

RESEARCH PAPER

Analysis of intraspecific seed diversity in *Astragalus aquilanus* (Fabaceae), an endemic species of Central Apennine

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Keywords

Interpopulation variability; intraspecific diversity; IR spectrum; morpho-colorimetric analysis; seed image analysis; trait–environment relationship.

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Editor

H. Pritchard

Received: 9 February 2018; Accepted: 14 May 2018

doi:10.1111/plb.12844

ABSTRACT

- This work aims to study seeds of the endemic species *Astragalus aquilanus* from four different populations of central Italy. We investigated seed morpho-colorimetric features (shape and size) and chemical differences (through infrared spectroscopy) among populations and between dark and light seeds.
- Seed morpho-colorimetric quantitative variables, describing shape, size and colour traits, were measured using image analysis techniques. Fourier transform infrared (FT-IR) spectroscopy was used to attempt seed chemical characterisation. The measured data were analysed by step-wise linear discriminant analysis (LDA). Moreover, we analysed the correlation between the four most important traits and six climatic variables extracted from WorldClim 2.0.
- The LDA on seeds traits shows clear differentiation of the four populations, which can be attributed to different chemical composition, as confirmed by Wilk's lambda test ($P < 0.001$). A strong correlation between morphometric traits and temperature (annual mean temperature, mean temperature of the warmest and coolest quarter), colorimetric traits and precipitation (annual precipitation, precipitation of wettest and driest quarter) was observed.
- The characterisation of *A. aquilanus* seeds shows large intraspecific plasticity both in morpho-colorimetric and chemical composition. These results confirm the strong relationship between the type of seed produced and the climatic variables.

INTRODUCTION

Astragalus aquilanus Anzal. (Fabaceae) is an endemic species of Italy, where it has been recorded in Abruzzo (Pignatti 1982). It can be found in arid grasslands with south exposure in natural pinewoods or in degraded oak sites (Pignatti *et al.* 2001). It is in the IUCN Red List and the Habitat Directive. One characteristic of this species is the presence of heterogeneity of seed in shape, colour and size. Higher plants have several strategies to perpetuate themselves under adequate ecophysiological conditions, and the production of heterogeneous seeds is one of these strategies. That is, to ensure the survival of the next generation, an individual plant might produce seeds that are heterogeneous with respect to the extent of dormancy, dispersion and persistence within the seed bank. Seed heterogeneity appears to be confined to a limited number of families of phylogenetically advanced angiosperms (Matilla *et al.* 2005), including *Astragalus* genus (Angosto & Matilla 1993).

The recent literature (Venora *et al.* 2009; Grillo *et al.* 2011; Smykalova *et al.* 2011; Santo *et al.* 2015) proves that morphological and colorimetric characterisation of seeds, as well as other plant structural parts, can be performed with computer vision techniques that give very accurate and repeatable measurements. Moreover, this technology allows us to rapidly implement precise, repeatable and above all non-destructive

measurements of seed colour, size and shape inside a digital image. In agreement with Chtioui *et al.* (1996), we combined colour (mean grey value) and seed size and shape variables to increase the power of discrimination analysis among populations. In particular, we compared four different populations, from geographic areas separated by at least 18 km from each other as the crow flies. All the investigated seeds were collected in the same year, carefully choosing the best moment for collection in each population. The morpho-colorimetric analysis was carried out on 200 seeds of each population, using ImageJ for measurements and Linear Discriminant Analysis (LDA) for successive data handling.

Infrared spectroscopy (IR) combined with step-wise LDA (swLDA) was used to investigate differences in chemical composition among populations of *A. aquilanus* seeds. In particular, 20 samples of each population, consisting of ten dark and ten light seeds, were analysed. The spectral region of the IR spectrum between 2000 and 1000 cm^{-1} was considered in discriminant analysis.

Finally, correlation between the four most discriminant morpho-colorimetric traits and bioclimatic variables was investigated. The bioclimatic variables were extracted from WorldClim 2.0 (Fick & Hijmans 2017).

The aims of the current study were to: (i) investigate the intraspecific diversity in seeds traits (morpho-colorimetric and

chemical composition) among populations and seed colour; (ii) understand if intraspecific diversity of both seed morphology and chemical composition can be explained in the same way; and (iii) investigate and understand the correlation among the most important morpho-colorimetric traits and bioclimatic variables.

MATERIAL AND METHODS

Study species and area

Astragalus aquilanus Anzal. (Fabaceae) is an Italian endemic species present in Abruzzo and Calabria regions (Anzalone 1970; Bernardo 1997; Pignatti *et al.* 2001; Conti *et al.* 2002; Conti & Bartolucci 2015, 2016); but in the Calabrian station this plant has not been confirmed recently (Ercole *et al.* 2016). It occurs in the Habitats Directive listed habitat 6210 "Semi-natural dry grasslands and scrubland facies on calcareous substrates (*Festuco-Brometalia*)" and the geographic range is 1439 km² (Peruzzi 2011; Pesaresi *et al.* 2014). *A. aquilanus* is listed as a priority species in Annex II of the Habitats Directive and under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention) and has been listed as Vulnerable in Italy (Pignatti *et al.* 2001). Furthermore, this species is classified as Endangered in the IUCN Red List of Italian Flora (Rossi *et al.* 2013) and is also a target species of Floranet LIFE project (LIFE15 NAT/IT/000946).

This species is a chamaephyte, with spring–summer flowering from late April to June, fruiting from June to early August and seed dispersal from July to August. *A. aquilanus* reproduces by means of entomophilous pollination and barochory dispersal. Di Martino *et al.* (2015) investigated *A. aquilanus* and reported physical seed dormancy, due to the waterproof tegument, which is a strategy to regulate the timing of germination. *A. aquilanus* is considered a heliophilous species, and lives preferentially in arid grasslands exposed to the south, at the margins of thermophilic forests, including road margins, but is less common in woody formations of *Pinus nigra* J.F. Arnold subsp. *nigra*, *Quercus pubescens* Willd. subsp. *pubescens* or *Q. ilex* L. subsp. *ilex*, on calcareous substrates, at an altitude of between 300 and 1050 m a.s.l. In particular, the grassland where *A. aquilanus* is found is part of the alliance *Phleboambigui–Bromion erecti*.

The species is found in relatively isolated stations, and threats are mostly in populations with a small number of

individuals. The main pressures on *A. aquilanus* result from anthropogenic activities, especially clearing of the vegetation of road margins, indiscriminate harvesting and other environmental alterations (Gigante *et al.* 2016). The effect on this species of natural recolonisation of woody vegetation also creates changes in available habitat. It must be noted that, at present, the species is not included in any national or international protection laws.

In this study we analysed four different populations: Monteluco di Roio (AQ), Piano La Roma (CH), San Colombo (AQ) and M. Scuncole (PE). All information on the location of the four different populations is given in Table 1. The populations are geographically isolated and the minimum direct distance between the investigated stations is 18 km, while the maximum distance is 76 km. All the investigated populations come from sites not included in protected areas (Fig. 1).

Seed lot details

Seeds of *A. aquilanus* were collected from the four Abruzzo populations, on the basis of international protocols (ISTA 2012); these represented <20% of the seeds available in the existing populations. The mother plants were randomly selected, and mature seeds collected during the fruiting period, immediately before dispersal (Baskin & Baskin 2014), during July and August 2016. The seeds were cleaned manually in the laboratory and were further dried at room temperature (≈20 °C) to prevent rotting during cold storage (Di Cecco *et al.* 2017) and finally stored in the Majella Seed Bank. They were then kept in the dark at 5 °C until April 2017, when the morpho-colorimetric and IR spectroscopy tests were performed. The morpho-colorimetric analyses were carried out in the Majella Seed Bank (Majella National Park), while chemical content and FT-IR characterisation was carried at the laboratory of the Department of Physical and Chemical Sciences of the University of L'Aquila.

Morpho-colorimetric analysis

Digital images of seed samples were acquired using a flatbed scanner (Epson GT-15000) with a digital resolution of 600 dpi and a colour depth of 24 bit. Following Bacchetta *et al.* (2008), the seeds were arranged singly on the scanner tray, avoiding any overlap. To clearly highlight the colour variations on the edge between the seeds and the background, a white paper cover was used to avoid environmental light interference. The

Table 1. Data for the four *Astragalus aquilanus* populations investigated in this work.

population code	locality	date collected	altitude (m a.s.l.)	fuse	longitude	latitude	Lat/Long method	GPS Datum	substrate	aspect	slope	distance between populations (km)			
												PLR	SNC	MLR	MSC
PLR	Piano La Roma – Casoli (CH)	27/07/2016	365	33T	438647	4665340	UTM	WGS 84	Limestone	SSE	10°	–	59	76	37
SNC	San Colombo – Barisciano (AQ)	11/08/2016	1150	33T	384048	4688096	UTM	WGS 84	Limestone	S	15°	59	–	18	24
MLR	Monteluco di Roio - L'Aquila (AQ)	13/08/2016	980	33T	366150	4688768	UTM	WGS 84	Limestone	S	5°	76	18	–	40
MSC	Monte Scuncole – Bussi (PE)	10/08/2016	630	33T	402466	4672696	UTM	WGS 84	Limestone	E	20°	37	24	40	–

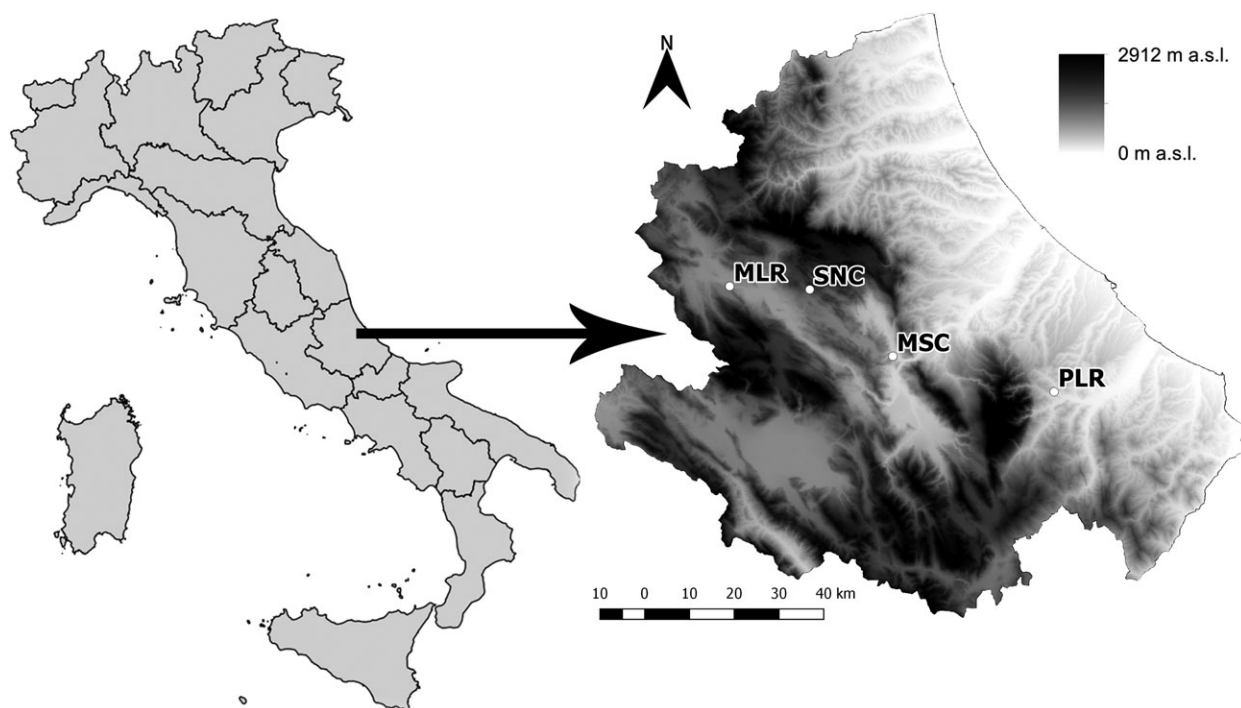


Fig. 1. Geographic distribution of the four investigated populations of *Astragalus aquilianus*: Montelucio di Roio (MLR), Monte Scuncole (MSC), Piano La Roma (PLR) and San Colombo (SNC). The Abruzzo region is represented with a Digital Terrain Model.

captured digital images were stored in TIFF (Target Image File Format) format (Smykalova *et al.* 2011). Before image acquisition, the scanner was calibrated for colour matching, using a colour calibrator (Datacolor Spydercheckr). Digital images of samples consisting of 100 seeds were acquired and used for successive analyses (Santo *et al.* 2015). Digital images of seeds were analysed using the free software ImageJ. Twenty-two descriptive parameters of shape, size and colour were measured for each single seed (Table 2), including: area, perimeter, mean grey value (average grey value within the caryopsis), width, height, circularity, aspect ratio, roundness and solidity.

Fourier transform infrared (FT-IR) spectroscopy analysis

Seeds were initially ground to a fine powder using an agate mortar and pestle. The powdered sample was mixed with KBr at a 1:180 ratio (w/w), homogenised and the mixture was then compressed under a pressure of ca. 200 MPa for 1 min to form a thin KBr disc. FT-IR spectra were recorded using a Spectrum Two Perkin Elmer (Perkin Elmer, Waltham, MA, USA) spectrometer that operates in the region 4000–400 cm^{-1} at room temperature, with a total of ten scans and 1 cm^{-1} resolution. Spectrum PerkinElmer software supplied from the manufacturer was used for spectra recording and pre-processing. Each spectrum was acquired in the absorbance mode against air background. A multipoint baseline operation was applied to set zero absorbance at 4000, 2000 and 400 cm^{-1} . All the spectra were normalised so that the absorbance at 3400 cm^{-1} , defining the maximum of the broad band due to OH and NH stretching, was 1. First derivative spectra were generated using nine-point Savitzky-Golay filters. Statistical analysis was limited to the spectral range 2000–1000 cm^{-1} .

Table 2. Morpho-colorimetric features of *Astragalus aquilianus* seeds, obtained by image analysis (software ImageJ).

feature	description
Area	Area of selection mm^2
Mean grey value	Average grey value within the selection
Standard deviation	SD of grey values used to generate mean grey value
Modal grey value	Most frequently occurring grey value within the selection
Max grey level	Maximum grey values within the selection
Min grey level	Minimum grey values within the selection
Perimeter	Length of outside boundary of the selection
Major ellipse	Primary axis of best fitting ellipse
Minor ellipse	Secondary axis of best fitting ellipse
Angle	Angle between primary axis and a line parallel to x-axis of the image
Circularity	$4\pi \times \text{area}/\text{perimeter}^2$. A value of 1.0 indicates a perfect circle
Aspect ratio	Major axis/minor axis of best fitting ellipse
Roundness	$4 \times \text{area}/(\pi \times \text{major_axis}^2)$, or inverse of the aspect ratio
Solidity	Area/convex area
Height	Longest distance between any two points along the selection boundary (maximum caliper)
Width	Minimum caliper diameter
Feret's angle	Angle between Feret's diameter and a line parallel to the x-axis of the image
Integrated Density	Product of area and mean grey value
RawIntDen	Sum of values of pixels in the image or selection
Median	Median value of pixels in the image or selection
Skewness	Third order moment about the mean
Kurtosis	Fourth order moment about the mean

Data analysis

Intraspecific diversity among the four populations was investigated using Linear Discriminant Analysis (LDA; Fisher 1936). Moreover, to evaluate the quality of the discriminant functions achieved for each statistical comparison, Wilks' Lambda tests were performed (Lo Bianco *et al.* 2017b). Before performing LDA analysis on morpho-colorimetric traits the variables not useful for seed shape and size description were excluded (Brandt *et al.* 2017), and Random Forest Method was successively applied to select among those left (15 out of 22), the most important predictor variables, namely those explaining most variance. For the four most important variables, the relationship with six bioclimatic variables was analysed using a linear model (lm). The bioclimatic variables were obtained through raster extrapolation of WorldClim 2.0 (Fick & Hijmans 2017). Using expert opinion (Brandt *et al.* 2017), six variables were selected: three for temperature, namely annual mean temperature (bio01), mean temperature of the warmest and coolest quarter (bio10, bio11, respectively), and three for precipitation, namely annual precipitation (bio12), precipitation of wettest quarter and driest quarter (bio16, bio17, respectively).

All the morpho-colorimetric data were standardised before statistical elaborations, and both normal distribution and homoscedasticity of each variable was tested. A step-wise algorithm was applied to the IR spectra to identify the most powerful variables able to provide the best class discrimination. To this end, the sequentially selected variables were those able to minimise the within-class distance and contextually maximise the between-class distance (Kuhn & Johnson 2013). To graphically highlight the differences among the four seed populations, the data samples were projected into two-dimensional plots, the axes being the first two discriminant functions (DFs). In contrast, separation of two single classes (*e.g.* light and dark seeds) is described with only one DF and two-dimensional representation is not required. All statistical analyses were performed using R software (R Development Core Team 2008).

RESULTS

The first analysis aimed at morpho-colorimetric differentiation of the four *A. aquilianus* populations. Morphological characterisation of seeds was based on ten descriptors, while five variables associated with the grey scale provided by the image analysis were selected for the colorimetric characterisation. The results using LDA on seed traits show clear differentiation of the four populations (Fig. 2), also confirmed with Wilk's lambda test ($P < 0.001$). The four main variables that explain the largest variability among populations are max grey value, width, mean grey value and height.

Figure 3A displays a typical FT-IR spectrum of an *A. aquilanus* seed. The broad band centred at ca. 3350 cm^{-1} can be mainly assigned to OH and NH stretching. Apart from proteins and sugars, water, present as moisture in the seeds, also contributes to this band. The signals in the region $3000\text{--}2850\text{ cm}^{-1}$ are typical of C-H stretching. The intense band at 1654 cm^{-1} is associated with the CONH group of proteins or polypeptides and is originated through C=O stretching (amide I), while the relatively intense band at 1546 cm^{-1} and the low-intensity band at 1247 cm^{-1} result from coupled C-N

stretching and N-H bending vibrations of the peptide group (amide II and amide III, respectively; Carbonaro *et al.* 2008). The band at 1745 cm^{-1} is attributed to stretching of the C=O group of esters (*e.g.* triglycerides). The large band around 1100 cm^{-1} is associated with C-O and C-C stretching and CCH, COH and HCO deformations of both proteins and polysaccharides (Schulz & Baranska 2007). The band at 1400 cm^{-1} falls in the region of C-H bending modes and can be attributed to vibrations of CH_2 groups; proteins, polysaccharides and lipids can contribute to this band.

To enhance the spectral differences, the first-derivative IR spectra were analysed rather than the original ones. Because of the expected large contribution of seed moisture to the broad band at about 3400 cm^{-1} and ubiquitous contribution of all the seed functional constituents to the bands at $3000\text{--}2850\text{ cm}^{-1}$ (C-H stretching), LDA was applied to the spectral range between 2000 and 1000 cm^{-1} . Ten variables providing the best discrimination of seeds based on population, identified as green points in Fig. 3B, were sequentially selected by step-wise algorithm.

A clear, although partial, separation among the populations based on step-wise LDA applied to the FT-IR spectra, confirmed with Wilk's lambda test ($P < 0.001$), was observed (Fig. 4), where the seeds are projected in the plane of the first two DFs. This suggests that the chemical compositions of seed from the four populations are significantly different. On the other hand, step-wise LDA carried out to investigate the difference in IR spectra in seeds of different colours permitted identification of five discriminant variables, reported in red colour in Fig. 3B, showing a slight but significant difference in chemical composition of light and dark seeds.

The correlation analysis among seed traits and climatic conditions showed a strong relationship of morphometric traits with temperature, and colorimetric traits with precipitation. In particular, seed height is negatively correlated with all the three bioclimatic variables of temperature as confirmed by a linear model ($p_{\text{bio01}} = 0.032$, $p_{\text{bio10}} = 0.029$, $p_{\text{bio11}} = 0.035$); in contrast, width is positively correlated (lm P -value, $p_{\text{bio01}} = 0.002$, $p_{\text{bio10}} = 0.003$, $p_{\text{bio11}} = 0.003$; Fig. 5). Moreover, the analysis showed no significant correlation between morphometric traits

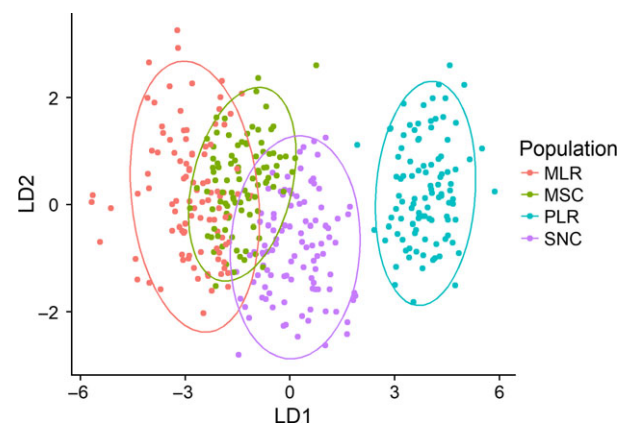


Fig. 2. Representation of discriminant analysis carried out on morpho-colorimetric traits for the four studied *A. aquilianus* populations: Montelucio di Roio (MLR), Monte Scuncole (MSC), Piano La Roma (PLR) and San Colombo (SNC). The ellipse represents 95% confidence level interval. The plot axes are the two main LDA discriminant functions (DFs).

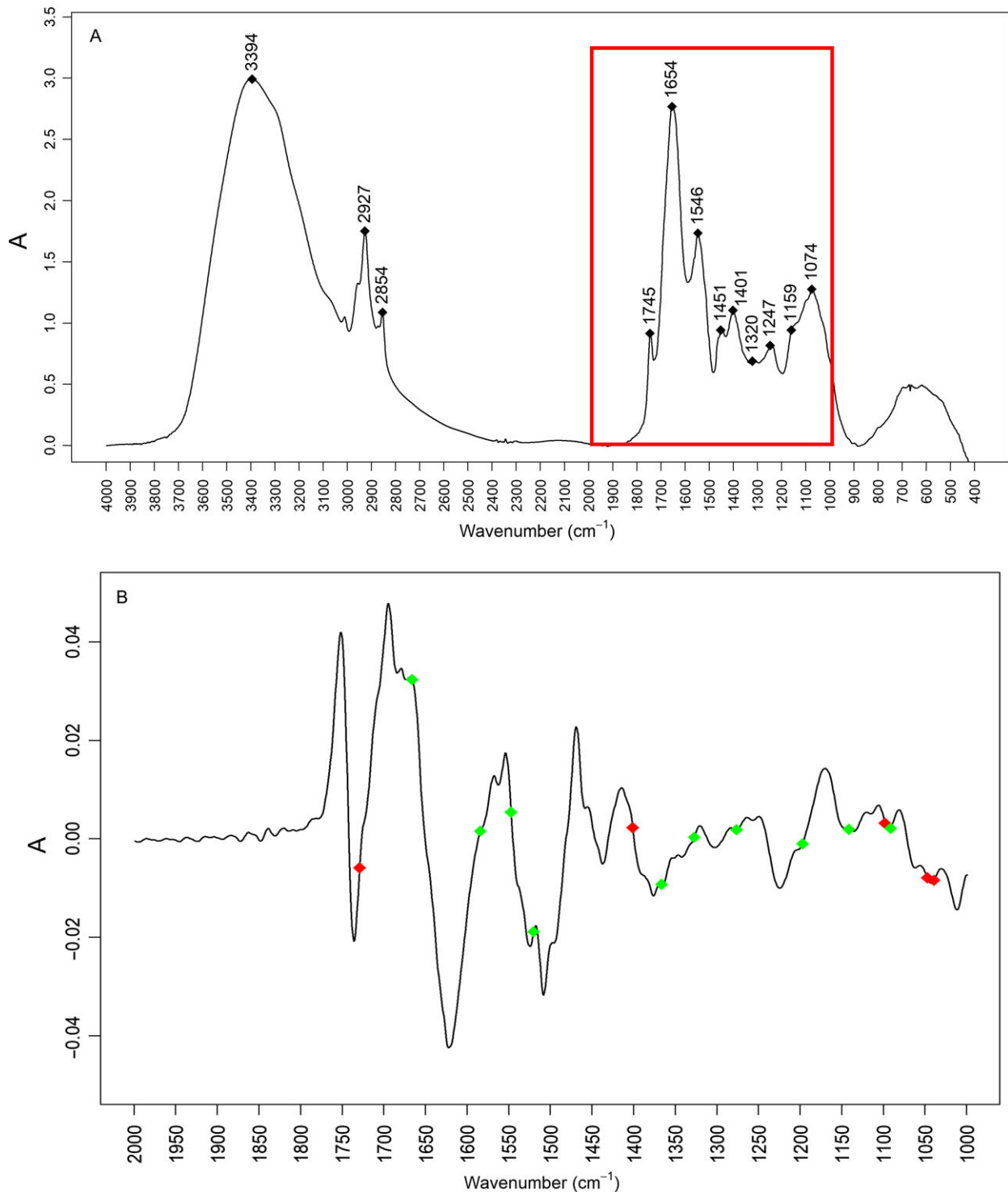


Fig. 3. A: Representative FT-IR spectrum of *A. aquilianus* seed, in red box is the spectral region considered in LDA; B: first-derivative FT-IR spectrum of spectral region considered in LDA with discriminant variables selected by swLDA, for populations (green points) or colour (red points).

and pluviometric variables. In contrast, both mean grey and max grey values show a positive interaction with pluviometry. In particular, both of these traits showed a strong relationship with precipitation of the wettest quarter: mean grey value (lm P -value, $p_{\text{bio12}} \ll 0.001$, $p_{\text{bio16}} \ll 0.001$, $p_{\text{bio17}} \ll 0.001$), max grey value (lm P -value, $p_{\text{bio12}} \ll 0.001$, $p_{\text{bio16}} \ll 0.001$, $p_{\text{bio17}} \ll 0.001$; Fig. 6). The linear regression of both mean and

max grey values against temperature data did not show any significant correlation.

DISCUSSION

The traits of seed shape are important for seed dispersal, probable loss and moisture imbibition (Balkaya & Odabas 2002;

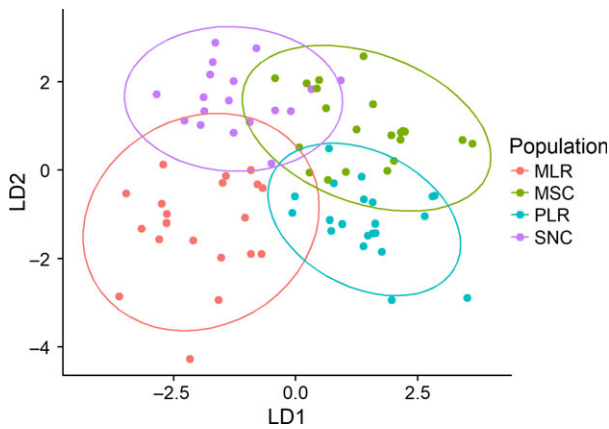


Fig. 4. Representation of discriminant analysis carried out on FT-IR spectra observed for the four studied *A. aquilianus* populations: Monteluco di Roio (MLR), Monte Scuncole (MSC), Piano La Roma (PLR) and San Colombo (SNC). The ellipse represents 95% confidence level interval. The plot axes are the two main LDA discriminant functions (DFs).

Cerdà & Garcia-Fayos 2002; Frattaroli *et al.* 2013) and also useful to study inter- and intraspecific diversity (Lo Bianco *et al.* 2017a,b).

The image analysis carried out on *A. aquilianus* seed showed large intraspecific plasticity in type of seed produced. Indeed,

clear separation of the four populations was observed and PLR, in particular, was completely isolated from the other three populations. This is due to the presence of ecological barriers, which are essentially the large urbanised inner valleys. The larger differentiation of the PLR population is due to the greater distance from the other populations (37 km from the nearest) and to the different altitude and stationary features.

Differences in seed shape determine variations in the surface area in contact with the external environment (Grundy *et al.* 2003) and influence the response to burial depth in different ways. There is a significant correlation between optimum emergence depth and seed shape (Thompson *et al.* 1993), where small and round seeds tend to persist in soil, while large and elongated or flattened seeds are transient in the soil (Thompson *et al.* 1994; Bekker *et al.* 1998). All the populations of *A. aquilianus* have elongated, small (mean seed area < 4 mm²) and compact seeds with a smooth surface; suggesting it could be considered a persistent species in the soil seed bank (Thompson & Grime 1979; Cerabolini *et al.* 2003). This agrees with the theory of Thompson *et al.* (2003) that shows a higher persistence in soil of seeds with physical dormancy (PY) as compared to species without PY. Moreover, Thompson *et al.* (1993) suggest that ease of burial and rates of predation could be the mechanism underlying the relationship between seed size and shape and seed persistence in the soil.

The interpopulation variability identified through morpho-colorimetric analysis was also observed with IR spectroscopy,

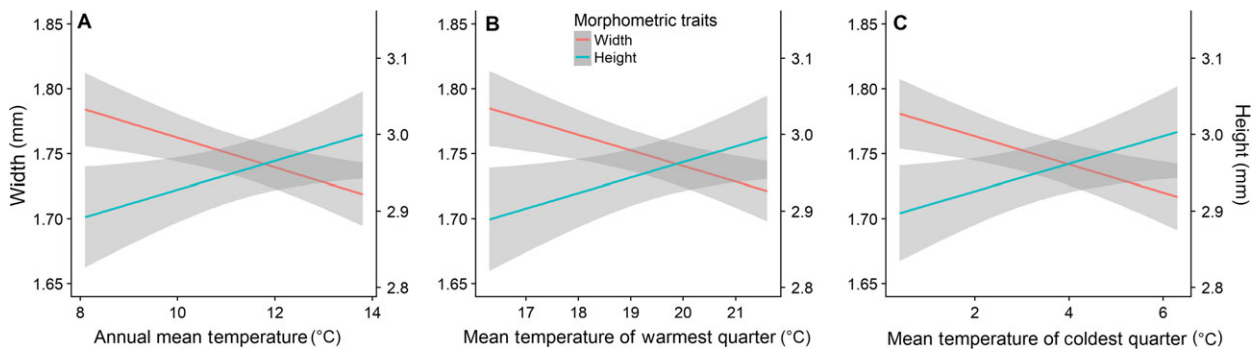


Fig. 5. Linear correlation models of seed morphometric traits, height (in red) and width (in teal), and bioclimatic variables. In each plot, y-axis on the left is width in mm and y-axis on the right is height in mm. The grey zone represents 95% confidence level interval for prediction.

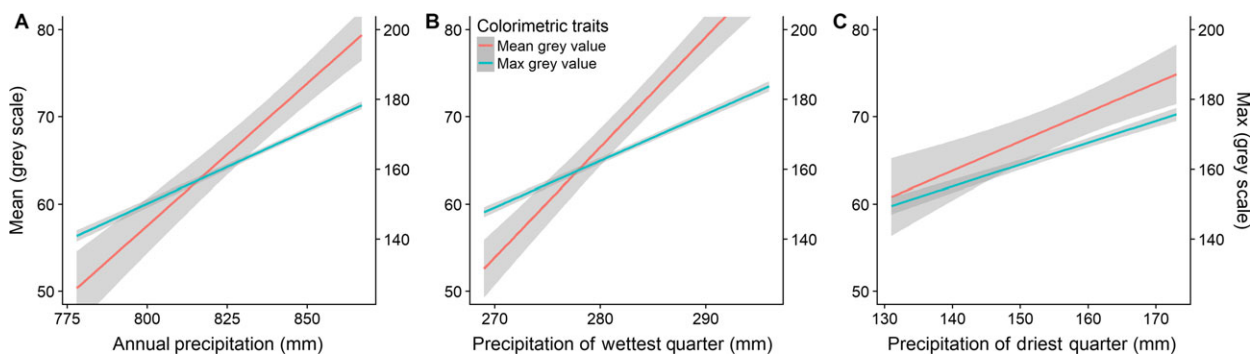


Fig. 6. Linear correlation models of seed colorimetric traits, mean grey value (in red) and max grey value (in teal) and bioclimatic variables. In each plot, y-axis on the left represents mean grey value and y-axis on the right represents max grey value. High values of mean grey value correspond to light colours (255 is white) and *vice versa*. The grey zone represents 95% confidence level interval for prediction.

and LDA data treatment confirms this differentiation. The variations in IR spectra responsible for population and colour differentiation are concentrated within the 1700–1500, 1400–1270 and 1140–1040 cm^{-1} spectral regions (Fig. 3B). This suggests that the differences in chemical composition of seeds of different colour or populations primarily involve amide-based compounds (proteins) responsible for the bands falling in the 1700–1500 cm^{-1} range, but variations in the relative content of other constituents (polysaccharides and lipids) cannot be excluded. It must be noted that one of the selected variables in LDA, colour differentiation (1729 cm^{-1}), is close to adsorption of the carbonyl group of free fatty acids (Schulz & Baranska 2007). However, except for two single values (1401 and 1547 cm^{-1}), the wave number values useful for population or colour discrimination do not correspond to absorption peak maxima of the FT-IR spectra, which are typical of specific chemical classes. Therefore, seeds of different colour or population do not exhibit distinct differences in terms of the global content of the main constituents (proteins, polysaccharides and lipids), but the observed variations seem due to slight differences in the relative concentrations of each functional group.

Differentiation in seed characters at intraspecific level is likely driven by geographic isolation (Grillo *et al.* 2013). According to Bacchetta *et al.* (2011), the observed evidence reveals that parameters related to seed colour are more discriminant than the size and shape descriptors. Accordingly, for the genus *Astragalus* chromatic features are among the best key parameters able to discriminate at specific or intraspecific level. The clear differentiation is due to grey values and seeds height and width, as confirmed by Random Forest algorithm. The change in grey value is due to an increase in the percentage of dark seeds; this means that seed colour is strongly affected by plasticity in seed production. This hypothesis was confirmed by a strong correlation between seed colour and rainfall variables. This suggests that a decrease in light seed quantity is due to an increase in precipitation. In particular, the max grey value is strictly correlated with precipitation in the wettest period, a key period for plant development and seed production (Moles & Westoby 2006; Baskin & Baskin 2014). However, seed colour does not show any significant relationship with temperature, underlining precipitation as the main driver of colour plasticity.

On the other hand, the most important change in seed morphology is due to height and width fluctuations. Because of these variations, there was a slight change in circularity of seeds. In this regard, height was negatively correlated with temperature, while there was a positive correlation between width

and temperature. Several studies have demonstrated that resource availability in the maternal environment is directly correlated with the next generation through seed size. In particular, the variation in seed characteristics is due to contact of the mother plant tissue with the embryo, which is responsible for seed nutrition (Fenner 1992). According to Luzuriaga *et al.* (2006), the observed correlation between the bioclimatic variables and the morphometric characteristics is due to a maternal effect (Stanton 1984; Crawley & Nachapong 1985; El-Keblawy & Lovett-Doust 1998).

Characterisation of *A. aquilianus* seeds with IR spectroscopy confirms the results of morphological analyses, revealing a clear difference among the investigated populations and highlighting the fact that interpopulation phenotypic plasticity also generates noteworthy changes in chemical composition.

CONCLUSION

The characterisation of *A. aquilianus* seeds showed large intraspecific plasticity, both in morpho-colorimetric and chemical composition. Moreover, a strong relationship between morphometric traits and climatic variables was found. In particular, a decrease in light seed quantity correlated with an increase in rainfall precipitation, while seed shape (height decrease and width increase) was positively correlated with temperature. These results confirm the strong mechanistic relationship between climate variables and characteristics of seeds (Harper 1977; Fenner & Thompson 2005). In this context, it is expected that climate changes will affect seed morphology and ecology and, indirectly, seed soil persistence, especially in species with PY (Ooi 2012). Hence, investigation of the relationship between seeds traits and environment is crucial to predict the possible negative effects of the different scenarios of climate change on species distribution, especially for rare and threatened species. This work highlights the importance of endemic populations of species and, at the same time, underlines the key role of all types of conservation and restocking strategies for protecting species that might be threatened by future changes in climate.

ACKNOWLEDGEMENTS

We thank everyone that contributed to this work, from seed collection to image and statistical analysis; special thanks to Antonella Grossi for help on IR spectrum analysis. This work was supported by Floranet LIFE project (LIFE15 NAT/IT/000946).

REFERENCES

- Angosto T., Matilla A. (1993) Germination, seed-coat structure and protein patterns of seeds from *Adenocarpus decoticans* and *Astragalus granatensis* growing at different altitudes. *Seed Science and Technology*, **21**, 317–326.
- Anzalone B. (1970) Su un nuovo «*Astragalus*» scoperto in Abruzzo: e osservazioni su «*Astragalus vesicarius*» L. (sensu latu). *Webbia*, **24**, 723–734.
- Bacchetta G., Grillo O., Mattana E., Venora G. (2008) Morpho-colorimetric characterization by image analysis to identify diaspores of wild plant species. *Flora*, **203**, 669–682.
- Bacchetta G., Fenu G., Grillo O., Mattana E., Venora G. (2011) Identification of Sardinian species of *Astragalus* section *Melanocercis* (Fabaceae) by seed image analysis. *Annales Botanici Fennici*, **48**, 449–454.
- Balkaya A., Odabas M.S. (2002) Determination of the seed characteristics in some significant snap bean varieties grown in Samsun, Turkey. *Pakistan Journal of Biological Science*, **5**, 382–387.
- Baskin C.C., Baskin J.M. (2014) *Seeds. Ecology, Biogeography, and Evolution of Dormancy and Germination*. Academic Press, New York, USA.
- Bekker R., Bakker J., Grandin U., Kalamees R., Milberg P., Poschold P., Thompson K., Willems J. (1998) Seed size, shape and vertical distribution in the soil: indicators of seed longevity. *Functional Ecology*, **12**, 834–842.
- Bernardo L. (1997) *Segnalazioni Floristiche Italiane. Informatore Botanico Italiano*, **28**, 837–842.
- Brandt L.A., Benschoter A.M., Harvey R., Speroterra C., Bucklin D., Románach S.S., Watling J.I., Mazzotti F.J. (2017) Comparison of climate envelope models developed using expert-selected variables versus statistical selection. *Ecological Modelling*, **345**, 10–20.
- Carbonaro M., Maselli P., Dore P., Nucara A. (2008) Application of Fourier transform infrared spectroscopy to legume seed flour analysis. *Food Chemistry*, **108**, 361–368.

- Cerabolini B., Ceriani R.M., Caccianiga M., De Andreis R., Raimondi B. (2003) Seed size, shape and persistence in soil: a test on Italian flora from Alps to Mediterranean coasts. *Seed Science Research*, **13**, 75–85.
- Cerdà A., Garcia-Fayos P. (2002) The influence of seed size and shape on their removal by water erosion. *Catena*, **48**, 293–301.
- Chtioui Y., Bertrand D., Dattée Y., Devaux M.F. (1996) Identification of seeds by colour imaging: comparison of discriminant analysis and artificial neural network. *Journal of the Science of Food and Agriculture*, **71**, 433–441.
- Conti F., Bartolucci F. (2015) *The vascular flora of the national park of Abruzzo, Lazio and Molise (Central Italy): an annotated checklist*. Springer International, Berlin, Germany.
- Conti F., Bartolucci F. (2016) The vascular flora of Gran Sasso and Monti della Laga National Park (Central Italy). *Phytotaxa*, **256**, 1–119.
- Conti F., Manzi A., Tinti D. (2002) Aggiunte alla Flora d'Abruzzo. *Informatore Botanico Italiano*, **34**, 55–61.
- Crawley M., Nachapong M. (1985) The establishment of seedlings from primary and regrowth seeds of ragwort (*Senecio jacobaea*). *Journal of Ecology*, **73**, 255–261.
- Di Cecco V., Paura B., Bufano A., Di Santo P., Di Martino L., Frattaroli A. (2017) Analysis of diaspore morphology and seed germination in *Bubon macedonicum* L., a rare species in Italy. *Plant Biosystems*, **152**, 738–748.
- Di Martino L., Di Cecco V., Di Santo M., Di Cecco M., Ciaschetti G., Marcantonio G., Frattaroli A.R. (2015) The Majella Seed Bank for the conservation of the endemic, rare or endangered species in Abruzzo: a tangible example of interaction between *ex situ* and *in situ* conservation. *RIBES SERIES*, **1**, 49–52.
- El-Keblawy A., Lovett-Doust J. (1998) Persistent, non-seed-size maternal effects on life-history traits in the progeny generation in squash, *Cucurbita pepo*. *New Phytologist*, **140**, 655–665.
- Ercole S., Giacanelli V., Bacchetta G., Fenu G., Genovesi P. (2016) Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie vegetali.
- Fenner M. (1992) Environmental influences on seed size and composition. *Horticultural Reviews*, **13**, 183–213.
- Fenner M., Thompson K. (2005) *The ecology of seeds*. Cambridge University Press, Cambridge, UK.
- Fick S.E., Hijmans R.J. (2017) WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology*, **37**, 4302–4315.
- Fisher R.A. (1936) The use of multiple measurements in taxonomic problems. *Annals of Human Genetics*, **7**, 179–188.
- Frattaroli A.R., Di Martino L., Di Cecco V., Catoni R., Varone L., Di Santo M., Gratani L. (2013) Seed germination capability of four endemic species in the Central Apennines in Italy: relationship between seed size and germination capability. *Lazaroo*, **34**, 43–53.
- Gigante D., Attorre F., Venanzoni R., Acosta A., Agrillo E., Aleffi M., ... Assini S. (2016) A methodological protocol for Annex I Habitats monitoring: the contribution of Vegetation science. *Plant Sociology*, **53**, 77–87.
- Grillo O., Miceli C., Venora G. (2011) Image analysis tool for vetch varieties identification by means of seeds inspection. *Seed Science and Technology*, **39**, 490–500.
- Grillo O., Mattana E., Fenu G., Venora G., Bacchetta G. (2013) Geographic isolation affects inter- and intra-specific seed variability in the *Astragalus tragacantha* complex, as assessed by morpho-colorimetric analysis. *Comptes rendus biologiques*, **336**, 102–108.
- Grundy A., Mead A., Burston S. (2003) Modelling the emergence response of weed seeds to burial depth: interactions with seed density, weight and shape. *Journal of Applied Ecology*, **40**, 757–770.
- Harper J.L. (1977) *Population biology of plants*. Academic press, London, UK.
- ISTA (2012) *International rules for seed testing*. The International Seed Testing Association (ISTA), Bassersdorf, Switzerland.
- Kuhn M., Johnson K. (Eds) (2013) Discriminant analysis and other linear classification models. *Applied predictive modeling*. Springer, New York, USA, pp 275–328.
- Lo Bianco M., Grillo O., Cañadas E., Venora G., Bacchetta G. (2017a) Inter- and intraspecific diversity in *Cistus* L. (Cistaceae) seeds, analysed with computer vision techniques. *Plant Biology*, **19**, 183–190.
- Lo Bianco M., Grillo O., Escobar Garcia P., Mascia F., Venora G., Bacchetta G. (2017b) Morpho-colorimetric characterisation of *Malva* alliance taxa by seed image analysis. *Plant Biology*, **19**, 90–98.
- Luzuriaga A., Escudero A., Pérez-García F. (2006) Environmental maternal effects on seed morphology and germination in *Sinapis arvensis* (Cruciferae). *Weed Research*, **46**, 163–174.
- Matilla A., Gallardo M., Puga-Hermida M.I. (2005) Structural, physiological and molecular aspects of heterogeneity in seeds: a review. *Seed Science Research*, **15**, 63–76.
- Moles A.T., Westoby M. (2006) Seed size and plant strategy across the whole life cycle. *Oikos*, **113**, 91–105.
- Ooi M.K. (2012) Seed bank persistence and climate change. *Seed Science Research*, **22**(S1), S53–S60.
- Peruzzi L. (2011) *Astragalus aquilanus*. The IUCN Red List of Threatened Species 2011: e.T161900A5511151.
- Pesaresi S., Galdenzi D., Biondi E., Casavecchia S. (2014) Bioclimate of Italy: application of the worldwide bioclimatic classification system. *Journal of Maps*, **10**, 538–553.
- Pignatti S. (1982) *Flora d'Italia*. Edagricole, Bologna, Italy.
- Pignatti S., Menegoni P., Giacanelli V. (2001) *Liste rosse e blu della flora italiana*. ANPA, Roma, Italy, 328 pp.
- R Development Core Team (2008) *A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Rossi G., Montagnani C., Gargano D., Peruzzi L., Abeli T., Ravera S., Cogoni A., Fenu G., Magrini S., Genai M., Foggi B., Wagensommer R.P., Venturella G., Blasi C., Raimondo F.M., Orsenigo S. (Eds), 2013. *Lista rossa della Flora italiana. 1. Policy Species e altre specie minacciate*. Comitato Italiano IUCN e Ministero dell' Ambiente e della Tutela del Territorio e del Mare Stamperia Romana, Rome, Italy.
- Santo A., Mattana E., Grillo O., Bacchetta G. (2015) Morpho-colorimetric analysis and seed germination of *Brassica insularis* Moris (Brassicaceae) populations. *Plant Biology*, **17**, 335–343.
- Schulz H., Baranska M. (2007) Identification and quantification of valuable plant substances by IR and Raman spectroscopy. *Vibrational Spectroscopy*, **43**, 13–25.
- Smykalova I., Grillo O., Bjelkova M., Hybl M., Venora G. (2011) Morpho-colorimetric traits of *Pisum* seeds measured by an image analysis system. *Seed Science and Technology*, **39**, 612–626.
- Stanton M.L. (1984) Seed variation in wild radish: effect of seed size on components of seedling and adult fitness. *Ecology*, **65**, 1105–1112.
- Thompson K., Grime J.P. (1979) Seasonal variation in the seed banks of herbaceous species in ten contrasting habitats. *Journal of Ecology*, **67**, 893–921.
- Thompson K., Band S., Hodgson J. (1993) Seed size and shape predict persistence in soil. *Functional Ecology*, **7**, 236–241.
- Thompson K., Green A., Jewels A. (1994) Seeds in soil and worm casts from a neutral grassland. *Functional Ecology*, **8**, 29–35.
- Thompson K., Ceriani R.M., Bakker J.P., Bekker R.M. (2003) Are seed dormancy and persistence in soil related? *Seed Science Research*, **13**, 97–100.
- Venora G., Grillo O., Ravalli C., Cremonini R. (2009) Identification of Italian landraces of bean (*Phaseolus vulgaris* L.) using an image analysis system. *Scientia horticultrae*, **121**, 410–418.