Wulfenia **29** (2022): 1–8

Wulfenia
Mitteilungen des
Kärntner Botanikzentrums
Klagenfurt

Myosotis arvensis and *M. ramosissima* differ in pollen size

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Summary: Myosotis arvensis (L.) Hill and *M. ramosissima* Rochel. are small-flowered forget-me-nots with similar morphology, distribution and ecology. Identifying species on fresh material is not difficult; however, determination of herbarised specimens might be problematic. We conducted a morphological analysis of all herbarium material of these two species in Herbarium LJU and measured some morphological characters that could be used on herbarised plants, too. Despite the small difference in chromosome number, we also tested the pollen length for discrimination between the species, and this character proved to be very reliable. Other useful characters are the corolla's diameter, the flower pedicels' length in fruiting time and some traits on nutlets. We included the discriminative morphological characters relevant for herbarised plants in the identification key. We also present the distribution map based on analysed specimens and confirm that *M. arvensis* is more frequent than *M. ramosissima*.

Keywords: flora, forget-me-not, herbarium, identification key, morphology, *Myosotis*, pollen length

In the recent revision of *Myosotis* (Boraginaceae) in Herbarium LJU, we discovered difficulties in differentiation between herbarised specimens of *Myosotis arvensis* (L.) Hill and *M. ramosissima* Rochel. Both species are representatives of the small-flowered group of forget-me-nots and have similar morphology, distribution and ecology. They are both widespread across Europe and the Mediterranean countries of North Africa (Valdes 2011). However, in Slovenia and its neighbouring countries, *M. arvensis* is more frequent than *M. ramosissima* (Jogan et al. 2001; Hartl et al. 1992; Poldini 2002; Nikolić 2005–; Bartha & Király 2015). Both species grow on dry, often ruderal sites; *M. arvensis* is a biennial, and *M. refracta* is an annual species (Grau & Merxmüller 1972). The chromosome number (2n) of *M. arvensis* is 52 and 48 of *M. ramosissima* (Grau & Merxmüller 1972).

The species identification on fresh material is not difficult in most cases. However, many morphological characters used in the identification keys (Grau & Merxmüller 1972; Fischer et al. 2008; Rottensteiner 2014), for example, the openness of calyx and the growth direction of pedicel in time of fruiting, are not reliably identifiable on most herbarium specimens, due to deformation of plant material during herbarisation. Additionally, some good characters for identification are in nutlets, which are often missing on herbarised material.

We conducted a morphological analysis of all herbarium specimens of these two species in Herbarium LJU and tested some additional morphological characters, including pollen size. The pollen size was already used for delimitation between species in *Myosotis sylvatica* agg. (Grau 1964) and *M. palustris* agg. (Štepankova 1993), but not in the *M. arvensis* group.

Materials and methods

We revised all available herbarium material from Herbarium LJU, identified as *M. arvensis* or *M. ramosissima*. We selected specimens with well-developed flowers and ripe nutlets for morphological observations and measurements.

We observed and measured the following characters:

(A) numerical characters in flowers: (1) CL – length of calyx, (2) CTL – length of calyx tube, (3) CLL – length of calyx lobes, (4) COD – diameter of the corolla, (5) HTL1–5 – lengths of the 5 longest hooked trichomes on the calyx;

(B) numerical and descriptive characters in calyces in time of fruiting: (6) FPL1–3 – lengths of first three pedicels in the inflorescence, (7) FCL1–3 – lengths of first three calyxes in the inflorescence, (8) FPA1–3 – angles at which the first three pedicels grow in the inflorescence, (9) CO – openness of calyces (character states: O – all calyces open, C – all calyces closed, OC – some calyces open and some closed);

(C) numerical and descriptive characters in nutlets: (10) NL – length of nutlet, (11) NW – width of nutlet, (12) NWM – distance between nutlet base and the position on nutlet with a maximum width, (13) NSW – width of the scar (nutlet attachment area), (14) NSH – height of the scar, (15) NM – nutlet maturity (character states: 0 – not mature, 1 – mature), (16) NC – colour of the nutlet (character states: LB – light brown, B – brown, BL – very dark brown or black), observed in ripe nutlets only;

(D) pollen size: (17) PS – average length of up to 20 pollen grains from one randomly selected flower.

The characters from groups A and C were observed or measured using a stereomicroscope with a maximum magnification of 64× (Stemi SV 11, Carl Zeiss, Germany, computer programme Axiovision 4.6). All the characters from group B were observed or measured macroscopically. The pollen size was measured with a microscope (Axioscope MOT, Carl Zeiss, Germany, computer programme Axiovision 4.6) at 640×. Pollen grains were extracted from a randomly selected flower from herbarised specimen. We soaked the flower in hot water for a few minutes and extracted the anthers. We put the anthers in a drop of water on a microscopic slide, covered them with a cover slide and squashed them with pressure on the cover slide using a preparation needle.

We collected all measurements and observations in a MS Excel table. We calculated four ratios: (1) CCL/CL to show the proportion of the length of calyx lobes to the length of calyx, (2) FPL/FCL to show the length of pedicels in relation to the calyx length in time of fruiting; the average of the measurements of the three oldest flowers on the plant was calculated, (3) NWM/NL to show the position of the widest part of the nutlet and (4) NSW/NSH to show the shape of the scar on the nutlet. Statistical analyses were conducted in the programme GraphPad Prism 9.0.

Results

We revised 206 herbarium sheets from Herbarium LJU: 192 from Slovenia, 11 from Croatia and one from Montenegro, Northern Macedonia and Kosovo. The comparison between original determinations and the situation after the revision is presented in Table 1. 33 herbarium sheets contained material from other aggregates of *Myosotis,* and we excluded them from further analyses.

We selected 173 specimens of *M. arvensis* and *M. ramosissima* for the pollen size measurement. In 31 cases, the measurement was impossible, because samples did not have open flowers, flowers were without pollen, or it was deformed. Some flowers contained less than 20 pollen grains. If they were well developed, we measured them anyway and calculated the average value of pollen size of the sample. The average lengths of pollen grains proved reliable for discrimination between *M. arvensis* and *M. ramosissima* (Fig. 1).

Table 1. The comparison between original determinations of revised herbarium material and the situation after the revision.

	Determination after the revision				
Original determination	Number of specimens	*Myosotis arvensis* (L.) Hill	*Myosotis ramosissima* Rochel.	*Myosotis sylvatica* agg.	*Myosotis* from other agg.
Myosotis arvensis (incl. subsp.)	124	93	1	24	6
Myosotis ramosissima (incl. subsp. and var.), *M. collina*, *M. hispida*	37	21	14	2	0
Myosotis arvensis agg.	9	9	0	0	0
Myosotis stricta	3	2	1	0	0
Myosotis discolor	2	2	0	0	0
Myosotis sparsiflora	1	1	0	0	0
Species from *Myosotis sylvatica* agg.	10	10	0	0	0
Myosotis sp.	19	12	6	1	0
other determinations	1	1	0	0	0

We used the average pollen lengths as an identification trait and then tested the usefulness of the other measured and observed morphological characters. We checked the outliers of *M. arvensis* with pollen lengths close to the values of *M. ramosissima* and confirmed the identification before further analyses. We show the results of selected morphological measurements in Fig. 2.

Characters on calyx in flowering time (CL, CTL, CLL, the ratio CLL/CL (Fig. 2B)), including the length of hooked trichomes (HTL), proved useless for differentiation between herbarised specimens of *M. arvensis* and *M. ramosissima,* as the measured character states overlapped. The diameter of the corolla (COD), measured in a herbarised (dry) form, can be used for the differentiation between species, because the flowers of *M. ramosissima* are much smaller (Fig. 2A). After flowering, the calyces remain on the plant, and the developing nutlets are within them. The ratio between the length of pedicels and the calyx length in time of fruiting (FPL/FCL) is a good

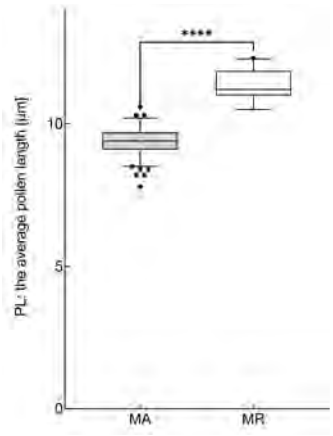

Figure 1. The average pollen lengths of analysed specimens of *Myosotis arvensis* (MA, N=123) and *M. ramosissima* (MR, N=21). The mean values of the samples are statistically different (unpaired t-test, P<0,0001).

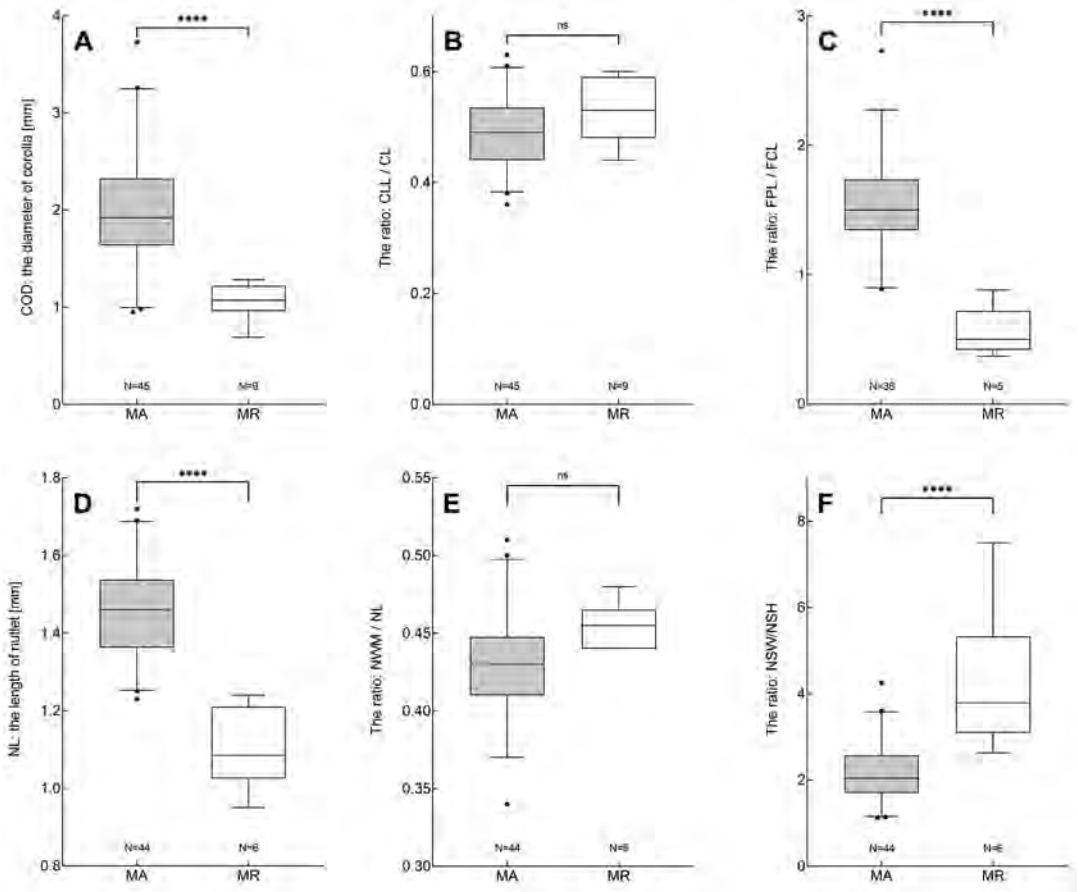

Figure 2. The results of measurements of selected morphological characters in herbarium material of *Myosotis arvensis* (MA) and *M. ramosissima* (MR) from Herbarium LJU. The box extends from the 25th to 75th percentiles, the middle line represents the median, and the whisker show 5th and 95th percentiles. Legend: COD – diameter of the corolla, CLL/CL – ratio between the length of calyx lobes and whole calyx, FPL/FCL – ratio between the length of pedicel and calyx in the time of fruiting, NWM/NL – ratio between the distance between nutlet base and the position of maximum width on nutlet and nutlet length, NSW/NSH – ratio between width and height of the scar on nutlet; * – statistically significant difference between species, ns – differences are not statistically different. In all cases, we used the unpaired T-tests.

character that can be used to identify herbarised specimens (Fig. 2C). The lowest three pedicels in the inflorescence of *M. ramosissima* are always shorter than associated calyces. *M. arvensis* has mostly much longer pedicels. When nutlets are available, their length could be used for differentiation (Fig. 2D). The nutlets of *M. ramosissima* are usually not longer than 1.2 mm, and the nutlets of *M. arvensis* are typically longer than 1.3 mm. The complete ripeness of nutlets is not obligatory, as the difference remained, when unripe nutlets were included in the analysis. The ratio NWM/NL (Fig. 2E) illustrates the shape of nutlets. The calculated value shows the position of the widest part on the nutlet measured from its base. The nutlets of *M. arvensis* are more ovate, and the nutlets of *M. ramosissima* are more elliptical, with the widest part closer to the middle. The shape of the scar on the nutlet is a minor character, but well visible under the stereomicroscope. It is illustrated with the ratio NSL/NSW (Fig. 2E), where higher values show the broader and narrower scar, more typical of *M. ramosissima.* The scar of *M. arvensis* is more isodiametric. The number of specimens with completely ripe nutlets was too small to confirm

nutlet's colour (NC) usability for species identification. The angle between the pedicel and stem (FPA) and calyx openness (CO) in fruiting time are not useful for identifying herbarised plants.

The discriminative morphological characters relevant for herbarised plants are included in the identification key:

1 Average pollen length (7.8)9.1–9.7(10.6) µm; diameter of the corolla (1.0)1.6–2.3(3.7) mm, oldest three flowers in the inflorescence with longer pedicel than calyx in fruiting time; ripe nutlets dark brown to black, ovate, (1.2)1.4–1.5(1.7) mm long, with a scar that is ca. 2× wider than high, well visible from the ventral side *M. arvensis*

– Average pollen length (10.5)11.0–11.9(12.3) µm; diameter of the corolla (0.7)1.0–1.2(1.3) mm, oldest three flowers in the inflorescence with distinctly shorter pedicel than calyx in fruiting time; ripe nutlets light to dark brown, ovate to elliptical, 1.0–1.2 mm long, with a scar that is 3.5–5× wider than high, clearly visible from the ventral side *M. ramosissima*

The localities of all revised herbarium specimens, except those from Montenegro, Northern Macedonia and Kosovo, are presented in the distribution map (Fig. 3). *M. arvensis* is much more common, and its localities are scattered all over the country. It grows mainly on grasslands, forest edges, arable and ruderal sites, on elevations 20–1100 m a.s.l. *M. ramosissima* is rarer than expected. Only 38% of specimens initially identified as *M. ramosissima* were identified correctly (Table 1). *M. ramosissima* grows in similar habitats as *M. arvensis*, but it seems likely that it prefers more natural sites. It was collected on sites from 2–830 m a.s.l. and in one site in Kosovo on 1250 m a.s.l. This species is more frequent in Slovenia's eastern and southern parts, and we did not find any locality in the alpine or dinaric phytogeographical region (Fig. 3).

Discussion

Herbarium collections are still a valuable source of floristic information. It was found out that only 16% of newly published species between 1970 and 2010 were described within five years after being collected for the first time and over 25% more than 50 years after the collecting time (Bebber et al. 2010). They predicted that more than half of the estimated 70 000 undescribed plant species are already collected in herbaria. Herbarium specimens also provide verifiable and citable evidence of the occurrence of plant species at particular localities and times and are vital resources for assessing species' extinction risk (Lughadha et al. 2018). As an example for these topics, we can show the discovery of an unexpected species for the flora of Slovenia, *Myosotis refracta* in Herbarium LJU, during the revision of herbarium material. The species was erroneously determined as *M. stricta* (Strgulc Krajšek et al. 2016).

The correct species determination is also crucial for all analyses of plant material as well as karyology, biochemistry and genetic analyses. The problem of erroneous determinations of plant material used for karyological studies of *Myosotis* was already shown by Merxmüller in 1970.

During herbarisation of plant material, many useful morphological characters, clearly visible on the fresh plant, deform. Such traits necessary for the identification of *M. arvensis* and *M. ramosissima* are the corolla's colour and size, the angles at which the flower pedicels grow and the openness of calyx in fruiting time. The identification keys often contain many such characters and are less appropriate for the identification of herbarium specimens.

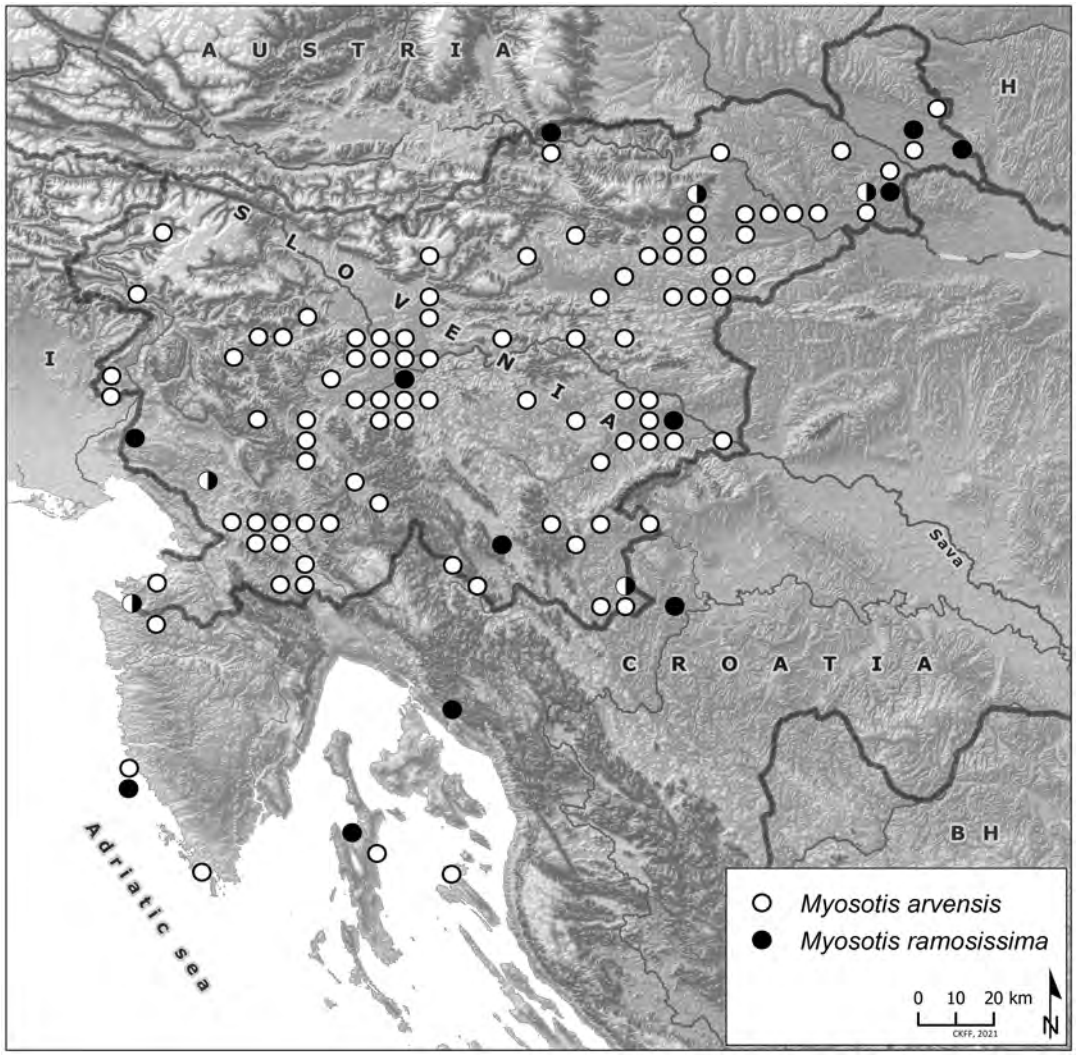

Figure 3. A map showing the distribution of analysed specimens of *Myosotis arvensis* and *M. ramosissima*. A symbol represents the presence of at least one *Myosotis* specimen in the quadrant of the MTB grid.

The main goal of our study was to find additional morphological characters to supplement those in the existing determination keys. Especially the pollen size that was already used for delimitation between species in *M. sylvatica* agg. (Grau 1964) and *M. palustris* agg. (Štepankova 1993) proved to be very useful also in the *M. arvensis* group. The difference in pollen size was unexpected, because the chromosome numbers of *M. arvensis* and *M. ramosissima* do not differ very much (Grau & Merxmüller 1972).

The pollen size was also convenient for finding specimens from *M. sylvatica* group that could look similar to *M. arvensis* in a herbarised state. Our measurements show that the average pollen size of *M. sylvatica* is 6 µm and of *M. decumbens* it is 7–7.5 µm (unpublished data). Additionally, many other morphological traits differentiate *M. arvensis* and *M. ramosissima* (see the identification key).

Another annual *Myosotis* that could be erroneously determined as *M. ramosissima* or *M. arvensis* is *M. stricta*. This species also has small flowers, but its pedicels are very short, almost absent.

Another good morphological trait that is not present in *M. ramosissima* and *M. arvensis* is the presence of hooked trichomes on the stem and the abaxial side of leaves (Grau & Merxmüller 1972). This species is rare in Slovenia, more frequent only in the NE part of the country (Jogan et al. 2001).

Acknowledgements

The Slovenian Research Agency partially supported this study financially, grant nr. P1-0212. We thank Ali Šalamun from the Centre for Cartography of Fauna and Flora for making the distribution map.

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