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An unexpected occurrence of *Alyssum rossetii* (*Brassicaceae*) in the Pyrenees, a new species for the Spanish flora

Stanislav Španiel¹, Lenka Mártonfiová² & Judita Zozomová-Lihová¹

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Abstract: *Alyssum rossetii* is recorded for the first time in Spain from a single locality in the Pyrenees. After the type locality in the Valpelline valley (Pennine Alps, Italy), it is only the second known locality of this species. Morphology, diploid chromosome number ($2n = 16$), relative genome size, and ITS sequences confirm that the Pyrenean plants are conspecific with the Alpine ones and should be classified as *A. rossetii*. Phylogenetic analyses placed *A. rossetii* within the clade of *A. sect. Gamosepalum* comprising species from Turkey and demonstrated that *A. rossetii* is only distantly related to other *Alyssum* species occurring in the Pyrenees and adjacent regions: *A. alyssoides*, *A. cacuminum*, *A. collinum*, *A. fastigiatum*, *A. granatense*, *A. montanum*, and *A. simplex*. An identification key to all *Alyssum* species known from the Pyrenees is presented.

Keywords: *Alyssum*, *Brassicaceae*, *Cruciferae*, flow cytometry, genome size, ITS of nrDNA, new record, Pyrenees, rare species, Spain, trichome morphology

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Introduction

The genus *Alyssum* L. includes about 114 species distributed mainly in Eurasia (Španiel & al. 2015). They are divided into two main phylogenetic clades (Flynn 2013; Rešetnik & al. 2013; Li & al. 2015; Cetlová & al. 2021). One of them includes almost all annual species and a nested monophyletic group of perennials belonging to the *A. montanum*–*A. repens* species complex with the centre of diversity in southern Europe (Zozomová-Lihová & al. 2014; Španiel & al. 2011b, 2017a, 2017b). The other of the two clades includes two annual taxa (*A. dasycarpum* Stephan ex Willd. and *A. macropodum* Boiss. & Balansa) and perennials, most of which were formerly classified in *A. sect. Gamosepalum* (Hausskn.) T. R. Dudley (hereafter referred to as the *Gamosepalum* lineage) and occur mainly in Asia. The *Gamosepalum* lineage comprises only a few taxa that occur in eastern Europe (e.g. *A. doerfleri* Degen, *A. lenense* Adams, *A. pulvinare* Velen., *A. taygeteum* Heldr.), including one (*A. doerfleri*) that was also recently discovered in southern Italy (Bernardo & al. 2018).

Alyssum rossetii Španiel & al. is a recently discovered and described perennial species known from a single site in the Valpelline valley (Pennine Alps, Italy) (Španiel & al. 2018b). The population is relatively small and until now no other localities have been recorded. Recent mo-

lecular studies revealed that it belongs to the *Gamosepalum* lineage (Španiel & al. 2023a, 2023b) and is phylogenetically placed closest to *A. aurantiacum* Boiss., *A. misirdalianum* Orcan & Binzet and *A. praecox* Boiss., all species known only from Turkey (Španiel & al. 2023a). The origin of such a large geographic disjunction (Alps versus Anatolia) is not clear. It might be a consequence of colonization events from Anatolia via the Balkans during glacial periods followed by range fragmentation and extinction in the intervening areas or a rare case of recent (Pleistocene) long-distance dispersal (Španiel & al. 2023a). All other perennial *Alyssum* species that occur in southwestern Europe belong to the *A. montanum*–*A. repens* complex. *Alyssum rossetii* differs from this species complex by a larger genome size and several morphological characters (Španiel & al. 2018b).

During a field trip in the Pyrenees in 2022, taxonomically unidentified plants resembling *Alyssum rossetii* were discovered by one of the authors on a rocky slope between Pic de Qüenca and Rocablanca near Alós d'Isil. The site is 620 km distant from the only known (and type) locality of *A. rossetii* in the Alps. According to available distribution data (Anthos 2023) and the most recent taxonomic treatments (Zozomová-Lihová & al. 2014; Španiel & al. 2015; Cetlová & al. 2019), seven species of *Alyssum* have been reported so far from the Pyrenees and adjacent regions.

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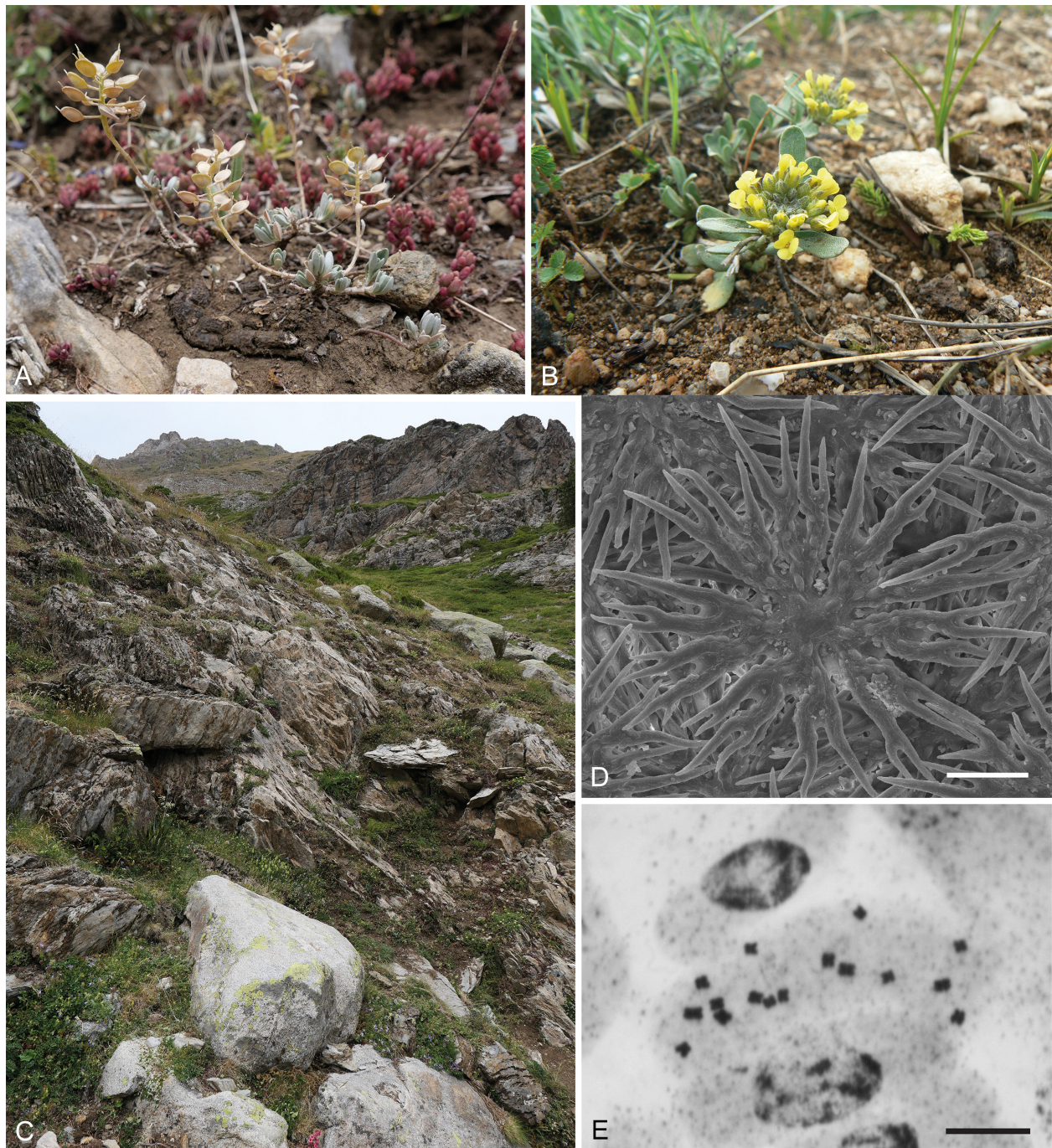


Fig. 1. *Alyssum rossetii* in the Pyrenees, Spain (A, C, E) and in the Pennine Alps, Italy (B, D). – A: fruiting plant at locality 684PQU between Pic de Qüenca and Rocablanca, 25 Jul 2022; B: flowering plant at locality 560FEU between La Tsa and Pas des Feuilles, 10 Jun 2017; C: habitat at locality 684PQU between Pic de Qüenca and Rocablanca, 25 Jul 2022; D: trichome on lower surface of stem leaves; E: metaphase mitotic cell with chromosome number $2n = 16$. – Photographs by S. Španiel (A, B, C); voucher specimens (D, E) deposited in SAV; scale bars: D = 50 μm ; E = 10 μm .

Four of these species are annual herbs: *A. alyssoides* (L.) L., *A. collinum* Brot., *A. granatense* Boiss. & Reut. and *A. simplex* Rudolphi. The other three taxa are perennial and belong to the *A. montanum*–*A. repens* complex: *A. cacuminum* Španiel & al., *A. fastigiatum* Heywood and *A. montanum* L. (Zozomová-Lihová & al. 2014). *Alyssum cacuminum* is endemic to the Pyrenees. Its populations were previously recognized under the name *A. cuneifolium* Ten.,

but recent studies revealed that this Apennine endemic is genetically and morphologically clearly different from the Pyrenean and Balkan plants for which this name was previously used (Zozomová-Lihová & al. 2014; Španiel & al. 2019). *Alyssum fastigiatum* was originally described as an endemic of Sierra de Cazorla, but molecular as well as morphological studies revealed that this name should be applied to all Iberian populations previously treated as *A.*

montanum (Zozomová-Lihová & al. 2014; Španiel & al. 2023b). Several other taxa formerly considered members of *Alyssum* but recently reassigned to other genera (Španiel & al. 2015; Salmerón-Sánchez & al. 2018) also occur in the Pyrenees (Jalas & al. 1996): *Hormathophylla lapeyrouseana* (Jord.) P. Küpfer, *H. pyrenaica* (Lapeyr.) Cullen & T. R. Dudley, *H. spinosa* (L.) P. Küpfer and *Odontarrhena serpyllifolia* (Desf.) Jord. & Fourr. (in the Pyrenees treated as *O. alpestris* (L.) Ledeb. or *A. alpestre* L. by some authors, e.g. Küpfer & Nieto Feliner 1993).

The aim of the present study is to clarify the taxonomic identity of the Pyrenean plants resembling *Alyssum rossetii* by inspecting genetic, karyological and morphological variability. More specifically, we aimed to (1) inspect their phylogenetic and morphological affinity to the Alpine *A. rossetii* and other relevant taxa of *Alyssum*, (2) determine their chromosome number and relative genome size, and (3) establish an identification key to *Alyssum* species occurring in the Pyrenees and adjacent areas.

Material and methods

Plant material

Several plants morphologically resembling *Alyssum rossetii* were sampled in July 2022 at the locality between Pic de Quença and Rocablanca near Alós d'Isil in the province of Lleida in the Pyrenees (684PQU, Fig. 1 and Appendix 1). They were used for phylogenetic analyses (ITS of nrDNA), genome size measurements (by flow cytometry), chromosome counting and morphological comparisons. Five living individuals were transferred to the Botanical Garden of P. J. Šafárik University in Košice and kept there until the anthesis in April 2023 (Fig. 2). Phylogenetic analyses and morphological evaluation included *A. rossetii* from the type locality in the Alps, and other perennial *Alyssum* species (*A. cacuminum*, *A. fastigiatum*, *A. montanum*) and annual species (*A. alyssoides*, *A. collinum*, *A. granatense* and *A. simplex*) previously reported from the Pyrenees and adjacent regions (Appendix 1). In addition, three species of the *Gamosepalum* lineage from Turkey, previously resolved as closest to *A. rossetii* (Španiel & al. 2023a), and *Odontarrhena muralis* (Waldst. & Kit.) Endl. and *O. tortuosa* (Waldst. & Kit. ex Willd.) C. A. Mey. (both as outgroups) were also included in the phylogenetic inferences. ITS sequences of nrDNA were generated for the present study or taken from GenBank and published studies (Appendix 1).

In contrast, *Alyssum* taxa occurring in France and Spain but neither in the Pyrenees nor in their foothills (annual *A. minutum* Schldtl. ex DC., perennial *A. flexicaule* Jord., *A. gallaecicum* (S. Ortiz) Španiel, Marhold & Lihová, *A. loiseleurii* P. Fourn., *A. orophilum* Jord. & Fourr., and *A. rhodanense* Jord. & Fourr.) were not included in the present study. Their phylogenetic relationships and morphological variation were analysed and dis-

cussed in detail in previous studies (Zozomová-Lihová & al. 2014; Španiel & al. 2019; Cetlová & al. 2021).

Chromosome counting and flow cytometry

Chromosome numbers were determined in mitotic metaphases of root tip cells. The root tips were taken from two adult plants identified as *Alyssum rossetii* collected at the locality 684PQU (Appendix 1) in the Pyrenees and kept in the Botanical Garden of P. J. Šafárik University in Košice. The root tips were pre-treated in a 0.002 M aqueous solution of 8-hydroxyquinoline for approximately 18 hours at 4° C, fixed in a mixture of 96 % ethanol and 98 % acetic acid (3:1) for 1–24 hours at 4° C, and macerated in 1-N HCl at 60° C for 6 min. Between each step, the root tips were washed in distilled water. Permanent squashes were prepared using the cellophane square method (Murín 1960) and stained in a 7 % Giemsa stock solution in Sørensen phosphate buffer for 1 h. The squashes were washed with distilled water, dried and observed in a drop of immersion oil using a Leica DM 1000 microscope equipped with HDCE-X5 camera and ScopeImage 9.0 software.

Flow cytometry using the AT-selective fluorochrome DAPI was employed to determine the relative genome size and ploidy level of six plants sampled in situ at the locality 684PQU in the Pyrenees, including the same individuals used for chromosome counting. *Solanum lycopersicum* L. “Stupické polní rané” (2C = 1.96 pg; Doležel & al. 1992) was used as an internal standard. The analyses were performed using a Partec Cyflow ML instrument equipped with an HBO-100 mercury arc lamp (Partec, Münster, Germany) at the Plant Science and Biodiversity Centre of the Slovak Academy of Sciences in Bratislava following the protocol described in Španiel & al. (2011a).

Ploidy levels of the other species were known from previous studies (Španiel & al. 2010, 2011a, 2018a, 2018b; Zozomová-Lihová & al. 2014; Cetlová & al. 2021; Appendix 1).

Phylogenetic analyses

The ITS region of nrDNA was amplified using the universal primers ITS4 and ITS5 (White & al. 1990) following the protocol of Melichárková & al. (2019). Purified PCR products were either sequenced directly or cloned if intraindividual polymorphisms were detected after direct sequencing, indicating the presence of multiple ITS copy variants within a genome. Molecular cloning was performed as described in Melichárková & al. (2017). Sequencing was carried out at Eurofins Genomics (Konstanz, Germany). The ITS sequences were edited and aligned in Geneious software v. R10.2.6 (Biomatters Ltd, Auckland, New Zealand). Two data partitions (for the non-coding ITS1+ITS2 and the coding 5.8S regions) were defined and the best-fit models of nucleotide substitutions were assessed separately for each of them in jModelTest version 2.1.10 (Darriba & al. 2012) using the Akaike in-

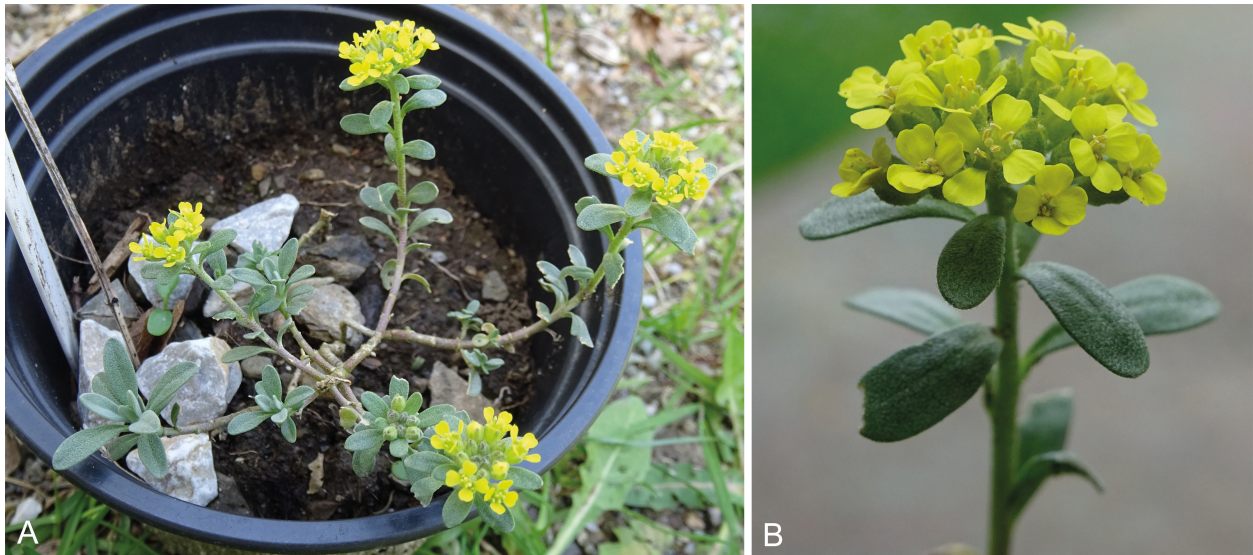


Fig. 2. *Alyssum rossetii* from the Pyrenees in cultivation. – A: flowering plant; B: inflorescence. – Slovakia, Košice, Botanical Garden of P. J. Šafárik University, 28 Apr 2023, photographs by L. Mártonfiová; voucher specimens deposited in SAV.

formation criterion. The phylogenetic tree was constructed using maximum-likelihood (ML) inference in GARLI v. 2.01 (Zwickl 2006) run at the CIPRES Science Gateway (Miller & al. 2010). Settings for ML phylogenetic computations followed Melichárková & al. (2017). All newly generated ITS sequences were submitted to GenBank (accession numbers OR016717–OR016761; Appendix 1).

Study of morphology and examination of herbaria

Morphology of the Pyrenean plants resembling *Alyssum rossetii* was evaluated on the basis of samples collected in situ and five ex situ flowering individuals in the Botanical Garden of P. J. Šafárik University in Košice. The examined morphological characters include those that unambiguously differentiate *A. rossetii* from taxa of the *A. montanum*–*A. repens* group and were identified in the previous study (Španiel & al. 2018b): presence/absence and shape of wings and teeth on the four inner (longer) filaments of stamens, presence/absence of purplish coloration on sepals, length of trichome rays of stellate trichomes on the lower surface of middle stem leaves and density (number per 0.5 mm²) of stellate trichomes on the lower and upper surface of middle stem leaves. Due to the small number of investigated individuals and the ex situ origin of the flowers, we did not carry out a thorough multivariate morphometric comparison of the Pyrenean and Alpine plants of *A. rossetii*. Values of morphological characters of almost all other taxa except *A. alyssoides* and *A. granatense*, were taken from previous studies (Zozomová-Lihová & al. 2014; Cetlová & al. 2019). For the latter two species, several individuals from the populations listed in Appendix 1 were examined (characters on fruits). Characters of indumentum on leaves and silicules were observed and measured using a stereomicroscope (Olympus SZ61) at magnification 80× and QuickPHOTO Micro 3.2 software.

The photo of the trichome of *A. rossetii* was taken using a scanning electron microscope (JEOL JSM-6390LV) at the Geological Institute of the Slovak Academy of Sciences in Banská Bystrica.

In order to find other potential localities of plants similar to *Alyssum rossetii* in the Pyrenees, we examined herbarium collections in BC, BCN and MA.

Results

Chromosome number, ploidy level and relative genome size

A diploid chromosome number ($2n = 16$) was determined in two plants of *Alyssum rossetii* from the locality 684PQU in the Pyrenees (Fig. 1). The total relative genome size ($2C$) of these two and additional four individuals was 0.916 ± 0.005 which corresponds to the monoploid relative genome size (Cx) of 0.458 ± 0.002 (mean \pm standard deviation in arbitrary units).

Phylogenetic analyses

The ITS alignment included 107 sequences from 48 individuals and was 629 bp long. It comprised 134 variable sites and a total of 62 different sequence variants (ribotypes) were identified. Intragenomic variation was frequently observed, within both diploid and polyploid accessions, with up to seven different ribotypes detected per individual. In contrast, all four analysed individuals of *Alyssum rossetii* from the Alps (560FEU) had a single ribotype that was identical to that found in the plants from the Pyrenees (four individuals from 684PQU). In the ML tree (Fig. 3), the ribotype of *A. rossetii* was placed in the *Gamosepalum* lineage, which was clearly distinct from

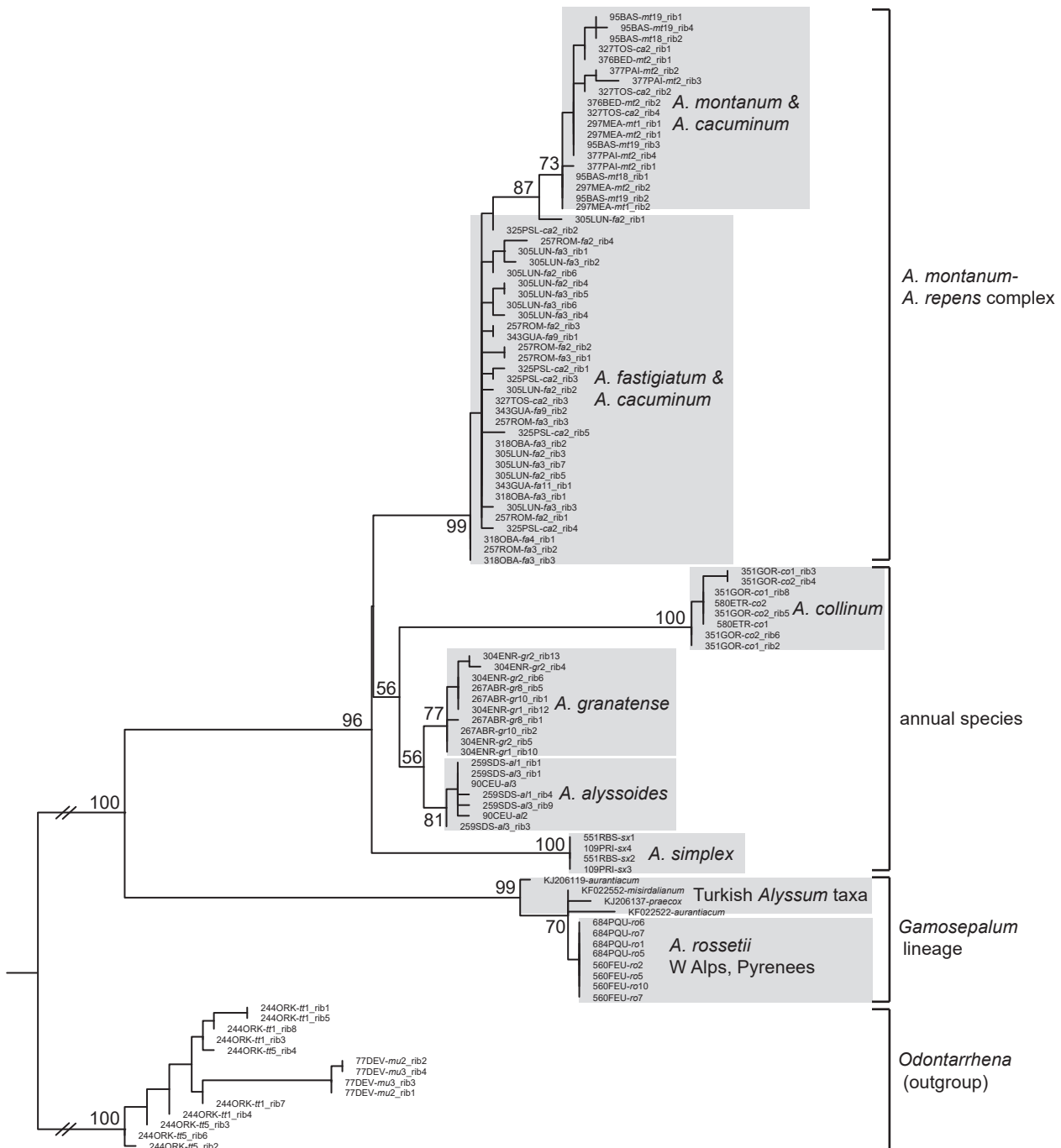


Fig. 3. Maximum-likelihood (ML) tree based on ITS sequence data of studied *Alyssum* taxa. Population codes follow Appendix 1. Bootstrap support values (> 50%) are indicated.

the clade comprising the other *Alyssum* species studied: annuals and the nested perennial *A. montanum*–*A. repens* complex, in congruence with the known phylogeny of the genus (see Introduction).

Morphology and herbarium data

Individuals found in the Pyrenees (684PQU), tentatively identified as *Alyssum rossetii*, have green sepals with a darker coloration at the apex, however not as dark and

purplish as in the plants from the type locality of *A. rossetii*. Wings and teeth on the four inner (longer) filaments of stamens are only slightly developed or absent (same as in the plants from the type locality of *A. rossetii*). Lower surface of middle stem leaves is covered by 31–45 stellate trichomes per 0.5 mm² with rays 0.11–0.13 mm long; upper surface of middle stem leaves is covered by 20–31 stellate trichomes per 0.5 mm². These values are within the range of those previously measured in plants from the type locality of *A. rossetii*. On the other hand, individuals

of *A. rossetii*, considering plants from both the Pyrenees and Alps, clearly differ from other perennial or annual species growing in the Pyrenees and adjacent regions, as presented in the Identification key below.

We found no herbarium specimens from the Pyrenees morphologically similar to *Alyssum rossetii* in the herbarium collections of BC, BCN and MA, and no earlier collections from the locality of 684PQU studied here.

Identification key of *Alyssum* species occurring in the Pyrenees and adjacent regions

The value ranges of the quantitative characters represent the 5th and 95th percentiles; asterisks (*) indicate mean values of three random counts per leaf surface or silicule valve. Indumentum of leaves should be inspected on leaves placed in a middle part of flowering stems, not on non-flowering shoots (therefore the term “middle stem leaf” is used throughout the key). Overall geographic ranges and habitats of species are reported according to Maire (1967), Küpfer & Nieto Feliner (1993), Jalas & al. (1996), Zozomová-Lihová & al. (2014), Španiel & al. (2018b) and Cetlová & al. (2019, 2021).

1. Annual plants, without non-flowering shoots **2**
 - Perennial plants, with several or many non-flowering shoots **5**
2. Sepals persistent until fruits are fully ripe (falling of only after fruits are ripe, straw-brown and dry) . . . **3**
 - Sepals caducous before fruits are ripe (falling of when fruits are still green and not full-grown) **4**
3. Silicules orbicular, but slightly longer than wide, with a monomorphic indumentum of ± symmetrical stellate trichomes with longest rays 0.15–0.20* mm long (Europe, North Africa, Asia; steppes, rocky slopes, quarries, disturbed ground, pastures, roadsides) ***A. alyssoides***
 - Silicules orbicular, but slightly wider than long, with a dimorphic indumentum with strigose trichomes (bifurcate or strongly asymmetrical stellate trichomes) with some rays conspicuously thick and elongated up to 0.75* mm in addition to ± symmetrical stellate trichomes with longest rays 0.15–0.20* mm long (Spain, Portugal, North Africa; gravelly pastures, open disturbed ground, roadsides) . . . ***A. granatense***
4. Style glabrous or with only 1–3 stellate trichomes at base, with long patent rays. Silicules with a dimorphic indumentum with strigose trichomes (bifurcate or strongly asymmetrical stellate trichomes with some rays thick and elongated) in addition to ± symmetrical stellate trichomes; longest rays of (strigose) trichomes on silicule valves 0.28–0.43* mm long (Spain, Portugal, North Africa; pastures, steppes, open dry sites, cultivated areas, roadsides, gravel, screes, rocks, sand) ***A. collinum***
 - Style hairy with 4–19 stellate trichomes all along style, with short adpressed rays. Silicules with a monomorphic indumentum of ± symmetrical stellate trichomes; longest rays of (stellate) trichomes on silicule valves 0.19–0.27* mm long (western Europe: France, Italy, Portugal, Spain; eastern Europe: Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Greece, Montenegro, Ukraine; western Asia: Turkey; pastures, open ground, rocky and gravelly slopes) ***A. simplex***
5. Wings and teeth on four inner (longer) filaments of stamens missing or only slightly developed; sepals purplish or green with a purplish coloration at least at apex; lower surface of middle stem leaves covered by 28–42 stellate trichomes per 0.5 mm² with rays 0.11–0.15 mm long; upper surface of middle stem leaves covered by 20–32 stellate trichomes per 0.5 mm² (Spain: Pyrenees, Italy: Pennine Alps; alpine rocks and dry grasslands) ***A. rossetii***
 - Wings and teeth on four inner (longer) filaments of stamens pronounced and well-developed; sepals yellowish to light green without major purplish coloration; lower surface of middle stem leaves covered by 3–23 stellate trichomes per 0.5 mm² with rays 0.20–0.47 mm long; upper surface of middle stem leaves covered by 3–13 stellate trichomes per 0.5 mm² . . . **6**
6. Silicules broadly elliptic; fruiting racemes mostly congested, umbel-like; stems mostly flexuous, slightly distorted in basal procumbent part; 8th stem leaf (counted downward) 2–4 times longer than wide (France and Spain: Pyrenees; alpine screes, rocks and dry grasslands; individuals of *A. fastigiatum* from Tozal de Guara in Pyrenees are very similar to this species) ***A. cacuminum***
 - Silicules suborbicular; fruiting racemes mostly not congested, elongated; stems mostly straight, firm, and ascending in basal part; 8th stem leaf (counted downward) 3.5–8 times longer than wide **7**
7. Upper surface of middle stem leaf green and contrastingly less hairy than lower surface, lower surface of middle stem leaf densely hairy, usually silvery-white and entirely covered by stellate trichomes, leaf epidermis often invisible underneath layer of trichomes, 14–27 trichomes per 0.5 mm² area with 16–27* rays 0.18–0.30 mm long (SW Germany, France, W Switzerland, not found in Spain; rocks, dry grassland, inland sand in lowlands and mountains, rarely sub-alpine pastures) ***A. montanum***
 - Upper surface of middle stem leaf of similar colour and indumentum as lower surface, lower surface of middle stem leaf sparsely to densely hairy, green, greyish green, rarely silvery-white, rarely entirely covered by trichomes, leaf epidermis usually at least partly visible underneath layer of trichomes, 3–15 trichomes per 0.5 mm² area with 8–20* rays 0.29–0.48 mm long (Spain, not found in France; montane to alpine dry grasslands, rocks and pastures; plants from Sierra de Mijas in southern Spain are a bit different, with leaves silvery-white and densely pubescent on both sides) ***A. fastigiatum***

Discussion

All data presented here, which include genome size values, morphological characters, and ITS sequences, are congruent and demonstrate that the Pyrenean plants growing between Pic de Qüenca and Rocablanca (684PQU) are conspecific with the population in the Valpelline valley (560FEU) in the Pennine Alps and should be classified as *Alyssum rossetii*. In concordance with previous phylogenetic inferences (Španiel & al. 2023a), *A. rossetii* is placed within the *Gamosepalum* lineage and is therefore phylogenetically distant from all other *Alyssum* species that occur both in the Pyrenees and in the Alps and adjacent regions. Previous studies on *Alyssum* have shown that the ITS region of nrDNA generally provides sufficient resolution to distinguish different species, that it often exhibits some intraspecific variation, and that intra-individual variation with multiple copy variants is also common (Melichárková & al. 2019; Cetlová & al. 2021; Španiel & al. 2023a), which is also demonstrated in the here presented results. Here, the species selection comprises both diploid and polyploid species, the latter including proven allopolyploids (see Cetlová & al. 2021; Španiel & al. 2023b), in which either homogenized ITS sequences toward one of the progenitors (*A. granatense*, *A. alyssoides* and *A. collinum*, Cetlová & al. 2021) or different parental copies were observed (*A. cacuminum*, present results). In *A. rossetii*, on the other hand, no variation in ITS sequences was detected either within the populations or between the two distant populations from the Alps and the Pyrenees. Species of the *Gamosepalum* lineage have rare and scattered occurrence in Europe and may represent relicts (discussed in Španiel & al. 2023b), whereas the centre of distribution and species diversity is in Asia (Irano-Turanian region). However, genetic variation patterns and phylogenetic relationships are poorly known in the *Gamosepalum* lineage (Rešetnik & al. 2013; Li & al. 2015). Further studies employing high-resolution genetic markers will be needed to understand the evolution and biogeographic history of the *Gamosepalum* lineage, to explain the lack of genetic variation within *A. rossetii*, its extreme disjunction, as well as the scattered occurrence of other species in Europe.

All investigated morphological characters of the plants from the locality 684PQU in the Pyrenees, preliminarily identified as *Alyssum rossetii*, were within the range of morphological variation previously detected in plants from the type locality of *A. rossetii* from the Pennine Alps (560FEU; Španiel & al. 2018b). The only exception is the dark coloration at the apex of sepals, which was not as pronounced and purplish as in the plants from the type locality. Nevertheless, this difference may be caused by their cultivation in the botanical garden. We assume that the darker coloration is more pronounced in situ (as observed in the plants from the Alps) due to high elevation and more intense sunlight. Plants in situ in the Alps and Pyrenees grow under direct sunlight in 2200–2320 m a.s.l. and flower in mid-June. In contrast, the investigated indi-

viduals were kept in the botanical garden in 250 m a.s.l. in partial shade and flowered in April.

Alyssum rossetii occurs at the type locality in the Pennine Alps at a single high-mountain site between La Tsa and Pas des Feuilles (population 560FEU). It grows on a steep south-facing slope in a subalpine-alpine grassland, especially on microsites with open soil and sparse vegetation along a footpath, but also in juniper bushes and in clumps with other alpine plants which include several calcicolous species. The bedrock in this region consists of the dominant paragneiss (kinzigits) with numerous small outcrops of calcareous rocks – ancient silicate-rich marbles (Španiel & al. 2018b). The habitat of *A. rossetii* in the Pyrenees between Pic de Qüenca and Rocablanca (684PQU) is similar. Here, plants of *A. rossetii* grow on south-facing rocky slopes on microsites with sparse vegetation and open soil, but also directly in rock crevices. The bedrock in this area consists of limestone, slate and siltstone (Sanz López & Palau Ramírez 1996). The Pyrenean population of *A. rossetii* is of similar size (few dozens individuals) to the Alpine population and occupies a relatively small area of approximately 100 m². The thorough research of herbarium specimens of *Alyssum* from the Pyrenees in the collections of BC, BCN and MAD revealed no other localities of this species. A detailed investigation in the broader surroundings of the locality has not yet been carried out. It should be noted that a distantly related *Odontarrhena serpyllifolia* (previously treated under *Alyssum*) grows only a few metres from the site of *A. rossetii*, but at first glance differs from *A. rossetii* by compound inflorescences.

Author contributions

S.Š. conceived the study, carried out the fieldwork, flow-cytometric and morphological analyses and prepared the identification key, S.Š. and J.Z.L. carried out the molecular laboratory work and wrote the manuscript, J.Z.L. performed molecular analyses, L.M. counted chromosomes.

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Appendix 1

List of *Alyssum* and *Odontarrhena* species and populations included in the study. References in the last column indicate the origin of ITS sequences of nrDNA unless generated in the present study. Ploidy levels were determined by flow cytometry in the present study (population 684PQU) or taken from previous studies as indicated by superscripts: ^a Španiel & al. (2010); ^b Španiel & al. (2011a); ^c Španiel & al. (2018a); ^d Španiel & al. (2018b); ^e Zozomová-Lihová & al. (2014); ^f Cetlová & al. (2021).

| Species | Population code | Ploidy | Locality and collection data | GenBank accession no. and place of publication of ITS sequences |
|--|-----------------|-----------------|---|---|
| Perennial species from <i>Alyssum montanum</i>–<i>A. repens</i> complex | | | | |
| <i>Alyssum cacuminum</i> Španiel & al. | 325PSL-ca | 4x ^c | Spain, Aragón, Huesca, E of Ampriu, under Tuca de Possolobino (2783 m), rocks and screes, 42.558333°N, 00.589833°E, 2362 m up to 42.565500°N, 00.594167°E, 2603 m, 5 Jul 2011, Španiel, Zozomová-Lihová & Letz (SAV) | OR016757–OR016761; present study |
| <i>A. cacuminum</i> | 327TOS-ca | 4x ^c | Spain, Catalunya, Barcelona, Pyrenees, La Tosa d'Alp, near the chalet Refugi Niu de l'Àliga, rocks and screes, 42.318667°N, 01.892500°E, 2511 m, 7 Jul 2011, Španiel, Zozomová-Lihová & Letz (SAV) | OR016753–OR016756; present study |
| <i>A. fastigiatum</i> Heywood | 257ROM-fa | 4x ^c | Spain, Euskadi, Álava, San Román de Campezo, Mt La Muela, calcareous rocks on the top, 42.672000°N, 02.431833°W, 1033 m, 25 May 2010, Španiel, Zozomová-Lihová & Marhold (SAV) | OR016739–OR016745; present study |
| <i>A. fastigiatum</i> | 305LUN-fa | 4x ^c | Spain, Castilla y León, León, Caldas de Luna, rocky slopes above the village, 42.930833°N, 05.866000°W, 1200 m and a pasture in the village 42.928167°N, 05.862500°W, 1168 m, 3 May 2011, Španiel, Zozomová-Lihová & Mereda (SAV) | OR016727–OR016738; present study |
| <i>A. fastigiatum</i> | 318OBA-fa | 2x ^c | Spain, Aragón, Huesca, Congosto de Obarra, rocks along the road near a tunnel, 42.400607°N, 00.599489°E, c. 1100 m, 26 Jun 2011, Španiel, Zozomová-Lihová & Letz (SAV) | OR016746–OR016749; present study |
| <i>A. fastigiatum</i> | 343GUA-fa | 2x ^c | Spain, Aragón, Huesca, Tozal de Guara, S exposed screes below the summit, 42.283833°N, 00.233333°W, 2077 m, 17 Aug 2011, La Harpe (SAV) | OR016750–OR016752; present study |
| <i>A. montanum</i> L. | 95BAS-mt | 2x ^b | Switzerland, Baselland, S of Basel, Aesch, calcareous rocks below the castle ruin of Pöfingen, 47.453000°N, 07.594167°E, 390 m, 13 Apr 2008, Brodtbeck, Marhold & Zozomová-Lihová (SAV) | MK096801–MK096806; Melichárková & al. (2019) |
| <i>A. montanum</i> | 297MEA-mt | 2x ^c | France, Rhône-Alpes, Isère, Gorges du Méaudret, rocks in the gorge along a road, 45.108167°N, 05.529833°E, 1000 m, 30 Apr 2011, Španiel, Zozomová-Lihová & Mereda (SAV) | ON159789–ON159792; Španiel & al. (2023a) |
| <i>A. montanum</i> | 376BED-mt | 2x ^c | France, Midi-Pyrénées, Ariège, Bèdeilhac-et-Aynat, rocks and screes above the hiking path, 42.868000°N, 01.561167°E, 720 m, 17 Jun 2012, Španiel & Závěská (SAV) | OR016725, OR016726; present study |
| <i>A. montanum</i> | 377PAI-mt | 2x ^c | France, Languedoc-Roussillon, Aude, Col de Pailhères, between Ax-les-Thermes and Mijanès, rocky sites on the horse pasture, 42.736000°N, 01.996333°E, 1979 m, 17 Jun 2012, Španiel & Závěská (SAV) | OR016721–OR016724; present study |
| Perennial species from the <i>Gamosepalum</i> lineage | | | | |
| <i>A. aurantiacum</i> Boiss. | – | – | Turkey, Antalya, Pisidis, Landrein & Borosova 44189 (K) | KJ206119; Li & al. (2015) |
| <i>A. aurantiacum</i> | – | – | Turkey, Antalya, Geyik Dağları, Akdaği, Döring, Parolly & Tolimir 697b (B 10 0132695) | KF022522; Rešetnik & al. (2013) |

| | | | | |
|--|-----------|-----------------|---|---|
| <i>A. misirdalianum</i> Orcan & Binzet | – | – | Turkey, Findikpinari, Deve Koyađı, <i>Binzet</i> (ANK) | KF022552; Rešetnik & al. (2013) |
| <i>A. praecox</i> Boiss. | – | – | Turkey, Içel, Elmali Dađ, <i>Landrein & Borosova 44214</i> (K) | KJ206137; Li & al. (2015) |
| <i>A. rossetii</i> Španiel & al. | 560FEU-ro | 2x ^d | Italy, Aosta Valley, the valley of Valpelline, N of Bionaz, NE of Chez Les Chenaux, between La Tsa and Pas des Feuilles, open soil with sparse vegetation cover, 2293–2320 m, 45.890722°N, 07.445444°E, 10 Jun 2017, <i>Španiel</i> (SAV) | ON159805–ON159808; Španiel & al. (2023a) |
| <i>A. rossetii</i> | 684PQU-ro | 2x | Spain, Cataluña, Lleida, W of Alós d'Isil, NNE of Pic de Qüenca, SSE of Rocablanca, along Barranc de Moredo, a rocky slope, 2206 m, 42.70723°N, 01.05927°E, 25 Jul 2022, <i>Španiel & Letz</i> (SAV) | OR016717–OR016720; present study |
| Annual species | | | | |
| <i>A. alyssoides</i> (L.) L. | 90CEU-al | 4x ^a | France, Provence-alpes-Côte d'Azur, Hautes-Alpes, Mt Ceüse, 1572 m, 44.51722°N, 05.86889°E, 15 Jun 2007, <i>Perný</i> (SAV) | MW022553, MW022554; Cetlová & al. (2021) |
| <i>A. alyssoides</i> | 259SDS-al | 4x ^f | Spain, Castilla y León, Burgos, Santo Domingo de Silos, 1100 m, 41.96473°N, 03.37332°W, 26 May 2010, <i>Španiel, Zozomová-Lihová & Marhold</i> (SAV) | MW022596–MW022601; Cetlová & al. (2021) |
| <i>A. collinum</i> Brot. | 351GOR-co | 4x ^f | Spain, Granada, N of Sierra de Baza, SW of Baza, E of Baúl, 1240 m, 37.43333°N, 02.90567°W, 13 Apr 2012, <i>Španiel, Meredá & Letz</i> (SAV) | MW022618–MW022623; Cetlová & al. (2021) |
| <i>A. collinum</i> | 580ETR-co | 4x ^f | Portugal, Évora, between Sousel and Estremoz, 350 m, 38.88720°N, 07.65070°W, 18 Mar 2018, <i>Španiel & Cetlová</i> (SAV) | MW022764, MW022765; Cetlová & al. (2021) |
| <i>A. granatense</i> Boiss. & Reut. | 267ABR-gr | 6x ^f | Spain, Andalucía, Granada, Sierra Nevada, Abrucena, 1864 m, 37.09125°N, 02.84642°W, 31 May 2010, <i>Španiel, Zozomová-Lihová & Marhold</i> (SAV) | MW022602–MW022605; Cetlová & al. (2021) |
| <i>A. granatense</i> | 304ENR-gr | 6x ^f | Spain, Castilla y León, León, Valverde-Enrique, 830 m, 42.311°N, 05.302°W, 3 May 2011, <i>Španiel, Zozomová-Lihová & Meredá</i> (SAV) | MW022612–MW022617; Cetlová & al. (2021) |
| <i>A. simplex</i> Rudolphi | 109PRI-sx | 2x ^f | Croatia, Šibensko-kninska županija, Primošten, 53 m, 43.5789°N, 15.93833°E, 20 Apr 2008, <i>Španiel & Perný</i> (SAV) | MW022555, MW022556; Cetlová & al. (2021) |
| <i>A. simplex</i> | 551RBS-sx | 2x ^f | Italy, Sicily island, Provincia di Palermo, Rocca Busambra, 1250 m, 37.85290°N, 13.14765°E, 23 May 2017, <i>Španiel & Cetlová</i> (SAV) | MW022751, MW022752; Cetlová & al. (2021) |
| Outgroup species | | | | |
| <i>Odontarrhena muralis</i> (Waldst. & Kit.) Endl. | 77DEV-mu | 2x ^f | Romania, Hunedoara, Munții Poiana Ruscă, hills near the village of Deva, 407 m, 45.89278°N, 22.88200°E, 10 Jul 2007, <i>Španiel, Majeský & Kolarčik</i> (SAV), 25 Jul 2016, <i>Španiel, Cetlová & Štenker</i> (SAV) | MW022549–MW022552; Cetlová & al. (2021) |
| <i>Odontarrhena tortuosa</i> (Waldst. & Kit. ex Willd.) C. A. Mey. | 244ORK-tt | 4x ^c | Hungary, Duna-Tisza köze (Danube-Tisza Interfluve), near the village of Orkeny, between Tatarszentgyörgy and Kunpeszer in puszta, sand dunes, 121 m, 47.107917°N, 19.395083°E, 15 Apr 2009, <i>Kolarčik & Šuvada</i> (SAV), 6 May 2016, <i>Španiel, Cetlová & Štenker</i> (SAV) | MW022582–MW022591; Cetlová & al. (2021) |