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COMPARATIVE SEED MORPHOLOGY IN RELATED HIGH-MOUNTAIN SPECIES OF THE GENUS *AQUILEGIA* (RANUNCULACEAE)

Abstract - L. PINZANI, S. BACCI, F. OLIVIERI, G. BEDINI, A. CARTA, *Comparative seed morphology in related high-mountain species of the genus Aquilegia (Ranunculaceae).*

Although the relevance of seed morphology in systematics is widely recognized, its contribution in the seed plant classifications is often neglected. In this study, we performed a comparative morphometric analysis of the internal and external morphology of seeds in closely related species of the genus *Aquilegia*. Seed length, width, thickness, sphericity, mass, embryo to endosperm ratio at dispersal and micro-ornamentation of the integument are examined in five high-mountain species of the northern Apennines and the western Alps. Multivariate ordination aggregates the species in two morphological groups: the first one brings together the populations of *Aquilegia lucensis* and *A. alpina*, characterized by larger, heavier, and more spherical seeds; the second group includes *A. bertolonii*, *A. ophiolitibica* and *A. reuteri*, exhibiting smaller, lighter, and ellipsoidal seeds. Altogether, our results do not support a clear separation of all the taxa based on seed morphology, nevertheless, each of the investigated species shows a specific combination of seed character states.

Key words - alpine species, Alps, Apennines, *Aquilegia*, comparative taxonomy, multivariate analysis, seed length, seed mass, SEM

Riassunto - L. PINZANI, S. BACCI, F. OLIVIERI, G. BEDINI, A. CARTA, *Morfologia comparativa dei semi in specie altomontane affini del genere Aquilegia (Ranunculaceae).*

Sebbene l'importanza della morfologia dei semi in sistematica sia ampiamente riconosciuta, il suo contributo nella classificazione delle piante da seme è spesso trascurato. In questo studio abbiamo condotto un'analisi morfometrica comparativa della morfologia interna ed esterna dei semi in specie strettamente affini del genere *Aquilegia*. Lunghezza, larghezza, spessore, sfericità, massa del seme, rapporto embrione-endosperma alla dispersione e micro-ornamentazione del tegumento sono stati esaminati in cinque specie alto-montane dell'Appennino settentrionale e delle Alpi occidentali. L'ordinamento multivariato aggrega le specie in due gruppi morfologici: il primo gruppo riunisce le popolazioni di *Aquilegia lucensis* e *A. alpina*, caratterizzate da semi più grandi, pesanti e sferici; il secondo gruppo comprende *A. bertolonii*, *A. ophiolitibica* e *A. reuteri* caratterizzati da semi più piccoli, leggeri ed ellissoidali. Complessivamente, i nostri risultati non supportano una chiara separazione di tutti i taxa basata sulle caratteristiche morfologiche dei semi, tuttavia, ciascuna delle specie studiate mostra una specifica combinazione di stati dei caratteri dei semi.

Parole chiave - Alpi, analisi multivariate, Appennino, *Aquilegia*, lunghezza dei semi, massa dei semi, SEM, specie alpine, tassonomia comparativa

INTRODUCTION

Seed morphological features like size, shape, colour, relative embryo length and external appendages are linked with many biological and ecological processes, such as seed dormancy (Baskin & Baskin, 2004), germination (Philipupillai & Ungar, 1984; Mølken *et al.*, 2005; Briggs & Mogan, 2011; Vandeloos *et al.*, 2012; Carta *et al.*, 2017), dispersal (Dehgan & Yuen, 1983; Augspurger, 1986; Willson, 1993; Nathan *et al.*, 2008) and persistence in soil seed banks (Thompson & Hodgson, 1993; Arditti & Ghani, 2000; Gioria *et al.*, 2020). Remarkable variability in seed morphology exists in angiosperm taxa, with relative constancy of seed structure in narrow taxonomic units (Esau, 1977). Seed structures can be also an informative taxonomic feature providing information on plant evolution and adaptations (Elisens, 1983; Linkies *et al.*, 2010).

The taxonomic relevance of seeds in the systematics of angiosperms has been highlighted by different authors who based their classifications primarily on seed characters (Corner, 1976; Crane, 1985; Jones & Luchsinger, 1986). In Ranunculales, seed shape varies considerably and their geometrical properties have been described by Martín-Gómez *et al.* (2019). In Papaveraceae, for example, seeds shape adjusts well to the cardioid model (Martín-Gómez *et al.*, 2019), whereas seeds in Ranunculaceae adjust well to other geometric shapes, including the oval, truncated cardioid, Fibonacci spiral and ellipse.

In Ranunculaceae, seed morphology has been widely considered for systematics and taxonomic purposes across different genera such as *Aconitum* (Cappelletti & Poldini, 1984; Molero & Puig, 1990), *Clematis* (Ghimire 2020), *Consolida* (Costantinidis *et al.*, 2001), *Delphinium* (Malyutin, 1987; Ilarsan *et al.*, 1997; Hadidchi *et al.*, 2020), *Nigella* (Karcz & Tomczok, 1987; Dadandi *et al.*, 2009; Heiss *et al.*, 2011) and *Thalictrum* (Ghimire *et al.*, 2016). According to these studies, seed size, shape, dimensions (of three main axes), coat micro-sculpture, colour, cell shape and hilum position are the most used morphological features. Interesting-

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ly, seed coat related characters are commonly informative diagnostic features at the family or genera level, but rarely at species level.

The genus *Aquilegia* (Ranunculaceae) is composed of about 70 species distributed in the temperate zone of the northern hemisphere (Munz, 1946). In recent years *Aquilegia* has been often investigated in plant evolution and development, and nowadays it represents a model system in evolutionary biology (Kramer, 2009; Kramer & Hodges, 2010). Traditionally, the systematic of *Aquilegia* is based on flower and adult plant morphology, with subjective choices of the taxonomic rank used as fundamental sorting model (Nardi, 2016). This aspect has determined contrasting treatments in floristic works in Europe (Nardi, 2016). Moreover, none of the proposed taxonomic frameworks are supported by phylogenetic data, as the European contingent is currently unresolved and placed in an extensive polytomy (Bastida *et al.*, 2010; Fior *et al.*, 2013). Indeed, the high genetic affinity of European species seems to result from a rapid and recent differentiation (Bastida *et al.*, 2010; Fior *et al.*, 2013). The most studied morphological traits in *Aquilegia* involve flower, fruit and vegetative parts of adult plants. Conversely, detailed information on the morphology of seeds and its contribution to the taxonomy of *Aquilegia* is still lacking, despite the importance of seeds in explaining diversity in Ranunculales (Martín-Gómez *et al.*, 2019).

To fill this gap, in this study we compare seed internal and external morphology in five related species of the genus *Aquilegia* from northern Apennines and western Alps. The aim of this paper is to explore the systematic relationships among *A. alpina* L., *A. bertolonii* Schott, *A. lucensis* E. Nardi, *A. ophiolithica* Barberis & E. Nardi and *A. reuteri* Boiss. from a seed morphology perspective. We discuss our findings in the light of the current taxonomic delimitation of the species.

MATERIAL AND METHODS

Sampling

Seeds of *Aquilegia alpina*, *A. bertolonii*, *A. lucensis*, *A. ophiolithica* and *A. reuteri* were collected in summer 2020 during the dispersal period (July-August) from 11 populations (Tab. 1). For each population, 30 plants at least 30 meters apart from one another were sampled to ensure statistical representativeness and to reduce spatial autocorrelation (ENSCONET, 2009). Sampling involved two fully mature and open follicles per plant. The fruits of *Aquilegia* are composed by five follicles. According to Nardi (2016), each follicle can contain from 10 to 50 seeds. The total number of seeds collected per population is therefore between 600 and 3000. Seeds used for analysis were randomly selected from the total number of seeds collected per population.

Measurement

All measurements were performed on seeds equilibrated at approximately 20°C and 50% RH. Seed length, width and thickness were measured with caliper on 40 seeds for each population; sphericity was then calculated as indicated in Nikam *et al.* (2013):

$$\text{Sphericity} = \sqrt{\frac{b \cdot c}{a^2}}$$

where a = Longest intercept (length), b = Longest intercept normal to a (width), c = Longest intercept normal to a and b (thickness).

Mass was measured by analytical balance by weighing 10 replicates of 50 seeds per population; embryo and endosperm lengths were measured on 20 seeds per population from seed longitudinal sections under a stereoscope. The embryo/endosperm ratio (E:S) was thereafter calculated. Micro-ornamentation of the integument was investigated on three seeds per population using Scanning Electron Microscope (SEM) images in panoramic (35×) and detailed (500×) views (the material was coated by a gold thin layer, then observed and photographed at 10 kV). For SEM analysis we selected two characters related to cell structure in accordance with the categorization proposed by Barthlott (1981): (1) tegument cells (related to cell shape and belonging to the “primary structure”), considering isodiametric or elongated cells; (2) tegument surface (related to the ornamentation of outer cell wall and belonging to the “secondary structure”), considering smooth or corrugated surface. Moreover, given the importance of seed shape in explaining diversity in Ranunculales (Martín-Gómez *et al.*, 2019) we selected two other characters related to this feature: (3) ridge, which evaluates the prominence of the integument extroflexion in the micropylar area (separating into prominent or scarce ridge), and (4) seed angularity, which evaluates the presence of reliefs in the integument that interrupt the regular curvature of the seed surface (separating into round and angular seeds).

Statistical analyses

Univariate analysis was performed by means of analysis of variance (ANOVA) and Tukey post-hoc tests for multiple comparisons among species. Multivariate analysis was conducted by means of Multiple Factor Analysis (MFA) allowing to simultaneously consider quantitative and qualitative characters. Indeed, the MFA is a multivariate analysis useful to summarize and visualize a complex data table in which individuals are described by several sets of variables (quantitative and /or qualitative) structured into groups. It takes into account the contribution of all groups of variables to define the distance between individuals (Pagès, 2002). For each population, seed quantitative

Table 1. Populations sampled for morphological analysis. Seed were collected from fully ripened fruits from July to August 2020.

species	locality	coordinates	elevation	habitat	collection date
<i>Aquilegia alpina</i>	Mont Avic (AO)	45.6497 N, 7.5953 E	2200 m	Larch woodlands	24 Aug 2020
<i>Aquilegia alpina</i>	Val Veny (AO)	45.7667 N, 6.8500 E	1980 m	Bushes	25 Aug 2020
<i>Aquilegia alpina</i>	Pian della Mussa (TO)	45.2833 N, 7.1500 E	1900 m	Larch woodlands	24 Aug 2020
<i>Aquilegia bertolonii</i>	Monte Corchia (LU)	44.0333 N, 10.2667 E	1280 m	Grassy ledges on marble	14 Aug 2020
<i>Aquilegia bertolonii</i>	Val Serenaia (LU)	44.1197 N, 10.2003 E	1510 m	Grassy ledges on marble	19 Aug 2020
<i>Aquilegia bertolonii</i>	Foce di Pianza (MS)	44.1117 N, 10.1467 E	1330 m	Grassy ledges on marble	14 Aug 2020
<i>Aquilegia lucensis</i>	Monte Gennaio (PT)	44.1093 N, 10.8442 E	1800 m	Grassland	16 Aug 2020
<i>Aquilegia lucensis</i>	Monte Gomito (PT)	44.1258 N, 10.6416 E	1880 m	Grassland	17 Aug 2020
<i>Aquilegia lucensis</i>	Monte Marmagna (MS)	44.3935 N, 10.0035 E	1720 m	Grassland	20 Aug 2020
<i>Aquilegia ophiolithica</i>	Monte Rama (GE)	44.4280 N, 8.6180 E	1100 m	Stony grassland	11 Aug 2020
<i>Aquilegia reuteri</i>	Montre Grai (IM)	43.9975 N, 7.6738 E	1920 m	Stony grassland	12 Aug 2020

variables were averaged and these values were used as input for the MFA. Quantitative, continuous variables were seed mass, E:S and sphericity; qualitative, categorical variables were those identified through SEM images: ridge, tegument surface, seed angularity, tegument cells.

To verify the relationship between embryo length and endosperm, a linear regression model was applied between the logarithm of the two variables. All the analyses were performed in R statistical environment (R Core Team, 2020). MFA was performed considering the continuous and the categorical variables using the FactoMineR package (Le *et al.*, 2008). Length, width and thickness were not included in the MFA, as they are used for the calculation of sphericity.

RESULTS

Overall, seeds of all species studied show a high degree of morphological similarity. Nevertheless, a moderate degree of differentiation can be observed.

Regarding size and shape, *Aquilegia lucensis* shows significantly ($P < 0.05$) longer and heavier seeds, followed by *A. alpina*, which has seeds of similar mean width; *A. bertolonii* and *A. ophiolithica* have shorter and lighter seeds; *A. reuteri* shows intermediate characters as it is similar to *A. alpina* in length and to *A. ophiolithica* in width (Tab. 2). Sphericity ranges from 0.44 to 0.56 with some variability among the species, as populations of *A. alpina* and *A. lucensis* show more spherical seeds, while seeds of *A. bertolonii*, *A. ophiolithica* and *A. reuteri* are more ellipsoidal. Nevertheless, the overall seed shape adjusts well to an oval. According to the Tukey Test no significant differences are observed among populations and species in E:S.

Concerning qualitative characters (ridge, tegument surface, seed angularity and cell shape) *A. lucensis* and *A. alpina* show rounded-smooth surfaces, elongated cells and prominent ridges, while *A. bertolonii* and *A. ophiolithica* show angular-corrugated surfaces, isodiametric cells and scarce ridges. *Aquilegia reuteri* shows intermediate qualitative character having rounded-smooth surfaces, prominent ridge but isodiametric cells.

The first dimension of MFA explains 55.07% of total variation showing a separation of species into two main groups (Fig. 1). One group, with positive correlation to the first dimension, brings together the populations of *A. lucensis* and *A. alpina* distinguished by heavier and more spherical seeds, prominent ridge, smooth tegument with less angularity; a second group, negatively correlated with the first dimension, includes *A. bertolonii*, *A. ophiolithica* and *A. reuteri*, with lighter and less spherical seeds, scarce ridge, corrugated tegument and higher angularity.

The second axis explains 21.44% of total variation, and is represented by the contribution of embryo size and shape of tegument cells. Along this dimension a moderate degree of differentiation can be observed: within the first group *A. lucensis* shows on average a larger embryo to endosperm ratio and elongated cells compare to *A. alpina* (Tab. 2). Within the second group *A. bertolonii* exhibits larger embryo to endosperm ratio, compared to *A. ophiolithica* and *A. reuteri*, having lower embryo to endosperm ratio (Tab. 2).

Within the first group *A. alpina* and *A. lucensis* show comparable seed features. In the second group instead, there is a higher morphological variability as *A. bertolonii* appears more separate from *A. ophiolithica* and *A. reuteri* displaying the lowest mass and sphericity.

Table 2. Seed morphology of *Aquilegia alpina*, *A. bertolonii*, *A. lucensis*, *A. ophiolithica* and *A. reuteri* populations with information about mass of 50 seeds (g), length (mm), width (mm), thickness (mm), E:S, sphericity, ridge, tegument surface, seed angularity, shape of tegument cells. Quantitative values are expressed as mean \pm SD.

species	population	mass of 50 seeds (g)	length (mm)	width (mm)	thickness (mm)	E:S	sphericity	ridge	tegument surface	seed angularity	tegument cells (shape)
<i>A. alpina</i>	Mont Avic (AO)	0.109 \pm 0.003	2.476 \pm 0.121	1.128 \pm 0.102	1.504 \pm 0.177	0.168 \pm 0.038	0.456 \pm 0.040	prominent	smooth	round	elongated
<i>A. alpina</i>	Val Veny (AO)	0.102 \pm 0.004	2.345 \pm 0.128	1.077 \pm 0.110	1.471 \pm 0.168	0.151 \pm 0.022	0.460 \pm 0.054	prominent	smooth	round	elongated
<i>A. alpina</i>	Pian della Mussa (TO)	0.110 \pm 0.003	2.345 \pm 0.138	1.127 \pm 0.106	1.499 \pm 0.176	0.166 \pm 0.019	0.481 \pm 0.047	prominent	smooth	round	elongated
<i>A. bertolonii</i>	Monte Corchia (LU)	0.062 \pm 0.003	2.254 \pm 0.156	0.874 \pm 0.147	1.280 \pm 0.107	0.175 \pm 0.039	0.389 \pm 0.069	scarce	corrugated	angular	elongated
<i>A. bertolonii</i>	Val Serenaia (LU)	0.065 \pm 0.002	2.376 \pm 0.190	0.876 \pm 0.114	1.259 \pm 0.100	0.161 \pm 0.025	0.370 \pm 0.045	scarce	corrugated	angular	elongated
<i>A. bertolonii</i>	Foce di Pianza (MS)	0.067 \pm 0.001	2.204 \pm 0.167	0.953 \pm 0.152	1.254 \pm 0.098	0.174 \pm 0.050	0.435 \pm 0.078	scarce	corrugated	angular	elongated
<i>A. lucensis</i>	Monte Gennaio (PT)	0.111 \pm 0.035	2.692 \pm 0.319	1.177 \pm 0.264	1.610 \pm 0.207	0.172 \pm 0.023	0.421 \pm 0.050	prominent	smooth	round	elongated
<i>A. lucensis</i>	Monte Gomito (PT)	0.124 \pm 0.004	2.620 \pm 0.187	1.127 \pm 0.144	1.589 \pm 0.201	0.185 \pm 0.029	0.430 \pm 0.053	prominent	smooth	round	elongated
<i>A. lucensis</i>	Monte Marmagna (MS)	0.113 \pm 0.004	2.605 \pm 0.128	1.124 \pm 0.098	1.500 \pm 0.176	0.172 \pm 0.029	0.432 \pm 0.037	prominent	smooth	round	elongated
<i>A. ophiolithica</i>	Monte Rama (GE)	0.074 \pm 0.003	2.177 \pm 0.144	0.979 \pm 0.123	1.327 \pm 0.123	0.167 \pm 0.021	0.452 \pm 0.067	scarce	corrugated	round	isodiametric
<i>A. reuteri</i>	Montre Grai (IM)	0.081 \pm 0.003	2.448 \pm 0.214	1.009 \pm 0.132	1.334 \pm 0.125	0.172 \pm 0.035	0.414 \pm 0.056	prominent	smooth	round	elongated

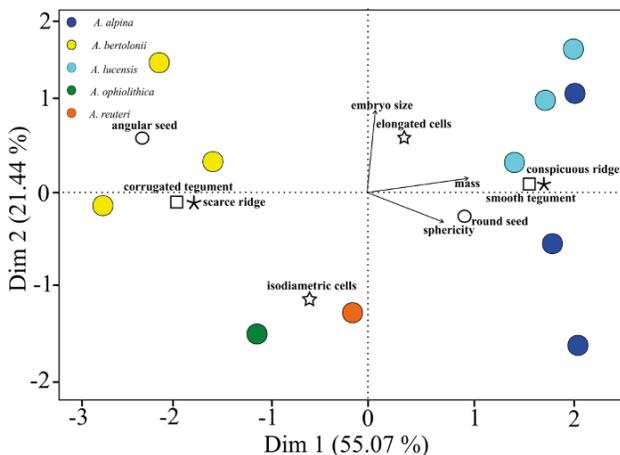


Figure 1. Multiple Factor Analysis (MFA) results. Populations are represented by coloured (online version) or grey (printed version) circles. Colours indicate species, the contribution of quantitative variables is expressed by the length of vectors. Qualitative variables are represented by unfilled symbols: circle (angularity), star (tegument cells), square (tegument surface) and asterisk (ridge).

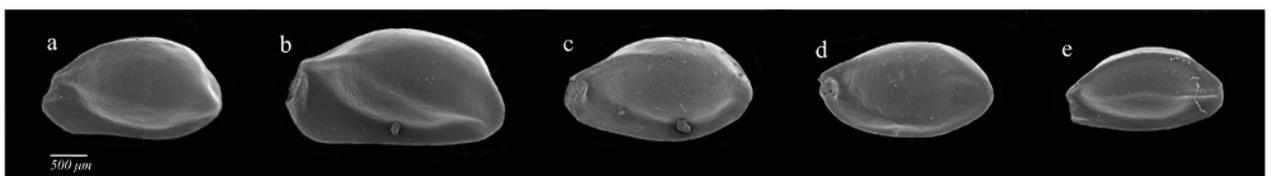


Figure 2. Seed external morphology on SEM for the taxa studied here. Each seed is represented in lateral view. (a) *Aquilegia alpina*; (b) *A. lucensis*; (c) *A. reuteri*; (d) *A. ophiolithica*; (e) *A. bertolonii*.

Considering all species together, the relationship between the logarithm of embryo length and the logarithm of endosperm length is a positive linear function (R-sq: 0.097; p-value: < 0.001). Considering each species separately, the relationship is not significant.

DISCUSSION

In this study, we have shown that overall there are clear distinction in seed morphological features exhibited by five closely related *Aquilegia* species. Indeed, the MFA divides the species into two main morphological groups along the first dimension, each of them moderately split in two additional groups along the second axis. The first group, along the first axis, brings together the populations of *A. lucensis* and *A. alpina* distinguished by heavier and more spherical seeds, conspicuous ridge, smooth tegument with less angularity; the second group includes *A. bertolonii*, *A. ophiolithica*, and *A. reuteri*, with lighter and less spherical seeds, scarce ridge, corrugated tegument, and higher angularity.

Seed mass is an important diagnostic character, as difference in weight between all taxa is statistically significant. Moreover, seed mass and elevation seem positively correlated, with species distributed at higher elevations showing heavier seeds (Tabs 1, 2). This result is consistent with those of Pluess *et al.* (2005) who tested the relationship between seed weight and elevation in 29 pairs of related species from open environments in Switzerland. The authors found that seed mass tends to be greater in high-elevation species than in lowland congeneric taxa. Moreover, the increase in mass does not appear to be significant among populations of the same species living at different altitudes. According to sphericity, the overall seed shape of the investigated species adjusts well to an oval, which is one of the most common seed shapes in Ranunculaceae (Martín-Gómez *et al.*, 2019).

Ridge prominence is an easily detectable character from which two types of seeds can be recognized: seeds with scarce ridge belonging to *A. bertolonii* and *A. ophiolithica*, and seeds with prominent ridge belonging to *A. lucensis*, *A. alpina* and *A. reuteri*. However, factors which determine the final shape of the seed can be climatic or related to the mother plant, for example, height and diaspore morphology (Traveset *et al.*, 2014). Indeed, despite variations in seed shape are classically interpreted as adaptations for dispersal, some features of shape could be affected by the conditions inside the ovary in which seeds develop (Harper *et al.*, 1970). Ridge prominence and sphericity in seeds of high-elevation *Aquilegia* species could result from different seed development within the fruit and may play a role in seed dispersal ability, but this possibility remains to be clarified.

The embryo length/endosperm regression considering all the studied species shows an allometric relationship likely ensuring proper functionality of pre- and post-germination processes in the seed. Indeed, according to the Tukey Test no significant differences are observed among populations and species in E:S. This result confirms that internal seed structure is usually a highly conservative trait (Nikolaeva, 1999; Forbis *et al.*, 2002).

The morphological differences detected among the investigated taxa do not seem sufficient to support the current separation into five species, whereas supporting the grouping of taxa into two distinct morphological clusters. Indeed, the seed morphology of *Aquilegia ophiolithica* and *A. reuteri* is more similar to *A. bertolonii* whereas *A. lucensis* is closer to *A. alpina*. Therefore, seed morphological differences of the species studied here do not support the current taxonomic scheme proposed by Nardi (2016) but are more consistent with Munz (1946), which summarized the five taxa studied here into two polymorphic species: *Aquilegia alpina* L., (including *A. lucensis*) and *Aquilegia bertolonii* Schott (including *A. reuteri* and *A. ophiolithica*).

Despite the high affinity of seeds within the two main morphological clusters, a moderate degree of differentiation can be observed. Along the second dimension of the ordination, the species show slightly different combinations of qualitative (shape of tegument cells) and quantitative (embryo to endosperm ratio) seed characters. These subtle differences within the main morphological groups, however, should be viewed with caution as differences in tegument micro-ornamentation were observed in a limited number of seeds, potentially affected by biotic or abiotic constraints which can determine high individual variability (Castellanos *et al.*, 2008). This aspect, along with the small number of populations observed for *A. ophiolithica* and *A. reuteri* may not fully account for the seed intraspecific variability of the species. Specifically, our results should be considered critically with respect to *Aquilegia alpina* as they may not be representative of the entire seed intraspecific variability of this species. According to Canne (1979), species with the largest distribution show the greatest seed morphological variation. It must also be considered that seed morphology is subject to the same uncertainties and ambiguities as any other attribute used in morphological studies (Newell & Hymowitz, 1978). Despite the significant differences observed among morphological groups, seeds show a high overall affinity. This aspect could reflect the high genetic affinity between the investigated species, which result from a rapid and recent radiative process in Europe (Bastida *et al.*, 2010; Fior *et al.*, 2013). Nevertheless, distinctly observable differences occur among the two clusters of taxa. The observed difference could re-

flect a real genetic divergence as plants epidermal characters are little affected by the environmental conditions and there is evidence for a strong genetic control over these features (Cutler & Brandham, 1977; Cutler, 1979). As indicated by Barthlott (1981) this pattern is not exclusive to the Ranunculaceae family, but involves most vascular plants. According to the author, many surface characters are of minor taxonomic importance and only of limited diagnostic significance at the specific rank. On the other hand, seed surface characters can generally be used to characterize groups of related species or genera at the family level.

In conclusion, the results found here, could be improved using a greater number of species and populations from different groups of European related *Aquilegia* species distributed in large mountain systems (e.g., Alps, Pyrenees, or Balkan Mountains). Moreover, to draw a more comprehensive picture of morpho-functional diversity of seeds in related taxa, the integration of morpho-physiological processes such as dormancy, embryo development and germination timing is advisable. The physiological component is intimately linked to seed morphology and complements its biological significance (Baskin & Baskin, 2014). Including seed variability in a morpho-functional perspective could help to understand its role in the differentiation process and therefore its contribution to taxonomic characterization.

Although seed morphology alone is generally insufficient to support strong separation of species, its contribution to the systematics of Ranunculaceae is crucial. Overall, our results indicate that the seed characteristics studied here are valuable tools to explore taxonomic relations among groups of related species in the genus *Aquilegia*.

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