Pollen and floral morphology of *Androsace brevis* (Hegetschw.) Ces. (Primulaceae), a vulnerable narrow endemic plant of the Southern European Alps

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ARTICLE INFO

ABSTRACT

Androsace brevis (Hegetschw.) Ces. (Primulaceae) is a narrow endemic plant of the Southern Central European Alps that lives only above 2000 m asl and flowers immediately after snowmelt for a very short period during which the plant must guarantee successful sexual reproduction. Despite the vulnerability of this species, mainly due to increasing temperature, competition with plant communities shifting to higher altitudes, and possible mismatches with pollinators, nothing is known about its pollen and floral morphology. In this study we aim to provide a detailed description of these traits and to investigate how they might be related to possible pollination strategies and vectors. Pollen and flower samples were collected from the Lepontine and Orobic Alps (Northern Italy) populations. The pollen grains are small (polar axis: 19.17 μm ± 0.09; equatorial axis: 10.84 μm ± 0.1) with a prolate shape. The morphology of the flower, with the location of both stamens and pistil inside the corolla tube, and the coloured corolla mouths, ranging from yellow to purple, suggest that *A. brevis* requires insect-mediated pollination. Moreover, floral morphology does not show a particular insect-selective pattern in that the presence of both stamens and pistil less than 1 mm below the corolla mouth (Ø: 0.76 mm ± 0.05) might allow many high-altitude flower-visiting insects to reach both nectar and pollen. A generalist pollination strategy could be fundamental in contests characterized by harsh environmental conditions as it could counteract potential plant-pollinator mismatches due to increase in temperatures.

1. Introduction

The genus *Androsace* L. (Primulaceae) includes 153 species distributed in temperate and cold regions of the world, with mostly high mountain or arctic species (Smith et al., 1997; Kelso, 1991). The biogeographic reconstruction suggests that this genus originated from an Asian ancestor about 35 million years ago (Boucher et al., 2012) from which it radiated widely across the mountain ranges of the Northern Hemisphere, reaching Europe first and North America afterwards (Schönswetter et al., 2003; Roquet et al., 2013).

In the European Alps, the *Androsace* genus consists of 17 species found mainly in prairies, rocky debris, and overhanging cliffs of the subalpine and alpine altitudinal belt (Aeschimann et al., 2004) and distributed up to 4200 m asl (Giacomini and Fenaroli, 1958). Among these species, eight are endemic to the Alps (Aeschimann et al., 2004): some of them are widely distributed along the chain (Conti et al., 2005), while others are only present in a very restricted area. *Androsace brevis* (Hegetschw.) Ces. (Fig. 1) belongs to the latter group, a narrow endemic species that is present in the Southern Central Alps (Aeschimann et al., 2004). In particular, the extent of its occurrence encompasses three Alpine sections: the Eastern Lepontine Alps (Como Province, Lombardy, Italy and Canton Ticino, Switzerland), Western Orobic Alps (Bergamo and Lecco Province, Lombardy, Italy), and Western Rhaetian Alps (Sondrio Province, Lombardy, Italy) (Provasi, 1922; Pignatti, 1982;

Mangili et al., 2014). The species grows only on siliceous peaks and ridges above 2000 m asl, with an estimated area of occupancy of 92 km² (Mangili et al., 2014).

Androsace brevis is a chamaephyte cushion plant (Fig. 1) characterized by a very short flowering period, starting as soon as the snow melts on the rocky outcrops and lasting about two weeks between late May and early June (Pignatti, 1982; Bonelli et al., 2020, 2022). During this short period, the plant must guarantee successful sexual reproduction, which is the most common strategy for *A. brevis* (unpublished data) as well for many other alpine species living in cold alpine environments, compared to vegetative propagation (Körner, 1999; Wirth et al., 2010; Hörandl et al., 2011; Brožová et al., 2019; Pegoraro et al., 2020).

Nowadays this species faces many threats: indeed, as reported by Mangili et al. (2014), *A. brevis* seems to be extremely vulnerable to increasing temperatures as it already grows on mountain peaks and ridges, thus being not able to shift to higher altitudes. This renders the species vulnerable to the upward shift of more competitive species from lower altitudes which could represent a serious threat in that it shows low competitive ability. Furthermore, populations of *A. brevis* are often located very close to mountain paths and huts as well as to high-altitude pastures, subjecting them to frequent trampling by hikers and grazing animals. In addition, the species has a very low seed/flower ratio which could lower fitness (Mangili et al., 2014). In this context, reproductive success could also be negatively impacted considering that climate change might also threaten its interactions with possible pollinators (Bonelli et al., 2020, 2022); indeed, the early season represents a very critical moment for plant-pollinator mismatches (Kudo and Cooper, 2019). Finally, *A. brevis* is classified as vulnerable ("VU") both in Italy and Switzerland (Mangili et al., 2014; Bornand et al., 2016), according to IUCN criteria "B" (B1 – extent of occurrence; B2 – Area of occupancy) and "C" (C2a(i) – no subpopulation estimated to contain more than 50 mature individuals), respectively.

Although the ecology of *A. brevis* is documented (Mangili et al., 2014), little is known about its pollen and floral morphology. Indeed, the pollen morphology of *A. brevis* has never been studied, even though Xu et al. (2016) described the pollen morphotypes of five species of the section Aretia (*A. alpina* (L.) Lam., *A. carnea* L., *A. obtusifolia* All*., A. wulfeniana* Steud. & Hochst.), to which *A. brevis* belongs. Regarding the floral morphology, only the external conformation of the flower has been described (Hegetschweiler, 1840; Cesati, 1844; Pignatti, 1982; Mangili et al., 2014): *A. brevis* corolla has a diameter of about 5–8 mm; it is gamopetalous and presents five limbs slightly fringed at the apex. The flower is pink with a yellow or purple mouth wrapped by a calyx about 3.5–4.5 mm long, which develops from a stem of 9–15 mm in length. Nothing has been reported about the inner morphology of the flower, i. e., the position of stigma and anthers, their distance from the mouth of the flower, and their implications for reproduction.

It is important to study both pollen and floral morphology as they are correlated with pollination vectors (Hesse, 2000; Wang et al., 2009; Zhao et al., 2016; Pacini and Franchi, 2020). In particular, pollen morphology could be the result of adaptations to specific pollinators (Ferguson and Skvarla, 1982; Proctor et al., 1997; Tanaka et al., 2004) while the morphology of the flower not only could derive from the need to protect pollen and nectar from nonoptimal weather conditions but also to attract specific insects for pollination (Armbruster, 1996; Aizen, 2003; Yon et al., 2017; Lawson and Rands, 2019). In this study, therefore, we aim to describe both the pollen and floral morphology of this narrow endemic Alpine species.

2. Materials and methods

2.1. Study sites and flower sampling

In 2021, full-anthesis flowers were sampled from *A. brevis* plants belonging to two different populations in the Lepontine (Como, Lombardy, Italy) and Orobic Alps (Bergamo, Lombardy, Italy) (Fig. 2). The first site is located on the border between Italy and Switzerland, on the Cugn Peak (UTM WGS84–32T E 512338, N 5112905, 2193 m asl), while the second site was located near the Mountain Hut "Cesare Benigni" (UTM WGS84–32T E 543496, N 5096577, 2222 m asl). The two sites are described in detail by Bonelli et al. (2022).

2.2. Pollen morphology

Morphometric analysis of *A. brevis* pollen grains was performed using light microscopy (LM), while the sporopollenin ultrastructure was investigated by scanning electron microscopy (SEM) in according with Androit et al. (2022). The pollen grains were obtained from 30 anthers of six flowers per site and subjected to acetolysis according to Erdtman (1960).

For LM observations, slides of pollen were mounted in glycerine jelly. At least 100 pollen grains in equatorial or in polar view were photographed for each population using the 100x immersion objective lens under a Leica DM – RD microscope equipped with a Leica MC170 HD camera (Leica Camera AG, Wetzlar, DE). To determine the pollen grain shape, the equatorial axis (E) and polar axis (P) were measured and the P/E ratio of 40 randomly selected pollen grains for each population was calculated. The measurements were performed with ImageJ software (Schneider et al., 2012). For SEM observations, acetolyzed and air-dried pollen was sputtered with a 25-nm layer of gold in argon plasma (AGAR Automatic Sputter Coater Mod. B7341, Scientific Limited, Stansted, UK) equipped with a quartz crystal thickness monitor. At least 30 pollen grains in equatorial and polar view were observed under the electron microscope (Model Leo-1430, Zeiss Inc., Thornwood, NY, USA).

Fig. 1. a. *Androsace brevis* on a rocky outcrop at San Jorio Pass – Cugn Peak (Como, Lombardy, Italy, 2200 m asl); **b.** Detail of *A. brevis* flowers, Scale bar: 1 cm.
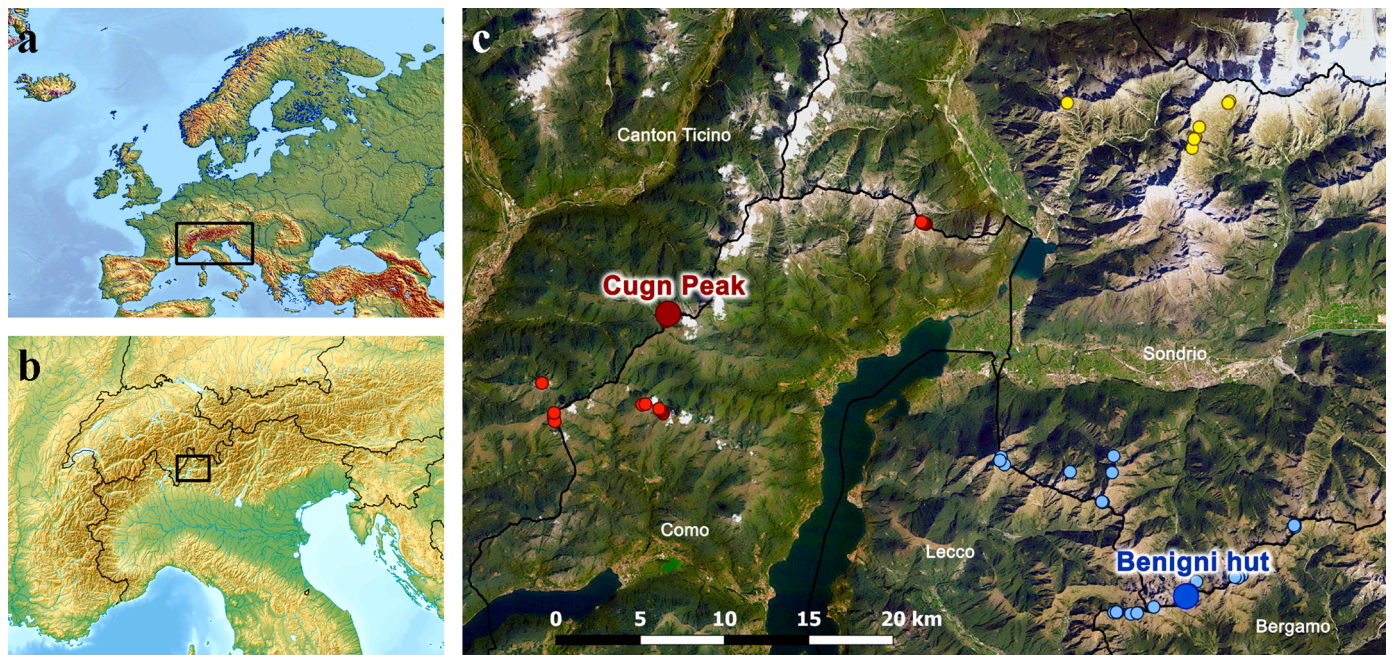
Fig. 2. a. Alps in Europe; **b.** *A. brevis* distribution range in the Southern Central Alps; **c.** Known populations of *A. brevis* (Red dots: Lepontine Alps; yellow dots: Rhaetian Alps; blue dots: Orobic Alps). The two sites chosen for this study (Cugn Peak and Benigni Hut) are highlighted with larger and darker dots.

We used palynological terminology according to Punt et al. (2007) and Hesse et al. (2009).

2.3. Floral morphology and phenology

To assess the number of flowers produced by *A. brevis*, the number of buds, open flowers, and withered blossoms carried by a single plant were counted, considering 15 plants from each site examined in this study. To describe the floral morphology, three open full-anthesis flowers of *A. brevis* were collected from each site. Fresh flowers were placed on graph paper, and corollas were photographed with Olympus Tough TG-4 (Olympus Corporation, Tokio, JP) camera. Then, longitudinal sections from the corolla mouth to the calyx of the flower were prepared, and pictures were taken with a stereomicroscope (Leica M50) equipped with a camera (Leica IC90) at 4x magnification. Floral parts were measured with ImageJ (Schneider, et al., 2012) software. In particular, the length and width of the limb and corolla tube, the diameter of mouth and corolla (Fig. 3a,b), the length and width of calyx limb, the length of calyx and calyx tube (Fig 3c), the length and the width of style, stigma, and anther, the length of stamen filament, the diameter of ovules, and the length of ovary (Fig. 3d,e) were measured. Moreover, the following distances were considered: anther – stigma, mouth – stigma, mouth – ovary, anther – ovary, and anther – anther (Fig. 3f). Based on these measurements, a scale drawing of the flower was rendered by using the software Adobe Photoshop CS6 (Adobe Inc., San Jose, CA, USA). A transverse section was also prepared to describe the structure and content of the ovary. In particular, three fresh flowers from each site were dissected along the upper wall of the ovary to observe the placentation and count the number of ovules. Moreover, the upper wall of the ovary was observed at 40x magnification under light microscope to evaluate the presence of structures involved in the nectar secretion.

Finally, to determine whether the different colors of the flower mouth are linked to the ripening stage of the anthers, three plants from each site bearing at least 10 flowers were observed from the beginning of anthesis to withering.

3. Results

3.1. Pollen morphology

The color of fresh *A. brevis* pollen is brownish-yellow. The mature pollen grains are released as free monads and have a prolate shape (mean $\pm$ SE: 10.84 μm $\pm$ 0.1 large and 19.17 μm $\pm$ 0.09 long) (Supplementary material S1). Grains are radially symmetric, isopolar, and zonocolporate with three colpori (tricolporate pollen grains) that are not fused at the polar edge (Fig. 4a,b,c). The tectum is nearly continuous on the apocolpium and perforated on the mesocolpium. Puncta (diameter < 0.5 μm) have different shapes and sizes (Fig. 4d,e). In many cases, the mesocolpium is not completely perforated by the puncta (Fig. 4f).

3.2. Floral morphology and phenology

Androsace brevis produces from two to about 200 (mean $\pm$ SE: 45 $\pm$ 9, Supplementary material S2) solitary, hermaphrodite, homostylous flowers. Flowers are held by erect pedicels and the corolla tube is enveloped by a gamosepalous, persistent calyx with five teeth. Inside the corolla tube, ca. 0.5 mm below the mouth, the androecium consists of one cycle of five yellow-green anthers held by a green filament inserted around the mouth of the corolla tube at the opposite side of the corolla lobes and surrounding the stigma. The gynoecium is simple: the stigma develops from a linear yellow-green style which starts from the ovary located at the base of the corolla tube. The ovary presents a free-central placentation and contains five ovules which are attached to the central axis without any partition between them (Fig. 5). Nectaries were not observed, but drops of a viscous substance were present both on the surface of the ovary and the inner side of the corolla tube.

The size (mean $\pm$ SE) of each trait of the *A. brevis* flower is reported below (Table 1), while the raw data are reported in Supplementary material S3.

Finally, it was observed that the color of the corolla mouth changes gradually from yellow to purple during the anthesis period. In particular, three main color shades can be distinguished (Fig. 6): 1) yellow, at the beginning of the flowering period, the moment when the anthers are brought straight close to the mouth; 2) yellow–purple, after the main
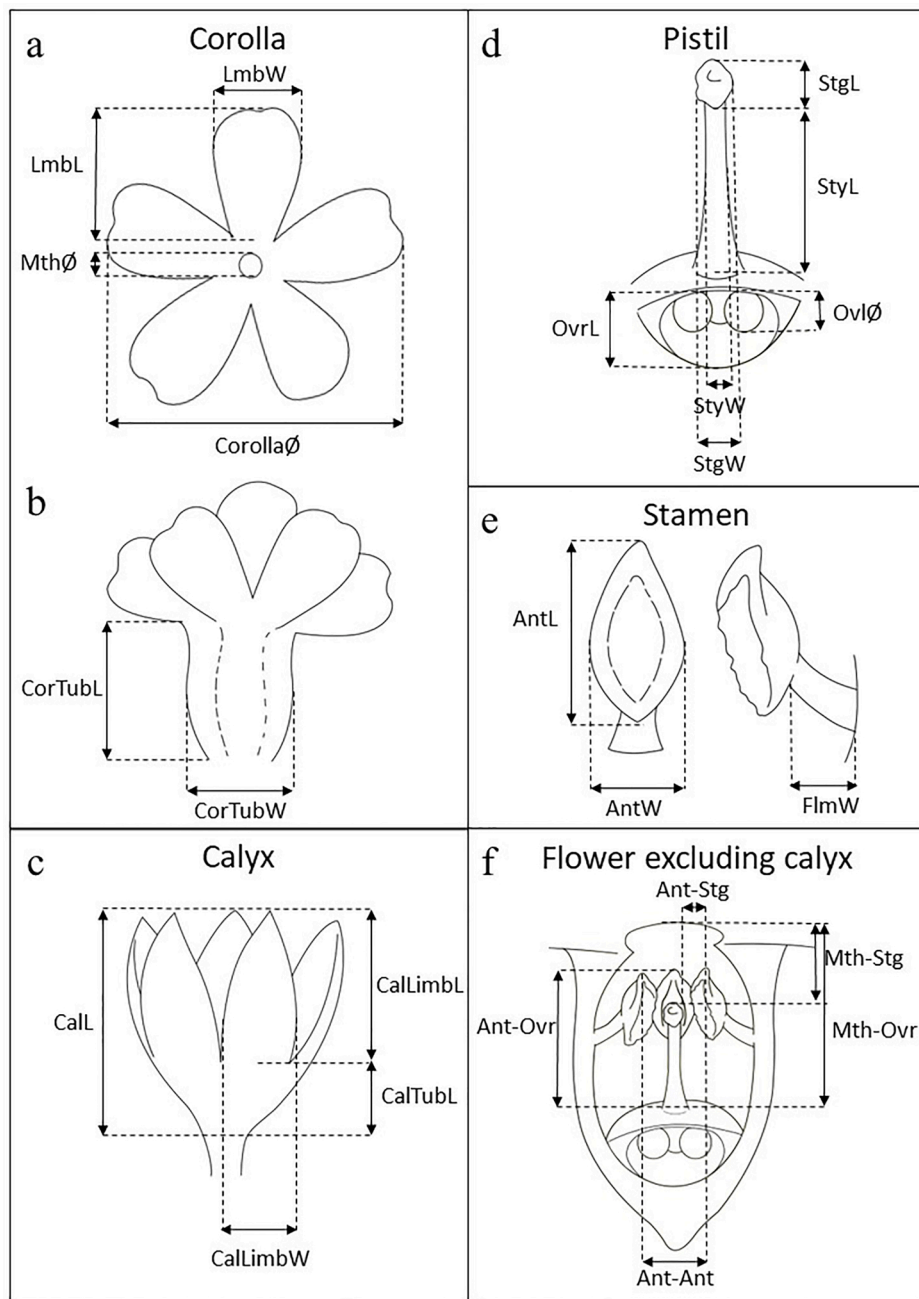
Fig. 3. Measurements of the flower parts (L = Length, W = Width, Ø = Diameter): **a.** Limb (Lmb), mouth (Mth), and corolla; **b.** Corolla tube (Cortub); **c.** Calyx (Cal), calyx limb (CalLimb), and calyx tube (CalTub); **d.** Style (Sty), stigma (Stg) and pistil (Pst), ovule (Ovl), and ovary (Ovr); **e.** Anther (Ant), filament (Flm); **f.** Distance anther – stigma (Ant-Stg), distance mouth – stigma (Mth-Stg), distance mouth – ovary (Mth-Ovr), distance anther – ovary (Ant-Ovr), and distance between anthers (Ant-Ant). Drawing by E. Eustacchio.

flowering period, when stamens begin to descend, revealing the corolla tube; and 3) purple, at the end of flowering period, when stamens are completely lowered, leaving the corolla tube visible.

4. Discussion

In relation to the six categories by which pollen grains can be classified according to their longest axis (Erdtman, 1952, 1969; Hesse et al., 2009), *A. brevis* produces small pollen grains (longest axis length ranging from 10 to 25 μm). Shape and exine ornamentation of the pollen grains are quite similar to those of other species belonging to the genus *Androsace* section Aretia described by Xu et al. (2016). However, *A. brevis* presents a larger polar axis but a similar equatorial one, resulting in a higher P/E ratio than other species reported by those authors. Although the small size of the pollen grains represents a feature of anemophilous pollen (Lu et al., 2022), the anthers are placed completely

inside the corolla tube and the morphology and color of the corolla exclude anemophilous pollination. This was confirmed by pollinator exclusion experiments conducted in 2016 (Supplementary material S4): even though flowers do not present heterostyly, as known for *Primula* genus (Lloyd and Webb, 1992; Barret and Shore, 2008), and anthers are located very close to the stigma (less than 0.5 mm apart), this species does not produce seeds if it is not visited by pollinators. This could suggest the occurrence of a mechanism which avoids self-fertilization, although the latter strategy is often advantageous in high-altitude environments because of low pollinator abundance (Kelso, 1987, 1991; Gugerli, 1998; Torres-Díaz et al., 2011; Goodwillie and Weber, 2018), especially for species that are characterized by small and fragmented populations (Van Rossum et al., 2006; Shao et al., 2008). However, plants that naturally live in small and scattered populations may have developed strategies to cope with long-term genetic isolation (Gonzalo-Turpin and Hazard, 2009; Becker et al., 2011; Hirao et al., 2019), as a
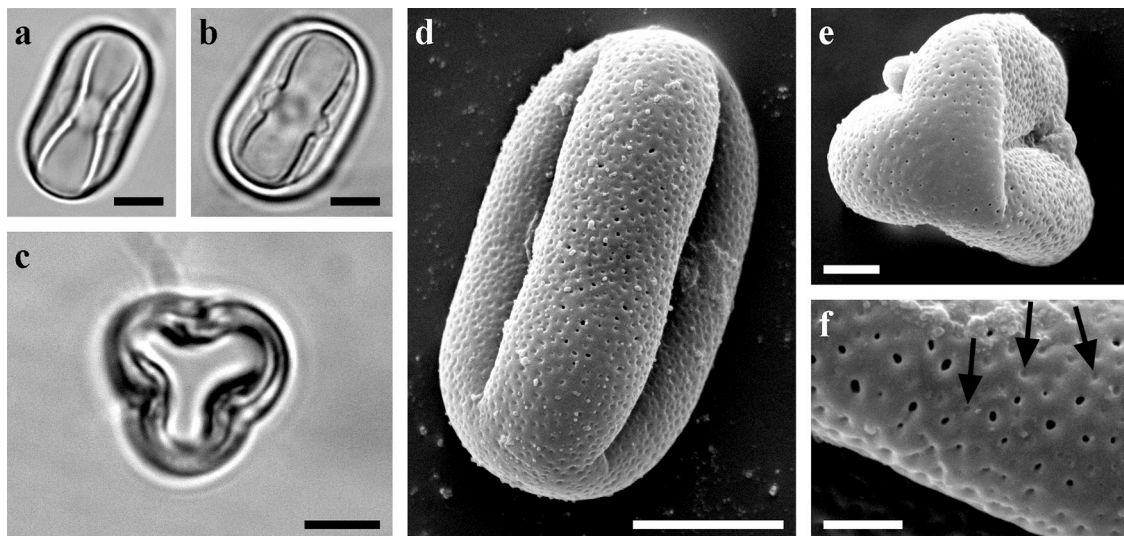
Fig. 4. a–c: LM micrographs. Scale bars: 5 μm. **a, b** Pollen grain in equatorial view at two different focal planes showing the equatorial pores; **c**. Pollen grain in polar view showing a triangular equatorial outline; **d–f**: SEM micrographs. Scale bars: d = 5 μm; e = 2 μm; f = 1 μm; **d**. Pollen grain in equatorial view showing a prolate shape and a perforate ornamentation; **e**. Pollen grain in polar view, showing a reduction in density of puncta on the apocolpium; **f**. In some cases, the tectum is not completely perforated by the puncta (arrows).
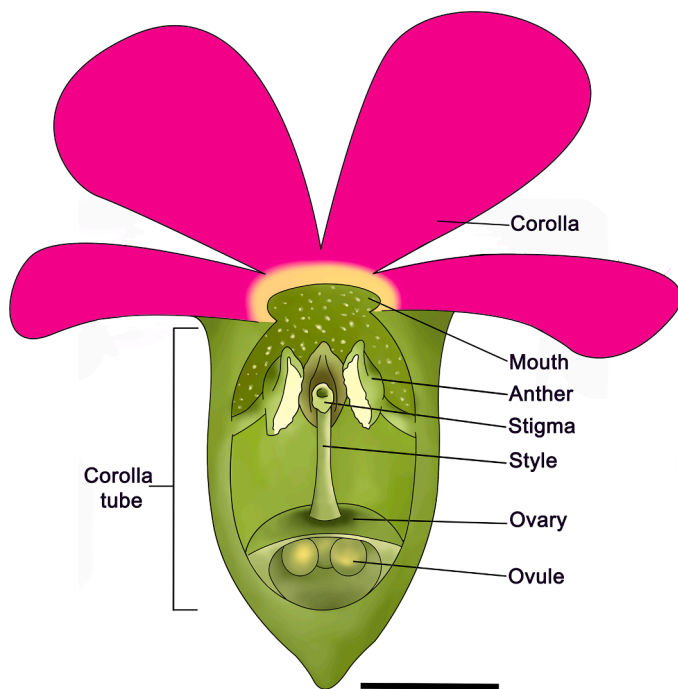
Fig. 5. Scale drawing of the longitudinal section of *A. brevis* flower based on data reported in Table 1. Scale bar: 1 mm. Drawing by E. Eustacchio.

Table 1

Mean value of the size of each trait of *A. brevis* flower with respective standard error (SE) and the number of measurements taken for each trait (n).

Section	Mean (mm)	SE	n
Anther length	0.58	0.02	6
Anther width	0.25	0.03	6
Anther-ovary distance	1.29	0.08	6
Anther-stigma distance	0.14	0.01	6
Anther-anther distance	0.27	0.05	6
Calyx length-	3.39	0.20	6
Corolla diameter	8.78	0.40	6
Flower length	3.93	0.06	6
Median calyx limb length	2.01	0.08	25
Median calyx limb width	0.96	0.05	25
Median calyx tube length	1.61	0.09	25
Median limb length	4.00	0.08	30
Median limb width	2.54	0.05	30
Mouth diameter	0.76	0.05	6
Mouth-anther distance	0.68	0.03	18
Mouth-ovary distance	2.14	0.10	6
Mouth-stigma distance	1.10	0.03	6
Ovary length	0.72	0.04	6
Ovule diameter	0.29	0.02	6
Stamen filament length	0.27	0.02	6
Stigma length	0.16	0.01	6
Stigma width	0.20	0.02	6
Style length	0.85	0.04	6
Style width	0.17	0.02	6

high outcrossing ability even in conditions of low pollinator activity (Kudo, 2022). In support of this, also other cushion plant species that live in alpine environments, such as *Silene acaulis* (L.) Jacq*., Saxifraga oppositifolia* L. and *Eritrichium nanum* (Vill.) Schrad. ex Gaudin, show an increase in seeds production when cross-pollination occurs (Stenström and Molau, 1992; Hermanutz and Innes, 1994; Delf and Carroll, 2001; Zoller et al., 2002). In addition to produce a high amount of nectar to be more attractive for pollinators (Hocking, 1968; Swales, 1979; Zoller et al., 2002), these species develop different strategies to limit self-pollination. For example, *S. acaulis* develops a variable number of hermaphrodite and female flowers within a population improving fitness in seeds production (Darwin, 1877; Hermanutz and Innes, 1994;

Delf and Carroll, 2001), while anthers and pistil of *E. nanum* ripen at different times (Zoller et al., 2002).

Given the low availability of pollinators in high-altitude environments (Holsinger, 1991; Barrett, 2002; Kalisz et al., 2004; Torres-Díaz et al., 2011; Goodwillie and Weber, 2018), plants often develop larger flowers to be more visible by insects (Proctor et al., 1997; Harder and Johnson, 2009). Indeed, as reported by Schimel (1995), the size of entomophilous plants tend to shrink with an increase in altitude since they invest more energy in reproductive structures. *Androsace brevis* develops relatively small solitary flowers whose diameter (~8.78 mm) is similar to those reported by Aeschimann et al. (2004) for *A. alpina* (L.) Lam and *A. wulfeniana* Steud. & Hochst (respectively Ø = ~8 mm; Ø = ~10 mm) while it is larger than that of other species belonging to section Aretia distributed along the Alpine chain (*A. hausmanni* Leyb., Ø = ~4

Fig. 6. Three different color shades of *A. brevis* corolla mouth within the same plant: 1: yellow, 2: yellow–purple, 3: purple.

mm; *A. helvetica* (L.) All., $\varnothing = \sim 5$ mm; *A. pubescens* DC.*, $\varnothing = \sim 5$ mm, *A. vandellii.* Sternb., $\varnothing = \sim 5$ mm). In alpine environments, many cushion plants develop small-sized flowers which potentially would hardly be visible by possible pollinators compared with those of other species flowering in the same area and period (Parachnowitsch and Kessler, 2010). For instance, in sites where *A. brevis* grows, other species that show a larger corolla surface (i.e., *Gentiana acaulis* L., *Primula hirsuta* All., *Pulsatilla alpina* (L.) Delarbre, and *Potentilla aurea* L.) are present and flower in the same period (Bonelli et al., 2022). However, *A. brevis* produces, on average, 50 flowers located very close to each other, and flowers have patent corolla limbs whose length is more than twice that of the calyx creating a homogeneous pink carpet with dots ranging from yellow to red. This pattern of a carpet of flowers with dots is very common in cushion alpine species as it helps them to compete with other alpine plants with larger flowers (Zoller et al., 2002; Parachnowitsch and Kessler, 2010). Showing a carpet of flowers leads pollinators to spend less flight energy moving from one flower to another searching for nectar and pollen and make greater gains in food rewards (Willson and Price, 1977; Heinrich, 1979; Kunin, 1993; Cartar et al., 2004; Dixit et al., 2020). The study of *A. brevis* floral morphology did not show the presence of nectaries above the gynoecium. Nevertheless, we observed a substance adherent to the inner wall of the corolla tube, above and under the stamens, allowing us to hypothesize that nectar is produced, but not secreted by specific structures as observed for *Androsace vitaliana* (L.) Lapeyr. by Abrahamczyk et al. (2017). In support of this, within the Primulaceae family, it is common that nectar is secreted by a nectariferous tissue which release a sugary substance through stomata located on the gynoecium (Percival, 1961; Vogel, 1986; Stevens, 2001; Sondenaa, 2000). Moreover, the presence of nectar is supported by the different color shade of the corolla mouth observed in different flowers within the same cushion, which may give a signal about the quality and quantity of floral rewards throughout the flowering period for potential pollinators (Melendez-Ackerman et al., 1997; Aragón and Ackerman, 2004). Color changes of the corolla mouth during anthesis were observed also in other *Androsace* species (Polunin, 1969; Zhang, 1982; Polunin and Stainton, 1984; Stainton and Polunin, 1988; Weiss, 1995; Sefali, 2021) as well as in *E. nanum*. The latter species, in particular, presents small ($\varnothing = \sim 6$–7 mm) blue flowers with five yellow epipetalous fornices that become visible when anthers begin to shed pollen and when nectaries at the base of the ovary start to produce fair amounts of nectar. When the pollination is concluded, the yellow color of the fornices become pale, and fructification takes place (Zoller et al., 2002).

Regarding the content of the ovary, species belonging to Primulaceae usually develop from few tens to hundreds of ovules and potentially the same number of seeds per flower (Constance, 1938; Anderberg and Ståhl, 1995; Andeberg, 2004). For *Androsace* species, to the best of our knowledge, only Douglas (1936) reported the presence of six ovules in *A. primuloides* Duby. *A. brevis* similarly shows five relatively large ovules

($\varnothing = \sim 0.29$ mm). They usually lead to the development of three or four seeds about 2.1 mm long, 1.4 mm wide and 0.5 mm thick (unpublished data), but one of them is frequently smaller than the others (Mangili et al., 2014) and probably not fertile. Investing a lot of energy to develop big seeds with a lot of storage could mean that *A. brevis* uses a K-strategy to cope with the harsh environmental conditions in which it lives. In fact, in alpine contest larger and heavier seeds increase the species fitness, giving a better chance to recruit and to withstand hazards (Jakobsson and Eriksson, 2003; Coomes and Grubb, 2003; Stöcklin et al., 2009).

Finally, floral morphology does not show a particular insect-selective pattern. The presence of both stamens and pistil less than 1 mm below the corolla mouth and the diameter of the mouth ($\varnothing = \sim 0.8$ mm) might allow many flower-visiting insects to reach both nectar and pollen (Conner et al., 1995) and possibly pollinate. Indeed, different flower-visiting taxa, such as families belonging to Diptera Brachycera and Hymenoptera Apoidea Anthophila, are known to visit *A. brevis* (Bonelli et al., 2022). According to the observations performed by Bonelli et al. (2022), our results confirm that *A. brevis* is a generalist pollinated species, with a possible plasticity in pollination. Indeed, if pollinator specificity can be advantageous for plants showing scattered populations (Wei et al., 2021), a generalist strategy may allows sharing of pollinators among different species that flower at the same time in a critical moment of the season. This strategy can be useful also to counteract potential plant-pollinator mismatches (Callaway et al., 2002; Kikvidze et al., 2005; Östman, 2018; Gérard et al., 2020), particularly in the context of climate change (Inouye, 2019).

CRediT authorship contribution statement

Elena Eustacchio: Conceptualization, Methodology, Validation, Formal analysis, Visualization, Writing – original draft, Investigation. **Marco Bonelli:** Conceptualization, Methodology, Validation, Formal analysis, Supervision, Writing – review & editing, Investigation. **Mario Beretta:** Conceptualization, Methodology, Validation. **Irene Monti:** Investigation. **Mauro Gobbi:** Conceptualization, Funding acquisition. **Morena Casartelli:** Conceptualization, Resources. **Marco Caccianiga:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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