

The conservation perspectives and value of small and isolated plant populations: preliminary clues for *Gentianella crispata* (Gentianaceae) at the western boundary of its range

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Received 27 Nov. 2007, revised version received 22 Jan. 2008, accepted 23 Jan. 2008

Gargano, D., Bellusci, F., Pellegrino, G., Palermo, A. M., Bernardo, L. & Musacchio, A. 2009: The conservation perspectives and value of small and isolated plant populations: preliminary clues for *Gentianella crispata* (Gentianaceae) at the western boundary of its range. — *Ann. Bot. Fennici* 46: 115–124.

We give the first account concerning the ecology, population size, breeding system and genetic variability of the unique Italian population of *Gentianella crispata*, a rare orophilous species with a disjunct distribution including Balkan peninsula and southern Italy. The population is relatively small (ca. 4000 individuals) and has a fragmented structure within a wider area covered by the grass-dominated vegetation *Seslerio nitidae*–*Brometum erecti*. The evaluated morphological traits (i.e. plant size, flower number per plant and length of corolla tube) greatly varied among individuals. Examination of the floral structures revealed that the stigma becomes receptive before anther dehiscence. Observations on the topological relationships between stigma and anther suggested that in young flowers protogyny is paralleled by herkogamy (i.e. hyperstigmatic condition). However, the anther dehiscence appeared to be accompanied by a reduction of the spatial separation between female and male organs. This suggested the occurrence of a mixed breeding system, as also sustained by ISSR analysis. The ITS-based phylogeny showed an interesting relationship with *G. caucasea*, conferring a peculiar evolutionary interest to the species and its westernmost, severely isolated Italian population. According to the IUCN Red List criteria, *G. crispata* in Italy meets the requirements for critically endangered species.

Key words: genetic diversity, dichogamy, herkogamy, phylogenetic relationships, population size

Introduction

Natural populations of rare and endemic plant species deserve our attention especially when they are small and isolated and as such exposed to the environmental and to stochastic demographic and genetic events with detrimental evolutionary consequences (Frankel *et al.* 1995). Indeed, small populations are more likely for a loss of genetic diversity and population fitness due to genetic drift and biparental inbreeding (Keller & Waller 2002, Reed & Frankham 2003). Since studies have evaluated the size enabling a population to preserve most of its fitness and genetic diversity (Reed 2005 and references therein), conservation managements of endangered species aim to maintain or increase population sizes. Otherwise, low levels or absence of gene flow enhance the genetic differentiation among populations and the divergence of evolutionary lineages may be favoured by local adaptations (Ellstrand & Elam 1993, Young *et al.* 1996). For this reason, conservation biologists have used ecological and genetic data in order to identify populations that represent evolutionarily significant units and deserve special management and conservation priority (Moritz 1994, Crandall *et al.* 2000).

Another factor influencing the genetic diversity of plant species is their breeding system, which may vary from an obligate outcrossing to a selfing or clonal mating (Hamrick & Godt 1996, Leimu & Mutikainen 2005). However, many plant species possess a flexible, mixed mating system and random fluctuations of ecological factors may affect their reproductive strategies by disturbing biotic interaction (i.e. pollination) and altering the relative proportion of self vs outcross matings (Oostermeijer *et al.* 1992, Lennartsson 2002). Hence, the reproductive system at population level might be considered as a key factor towards optimal conservation strategies (Weller 1994).

Gentianella crispata is a rare orophilous Mediterranean species typical of southwestern Balkans, aside the single population growing on the southern Apennines (Calabria region) of Italy (Cavara & Grande 1913, Gavioli 1947), which is included in the Regional Red List of threatened plants (Conti *et al.* 1997). Little is known of the

population biology and genetics of the species. According to the above-mentioned considerations, the small area of occurrence and the geographical isolation make the Italian population of *G. crispata* likely for disturbance due to genetic and stochastic events. However, at present there is no information stating whether and in what measure such events have really occurred.

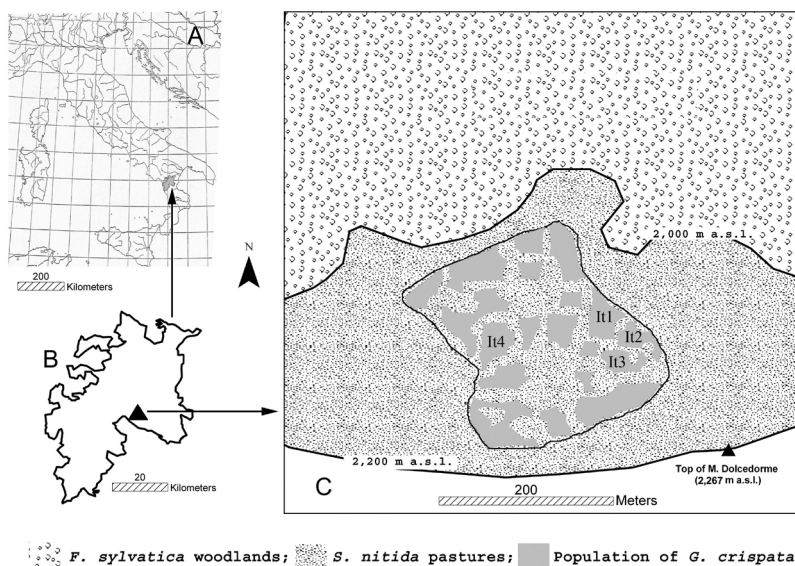
Based on field surveys and molecular analysis we give a first framework of the ecological, demographical and genetic aspects influencing the conservation of an isolated population of *G. crispata*. Hence, we provide preliminary clues for its conservation at the regional level. To this end, the Italian site of *G. crispata* was accurately checked, the population size was evaluated and the main morphological variations of plants and flowers were measured. To explore the mating system of *G. crispata*, we surveyed the floral topology for the occurrence of mechanism separating the female and male function either in time (dichogamy) or/and in space (herkogamy). Molecular analysis were carried out in order to assess the levels and distribution of genetic variability of the Italian population. The molecular tools were also used to check for the phylogenetic relationships of *G. crispata* within the section *Gentianella*, as a way to evaluate the “evolutionary value” of the species. Finally, based on the discussed data, we assessed the extinction risk for *G. crispata* at the national level according to the recent IUCN protocols (IUCN 2001, 2003).

Material and methods

The study species

Gentianella crispata belongs to the sect. *Gentianella* (= sect. *Amarella*) but is considered distinct from the three morphological groups in which the European taxa in the section (ca. 20) have been arranged, and from the two problematic species *G. columnae* and *G. caucasea* (Pritchard & Tutin 1972, Greimler *et al.* 2004). The disjunct distribution area of *G. crispata* includes mountains of the southwestern Balkan peninsula and a unique Italian stand (Pritchard & Tutin 1972), that has been reported from

Fig. 1. — **A:** Geographical position of the Pollino National Park in southern Italy. — **B:** Position of the studied stand within the boundaries of the Park. — **C:** Diagrammatic map representing the vegetation framework of the Italian stand of *G. crispata*. It₁, It₂, It₃ and It₄ are the positions of the subpopulations sampled for the molecular analysis.



Mt. Dolcedorme (southern Apennines, Calabria region) (Cavara & Grande 1913, Pignatti 1982). *Gentianella crispata* is a biennial herb of small size (2–20 cm), producing pentamerous flowers with a violet/whitish corolla, 12–20 mm long (Pritchard & Tutin 1972, Pignatti 1982). The fruits are capsules containing minute seeds. Recently, a karyological analysis has shown that the Italian plants of *G. crispata* are polyploid ($2n = 56$; Peruzzi & Cesca 2002).

The study site

The Italian site of *G. crispata* was mapped (scale 1:10 000) using GPS data and aerial photographs and its extension was measured using the ArcGis® 9.0 software. The Italian population of *G. crispata* occurs on a N-facing slope of the Mt. Dolcedorme and occupies an area of 0.03 km² (Fig. 1) extending from 2000 to 2200 m and is close to the upper boundary of *Fagus sylvatica* woods. According to Rivas-Martinez (1982, 1987, 1990) the climate of the site is lower alpine. The plant community is dominated by two grasses, *Sesleria nitida* and *Bromus erectus*, which characterize the *Seslerio nitidae*–*Brometum erecti* Bruno, 1968, a widespread phytocenosis in the high montane belts of southern Apennines (Biondi *et al.* 1995). The population

of *Gentianella crispata* is patchily distributed and is absent from the areas in which dense hummocks of *Sesleria nitida* occur (Fig. 1).

In the summer of 2004, the population size reached ca. 4000 flowering individuals; mainly, the individuals were concentrated in small areas; in three 2 × 2 m plots we counted up to 68 plants/m².

Plant and flower morphology

The plant size was measured and the number of flowers per plant was recorded for 130 individuals. Plant specimens were randomly selected from three plots with a similar plant density, and the measurements were made without detaching the plants from the soil, avoiding any damage to the population. For 35 randomly chosen plants the length of corolla tube was measured and the floral topology was examined for inferring the occurrence of dicogamy and/or herkogamy. The flowers were considered female when a papillose and glutinous stigma was observed; while the male phase was considered operating when at least one dehiscent anther was found; finally, the flowers in which the stigmas had closed and dry branches and the ovary appeared to be enlarged were classified as pollinated. To evaluate the occurrence of herkogamy, the heights of

the pistil and stamens and the distances between the anthers and stigmas were measured with a digital caliper (resolution 0.01 mm). According to the relative position of stigma and anthers, flowers were categorized as hypo-, iso- or hyper-stigmatic and tested for correlation with plant size and flowers number.

A Pearson (r) correlation with a two-tailed test of significance was always applied to statistically evaluate relationships among the measured characters.

Molecular analysis

In order to assess the levels of genetic diversity and to check the existence of a possible genetic structuring in the population of *G. crispata* we selected four distinct patches or sub-populations (Fig. 1). Three of them (It_1 , It_2 and It_3) were separated by short distances (from 10 to 20 m), while the fourth (It_4) was ca. 150 m apart. Small quantities of leaf tissue were collected from 20 individuals per subpopulation and stored in tubes with silica gel.

DNA extraction from 100 mg of leaves for each specimen was carried out using CTAB method (Doyle & Doyle 1990). Thirteen ISSR primers were screened and reactions, repeated at least three times, were performed in an automated cycler (PTC-100, MJ Research, Watertown, MA) programmed for 35 cycles (30 sec at 95 °C, 45 sec at 60 °C, 2 min at 72 °C). PCR products were electrophoresed in 1.8% agarose (SIGMA) gel, visualised under UV light, photographed using a Kodak EDAS-290 camera and qualitatively screened for their presence or absence in each sample.

The total number of fragments (N), the mean number of fragments per population (A), the percentage of polymorphic loci (%P), Nei's (1978) genetic diversity (h), and genetic differentiation between populations (G_{ST}) were calculated with POPGENE ver. 1.31 developed by Yeh *et al.* (1997). Moreover, ISSR data were analysed with GenAEx (Peakall & Smouse 2001), available at <http://www.anu.edu.au/BoZo/GenAEx/>, which performs a Molecular Variance Analysis (AMOVA) to calculate hierarchical distribution of variance components, the coefficient of

genetic differentiation between pairs of populations (Φ_{st}) and their significance levels.

To investigate the relationships among the European taxa of *Gentianella* section *Gentianella*, we amplified the internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA by means of a specific pair of primers (White *et al.* 1990) using a thermal cycler (Perkin Elmer 2600). PCR fragments were then double-strand sequenced in both directions loading into a 373A Applied Biosystems Automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.). The programme Sequence Navigator (Perkin Elmer, Applied Biosystems) was used to edit and to assemble the complementary strands. The boundaries of ITS1 and ITS2 were determined by comparison with sequences of other *Gentianella* spp. deposited in GenBank. The maximum parsimony (MP) analyses of the ITS dataset were performed using PAUP ver. 4.0b (Swofford 2000). To this end, we used sequences deposited in EMBL of species belonging to *Gentianella* section *Gentianella*, *Gentianella* ssp. growing in North and South America and Australia, and *Swertia perennis* and *Comostoma tenellum* as outgroups. Substitutions at each nucleotide position were treated as independent, unordered characters of equal weight. Bootstrap analyses (Felsenstein 1985) were performed to estimate internal node support using one thousand bootstrap replicates.

Results and discussion

Plant and flower features

The morphological features of the *G. crispata* individuals are listed in Table 1. Plant size varied from 3.5 to 14.0 cm and the number of flowers per plant ranged between 1 and 26; however, 26% of the examined individuals were one-flowered. The plant size was significantly correlated to flower number per plant ($r = 0.74$, $P \leq 0.0001$); no significant correlation was found between plant sizes and length of corolla tube ($r = 0.05$, $P = 0.78$). In relation to sexual function, within the 35 flowers examined, three different conditions were detected (Table 2); 23% had a receptive stigma, while dehiscent anthers

were lacking. That fraction was constituted by very young flowers (late buds nor just opened); therefore, the flowers resulted as functionally protogynous. A fraction of 46%, mainly including mature flowers, had both a receptive stigma and dehiscent anthers. Finally, 31% of the specimens, including aged flowers, were already pollinated; they had a dry stigma and an ovary with clear traces of maturation; however, such flowers had still dehiscent anthers. In mature and pollinated flowers the pistil and the stamens were more than twice as long as in young flowers with only receptive stigma (Table 2). As for the spatial arrangement of anthers and stigma, 71% of the flowers were hyper-stigmatic, while 20% were iso-stigmatic and only 9% (three flowers) were hypo-stigmatic. The hyper-stigmatic flowers were more frequent among the protogynous than among homogamous flowers (Table 2); also in pollinated flowers the stigma surface was often above the anthers, however such a condition appeared mainly related to the ovary growth consequent to pollination.

Genetic diversity and molecular relationships

Six primers gave polymorphic bands and were used to analyze 100 samples of *G. crispata*, producing a total of 89 reproducible scorable bands, of which 83% were polymorphic. The number of fragments per primer ranged from 12 to 18 (mean 14.7 loci per primer) and the size of fragments varied from 300 bp to 1400 bp.

All genetic diversity parameters indicated that the subpopulations showed relatively low levels of genetic diversity. The percentage of polymorphic loci (%P) per subpopulation ranged from 50.56 to 55.06%; while the percentage of polymorphic loci for pooled subpopulations was 78.65% (Table 3). Interestingly, 28% of the bands were exclusive among subpopulations (Table 3), five bands in It_1 , four in It_2 , one in It_3 and three in It_4 .

A clear-cut differentiation among the subpopulations was detected. Indeed, the G_{ST} value based on allele frequencies was 0.22. Partitioning

Table 1. Minimum, maximum and average values of the measured morphological traits.

	Sample size	Min	Max	Average \pm SD
Plant size (cm)	130	3.5	14	7.20 \pm 2.21
Flower number	130	1	26	4.67 \pm 5.16
Anter-stigma distance (mm)	35	0.55	1.6	0.94 \pm 0.29

Table 2. Sexual phase, number of samples (N), length (min–max) of stamens (S) and pistils (P) and topological arrangement of female and male organs detected in 35 flowers of *G. crispata*.

Sexual phase	N	S (cm)	P (cm)	Iso-stigmatic	Hypo-stigmatic	Hyper-stigmatic
♀	8	0.05–0.50	0.75–1.60	1	0	7
♀/♂	16	0.40–0.70	0.95–2.20	5	1	10
♂-Pollinated	11	0.45–0.75	1.20–2.20	1	2	8
Total	35			7	3	25

Table 3. ISSR genetic variability parameters found in the subpopulations of *G. crispata*.

Subpopulation	Mean number (\pm SE) of bands	Percentage of polymorphic bands	Nei's genetic diversity (h) (\pm SE)	Exclusive bands
It_1	1.30 \pm 0.34	53.93	0.18 \pm 0.16	5
It_2	1.31 \pm 0.37	55.06	0.19 \pm 0.17	4
It_3	1.28 \pm 0.32	55.06	0.17 \pm 0.15	1
It_4	1.28 \pm 0.35	50.56	0.17 \pm 0.14	3
Pooled subpopulations	1.34 \pm 0.32	78.65	0.21 \pm 0.17	13

Table 4. Analysis of molecular variance (AMOVA) based on ISSR data and partitioning the variation among *G. crispata* subpopulations. (SS = sum of squares, MS = mean squares, $F = F$ -ratio).

Source of variation	df	SS	MS	F	Percentage of variation	$P <$
Among subpop.	3	97.02	32.34	2.21	18.0	0.001
Within subpop.	75	367.78	10.21	10.21	82.0	0.001

the variation using AMOVA revealed a highly significant ($P < 0.001$) variability (82%) within the subpopulations, with 18% of the total variation partitioned among subpopulations (Table 4). Values of pairwise Φ_{st} showed highly significant levels of genetic differentiation among all pairs of subpopulations (Table 5).

The length of ITS1 (DQ384062) and ITS2 (DQ384064) sequences is 225 bp and 229 bp respectively, with a G + C content of 38.8% (ITS 1) and 46.15% (ITS 2). Among the aligned nucleotide positions analysed, 345 are constant, 87 are variable but uninformative, and 34 are potentially informative. The Branch and Bound search resulted in one most parsimonious (MP) tree with a Consistency Index of 0.858 and a Retention Index of 0.786. The maximum parsimony tree shows that *G. crispata* is phylogenetically strictly related to *G. caucasea* forming a sister group (83% bootstrap) to a clade comprising all other accessions of section *Gentianella* (Fig. 2).

The value of Nei's genetic index (0.17) is slightly lower than the average value reported by Nybom (2004) for the narrow (0.28) and endemic (0.20) plant species. In addition, a size of ~4000 flowering individuals is below the threshold indicated as sufficient to maintain both the equilibrium between loss of genetic diversity via drift and its replacement via mutation (4500 individuals; Franklin 1980), as well as below the majority of the original population fitness

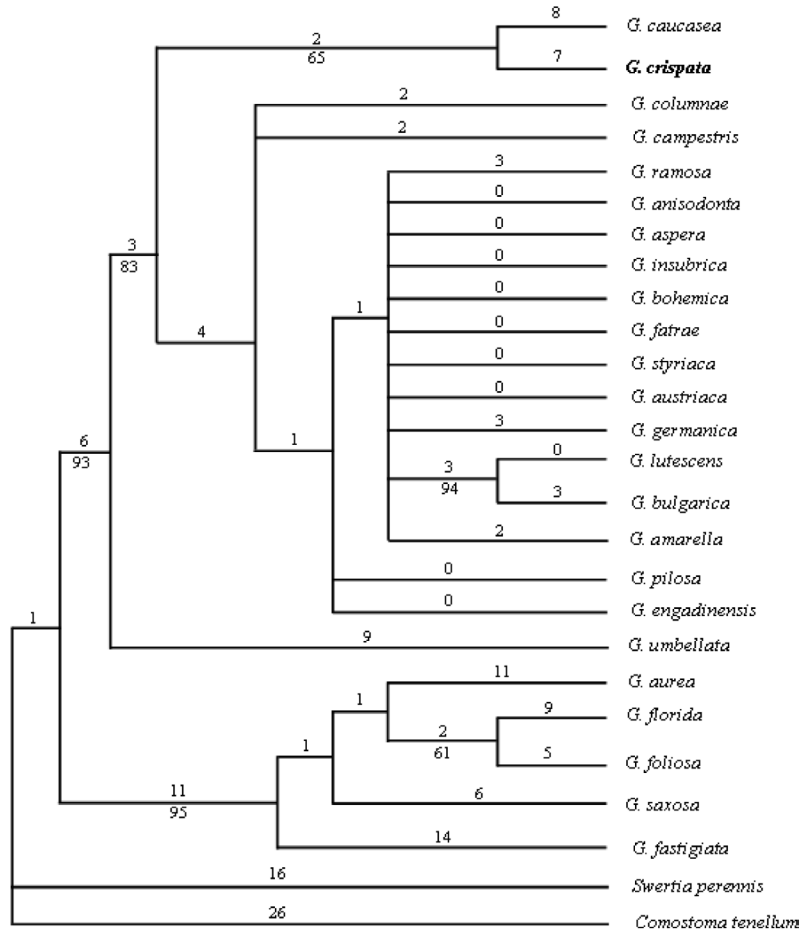
(5000 individuals; Reed 2005). Furthermore, field observations (L. Bernardo unpubl. data) suggest that the number of flowered individuals in the Italian population of *G. crispata* fluctuate annually, as also reported for the biennial *G. germanica* (Fischer & Matthies 1998). Thus, the population may be retained in a subtle equilibrium condition and any further size reduction could be deleterious for its persistence. Indeed, since exclusive bands and high pairwise Φ_{st} values have been detected among close patches, our ISSR analysis clearly highlighted the species' sensitivity to small-scale fragmentation. These results may be due to low gene flow (via pollen/seed) within the population, occurrence of selfing or biparental inbreeding, and fixation of alleles via genetic drift. The occurrence of individuals in dense patches may reflect a short-distance seed dispersal for *G. crispata*. This is also suggested by the Nei's genetic index, whose value ($h = 0.17$) is congruent for plant species with gravity-dispersed seeds (Nybom 2004). Furthermore, short-distance seed dispersal (< 1 m) has already been demonstrated for *G. germanica* (Verkaar & Scenkeveldt 1984), which has a similar habitus and fruits and seed size/shape as *G. crispata*.

The low genetic diversity and the small-scale differentiation observed in the population may also be influenced by biparental inbreeding and selfing. Indeed, the concentration of many individuals in small patches, due to limited seed dispersal, increases the chances for mating between close relatives. Furthermore, as recognized in other European ephemeral *Gentianella* (Petanidou et al. 1998, Luijten et al. 1999, Lennartsson et al. 2000), in the population of *G. crispata* the occurrence of selfing appears also likely, since the dynamics involving the floral structures do not exclude autogamy. In fact, examination of the sampled flowers allows us to

Table 5. Pairwise Φ_{st} of *G. crispata* subpopulations.

	It ₁	It ₂	It ₃	It ₄
It ₁		0.160	0.113	0.252
It ₂	0.001		0.124	0.279
It ₃	0.001	0.003		0.120
It ₄	0.001	0.001	0.003	

Fig. 2. Most parsimonious tree obtained from MP analysis of our ITS1 and ITS2 sequence data of *G. crispata* combined with sequence data of *Gentianella* spp. deposited in GeneBank by Jang *et al.* (2005). Numbers above branches are estimated branch lengths, numbers below branches are bootstrap percentages (> 50%).



suppose the occurrence of an initial herkogamy (Robertson & Lloyd 1991). Hence, the temporal and spatial separation of sexual functions may act in favouring outcrossing mainly in young *G. crispata* flowers, while increasing selfing rates would be likely with flower aging. According to Bawa and Beach (1981), protogyny would favour outcrossing and, in parallel, the co-occurrence of an initial herkogamy may assure self-fertilization. Furthermore, the high frequency of multi-flowered individuals may contribute to the reproductive success either by extending anthesis duration (Nishikawa 1998) or by facilitating selfing via geitonogamy (Snow *et al.* 1996). Hence, a series of morphological features makes the individuals of *G. crispata* likely for selfing, and this would be congruent to an unpredictable pollinator dynamic due to both elevation and isolation of the Italian stand (Luijten *et al.* 1999,

Takebayashi & Delph 2000, Blionis & Vokou 2001, Lennartsson 2002).

The clues just discussed suggest the occurrence of a mixed breeding system in the studied population of *G. crispata*; as pointed out by Nybom (2004), such a condition would fit well with the value of ISSR Nei's genetic diversity index.

Although a mixed breeding system may give some ecological and genetic advantages to plant species (Lloyd 1979), it may also promote genetic erosion (Charlesworth & Charlesworth 1987). As recently stressed by Takebayashi and Delph (2000) and Lennartsson (2002), small and isolated or fragmented populations show a shift toward increasing selfing rates. Such an event in combination with the severe isolation the species meets in Italy, would further enhance phenomena of genetic drift and population divergence.

The interesting molecular relationships with *G. caucasea* (Fig. 2) give further relevance to the populations of *G. crispata*. Indeed, such a species, traditionally assigned to the *G. amarella* group (Greuter et al. 1986), has an uncertain systematic position relative to the other European *Gentianella*, both from morphological (Greimler et al. 2004) and molecular viewpoints; also because the unit was excluded by the most recent studies aiming to clarify the phylogenetic relationships within the genus (Jang et al. 2005). Therefore, our finding represents a novelty.

In conclusion, although further knowledge is necessary, the Italian *G. crispata* population appears worthy of consideration in the future national plans for biodiversity conservation. The species has a limited and fragmented distribution within a larger area dominated by the *Seslerio nitidae*–*Brometum erecti*, a phytocenosis widespread on the Apennines near the limit of woody vegetation (Biondi et al. 1995). Little is known of the current dynamics of this vegetation type in the stand, but its expansion would reduce the ecological space of *G. crispata*. This appears as a key factor concerning the fate of the studied population, whose genetic structure indicates *G. crispata* is sensitive to small-scale fragmentation and isolation. Two phenomena play a major role in controlling the dynamic of the *Seslerio nitidae*–*Brometum erecti* vegetation. The first is grazing, because an alteration in the grazing pressure may increase the grass cover (Cadenasso et al. 2002). The second refers to the climate change that, as described for the central Apennines (Stanisci et al. 2005), may be detrimental for high-mountain communities by promoting the rising up of the present lower-altitude vegetation. According to the IUCN Red List protocols (IUCN 2001, 2003, 2006), *G. crispata* is in Italy a critically endangered species under the criterion B2. Indeed, the species exhibits an area of occupancy less than 10 km² and (1) it occurs in only one location (option a); (2) based on the concern highlighted for alpine communities on central Apennines (Stanisci et al. 2005), we can infer that climate warming can induce future decline of habitat quality through small-scale fragmentation and alteration of floristic composition (option b(iii)). In completing the risk assessment at the regional level (IUCN 2003), the high risk level of the

Italian *G. crispata* population is justified by the isolation, which makes it very unlikely for recruitment from other populations. Therefore, future efforts should be directed to monitoring the vegetation dynamics in the site, with a particular emphasis on the highly competitive grass such as *S. nitida*. Indeed, the expansion of dense grass clumps may induce genetic harm to the *G. crispata* population by promoting a further small-scale fragmentation.

Acknowledgements

This study was supported by a grant from the Pollino National Park. The authors wish to thank Dr. Lorenzo Peruzzi for valuable comments on the manuscript.

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