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A morphometric and karyological study of *Onobrychis calabrica* (*Fabaceae*), a neglected species endemic to Calabria, S Italy

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Abstract: A morphometric (multivariate and univariate) and karyological study of *Onobrychis calabrica* Širj. (*Fabaceae*), compared to *O. alba* subsp. *echinata* (Guss.) P. W. Ball and *O. supina* (Chaix ex Vill.) DC., was carried out. According to our results, twelve morphological characters clearly support the distinctiveness of *O. calabrica*. In addition, the latter species is tetraploid with $2n = 4x = 28$ chromosomes, whereas the other two taxa are diploid with $2n = 2x = 14$ chromosomes. The names *O. calabrica* and *O. handel-mazzettii* Širj. (a heterotypic synonym of *O. supina*) are lectotypified here. *Onobrychis calabrica*, a narrow endemic to SE Calabria, S Italy, is here assessed as Critically Endangered CR B1ab(iii)+B2ab(iii) following IUCN Red List categories and criteria.

Key words: Calabria, *Fabaceae*, Italy, karyology, *Leguminosae*, morphometry, *Onobrychis*, taxonomy

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Introduction

The genus *Onobrychis* Mill. (*Fabaceae*) includes nearly 170 annual and perennial species, which are distributed mainly in the N temperate regions, with the centres of diversity in the E Mediterranean area and SW Asia (Ranjbar & al. 2012, 2013, 2018; Safaei Chaei Kar & al. 2012; Abuş & Avci 2018). Based on features of indumentum and corolla, supported by molecular data, the genus is subdivided in two subgenera: *O.* subg. *Onobrychis*, including two sections, and *O.* subg. *Sisyrosema* (Bunge ex Boiss.) Širj., including seven sections (Amirahmadi 2016). According to Širjaev (1925, 1926), these sections can be further subdivided in a total of nineteen subsections.

In Italy, only *Onobrychis* subg. *Onobrychis* occurs, with 13 specific and subspecific taxa (Bartolucci & al. 2018). Two species, included in *O.* sect. *Lophobrychis* Hand.-Mazz., are annual herbs: *O. aequidentata* (Sm.) d'Urv. and *O. caput-galli* (L.) Lam. All other taxa, included in *O.* sect. *Onobrychis*, are perennials: *O. alba* (Waldst. & Kit.) Desv. subsp. *alba*, *O. alba* subsp. *echinata* (Guss.) P. W. Ball, *O. alba* subsp. *pentelica* (Hausskn.) Nyman, *O. arenaria* (Kit.) DC. subsp. *arenaria*, *O. arenaria* subsp. *taurerica* Hand.-Mazz., *O. arenaria* subsp. *tommasinii* (Jord.) Asch. & Graebn., *O. calabrica* Širj.,

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O. montana DC. subsp. *montana*, *O. saxatilis* (L.) Lam., *O. supina* (Chaix ex Vill.) DC. and *O. viciifolia* Scop.

Onobrychis handel-mazzettii Širj. was described based on specimens from Liguria and Calabria, Italy (Širjaev 1925). It is currently considered as a heterotypic synonym of *O. supina*, a species distributed from E Spain to NW Italy (Ball 1968). Širjaev attributed *O. handel-mazzettii* and *O. supina* to *O.* subsect. *Macrosemiae* Hand.-Mazz., characterized by flowers in which the standard is longer than the keel. Later, Širjaev (1931) changed his view, restricting the application of the name *O. handel-mazzettii* (as *O. supina* subsp. *handel-mazzettii* (Širj.) Širj.) to plants from Liguria, while plants from Calabria (collected by Huter, Porta and Rigo and identified as "*O. echinata* Dietr.") were attributed to a different species: *O. calabrica* Širj.

According to Širjaev (1931), *Onobrychis calabrica* has a shorter standard than other species of *O.* subsect. *Macrosemiae* and represents a transitional form toward *O.* subsect. *Vulgatae* Hand.-Mazz. Moreover, *O. calabrica* has been treated as a heterotypic synonym of *O. supina* (Ball 1968; Euro+Med 2006+; Roskov & al. 2019), although it was neglected by Greuter & al. (1989), Conti & al. (2005) and Pignatti (1982, 2017; *O. supina* is doubtfully reported from Calabria). Peruzzi & al. (2015) and Bartolucci & al. (2018) resurrected the name *O. calabrica* as applying to a taxonomically doubtful species.

Onobrychis alba subsp. *echinata*, an Italian endemic that occurs close to the *locus classicus* of *O. calabrica* (Brullo & al. 2001), was included by Širjaev in *O.* subsect. *Albae* Hand.-Mazz., because of the standard being distinctly shorter than the keel. Despite this, non-flowering plants share very similar vegetative habit and hair-coverage with *O. calabrica*. The original samples of *O. calabrica* collected by Huter, Porta and Rigo were attributed to *O. echinata* by mistake likely for this reason.

The aim of the present work is to clarify the taxonomic position of *Onobrychis calabrica* with respect to *O. supina* and to *O. alba* subsp. *echinata*, using morphometric and karyological approaches.

Material and methods

The protologues of *Onobrychis handel-mazzettii* and *O. calabrica* and digital images of their original material (in FI, JE, W and WU; herbarium codes according to Thiers 2019+) were studied, in order to designate nomenclatural types and fix the application of names.

In spring 2018, *Onobrychis calabrica* (Fig. 1) was collected in its *locus classicus* (Gerace, Reggio Calabria, S Italy, 38°16'N, 16°12'E), an area characterized by arenaceous detrital deposits at the base of calcarenitic sandstone. We collected 20 well-preserved individuals, complete with leaves, flowers and ripe pods, to represent the overall morphological variation at population level. For each individual, a herbarium specimen was prepared and conserved in CLU. Thirteen morphological characters (Table 1) were measured on these herbarium specimens. All the characters were measured on dried and pressed specimens under a stereomicroscope, using a digital calliper with 0.1 mm precision. For comparative purposes, the same characters were measured on 20 herbarium specimens (= 20 individuals) of *O. alba* subsp. *echinata* from Calabria, conserved in CLU, and on 19 herbarium specimens (= 19 individuals) of *O. supina* collected in France and Liguria (NW Italy), conserved in FI and PI (see Appendix 1).

Table 1. Comparison between *Onobrychis alba* subsp. *echinata*, *O. calabrica* and *O. supina* of characters used in the morphometric analyses. Quantitative numerical values are expressed as mean ± SD. Character states marked by different superscript letters are significantly different ($P < 0.01$). Significantly different character states among *O. calabrica* and the other two taxa are shown in **bold** here and in Fig. 3.

Abbreviation	Character	*O. alba* subsp. *echinata*	*O. calabrica*	*O. supina*
NL	number of leaflets of middle cauline leaf	13.75 ± 2.19^{b}	18.00 ± 2.10^{a}	20.63 ± 4.58^{a}
LL	length of longest leaflets of middle cauline leaf (mm)	$\mathbf{10.01 \pm 1.04^{b}}$	$\mathbf{21.04 \pm 2.84^{a}}$	$\mathbf{12.10 \pm 5.25^{b}}$
WL	width of widest leaflets of middle cauline leaf (mm)	3.04 ± 0.83^{a}	2.55 ± 0.54^{a}	1.67 ± 0.52^{b}
CL	calyx length (mm)	$\mathbf{6.43 \pm 0.69^{b}}$	$\mathbf{5.77 \pm 039^{a}}$	$\mathbf{5.25 \pm 0.77^{c}}$
LCT	length of longest tooth of calyx (mm)	4.50 ± 0.58^{b}	3.90 ± 0.49^{a}	3.56 ± 0.68^{a}
WCT	width of longest tooth of calyx (mm)	$\mathbf{0.95 \pm 0.13^{b}}$	$\mathbf{1.24 \pm 0.16^{a}}$	$\mathbf{0.48 \pm 0.18^{c}}$
SL	standard length (mm)	9.12 ± 0.67^{a}	8.64 ± 0.58^{a}	9.10 ± 1.02^{a}
KL	keel length (mm)	$\mathbf{9.44 \pm 0.61^{b}}$	$\mathbf{6.48 \pm 0.51^{a}}$	$\mathbf{7.50 \pm 1.09^{c}}$
PL	pod length without spines (mm)	$\mathbf{4.55 \pm 0.34^{b}}$	$\mathbf{6.62 \pm 0.53^{a}}$	$\mathbf{4.28 \pm 0.73^{b}}$
PW	pod width without spines (mm)	$\mathbf{3.49 \pm 0.44^{b}}$	$\mathbf{4.73 \pm 0.32^{a}}$	$\mathbf{3.31 \pm 0.59^{b}}$
NPS	number of spines on pod	$\mathbf{10.85 \pm 1.56^{b}}$	$\mathbf{23.15 \pm 2.85^{a}}$	$\mathbf{15.00 \pm 3.80^{c}}$
NSC	number of spines on pod crest	$\mathbf{4.1 \pm 0.30^{b}}$	$\mathbf{6.55 \pm 0.75^{a}}$	$\mathbf{5.00 \pm 1.29^{b}}$
LPS	length of longest spine on pod crest (mm)	$\mathbf{2.12 \pm 0.36^{b}}$	$\mathbf{1.79 \pm 0.41^{a}}$	$\mathbf{0.63 \pm 0.36^{c}}$

Fig. 1. *Onobrychis calabrica* on calcarenitic sandstone near the town of Gerace, SE Calabria, S Italy. – A: flowering individual; B: detail of inflorescence. – Photographed on 21 April 2018 by L. Bernardo.

The quantitative data were subjected to discriminant analysis after logarithmic transformation, with the individuals *a priori* assigned to the three taxa based on geographical distribution and morphological features. Each character was also subjected to univariate analysis (ANOVA or Kruskal-Wallis test with corrections for multiple comparisons, Tukey HSD test and Bonferroni, respectively, after checking for homoscedasticity of each variable). All analyses were carried out with PAST 3.22 software (Hammer & al. 2001; Hammer 2018). Boxplots were built by means of Plotly Technologies Inc. (2015).

Concerning karyological analysis of *Onobrychis calabrica* (from the *locus classicus*) and of *O. alba* subsp. *echinata* (from Fiumara Avena, Cosenza, N Calabria), squash preparations were made on root tips obtained from germinating seeds. Seeds were obtained from the same herbarium specimens used for morphometric analyses. For germination, seeds were sown in 90 mm-diam. Petri dishes containing 1% distilled water agar and maintained in

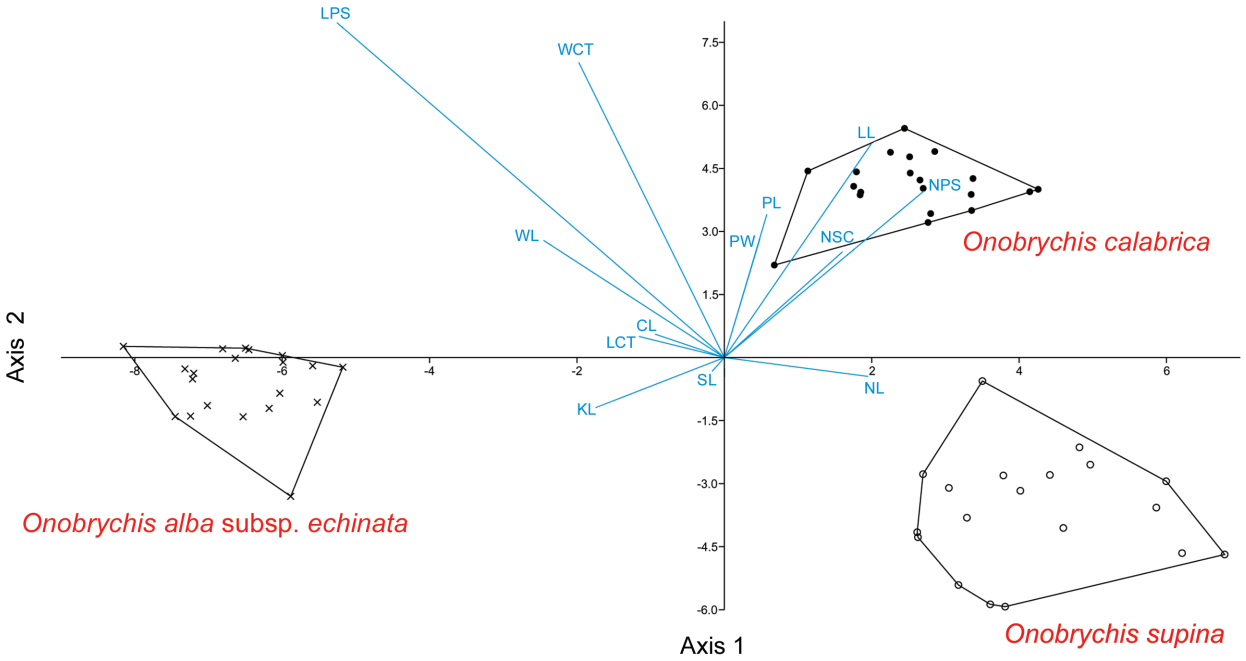

Fig. 2. Discriminant analysis based on 13 quantitative continuous characters. In blue, the relative contribution of each variable is reported.

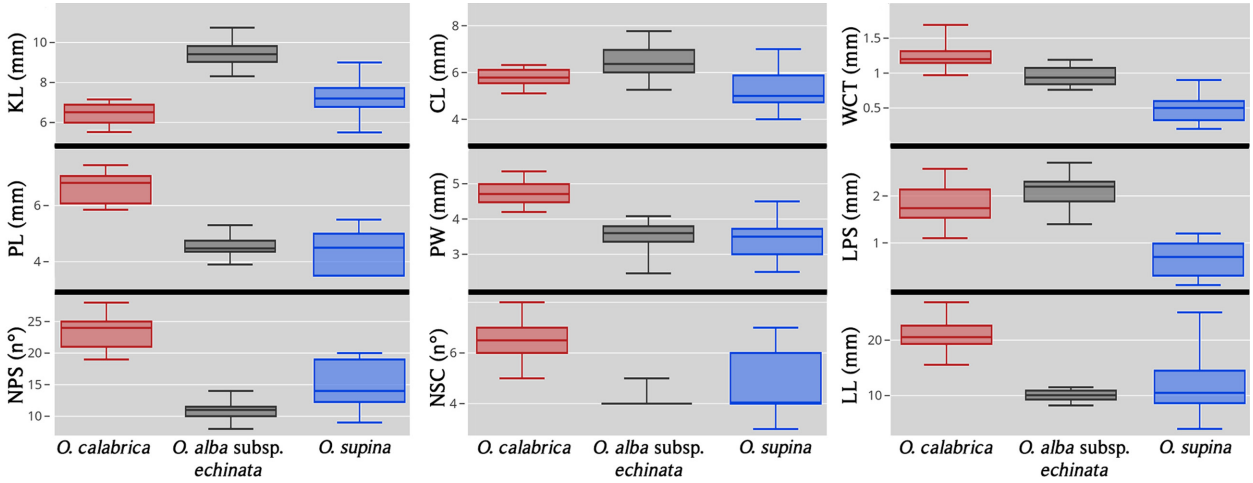

Fig. 3. Boxplots showing those statistically significant morphological characters less overlapping among the three taxa. Character abbreviations follow Table 1.

temperature- and light-controlled incubators, using respectively a 20/10°C and 12 h daily photo- and thermoperiod. Root tips were excised when c. 5 mm long and pre-treated with 0.4% colchicine at room temperature for 3 hours and then fixed in Carnoy's fixative solution for 1 hour. After hydrolysis in HCl 1N at 60°C for 9 minutes, the tips were stained in leuco-basic fuchsine (Sigma). For karyotype reconstruction, three or four metaphase plates from different seeds were photographed and then measured by means of the software KaryoType (Altınordu & al. 2016). The same software was also used to estimate karyotype asymmetry parameters (Peruzzi & Eroğlu 2013). Karyotype asymmetry parameters were also calculated for *O. supina*, based on published metaphase plate data (Sacristán 1965; Verlaque & al. 1987; Garnatje & Cardona 1988).

Results

Morphometric analysis

Discriminant analysis resulted in 98.31% (jackknifed) correct classification of individuals, *a priori* attributed to the three taxa (Fig. 2). No overlapping among individuals of *Onobrychis alba* subsp. *echinata*, *O. calabrica* and *O. supina* was found, showing a clear distinction between these units. All characters except SL (loadings on both axes with value < |0.0025|) contribute neatly to the discriminant function. In particular, high values of LL, PL, PW and NSC contribute to separate *O. calabrica* from the other two taxa. Furthermore, high values of WL, NPS and LPS clearly separate *O. calabrica* from *O. supina* and low values of LCT and KL separate *O. calabrica* from *O. alba* subsp. *echinata*.

Results of univariate analysis of continuous characters are summarized in Table 1 and shown in Fig. 3. The states of five characters (CL, WCT, KL, NPS and LPS) show significant differences among the three taxa ($P < 0.01$). In addition, the states of further four characters (LL, PL, PW and NSC) are significantly different between *Onobrychis calabrica* and the other two taxa ($P < 0.01$). As mentioned above, no significant difference in SL was found.

Karyological analysis

Onobrychis calabrica from the *locus classicus* is tetraploid, with $2n = 4x = 28$ chromosomes, while *O. alba* subsp. *echinata* from Fiumara Avena is diploid, with $2n = 2x = 14$ chromosomes (Fig. 4). Total haploid chromosome length (THL) ranged from 9.54–18.36 µm in the former taxon, and from 10–15.2 µm in the latter. Despite the difference in chromosome number, karyotype asymmetry is more or less overlapping among the studied taxa (Fig. 5).

Discussion

Širjaev (1931) described *Onobrychis calabrica* based on samples collected by Huter, Porta and Rigo, which they provisionally attributed to *O. echinata*. However, quantitative morphological comparisons show that *O. calabrica* is clearly distinct from *O. echinata*, especially based on floral and fruit features (LCT, KL, PL, NPS and LPS). We expected this result because the two species belong to two different subsections basing on standard and keel size ratio (Handel-Mazzetti 1909; Širjaev 1925).

The discriminant analysis also highlights the differences between *Onobrychis calabrica* and *O. supina*. In particular, *O. calabrica* shows leaflets and calyx (LL and CL) significantly longer and pods always larger (PL and PW), with longer and more numerous spines (NPS and LPS).

The relevance of fruit features went unnoticed by Širjaev (1931), because pods were absent from the sampled specimens: "*Valde doleo, quod legumina huius speciei non vidi.*" [I am very sorry that I have not seen the pods of this species].

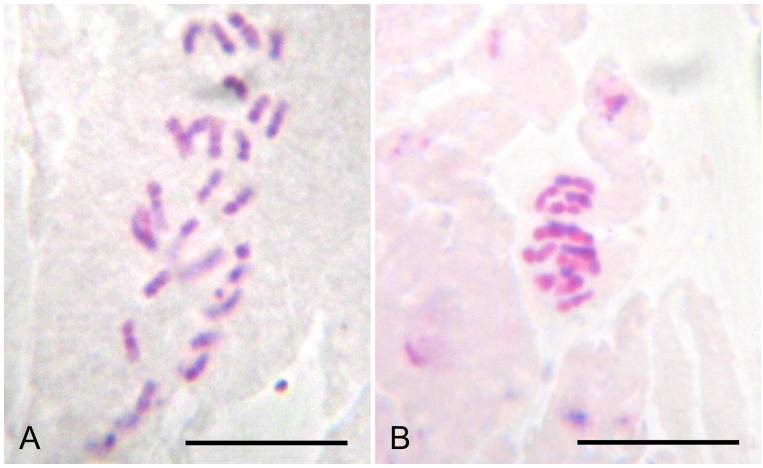

Fig. 4. Metaphase plates. – A: *Onobrychis calabrica* from the *locus classicus*, $2n = 4x = 28$. – B: *Onobrychis alba* subsp. *echinata* from Fiumara Avena, Calabria, $2n = 2x = 14$. – Scale bars: A, B = 10 µm.

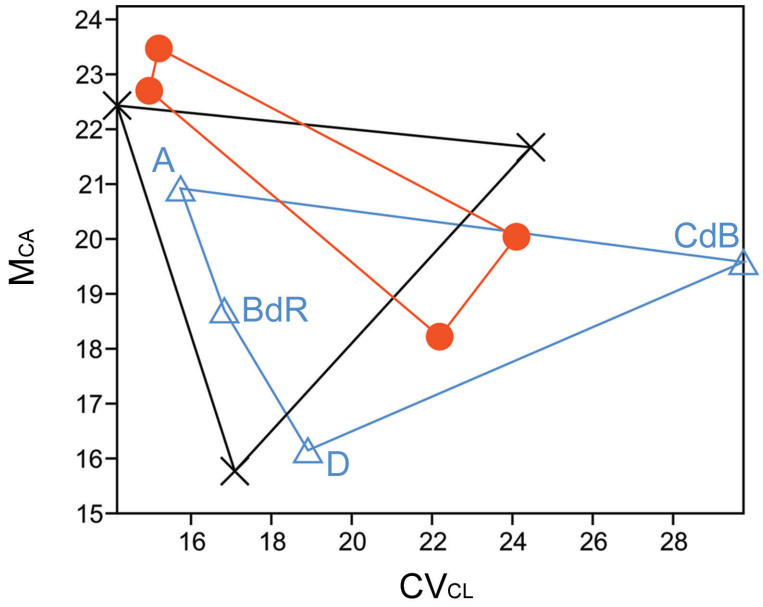

Fig. 5. Scatter plot of CV_{CL}, coefficient of variation of chromosome length (x axis) over M_{CA}, mean centromeric asymmetry (y axis) in karyotypes of *Onobrychis calabrica* from the *locus classicus* (filled circles), *O. alba* subsp. *echinata* from Fiumara Avena (crosses) and *O. supina* from literature (empty triangles). – A = Aineto, Spain (data from Sacristan 1965); BdR = Bouches du Rhône, France (data from Verlaque & al. 1987); D = Durro and CdB = Caldes de Boi, Spain (data from Garnatje & Cardona 1988).

Onobrychis calabrica is also clearly distinct from *O. alba* subsp. *echinata* and *O. supina* on karyological grounds, given that the former species is polyploid and the others are diploid (see also Sacristán 1965; Verlaque & al. 1987; Garnatje & Cardona 1988; Cenci & al. 2000; Astuti & al. 2019). However, karyotype asymmetry highlights a general resemblance in karyotype structure among these species (Fig. 5). Other E Mediterranean species belonging to the *O. supina* aggregate (Greuter & al. 1989) show a tetraploid complement with $2n = 28$ chromosomes, such as *O. gracilis* Besser and *O. pindicola* Hausskn. (Kozuharov & al. 1972; Pogan & al. 1980; Strid 1986), but these are clearly distinct from *O. calabrica* on morphological grounds, because *O. gracilis* shows a glabrous calyx and an unarmed pod crest, and *O. pindicola* shows a pod crest with teeth to 3.5(–5) mm long.

Accordingly, all the available morphological and karyological data concur in providing evidence that *Onobrychis calabrica* should be regarded as a good species, narrow endemic to SE Calabria, S Italy.

Taxonomic treatment

Onobrychis calabrica Širj. in Izv. Bulg. Bot. Druzh. 4: 10. 1931. – **Lectotype (designated here):** Italy, "Calabria orient. I. parte austr occid. praeruptorum urbis Gerace, sol. cretaceo", 400 m, 26 May 1877, *R. Huter, P. Porta & G. Rigo* ex itinere italico III No. 245 [under name *O. echinata*] (W-Rchb. No. 1889-0060084! http://www.jacq.org/detail.php?ID=507373; isolectotypes: WU No. 0102743!, FI!). – Syntypes: Calabria, Prov. Reggio, Gerace, in pascuis, solo cretaceo, 7 Jun 1898, *G. Rigo*, Iter quartum anni 1898 n. 333 [under name *O. echinata*] (W No. 1898-0008811!, WU No. 0102741!, WU No. 0102744!, WU No. 0102745!).

Description based on studied material — *Perennials*, herbaceous, (35–)50(–65) cm tall at fruiting, grey pubescent; *rootstock* ± woody; *flowering stem* erect. *Leaves* imparipinnate; *leaflets* in 7–10 pairs, linear to elliptic (18.3–)21(–26.8) × (1.5–)2.5(–3.2) mm, sericeous-pubescent. *Flowers* in fairly dense racemes. *Calyx* (5.1–)5.7(–6.3) mm long, hairy; teeth 2–3 × as long as tube, margin ciliate. *Corolla* pink-salmon with darker veins, (7.7–)8.6(–9.6) mm long, glabrous; *standard* 1.2–1.4 × as long as keel, c. 1.5 × as long as calyx. *Pod* (5.8–)6.6(–7.4) × (4.2–)4.7(–5.3) mm (not including spines), indehiscent, pubescent, with 19–28 *spines*, 5–8 on margin, longest one (1.3–)1.8(–2.6) mm long.

Phenology — Flowering in April–May, fruiting in May–June.

Distribution — Presently known from a single population near the town of Gerace, SE Calabria, S Italy (38°16'N, 16°12'E), the *locus classicus* of this species.

Habitat — Arenaceous detrital deposits and on ledges of calcarenitic cliffs.

Conservation status — This species is assessed here as Critically Endangered CR B1ab(iii)+B2ab(iii), according to IUCN categories and criteria (IUCN 2012), because of the restricted extent of occurrence (< 100 km²), the small area of occupancy (< 10 km²) and the occurrence in a single location, which is subjected to habitat alteration due to coniferous afforestation, grazing and accidental fire.

Additional specimens examined — Italy: Calabria: Gerace, Rupi del Castello, su rupe arenacea esposta a W-SW, 425 m, 21 Apr 2018, *G. Maiorca & L. Bernardo s.n.* (CLU 26259!, Herb. Maiorca-Caprio 5023!); *ibidem*, depositi detritici consolidati alla base di rupi calcarenitiche e arenacee, 475 m, 8 Jun 2018, *G. Maiorca & L. Bernardo s.n.* (CLU 26260!, Herb. Maiorca-Caprio 5075!); Gerace (Reggio Calabria), appena fuori dalla zona di rimboschimento (38.275425°N, 16.212115°E), rupe calcarenitica con alta esposizione, 2 May 2019, *J. Franzoni & D. Scalzo s.n.* (PI 026682!). See also Appendix 1.

Hedysarum supinum Chaix ex Vill., Prosp. Hist. Pl. Dauphiné: 41. 1779 ≡ ***Onobrychis supina*** (Chaix ex Vill.) DC. in Lamarck & Candolle, Fl. Franç., ed. 3, 4: 612. 1805. – Original material: à Gap, à Veynes, à Laric, à Serres, s.d., [*Villars*] (GRM No. MHNGr.1837.28299!).

= *Onobrychis handel-mazzettii* Širj. in Spisy Přír. Fak. Masarykovy Univ. 56: 90. 1925 ≡ *Onobrychis supina* subsp. *handel-mazzettii* (Širj.) Širj. in Izv. Bulg. Bot. Druzh. 4: 10. 1931. – **Lectotype (designated here):** Italy, "in coll. S. Angelo pr. Genuam", 16 Apr 1893, *C. Haussknecht s.n.* (JE barcode JE00024506! http://www.jacq.org/detail.php?ID=1415460; isolectotype: JE barcode JE00024507!). – Syntypes: Genua, s.d., *C. Haussknecht s.n.* (JE barcodes JE00024508!, JE00024509!); Genua in Campo Santo, 25 Mar 1898, *C. Haussknecht s.n.* (JE barcode JE00024510!); Gènes (Ligurie), s.d., *Miciol s.n.* [under name *O. tommasinii*] (WU WU-Halácsy-Europaeum 0102742!); Italy, "Calabria orient. I. parte austr occid. praeruptorum urbis Gerace, sol. cretaceo", 400 m, 26 May 1877, *R. Huter, P. Porta & G. Rigo* ex itinere italico III No. 245 [under name *O. echinata*] (W-Rchb. No. 1889-0060084!, WU No. 0102743!, FI!); Calabria, Prov. Reggio, Gerace, in pascuis, solo cretaceo, 7 Jun 1898, *G. Rigo*, Iter quartum anni 1898 n. 333 [under name *O. echinata*] (W No. 1898-0008811!, WU No. 0102741!, WU No. 0102744!, WU No. 0102745!).

Note — The typification of the name *Onobrychis handel-mazzettii* was crucial, because among the elements of original material for this name (Širjaev 1925), which was published six years before *O. calabrica*, there are also those specimens from Calabria later used by Širjaev to describe the latter species, so that the name *O. handel-mazzettii*, in the absence of a proper lectotypification, could even apply to Calabrian plants. On the other hand, it is clear from Širjaev (1931) that the author implicitly restricted the application of the name *O. handel-mazzettii* to plants from Liguria, when separating those from Calabria in a distinct species. After our typification, *O. handel-mazzettii* becomes a heterotypic synonym of *O. supina*.

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Appendix 1. Specimens seen and studied for the morphometric analyses

Onobrychis alba subsp. ***echinata*** (Guss.) P. W. Ball — Italy: Calabria: Montegiordano (Cosenza), nei pressi di Monte Rotondella, poco oltre contrada S. Filippo, pendio eroso al margine della macchia a ginepri, 600 m, 25 Jun 2019, *L. Bernardo & D. Gargano s.n.* (CLU Nos. 26286, 26287, 26289, 26290, 26291, 26292, 26293, 26294, 26295, 26296, 26297, 26298, 26299, 26300); Fiumara Avena (Albidona-Amendolara, Cosenza), greto ciottoloso, 17 m, 27 May 2018, *L. Bernardo & D. Gargano s.n.* (CLU Nos. 26280, 26281, 26282, 26283, 26284, 26285).

Onobrychis calabrica Širj. — Italy: Calabria: Gerace, Rupi del Castello, depositi detritici consolidati alla base di rupi calcarenitiche e arenacee, 475 m, 8 Jun 2018, *G. Maiorca & L. Bernardo s.n.* (CLU Nos. 26260, 26261, 26262, 26263, 26264, 26265, 26266, 26267, 26268, 26269, 26270, 26271, 26272, 26273, 26274, 26275, 26276, 26277, 26278, 26279).

Onobrychis supina (Chaix ex Vill.) DC. — Italy: Liguria: Bordighera, 18 Jun 1897, *C. Bicknell s.n.* (FI [2 sheets]); Bordighera, in aridis collium, 60 m., solo conglomeratico, 2 Jun 1912, *C. Bicknell & L. Pollini s.n.* (FI [3 sheets]); in pascuis prope Genuam, Jun 1876, *H. Groves s.n.* (FI [2 sheets]). — France: Frequentissima nei luoghi aridi di Mentone, 1851, *Ardoino s.n.* (FI); Mentone, in aridis frequens, 1851, *Ardoino s.n.* (FI); Col de Glaise près Gap, Hautes-Alpes, 12 Jul 1868, *A. Burle s.n.* (FI [2 sheets]); Aveyron, Saint-Paul-des-Fonts, coteaux, 500 m, 14 Jun 1903, *H. Coste s.n.* (FI [2 sheets]); Hyères, prope Carcassonne (Gallia mediterr.), in collibus incultis, 22 Mai 1876, *S. Sommier s.n.* (FI); Montagnes du Luc, […] (Var), 27 Apr 1873, *[without collector or number]* (FI); Montagne de la Cisé près Carcassonne, 17 Jun 1864, *Gaudesfroy s.n.* (FI); Alzon (Gard), 29 Jun 1863, *Le Jolis s.n.* (FI); Crest (Drôme), taillis, 1 Jun 1870, *J. Hervier-Basson s.n.* (PI); Alzon (Gard), 29 Jun 1863, *[collector's name illegible] s.n.* (PI).

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