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Population biology and conservation of Mediterranean endemics: studies on the rare *Dianthus guliae* Janka.

D. Gargano

Dipartimento di Ecologia dell'Università della Calabria. Current address: Museo di Storia Naturale della Calabria ed Orto Botanico dell'Università della Calabria, Loc. Polifunzionale, I-87030 Arcavacata di Rende (CS), e-mail: gargano@unical.it

Abstract

The paper provides insights into habitat features, breeding system and sensitivity to inbreeding depression of *Dianthus guliae* Janka, an endangered carnation endemic to Italian Peninsula. Since its discovery, *D. guliae* disappeared in various sites of historical occurrence. Consequently, the species currently occurs in a few stands scattered on the Cilento mountains, and in a severely depleted population found in north-eastern Calabria. In order to improve the conservation status of *D. guliae*, the species was subject to an array of empirical investigations on the possible reasons of its proneness to extinction. Much work was focused on biological traits potentially related to a low persistence ability under adverse demographic contexts. Subsequently to preliminary tests, improved work on reproductive traits confirmed that *D. guliae* is unable for effective seed production under scarce pollen delivery. Moreover, fitness assessments in crossed and selfed progenies highlighted the plant's sensitivity to inbreeding depression. The cultivation of selfed and crossed offspring under different regimes (including field conditions) showed that the extent of inbreeding depression increases with environmental harshness. Information on community structure and composition suggest that in Calabria the species is prone to forest recovery and clumping. In absence of conservation measures focused on both habitat and population, it is likely that *D. guliae* will undergo a further extinction in the southernmost stand.

Key words: breeding system, conservation, endemic, forest recovery, inbreeding depression.

Riassunto

Viene presentato uno studio inerente habitat, sistema d'incrocio ed esposizione alla depressione d'inincrocio di *Dianthus guliae* Janka, un raro garofalino endemico dell'Italia Peninsulare. Questa specie risulta scomparsa in numerosi siti storici di presenza, ed oggi persiste in un numero limitato di località dislocate nelle aree montane del Cilento, cui si aggiunge una singola popolazione, estremamente ridotta, in Calabria nord-orientale. Al fine di migliorarne lo stato di conservazione, *D. guliae* è stato oggetto di una serie di studi empirici volti a comprenderne l'apparente propensione all'estinzione. In particolare, sono state indagate le caratteristiche biologiche della specie potenzialmente correlate alla sua scarsa capacità di persistenza in contesti demografici svantaggiosi. Ricerche approfondite su vari aspetti della biologia riproduttiva di *D. guliae* hanno evidenziato le difficoltà della pianta a sopperire ad una scarsa efficienza dei processi d'impollinazione. Inoltre, la comparazione di parametri di fitness in piante ottenute attraverso impollinazione incrociata ed auto-fecondazione ha evidenziato in quest'ultime forti limitazioni dovute a depressione da inincrocio. Protocolli sperimentali basati su coltivazione in diversi ambienti di crescita (ivi incluso l'habitat naturale) hanno mostrato che l'incidenza della depressione da inincrocio viene accentuata da condizioni ecologiche avverse. La composizione e struttura delle comunità che ospitano la specie indicano che, in Calabria, *D. guliae* risente della trasformazione dell'habitat dovuta all'espansione della copertura forestale. In assenza di adeguate strategie di gestione a livello di popolazione ed habitat, sarà probabile assistere ad un ulteriore scomparsa della specie dal sito che ne rappresenta il limite meridionale d'areale.

Parole chiave: sistema d'incrocio, conservazione, endemismo, espansione forestale, depressione da inincrocio.

Introduction

Mediterranean habitats show high spatial heterogeneity, and are often subject to rapid vegetation dynamics. This often leads to a fragmentation of plant populations in small and isolated remnants. In some cases such processes may enhance the divergence of populations toward different evolutionary trajectories (Thompson, 2005). On the other hand, small population fragments may undergo a higher extinction risk (Reed, 2005; Leimu *et al.*, 2006). Indeed, a reduced population size and/or density may constrain the reproductive fitness by affecting ecological and genetic components of plant reproduction (Berec *et al.*, 2006). However, an array of mating-related traits may influence the ability of plants to persist under adverse demographic conditions. According to the Baker's law, adaptation to selfing may have a crucial role. Trough self-fertilization plants may prevent loss of pollination efficiency and subsequent recruitment that are consequent to ecological/demographic contexts unsuitable for cross-pollination (Lennartsson, 2002; Kennedy & Elle, 2008; Wagenius, 2006; Zorn-Arnold & Howe, 2007). Moreover, a durable history of selfing can also favor gene purging, and reduce the extent of inbreeding depression in progenies obtained by self-fertilization (Barret & Charlesworth, 1991; Takebayashi & Delph, 2000). Overall, the adoption of a flexible mating system allows many hermaphrodite plants to reproduce at varying rates of self- and cross-fertilization in relation to the pollination context (Lennartsson, 2002; Goodwillie *et al.*, 2005; Kalisz *et al*., 2004). Especially, ability for delayed selfing was found to greatly improve fail-safe mechanisms to ensure fertilization (Lloyd, 1992; Jacquemyn & Brys, 2008).

The extent at which plants may recruit under unfavorable ecological/genetic is recognized as reproductive assurance. Based on the concept of reproductive assurance, we can expect that selfing ability, accompanied by minimum pollen/seed discounting and little viability loss in inbred offspring, may significantly enhance the persistence of small plant populations (Thompson, 2005).

The aforementioned considerations highlight how reproductive traits (e.g selfing ability, sexual phenology) might influence various aspects that are crucial for long-time persistence in plants (e.g. pollination/recruitment rates, sensitivity to inbreeding depression). Therefore, knowledge regarding plant reproductive biology is a basic information for understanding the varying species/populations responses to similar detrimental conditions (i.e. small population size, isolation, fragmentation), and for improving the soundness of risk assessment procedures and conservation measures.

Possible relationships between reproductive biology and extinction proneness in *Dianthus guliae* Janka are under study (Gargano *et al*., 2009). This rare Italian endemic disappeared from many stands of historical occurrence, and in spite of a clear range decline it does not appear in the Italian Red Lists (Conti *et al*., 1992, 1997). Here, it is provided improved empirical evidence that the persistence of *D. guliae* under demographically unsuitable conditions (e.g. small populations, low pollination efficiency) is severely constrained by a little ability to gain reproductive assurance. Moreover, based on community and habitat information, we will try to furnish possible ecological explanations on the ecological processes that are threatening the species at the southern boundary of its range.

Materials and methods

Species and stand characteristics

D. guliae is an endemic to southern Italy closely related to the *Dianthus balbisii*-group (Peruzzi & Gargano, 2006). This perennial species has a woody stock with numerous stems; flowers are in terminal multi-flowered heads (normally, 2-8 flowers/head); the calix is surrounded by obovate and long-aristate bracts; the color of the corolla varies from citrine to orange-yellow (Tutin & Walters, 1993; Peruzzi & Gargano, 2006). With regard to the species range, *D. guliae* disappeared from historical sites of occurrence in Abruzzi (*locus classicus*) and Tuscany; currently, this rare carnation is recognized just in a few stands located in the Campania and Calabria regions (Fig. 1). The ecological and population data showed and discussed in this paper refer to the Calabria population. In Calabria, a unique small population (less than 50 individuals) of *D. guliae* was found by Bernardo (1996) in the eastern part of the Pollino National Park (N= 39°51', E= 16°21'). In this stand *D. guliae* occurs at elevation ranging between 950 and 1100 m a.s.l., on a surface less than 0.5 Km^2 that is subject to intensive grazing by sheep and cows. The species is found in partially open communities that have a very composite flora. To give an idea about such a floristic and vegetation context, some phytosociological relevés were taken in the stand by using the classic Braun-Blanquet method as modified by Pignatti & Mengarda (1962). Plant names follow Conti *et al*. (2005).

Reproductive biology and breeding system

Basic aspects of the reproductive biology of *D. guliae* were investigated under cultivation in a semi-natural habitat (between an olive yard and a recovering *Q. pubescens* forest) available at the Botanical Garden

Fig. 1 - Italian regions (in gray) with historical (E) and current (black stars) presence of *Dianthus guliae*.

of the Università della Calabria. All the cultivated individuals were permanently marked. During the blooming period the flowers in the population were checked every day for assessing sexual expression, and measuring their diameter by a digital calliper. Each flower was marked by reporting an univocal code on the calyx with a felt tip pen. Over two seasons (2008 and 2009), more than 500 flowers were followed from the bud stage up to fruit maturation. The breeding system was empirically studied in order to verify self-compatibility and ability for autonomous self-pollination. Such studies were based on self- and cross-pollinations performed by hand, and on pollinators-exclusion experiments. The latter were carried out by isolating plants before blooming with bags of a fine nylon mesh. At the end of the flowering season the fruits were harvested to define fruit-set and seed-set. In addition the seed/ovule ratio (S/O) was defined for each fruit, and such a parameter was used to compare the outcomes of the different pollination experiments.

Inbreeding depression

The sensitivity of *D. guliae* to inbreeding depression was evaluated by measuring fitness traits at various life stages on crossed (N = 152) and selfed (N = 152) progenies obtained from hand-pollinations. Possible influence of the growth context on the extent of inbreeding depression was studied by cultivating plants under six different regimes that simulated increasing environmental harshness: 1) common garden, in pot with nutrient addition (CG_{p+f}); 2) common garden, in pot without nutrient addition (CG_p); 3) common garden, on the ground with nutrient addition (CG_{g+f}); 4) common garden, on the ground without nutrient addition (CG_g); 5) in the wild stand, on the ground with nutrient addition (W_{g+f}); 6) in the wild stand, on the ground without nutrient addition (W_g). At the seed stage the considered fitness traits were: seed weight (*SW*), time required to germination (*TG*), germination rate (*GR*). Subsequently germination, 30 days-old plants were transplanted and monthly checked by recording survival rate (*S*), plant size (*PS*), number of leaves (*NL*), length of the longest leaf (*Ll*). Such measures were used to derive the monthly growth rate (*mGw*) by adapting the equation of Mustajärvi *et al.* (2005), then: $mGw = [\Sigma (PS+NL+Ll)/t_i]/N$, where t_i= time (in days) from the beginning of the experiment, and N = number of measurements. Finally, the traits measured during the flowering season were: number of flowers (*NF*), flower size (*FS*), pollen viability (*PV*). Pollen viability was tested by the MTT staining technique (Norton, 1966; Khatun & Flowers, 1995), by counting a sample of pollen grains from a flowers collected on 3 individuals of the crossed- and selfed- progeny. For each fitness trait the magnitude of inbreeding depression (δ) was measured as $\delta = (W_o-W_s)/W_o$, being W_o and W_s the mean trait values of crossed and selfed offspring respectively (Donohue 1998).

Results

Stand characteristics

The phythosociological relevés taken in the stand of *D. guliae* are showed in Tab. 1. The floristic composition of the two relevés is quite different, and mirrors relevant structural differences. The first relevés shows a higher cover of the shrubby and herbaceous layers, while in the second one the woody layer covers most of the sampled surface. Main woody species are *Quercus pubescens*, *Q. ilex* and *Fraxinus ornus*; the shrubby layer is dominated by *Lonicera etrusca*, *Cytisophyllum sessilifolium* and *Spartium junceum*; finally, *Brachypodium sylvaticum*, *Sesleria autumnalis*, *Festuca circummediterranea*, and *Dactylis glomerata* subsp. *hispanica* characterize the herbaceous component. However, forestry practices have also deeply influenced the flora of the area, as testified by the presence of some species that were introduced by man (*Cupressus sempervirens* L., *Fraxinus angustifolia* Vahl. subsp. *oxycarpa* (Willd.) Franco & Rocha Afonso).

Reproductive biology and breeding system

As typical in the genus, the recognitions regarding the sexual phenology of flowers showed that in *D. guliae* occurs a proterandrous hermaphroditism. Furthermore, after numerous observations, the presence of some male-sterile flowers was recorded for the first time in this species. Female flowers are smaller than perfect ones (respectively 15.1±2.2 mm and 18.5±2.2 mm, values from 30 female and 274 hermaphrodite flowers), and their frequency appears to be higher in the early blooming period (Fig. 2). Moreover, along with perfect and female flowers, in some cases it was recognized a partial male-sterility (e.g. small flowers in which the stigmas are exerted contemporaneously with one or two anthers) indicating an extreme lability of the patterns of sexual expression in the species. Based on hand-pollinations *D. guliae* is fully self-compatible, being the outcomes of self- and cross-pollinations highly comparable (self-pollination: S/O=0.53±0.13, N=50; cross-pollinations: S/O=0.47±0.18, N=50; t test $_{(S/Oself\ -\ S/Ocross)}$: t=1.936, P=0.6, N=50). However,

Tab. 1 - Phytosociological réléves taken in the Calabria stand of *D. guliae*.

Rel. numb.	1	2
Elevation (m a.s.l.)	963	1072
Slope (°)	30	25
Surface (m^2)	100	100
Height woody layer (m)	5	5
Cover woody layer (%)	10	60
Height shrubby layer (m)	1,5	1,5
Cover shrubby layer (%)	80	20
Cover herbaceous layer (%)	90	80
Dianthus guliae Janka	+	+
Querco-Fagetea		
Quercus pubescens Willd.	+	2
Crepis leontodontoides All.	+	.
Lilium bulbiferum L. subsp. croceum (Chaix) Jan	.	+
Quercetea ilicis		
Lonicera etrusca Santi	+	.
Fraxinus ornus L.	.	2
Quercus ilex L.	.	+
Cytision sessilifolii		
Cytisophyllum sessilifolium (L.) O. Lang	+	2
Crataegus monogyna Jacq.	+	+
Spartium junceum L.	+	.
Geranion sanguinei and higher units		
Sesleria autumnalis (Scop.) F. W. Schultz	.	4
Brachypodium sylvaticum (Huds.) P. Beauv.	3	+
Stachys officinalis (L.) Trevis.	+	+
Silene italica (L.) Pers. subsp. sicula (Ucria) Jeanm.	+	+
Geranium sanguineum L.	+	.
Teucrium chamaedrys L.	+	.
Syderidenion italicae and higher units		
Festuca circummediterranea Patzke	+	1
Bromus erectus Hudson	+	+
Galium lucidum All.	.	+
Sideritis italica (Miller) Greuter & Burdet	+	.
Eryngium campestris L.	+	.
Trifolium campestris Schreb.	+	.
Phleum hirsutum Honck. subsp. ambiguum (Ten.) Tzvelev	+	.
Petrorhagia saxifraga (L.) Link subsp. gasparrinii (Guss.) Greuter & Burdet	+	.
Cynosurion cristati and higher units		
Lolium perenne L.	.	+
Dactylis glomerata L. subsp. hispanica (Roth) Nyman	3	1
Poa sylvicola Guss.	+	.
Anthoxanthum odoratum L.	+	.
Cynosurus echinatus L.	1	.
Cynosurus cristatus L.	+	.
Carduus nutans L.	+	.
Bromus hordeaceus L.	+	.

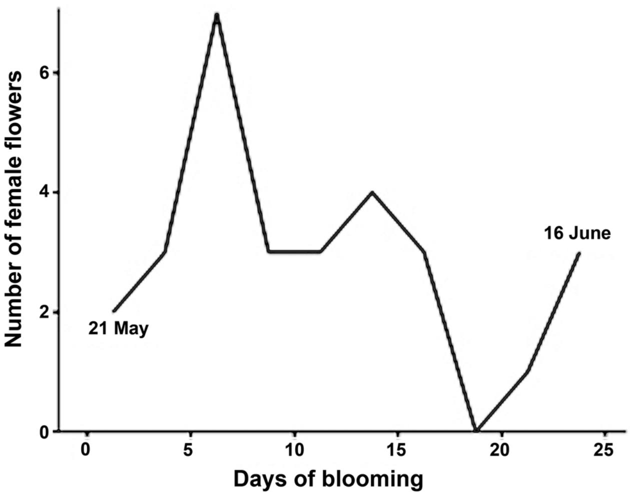

Fig. 2 - Frequency of female flowers over the blooming period of plants cultivated in common garden.

the experiments of pollinators exclusion showed a little occurrence of autonomous selfing (percent of pollinated flowers=9.2, N=65), which induced low fertilization rates (S/O=0.29±0.16; N=6). Overall, such findings suggest that in *D. gulie* both self- and cross-fertilization rely on effective pollen vectors.

Inbreeding depression

As far as early life-stages are concerned, almost all the considered traits revealed high inbreeding depression. Seeds produced by crossing germinated faster and at higher rates than those obtained via selfing (*TG*: δ = 0.39; *GR*: δ = 0.26); in addition, during the first months of life, the individuals belonging to the selfed progeny grew much more slower than plants of the crossed lineage (*mGw*, δ = 0.68). In contrast, the reproductive traits evaluated during the first reproductive season did not show any significant inbreeding depression. Therefore, inbreeding effects on pre-flowering stages seem the main responsible for the strong loss of overall fitness (fraction of individuals reaching flowering from the seed stage) detected in the inbred progeny ($\delta_{overall}$ = 0.54, Fig. 3) under common garden conditions. For most of the considered traits the extent of inbreeding depression increased under harsher conditions, by also affecting sexual traits (Tab. 2). Field conditions severely constrained plant growth in both progenies, and no plants reached flowering in the first season. Overall, in the wild stand, a very high inbreeding depression affected the survivorship of the selfed progeny after seven months (δ = 0.54; Fig. 3). However, by considering the mortality due to

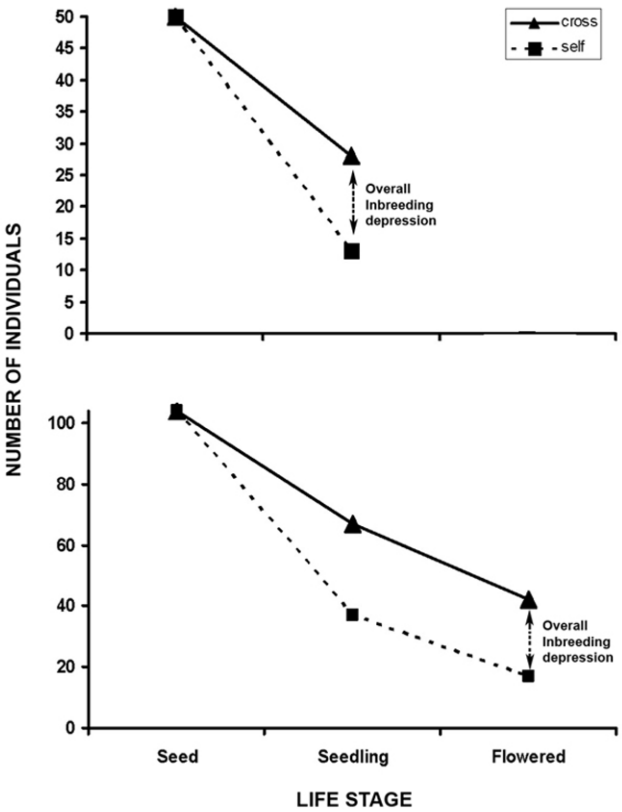

Fig. 3 - Curves of overall fitness across life stages in selfed and crossed *D. guliae* offspring cultivated under wild (upper side) and common garden conditions (lower side). The gap between curves represents overall inbreeding depression. Time frame = 240 days. No plants reached flowering in the wild in the considered period.

trampling and grazing, the latter measure of inbreeding depression may have been underestimated. Indeed, the daily presence of roaming sheeps in the site caused 16% of mortality in the selfed lineage, and more than 50% of mortality in the most numerous crossed progeny.

Discussion

Overall, data in Tab. 1 represent sparse forests with an intermediate composition between the classes *Querco-Fagetea* and *Quercetea ilicis*, as congruent to the elevation. Among the recognized shrubs, there are species related to the mantel vegetation of degraded sub-mountain woods (Biondi *et al*., 1988; Maiorca & Spampinato, 1999), this may suggest recent clumping of the tree cover. As a consequence, the herb layer is very heterogeneous. Accordingly to authors who studied forest edges in central and northern regions of Apennine (Biondi *et al.,* 2001; Vagge & Biondi, 2004), most of the recognized herbaceous taxa may be referred to the class *Trifolio-Geranietea*, which represents the vegetation established in transition zones between forest and open communities. In addition, as usually found in such ecotones (Biondi *et al*., 2001), the plant richness is improved by the inclusion of many species plants from surrounding pastures (e.g. *F. circummediterranea*, *B. erectus*) and meadows (e.g. *Lolium perenne* L., *Cynosurus cristatus* L.). Then, in Calabria, structural and floristic characteristics of the habitat of *D. guliae* suggest a high spatial-temporal heterogeneity, probably enhanced by both man and natural dynamics. In such conditions the ability for reliable reproductive outcome may be crucial in permitting long-term persistence of rare plants (Kalisz *et al*., 2004). On this respect, work on *D. guliae* is demonstrating the inadequacy of the species to gain conspicuous reproductive fitness in adverse contexts (Gargano *et al*., 2009). In other words, the plant is resulting unable to achieve reproductive assurance, as proved by various circumstances. In spite of a full self-compatibility, bagging experiments highlighted a little ability for self-fertilization in *D. guliae*. This may be explained by functional reasons. Indeed, flower dichogamy strongly reduce chances for autonomous selfing, and selfing difficulties may be further exacerbated by occurrence of male-sterility (Collin & Shykoff, 2003; López-Villavicencio *et al*., 2005). However, proterandry and partial dioecy (i.e. gynomonoicism) do not prevent selfing due to geitonogamy (Petterson 1992; Hidalgo & Hubera 2001; Collin & Shykoff 2003). Unfortunately, geitonogamy provides little reproductive assurance, because it involves both pollen and seed discounting and, most important, relies on pollinators as normal cross-pollination (Lloyd 1992; Lloyd & Schoen 1992; Herlihy & Eckert 2002). Moreover, according to Thompson (2005), minimizing the genetic detriments of inbreeding is a further condition for obtaining a true reproductive assurance. Although results on this matter have to be retained as preliminary, there is evidence to suppose that *D. guliae* is highly exposed to inbreeding depression. This appears also supported by considering the interrelationships between sensitivity to inbreeding depression and selfing ability. Indeed, infrequent selfing over the population history reduces gene purging, by causing over-expression of deleterious recessive alleles when the frequency of inbreeding becomes unusually high (Barrett & Charlesworth 1991; Knight *et al*. 2005). In *D. guliae*, inbreeding depression appears mainly affecting the

Traits	CG_{p+f}	CG_p	CG_{g+f}	CG_g	W_{g+f}	W_g
Survival	0.06	0.06	0.23*	0.30*	0.50*	0.20*
Growth rate	0.11	0.22	0.23*	0.28*	0.19	0.15
Flower/Individuals	-0,46	-0.20	0.19	0.47*	//	//
Flower size	0.01	0.10	0.02	0.08	//	//

Tab. 2 - Extent of inbreeding depression detected on different fitness components under varying growth conditions. In the wild stand no plants reached flowering in the first season. Values of $\delta \geq 0.20$ are indicated by an asterisk.

early developmental phases, that are recognized as critical for a successful establishment (Ramsey & Vaughton 1998; Mustajärvi *et al*. 2005); in addition, as expected, the environmental stress proper of natural contexts may significantly emphasize the genetic constraints in inbred offspring of *D. guliae* (Pray *et al*. 1994; Ramsey & Vaughton 1998).

Work on *D. guliae* is providing evidence on the plant's inadequacy to face the consequences of population reduction and fragmentation, by also supplying possible biological explanation for the high proneness to extinction revealed by the species history. Future perspectives are further concerning; it is very likely that *D. guliae* is experiencing in Calabria new patterns of habitat dynamics (i.e. forest clumping), which are conducing the species toward a further local extinction. The conservation *in situ* of this rare endemic at the southern border of the range require both habitat and population management. Conservation measure should consist in: favoring the presence and maintenance of forest clearings, and, at the same time, limiting trampling and grazing in the stand; trying population reinforcement by introducing adult plants (young individuals appear to be more constrained by genetic detriments); evaluating chances for using material from other populations to attempt a genetic improvement. The latter option implicate further research effort also accounting for the populations of *D. guliae* established in the Cilento area.

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