ORIGINAL ARTICLE

Flower characteristics and breeding system of two phenological ecotypes of *Cyclamen persicum* Mill. (Myrsinaceae) in Israel

R. Schwartz-Tzachor · D. Eisikowitch ·
A. Dafni

Abstract *Cyclamen persicum* Mill. in Israel consists of two discrete population types that differ in their flowering (fall versus winter) as well as leafing (hysteranthous versus synanthous) phenology. The two populations have similar pollen:ovule ratios, index of self incompatibility, stigma receptivity duration, pollen longevity, flower longevity, floral morphology and seed production rate. These data indicate no apparent selective pressure on floral characteristics or on the breeding system, which may be exerted by the pollinators. The two populations retained their autonomous blooming rhythm even after transplantation, which indicated that this character was genetically determined. There are no genetic barriers and the populations may share some pollinators (Schwartz-Tzachor et al. 2006). Thus the main potential restriction on gene flow is the incomplete phenological barrier. These findings may indirectly support our hypothesis that the phenological shift is due to environmental cues. Thus it was concluded that these two types are 'phenological ecotypes' in the sense of Turesson (1922) due to the unequivocal delineation of both types, as revealed by the timing of leaf appearance (synanthous versus hysteranthous) without any evidence of any intermediates.

Keywords *Cyclamen* · Phenology · Synanthous · Hysteranthous · Ecotypic differentiation

R. Schwartz-Tzachor
Ramat-Hanadiv Nature Park, PB, Binyamina 246 5089,
Zichron-Yaacov 30900, Israel

D. Eisikowitch
Department of Plant Sciences the George,
S. Wise Faculty of Sciences, Tel Aviv University,
Ramat Aviv 69978, Israel
e-mail: eisik@post.tau.ac.il

A. Dafni (✉)
Laboratory of Pollination Ecology, Institute of Evolution,
Haifa University, Haifa, Israel
e-mail: adafni@research.haifa.ac.il

Introduction

Cyclamen persicum Mill. (Myrsinaceae, formerly Primulaceae, Källersjö et al. 2000) is a common geophyte growing in the East Mediterranean countries of Lebanon, Syria, Turkey, Cyprus, Greece and Israel with additional enclaves in North Africa (Grey-Wilson 2003; Yesson and Culham 2006). In Israel this species occupies rocky hills of the Mediterranean region with several enclaves in the desert (Zohary 1978; Danin 1998).

Most of the *C. persicum* populations in Israel have synanthous leaves (i.e. in which flowering and leafing are simultaneous), and these populations bloom throughout the winter and the spring (December–May); however, there are rare populations in Samaria and the Judean Desert; (Zohary and Livneh 1983) the Golan Heights, Mt. Carmel, (A.D. personal observations), and Mt. Gilboa in the Upper Galilee (Uzi Paz Pers. Comm.) that have hysteranthous leaves (i.e. plants in which the flowering period is distinct from the leaves' appearance time). These populations flower only in the autumn (October–November, Dafni et al. 1981). Although these two population types may be adjacent at Mt. Carmel, our preliminary observations showed that each population is uniform and retains its own blooming and vegetative phenology from year to year.

Analysis of the flowering periods of 33 species, subspecies and varieties of the whole genus *Cyclamen* shows the following patterns of flowering rhythms: four species

are summer-flowering (June–August), 13 are autumn-flowering (September–November), nine are winter-flowering (December–February) and nine spring-flowering (March–May) (Grey-Wilson 2003: 37–38). Unfortunately, this author did not mention the timing of leaf appearance in relation to the time of flowering among these groups.

According to Debussche et al. (2004), the combination of leafing and flowering phenology in *Cyclamen* shows a gradient from strictly hysteranthous species [e.g. *C. africanum*, *C. hederifolium* and *C. rohlfsianum*, all of which are 'autumn flowering' according to Grey-Wilson (2003): 37] to strictly synanthous species (e.g. *C. persicum*, *C. coum*, which are 'winter flowering' l.c.) and *C. repandum* (which is 'spring flowering' l.c.), while in some species leaves appear before or at the end of the flowering.

Species which bear hysteranthous leaves in the East Mediterranean occur in several unrelated genera [e.g. *Colchicum* (Colchicaceae) and *Urginea* (Hyacinthaceae), *Crocus* (Iridaceae), *Pancratium* and *Sternbergia* (Amaryllidaceae), *Biarum* (Araceae), all of which flower at the end of the summer or the beginning of the fall, before the first rains (Dafni et al. 1981)]. In none of these species are there any individuals bearing synanthous leaves at the time of flowering. All the species with synanthous leaves, (in the above mentioned genera) flower after the rains, from December onwards without any individuals with hysteranthous leaves.

Dafni et al. (1981) considered the hysteranthous species in *Crocus* and *Colchicum* as an adaptation to high elevations in which the flowering season is at the end of summer and the vegetative season is in the spring after the snow melting. None of the three hysteranthous *Cyclamen* species follow this pattern.

Grey-Wilson (1988: 25) generalized the breeding system in the genus *Cyclamen*: 'The different levels of the anthers and stigma preclude self-pollination initially and self-incompatibility prevents self-pollination should pollen fall from the anthers on the stigma of the same flower'. In a later edition (Grey-Wilson 2003: 40), he considers *Cyclamen* 'as basically geared to outbreeding…however that is not to say that some inbreeding does occur. Self-fertilization is precluded primarily by protandry.' In contrast, Affre and his co-workers show that various species of *Cyclamen* differ in their degree of self-incompatibility, inbreeding and spontaneous self-pollination (Affre et al. 1995, 1997a, b; Affre and Thompson 1999).

The aim of the present paper is to compare the morphology, phenology, duration of stigma receptivity, pollen longevity, and breeding system of the two populations of *C. persicum* which differ in flowering and leafing phenologies to elucidate whether these populations are two distinct phenological ecotypes or one variable taxon with a continuous variation.

Materials and methods

The habitat

The study was carried out in Ramat-Hanadiv, near Zichron-Yaacov, a protected reserve on the southern slopes of Mt. Carmel, 686/604 U.T.M. This reserve is 125–150 a.s.l. and has a typical Mediterranean habitat. The winter is rainy with ca. 500–600 mm per annum. The summer is hot and dry with no rain. (Kaplan 1989). The reserve comprises natural mixed Mediterranean maquis especially of *Phyllirea latifolia* L. with garigue and phrygana elements.

C. persicum plants are spread all over the reserve plateau. The more common synanthous winter population occupies mainly light calcareous Rendzina soil and usually grows in the shade. The less common hysteranthous fall population is restricted to north-west facing cliffs on non-calcareous Terra-Rossa soil pockets (Kaplan 1989). This rocky habitat is exposed to direct sun and to winds from the Mediterranean Sea and thus has more severe temperature fluctuations than the habitat of the synathous winter population.

Floral morphology

From 40 randomly-collected fresh flowers, each from a different plant, in each population, we measured the following morphological floral parameters: petal length, petal width, the distance between the inner corolla and the anthers, and the distance between the stigma and the anthers. The measurements were carried out using an electronic caliper (Model CD-6''R by Mitutoyo Corporation). The data were compared with two-sample *t* test: assuming unequal variances (Sokal and Rohlf 1995).

Flowering phenology and transplantations

To determine the individual flower longevity, the average number of flowers per plant, and the blooming duration of each population, we individually tagged 20 hysteranthous fall plants (in 1996) and 20 synanthous winter plants (in 1997), and monitored them daily.

To test for environmental determination of flowering time, we dug and reciprocally transplanted into the other population's site 20 corms of each population (in July 1997) We dug and replanted in their original habitats another 10 corms of each population as a control to rule out any possibility that digging and uprooting by itself might affect the plants' blooming rhythm.

The number of flowers per plant and individual flower longevity were examined by sampling 20 hysteranthous, fall plants (1996) and 20 synanthous winter plants (1997). The plants were tagged and monitored daily for the average

number of flowers in each corm of each population and for the blooming longevity of each population as well as of that of the individual flower.

The differences between the two populations for all variables were statistically analyzed using two-sample *t* tests.

Stigma receptivity

Stigma receptivity of the flowers of the two populations was examined in the field by pollination of flowers of different ages. Sixteen plants of the hysteranthous fall population (1996) and 20 plants of the synanthous winter population (1997), were tagged and bagged, before anthesis, with fine mesh bags that excluded any possible pollinators. In addition, eight open flowers of different ages (1–16 days old) in each population were hand-pollinated with 5-day-old pollen (previously found to be the peak of viability). All pollen was taken from other plants of the same population. Immediately after the hand-pollination was carried out, the pollinated flowers were rebagged. At the end of the season, fruit set was assessed as an indicator of stigma receptivity.

Pollen viability

Pollen viability (as assessed by in vitro germinability) was examined in the two populations by using a modification of the 'hanging drop method' (Stanley and Linskens 1974). Pollen grains from six flowers of ages 1, 4, 8, 12, 16, and 20 days were incubated in 2 µl of 10% sucrose solution in 3 cm Petri dishes. Pollen was stirred into the sucrose solution with a toothpick and left for incubation at 22°C for 48 h. Pollen in each drop was stained in methyl green + phloxine (Dafni 1992) and scored for germination (only those pollen grains that had tubes longer than the pollen diameter were considered). In parallel to this test, 16 potted plants of the hysteranthous fall population (1996) and 20 potted plants of the synanthous winter population (1997) were taken into the lab prior to blooming and hand pollinated in groups of six plants of the same age with pollen of different ages (1–20 days old). Sixty days later the pollinated flowers were examined for fruit and seed set.

Crossability between the two populations

Twenty plants of the synanthous winter population were randomly chosen and five flower buds of each plant were bagged prior to anthesis. Immediately after these flowers opened (between 20 December 1996 and 5 January 1997), they were hand-pollinated with fresh pollen taken from plants of the hysteranthous fall population and rebagged. As a control the same procedure was performed with eight

flowers of the synanthous winter population and pollinated with pollen from different plants of the same population. Total number of fruit set from each procedure was examined.

Breeding system

In order to assess the breeding system of the two populations, 16 potted plants of the hysteranthous fall population in 1996 and 20 potted plants of the synanthous winter population in 1997 were transferred into a greenhouse prior to their blooming. Sixteen 1 day old flowers were pollinated as follows: eight flowers were selfed by pollen taken from flowers of the same plant, another eight flowers were outcrosses by pollen taken from flowers of another plant of the same population. All pollen used for pollinations was taken from 5-day-old flowers. This procedure was continued every 2 days until the twelfth day, for a total of 56 flowers in each pollination treatment.

As a control, 40 flowers in each population were left covered to check the possibility of spontaneous selfing. Sixty days later, fruits were examined for seed number and weight. These data were used to evaluate the ISI (index of self incompatibility) as follows:

ISI = percent fruit set derived from self-pollination/percent fruit set derived from cross-pollination. The results were compared to key values presented by Zapata and Arroyo (1978).

Pollen:ovule ratio

Twelve flowers from each population, each from a different plant, were dissected and the number of ovules counted under a stereoscopic microscope. For counting pollen grains, the ten anthers of each flower were immersed separately in 1 ml tubes in 70% ethanol with three drops of detergent and one drop of safranin and were sonicated for 7 min in 35 kHz. From each tube, six aliquots of 2 µl sample were taken to examine and calculate number pollen grains per flower. The pollen: ovule (P:O) ratios of the two populations of were assessed according to Cruden (1977) and statistically compared using a paired *t* test.

Results

Floral morphology

In general, the flowers of the two populations were morphologically very similar; however, some minor differences were found between them (Table 1). In the synanthous winter population, both flower petal length and the distance between the stigma and the stamens were
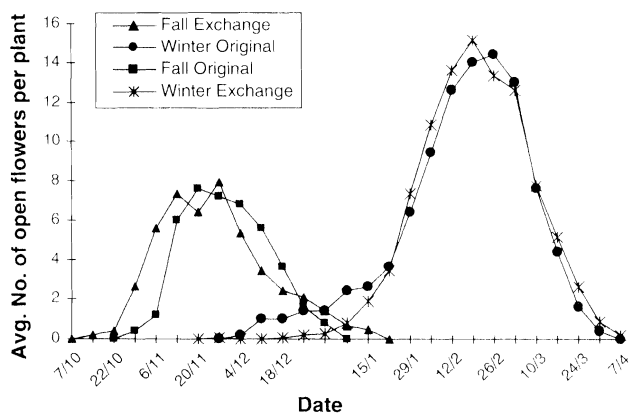
Table 1 Morphological characteristics of two population of *C. persicum* (1996–1997) Two-Sample *t*)

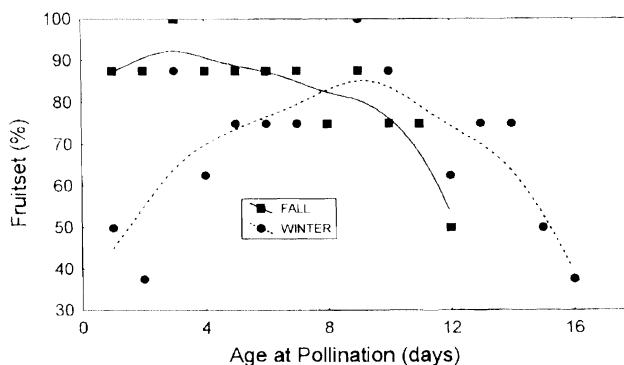|	Petal length		Petal width		Stamen–Corolla dist.		Stamen–Stigma dist.	
	Fall	Winter	Fall	Winter	Fall	Winter	Fall	Winter
Mean	2.64	2.93	0.92	0.94	2.28	2.13	1.72	1.93
Variance	0.102	0.138	0.017	0.019	0.165	0.128	0.191	0.156
Observations	40	40	40	40	40	40	40	40
Hypothesized mean difference	0		0		0		0	
df	76		78		77		77	
t Stat	−3.642		−0.573		1.751		−2.278	
P (*T* < = *t*) one-tail	0.00024		0.28399		0.04189		0.01273	
t Critical one-tail	1.665		1.664		1.664		1.664	
P (*T*< = *t*) two-tail	0.000491		0.567981		0.083787		0.025468	
t Critical two-tail	1.991		1.990		1.991		1.991	

significantly longer than in the hysteranthous fall flowers ($P < 0.05$). On the other hand petal width and the distance between stamens and the inner side of the corolla were the same.

Flowering phenology

Each population retained its original blooming season after transplantation into the other's habitat (Fig. 1). The winter population bloomed for a longer period (70.0 ± 3.4 days) than the fall population (55.0 ± 4.1 days) and the average number of flowers per plant was higher than in the fall population (38.3 ± 4.6 versus 18.5 ± 2.0, respectively, $P < 0.05$). Longevity of individual unpollinated flowers was also longer in the winter population than in the fall (20.1 ± 0.6 versus 18.4 ± 0.5, respectively, $P < 0.05$).

Fig. 2 Stigma receptivity as expressed by fruit set as a result of pollination of different ages of flowers in fall (hysteranthous) and winter (synanthous) populations of *C. persicum*

Stigma receptivity

The peak of receptivity was on the third or fourth day in the fall hysteranthous population and on the eight or ninth day in the synanthous population. However, the fall population retained stigma receptivity for a longer period (Fig. 2).

Pollen viability

In both populations, pollen viability, as inferred from pollen germination in vitro, showed similar longevity: pollen retained relatively high germinability for up to 16 days (Fig. 3). Similar results were obtained by using fruit set as an indicator of pollen viability (Fig. 4).

Cross pollination between the two populations

Fig. 1 Blooming period of two populations of *C. persicum* in Ramat-Hanadiv under original (untreated) and transplanting conditions (1996–1997)

There was no significant difference in fruit set in winter synanthous flowers pollinated by fall, hysteranthous pollen
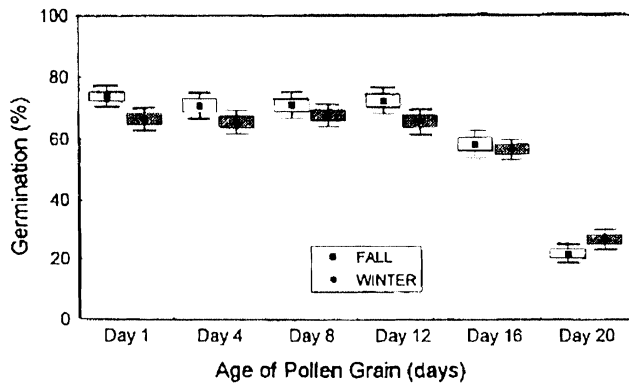
Fig. 3 Germination of pollen at different ages in 10% sucrose solution in two populations of *C. persicum*
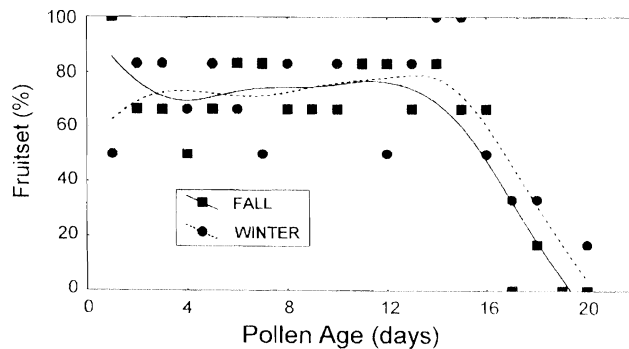
Fig. 4 Fruit set as a result of the day of cross pollination between fall (hysteranthous and winter (synanthous) populations of *C. persicum*

as compared to those pollinated by their own (winter, synanthous) pollen ($G = 0.074$, $P > 0.05$).

Breeding system

No seeds were found from spontaneous selfing, and very low yield of seeds was found from artificial selfing. Only cross-pollination resulted in high seed set in each population (fall × winter, $n = 8$, fruit set = 50%; winter × winter, $n = 100$, fruit set = 55%, fall × fall, $n = 24$, fruit set = 60%).

The ISI calculations resulted in values of 0.025 for the fall population and 0.068 for the winter. Both populations, thus, were 'mostly self-incompatible' according to the scale in Zapata and Arroyo (1978).

Pollen:ovule ratio

Examination of the P:O ratios (fall 17,340 versus winter 16,564, pollen number = 1,442,444 ± 440,353 versus 1,501,333 ± 478,416; ovule number = 86.6 ± 11.33 versus 87 ± 12.9, respectively) showed no significant difference between the two populations. Both populations

are, thus, may be included within the range of 'obligate xenogamy' according to Cruden (1977).

Discussion

Although there are some significant morphological differences between the two populations of *C. persicum*, it seems that the only one that may have any biological significance is the shorter distance between the stigma and the anthers in the fall population. Affre and Thompson (1999) found that the proximity of the stigma to the anthers in *C. balearicum* confers a high degree of self-pollination. In contrast, our data show no spontaneous self-pollination in either type of *C. persicum*.

The two populations of *C. persicum* are essentially sympatric, often as close as 100 m (Schwartz-Tzachor 1998). However, the two populations occupy different ecological habitats, which have different soil types (Kaplan 1989) and are located on different aspects (north-west facing slopes versus horizontal plateau area), which have different temperature fluctuations as well as different light and wind regimes. According to Sagiv (2001), the irradiation on the south facing aspect, at the research area, is 1.5 times that on the north facing aspect.

Despite the close physical proximity, the populations retain their own vegetative and blooming phenologies (Fig. 1) even when transplanted between populations. Our results show that there is no genetic barrier between the two populations, which also share some common pollinators (Schwartz-Tzachor 1998; Schwartz-Tzachor et al. 2006). Thus, the main restriction on gene flow is the incomplete phenological barrier. Reciprocal transplantations (Fig. 1) showed that plants of each habitat retained their flowering regime when planted in the other habitat, which constitutes indirect evidence that the phenological types are genetic and not environmentally determined.

The phenological shift from a synanthous winter bloom to a hysteranthous fall bloom is likely related to temperature fluctuations in north-west facing slopes, the habitat of the fall population. The change in the temperature regime may push flowering earlier (Dafni et al. 1981), even to the point of ecotypic differentiation of 'hysteranthous' versus 'synanthous' types as in *Narcissus tazetta* in Israel (which also differ in pollinators; Arroyo and Dafni 1995). In *N. tazetta* the two ecotypes are separated spatially (hills versus valley marshes) as well as temporally (flowering October–November versus December–January, respectively) while in *C. persicum* both populations are adjacent and the flowering season is somewhat overlapping (Fig. 1). If we consider the winter type with synanthous leaves as the ancestral type (Dafni et al. 1981), one may reconstruct the events leading to the differentiation of the phenological shift

of the hysteranthous types in *C. persicum*, in Israel, as follows: temperature fluctuations on the northwest facing slope at the microclimate level earlier flowering recruiting of any available unspecialized fall pollinators partial replacement of winter pollinators by fall pollinators restrictions on gene flow (also by the incomplete flowering season overlap) genetic establishment of the two phenological types.

Our results are not consistent with the results of Debussche et al. (2004), who grew 16 spp. of *Cyclamen* under uniform condition in southern France and concluded that 'the major phenological shifts in the genus *Cyclamen* occurred prior to the diversification of species in each subgenus and that the species are more sensitive to selection pressure on morphological traits than on phenological traits'. Thompson (2005: 123) summarizes this point: 'flowering phenology (in this genus) no doubt evolved prior to onset of the Mediterranean climate'.

In the two populations of *C. persicum* there was a complete lack of spontaneous self-pollination. The ISI indices of the hysteranthous population (0.025) and the synanthous population (0.068) were in the range of 'mostly self-incompatible' (Zapata and Arroyo 1978). The P:O ratios of 17.340 for the fall population and 16.564 for the winter population were not significantly different; $t = -0.549$, $P > 0.05$. This P:O ratios fits a situation of obligate xenogamy (Cruden 1976).

Affre and his co-workers have studied four species of *Cyclamen* in relation to floral morphology, P:O ratios, inbreeding, self-pollination and chances of pollination. Of these four, only *C. balearicum* Willk. showed spontaneous self-pollination (79.4%) and a high index of self-pollination (0.72) (Affre and Thompson 1999). Relative to the other three species studied, it also had a low P:O ratio (ca 6000, Affre and Thompson, 1998) and a high level of

inbreeding in natural populations (Affre et al. 1997b). The pollen longevity and stigmatic receptivity duration (Figs. 2, 3, respectively) were low in comparison to the three non-selfing species (Affre and Thompson 1998). Moreover, the stigma of *C. balearicum* is close to the stamens to allow spontaneous self-pollination (Affre and Thompson 1999) and the species is subjected to scarcity of pollinators (Affre et al. 1995).

Cyclamen hederifolia Kotshy is closer to *C. balearicum* in its P:O values (5000) and in the stigma receptivity duration (Affre and Thompson 1998), but like *C. creticum* and *C. repandum* (Affre 1996), it approaches herkogamy, which may account for the low self-pollination in all three species (Affre and Thompson 1999).

Cyclamen creticum (Dörfler) Hildebr. and *C. repandum* Sm. have longer stigma receptivity durations and pollen longevity as well as higher P:O ratios than the former two species (Affre and Thompson 1998). Affre and Thompson (1998, 1999) interpreted their results, especially the occurrence of spontaneous self-pollination, in terms of reproductive assurance under fluctuating pollinator availability.

In the present case of *C. persicum*, the two populations have similar P:O ratios; ISI values, stigma receptivity duration (Fig. 2), pollen longevity (Fig. 3), flower longevity (Table 2), some of the floral morphology measures (Table 1) and seed production rate and also similar pollination success (Schwartz-Tzachor 1998). In additional, the relative reproductive success (RRS) of the two populations was similar and typical of perennial plant species (l.c.).

All these data indicate no apparent selective pressure on floral characteristics or on the breeding system, which might have been exerted by the pollinators. These findings

Table 2 Blooming duration and average number of flowers per plant in natural and lab conditions of two *C. persicum* populations (field observations)

	Fall-pots		Fall-field		Winter-pots		Winter-field	
	Days in flower	Number of flowers	Days in flower	Number of flowers	Days in flower	Number of flowers	Days in flower	Number of flowers
Mean	58.88	26.88	55.05	18.45	53.15	24.35	70	38.3
Standard deviation	15.85	15.67	14.43	9.06	10.03	18.39	15.53	20.56
Sample variance	251.36	245.61	208.26	82.15	100.76	338.34	241.47	422.85
Kurtosis	−0.7346	0.1344	−1.1922	−1.032	0.7047	2.2961	0.3107	−0.4517
Skewness	0.1172	0.7296	0.1966	0.3411	−0.5359	1.5731	−0.8391	0.7371
Range	53	57	47	29	43	69	57	70
Minimum	31	5	32	5	29	5	34	12
Maximum	84	62	79	34	72	74	91	82
Sum	1001	457	1101	369	1063	487	1400	766
Count	17	17	20	20	20	20	20	20
Confidence level (95.000%)	7.536	7.449	6.324	3.972	4.399	8.061	6.810	9.012

may support, indirectly, our hypothesis that the phenological shift is due to environmental cues.

Our results show that there are no genetic barriers and that the populations share some common pollinators (Schwartz-Tzachor 1998; Schwartz-Tzachor et al. 2006). Thus the main (partial) restriction on possible gene flow is the incomplete phenological barrier.

The autumnal (fall) and winter pollination "markets", under Mediterranean circumstances, are considered "oligopolistic markets," which implies very few pollinators as well as few flowers (Shmida and Dafni 1989; Cohen and Shmida 1993). In these seasons there are also harsh conditions in terms of exposure risks to heat (end of the summer, beginning of the fall) and/or rain damage (fall/winter, Dafni 1996).

An increase in pollination success, in a harsh pollination environment (from the plant's point of view) may be achieved by several independent devices: long floral exposure (on individual flowers as well as the whole plant/population level), combined pollination mechanisms, wind pollination, and self-pollination (Herrera 1982; Dafni 1996). In both populations, floral longevity exceeded 18 days, which is quite rare generally (Primack 1985), but is known in other *Cyclamen* species: 20 days in *C. balearicum* and 37 days in *C. creticum* (Affre et al. 1997a). The long-lived flowers of all these species retain stigma as well as pollen function for most of their life cycle (Figs. 3, 2), as was also found by Affre et al. (1997b) in other *Cyclamen* species.

Affre and Thompson (1998) have already related the *Cyclamen* floral life cycle and breeding system as adaptations to 'maximize chances for being visited and the proportion of outcross pollen deposit on stigma'. The same view was expressed by Herrera (1982) and Dafni (1996) considering floral adaptations under unfavourable pollination environments and low chances of pollination, as is found in *Cyclamen*.

All these data indicate that the pollination of *C. persicum* fits into the 'sit and wait strategy' (Ashman and Schoen 1994) to face the scarcity of pollinators. This strategy involves extended flower longevity as well as a long flowering season for an individual plant with extended pollen longevity and stigma receptivity—all of which may compensate for the low pollinator visitation rate of the flowers (Schwartz-Tzachor et al. 2006). *C. persicum* has also a low degree of wind pollination (Schwartz-Tzachor 1988) and is self-incompatible.

The two populations of *C. persicum*, in Israel, varied in their flowering (fall versus winter) as well as leafing (hysteranthous versus synanthous) seasons as a result of a phenological shift. The incomplete reproductive isolation and the partial sharing of pollinators show that these two types are in a process of separation into two discrete phenological units as well as genetic entities.

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