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Adaptation to habitat in *Aquilegia* species endemic to Sardinia (Italy): Seed dispersal, germination and persistence in the soil

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Abstract
The autecology of the Sardinian endemics *Aquilegia barbaricina* Arrigoni *et* Nardi and *A. nugorensis* Arrigoni *et* Nardi were investigated. Peaks of anthesis and seed dispersal were recorded for five populations occurring in two distinct habitats, one riparian and one rupicolous. Germination tests were carried out on seed lots belonging to each population by sowing seeds at 10, 15, 20, 25 and 25/15°C. In addition, seeds were incubated for 2 months at either 25°C (summer), 5°C (winter) or 25°C for 2 months plus 2 months at 5°C (summer followed by winter–SW), and then moved to the germination temperatures. Embryo measurements were taken during pre-treatments and germination. Experimental seed burials were carried out for two populations of each species. Both species dispersed in summer. The population of *A. nugorensis* occurring on rocky outcrops differed in phenology from both the other *A. nugorensis* population from riparian vegetation and from *A. barbaricina*. Both species showed morphophysiological seed dormancy, with $<50\%$ germination under laboratory conditions. All riparian populations germinated only after the SW pre-treatment, while the rupicolous population germinated at 25°C, without any pre-treatment. Low germination percentages were observed in the experimental seed burials, suggesting the ability for both species to form a persistent soil seed bank.

Keywords: *Mediterranean region, morphophysiological dormancy, narrow endemics, persistent soil seed bank, seed dispersal*

Introduction

In the western Mediterranean region, narrow endemic species often have particular habitat requirements in local refuges that constitute ''ecological islands'' in a landscape dominated by deciduous or evergreen forest and shrubland (Lavergne et al. 2004). Medrano et al. (2006) found that local populations of two European *Aquilegia* species [*A. vulgaris* L. and *A. pyrenaica* DC. subsp. *cazorlensis* (Heywood) Galiano *et* Rivas-Martínez] are isolated by both the rugged topography of the population areas and ecological barriers, since their favourable, humid microhabitats occur as sparse, small islands amongst an extensive dry Mediterranean matrix. This distribution is similar to that reported by different authors for Sardinian *Aquilegia* species that are isolated both from each other (by the intervening matrix of ''typical'' Mediterranean vegetation) and from populations of their ancestors that are not present on the Island (Pignatti 1982; Arrigoni 2006).

Three species of *Aquilegia* are reported in Sardinia: *A. barbaricina* Arrigoni *et* Nardi, *A. nugorensis* Arrigoni *et* Nardi and *A. nuragica* Arrigoni *et* Nardi; all are restricted to this Italian island (Arrigoni & Nardi 1977, 1978; Pignatti 1982; Arrigoni 2006). Two of these species (*A. barbaricina* and *A. nuragica*) are also included in the International Union for Conservation of Nature (IUCN) Red Lists where they are classified as Critically Endangered (Camarda 2006a, b).

Rare species conservation is closely related to understanding the key factors determining their distribution and abundance. Knowledge of their life-cycle and reproductive traits (such as flowering phenology, seed dormancy and germination and seed persistence in the soil) is essential for identifying limits to population growth and persistence (Bevill &

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Louda 1999). Information on survival strategies of threatened species is critical for optimising and determining the success of *in situ* and *ex situ* conservation efforts. Knowledge of their germination behaviour, in particular, is vital in promoting *ex situ* conservation measures (Perez-Garcia et al. 1995; Cerabolini et al. 2004; Flores et al. 2008; Kadis & Georghiou 2010).

Under a Mediterranean climate, characterised by a highly seasonal alternation of favourable and unfavourable conditions, plant growth and reproduction must occur in a window of favourable conditions that may vary in length and in which environmental cues and constraints play a central role (Debussche et al. 2004). For example, both flowering (Rathke & Lacey 1985) and seed germination are highly influenced by environmental conditions, such as photoperiod, moisture and temperature.

Aquilegia seeds have rudimentary embryos (Martin 1946), and the recommended pre-treatment for germination of seeds of *Aquilegia* spp. is stratification at 3–5°C for 2–4 weeks before sowing (Ellis et al. 1985). However, Grime et al. (1981) reported 4% germination for freshly collected *A. vulgaris* seeds, 53% germination after 12 months of dry storage at 5°C and 38% germination after 6 months of moist stratification at 5°C. Furthermore, pre-treatments, such as pre-chilling, scarification and with gibberellic acid (GA_3), can lead to high germination percentages (78–100%) in some seed lots of *A. vulgaris* (Liu et al. 2008). However, little is known about germination patterns in endemic *Aquilegia* species.

The Ranunculaceae is reported to contain species exhibiting both morphological dormancy (MD) and morphophysiological dormancy (MPD) (Baskin & Baskin 1994, 1998; Walck et al. 1999). The ''move-along experiment'' (or double germination phenology technique) is a method designed to determine the temperature, or temperature sequence, for seed dormancy break. This kind of ecophysiological approach is likely to result in successful germination based on mimicking the environment into which dormant seeds are dispersed and subsequently exposed and is applicable to both temperate and tropical environments (Baskin & Baskin 2003; Hoyle et al. 2008).

Germination patterns can differ between closely related species due to habitat factors (Daws et al. 2002; Karlsson & Milberg 2008; Karlsson et al. 2008; Vandelook et al. 2008). Even within the same species, several cues and responses may operate with differing importance in distinct populations or ecotypes (Milberg & Andersson 1998; Giménez-Benavides et al. 2005; Mondoni et al. 2008).

The ability to form a persistent soil seed bank (PSB) is crucial to the survival of many rare or declining species (Keddy & Reznicek 1982; Quilichini & Debussche 2000; Eckstein et al. 2006). In a study on persistence of 259 species of the Italian flora, Cerabolini et al. (2003) found that seed persistence in the soil for the Ranunculaceae was generally associated with small seed size and a low variance in seed dimensions. However, they were unable to classify the two investigated *Aquilegia* species (*A. atrata* W.D.J. Koch and *A. einseleana* F.W. Schultz) in their proposed scheme owing to insufficient or contradictory data.

In this study, the autecology of two of the three *Aquilegia* species of Sardinia (*A. barbaricina* and *A. nugorensis*) were investigated (*A. nuragica* was not included in this study to avoid damaging the only known population) by phenological observations, germination tests and experimental seed burials. These data may help to explain the narrow and scattered distribution of the Sardinian *Aquilegia* species and to implement conservation measures for them.

Materials and methods

Study species and seed lot details

A. barbaricina is exclusive to the Gennargentu massif and Supramonte region (CE-Sardinia), growing from 1100 to 1400 m a.s.l. under riparian woods (Arrigoni & Nardi 1977; Arrigoni 2006; Camarda 2006a). *A. nugorensis* is considered the most common columbine of the Island; it is reported for the Tacchi region and Mountains of Oliena (CE-Sardinia), growing from 1000 to 1400 m a.s.l. under riparian woods and on rocky outcrops (Arrigoni & Nardi 1978; Bacchetta et al. 2004; Arrigoni 2006). *A. barbaricina* is reported to be a silicicolous species, while *A. nugorensis* to be indifferent to substrate (Arrigoni & Nardi 1978). Phenological data indicate a similar behaviour for the two species, with a flowering period from May to June and a fruiting period from June to July (Arrigoni & Nardi 1977, 1978; Arrigoni 2006).

Seeds were collected in the summers of 2006 and 2007 from three populations of *A. barbaricina* and two of *A. nugorensis* (Table I), and either buried directly in the soil for experimental seed burials or stored at 15% RH and 15°C before use in laboratory germination tests (commenced November 2007). Seed mass was determined for each seed lot by weighing three replicates of 50 seeds each. Details on each seed lot and the average seed mass are reported in Table II.

Phenological observations

In each of the five populations (Table I), 15 mature individuals were monitored, following the

Table I. Geographical location and habitat characteristics of the studied populations of *Aquilegia*.

Taxon	Population	Code	Altitude (m a.s.l.)	Slope (°)	Aspect (°)	Substrate	Habitat (plant community)
A. barbaricina	Monte Spada, Fonni (NU)	ABA1	1340–1345	0–20	NW 340	Metamorphites	Riparian woods with *Alnus glutinosa* (L.) Gaertn. and *Ilex aquifolium* L. (*Salici purpureae-Populetea nigrae* Rivas-Martínez *et* Cantó *ex* Rivas-Martínez, Báscones, T.E. Díaz, Fernández-González *et* Loidi 2001)
A. barbaricina	Rio Olai, Orgosolo (NU)	ABA2	849–961	0–10	NE 65	Metamorphites	Riparian woods with *Alnus glutinosa* (L.) Gaertn. and *Rhamnus persicifolia* Moris (*Salici purpureae-Populetea nigrae* Rivas-Martínez *et* Cantó *ex* Rivas-Martínez, Báscones, T.E. Díaz, Fernández-González *et* Loidi 2001)
A. barbaricina	Pischina Urtaddala, Urzulei (OG)	ABA3	700	0	–	Limestones	Riparian vegetation (*Phragmito-Magnocaricetea* Klika *in* Klika *et* Novák 1941)
A. nugorensis	Monte Corrasi, Oliena (NU)	ANS1/2	1331	5	E 80	Limestones	Rupicolous vegetation [*Asplenietea trichomanis* (Br.-Bl. *in* Meier *et* Br.-Bl. 1934) Oberd. 1977]
A. nugorensis	Funtana Sa Cerasia, Seui (OG)	ANS3	905–1058	20–50	N 5	Travertines	Riparian mesophilous woods with *Ostrya carpinifolia* Scop. (*Querco roboris - Fagetea sylvaticae* Br.-Bl. *et* Vlieger *in* Vlieger 1937)

Table II. Seed lot details and experimental trials carried out for each seed lot.

Population	Code	Collection date	Seed mass (mg)	Viability (%)	Initial E:S ratio	Trials
Monte Spada, Fonni (NU)	ABA1	18/07/07	1.43 ± 0.05	99.5 ± 1.6	0.10 ± 0.02	GT/SB
Rio Olai, Orgosolo (NU)	ABA2	01/07/07	1.36 ± 0.06	97.8 ± 6.2	0.12 ± 0.06	GT/SB
Pischina Urtaddala, Urzulei (OG)	ABA3	23/07/07	1.15 ± 0.06	89.5 ± 6.7	0.10 ± 0.01	GT
Monte Corrasi, Oliena (NU)	ANS1	29/07/06	1.37 ± 0.09	96.2 ± 4.7	0.10 ± 0.02	GT
Monte Corrasi, Oliena (NU)	ANS2	04/08/07	1.47 ± 0.09			SB
Funtana Sa Cerasia, Seui (OG)	ANS3	18/07/07	1.00 ± 0.04	94.5 ± 5.6	0.11 ± 0.03	GT/SB

Note: GT, germination test; SB, experimental seed burial.

semi-quantitative method of Montserrat-Martí and Pérez-Rontomé (2002), to describe their phenology of flowering and seed dispersal during the 2006–2009 period. According to Milla et al. (2010), a phenophase was present when >5% of the plants for each population showed the same pattern. We complemented the data with observations made on other randomly selected individuals to provide a more precise estimation of each phenophase. During phenological observations, we also noted altitudinal range, slope, aspect, substrate and habitat type for each population (Table I).

Germination tests

Three replicates of 18 seeds each per treatment, belonging to each investigated population (Table I), were sown in November 2007, in 60-mm plastic Petri dishes on the surface of 1% solid water-agar, and incubated in the light for 6 months at a range of germination temperatures (10, 15, 20, 25 and 25/15°C). The water-agar substrate was chosen in order to provide a solid, non-sterile medium for germination. The light period was 8 h of irradiance per day, with a ratio of red: far-red light of ca. 2.0, and a light quantity of ca. 7 μmol $m^{-2} \cdot s^{-1}$; in the alternating temperature regime, the 8-h light period coincided with the high temperature period. In addition, dishes were incubated for 2 months at either 25°C (S, to simulate summer), 5°C (W, to simulate winter) or 25°C, for 2 months, followed by an additional 2 months at 5°C (SW, to simulate summer followed by winter), and then moved to the germination temperatures listed above (see Table III

Table III. Experimental designs used in the germination phenology experiments.

	Pre-treatments	Germination temperatures (°C)	Embryo growth measurements
0	None	10, 15, 20, 25 or 25/15 for 6 months	2 measurements: before sowing and after two months
S	2 months at 25°C (warm stratification = summer)	10, 15, 20, 25 or 25/15 for 4 months	1 measurement after 2 weeks at the germination conditions
W	2 months at 5°C (cold stratification = winter)	10, 15, 20, 25 or 25/15 for 4 months	1 measurement after 2 weeks at the germination conditions
SW	2 months at 25°C and 2 months at 5°C (warm + cold stratification = summer and winter)	10, 15, 20, 25 or 25/15 for 2 months	1 measurement after 2 weeks at the germination conditions

for experimental design). The low number of seeds per replicate (18) was due to limited seed availability resulting from these species being rare with small populations. All pre-treatment/germination combinations ran for a total of 180 days, with germination defined as visible radicle emergence. During germination scoring, minimal microbial growth was observed. At the end of germination tests, the viability of any remaining seeds (Table II) was checked using a cut test (ISTA 2006), and the final germination percentage calculated as the mean of the three replicates (± 1 standard deviation) on the basis of the total number of filled seeds.

Embryo growth

To examine growth of the embryo during pre-treatments and during germination conditions, an additional five seeds per treatment were included for embryo measurements; timing of measurements is reported in Table III. Seeds were sectioned at -20°C using a cryotome (Leica CM3050S) to expose the embryo. Images were taken at 6.6 × magnification using a Stemi SV 11 Microscope (Carl Zeiss, Welwin Garden City, Herts, UK) equipped with a Carl Zeiss Axiocam Colour, and measurements were carried out using the image analysis software Axiovision 3.1.2.1 (Carl Zeiss Vision GmbH). The embryo/seed (E:S) ratio was calculated as embryo length/seed length. Total seed length was determined as mean length of 100 seeds for each seed lot. The E:S Ratio for seeds that had visibly germinated during the treatments was assumed to be 1.

Experimental seed burial

Experimental seed burials were carried out at the time of natural seed dispersal following a modification of the protocol described by Arroyo et al. (2004). Sets of five replicates containing 10 fresh seeds each were put into fine nylon mesh envelopes, which were then placed in plastic nets. The five envelopes were filled with sieved local soil and buried at the original population locations (Table II), so that they were at a 5-cm depth at the four vertices and at the centre of a square (1-m sides). Seeds were buried at this depth rather than closer to the surface since the purpose was to test whether or not they remained viable. After 1 year, the replicates were exhumed. All intact, non-germinated seeds were sown immediately in 60-mm plastic Petri dishes on the surface of 1% solid water-agar and incubated in the light at 25/15°C to check their viability and germination capacity.

Statistical analysis

Arcsine transformed germination percentages were analysed using analysis of variance (ANOVA) within and across treatments (one-way ANOVA), followed by Fisher's *post hoc* least significant difference test. The non-parametric Mann–Whitney *U* test was used to analyse E:S ratio values using Minitab© release 11.21 (Minitab Inc.). As a result of multiple comparisons, a Bonferroni correction was applied to the critical value of α for the Mann–Whitney *U* test (Sokal & Rohlf 1995). Sigmoidal regressions of the germination curves were fitted by using SigmaPlot© 2002 for Windows version 8.0 (SPSS Inc.).

Results

Phenological patterns

The two species showed a different behaviour in their flowering periods (Figure 1). For all three *A. barbaricina* populations, anthesis peaked in May, whereas in the two *A. nugorensis* populations it peaked 1 month later. No differences were detected among populations for this phenophase in either species. The peak of seed dispersal was in July in all *A. barbaricina* populations and in the ANS3 population (see Table I for population codes), whereas for plants of ANS1/2 it peaked in August (Figure 1). The time between the peak of flowering and seed dispersal (seed development period) was 3 months for all the *A. barbaricina*

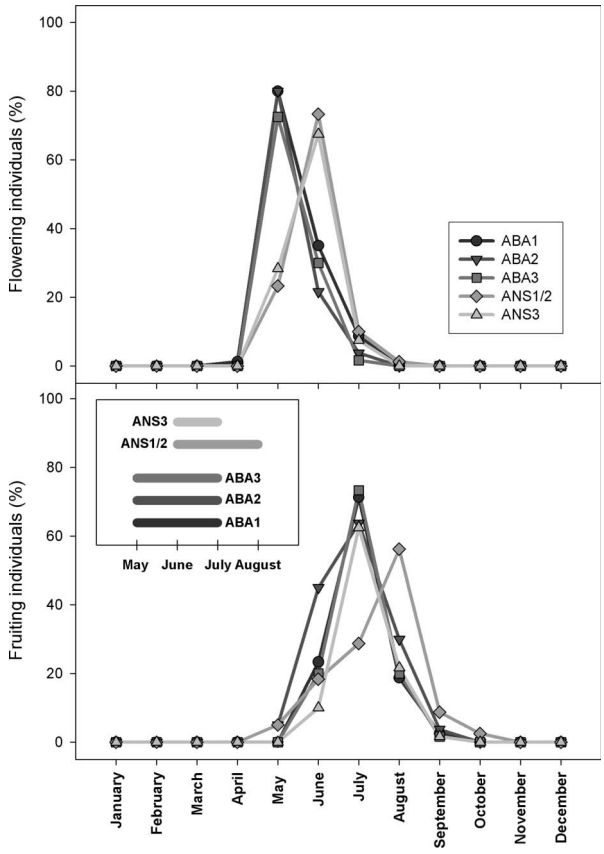

Figure 1. Flowering and seed dispersal periods for each of the five monitored populations in 2006–2009. Data are the mean of 4 years, calculated on the basis of 15 individuals. In the inset plot, time between the peaks of flowering and seed dispersal for each population is reported.

populations and ANS1/2 and 2 months for ANS3 (Figure 1).

Germination requirements

All populations germinated best after the SW pre-treatment ($p < 0.001$), while there was no statistical difference ($p > 0.05$) between control (0) and S or W pre-treatments (Figure 2). All three *A. barbaricina* seed lots showed similar germination patterns, even if they exhibited different final germination percentages. Whereas $< 10\%$ germination was recorded after the 0 (control), S and W pre-treatments, the three *A. barbaricina* populations germinated to higher percentages after SW (summer + winter), reaching about 40% germination and showing a preference ($p < 0.05$) for the lower temperatures (10 and 15°C). Alternating temperatures did not significantly improve the final germination percentages ($p > 0.05$) (Figure 2).

A seed lot of *A. nugorensis* (ANS3) showed a similar pattern of response to temperature as *A. barbaricina* populations, while ANS1 germinated at 25°C and 25/15°C without any pre-treatments ($38.9 \pm 5.6\%$ and $35.2 \pm 12.8\%$, respectively). After S and W, this seed lot germinated at all conditions with a preference ($p < 0.05$) for the warmer temperatures ($48.1 \pm 12.8\%$ after S and $50.0 \pm 5.56\%$ after W); after SW, it germinated quite well at all the conditions without significant differences ($p > 0.05$) between temperature. While *A. barbaricina* seed lots and ANS3 generally started to germinate during incubation at the germination temperature after the pre-treatments, ANS1 started to germinate during warm stratification at 25°C both during the S and SW pre-treatments (Figure 3).

Embryo development

Before starting germination tests, seeds had an initial E:S ratio of 0.10 ± 0.03 and 0.11 ± 0.02 for *A. barbaricina* and *A. nugorensis*, respectively (Figure 4). These values were not statistically different between species and seed lots ($p > 0.05$). Final E:S ratios increased significantly for all seed lots and treatments ($p < 0.0001$, with $\alpha = 0.0083$) achieving mean values ranging from 0.15 ± 0.02 and 0.16 ± 0.04 (control) to 0.19 ± 0.03 and 0.21 ± 0.02 (after SW pre-treatment) for *A. barbaricina* and *A. nugorensis,* respectively (Figure 4). Final E:S ratios were not statistically different ($p > 0.05$ and $p > 0.01$, respectively, with $\alpha = 0.0083$) for *A. nugorensis* after S (0.16 ± 0.04) and W (0.21 ± 0.20) to the control treatment, while they were significantly higher ($p < 0.01$ with $\alpha = 0.0083$) for *A. barbaricina*, with a mean value of 0.16 ± 0.03 and 0.16 ± 0.02 for S and W, respectively (Figure 4).

Seed persistence in the soil

After 1 year of burial, 76 to 94% of the seeds were retrieved intact for ABA1, ANS2 and ANS3, but only 32% for ABA2 (Figure 5). Between-population differences were detected in the laboratory germination percentages of intact seeds after incubation at 25/15°C (Figure 5). Germination percentages for ABA2 ($95.0 \pm 11.2\%$) and ANS2 ($87.3 \pm 12.9\%$) were high, with $< 10\%$ of the seeds failing to germinate at the end of the test. ABA1 and ANS3 achieved $23.3 \pm 22.4\%$ and $14.9 \pm 12.5\%$ germination, respectively, with ca. 70% of seeds not germinating but still appearing to be viable (assessed by a cut-test). There were no statistical differences ($p > 0.05$) in the final germination percentages at 25/15°C after SW pre-treatment and 1 year burial in the field in ABA1 and ANS3, whereas final germination differed significantly ($p < 0.001$) for ABA2 (Figure 6). No comparison was carried out for the Monte Corrasi population (ANS1/2), as two different seed lots were used for laboratory germination tests and experimental seed burial (Table II).

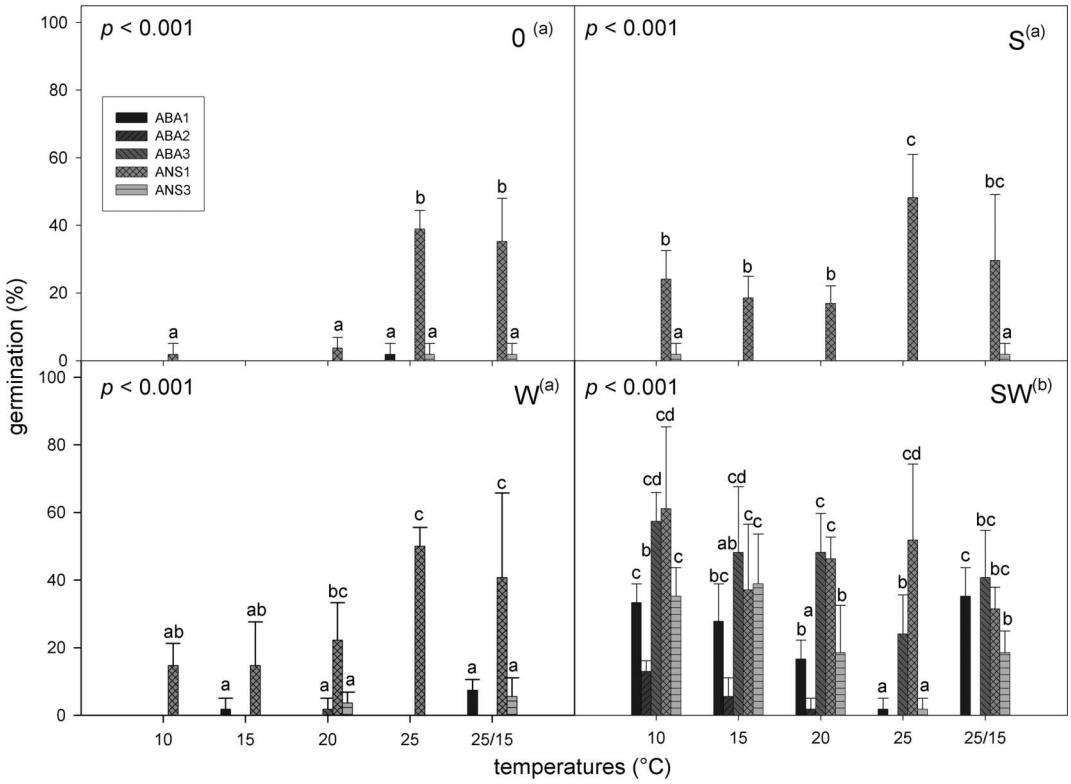

Figure 2. Final germination percentages after each pre-treatment (0, S, W and SW refer to the control, simulated summer, simulated winter and simulated winter after summer, respectively; see Table III) for the *Aquilegia* populations. ANOVA between pre-treatments: $p < 0.001$ by one-way ANOVA followed by Fisher's *post hoc* LSD test; graphs with the same letters in brackets are not significantly different at $p > 0.05$. Within each pre-treatment, bars with the same letters are not significantly different at $p > 0.05$ by one-way ANOVA followed by Fisher's *post hoc* LSD test. Data are the means of three replicates (± 1 standard deviation).

Discussion

Phenological trends

Flowering period may vary between years, and onset of flowering may be strongly affected by yearly climatic variability (Stenström et al. 1997; Price & Waser 1998; Makrodimos et al. 2008). Temperature may also change drastically along elevation gradients resulting in phenological shifts, such as a delay in the onset of flowering along elevation gradients (Reader 1984; Blionis et al. 2001). Nonetheless, the peak of anthesis was in May for all *A. barbaricina* populations and in June for all *A. nugorensis* populations (Figure 1), with no correlation with altitude (see Table I). Our observations confirm the data already reported by Arrigoni and Nardi (1977, 1978), highlighting a similar trend for all the populations. ABA3, a population which occurs below the altitudinal range previously reported for *A. barbaricina* (Arrigoni & Nardi 1977; Arrigoni 2006), confirmed this trend as well. The overlap period detected between the two flowering peaks (June to July, with more than 20% of individuals per population flowering) suggests the absence of phenological barriers between the two species.

While no differences were detected among dispersal periods for any of the *A. barbaricina* populations, the two *A. nugorensis* populations showed different trends for dispersal. In particular, while ANS3 dispersed in July, similar to *A. barbaricina*, ANS1/2 did so in August. Also in this case, no correlation was found with altitude and timing of dispersal. Instead, these trends may be explained by their different habitats. Kadis and Georghiou (2010) detected a delayed seed dispersal for three endemic plants of Cyprus. These authors found that although seeds matured by mid to late summer, dispersal was delayed until after the beginning of the rainy season in mid-autumn, when chances for seedling survival under Mediterranean climates are maximal. In contrast, all *Aquilegia* populations in our study dispersed in summer (July to August). In all *A. barbaricina* populations and for ANS3, plants occur in habitats characterised by riparian vegetation, with high water availability even during the harsh Mediterranean summer, while ANS1/2 occurs in limestone rocky slopes characterised by rupicolous vegetation.

Germination requirements

Seeds of these two species have underdeveloped embryos and, therefore, are morphologically

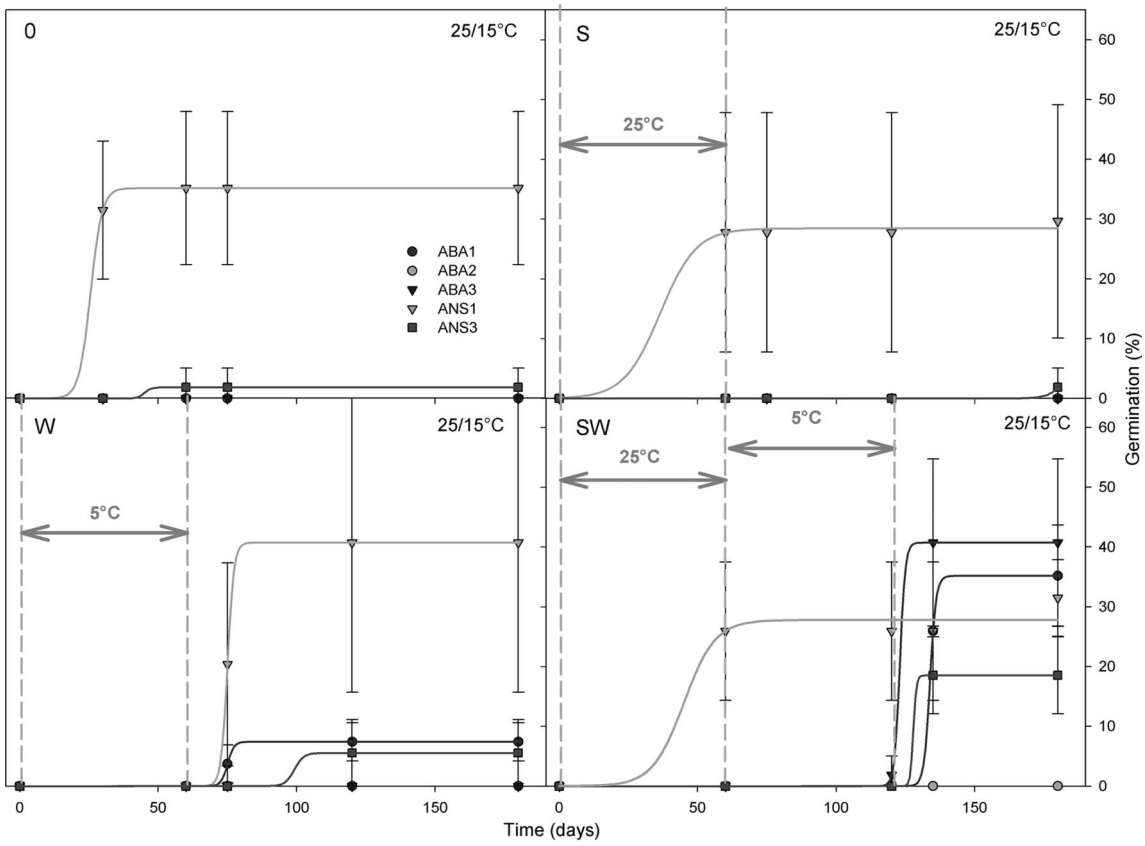

Figure 3. Germination trends at 25/15°C after each pre-treatment for the *Aquilegia* populations. Points correspond to the actual data, and solid lines indicate the fitted lines from the sigmoidal regressions. Data are the means of three replicates (±1 standard deviation).

dormant (MD) *sensu* Baskin and Baskin (2004). However, the positive effect on embryo growth and germination detected after pre-treatment with warm and cold stratification (SW) indicates a physiological component *sensu* Baskin and Baskin (2004); hence, these seeds have a MPD.

The three *A. barbaricina* seed lots and ANS3 showed an intermediate or deep (depending on the ability of GA_3 to overcome the physiological dormancy) simple MPD. The combination of both warm and cold pre-treatment (SW) was needed to break dormancy, and germination started after transfer of the seeds to the germination conditions. In addition, no germination was detected after the control, S or W pre-treatments, whereas after SW, the four seed lots germinated at all temperatures, with a preference for the lower temperatures (10–15°C). Seeds of these populations germinate in spring/early summer after seed dispersal in the previous summer (i.e. after dispersal, seeds experience warm stratification during the summer/autumn, cold stratification during the autumn/winter and then germinate during the early spring before tree canopy closure). From an ecological perspective, the low temperature requirement for dormancy loss and germination ensures that seedlings emerge only in late winter or early spring and grow during the summer under the closed canopy of *Alnus glutinosa* (L.) Gaertn. (*A. barbaricina*) and *Ostrya carpinifolia* Scop. (*A. nugorensis*) where, presumably, seedlings are buffered against the dry summer. Summer drought is a limiting factor in Mediterranean regions for plant growth and reproduction, and is more severe at lower altitudes, with higher temperatures, higher evapotranspiration and lower soil water content (Giménez-Benavides et al. 2007).

ANS1 showed different germination requirements. Seed germination largely occurred at warm temperatures, also without any pre-treatment; on account of the fact that embryos were underdeveloped before sowing, seeds of this population could be considered as MD. This response to high temperatures confirms the warm preference for germination detected for Mediterranean orophytic plants by Giménez-Benavides et al. (2005), as opposed to the cold adaptation of ''typical'' Mediterranean plants (Thanos et al. 1989, 1995). Germination of alpine species is restricted to short periods in early summer, when temperatures are high enough, and soil water from snowmelt is still available (Bliss 1971). However, in the very dry habitat of ANS1 (limestone rocky cliffs with *Asplenietea trichomanis* vegetation), there may be insufficient water availability during summer despite

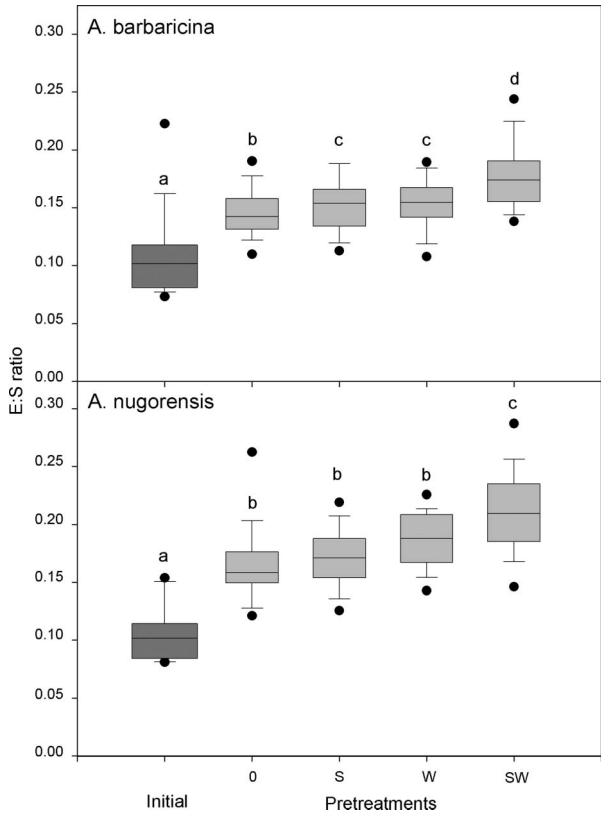

Figure 4. E:S ratio after each pre-treatment, 0: Control, S: 2 months at 25°C, W: 2 months at 5°C and SW: 2 months at 25°C then 2 months at 5°C (Table III). Data are the means of five seeds for initial values, and for 75 seeds after the pre-treatments; points represent outliers. Within each species, values after pre-treatments with the same letters are not significantly different at $p > \alpha = 0.0083$ (Bonferroni's correction critical value).

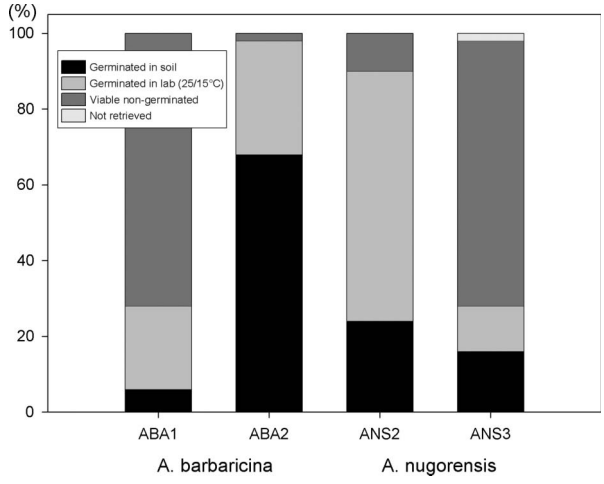

Figure 5. Cumulative seed germination and seed viability after 1 year burial and germination at 25/15°C in the light carried out in the laboratory on the retrieved seeds. Viability was assessed by a cut-test. Data are the means of five replicates.

suitable germination temperatures. Consequently, these seeds may not actually germinate until autumn or the following spring when, after experiencing

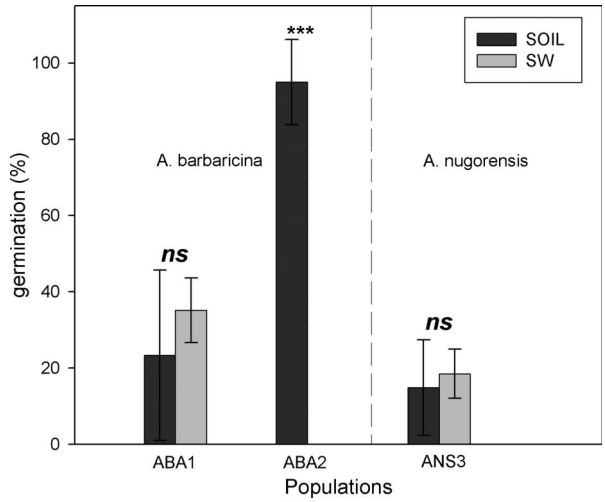

Figure 6. Germination tests at 25/15°C after the SW pre-treatment and after 1 year of burial in the soil (germinated in the lab). Data are the mean of 15 replicates (±1 standard deviation) for the germination test after SW and five replicates for the test after burial. (ns: $p > 0.05$ and ***: $p < 0.001$, by one-way ANOVA followed by Fisher's *post hoc* LSD test).

warm or cold stratification, respectively, they can germinate at lower temperatures.

Soil seed bank persistence

The experimental seed burials carried out on four populations were in accordance with the results achieved in the laboratory germination experiments. Seeds of both species had very low germination in the soil before being exhumed except for the ABA2 population. After 1 year of burial, seeds of ABA1 and ANS3, both from populations growing in deciduous woodland (*Salici purpureae-Populetea nigrae* and *Querco roboris-Fagetea sylvaticae* classes, respectively) reached similar germination percentages to those obtained in the laboratory under the same germination conditions, after SW pre-treatment. Non-germinated seeds were still viable, confirming the ability to form a PSB for more than 1 year. Seeds from ANS2 germinated quite well in the laboratory after 1 year of burial, confirming the ability to form a soil seed bank for at least 1 year. The lower germination percentage detected for ANS3 as compared with ANS2 may be explained by an adaptation to the Mediterranean climate, characterised by considerable unpredictability of temperature and, particularly, rainfall between consecutive years (Doussi & Thanos 2002).

Seed burial results for the ABA2 population could not be explained by the germination requirements of this species, suggesting that the high germination percentage in the soil before exhumation and then during the germination test may due to unknown *in situ* events occurring during burial (e.g. excavation by

animals, temporary floods, etc.). Consequently, further investigation of the regulation of seed germination in these species may be necessary.

Experimental seed burial results were also consistent with the seed mass of these species. Thompson et al. (1993) found that compact diaspores weighing less than 3 mg were all persistent in the soil, and fresh seed mass of the seed lots investigated in this study ranged from 1.00 to 1.47 mg.

Conclusions

Although further studies, particularly on fresh seeds (Baskin & Baskin 1998), are needed to characterise seed dormancy and germination in these two species of *Aquilegia*, the identified phenological patterns and germination requirements, which differ from those of ''typical'' Mediterranean plants, and the intraspecific variability detected among populations, suggest that they are strictly adapted to their microhabitat. Seed dispersal time and germination traits, particularly the germination season, could explain the narrow and scattered distributions of *A. barbaricina* and *A. nugorensis* with plants being restricted by the need for water availability during the warmer season, when seed development and seed germination/seedling establishment occur.

Furthermore, the adaptation of these two species to their microhabitats confirms the findings of Medrano et al. (2006) and Bastida et al. (2010) on the pivotal role of abiotic factors, as compared with pollination-related ones, in species differentiation of the *Aquilegia* genus in Europe, and in the Mediterranean region in particular, despite the overlap detected in the flowering periods of the two species.

The data reported in this study, particularly the seed dispersal period and germination requirements for different populations, may be useful in activating a long-term effective conservation programme for these threatened species by *ex situ* seed conservation and subsequent reinforcement measures.

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References

Arrigoni PV. 2006. Flora dell'Isola di Sardegna. Vol. 1. Sassari: Carlo Delfino Editore.

Arrigoni PV, Nardi E. 1977. Le piante endemiche della Sardegna: 1. Boll Soc Sarda Sci Nat 16: 265–268.

Arrigoni PV, Nardi E. 1978. Le piante endemiche della Sardegna: 19–20. Boll Soc Sarda Sci Nat 17: 215–225.

Arroyo MT, Cavieres LA, Humaña AM. 2004. Experimental evidence of potential for persistent seed bank formation at a subantartic alpine site in Tierra del Fuego, Chile. Ann Mo Bot Gard 91: 357–365.

Bacchetta G, Iiriti G, Mossa L, Pontecorvo C, Serra G. 2004. A phytosociological study of the *Ostrya carpinifolia* Scop. woods in Sardinia (Italy). Fitosociologia 41: 67–75.

Baskin CC, Baskin JM. 1994. Deep complex morphophysiological dormancy in seeds of the mesic woodland herb *Delphinium tricorne* (Ranunculaceae). Int J Plant Sci 155: 738–743.

Baskin CC, Baskin JM. 1998. Seeds – Ecology, biogeography and evolution of dormancy and germination. San Diego: Academic Press.

Baskin CC, Baskin JM. 2003. When breaking seed dormancy is a problem. Native Plants J 4: 17–21.

Baskin JM, Baskin CC. 2004. A classification system for seed dormancy. Seed Sci Res 14: 1–16.

Bastida JM, Alcántara JM, Rey P, Vargas P, Herrera CM. 2010. Extended phylogeny of *Aquilegia*: The biogeographical and ecological patterns of two simultaneous but contrasting radiations. Plant Syst Evol 284: 171–185.

Bevill RL, Louda SM. 1999. Comparisons of related and common species in the study of plant rarity. Conserv Biol 13: 493–498.

Blionis GJ, Halley JM, Vokou D. 2001. Flowering phenology of *Campanula* on Mt Olympos, Greece. Ecography 24: 696–706.

Bliss LC. 1971. Arctic and alpine plant life cycles. Ann Rev Ecol Syst 2: 405–438.

Camarda I. 2006a. *Aquilegia barbaricina*. In: IUCN. 2010. IUCN Red List of threatened species. Version 2010.1. Available: www.iucnredlist.org. Accessed May 2010 30.

Camarda I. 2006b. *Aquilegia nuragica*. In: IUCN. 2010. IUCN Red List of threatened species. Version 2010.1. Available: www.iucnredlist.org. Accessed May 2010 30.

Cerabolini B, Ceriani RM, 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 Sci Res 13: 75–85.

Cerabolini B, De Andreis R, Ceriani RM, Pierce S, Raimondi B. 2004. Seed germination and conservation of endangered species from the Italian Alps: *Physoplexis comosa* and *Primula glaucescens*. Biol Conserv 117: 351–356.

Daws MI, Burslem DF, Crabtree LM, Kirkman P, Mullins CE, Dalling JW. 2002. Differences in seed germination responses may promote coexistence of four sympatric *Piper* species. Funct Ecol 16: 258–267.

Debussche M, Garnier E, Thompson JD. 2004. Exploring the causes of variation in phenology and morphology in Mediterranean geophytes: A genus-wide study of *Cyclamen*. Bot J Linn Soc 145: 469–484.

Doussi MA, Thanos CA. 2002. Ecophysiology of seed germination in Mediterranean geophytes. 1. *Muscari* spp. Seed Sci Res 12: 193–201.

Eckstein RL, Hölzel N, Danihelka J. 2006. Biological flora of central Europe: *Viola elatior, V. pumila* and *V. stagnina*. Perspect Plant Ecol 8: 45–66.

Ellis RH, Hong TD, Roberts EH. 1985. Handbook of seed technology for genebanks n. 3. Vol. II. Compendium of specific germination information and test recommendations. Reading, UK: University of Reading.

Flores J, Jurado E, Himenez-Bremont JF. 2008. Breaking seed dormancy in specially protected *Turbinicarpus lophophoroides* and *Turbinicarpus pseudopectinatus* (Cactaceae). Plant Spec Biol 23: 43–46.

Giménez-Benavides L, Escudero A, Iriondo JM. 2007. Local adaptation enhances seedling recruitment along an altitudinal gradient in a high mountain Mediterranean plant. Ann Bot 99: 723–734.

Giménez-Benavides L, Escudero A, Pérez-García F. 2005. Seed germination of high mountain Mediterranean species: Altitudinal, interpopulational and interannual variability. Ecol Res 20: 433–444.

Grime JP, Mason G, Curtis AV, Rodman J, Band MA, Mowforth MA, et al. 1981. A comparative study of germination characteristics in a local flora. J Ecol 69: 1017–1059.

Hoyle GL, Daws MI, Steadman KJ, Adkins SW. 2008. Mimicking a semi-arid tropical environment achieves dormancy alleviation for seeds of Australian native Goodeniaceae and Asteraceae. Ann Bot 101: 701–708.

ISTA. 2006. International rules for seed testing. 2006 ed. Bassersdorf, CH-Switzerland: The International Seed Testing Association (ISTA).

Kadis C, Georghiou K. 2010. Seed dispersal and germination behaviour of three threatened endemic labiates of Cyprus. Plant Spec Biol 25: 77–84.

Karlsson LM, Milberg P. 2008. Variation within species and interspecies comparison of seed dormancy and germination of four annual *Lamium* species. Flora 203: 409–420.

Karlsson LM, Tamado T, Milberg P. 2008. Inter-species comparison of seed dormancy and germination of six annual Asteraceae weeds in an ecological context. Seed Sci Res 18: 35–45.

Keddy PA, Reznicek AA. 1982. The role of seed banks in the persistence of Ontario's coastal plain flora. Am J Bot 69: 13–22.

Lavergne S, Thompson JD, Garnier E, Debussche M. 2004. The biology and ecology of narrow endemic and widespread plants: A comparative study of trait variation in 20 congeneric pairs. Oikos 107: 505–518.

Liu K, Eastwood RJ, Flynn S, Turner RM, Stuppy WH. 2008. Seed information database (release 7.1, May 2008). Available: http://www.kew.org/data/sid. Accessed Nov 2008 20.

Makrodimos N, Blionis GJ, Krigas N, Vokou D. 2008. Flower morphology, phenology and visitor patterns in an alpine community on Mt Olympos, Greece. Flora 203: 449–468.

Martin AC. 1946. The comparative internal morphology of seeds. Am Midl Nat 36: 513–660.

Medrano M, Castellanos MC, Herrera CM. 2006. Comparative floral and vegetative differentiation between two European *Aquilegia* taxa along a narrow contact zone. Plant Syst Evol 262: 209–224.

Milberg P, Andersson L. 1998. Does cold stratification level out differences in seed germinability between populations? Plant Ecol 134: 225–234.

Milla R, Castro-Díez P, Montserrat-Martí G. 2010. Phenology of Mediterranean woody plants from NE Spain: Synchrony, seasonality, and relationships among phenophases. Flora 205: 190–199.

Mondoni A, Probert R, Rossi G, Hay F, Bonomi C. 2008. Habitat-correlated seed germination behaviour in populations of wood anemone (*Anemone nemorosa* L.) from northern Italy. Seed Sci Res 18: 213–222.

Montserrat-Martí G, Pérez-Rontomé C. 2002. Fruit growth dynamics and their effects on the phenological pattern of native *Pistacia* populations in NE Spain. Flora 197: 161–174.

Perez-Garcia F, Iriondo JM, Gonzalez-Benito ME, Carnes L, Tapia J, Prieto C, Plaza R, Perez C. 1995. Germination studies in endemic plant species of the Iberian Peninsula. Isr J Plant Sci 43: 239–247.

Pignatti S. 1982. Flora d'Italia, Vol. 1. Bologna: Edagricole.

Price MV, Waser NM. 1998. Effects of experimental warming on plant reproductive phenology in a subalpine meadow. Ecology 79: 1261–1271.

Quilichini A, Debussche M. 2000. Seed dispersal and germination patterns in a rare Mediterranean island endemic (*Anchusa crispa* Viv., Boraginaceae). Acta Oecol 21: 303–313.

Rathke B, Lacey EP. 1985. Phenological patterns of terrestrial plants. Annu Rev Ecol Evol Syst 16: 179–214.

Reader RJ. 1984. Comparison of the annual flowering schedules for Scottish heathland and mediterranean-type shrublands. Oikos 43: 1–8.

Sokal RR, Rohlf FJ. 1995. Biometry. 3rd ed. New York: WH Freeman and Company.

Stenström M, Gugerli F, Henry HB. 1997. Response of *Saxifraga oppositifolia* L. to simulated climate change at three contrasting latitudes. Glob Change Biol 3: 44–54.

Thanos CA, Georghiou K, Skarou F. 1989. *Glaucium flavum* seed germination: An ecophysiological approach. Ann Bot 63: 121–130.

Thanos CA, Kadis CC, Skarou F. 1995. Ecophysiology of germination in the aromatic plants thyme, savory and oregano. Seed Sci Res 5: 161–170.

Thompson K, Band SR, Hodgson JG. 1993. Seed size and shape predict persistence in soil. Funct Ecol 7: 236–241.

Vandelook F, Van de Moer D, Van Assche JA. 2008. Environmental signals for seed germination reflect habitat adaptations in four temperate Caryophyllaceae. Funct Ecol 22: 470–478.

Walck JL, Baskin CC, Baskin JM. 1999. Seeds of *Thalictrum mirabile* (Ranunculaceae) require cold stratification for loss of nondeep simple morphophysiological dormancy. Can J Bot 77: 1769–1776.