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## Male individuals in cultivated *Fritillaria persica* L. (Liliaceae): real androdioecy or gender disphasy?

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**Abstract:** In the last twenty years the growing number of studies about the reproductive biology in angiosperms has brought to light new cases of andromonoecy and androdioecy, the rarest sexual models among flowering plants. Female-sterile sexual systems, especially within order Liliales and family Liliaceae, often seem to occur in the particular form of size/age dependent sex allocation, known as “gender disphasy”. The presence of male individuals of *Fritillaria persica* (Liliaceae) is here documented. Comparative morphological and functional sexual expression of this species, among males and hermaphrodites, was investigated by means of flowers counting, morphometric measurements of plants and pollen-grains, pollen viability and germinability tests, and crossing experiments. The results show that hermaphrodite plants are significantly bigger and produce a higher number of flowers than males. On the other hand, there is no difference either in terms of pollen size or potential male fitness, between the 2 sex types. This suggests the occurrence of gender disphasy in this species, even if our preliminary crossing experiments seem to show an effective higher fitness of male individuals in fertilization. *F. persica* resulted also partially self-compatible. Our results are discussed in relation to recent findings about andromonoecious and androdioecious breeding systems within Liliales.

**Key words:** Breeding systems, fitness, Liliales, self-compatibility

### Introduction

The adoption of subdioecious and submonoecious sexual models is rare among angiosperms. In particular, the female-sterile reproductive systems – andromonoecy and androdioecy – are considered the rarest strategies (Charlesworth, 2002), being known for about 4000 angiosperm species (Vallejo-Marín & Rausher, 2007), which are approximately 1% of the total number of species. On the contrary, male-sterile systems are much more frequent, occurring in ca. 7%-10% of angiosperms (Delannay, 1978; Jong & Klinkhamer, 2005). The rarity of andromonoecy and

androdioecy may be explained by a negative condition, in terms of genetic transmission from male flowers/individuals. Since an equal investment among the 2 sexual functions is expected in hermaphrodites (Fisher, 1930; Lloyd, 1983), theoretical models indicate that, in species with female-sterile systems, male individuals should provide a genetic contribution at least twice as much as hermaphrodites to compensate for the loss of female function (Pannel, 2002a). Moreover, such a condition becomes more problematic in self-compatible hermaphrodites, where instead female fitness is not affected.

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There are also peculiar cases that, although not unanimously, are considered to fall within andromonoecy/androdioecy. Among them, there is the “gender disphasy”, which is a particular case of size/age dependent sex allocation, where smaller/younger individuals allocate their resources only to male function, less expensive in energetic terms, than to the female one. On the contrary, larger/older individuals express both the sexual functions thanks to a larger amount of stored resources (Lloyd & Bawa, 1984). However, according to some authors, this phenomenon should not be considered as a real case of androdioecy, since each individual is expected to make an equal genetic contribution during its lifetime by means of each sexual function (Pannel, 2002b). On the contrary, “real” androdioecy – evolutionarily connected with dioecy and sexual chromosomes (but see Meagher, 2007) – is derived solely from genetic sex determination of the individuals (Barrett, 2002; Charlesworth, 2002). Similarly, the phenomenon called “cryptic dioecy”, even morphologically appearing like androdioecy, is not considered a dimorphic strategy because the plants have an androdioecious phenotype but they are functionally dioecious, having hermaphrodite individuals not functional male structures or however failing to fertilize ovules (Mayer & Charlesworth, 1991). Despite the general rarity of female-sterile reproductive systems, even including particular cases, such as gender disphasy, in the last 20 years a growing number of studies are emphasizing the occurrence of these strategies within the monocot order Liliales, where about 20% of the ca. 1600 species are known to be dioecious (i.e. the whole family Smilacaceae, Kong et al., 2007; *Chamaelirium luteum* – Melianthaceae, Meagher & Thompson, 1987; Smouse & Meagher, 1994; Smouse et al., 1999), about 1% is known to show female-sterile systems (Colchicaceae: *Wurmbea dioica*, Barret, 1992; *Colchicum stevenii*, Dafni & Shmida, 2002; Melanthiaceae: *Veratrum nigrum*, Liao et al., 2006; Liao & Zhang, 2008; *Zigadenus paniculatus*, Emms, 1993, 1996; Liliaceae: see over) and only 0.2% to show male-sterile systems (*Chionographis*; Maki 1992, 1993; Maki & Masuda, 1993).

Accordingly, Liliales show an inverted proportion in occurrence of male-sterile and female-sterile systems, with respect to other angiosperms, where gynomonoecy/gynodioecy is much more frequent than andromonoecy/androdioecy, for the reasons explained above. Increasingly frequent observations of female-sterile systems within the order, and particularly in Liliaceae, suggest that they could have an evolutionary significance. Within Liliaceae, many species have been reported to be andromonoecious and/or androdioecious, like for instance several species in the genus *Gagea* (Nishikawa, 1998; Wolfe, 1998; Jones & Gliddon, 1999; Manicacci & Desprès, 2001; Peruzzi et al., 2008) and *Fritillaria camtschatcensis* (Matsuura, 1935; Shimizu et al., 1998). *F. persica* was already incidentally reported to bring flowers with “reduced ovaries” (“pistilli ridotti”) in a seminal – mainly embryological – study of Bambacioni (1928). This led us to observe more closely flowering individuals of this species, grown in the Botanical Garden of Pisa, which showed indeed 2 different sexual phenotypes, apparently resulting androdioecious.

Our preliminary study aims to address the following questions: 1) Which is the functional sexual expression of the individuals? 2) Are there any differences in pollen size and male fitness between hermaphrodite and male individuals? 3) Is the sex expression related to the age/size of the plants? 4) Is *F. persica* a self-incompatible species?

## Materials and methods

*The species.* *Fritillaria persica* L. (Liliaceae) is a perennial non-clonal geophyte native to Western Asia (Iran, Turkey, Syria and Cyprus). It is a diploid species, with  $2n = 24$  chromosomes, belonging to Liliaceae subfam. Lilioideae tribe Lilieae (D’Arienzo & Bambacioni-Mezzetti, 1967; Khaniki, 1997; Peruzzi et al., 2009). The flowering stem is 60-100 cm tall, with numerous linear leaves and a terminal pyramidal raceme of 10-50 small nodding flowers; the perigon, 10-20 mm long, is violet-purplish and campanulate. The species occurs in Italy as cultivated ornamental and casual alien species (Pignatti 1982), although not recorded outside cultivation since 1950 (Celesti-Grapow et al., 2009). During spring 2009, we studied

8 flowering individuals (5 resulting hermaphrodites, 3 resulting males) grown in the Botanical Garden of Pisa from bulbs originally collected (June 1994) in southern Anatolia, Manavgat district, 600-700 m a.s.l., by Prof. Canio G. Vosa.

*Sexual expression and plant size.* The investigation of sexual expression modality was carried out for each flowering plant, taking note of the sex of all the flowers. They were considered hermaphrodite (H) when they had both well developed stamens and pistils and male (M) when they had only well developed stamens. At the same time, together with the sexual type, the number of flowers and the diameter of the stem at the ground level (a good indicator of plant age, according to Shimizu et al., 1998) was recorded for each individual, by means of a manual calliper (accuracy 0.01 mm). Pollen grain size (both polar and equatorial axes) was measured from microphotographs obtained by means of optic microscopy, with 100 repetitions for each sexual type (H and M).

*Male fitness.* Male fitness between male and hermaphrodite individuals was compared through pollen viability and pollen germinability tests. For the viability test a 1% solution of MTT (2,5-diphenyltetrazolium bromide or thiazolyl blue) (Dafni, 1992; Khatum & Flowers, 1995; Rodriguez-Riano & Dafni, 2000) was prepared in a 5% sucrose solution. Pollen was transferred from freshly dehisced anthers onto microscope slides, where few drops of MTT solution were subsequently added. After about 30-45 min of incubation, 300-400 pollen grains were randomly counted for each slide, scoring the grains by their colour. Red grains were considered viable, while dark and pink-transparent ones were considered not viable (Rodriguez-Riano & Dafni, 2000). The test was performed 5 times, using 5 different flowers for each sexual type (male vs. hermaphrodite).

For pollen germinability test, the Brewbaker and Kwack's nutrient solution (Dafni, 1992) was prepared and used in solutions at 0%, 5%, 10%, 20%, and 50% of sucrose. Anthers from each sexual type were dipped into a small amount of the solutions, in order

to promote the release of pollen grains. Then, a drop for each combination of sexual type vs. kind of solution – containing a small quantity of pollen grains – was transferred into a Petri's capsule. After 24 h of incubation, the number of germinated and not germinated grains was scored. This test was performed 10 times.

*Compared fertilizing power and self-compatibility.* To compare the fertilizing power in *F. persica* and to determine the self-compatibility rate, we made pollination experiments. Flower racemes of 4 hermaphrodite individuals, bearing 18-41 flowers each, were pollinated manually in the following combinations: 1. self-pollination (pollen from the same flower), 2. geitonogamous pollination (pollen from different flowers in the same raceme); 3. H-H outcross pollination; 4. H-M outcross pollination. The single hermaphrodite individual left was used as control for possible spontaneous self-pollination. Each plant was marked in order to later recognize the corresponding crossing type. After about 60 days, the number of capsules produced by each plant, and the number of seeds per fruit was scored.

*Data analysis.* To compare quantitative data, as the number of flowers per plant, the diameter of the stem, pollen size and the number of seeds per fruit, we used the non-parametric Mann-Whitney test for 2 independent samples. The Chi-Squared test was used to analyze data of pollen viability and germinability, consisting of frequencies within the 2 groups.

## Results

*Sexual expression and plant size.* According to the observations on the studied plants, *F. persica* is morphologically androdioecious, given that flowering individuals had racemes composed only by flowers of the same sexual morph, either hermaphrodite or male. Both the stem diameter in mm ( $P = 0.036$ ) and the number of flowers per plant ( $P = 0.036$ ) were significantly higher in hermaphrodite individuals than in male ones (Figure 1), while pollen size in  $\mu\text{m}$  is not statistically different among M and H flowers ( $P = 0.218$  for polar axis,  $P = 0.227$  for equatorial axis; Figure 2).

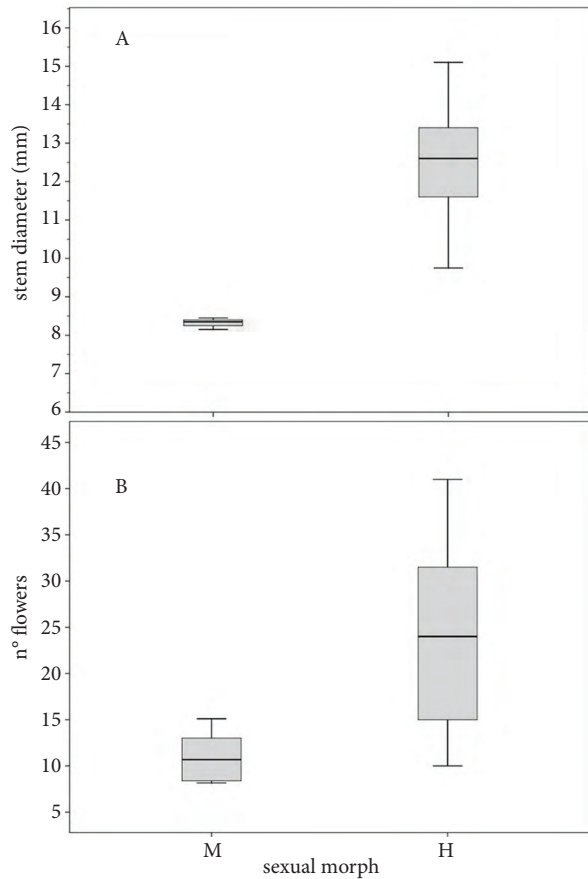


Figure 1. Boxplots illustrating the variability of stem diameter (A) and flowers number (B), among male (M) and hermaphrodite (H) individuals. The outlined central box depicts the middle 50% of the data extending from upper to lower quartile; the horizontal bar is at the median. The ends of the vertical lines (“whiskers”) indicate the minimum and maximum data values, unless outliers are present in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range. Circles indicate outliers.

*Male fitness.* There were no statistically significant differences in pollen viability between male and hermaphrodite plants ( $\chi^2 = 1.38$ ,  $df = 1$ ,  $P = 0.24$ ; mean  $\pm$  SD pollen germination %:  $75.97 \pm 9.25$  for M pollen,  $74.51 \pm 2.35$  for H pollen), while pollen germinability was significantly higher in male individuals only at the sucrose concentration of 5% ( $\chi^2 = 42.35$ ,  $df = 1$ ,  $P < 0.01$ ); (Figure 3).

*Compared fertilizing power and self-compatibility.* Three out of the 5 plants used for the pollination experiments produced fruits (Table). We obtained 5

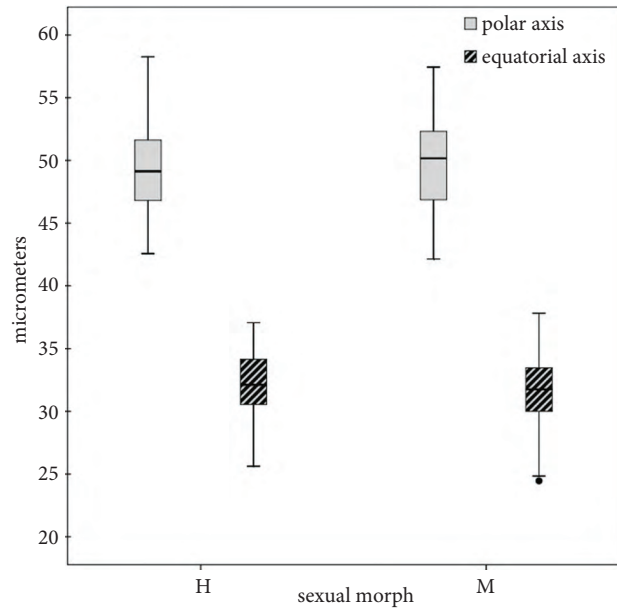


Figure 2. Boxplots illustrating the variability of pollen size, among male (M) and hermaphrodite (H) individuals. See Figure 1 for more explanations concerning the boxplots.

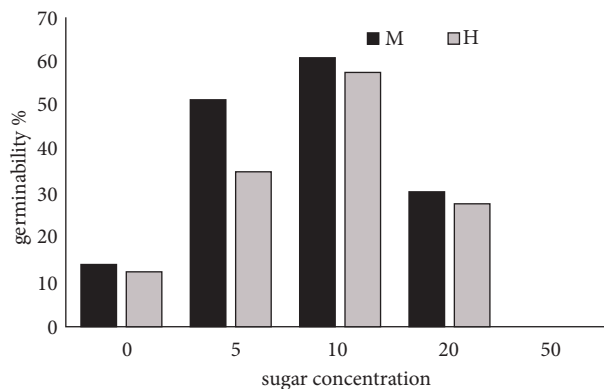


Figure 3. Histogram illustrating the mean % values of pollen germination, at different sucrose concentrations for male (M) and hermaphrodite (H) individuals.

capsules from forced self-pollination; 1 capsule from the outcross between different hermaphrodite individuals, and 20 capsules from the outcross between a male and a hermaphrodite individual. There were no significant ( $P = 0.336$ ) differences in the mean number of seeds per fruit between capsules produced by manual self-pollination and those produced by hermaphrodite-male cross.

Table. Capsules and seeds of *F. persica* produced in crossing experiments. For the combination H-H, the single capsule produced was damaged before its ripening.

	n° capsules/flowers	mean n° seeds	DS
Spontaneous self-pollination	0/41	0	0
Forced self-pollination	5/32	47.60	23.80
Geitonogamy	0/18	0	0
H-H	1/31	–	–
H-M	20/30	61.20	20.43

## Discussion

Our preliminary results confirm that female-sterile strategies are not an isolated exception within the genus *Fritillaria*. *F. persica* is confirmed here to bring male flowers – as stated first by Bambacioni (1928) – and results morphologically androdioecious. Morphometric measurements show that the sex expression seems related to the plant size/age, being male individuals smaller and having fewer flowers. The absence of differences in pollen size and viability, together with the comparable pollen germination rates between the 2 sexual morphs, clearly indicates that male and hermaphrodite plants do not differ in the potential male fitness.

Nonetheless, this result seems to contrast with data from pollination experiments, given that the H-M outcross combination produced 20-fold higher number of fruits than H-H outcross. This point, which could lead us to suspect a partial occurrence of cryptic dioecy in *F. persica*, is not statistically representative and needs therefore further investigation.

The production of 5 fruits by forced self-pollination indicates that *F. persica* is – at least partially – self-compatible, but without evidence of spontaneous self-pollination ability. Indeed, also *Erythronium dens-canis* (Liliaceae, subfam. Lilioideae, tribe Tulipeae) is known to be potentially auto-compatible, but only after fertilization induced by insect visits or by experimentally forced self-pollination (Gutián et al., 1999).

All things considered, a case of full cryptic dioecy in *F. persica* can be excluded, since hermaphrodite flowers resulted functionally bisexual, and, to conclude, this species could represent a new case of androdioecy and particularly of size dependent sex determination (gender disphasy). The occurrence of this phenomenon also in *F. camtschatcensis* (Shimizu et al., 1998) suggests a possible evolutionary and/or adaptive significance. In this respect, a study of the monocarpic hermaphrodite *Cardiocrinum cordatum* – also belonging to the tribe Lilieae – shows a facultative size-dependent sex allocation favouring male function in smaller plants, which seems to be adaptive in that case (Cao & Kudo, 2008). It would be interesting to extend the investigation to natural populations of *F. persica*, as well as to other Liliaceae species, in order to properly address these interesting issues.

Moreover, *F. persica* is a well known and frequently used ornamental plant and, as recently shown for the medicinal herb *Aconitum heterophyllum* Wall. (Ranunculaceae), basic knowledge of floral biology could have positive economical and conservational consequences, improving cultivation practices (Nautiyal et al., 2009).

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