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A comparative assessment of fruit formation in some orchid species from the southern Caucasus region

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Abstract: The distribution and population size of 5 orchid species (*Cephalanthera longifolia*, *Orchis simia*, *Platanthera bifolia*, *Dactylorhiza romana*, and *Ophrys scolopax*) were studied in the southern Caucasus based on the individual numbers in a given area. The fruit set was recorded for the latter 3 species and compared in natural and control groups (pollinators prevented) for *D. romana* and *O. scolopax*. We compared the obtained fruit set values with those reported from other regions. The distributions of the 5 species were completely fragmented and the population sizes were extremely small. This is the first report of *O. scolopax* from the region, recorded only in a remote location with a population of about 44 plants. The average fruit sets of *D. romana* and *O. scolopax* found in nature were significantly higher than those of the control groups (27% compared to 16%, N = 67, P < 0.001; and 10.4% compared to 3.04%, N = 44, P < 0.02, respectively). We located 20 plants of *P. bifolia* in 2 different locations with an average fruit set of 62.7%. Only 2 *O. simia* plants were found in the region. Our data show that pollinators have a significant role in the fruit sets of *D. romana* and *O. scolopax* and that the orchids studied require urgent conservation action.

Key words: *Cephalanthera longifolia*, *Dactylorhiza romana*, *Ophrys scolopax*, *Orchis simia*, *Platanthera bifolia*, fruit set

Introduction

Over 90% of the 250,000 extant angiosperm species are pollinated by animals, primarily insects (Buchmann & Nabhan, 1996). Pollination failure is common in plant species (Wilcock & Neiland, 2002), with its level sometimes varying among closely related species (Burd, 1994). Variation in the levels of pollination failure occurs within a species at different times and places, indicating environmental effects (Burd, 1994; Larson & Barrett, 2000). Experimental studies have shown that pollination failure occurs at various stages, including during the release, transport, and deposition of pollen onto the stigma (Wilcock & Neiland, 2002). In plants pollinated by animals, pollinator limitation is the main reason for failure of reproductive success, either through a lack of pollinator activity owing to environmental conditions such as temperature, humidity (Seçmen et al., 2010), or cold weather in the Arctic (Wada, 1999), or through a loss of pollinators in the community (Wilcock & Neiland, 2002). Resource limitation can also cause reproductive failure when sufficient resources are not available to produce the maximum fruit set (Bierzychudek, 1981; Stephenson, 1981).

With approximately 25,000 species, Orchidaceae is one of the largest plant families, with its highest diversity in temperate and tropical regions (Chase, 2005). Low levels of fruit formation, a characteristic

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of the family (Ackerman, 1986; Gill, 1989; Neiland & Wilcock, 1998), have been attributed to limited pollination (Darwin, 1877; Ackerman, 1989; Calvo & Horvitz, 1990; Neiland & Wilcock, 1998), insufficient resources to set fruit (Montalvo & Ackerman, 1987; Zimmerman & Aide, 1989), or a low agamospermy rate in the family (Catling & Catling, 1991). Fruit formation is the most widely used measure for reproductive success in orchids and has been widely applied in most ecological studies (e.g. Proctor & Harder, 1994; Neiland & Wilcock, 1998). In recent years, there has been a remarkable increase in the amount of literature recording fruit sets in orchids, most of which has been carried out in North America, Europe, and the tropics, indicating an increasing interest in the fruit formation of these plants (Neiland & Wilcock, 1998). Some orchid species that have species-specific pollinators are at particular risk of reproductive failure due to pollen limitation as a result of declines in the pollinator community. Such declines are often caused by environmental changes or disturbances from human activities (Wilcock & Neiland, 2002).

We have recorded the distribution and population size of 5 orchid species, 3 of which were studied for the levels of fruit set. We also carried out a comparative study on the level of fruit set in natural and control groups (pollinators prevented) for 2 species in order to investigate the role of pollinators on fruit formation. In addition, we compared the fruit set values obtained from the orchid species studied in the Arasbaran Protected Area of the southern Caucasus region of northwestern Iran with those reported from other regions.

Materials and methods

Site and species study

This study was carried out in the Arasbaran Protected Area, managed by the Natural Resources Centre in northwestern Iran. This is located in the southern part of the Caucasus, a region covering 440,000 km^2; it is located between the Black and Azov seas and the Caspian Sea and delimited by the Kuma-Manych Canal to the north and the Turkish-Iranian border to the south. The Caucasus is home to over 6300 plant species, about 20% of which are restricted to the region (Gagnidze et al., 2002). The destruction of forests in different parts of the Caucasus by human activities has negatively affected the floristic and faunistic diversity of the area (Krever et al., 2001). The study site comprises of over 78,000 ha with altitudes varying from 250 to 2900 m, and it has been listed as a UNESCO wildlife refuge since 1976 as a result of the richness of fauna and flora recorded (1000 taxa), including some endemic exclusive species.

This study examined 5 orchid species: *Cephalanthera longifolia* (L.) Fritsch, *Orchis simia* Lam., *Dactylorhiza romana* (Sebast. & Mauri) Soó, *Ophrys scolopax* Cav., and *Platanthera bifolia* (L.) Rich. The distribution and population sizes of these species were studied based on the numbers of individual plants in a given area and the levels of fruit set were recorded for the latter 3 species. Moreover, the percentage of fruit set was compared between the natural and control groups (pollinators prevented) in *D. romana* and *O. scolopax* to investigate the role of pollinators on fruit formation in the study site. In control groups, the flowers were bagged in a double-layer of fibre fleece before opening in order to prevent visitation by pollinators. Field studies and inspections were carried out in 2009 over the course of several days between April and June (9-11 and 21-23 April, 3-4 and 16-17 May, 2-3 and 17-18 June). We also compared the obtained fruit set values in *D. romana*, *O. scolopax*, and *P. bifolia* with those reported from other regions. The percentage of fruit set was calculated for each individual plant based on the proportion of flowers set fruit. The fruit set value for each species was based on the mean of the percentage values of individuals.

Dactylorhiza romana is a food-deceptive orchid (Sabat & Ackerman, 1996) widely distributed across Eurasia (Pillona et al., 2006). *D. romana* displays a floral colour polymorphism with yellow, red, and intermediate orange (Salzmann & Schiestl, 2007), and, rather than providing any reward to its pollinators, relies on general food advertising signals to deceive them. The dominant pollinators are bumblebee queens and various other bees (Nilsson, 1980). *Ophrys scolopax* is widespread across southern Europe and the Mediterranean region. The pollinator attraction in this species is based on mimicking sexual mates and pseudocopulation, and the pollinators include male *Eucera elongatula* Vachal, 1907 (Apidae) (Kullenberg, 2009) and male *E. barbiventris* Pérez, 1902 (Apidae) (Paulus & Gack, 1986). *Platanthera bifolia* is distributed throughout moist habitats in temperate and subtropical regions. The flowers of *P. bifolia* have a strong, sweet scent and produce nectar to attract long-tongued moths from Sphingidae and Noctuidae (Nilsson, 1992; Maad, 2000). *Orchis simia* occurs widely in western and southern Europe and the Mediterranean region, including North Africa and the Caspian Sea (Willems, 1982). The plants are pollinated by *Cidnopus pilosus* (Coleoptera: Elateridae) and *Hemaris fuciformis* (Lepidoptera: Sphingidae) (Schatz, 2006). *Cephalanthera longifolia*, a Eurasian herb displaying long-spurred flowers, is pollinated by solitary bees (*Halictus* sp.) and offers no reward to its pollinators (Dafni & Ivri, 1981).

The percentages of fruit set for *Dactylorhiza romana* and *Ophrys scolopax* recorded in natural conditions were compared with those determined from the control groups. In *D. romana* and *O. scolopax*, 37 and 25 plants were assessed for fruit set in nature, respectively, and 30 and 19 plants represented the control groups. The percentages of fruit set recorded from these 2 different conditions were compared for each species using the Mann-Whitney U-test in SPSS, since the percentage values were not normally distributed. For *Platanthera bifolia*, the level of fruit set was recorded only in nature, while recording the fruit set of *Cephalanthera longifolia* was not possible since fruit fell from the plants after formation. We located only 2 plants of *Orchis simia* during the 14 days of our regional field inspections from April to the end of June.

Results

The distributions of *Cephalanthera longifolia*, *Dactylorhiza romana*, *Ophrys scolopax*, *Orchis simia*, and *Platanthera bifolia* in the area were completely fragmented, and the population sizes were small. *Dactylorhiza romana* was found at 8 locations, 5 of which had not been previously recorded (Table 1). The numbers of individual plants in each population were few, ranging from 8 to 23, and each population covered a small area of approximately 300-1000 m^2. The existence of natural barriers such as high mountains and large geographical distances make interbreeding among populations unlikely. Recorded in 37 plants, the average fruit set for this species in natural conditions was 27% (StError = 2.3), while this value for 30 control plants was 16% (StError = 2.3) (Table 2). The levels of fruit set recorded in these 2 conditions were found to be significantly different ($N = 67$, $P < 0.001$). Some 44 plants of *Ophrys scolopax* were observed in a small area (about 40 × 200 m) of a remote part of the southern Caucasus near the village of Taimour-Bayly. To our knowledge, this is the first report of *O. scolopax* from the region. The level of fruit set in 25 plants studied in nature ranged from 0.0% to 40% with a mean value of 10.4% (StError = 2.4). Almost half of the plants surveyed

Table 1. The distribution and locations of 5 orchid species in the southern Caucasus (Arasbaran Protected Region, northwestern Iran).

No.	Species	New locations*	Locations already reported
1	*Cephalanthera longifolia*	Haft-Cheshmeh, MardanaGhom, Ojdag-Kendi, Taimour-Baylu	between Makidi and Vinak, Vayan, Aynali
2	*Dactylorhiza romana*	Aynali, Taimour-Baylu, Haft-Cheshmeh, MardanaGhom, Ojdag-Kendi	between Kaleybar and Makidi, Kalaleh, Vinak
3	*Ophrys scolopax*	Taimour-Bayly (Soli Dara)	
4	*Orchis simia*	Galeye Babak, Aynali	between Makidi and Vinak
5	*Platanthera bifolia*	Aynali, Kalaleh	

*New locations are those reported for the first time.

did not set any fruit. The mean value for the control group was 3% from 19 plants, of which 15 did not set any fruit (Table 2). The level of fruit set in the *O. scolopax* control group was significantly lower than that of the unbagged plants (n = 44, P < 0.02). We located 20 plants of *Platanthera bifolia* in 2 distant locations (having 12 and 8 plants) with a distance of approximately 17 km between them. The average level of fruit set recoded in 14 plants was 62.7%, ranging from 45% to 87.5% (Table 2). Small populations of 6 to 25 plants of *Cephalanthera longifolia* were located in 7 locations, covering areas ranging from about 500 to 1200 m^2. It was impossible to record the level of fruit set since the capsules fell off the scape after ripening. At present, we cannot conclude the reason for this and further investigations are required. After 14 days of field inspections in the region at different times between April and the end of June, we managed to locate only 2 plants of *Orchis simia* at 2 different sites with 24 km between them.

Discussion

Our data on *Dactylorhiza romana* show that the plant does not depend solely on self-pollination; instead, pollinators have a significant impact on the percentage of fruit formation through promoting cross-pollination. The average level of fruit set we recorded for nectarless *D. romana* in nature (27%) fell within the range (10%-39%; mean = 25%) reported for 6 different nectarless *Dactylorhiza* species (Neiland & Wilcock, 1998). In general, the low level of fruit formation in this genus could be attributed to pollination limitation resulting from a lack of nectar since the absence of floral rewards can increase pollination limitations in insect-pollinated plants (Wilcock & Neiland, 2002). Comparing the impact of both pollination and resource limitations on reproductive success in *Dactylorhiza incarnate*, Mattila & Kuitunen (2000) showed that pollination limitation was the only factor limiting reproduction of this species within a year, because hand pollination was found to increase capsule production.

The average level of fruit set recorded for the sexual mimic *Ophrys scolopax* in this study (10.4%) was slightly higher than the values obtained for some European nectarless mate-mimicking orchids such as *O. insectifera* L. (8.7%) and *O. vernixia* Brot. (7.6%), but much lower than values recorded for other sexual mate-mimicking species such as *O. bombyliflora* Link (21.3%), *O. sphegodes* Mill. (21.1%), and *O. tenthredinifera* Willd. (55.5%) (Neiland & Wilcock, 1998). Our data indicate that *O. scolopax* has a low fruit set compared to many other sexual mimic orchids (see review by Neiland & Wilcock, 1998). Since the only existing population of this species in the region had 44 plants, the lower level of fruit formation may result from either pollinator limitation or inbreeding depression (Wilcock & Neiland, 2002). There have been few studies on inbreeding depression in Orchidaceae (Trembaly et al., 2005; Smithson 2006). In early life history stages, significant inbreeding depression was reported from 3 orchid species: *Barlia robertiana* (Loisel.) Greuter, *Anacamptis morio* (L.) Bateman, and *Anacamptis fragrans* (Smithson, 2006). The high inbreeding depression in *Cypripedium acaule* prevents the evolution of autogamy from improving its current inefficient mating system, which results in a natural fruit set of less than 2% (Gill, 1989, as cited by Tremblay et al., 2005).

Based on the presence of just a single population, it is predicted that *Orchis simia* may be on the verge of extinction in this region. Not only is conservation action urgently required to protect the remaining few plants of *O. simia*, but it is also necessary to apply ex situ conservation for *O. simia* if this species is to survive in the region. *O. simia* is classified as very rare, vulnerable, and fully protected in several countries such as Great Britain (Godfery, 1933; Brooke, 1950; Preston et al., 2002; Sumpter et al., 2004) and the Netherlands (Willems, 1982). There have been reports of populations of *O. simia* that comprise only a single plant in Europe. Single-plant plant populations of *O. simia* have been reported from Oxfordshire (Godfery, 1933); from East Yorkshire, England, more than 250 km north of the nearest site in the Thames valley (Crackles, 1975); from Kent, England (Lang, 1980); and from the Netherlands (Willems, 1982). The excessive rarity of *O. simia* in England has been attributed to the very seldom fertilisation of the flowers (Brooke, 1950).

The level of fruit set we recorded in *Platanthera bifolia* (62.7%) was generally lower than those reported from populations in Sweden (96%; Maad,

Table 2. A comparison of the fruit set in *Dactylorhiza romana Ophrys scolopax* and *Platanthera bifolia* (for which the fruit set was recorded only in nature).

Dactylorhiza romana								*Ophrys scolopax*								*Platanthera bifolia*			
Natural group				Control group				Natural group				Control group				Natural group			
No.	No. of flowers	No. of fruit	% of fruit set	No.	No. of flowers	No. of fruit	% of fruit set	No.	No. of flowers	No. of fruit	% of fruit set	No.	No. of flowers	No. of fruit	% of fruit set	No.	No. of flowers	No. of fruit	% of fruit set
1	29	0	0	1	27	0	0	1	6	0	0.0	1	5	0	0.0	1	20	9	45.0
2	30	1	3	2	29	0	0	2	5	0	0.0	2	3	0	0.0	2	13	6	46.2
3	23	2	9	3	18	0	0	3	3	0	0.0	3	6	0	0.0	3	15	7	46.7
4	16	2	13	4	18	0	0	4	6	0	0.0	4	4	0	0.0	4	21	10	47.6
5	47	7	15	5	42	1	2	5	5	0	0.0	5	4	0	0.0	5	27	14	51.9
6	25	4	16	6	28	1	4	6	4	0	0.0	6	5	0	0.0	6	28	16	57.1
7	32	6	19	7	17	1	6	7	3	0	0.0	7	3	0	0.0	7	24	14	58.3
8	37	7	19	8	12	1	8	8	3	0	0.0	8	5	0	0.0	8	16	10	62.5
9	26	5	19	9	32	3	9	9	4	0	0.0	9	7	0	0.0	9	20	14	70.0
10	25	5	20	10	28	3	11	10	6	0	0.0	10	7	0	0.0	10	21	15	71.4
11	29	6	21	11	23	3	13	11	4	0	0.0	11	5	0	0.0	11	19	14	73.7
12	43	9	21	12	28	4	14	12	7	0	0.0	12	7	0	0.0	12	28	21	75.0
13	33	7	21	13	26	4	15	13	8	1	12.5	13	6	0	0.0	13	13	11	84.6
14	14	3	21	14	38	6	16	14	8	1	13.0	14	6	1	16.7	14	16	14	87.5
15	23	5	22	15	24	4	17	15	7	1	14.0	15	7	1	14.3				
16	27	6	22	16	30	5	17	16	7	1	14.3	16	8	0	0.0				
17	18	4	22	17	16	3	19	17	7	1	14.3	17	6	0	0.0				
18	21	5	24	18	21	4	19	18	6	1	16.7	18	8	1	12.5				
19	29	7	24	19	25	5	20	19	6	1	17.0	19	7	1	14.3				
20	29	7	24	20	25	5	20	20	6	1	17.0								
21	33	8	24	21	34	7	21	21	5	1	20.0								
22	24	6	25	22	38	8	21	22	5	1	20.0								
23	32	8	25	23	19	4	21	23	7	2	28.6								
24	31	8	26	24	18	4	22	24	6	2	33.3								
25	29	8	28	25	21	5	24	25	5	2	40.0								
26	27	8	30	26	36	9	25												
27	27	8	30	27	24	6	25												
28	26	8	31	28	28	8	29												
29	22	7	32	29	14	4	29												
30	17	6	35	30	11	7	64												
31	19	8	42																
32	45	19	42																
33	19	9	47																
34	26	13	50																
35	20	10	50																
36	30	18	60																
37	18	11	61																
Standard Error			**2.3**	**Standard Error**			**2.3**	**Standard Error**			**2.4**	**Standard Error**			**1.4**				
Average			**26.8%**	**Average**			**16.4%**	**Average**			**10.4%**	**Average**			**3.04%**		**Average**		**62.7%**

2000) and Poland (62.3%-100%; Brzosko, 2003). The higher level of fruit set in this species has been attributed to a high autogamy rate, making the plant partially independent of pollinators (Brzosko, 2003). However, both hand pollination and fertiliser treatment increased the percentage of matured fruit in this species within a year, indicating that *P. bifolia* is both a resource- and pollination-limited species (Mattila & Kuitunen, 2000). In this study, the average level of fruit set recorded for the nectariferous *P. bifolia* (62.7%) was higher than the average value for 8 nectariferous *Platanthera* species (43.7%) (Neiland & Wilcock, 1998).

The low level of fruit set observed in some of the species studied could be attributed to small population sizes. In all 5 of the orchid species studied here, the populations sizes were small, mostly comprising a few surviving plants. The patterns proposed for impact of population size and density on pollination success and fruit set in orchids vary from neutral to positive (see below). Experimental studies and observations of natural populations show that both pollination failure and pollen limitation occur in isolated habitat fragments (Wilcock & Neiland, 2002). Plants with a small population size are generally less attractive to pollinators when compared to larger populations, leading to a reduction in pollinator visitation (Jennersten, 1988). However, Galetto et al. (2007) stated that pollinator and plant responses to fragmentation may be more complex than previously believed. They have reported on the lack of a direct relationship between fragment areas and pollinator richness or the frequency of floral visits. Moreover, in a review of literature published on deceptive orchid species in Africa, Australia, North America, and the Caribbean, Tremblay et al. (2005) concluded that the percentage of fruit set or flowers pollinated generally appears to be unrelated to most population sizes encountered. Sexual seed production is essential for the long-term sustainability of plant populations because it increases genetic diversity and the potential to adapt to new environments. As a result, pollination failure can be seen to directly impact population ecology and demographics (Wilcock & Neiland, 2002).

Further studies are required in the region to reveal the causes for fruit set failure in the orchid species we studied. In small populations, a lack of pollination is the main cause of a low level of fruit formation (Wilcock & Neiland, 2002), although inbreeding depression has also been reported as the main cause of reproductive failure in some orchid species (Gill, 1989; Smithson, 2006). Therefore, the next step in the investigation of the species studied should focus first on pollination failure through assessing the levels of pollinia deposited on the stigma. If this is the case, conservation attempts may focus on artificial methods such as hand pollination, which could be the utilised to improve pollination success and subsequently to increase the levels of the fruit set. Secondly, the existence of inbreeding depression could be examined through experimental crossings using pollen from genetically distant genotypes. Meanwhile, we suggest that conservation actions be undertaken for *Orchis simia* similar to those applied in the UK (Sumpter et al., 2004) and the Netherlands (Willems, 1982). In the UK, a single plant of *O. simia* has been protected and monitored for a period of 29 years since 1975, and as a result, the number of specimens reached over 200 flowering plants in 2001 and 300 plants in 2004. The reasons for such a dramatic increase in the number of plants were appropriate management of the sites, including deer- and rabbit-proof fencing, and hand pollination of *Orchis simia* specimens for 12 years, which played a significant role in the expansion its population (Sumpter et al., 2004). As a further step, we plan to carry out an investigation on the genetic variation within and among populations of these orchids in the region using DNA markers (e.g. RAPD, ISSR) to propagate these species through seed germination, and, finally, to reintroduce the obtained plants into the region.

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