

## Evidences of a mixed pollination strategy in Vietnamese species of *Thismia* (Thismiaceae: Dioscoreales)

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**Summary:** While reproductive biology in the mycoheterotrophic family Thismiaceae has long been discussed, only fragmentary knowledge on this issue has been accumulated to date. Direct investigations performed for several species of the family indicated xenogamous mating system and pollination by various dipteran insects. Data on dichogamy, floral nectar, floral odour and other important traits remain controversial. We conducted field studies of pollination ecology in *Thismia mucronata* and *T. puberula*, accompanied by estimation of pollen-ovule ratio in *T. annamensis*, *T. javanica* and *T. mucronata*. Flowers are visited by a wide range of invertebrates, among which we identified scuttle flies (Phoridae, Diptera) as possible pollinators in accordance with the presence of *Thismia* pollen on their bodies. Pollen of *Thismia* was also found to be carried by several parasitic species of Hymenoptera, which are generally considered not to be capable of effective pollen transfer. Anther thecae dehisce at the stage of immature flower bud, when the floral chamber is isolated from the external environment. The pollen-ovule ratios range within 26 and 84; we interpret these low values as an indication of highly efficient pollen transfer, but not necessarily of reproductive strategy with predominance of self-pollination. We demonstrate that there are evidences in favor of both xenogamy and autogamy in the studied species of *Thismia*, and we suppose that various mechanisms are employed in order to ensure the sufficient fruit set, including insect-mediated pollination within the same flower.

**Keywords:** autogamy, Chu Yang Sin National Park, mycoheterotrophy, pollen-ovule ratio, reproductive ecology, sapromyophily, xenogamy

*Thismia* Griff. is an intriguing genus of small mycoheterotrophic non-photosynthetic herbs. Species of *Thismia* (commonly called ‘fairy lanterns’) are remarkable for their complex floral morphology and unusual ecology. *Thismia* is characterized by a trimerous pentacyclic flower, which is typical for monocots. At the same time, the genus shows several peculiar floral features such as a long hypanthium forming a floral chamber, a ring-like structure called annulus covering the floral chamber, long appendages of tepals, occasional absence of outer tepals, a roof-like structure (either a mitre or a loose dome) formed by the inner tepals, a tube of six postgenitally fused stamens hanging down from the annulus (MAAS et al. 1986; LARSEN & AVERYANOV 2007; CHANTANAORRAPINT et al. 2015; HRONEŠ et al. 2015; SUETSUGU et al. 2018; NURALIEV et al. 2020, 2021). The ovary is inferior, unilocular, usually with three parietal column-like placentas (NURALIEV et al. 2021). To date, approximately 85 currently accepted species of *Thismia* have been described (DANČÁK et al. 2020; SHEPELEVA et al. 2020). Information on diversity and biology of *Thismia* has been rapidly accumulated during the past decades. Species of *Thismia* are distributed in tropical, subtropical and temperate areas of Asia, Australia and America, where they predominantly inhabit primary rainforests growing among leaf litter (MAAS et al. 1986; MERCKX et al. 2013; MERCKX & SMETS 2014; COOPER 2017; KUMAR et al. 2017; SOCHOR et al. 2018; NURALIEV et al. 2020). *Thismia* is involved into a symbiosis with fungi belonging to the genus *Rhizopogon* Herbst. (Glomeraceae) (MERCKX et al. 2017; GUO et al. 2019).

The family Thismiaceae is particularly understudied with respect to reproductive ecology. Most of the ideas on pollination of Thismiaceae were based exclusively on floral morphology, whereas only a few studies employed direct observations (MAR & SAUNDERS 2015; CHEEK et al. 2019; GUO et al. 2019). Species of *Thismia* were generally considered to be myiophilous plants pollinated by small dipteran insects such as fungus gnats, and sometimes also supposed to be autogamous (STONE 1980; MAAS et al. 1986; MAAS-VAN DE KAMER 1998; WATERMAN et al. 2013; GUO et al. 2019). The first hypothesis on myiophily of *Thismia* was suggested by GROOM (1895). GROOM (1895) supposed that small flies enter the flower of *T. aseroe* Becc. through the annulus, descend along the abaxial (inner) side of the stamens to the stigmas, crawl up the hypanthium wall (supposedly, to the nectar produced by the stamen glands) and eventually leave the flower through the gaps between free stamen filaments. VOGEL (1962, 1978a, 1978b, cit. after MAAS et al. 1986), proposed small Diptera as candidate pollinators for the Brazilian *T. caudata* Maas & H. Maas and *T. macahensis* (Miers) F. Muell. and particularly fungus gnats for the Brazilian *T. fungiformis* (Taub. ex Warm.) Maas & H. Maas, attracted in the latter case by the opportunity to lay eggs in the floral chamber (although we were unable to find any mentioning of Thismiaceae in the original works by VOGEL 1978a, 1978b). The idea of pollination of *Thismia* by fungus gnats was supported by THIELE & JORDAN (2002) for *T. clavarioides* K.R. Thiele and by NURALIEV et al. (2014) for *T. mucronata* Nuraliev, who, however, have not provided any direct evidences in favor of this hypothesis. A small dipteran species was recorded as a floral visitor of *T. gongshanensis* Hong Qing Li & Y.K. Bi (LI & BI 2013), and a fungus gnat (Sciaridae or Mycetophilidae, Diptera) and a wing of a scuttle fly (Phoridae, Diptera) were found in a flower of *T. hongkongensis* Mar & R.M.K. Saunders by MAR & SAUNDERS (2015). MAR & SAUNDERS (2015) proposed protandry for *T. hongkongensis*. It is important that many species of *Thismia* show deviation from the typical myiophilous floral syndrome, as their flowers lack any noticeable odour (e.g. STONE 1980 and authors' personal observations; but see KIEW 1999; GUO et al. 2019; SITI-MUNIRAH & DOME 2019; SUETSUGU & SUEYOSHI 2021).

A recent wide-scale study of *Thismia tentaculata* K. Larsen & Aver. conducted by GUO et al. (2019), which involved two years of observations, shed some light on the reproductive biology of *Thismia*. The study showed close synchrony of anthesis among the individuals within a population, with flowers within an inflorescence developing successively, and individual flowers functioning for 13–17 days (GUO et al. 2019: Fig. S1). The absence of dichogamy in *T. tentaculata* was demonstrated, i.e. its anthers and stigmas within a flower are functionally synchronous. An exudate was found on tips of the hairs that terminate stamen supraconnectives. The authors suppose that the exudate functions as a nutritive reward for pollinators. GUO et al. (2019) reported this species to be self-compatible but predominantly xenogamous, which follows from their experiments on artificial pollination (although these experiments do not provide evidences against insect-mediated autogamy). The authors recognized a species of fungus gnats from the genus *Corynoptera* (Sciaridae, Diptera) attracted by yellow colour of the flower as a pollinator of *T. tentaculata*. Having once entered the flower, the fungus gnats were observed to be temporarily restrained within the floral chamber (GUO et al. 2019), which indicates a mechanism similar to those in typical trap flowers.

Little is known about pollination ecology in the other genera of Thismiaceae. *Tiputinia foetida* P.E. Berry & C. Woodw. produces rotten fish-like odour, which allows to suppose sapromyiophily

in this species (WOODWARD et al. 2007). Field examination revealed a spectrum of various floral visitors of *T. foetida* including ants, beetles, small bees and flies. The authors were unable to indicate the pollinator, although their observations detected insects walking over the anthers and appearing inside the floral tube (WOODWARD et al. 2007), possibly performing the movements inside the flower similar to those suggested by GROOM (1895). Several findings of floral visitors were made for species of the African genus *Afrothismia* Schltr. ENGLER (1905) found small dipterans in the lower part of the floral tube of *A. winkleri* (Engl.) Schltr., and CHEEK & WILLIAMS (1999) observed dipterans leaving the flower of *A. pachyantha* Schltr. FRANKE (2004) observed a drosophilid fly inspecting tepals of an unknown species of *Afrothismia* for several minutes. Accurate observations on *A. kupensis* Cheek & S.A. Williams revealed females of scuttle flies *Megaselia* (Phoridae, Diptera) as possible pollinators of this species (CHEEK et al. 2019). In the study by CHEEK et al. (2019), the scuttle flies were the only floral visitors of *A. kupensis* during the investigation period; they entered the floral chamber and spent several seconds there before departing, and pollen of Thismiaceae predominated in washings from their bodies. CHEEK et al. (2019) concluded that the scuttle flies *Megaselia* might be the only pollinators of *A. kupensis* at that site and that time.

In the present study, we provide new data on pollination biology of four Vietnamese species of *Thismia*. We report on timing of anthesis and records of flower visitations by invertebrates for *T. mucronata* and *T. puberula* Nuraliev as results of our field observations. We estimate pollen-ovule ratio for *T. annamensis* K. Larsen & Aver., *T. javanica* J.J. Sm. and *T. mucronata* and use this index as an indirect evidence of their reproductive systems.

Floral structure of the species under study was described and illustrated in details by NURALIEV et al. (2014, 2015, 2020), LARSEN & AVERYANOV (2007) and SITI-MUNIRAH & DOME (2019). *Thismia annamensis* and *T. javanica* are characterized by a rotate perianth with free inner tepals, with the annulus orifice being readily visible from the outside. Flowers of *T. mucronata* and *T. puberula*, in contrast, possess a mitre, which hampers observation of the floral entrance and the inner space of the floral chamber.

## Materials and methods

Information on the studied living plants and fixed material is presented in Table 1. For terminology of reproductive systems, we follow CARDOSO et al. (2018).

### Study site

Direct observations of *Thismia mucronata* and *T. puberula* were performed in Chu Yang Sin National Park (Vietnam, Dak Lak province) during 16–28 May 2019. Both species were found among leaf litter on river banks in monsoon tropical mountain forests of middle elevation. *Thismia mucronata* (voucher: Yudina, Nuraliev 6) was found in the amount of five small groups each comprising 1–4 individuals; they occupied a flat place ca 10 m in diameter. Three individuals of *Thismia puberula* (voucher: Yudina, Nuraliev 15) were found at the type locality of this species; only one of them was found in the beginning of the field study and involved in the observations. The distance between study sites of *T. mucronata* and *T. puberula* is approximately 4 km. Additional information on the habitats of the studied populations is provided by NURALIEV et al. (2015, 2020).

**Table 1.** Species and specimens examined, voucher information and performed studies.

| Species                                        | Voucher                                                   | Location and date of collection                                                                                                                                                                              | Investigation                                                              |
|------------------------------------------------|-----------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| <i>Thismia annamensis</i><br>K. Larsen & Aver. | <i>M.S. Nuraliev 1562</i><br>[MW]                         | Vietnam, Gia Lai Province, K'Bang District, Son Lang Municipality, Kon Chu Rang Nature Reserve, 29 km ESE of Mang Den town, forest, river bank, N 14°30'55", E 108°32'50", 1000 m a.s.l., 26.05.2016         | pollen-ovule ratio                                                         |
|                                                | <i>S.V. Yudina, M.S. Nuraliev 16</i><br>[MW]              | Vietnam, Dak Lak Province, Lak District, Bong Krang municipality, Chu Yang Sin National Park, N 12°23'41", E 108°20'55", 1098 m a.s.l., 27.05.2019                                                           | floral visitor<br>(in fixed flower)                                        |
| <i>Thismia javanica</i><br>J.J. Smith          | <i>Do Thi Xuyen, Nguyen Tan Hieu BHH 800</i><br>[HNU, MW] | Vietnam, Quang Tri Province, Huong Hoa District, Huong Phung commune, Bac Huong Hoa Nature Reserve, Sa Mu mountain, 1012 m a.s.l., 11.10.2015                                                                | pollen-ovule ratio                                                         |
| <i>Thismia mucronata</i><br>Nuraliev           | <i>M.S. Nuraliev 1009</i><br>[MW]                         | Vietnam, Dak Lak Province, Lak District, Bong Krang Municipality, Chu Yang Sin National Park, 10 km S of Krong Kmar village, forest, not far from river, N 12°25'35", E 108°21'58", 970 m a.s.l., 21.05.2014 | pollen-ovule ratio                                                         |
|                                                | <i>S.V. Yudina, M.S. Nuraliev 6</i><br>[MW]               | Vietnam, Dak Lak Province, Lak District, Bong Krang municipality, Chu Yang Sin National Park, in the forest, near stream, N 12°25'34", E 108°21'58", 963 m a.s.l., 26.05.2019                                | floral phenology,<br>observation of<br>pollinators, pollen-<br>ovule ratio |
| <i>Thismia puberula</i><br>Nuraliev            | <i>S.V. Yudina, M.S. Nuraliev 15</i><br>[MW]              | Vietnam, Dak Lak Province, Lak District, Bong Krang municipality, Chu Yang Sin National Park, N 12°23'41", E 108°20'55", 1098 m a.s.l., 27.05.2019                                                           | observation of<br>pollinators                                              |

## Phenology of flowers and inflorescences

For phenological observations, photos of all flowers in the five groups of individuals of *T. mucronata* were taken using Canon EOS 750D and Canon EOS 70D (Japan) digital cameras. Flowers were photographed twice a day, in the morning (at 8:00–9:00 a.m.) and in the evening (at 4:00–5:00 p.m.).

In order to establish the stage of flower anthesis at which the thecae dehisce, we dissected living and ethanol-fixed flower buds by blade and tweezers, which made the observation of anthers available, and measured length of the flower buds using calipers.

## Floral visitors

We conducted observations of flowering individuals of *Thismia mucronata* and *T. puberula* between 5 a.m. and 8 p.m. The sunrise and sunset time was approximately 5:30 a.m. and 6:10 p.m., respectively (<https://www.timeanddate.com>). We performed direct visual observations mainly from 8 a.m. to 5 p.m., and we used digital video cameras for observations at 5 a.m. to 8 a.m. and 5 p.m. to 8 p.m. The cameras HC-VX1EE-K (Panasonic, Japan) and GZ-R495BEU (JVC, Japan) were used for this purpose. During camera operation, the plants were highlighted with a

red flashlight to avoid influence on visitor behaviour (FINLEY 1959; SUETSUGU 2018). Primary examination has not detected any insect activity in the proximity of the flowers of *Thismia* at night time, and therefore further night observations (from 8 p.m. to 5 a.m.) were not performed. Total observation time was 25.5 hours for *T. mucronata* and 41.4 hours for *T. puberula*.

Since the inner space of the observed flowers was invisible from the outside, an attempt to capture the visitors was performed each 10–15 min. by exhaustor through the ‘windows’ between the mitre and the annulus. We considered all insects captured this way as visitors. Captured visitors were killed with ethyl acetate and fixed in 70% alcohol. Taxonomic identity of the captured insects was evaluated by Alexander Timokhov for Hymenoptera, Tatiana Galinskaya for Diptera and Alexander Prosvirov for Coleoptera at Department of Entomology, M.V. Lomonosov Moscow State University.

Examination of pollen grains attached to visitor bodies was performed in Moscow State University. Captured visitors were washed in 70% ethanol, which was then centrifuged for 5 min. at 3000 rpm. After that, 50 µl of sediment was taken from each sample for investigation under a Micromed-2 light microscope (Ningbo Sheng Heng Optics & Electronics, Zhejiang, China). Identification of pollen grains of *Thismia* was performed by their comparison against pollen observed in the studied specimens.

### Pollen-ovule ratio

Counting of pollen and ovules in flowers of *Thismia* was performed in Moscow State University, using plants fixed in 70% ethanol. Flowers of *T. annamensis* (Nuraliev 1562; four individuals), *T. javanica* (Do Thi Xuyen, Nguyen Tan Hieu BHH 800; two individuals) and *T. mucronata* (Nuraliev 1009, Yudina, Nuraliev 6; four and five individuals, respectively) were studied.

The pollen and ovules were mainly counted in different flowers, because their investigation requires different stages of floral development. For counting of pollen grains, flower buds with undehisced thecae (less than 8 mm in height) were used. Preparations of buds were made under an Olympus SZX7 (Japan) stereomicroscope. The thecae were extracted by tweezers, placed on a microscope slide in a drop of glycerol, opened with needles to release the pollen and then covered by a glass. The slides were studied using the Micromed-2 and Olympus BX53 (Japan) microscopes. Pollen grains in the entire slide were counted; the slide was browsed in a zigzag manner in order to cover its entire area. For counting of ovules in the entire placenta, flowers and flower buds exceeding 8 mm in height were used. Preparation and investigation of flower parts for ovule counting were performed similarly to those for counting of pollen grains. We studied one to three (out of twelve) thecae and one to three (out of three) placentas in a flower.

Calculation of pollen-ovule index was performed according to the method described by CRUDEN (1977). We estimated the number of pollen grains produced by a given flower by multiplying the average number of grains in the studied thecae of this flower by 12, according to the presence of six stamens in a flower and the dithecal structure of anthers. We estimated the number of ovules in a given flower of *T. mucronata* by summing up the numbers of ovules in all three placentas of the flower. For *T. annamensis* and *T. javanica*, the number of ovules in a given flower was determined by multiplying the number of ovules in one placenta by three. The pollen-ovule ratio for a species was calculated by dividing the average number of pollen grains in a flower by the average number of ovules in a flower.

## Results

### Flowering season of *Thismia* in Chu Yang Sin National Park

In our field investigations in Chu Yang Sin National Park in 2019, we apparently observed the very beginning of the flowering period of the three species of *Thismia*. We recorded a synchronous emergence of several groups of individuals of *T. mucronata* on May 21. All individuals of *T. annamensis* and two of the three individuals of *T. puberula* were found on May 27, in spite of intensive search in the previous days.

### Phenology of flowers and inflorescences of *Thismia mucronata*

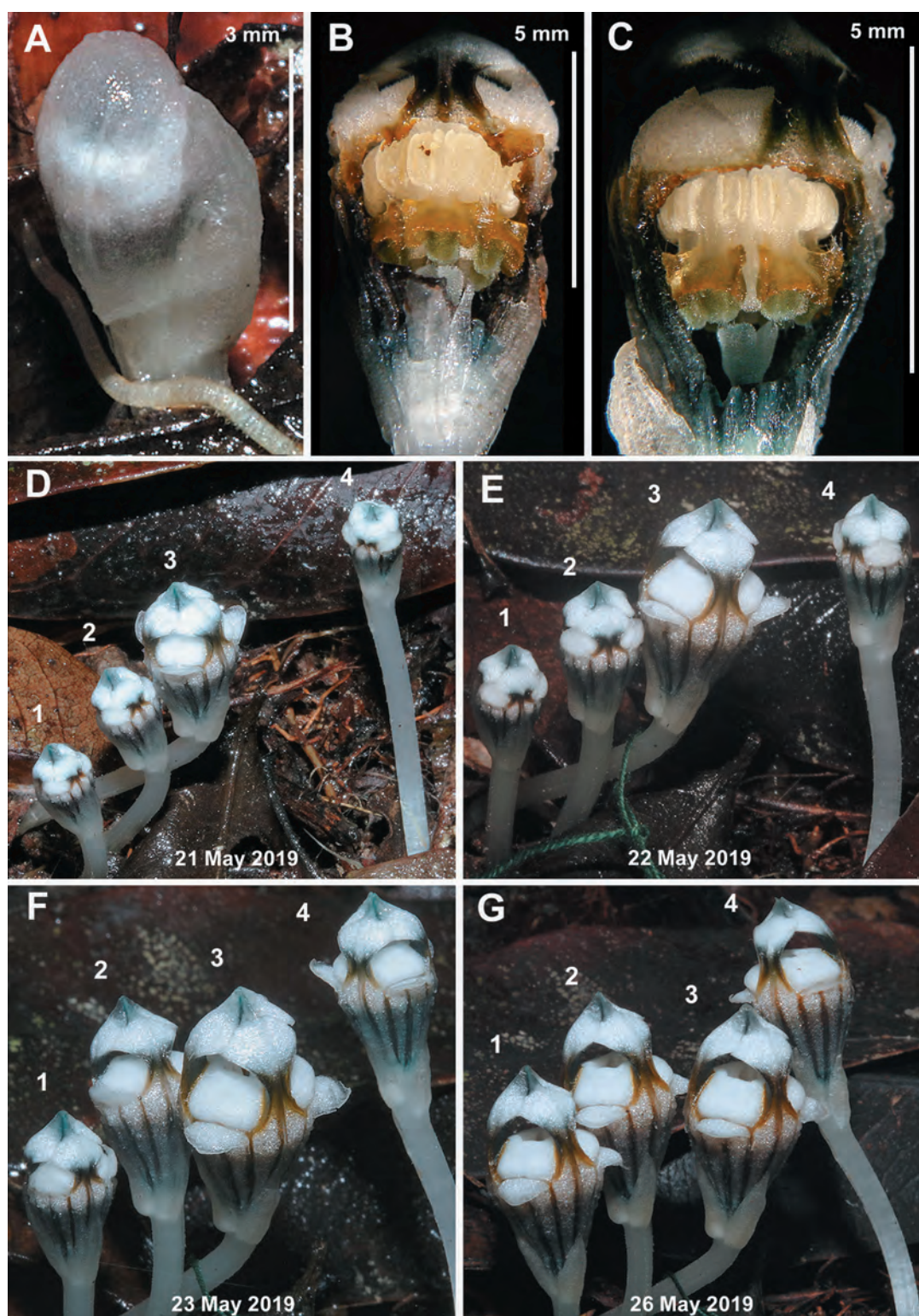
We describe the detailed floral phenology for *T. mucronata*, because for this species the most comprehensive data were obtained. The overall duration of flower growth from definitive bud to anthetic flower was estimated by summarizing durations of phenological stages observed in different flowers.

The first part of the inflorescence becoming visible above leaf litter is the terminal floral bud, which is initially fully covered by three involucre bracts (Fig. 1A). At the moment of plant emergence from the leaf litter, the floral bud is 3 mm high. At this stage, all the floral parts are completely formed, but free basal parts of the inner tepals have yellow coloration and remain short, so that the mitre is tightly appressed to the annulus, the outer tepals are appressed to the mitre, the mitre appendages and the hypanthium are dark-coloured, and the hypanthium has well distinguishable brown vertical ribs.

Since the stage of 3 mm in height, the floral bud enlarges during five days until it outgrows the involucre bracts and becomes ca 6 mm in height (Fig. 1D: flowers 1, 2, 4). At this stage, the bases of the inner tepals become brown, their free parts slightly elongate and the mitre rises insignificantly above the annulus. The outer tepals remain appressed to the mitre. The ribs on the hypanthium become black.

The flower continues to increase in size during its further development for two or three days. During this period, the outer tepals bend away from the mitre and occupy a vertical position; after this event, the flower reaches ca 9 mm in height. Extension of free parts of the inner tepals leads to some further elevation of the mitre over the annulus. Diameter of the mitre base is equal to the width of the hypanthium at this stage (Fig. 1E: flower 4, 1F: flower 1). When the bud is 6–7 mm in height, the anther thecae dehisce (Fig. 1B, C; see also Table 7), but the flower is most probably still unavailable for visitors because of the limited space for entrance between the mitre and the annulus, and also because of the vertical position of the outer tepals which close the ‘windows’ between the mitre and the annulus. The dehiscence of thecae at the stage of underdeveloped floral bud was also recorded in *T. annamensis* based on investigation of fixed material.

Another five days are needed for the flower to open after resuming the previous stage (i.e. since it is ca 9 mm in height). During this final period, the outer tepals occupy a spreading position, and free parts of inner tepals extend resulting in the opening of the ‘windows’ between the mitre and the annulus. Hypanthium enlarges in width and height, so that its upper part becomes wider than the mitre base (Fig. 1E: flower 3, 1F: flower 3, 1G). During this stage, the flower reaches up to 17 mm in height, and its appearance undergoes no further transformations after that. Flowers of the next order are well distinguishable at this stage; when the terminal flower is 6–9 mm or smaller, the flower bud of the next order is less than 2 mm high.



**Figure 1.** Floral phenology of *Thismia mucronata*. A – terminal floral bud ca 3 mm high covered by three involucral bracts; B – artificially opened floral bud with undeveloped thecae (height of bud 8 mm); C – artificially opened floral bud with developed thecae (height of bud 10 mm); D–G – group of four individuals (indicated as 1–4) depicted on several subsequent days.

Thus, the growth of the flower from the definitive bud (3 mm high) to its opening (at the size of up to 17 mm high) takes approximately 13 days. The recorded duration of anthesis (beginning from the completely opened flower) is 7 days; not a single flower has been traced from its opening till withering, suggesting that the actual anthesis is considerably longer.

### Floral visitors

Summarized information on floral visitors and study time is presented in Table 2 for *Thismia mucronata* and in Table 3 for *T. puberula*.

Visitors from the following groups were captured during direct observations of anthetic *T. mucronata*: moss mites (Acari), sap beetles (Nitidulidae, Coleoptera), gall midges (Cecidomyiidae, Diptera), fungus gnats (Mycetophilidae, Diptera), scuttle flies (Phoridae, Diptera), Ceraphronidae, Diapriidae, Mymaridae (Hymenoptera), ants (Formicidae, Hymenoptera) and some unidentified insects. For *T. puberula*, a similar set of visitors was captured: fungus gnats (Mycetophilidae, Diptera), Scatopsidae (Diptera), Braconidae, Ceraphronidae, Diapriidae, Figitidae, Scelionidae (Hymenoptera) and some unidentified insects. All captured Scelionidae are male individuals of the genus *Telenomus* (Telenomini, Telenominae). In addition, an individual scuttle fly (Phoridae, Diptera) was found in a fixed flower of *T. annamensis* (Yudina, Nuraliev 16).

The captured beetles and moss mites were observed to visit the tepals, but they apparently have not intended to enter the flower chamber. Fungus gnats and an individual of Cecidomyiidae probably entered the flower because they were captured during the regular catching with exhaustor; their presence inside the flower could not be confirmed because the inner space of the floral chamber is invisible from the outside. The ants (registered by video camera) and scuttle flies (found in the alcohol-preserved flower) are the only taxa confirmed to visit the floral chamber.

The results of investigations of pollen in the washings from insect bodies are presented in Table 4. Among the visitors of *T. mucronata*, pollen grains belonging to *Thismia* were found only in the washing from a single scuttle fly (Phoridae, Diptera). This fly was recorded to carry six pollen grains of *Thismia*. Totally 35 *Thismia* pollen grains were found in washings from Braconidae, Diapriidae and Scelionidae (Hymenoptera) that visited the flower of *T. puberula*. Many of the visitors of both *T. mucronata* and *T. puberula*, including those confirmed to bear pollen of *Thismia*, were also found to carry pollen belonging to other (unidentified) angiosperm taxa.

### Pollen-ovule ratio

Complete data on countings of pollen and ovules are presented in Tables 5 and 6. Final results are given in Table 7.

Pollen-ovule ratio is estimated as 26.26 for *T. annamensis*, 83.51 for *T. javanica*, 29.98 for *T. mucronata*. Thus, the highest ratio was obtained for *T. javanica*, and the ratios of *T. annamensis* and *T. mucronata* are essentially similar.

## Discussion

### Floral phenology: Possible dichogamy in *Thismia*

Our observations on *Thismia annamensis* and *T. mucronata* showed that the anther thecae of these species dehisce at the stage when the flower bud is 6–7 mm long (see Table 7), whereas the anthetic flower is 11–22 mm and 12–17 mm long, correspondingly (NURALIEV et al. 2020).

**Table 2.** Numbers of visitors captured on flowers of *Thismia mucronata*.

| Interval of observation        | 08:00 – 10:00 | 10:00 – 12:00 | 12:00 – 14:00 | 14:00 – 16:00 | 16:00 – 18:00 | 18:00 – 20:00 |
|--------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Duration of observation (min.) | 210           | 570           | 480           | 150           | 60            | 60            |
| ACARI                          |               | 1             |               |               |               |               |
| COLEOPTERA:                    |               |               |               |               |               |               |
| Nitidulidae                    | 1             |               |               |               |               |               |
| DIPTERA:                       |               |               |               |               |               |               |
| Cecidomyiidae                  |               |               | 1             |               |               |               |
| Mycetophilidae                 | 1             |               |               |               |               |               |
| Phoridae                       |               | 1             |               |               |               |               |
| Undetermined Diptera           | 1             |               |               |               |               |               |
| HYMENOPTERA:                   |               |               |               |               |               |               |
| Ceraphronidae                  |               | 1             |               |               |               |               |
| Diapriidae                     |               | 1             | 1             |               |               |               |
| Formicidae                     | 2             |               |               | 1             |               |               |
| Mymaridae                      |               | 1             |               |               |               |               |
| Undetermined tiny insects      | 1             | 2             |               |               |               |               |

The precocious beginning of stamen functioning is indicative of protandry, although we have not investigated the timing of stigma receptivity. An intriguing fact is that the flower opens (and both anthers and stigmas become available for visitors) significantly later than the thecae dehisce. This pattern is common for plants with secondary pollen presentation, but in *Thismia* the pollen is apparently uniformly presented directly from the thecae. The functional significance of early theca dehiscence in *Thismia* remains thus unclear.

**Table 3.** Numbers of visitors captured on the flower of *Thismia puberula*.

| Interval of observation        | 04:00 – 06:00 | 06:00 – 08:00 | 08:00 – 10:00 | 10:00 – 12:00 | 12:00 – 14:00 | 14:00 – 16:00 | 16:00 – 18:00 | 18:00 – 20:00 |
|--------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Total observation time (min.)  | 32            | 192           | 432           | 306           | 856           | 392           | 146           | 128           |
| DIPTERA:                       |               |               |               |               |               |               |               |               |
| Mycetophilidae                 |               |               | 4             |               |               |               |               |               |
| Scatopsidae                    |               |               |               |               | 1             |               |               |               |
| HYMENOPTERA:                   |               |               |               |               |               |               |               |               |
| Braconidae                     |               |               |               |               |               | 1             |               |               |
| Ceraphronidae                  |               |               |               |               | 1             |               |               |               |
| Diapriidae                     |               |               | 1             |               |               | 1             |               |               |
| Figitidae                      |               |               |               |               |               | 1             |               |               |
| Scelionidae (Telenomus, males) |               |               | 5             | 1             |               |               |               |               |
| Undetermined tiny insects      |               |               |               | 1             |               | 1             |               |               |

**Table 4.** Numbers of pollen grains found in washings from floral visitors of *Thismia mucronata* and *T. puberula*.

|                          | Visitors                  | Number of individuals       | $\Sigma$ pollen grains of <i>Thismia</i> | $\Sigma$ other pollen grains |
|--------------------------|---------------------------|-----------------------------|------------------------------------------|------------------------------|
| <i>Thismia mucronata</i> | ACARI                     | 1                           | 0                                        | 0                            |
|                          | COLEOPTERA                | 1                           | 0                                        | 0                            |
|                          | DIPTERA:                  |                             |                                          |                              |
|                          | Cecidomyiidae             | 1                           | 0                                        | 1                            |
|                          | Mycetophilidae            | 1                           | 0                                        | 1                            |
|                          | Phoridae                  | 1                           | 6                                        | 4                            |
|                          | Undetermined Diptera      | 1                           | 0                                        | 3                            |
|                          | HYMENOPTERA:              |                             |                                          |                              |
|                          | Ceraphronidae             | 1                           | 0                                        | 5                            |
|                          | Diapriidae                | 2                           | 0                                        | 2                            |
|                          | Formicidae                | 1                           | 0                                        | 0                            |
|                          | Mymaridae                 | 1                           | 0                                        | 0                            |
|                          | Undetermined tiny insects | 1                           | 0                                        | 18                           |
| <i>Thismia puberula</i>  | DIPTERA:                  |                             |                                          |                              |
|                          | Mycetophilidae            | 4                           | 0                                        | 2                            |
|                          | Scatopsidae               | 1                           | 0                                        | 0                            |
|                          | HYMENOPTERA:              |                             |                                          |                              |
|                          | Ceraphronidae             | 1                           | 0                                        | 4                            |
|                          | Braconidae                | 1                           | 14                                       | 0                            |
|                          | Diapriidae                | 2 (1 carried pollen grains) | 10                                       | 7                            |
|                          | Figitidae                 | 1                           | 0                                        | 0                            |
|                          | Scelionidae               | 6 (2 carried pollen grains) | 11                                       | 7                            |
|                          | Undetermined tiny insects | 2                           | 0                                        | 0                            |

MAR & SAUNDERS (2015) tentatively described *T. hongkongensis* to be protandrous; however, their assumption is based on the absence of pollen in thecae of flowers at late anthesis, when both male and female functionality of the flower could possibly be over. GUO et al. (2019) revealed synchronous functioning of thecae and stigmas (the latter estimated by a receptivity test) in *T. tentaculata*. In total, there are currently no reliable evidences of dichogamy in *Thismia*, but the available data indicate presence of some unusual patterns in timing of functioning of its floral parts.

### Possible pollinators of *Thismia*

In our study, flowers of *Thismia* were visited by a wide taxonomic range of insects and other small invertebrates. Of them, individuals belonging to a species of scuttle flies (Phoridae, Diptera) and

**Table 5.** Numbers of pollen grains in flowers of *Thismia* (all studied flowers had undehisced thecae).

| Specimen                                                        | No of flower | Height of flower bud, mm | No of theca | Number of pollen grains in theca | Number of pollen grains in flower |
|-----------------------------------------------------------------|--------------|--------------------------|-------------|----------------------------------|-----------------------------------|
| <i>Thismia annamensis</i> (Nuraliev 1562)                       | 1            | 6.3                      | 1.1         | 3528                             | 42336                             |
|                                                                 | 2            | 6.8                      | 2.1         | 4161                             | 49932                             |
| <i>Thismia javanica</i> (Do Thi Xuyen, Nguyen Tan Hieu BHH 800) | 1            | 6.2                      | 1.1         | 6827                             | 81924                             |
| <i>Thismia mucronata</i> (Nuraliev 1009)                        | 1            | 7.5                      | 1.1         | 3454                             | 41448                             |
| <i>Thismia mucronata</i> (Yudina, Nuraliev 6)                   | 2            | 5.1                      | 2.1         | 2831                             | 35406                             |
|                                                                 |              |                          | 2.2         | 3070                             |                                   |
|                                                                 | 3            | 5.9                      | 3.1         | 4355                             | 58492                             |
|                                                                 |              |                          | 3.2         | 5074                             |                                   |
|                                                                 |              |                          | 3.3         | 5194                             |                                   |

three families of Hymenoptera were shown to carry pollen of *Thismia*. At the same time, only ants (direct observations) and a scuttle fly (found in the alcohol-preserved flower of *T. annamensis*) are confirmed to visit the floral chamber, while the other insects were captured by exhaustor and could possibly visit the outer flower surface or even fly extremely close to it (although we have not intended to capture insects in such cases).

The scuttle flies (Phoridae) are widely known to pollinate tropical plants characterized by a flower with a narrow entrance. For example, they act as pollinators of *Aristolochia littoralis* D. Parodi (Aristolochiaceae) (HALL & BROWN 1993), *Aspidistra phanluongii* Vislobokov (Asparagaceae) (VISLOBOKOV et al. 2013), *Pleurothallis ochreatea* Lindl. and *P. teres* Lindl. (Orchidaceae) (BORBA 2001). The family Phoridae has already been discussed with respect to pollination of Thismiaceae: CHEEK et al. (2019) suggested *Megaselia* as pollinators for *Afrothismia kupensis*; in addition, a wing of a scuttle fly was found in the floral chamber of *T. hongkongensis* by MAR & SAUNDERS (2015). In our investigation, two individuals of Phoridae were recorded, one of them captured from a flower of *T. mucronata* with pollen of *Thismia* on its body, and the other found in a flower of *T. annamensis*. Although it is impossible to quantitatively estimate the effectiveness of these insects as pollinators based on the available data, our results together with other findings regarding the role of Phoridae in reproductive ecology of various plants lead to a conclusion that this group is the most possible pollinator of *Thismia* within our study.

Contrary to expectations, only five individuals of fungus gnats (Mycetophilidae) were captured during our observations of *Thismia*, and only non-*Thismia* pollen was found in their washings. In this respect, our results contradict those obtained by GUO et al. (2019) who revealed fungus gnats as the most probable pollinators of *T. tentaculata*. Fungus gnats are known to pollinate a wide range of angiosperms, e.g. *Arisaema serratum* (Thunb.) Schott (Araceae) (SASAKAWA 1994), *Scoliopus bigelovii* Torr. (Liliaceae), *Listera cordata* (L.) R. Br. (Orchidaceae) (ACKERMAN & MESLER 1979; MESLER et al. 1980), including those with flowers placed at the ground level among leaf litter and having narrow entrance, such as *Aspidistra formosa* Aver. & Tillich and *A. subrotata*

**Table 6.** Numbers of ovules in flowers of *Thismia*. The two doubtful figures are supposed to be erroneous, and were excluded from the countings.

| Specimen                                                           | Nº of flower | Nº of placenta | Number of ovules in placenta | Number of ovules in flower |
|--------------------------------------------------------------------|--------------|----------------|------------------------------|----------------------------|
| <i>Thismia annamensis</i><br>(Nuraliev 1562)                       | 3            | 3.1            | 563                          | 1689                       |
|                                                                    | 4            | 4.1            | 608                          | 1824                       |
| <i>Thismia javanica</i><br>(Do Thi Xuyen, Nguyen Tan Hieu BHH 800) | 2            | 2.1            | 327                          | 981                        |
| <i>Thismia mucronata</i><br>(Nuraliev 1009)                        | 4            | 4.1            | 115 (doubtful figure)        | 1452                       |
|                                                                    |              | 4.2            | 484                          |                            |
|                                                                    |              | 4.3            | 145 (doubtful figure)        |                            |
|                                                                    | 5            | 5.1            | 604                          | 1836                       |
|                                                                    |              | 5.2            | 622                          |                            |
|                                                                    |              | 5.3            | 610                          |                            |
|                                                                    | 6            | 6.1            | 527                          | 1449                       |
|                                                                    |              | 6.2            | 439                          |                            |
|                                                                    | 7            | 7.1            | 464                          | 1282                       |
|                                                                    |              | 7.2            | 416                          |                            |
|                                                                    |              | 7.3            | 402                          |                            |

Y. Wan & C.C. Huang (Asparagaceae) (VISLOBOKOV 2017). Thus, it is highly probable that Mycetophilidae are important pollinators of various species of *Thismia*, although our results do not provide a direct confirmation of this idea. It is noteworthy that in the experiments conducted by Guo et al. (2019) yellow was the most attractive colour for fungus gnats, whereas white was the least attractive. Given that both *Thismia mucronata* and *T. puberula* studied here are characterized by predominantly white flowers, the colour preferences can be assumed as a possible explanation of the difference in results, and in general, of the uneven role of fungus gnats in reproductive biology of *Thismia* with different floral types.

The ants (Formicidae) are known to be hardly capable of pollination, because they produce myrmicacin, which inhibits growth of pollen (BEATTIE et al. 1984). However, ants were identified as pollinators of species of *Borderea* Miégev. (Dioscoreaceae, Dioscoreales) (GARCÍA et al. 1995, 2012). Although ants were registered to enter the floral chamber of *Thismia mucronata*, we assume them to unlikely pollinate *Thismia* based on the absence of pollen of *Thismia* in the washing from a single captured individual, as well as on the general idea of rarity of ant pollination.

Our finding of pollen of *Thismia* in washings of several individuals of Hymenoptera is remarkable. All the captured Hymenoptera (with the exclusion of ants), i.e. Braconidae, Ceraphronidae, Diapriidae, Figitidae, Mymaridae and Scelionidae, are parasitic upon other insects (MATTEWS 1974; JOHNSON 1984; NOYES & VALENTINE 1989; WHITFIELD 1998; MASNER & GARCÍA 2002; BUFFINGTON et al. 2007). Parasitic Hymenoptera in general are not known to be involved into pollination of any plants. Moreover, all six studied individuals of Scelionidae are males

**Table 7.** Pollen-ovule ratios in species of *Thismia*, and timing of thecae dehiscence.

| Species              | Number of studied flowers | Maximum height of flower with undehisced thecae | Minimum height of flower with dehisced thecae | Number of studied thecae | Average number of pollen grains in flower | Numbers of studied placentas | Average number of ovules in flower | Pollen-ovule ratio |
|----------------------|---------------------------|-------------------------------------------------|-----------------------------------------------|--------------------------|-------------------------------------------|------------------------------|------------------------------------|--------------------|
| <i>T. annamensis</i> | 4                         | 6.8                                             | 6.3                                           | 2                        | 46134                                     | 2                            | 1756.5                             | 26.26              |
| <i>T. javanica</i>   | 2                         | 6.2                                             | no data                                       | 1                        | 81924                                     | 1                            | 981                                | 83.51              |
| <i>T. mucronata</i>  | 9                         | 5.9                                             | 7.2                                           | 6                        | 45115.4                                   | 9                            | 1504.75                            | 29.98              |

of *Telenomus* (Telenominae: Telenomini) captured in a single four-hour interval (and five of them in a two-hour interval); they have probably developed from the same host egg-laying (Alexander Timokhov, personal communication), which may be the reason of the presence of several individuals at the studied place at the same time. Thus, the biology of the captured Hymenoptera precludes us from considering them as possible pollinators of *Thismia*. At the same time, the finding of angiosperm pollen (and especially that of *Thismia*) on their bodies remains unexplained.

### Pollination syndrome of *Thismia*

Earlier investigations (see above) together with the current study are in agreement with respect to identification of *Thismia* as dipteran-pollinated plants. This idea is based on myiophilous syndrome of floral features exhibited by *Thismia*, as well as on direct observations. More specifically, representatives of *Thismia* appear to closely meet the syndrome of sapromyiophily outlined by FAEGRI & VAN DER PIJL (1979) in having the deep lantern-like floral chamber with reproductive organs hidden inside it, ‘windows’ between the mitre and the annulus, filiform appendages of tepals, narrow gaps between stamen filaments, various appendages and hairs on stamens and a dull flower coloration with dark spots. FAEGRI & VAN DER PIJL (1979) listed Burmanniaceae (which at that time was commonly considered to comprise Thismiaceae) among the typical sapromyiophilous families. One more remarkable trait typical of sapromyiophilous pollination units is the presence of a trap (FAEGRI & VAN DER PIJL 1979). Direct observations of fungus gnats restrained within flowers of *T. tentaculata* (GUO et al. 2019) confirm that the floral chamber acts as a trap in *Thismia*.

Sapromyiophilous plants with trap flowers are known to be pollinated by representatives of various dipteran families, including Ceratopogonidae (HEIDUK et al. 2017), Chloropidae (AUTTAMA et al. 2018), Drosophilidae (HEIDUK et al. 2017), Milichiidae (HEIDUK et al. 2010, 2017; AUTTAMA et al. 2018), Mycetophilidae (PHILLIPS et al. 2014), Phoridae (HEIDUK et al. 2017; MARTIN et al. 2017), Scatopsidae (HEIDUK et al. 2017) and Sciaridae (HEIDUK et al. 2010). The recorded (putative) pollinators of various species of *Thismia* fall within this diversity: representatives of Phoridae (MAR & SAUNDERS 2015; this study) and Sciaridae (GUO et al. 2019), as well as several unidentified small dipteran insects (LI & BI 2013; MAR & SAUNDERS 2015), have been reported. Thus, the known interactions with the insects support the assignment of *Thismia* to the ecological group of sapromyiophilous trap-flowered plants.

## Pollination ecology of *Thismia* inferred from the pollen-ovule ratio

As it was shown by CRUDEN (1977), pollen-ovule ratios are correlated with breeding systems in angiosperms. Within CRUDEN's (1977) sampling, cleistogamous species are characterized by the lowest ratios ( $4.7 \pm 0.7$ ), followed by obligately autogamous species ( $27.7 \pm 3.1$ ), then by facultatively autogamous species ( $168.5 \pm 22.1$ ). Facultative xenogamy and (strict) xenogamy are associated with the highest ratios ( $796.6 \pm 87.7$  and  $5859.2 \pm 936.5$ , respectively). The ratios estimated in this study for *T. annamensis* (26.26) and *T. mucronata* (29.98) evidence in favor of obligate autogamy. The ratio obtained for *T. javanica* (83.51) is intermediate between those indicated by CRUDEN (1977) for obligately autogamy and facultative autogamy. Given that the number of studied flowers is small (15 in total) and the difference between the ratios of *T. javanica* and the other species is not so crucial, we conclude that the three studied species are characterized by a similar reproductive system. Thus, our data, representing the first estimation of pollen-ovule ratio for *Thismia*, predict autogamy in this genus.

Autogamy is known to occur rather widely among Dioscoreales; for example, it has been reported for *Burmannia larseniana* D.X. Zhang & R.M.K. Saunders (ZHANG & SAUNDERS 1999), *B. wallichii* Hook. f. (ZHANG & SAUNDERS 2000), several species of Taccaceae (ZHANG et al. 2005; CHUA et al. 2020). Taccaceae are at the same time the only lineage within Dioscoreales hitherto studied with respect to pollen-ovule ratio. These studies revealed a considerable diversity of reproductive strategies in *Tacca* J.R. Forst. & G. Forst., the only genus of Taccaceae. Whereas *Ta. bibracteata* Drenth is facultatively xenogamous according to its pollen-ovule ratio (221.0), *Ta. borneensis* Ridl. (p/o 42.1), *Ta. cristata* Jack (p/o 53.8), *Ta. havilandii* S.Y. Wong & K.S. Chua (p/o 74.3), *Ta. leontopetaloides* (L.) Kuntze (p/o 144.1) and *Ta. reducta* P.C. Boyce & S. Julia (p/o 93.1) are considered to be facultatively autogamous (CHUA et al. 2020). All these species of *Tacca* are protogynous, and all of them are pollinated by female biting midges (Ceratopogonidae, Diptera), except for *Ta. bibracteata* for which the pollinators are unknown (CHUA et al. 2020). One can infer that in *Tacca* the pollen-ovule ratio is indicative of the efficiency of pollen transfer, rather than the autogamy vs xenogamy, especially if the presence of dichogamy, a mechanism preventing autogamy, is considered (also see CHUA et al. 2020). On the other hand, *Ta. chantrieri* André has been demonstrated to be actually predominantly autogamous, consistently with its low pollen-ovule ratio (49): its bagged flowers had high seed set, and the only floral visitors revealed during two years of observations were bees (Hymenoptera) of the genus *Trigona* (ZHANG et al. 2005). The authors suggest explanations of the discrepancy between the reproductive strategy of *Ta. chantrieri* and its sapromyiophilous appearance by a relict nature of sapromyiophily for this species and by variability of breeding systems in its different populations (ZHANG et al. 2005).

The example of *Tacca* shows that in *Thismia* possibly also only indirect relationships exist between the pollen-ovule ratio and the aspects of species pollination, and low values are not necessarily related to autogamy. Apparently, it is premature to judge about pollination biology of *Thismia* solely on the basis of the pollen-ovule ratio.

## Pollination system of *Thismia*: autogamy vs xenogamy

A set of features of floral morphology indicate xenogamy in *Thismia*. In particular, extrorse anthers, presence of supraconnectives, stamen tube, distant arrangement of anthers and stigmas known in *Thismia* (MERCKX et al. 2013; NURALIEV et al. 2021) were evaluated by ORNDUFF (1969) as associated with xenogamous plants. The possible presence of protandry in *Thismia* is

also consistent with the idea of xenogamy. Xenogamy has already been confirmed for a single species of the genus, *T. tentaculata* (GUO et al. 2019).

On the other hand, there are several indirect indications in favor of autogamy in *Thismia*. First, one can consider our data on pollen-ovule ratio as indicative of autogamy, although other reasons of this phenomenon are possible, as discussed above. Second, *Thismia* was demonstrated to be self-compatible (GUO et al. 2019). Self-compatibility does not necessarily contradict specialized zoophilous pollination syndrome (such as sapromyophily proposed for *Thismia* above): in fact, a number of self-compatible (and at least partly autogamous) mycoheterotrophic plants are supposed to employ pollinators as mediators for self-pollination (e.g. TAKAHASHI et al. 1993; LEHNEBACH et al. 2005; WATERMAN et al. 2013), thus performing facilitated autogamy (sensu CARDOSO et al. 2018). Furthermore, self-pollination is known in several xenogamous mycoheterotrophic species, where it is believed to serve as a reserve mechanism for situations, when outcrossing is unsuccessful (HENTRICH et al. 2010; SUETSUGU 2013, 2015).

Finally, a recent palynological research of several Asian species of *Thismia* reported them to possess a number of features typical for non-zoophilous angiosperms, including small size of pollen grains and the lack of sporoderm sculpture (SEVEROVA et al. 2021). This finding is in contradiction with the supposed myiophilous syndrome in *Thismia*. Since abiotic cross-pollination is apparently impossible in *Thismia* due to its complicated floral structure, pollen morphology supports the idea of autogamy in this genus.

In addition, it should be noted that vegetative reproduction is considered to be important for mycoheterotrophic plants as a way to fulfill their specialized habitat requirements and maintain symbiosis with fungi for long periods of time (LEAKE 1994). Species of *Thismia*, including *T. annamensis*, *T. javanica*, *T. mucronata* and *T. puberula*, are known to reproduce by root suckers (MAAS et al. 1986; MAAS-VAN DE KAMER 1998; NURALIEV et al. 2020). There is thus a possibility that in *Thismia* the vegetative reproduction prevails over sexual reproduction. This assumption is in concordance with the scarcity of known populations of *Thismia* and the apparent rarity of pollination events. On the other hand, available images of post-anthetic individuals of *Thismia* (LARSEN & AVERYANOV 2007: Fig. 2; MAR & SAUNDERS 2015: Fig. 3; GUILHERME et al. 2016: Fig. 2; SITI-MUNIRAH & DOME 2019: Fig. 4) show that they have a significant fruit set, and a fruit set of 21% was reported for *T. tentaculata* by GUO et al. (2019), which indicates considerable level of successful fertilization.

All in all, a considerable set of the evidences are in concordance with the presence of xenogamy in *Thismia*. At the same time, a certain role of autogamy (with insect-mediated and/or pollinator-independent self-fertilization) cannot be excluded. Vegetative reproduction by root suckers possibly also plays an important role in the maintenance of the population size in *Thismia*.

## Conclusions

We provide for the first time results of direct observations of the flowering of two Asian species of *Thismia*, *T. mucronata* and *T. puberula*, as well as the first attempt to infer pollination system from the pollen-ovule ratio in Thismiaceae. Among the taxonomically diverse floral visitors observed and captured, we recognized scuttle flies (Phoridae, Diptera) as possible pollinators, in accordance with the presence of pollen of *Thismia* on their bodies and the general ideas on biology

of these insects. *Thismia annamensis* and *T. mucronata* are found to have precocious dehiscence of anther thecae, i.e. before the flower is fully developed and become available for pollinators; this phenomenon possibly indicates protandry in these species.

We report the pollen-ovule ratio of three species of *Thismia* to range within 26 and 84. We argue that such low values, which are generally characteristic of autogamous plants, can also indicate some other reasons of high efficiency of pollen transfer in *Thismia*.

Species of *Thismia* exhibit sapromyiophilous pollination syndrome with trap flowers, which is in concordance with their known interactions with dipteran insects. Important evidences in favor of both autogamy and xenogamy in *Thismia* are found. It is likely that both types of fertilization occur in *Thismia*, with the ratio between them depending on pollinator behaviour, which, in turn, is influenced by environmental factors (air temperature, humidity, rain, wind, cloudiness) (McCALL & PRIMACK 1992; INOUE et al. 2015; AUTTAMA et al. 2018). In addition, vegetative reproduction by root suckers probably plays an important role in maintenance of the population size in *Thismia*.

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