | AAS analysis of field-collected soil and plant samples

Elemental concentrations in shoots, roots, and soil of *O. vourinensis* collected from the lower elevation site are shown in Table 4. Elemental concentrations were higher in shoots than in roots for all the elements, except for K and Cr. Compared with the soil, shoot concentration was higher for K, Ca, and Ni. Both roots and shoots showed a Ca:Mg mol ratio  $>1$ , whereas in the soil this was  $<1$ . Overall, leaf metal concentration measured with AAS in three summit plants were in a similar range

with K = 15,133  $\pm$  2302  $\mu\text{g g}^{-1}$ , Ca = 26,581  $\pm$  407, Ni = 18,699  $\pm$  2932, and Co = 15.0  $\pm$  3.

Adding the lower elevation accession of *O. vourinensis* to the linear regression analysis of soil-shoot Ni concentration in other *Odontarrhena* taxa from the Balkan ultramafics showed its position close to two accessions of *O. decipiens* (Figure 6).

### 3.10 | Ni-hydroponics study

Root and shoot length in *O. vourinensis* treated with increasing  $\text{NiSO}_4$  concentrations is shown in Figure 7a. Root growth was significantly higher than the control for the 50 and 150  $\mu\text{M}$   $\text{NiSO}_4$  treatment. Shoot growth was significantly higher than the control for the 150  $\mu\text{M}$  treatment. Root growth was significantly lower than the control at and above the 1000  $\mu\text{M}$   $\text{NiSO}_4$  treatment. Shoot growth was significantly lower than the control at the 3000  $\mu\text{M}$  (maximum) treatment. Ni accumulation increased with increasing metal concentration in the substrate, closely following a saturation trend curve, and element concentration was always higher in shoots than in roots (Figure 7b). The root and shoot dose-response analysis showed significant data fitting to the hormetic model of Brain-Cousens, allowing for calculation of the hormetic and tolerance parameters (Table 5).

## 4 | DISCUSSION

*Odontarrhena vourinensis* is an obligate endemic of ultramafic soil that probably arose through in-situ isolation and divergence from *O. decipiens*, towards the southeastern margin of its range. The parapatric speciation mode as illustrated in O'Dell and Rajakaruna (2011) provides a plausible explanatory model for the origin of this geographically isolated species. *Odontarrhena decipiens* was described from Mt. Smolikias, ca. 60 km to the west of Mt. Vourinos, and is frequent in the ultramafic areas of North Pindos as well as those of Albania, Macedonia, and Kosovo, at 220–1900 m a.s.l. (Cecchi et al., 2018). *Odontarrhena decipiens* is considered to be of homoploid

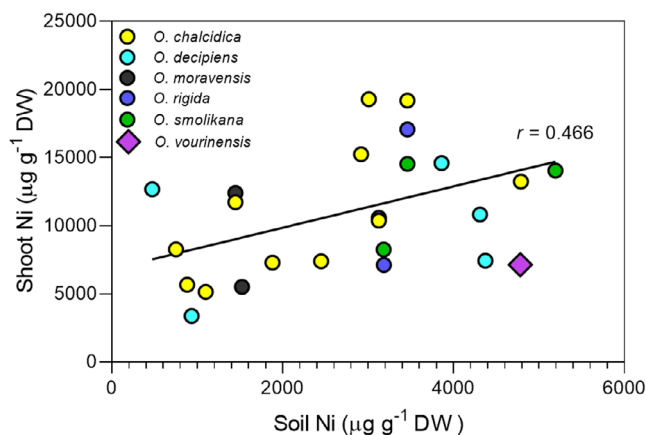
3 novembre 2023



FIGURE 5 Holotype of *Odontarrhena vourinensis* Selvi, Echevarria, and Bianchi (FI078193).

**TABLE 4** Total elemental concentrations ( $\mu\text{g g}^{-1}$  DW) in shoots, roots, and soil for *Odontarrhena vourinensis* sp. nov. ( $n = 5$ ); values are means  $\pm$  standard errors.

	K	Ca	Mg	Ca:Mg	Ni	Cr	Co
Shoot	10,872 $\pm$ 1115	51,371 $\pm$ 3110	3029 $\pm$ 219	17.5 $\pm$ 2.1	7133 $\pm$ 461	4.2 $\pm$ 0.6	9.9 $\pm$ 0.7
Root	12,444 $\pm$ 962	1724 $\pm$ 408	1842 $\pm$ 344	1.2 $\pm$ 0.5	5941 $\pm$ 257	11.3 $\pm$ 2.6	3.4 $\pm$ 0.7
Soil	3627 $\pm$ 99	13,452 $\pm$ 1651	90,130 $\pm$ 3657	0.15 $\pm$ 0.02	4781 $\pm$ 127	660 $\pm$ 28	187.0 $\pm$ 9.0



**FIGURE 6** Relationship between shoot and soil Ni concentrations in *Odontarrhena vourinensis* sp. nov. and other Albanian congeneric Ni-accumulator taxa (analyzed in Bettarini et al., 2019) as resulting from linear regression analysis ( $p < 0.05$ ). The star indicates the accession of *O. vourinensis* from lower altitude (1530 m a.s.l.).

hybrid origin between the tetraploid *O. smolikana* (Nyár.; Španiel et al., 2015) and a taxon of the *O. muralis* group, probably the tetraploid *O. chalcidica* (Janka; Coppi et al., 2020; Hartvig, 2002; Španiel et al., 2015).

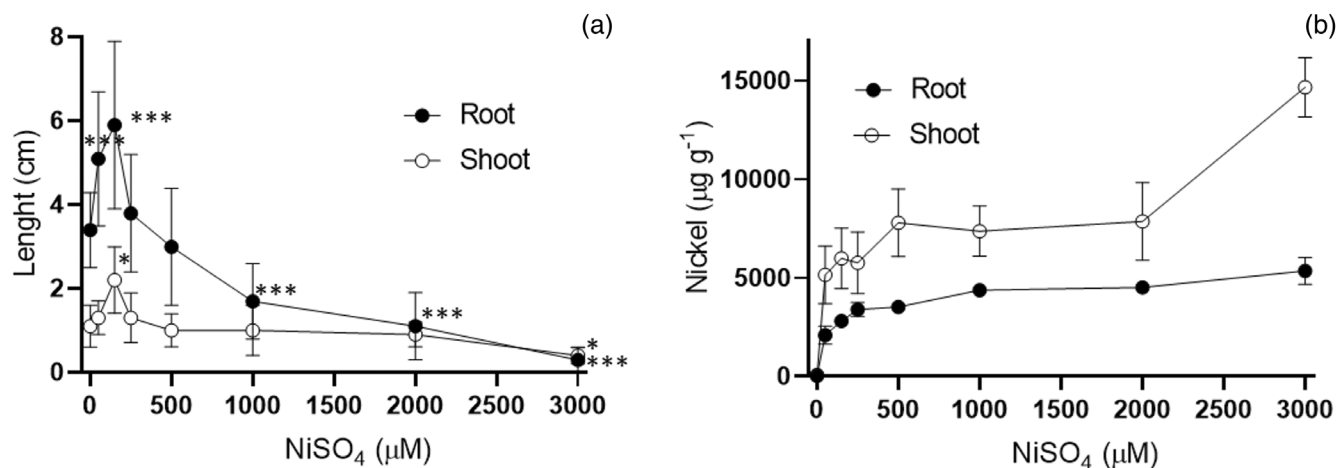
Karyotype and sequences from the nrDNA ITS region point to a close relationship between *O. vourinensis* and *O. decipiens*. In fact, a very similar chromosome complement ( $2n = 32$ ) was observed in two accessions of *O. decipiens* from North and central Albania (Cecchi et al., 2018), while “*A. pichleri*” showed a diploid level ( $2n = 16$ ), in line with previous reports for *O. muralis* (Cecchi et al., 2018; Hartvig, 2002). However, differentiation of *O. vourinensis* from *O. decipiens* is supported by the presence of four degenerate positions in the ITS1 region. The degeneracy at different positions in the genome reflects the likelihood that structurally different elements may perform alternative functions depending on environmental factors. This process is nowadays considered as a significant component in biological evolution at all levels of organization (Edelman & Gally, 2001). Moreover, intraspecific variation of ITS pseudogenes has been reported in angiosperms (Álvarez & Wendel, 2003; Buckler IV et al., 1997; Hartmann et al., 2001), often

associated with hybridization and consequent allopolyploidy (Wendel et al., 1995).

Phenological characters also support the differentiation between *O. vourinensis* and *O. decipiens*, since both flowering and fruiting were earlier and more synchronous in the former, whereas in the latter these processes were temporally more extended towards the summer and autumn (Cecchi et al., 2018).

Mount Vourinos covers an area of ca. 450 km<sup>2</sup> and belongs to the NNW–SSE trending belt within the orogen of the Hellenides, that extends further north into the Dinarides; it is formed by ophiolitic rocks (mainly depleted harzburgites) of Mid- to Late-Jurassic age, similarly to the ophiolitic rocks of the Pindos (Liat et al., 2004). It is an important center of ultramafic endemism in continental Greece, due to geographical isolation, wide altitudinal range, and different microclimatic conditions on the southern and northern slopes (Akeroyd & Preston, 1981; Stevanović et al., 2003). The area is inhabited by three other species of *Odontarrhena*; (i) *O. chalcidica* has a clear affinity for ultramafic soils and occurs at lower altitudes on both sides of Mt. Vourinos, usually on disturbed ground; (ii) *O. heldreichii* (Hausskn.) Španiel et al. is a strict ultramafic endemic of northern Greece that also occurs on both sides of Mt. Vourinos in more natural communities over a broad altitudinal range; (iii) *O. corymbosoidea* (Form.) Španiel, Al-Shehbaz, D. A. German, and Marhold occurs in a few sites at the southern foot of Mt. Vourinos, at the geologic boundary between ultramafic and calcareous rock formations (L. Cecchi & F. Selvi 08.25, FI!; F. Selvi & I. Bettarini FI055813!). The phytogeographic relevance of Mt. Vourinos is highlighted by other local ultramafic endemics, such as *Onosma elegantissima* Rech. fil. and Goulimy, *Viola vourinensis* Erben, *Anthyllis serpentinicola* Rech. fil. and Goulimy, *Inula serpentinicola* Rech. fil. and Goulimy, *Silene parnassica* subsp. *vourinensis* Greuter, and *Nepeta argolica* subsp. *vourinensis* (Baden) Strid and Kit Tan.

Based on metal concentration analyses, *O. vourinensis* showed the typical features of Ni-accumulating plants. Shoot Ni, Ca, and K concentrations were higher than in the corresponding soil, as usually reported (Brooks, 1987;



**FIGURE 7** Length (cm) (a) and Ni concentration ( $\mu\text{g g}^{-1}$  d w) (b) in shoots and roots of *Odontarrhena vourinensis* sp. nov. treated with eight  $\text{NiSO}_4$  concentrations for 7 days ( $N = 12$ , mean  $\pm$  standard deviation). Asterisks indicate the significant differences in shoot and root length compared with control conditions according to Tukey's test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**TABLE 5** Parameters of the root and shoot dose–response curves of *Odontarrhena vourinensis* sp. nov. inferred from Brain-Cousens hormetic model in the presence of eight  $\text{NiSO}_4$  concentrations.

	Lack-of-fit test	$\text{MSD}_{\text{ext}}$ ( $\mu\text{M}$ )	MAX (cm)	HP (%)	$\text{EC}_{50\text{ext}}$ ( $\mu\text{M}$ )
Shoot	0.055	123.5	1.7	152.9	$2824.8 \pm 417.8$
Root	0.057	94.4	5.7	168.2	$946.6 \pm 177.7$

Abbreviations: HP, hormetic percentage; MSD, maximum stimulation dose.

Kazakou et al., 2008), while Co, Cr, and Mg were lower. The levels of the examined metals in roots and shoots were in the range of those found in other Ni-hyperaccumulating taxa of *Odontarrhena* from the Albanian serpentine soils (Bettarini et al., 2019). Our findings, however, point to a significant variation, perhaps dependent on inherent individual differences or on local climate and soil conditions. Plants from higher altitude apparently accumulated more Ni possibly in relation to variation in soil characteristics, such as organic matter content and pH. The latter variable is in fact known to affect Ni accumulation in obligate and facultative hyperaccumulators of *Odontarrhena* and other genera (Bettarini et al., 2020; Kukier et al., 2004; Reeves et al., 2015; Selvi et al., 2017). From the analysis of the Ni plant–soil relationship, the accession of *O. vourinensis* from lower elevation was closer to one of the populations of *O. decipiens* than to any other accession of Balkan hyperaccumulators analyzed in Bettarini et al. (2019). On the other hand, our data show that *O. vourinensis* is also able, like *O. decipiens* and several other congeneric serpentine taxa, to reach concentrations that are typical of the so-called “hypernickelophores,” for example, taxa exceeding 1 wt% ( $10,000 \mu\text{g g}^{-1}$ ) in their shoots (Boyd & Jaffré, 2009). In controlled conditions, the behavior of *O. vourinensis* under an increasing series of Ni

concentrations largely corresponded to that observed in other hyperaccumulating species of *Odontarrhena*, consisting in a stimulatory effect of the metal at low concentrations (Bettarini et al., 2021; Colzi et al., 2023). Consequently, the dose–response curve significantly fitted the hormetic model of Brain and Cousens (1989) and allowed for the calculation of hormetic parameters with values like those of the congeneric species previously examined in similar experiments.

In summary, *O. vourinensis* possesses all the physiological characteristics of Ni-hyperaccumulator species and considering its substantial shoot biomass, it appears to be a promising candidate for phytomining, agromining, and other biotechnological field applications.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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