Plant Ecology and Evolution 154 (1): 39–48, 2021
https://doi.org/10.5091/plecevo.2021.1747

RESEARCH ARTICLE

Poor fruit set due to lack of pollinators in *Aristolochia manshuriensis* (Aristolochiaceae)

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Background and aims – Interactions of insects with trap flowers of *Aristolochia manshuriensis*, a relic woody liana with fragmented natural populations from south-eastern Russia, were studied. Pollination experiments were conducted to identify the causes of the poor fruit set in this plant.
Material and methods – The study was carried out at two ex situ sites within the natural range of *A. manshuriensis* in the suburban zone of the city of Vladivostok (Russia). The floral morphology was examined to verify how it may affect the process of pollination in this species. To test for a probability of self-pollination, randomly selected flowers at the female phase of anthesis (day 1 of limb opening) were hand-pollinated with pollen from the same plant. The daily insect visitation was studied. The pollen limitation coefficient and the number of visitors to the flowers were determined. To identify insects that lay eggs on the flowers, the insects were reared from eggs collected from fallen flowers. Both caught and reared insects were identified.
Key results – The floral morphology and the colour pattern of *A. manshuriensis* are adapted to temporarily trap insects of a certain size. The hand-pollination experiment showed that flowers of this plant are capable of self-pollination by geitonogamy and require a pollinator for successful pollination. The positive value (2.64) for the pollen limitation coefficient indicates a higher fruit set after hand-pollination compared to the control without pollination. The number of visitors to the flowers was low (0.17 visitors per flower per day). Insects from three orders were observed on the flowers: Diptera (up to 90.9%), Coleoptera (8.3%), and Hymenoptera (0.8%). Four species of flies (*Scaptomyza pallida*, *Drosophila transversa* (Drosophilidae), *Botanophila fugax*, and *Botanophila* sp. 1 (Anthomyiidae)) are capable of transferring up to 2500–4000 pollen grains on their bodies and can be considered as pollinators of *A. manshuriensis*. Data of the rearing experiment indicate that flies of the families Drosophilidae (*S. pallida*, *D. transversa*), Chloropidae (*Elachiptera tuberculifera, E. sibirica*, and *Conioscinella divitis*), and Anthomyiidae (*B. fugax*, *B.* sp. 1) use *A. manshuriensis* flowers to lay eggs. Beetles were also collected from the flowers, but they were probably not involved in pollination, because no pollen grains were observed on them during our study.
Conclusions – Pollinators of *A. manshuriensis* include mainly Diptera that lay eggs on the flowers. The poor fruit set (2%) in *A. manshuriensis* is associated with pollen limitation due to the lack of pollinators, as the number of visitors to flowers was extremely low. This may be due to the fact that the flowers of this species are highly specialized on insects of a certain size for pollination.

Keywords – *Aristolochia manshuriensis*; fruit set; insect rearing; plant-insect interactions; pollination.

Plant Ecology and Evolution is published by Meise Botanic Garden and Royal Botanical Society of Belgium
ISSN: 2032-3913 (print) – 2032-3921 (online)

INTRODUCTION

Aristolochia L., a genus of the angiosperm family Aristolochiaceae Juss. (Chevallier 1996; Kelly & González 2003; González et al. 2014), is characterized by a highly specialized pollination system. Flowers of many *Aristolochia* species are adapted in structure and colour to sapromyophily (carrion-fly pollination). The floral features of sapromyophily can be identified as follows: the brightly coloured inner side of perianth and the discrete (or dim) colouring of the outer side (dark brown, purple, or green); a perianth with a window area; the presence of osmophores (odorous glands); the missing nectar paths and nectar; etc. (Faegri & van der Pijl 1979; Vogel 1990; Proctor et al. 1996; Burgess et al. 2004). *Aristolochia* species have peculiar, protogynous flowers which can temporarily trap their pollinators, small dipteran insects from different families (Wolda & Sabrosky 1986; Razzak et al. 1992; Sakai 2002; Burgess et al. 2004; Murugan et al. 2006; Trujillo & Sérsic 2006; Valdivia & Niemeyer 2007; Rulik et al. 2008; Berjano et al. 2009; Hipólito et al. 2012; Stotz & Gianoli 2013; Oelschlägel et al. 2015, 2016; Aliscioni et al. 2017; Martin et al. 2017). These flowers attract flies primarily by their specific scent (Vogel 1990) and by mimicking sex-specific pheromones (Wolda & Sabrosky 1986) or the same scent components that insects (chloropids) use to find their food sources (Oelschlägel et al. 2015). The perianth in *Aristolochia* species consists of a modified tubular calyx (tube) with a chamber at its base (utricle) which surrounds the fused styles, stigmas, and anthers, collectively referred to as gynostemium (Pabón-Mora et al. 2015). The U-shaped tube, expanding at the terminal end, is often equipped with various trap-and-release mechanisms to keep insects inside the utricle until the anthers burst. Some *Aristolochia* species are described as adapted for capturing flies of a certain size (Brantjes 1980), or even a certain insect species (Razzak et al. 1992). Other species have developed peculiar mutualistic relationships, e.g. plants that provide breeding sites as a reward for pollination (Sakai 2002). Though flowers of the family Aristolochiaceae are adapted to cross-pollination, several species were shown to utilize self-pollination as a spare strategy (Sakai 2002; Trujillo & Sérsic 2006; Berjano et al. 2006, 2009; Bliss et al. 2013; Nakonechnaya et al. 2015). Moreover, cleistogamy was found in *A. serpentaria* L. (Pfeifer 1966).

Aristolochia species are generally distributed in tropical and subtropical regions, but some of them reach the temperate zone (Cronquist 1981). They have been found in North Korea, north-eastern China, and the south-eastern part of Russia. In the latter region, which remained ice-free during the Pleistocene glaciation, some members of the tropical flora, including two species of this genus, *A. manshuriensis* Kom. and *A. contorta* Bunge, have survived in refugia (Kurentsova 1968; Kozhevnikov et al. 2005). The former species is a woody liana relict, endemic to the Manchurian floristic region (Kitagawa 1979). It is a rare plant with fragmented natural populations. In Russia, *A. manshuriensis* is listed on the Russian Red Data Book as an endangered species (Nesterova 2008). It is a valuable medicinal plant (Akulova & Aleksandrova 1996). In southern Primorsky Krai, the known habitats of *A. manshuriensis* are located in the valleys of the Borisovka, Nezhinka, and Anan'evka rivers with their tributaries separated by ridges (Slizik 1978a, 1978b). In its natural habitat, *A. manshuriensis* grows in small groups of up to 20 lianas within an area of approximately up to 300 m^2 each (Nakonechnaya et al. 2014).

The perianth of *A. manshuriensis* is U-shaped, up to 9 cm in length, with a colourful limb. The limb colour varies between lianas from different populations: it can be either purplish or yellowish. No variation in limb colour on the same liana has been observed. The limb is 3-lobed, approximately 22–24 mm in diameter (Nakonechnaya et al. 2014). The variability in limb colouration may play a role in pollination by attracting more visitors (Nakonechnaya & Nesterova 2013). Moreover, the flower structure in *A. manshuriensis* is strictly adapted to cross-pollination. However, due to self-compatibility, there is a possibility of self-pollination by insects (geitonogamy) in this species (Nakonechnaya et al. 2008).

Aristolochia manshuriensis propagates via seeds only, with species reproduction rate being very low (Kurentsova 1968; Nakonechnaya et al. 2005, 2014). Previously, we found a remarkably poor fruit set in an *A. manshuriensis* plant grown ex situ (Nakonechnaya et al. 2014). Considering fruit set, at least two factors are remarkable in this species: the high bud loss (up to 50%) at early stages of development and the low level of successful pollination despite normal functioning of the reproductive organs (Nakonechnaya et al. 2005, 2006).

The aim of this study is to test if the poor fruit set is due to the lack of pollinators in *A. manshuriensis* (Aristolochiaceae). We therefore provide new data on its floral morphology, anthesis, and mode and efficiency of pollination. To identify the floral attractants, we study the inner perianth colouration and the time of anther opening and stigma receptivity. These data can be useful to better understand how the inner perianth morphology and colouration as well as the timing of floral receptivity may affect the process of pollination in this species. To verify the assumption of pollinator deficiency, we set up a hand-pollination experiment, conduct pollinator observations, compiling a list of visitors and finally compare both. We also determine which insects oviposit on the flowers by rearing insects from eggs collected from fallen flowers.

MATERIAL AND METHODS

Study sites and plants

The study was carried out at two sites in the suburban zone of the city of Vladivostok: (1) the Botanical Garden-Institute, Far Eastern Branch, Russian Academy of Sciences (43º22.38′N, 131º59.37′E), and (2) a private garden in the vicinity of the Sputnik railroad station (43º24.93′N, 132º04.19′E). Both sites are located within the native distribution range of *A. manshuriensis*. In the Botanical Garden, *A. manshuriensis* has been growing at two localities, Bg1 and Bg2 since 1990. In the private garden (Sp), *A. manshuriensis* has been growing since 1995. Six plants from the Botanical Garden and two from the private garden were used as the material for this study. Both sites have the same climate conditions. Due to the conservation status of *A.*

manshuriensis, no studies can be conducted within its natural habitat (Nesterova 2008).

Floral morphology

A total of 50 buds from three plants were randomly labelled for phenological studies of changes in the morphology of reproductive organs from the flower opening to the fall of the perianth. Every day, from flower opening till day 7, eight flowers out of the 50 labelled buds were cut in half. Images of the colour pattern inside the utricle, tube, and gynostemium were taken through a Stemi 2000C stereomicroscope (Carl Zeiss) and using the AxioVision 4.8 software.

To determine the phase of anthesis at each site, utricles of 20 randomly labelled flowers were carefully incised with a scalpel to make a T-shaped slit close to the gynostemium. Then, the slit was carefully opened to evaluate the state of anthers once a day for five days. To determine the time when stigmas become receptive to pollen during flower development, 25 stigmas at different stages of development and flowering were placed in a 1–2% $KMnO_4$ solution for 1–2 min (Robinsohn 1924). Finally, the stigmas were rinsed in distilled water for 5–10 min and examined under a compound microscope. Stigmas were considered receptive to pollen when they acquired a brownish hue after staining; non-receptive ones remained unstained.

Pollination experiment

A hand-pollination experiment was set up in 2017 on a single liana from the Botanical Garden (Bg1 locality), since no other lianas were available for experiments at that time. To test for the probability of self-pollination, 25 randomly selected flowers in the female phase of anthesis (day 1 of limb opening) were hand-pollinated with the pollen from the flowers in the male phase of anthesis collected from the same plant. The pollen was transferred to a stigma with a thin long wooden wand through the T-shaped slit close to the gynostemium. To prevent insects from entering the experimental flowers before the pollination experiment, the flowers with closed perianth were enclosed in a fine-meshed bag. After pollination, the flowers were also immediately enclosed in bags against insects. Two control groups were used in the experiment. In the first group (control 1), 50 randomly selected closed buds were bag-enclosed before the onset of anthesis to prevent insect visitation and left bag-enclosed for a month. In the second group (control 2), 150 randomly selected buds were left intact and insects were allowed to freely enter the flowers. The fraction of flowers that developed into fruit in the control 2 group can be considered as a natural fruit set at the study site.

The pollen limitation coefficient was calculated as the natural logarithm of the response ratio (R: $\ln R = \ln E - \ln C$, where E is the mean fruit set of the plants in the experiment and C is the mean fruit set of the control plants) (Vamosi et al. 2006). In our case, the hand-pollinated plants were the experimental group, and the intact flowers were used as the control.

Insect visitation

To identify species of insects that visited *A. manshuriensis*, insects trapped inside the flowers were randomly collected three days a week during three weeks of flowering from 20 randomly selected flowers from the Botanical Garden (Bg2) during the flowering seasons of 2009–2018. A Zeiss SteREO Discovery.V12 microscope with a digital video camera AxioCam MRc was used for the identification of the caught insects. The insects were identified by Drs Vasilii Sidorenko, Tatyana Markova, Sergey Shabalin, and other colleagues listed in the Acknowledgements.

The number of visitors to the flowers was calculated as the ratio of the total number of insects collected from the flowers during a day to the total number of inspected flowers during the same day. To determine the pollen load on visitors, all pollen grains were collected from each visitor with a needle. Unstained grains were placed on a microscope slide, covered with a cover slip, identified to species level, and counted under a Zeiss Stemi 2000C stereomicroscope. Then, the data on pollen load for each insect family was expressed in terms of mean and standard error.

According to our previous data (Nakonechnaya et al. 2014) over a few years of observations, the minimum number of viable seeds per fruit was approximately 50. Thus, this number of ovules should be successfully fertilized for the ovary to develop into fruit. If the number of ovules is fewer than 50, the fruit fails to develop. Hence, a visitor was considered as a pollinator when the pollen load was more than 50 pollen grains (for more details see Discussion).

Additionally, the daily insect visitation was studied in 100 randomly selected flowers for five days during the flowering season in May 2010. The flowers were randomly selected from six plants at two sites in the Botanical Garden (Bg1 and Bg2). Each day the flowers were inspected for visitors three times a day, between 10:00 and 11:00, 13:00 and 14:00, and 16:00 and 17:00. The insect visitors trapped in the utricles were collected from flowers without damaging the perianth and were fixed in 70% ethanol for subsequent identification.

Insect rearing

To identify species of the insects that had oviposited on the *A. manshuriensis* flowers, 50 freshly fallen flowers from three lianas (one liana per site) were collected from each locality and placed in sterile glass containers with wet sand. The insects that emerged from the flowers were collected and fixed in 70% alcohol for further identification.

Statistical analysis

The statistical analysis was performed in R v.3.6.1 (R Core Team 2018). To determine whether the fruit sets in hand-pollinated and control flowers differ from equal proportions, these data were analysed by the χ^2 goodness-of-fit test. The same test was used to assess the proportions of the number of visitors at different times of the day (morning, 10:00–11:00; noon, 13:00–14:00; and evening, 16:00–17:00). The differences were considered as statistically significant at a level of $p < 0.05$.

RESULTS

Floral coloration and morphology

Flowers of *A. manshuriensis* have a greenish perianth with purple or brownish venation (fig. 1). Three sepals are fused into a tubular calyx in which three parts may be distinguished: a limb, a tube, and a perianth chamber (fig. 2). The yellowish or purplish three-lobed limb has small separate trichomes on the surface (fig. 2B). There are several lines of dark maroon spots inside the perianth tube (fig. 2C). The inner surface of the perianth tube is covered by a waxy layer. The perianth chamber consists of two parts. One part, located near the tube, is white with small burgundy spots (fig. 2D). The other part (utricle), where the reproductive organs are located, is burgundy in colour and is covered by multicellular hairs (fig. 2E). The reproductive organs are surrounded by a light-yellow ring. The style, the stigma, and the stamens fuse together to form a gynostemium (figs 1, 2F, G). The tips of the stigma lobes are narrowed and bent over the stamens. Flowers are protogynous; the flower anthesis of *A. manshuriensis* can be divided into two phases. The first (female) phase lasts for three days after flower opening. During this period, the anthers are closed; the stigma lobes are receptive to pollen and covered by a secretion that is stained well with $KMnO_4$ for three days after flower opening (fig. 1A–B inset). During the second (male) phase of anthesis that starts on day 4 after flower opening, the stigma lobes adjoin each other, and the anthers open (fig. 2G). The stigma lobes lose their receptivity, the secretion almost disappears from their surface, and almost no $KMnO_4$ staining occurs (fig. 1C inset).

Pollination experiment

During the hand-pollination experiment, eight flowers of *A. manshuriensis* out of 25 fully developed into fruits (fruit set 32%). In the control groups, none of the 50 flowering ovaries in bags developed and only three out of 150 intact flowers open to natural pollination developed into fruits (fruit set 2%). The χ^2 goodness-of-fit test showed that the proportion of fruit set in the hand-pollinated flowers and in the naturally pollinated flowers differed from equality ($\chi^2 = 26.47$, d.f. = 1, $p < 0.001$). The pollen limitation coefficient was 2.64.

Flower visitors

A total of 253 insects from three orders (Diptera, Coleoptera, and Hymenoptera) were collected from flowers of *A. manshuriensis* (table 1). Diptera were represented by 14 families, while Coleoptera and Hymenoptera by two families each. Dipterans were the most numerous visitors (230 specimens, 90.9%). For the list of flower visitors split by sex, see table 2. The number of visitors was on average 0.17 per flower per day. The highest activity of visitors was observed in the morning between 10:00 and 11:00 (table 3). The number of insect visitors gradually declined towards the evening (table 3). The χ^2 goodness-of-fit test showed that the number of visitors differed from equality between morning and noon ($\chi^2 = 12.9$, d.f. = 1, $p < 0.001$) and between morning and evening ($\chi^2 = 19.7$, d.f. = 1, $p < 0.001$). The numbers of visitors at noon and in the evening were equal ($\chi^2 = 1.14$, d.f. = 1, $p = 0.29$).

The insects collected from *A. manshuriensis* flowers, except for the species of Drosophilidae and Anthomyiidae, had fewer than 10 pollen grains on their bodies. Two species of Drosophilidae, *Scaptomyza pallida* and *Drosophila transversa*, carried up to 2500 pollen grains on their bodies (mean ± SD: 1982 ± 518, n = 30). The highest pollen load was recorded from *Botanophila fugax* and *Botanophila* sp. 1 (family Anthomyiidae), which had up to 3500 (2777 ± 723, n = 30) pollen grains on their bodies. Most pollen grains adhered to the thorax of the insects, whereas few pollen grains were found on their abdomen, wings, legs, and head. It should be noted that all pollen grains collected from the insects belong to *A. manshuriensis*. No pollen grains were

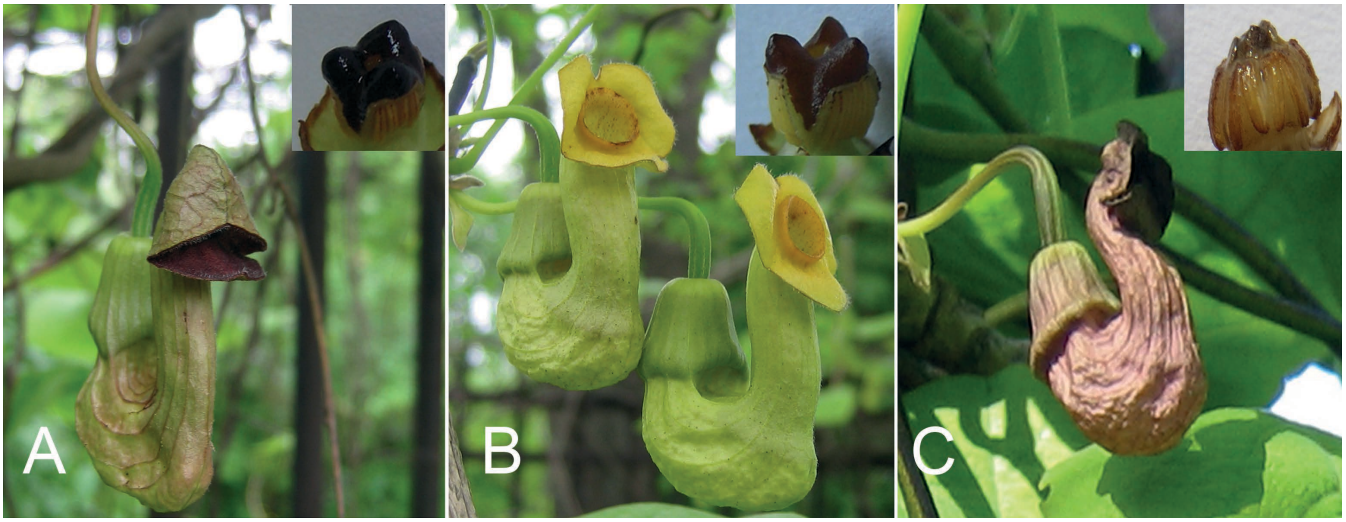

Figure 1 – Flowers of *Aristolochia manshuriensis* from different individuals with either purplish (A and C) or yellow perianth (B) (different colour morphs). **A**. A recently opened flower at the female phase of anthesis. **B**. A flower on day 3 after opening, the female phase of anthesis. **C**. A flower on day 6 after opening, the male phase of anthesis. Insets in each figure depict the gynostemium state in a recently open flower, as well as on days 3 (female phase) and 6 (male phase) after opening, respectively. Photographs by O.V. Nakonechnaya and V.M. Loktionov.

Table 1 – Number of insects collected from *Aristolochia manshuriensis* flowers during the flowering seasons of 2002–2018. *According to Nakonechnaya et al. (2008).

Order/ Family	**2002–2008***	**2009–2018**	**Total number**	**Proportion (%)**
Diptera				
Anthomyiidae	73	37	**110**	**43.5**
Drosophilidae	5	36	**41**	**16.2**
Calliphoridae	1	4	**5**	**2.0**
Chloropidae	25	5	**30**	**11.8**
Ceratopogonidae	0	6	**6**	**2.4**
Lauxaniidae	1	0	**1**	**0.4**
Lonchaeidae	9	0	**9**	**3.5**
Muscidae	11	0	**11**	**4.3**
Phoridae	2	5	**7**	**2.8**
Sarcophagidae	2	0	**2**	**0.8**
Sciaridae	1	2	**3**	**1.2**
Sepsidae	1	0	**1**	**0.4**
Syrphidae	1	0	**1**	**0.4**
Tachinidae	3	0	**3**	**1.2**
Hymenoptera				
Braconidae	1	0	**1**	**0.4**
Formicidae	0	1	**1**	**0.4**
Coleoptera				
Lathridiidae	2	0	**2**	**0.8**
Nitidulidae	0	19	**19**	**7.5**
Total	**138**	**115**	**253**	**100**

observed on the beetles collected from *A. manshuriensis* flowers.

Insect rearing

Seven species of insects were reared from eggs collected from the fallen flowers of *A. manshuriensis* (table 4). Eggs of *Elachiptera sibirica*, *E. tuberculifera* (Chloropidae), and *Botanophila fugax* (Anthomyiidae) were found on the inner surface of the utricle and around the gynostemium. Most of the hatched flies were from the family Drosophilidae (table 4), with *Scaptomyza pallida* reaching the largest proportion (93.2%). Anthomyiidae and Chloropidae accounted for 4.5% and 2.25% of the total number of hatched insects, respectively. The number of eggs per flower counted during our study was up to 10–20. The larvae of all insects hatched from eggs within 2–3 days after the flowers were placed into glass containers. They developed inside the calyx and fed on decomposing tissues of the perianth. The small-sized flies from the families Drosophilidae and Chloropidae pupated on days 7–9, and the larger flies from the family Anthomyiidae pupated on days 14–16 after being placed into glass containers. Pupation often occurred on rotten flowers and rarely on the wall of the glass container. The numbers of pupated males and females were approximately equal (table 4).

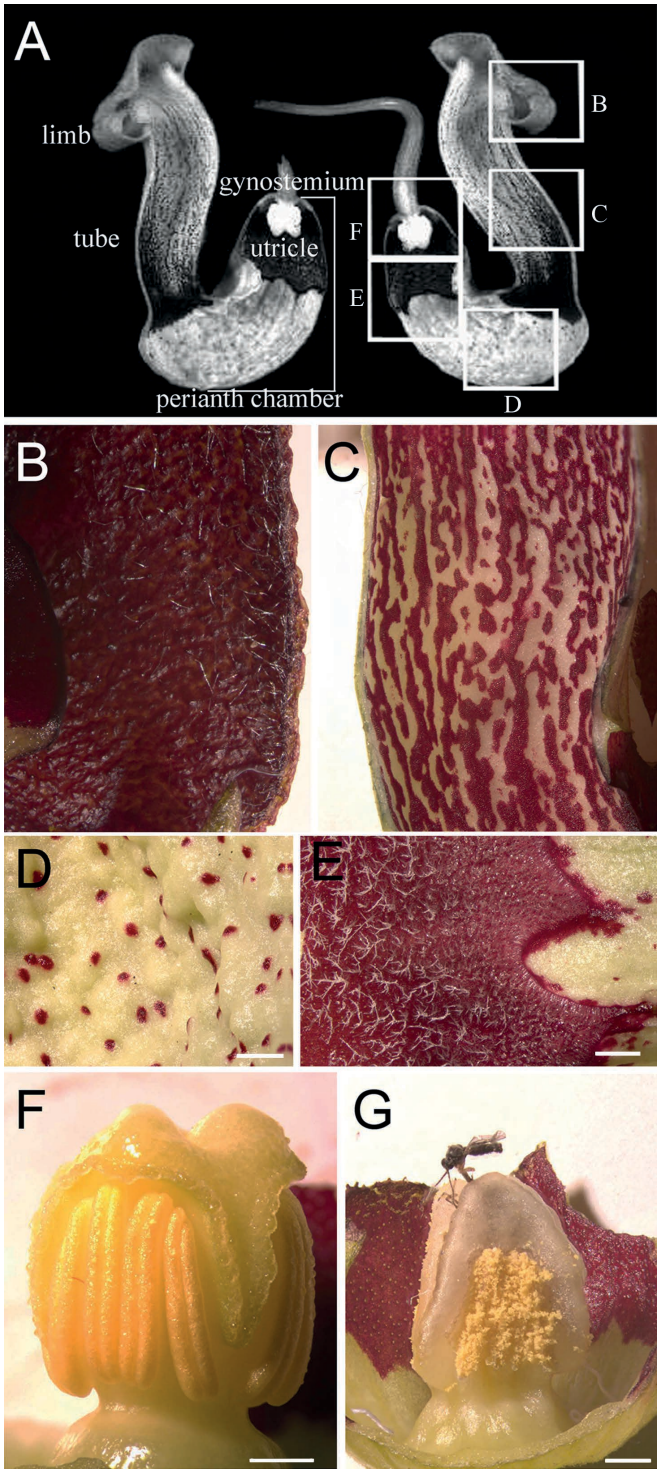

Figure 2 – Colour pattern of the limb and the interior of *Aristolochia manshuriensis* flowers belonging to the purplish colour morph as only the colour but not the pattern differs among different colour morphs. **A**. Cross-section through a flower at the female phase of anthesis. **B**. Limb with hairs. **C**. Colour pattern inside the tube. **D–E**. Colour pattern inside the perianth chamber. **F–G**. Gynostemium in the female and male phases, respectively. Scale bars D–G = 1 mm.

Table 2 – List of insect visitors split by sex, collected from *Aristolochia manshuriensis* flowers. A question mark means that sex could not be determined.

Family / Species or subgenus	**Males**	**Females**	**Total number**
Anthomyiidae			
Adia cinerella (Fallén, 1825)	4	2	6
Anthomyia avisignata Suwa, 1987	1	0	1
Botanophila fugax (Meigen, 1826)	?	?	1
Botanophila striolata (Fallén, 1824)	3	1	4
Egle ciliate (Fallén, 1824)	10	4	14
Delia linearis (Stein, 1898)	1	0	1
D. tenuiformis Suwa, 1977	2	1	3
Paregle audacula (Harris, 1780)	1	0	1
Pegomya geniculata (Bouche, 1834)	1	0	1
Pegoplata virginea (Meigen, 1826)	1	0	1
Zaphne ambigua (Fallen, 1823)	6	3	9
Anthomyiidae sp. 1	0	2	2
Anthomyiidae sp. 2	0	1	1
Chloropidae			
Elachiptera tuberculifera (Corti, 1909)	?	?	14
Elachiptera sibirica (Loew, 1858)	?	?	8
Chloropidae sp.	0	1	1
Ceratopogonidae			
Dasyhelea sp.	0	3	3
Subgenus *Dicryptoscena*	?	?	1
Subgenus *Pseudoculicoides*	?	?	2
Phoridae	0	1	1
Nitidulidae	14	5	19
Total number	44	24	94

Table 3 – Number of insects that visited *Aristolochia manshuriensis* flowers during five days of observation.

Family	**10:00–11:00**	**13:00–14:00**	**16:00–17:00**
Anthomyiidae	9	6	2
Chloropidae	3	0	0
Drosophilidae	15	2	1
Calliphoridae	1	0	0
Nitidulidae	4	1	2
Total number	32	9	5

DISCUSSION

Flowers of *A. manshuriensis* are adapted in structure and colour to sapromyophily, thus to pollination by flies and beetles. One adaptative trait is the sophisticated colour pattern of the perianth. It is completely pale outside and bright inside (fig. 2C–E). The pallid ring around the gynostemium presumably guides pollinators to the reproductive organs. Insects are attracted by light at the top of the utricle, indicating a false exit from the trap (Oelschlägel et al. 2009). As shown by monitoring the dynamics of daily visitors to *A. manshuriensis* flowers, flies of the families Anthomyiidae, Drosophilidae, and Chloropidae visit flowers during the first half of the day (table 3). This is probably explained by the diurnal rhythm of flower opening. According to our study, the flowers mainly open in the first half of the day (08:00–12:00) (Nakonechnaya et al. 2014). Most saprophagous flies and beetles are attracted to flowers by the specific smell that is especially strong in the first hours after flower opening (Burgess et al. 2004; Berjano et al. 2011). It is possible that flowers of *A. manshuriensis* can also attract insects by smell, but this assumption requires further study.

Table 4 – Number and sex of flies that emerged from *Aristolochia manshuriensis* flowers, collected at three localities (Sp, Bg1, Bg2).

Family / Species	**Sex**	**Sp**	**Bg1**	**Bg2**	**Total**
Drosophilidae					
Scaptomyza pallida (Zetterstedt, 1847)	M	870	16	152	1038 (47.4%)
	F	920	23	211	1154 (52.6%)
Drosophila transversa Fallén, 1823	M	0	0	0	0
	F	1	0	0	1
Chloropidae					
Elachiptera tuberculifera (Corti, 1909)	M	16	0	0	16 (47.1%)
	F	17	0	1	18 (52.9%)
Elachiptera sibirica (Loew, 1858)	M	7	0	0	7 (41.2%)
	F	10	0	0	10 (58.8%)
Conioscinella divitis Nartshuk, 1971	M	1	0	0	1
	F	1	0	0	1
Anthomyiidae					
Botanophila fugax (Meigen, 1826)	M	53	0	0	53 (50.5%)
	F	52	0	0	52 (49.5%)
Botanophila sp. 1	M	0	0	0	0
	F	1	0	0	1
Total					2352

Aristolochia manshuriensis flowers are able to hold insects captured inside their flowers. This is due to the structure of the vertical perianth tube, which serves as a springboard for rapid contact with the perianth chamber. Hence, the vertical orientation of the perianth tube and the waxy layer on its inner side prevents an insect from leaving the flower. This contrasts to the other species of the genus *Aristolochia* in which trichomes within the perianth tube perform this function (Oelschlägel et al. 2009; González & Pabón-Mora 2015).

Despite the special elements to attract insects, only few insects became trapped in the examined flowers. Many flowers were empty during the monitoring period. The number of visitors was 0.17 per flower per day. For other *Aristolochia* species, a significant number of visitors per flower was recorded: 0.4 to 0.6 for *A. chilensis* Bridges ex Lindl. (Valdivia & Niemeyer 2007; Stotz & Gianoli 2013), 0.39 for *A. baetica* L., 0.18 for *A. paucinervis* Pomel (Berjano et al. 2006), 2.1–6.0 for *A. pilosa* Kunth (Wolda & Sabrosky 1986), 3.6 for *A. littoralis* Parodi (Hall & Brown 1993), and even 454 insects for *A. grandiflora* Sw. (Burgess et al. 2004). An insect captured by an *A. manshuriensis* flower sometimes stays inside and dies, with parts of its body remaining clamped between the stigma's tops (fig. 2G). Small-sized Drosophilidae can escape the flower trap easily through the wide (1 cm) perianth tube.

After the opening of the anthers, we found that some amount of pollen had spilled out and got onto the insect's back, head, and legs. The scabrate surface of the pollen grains enable their attachment to the body of a potential pollinator, as we found many pollen grains (ca 100) on the bodies of even small-sized (2–3 mm) Drosophilidae. We suggest that these small insects can probably enter and leave a flower easily, and, hence, they can make some contribution to the pollination of *A. manshuriensis*. Much more pollen grains (ca 3500 per individual) were found on bodies of the larger (5–7 mm) insects (Anthomyiidae) that were trapped in the *A. manshuriensis* flowers. Our previous data indicate that fertilization of a single *A. manshuriensis* flower requires approximately at least 50 to 200 pollen grains, because a fruit typically contains as many as 99 ± 5 (up to 170) seeds (Nakonechnaya et al. 2014). This difference in number of grains is due to the fact that the number of viable seeds per fruit ranged from 50 to 200. It seems likely that a visit of one big insect such as an anthomyiid can result in pollination success for a single flower, while several small-sized insects are required for this. However, the probability that several small-sized insects contribute significantly to pollination is low if we take into account how many visitors per flower per day (0.17) were found. Of all the visitors collected during our study, four species of flies (*Scaptomyza pallida*, *Drosophila transversa* (Drosophilidae), *Botanophila fugax*, and *Botanophila* sp. 1 (Anthomyiidae)) are capable of transferring up to 2500–4000 pollen grains on their bodies and, therefore, these flies can be considered as pollinators of *A. manshuriensis*.

The fact that fruit fully developed in 32% of the flowers that were hand-pollinated with own pollen indicates that flowers of *A. manshuriensis* are capable of self-pollination by geitonogamy. However, a low fruit set (2%) was observed in the free pollination experiment (control 2). This can be caused by several factors. Ovaries in the bagged unpollinated

flowers could dry out due to the low number of pollen grains on the stigma that was insufficient to pollinate the flowers and initiate the fruit development. The positive value of the pollen limitation coefficient indicates a higher fruit set in the experimental plants as compared to the control ones (Vamosi et al. 2006). Earlier we found a significant number of pollen grains (up to 12 000) per flower and a low number (approximately 3%) of defective pollen grains (Nakonechnaya & Kalachev 2018). Thus, we suggest that the pollen deficiency is probably related to an insufficient number of effective pollinators (0.17 per flower per day).

There is a probability that outcross-pollination in *A. manshuriensis* results in a higher fruit set than geitonogamous hand-pollination. According to Berjano (2006), the fruit set during the xenogamy in *A. baetica* was approximately 20%, while during the geitonogamy it was only 8%. However, it is unclear whether this is the case for *A. manshuriensis*, and this question requires further study.

One can assume that high viable fruit set (Nakonechnaya et al. 2014) and high percentage of seed germination, which germinate without cold stratification (Nakonechnaya et al. 2018), as well as certain level of heterozygosity in populations of *A. manshuriensis* (Koren et al. 2009) contribute to maintaining the number of plants in natural populations of this species. Although there is the possibility of self-pollination by insects, no inbreeding at species level in natural populations was observed (Koren et al. 2009). This indicates that events of self-pollination in *A. manshuriensis* are rare and do not increase genetic load.

In our rearing experiment, flies from three families (Anthomyiidae, Chloropidae, and Drosophilidae) were found. These are the most common visitors of *A. manshuriensis* that constituted 71.5% of the total number of insects collected from flowers during our study (table 1). Of these species, *S. pallida*, *D. transversa* (Drosophilidae), *B. fugax*, and *Botanophila* sp. 1 (Anthomyiidae) visited flowers and could transfer many pollen grains on their bodies. As their eggs were observed in the perianth, we may conclude that these flies use flowers to lay eggs and can potentially serve as cross-pollinators. When visiting flowers of *A. manshuriensis*, the flies get trapped inside and are involved in pollination. Although they cannot leave the flower again, it is likely that these flies can transfer pollen grains from the anther to the stigma within the same flower and act as a mediator for self-pollination. In contrast, insects of the family Chloropidae are smaller (up to 2 mm) than those of Anthomyiidae (5–7 mm) or Drosphilidae (up to 3 mm), they can leave *A. manshuriensis* flowers and, probably, do not participate in pollination. After flowering, the perianth becomes separated from the ovary and falls off. Larvae of all fly families feed on fallen flowers without causing damage to developing seeds.

The absence of eggs belonging to Drosophilidae flies on the inner and outer surfaces of the perianth, and on the surface of stigma and style can be explained by the fact that these flies can lay eggs within floral tissues (Sakai 2002). According to data published in literature, flies of the family Phoridae pupate in flowers of Aristolochiaceae (Disney & Sakai 2001; Rulik et al. 2008). For example, *Megaselia metropolitanoensis* Disney, 2001 and *Puliciphora pygmaea* (Borgmeier, 1960) were reared from shed *A. pallida* Willd. flowers collected from the forest floor (Rulik et al. 2008). Female *M. sakaiae* Disney, 2001 lay eggs inside flowers of *A. inflata* Kunth and *A. maxima* Jacq. during blooming. Reared larvae fed on sepals and gynostemium inside flowers, completed their development in fallen and decaying flowers on the ground, and became adult on day 15 after oviposition (Disney & Sakai 2001). Hime & Costa (1985) reported that 102 adult females of *M. aristolochiae* Prado laid eggs inside *A. labiata* Willd. flowers in Brazil. Their larvae developed in cavities within the utricle wall. The authors found almost 400 phorid larvae in 3-day-old flowers of *A. grandiflora* Sw. in Mexico, which did not develop inside the flowers and died (Burgess et al. 2004). Under the rearing conditions, *M. sakaiae* pupated within one week after oviposition, often on the wall of a plastic container, and less frequently on rotten flowers. During our study, the Phoridae species visited flowers of *A. manshuriensis*, but we did not observe any flies of this family in our rearing experiment. This may indicate that these flies do not lay eggs in *A. manshuriensis* flowers, or their larvae did not develop in our rearing conditions. Larvae of Drosophilidae were observed feeding on the inner surface of the calyx in *A. inflata* and *A. maxima*, and imagoes emerged on day 15 after flowering (Sakai 2002). Our data on the development of Drosophilidae in flowers of *A. manshuriensis* agree with that reported by Sakai (2002).

CONCLUSION

Aristolochia manshuriensis is a Tertiary relict that evolved as a species in a climate similar to the modern tropical one. There are a number of adaptations in *Aristolochia* species such as the presence of gynostemium and the pollination by a narrow range of pollinators that prevent inbreeding and allow species renewal. Today, as climate conditions have changed, the reproductive strategy of *A. manshuriensis* rather hampers the species renewal. The adaptation to pollination by certain insects leads to a decrease in fruit set when these pollinator species are absent. The fragmentation of the *A. manshuriensis* range increases the probability of crossing within small groups, thus, inevitably raising the level of segregational genetic load and potentially resulting in the degradation of its populations. However, the high percentage of germination of *A. manshuriensis* seeds, which germinate without cold stratification, maintains the population size and prevents the species from a negative scenario.

ACKNOWLEDGEMENTS

We are grateful to S.F. Vasyukevich (Far Eastern Geological Institute, FEB RAS, Vladivostok) for the opportunity to carry out experiments with her private flower collection, as well as to E.P. Narchuk, A.A. Przhiboro, V.A Richter (Zoological Institute, RAS, St. Petersburg), Z.A. Fedotova (Samara State Agricultural Academy, Ust-Kinelsky, Samara Oblast), N.V. Repsh (Far Eastern Federal University, Ussuriysk), and V.A. Mutin (Amur State University, Blagoveshchensk) for their help with the identification of some Diptera species. We also thank the following researchers of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, FEB RAS, Vladivostok: A.N. Kupianskaya for the identification of

Formicidae, A.S. Lelej for the advice and identification of Hymenoptera, and V.M. Loktionov for his help with microscope images. Special thanks are due to S.V. Nesterova (Botanical Garden-Institute, FEB RAS, Vladivostok) for her assistance with the pollination of the flowers in the Botanical Garden. Dr V.S. Sidorenko has seen and approved the final manuscript. All microscopic studies were carried out at the Instrumental Center for Biotechnology and Gene Engineering, Federal Scientific Center of the East Asia Terrestrial Biodiversity, FEB RAS. This work was supported by the Far Eastern Branch, Russian Academy of Sciences (FEB RAS), grant no. 18-4-049.

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Communicating editor: Alexandra Ley.

Submission date: 18 Apr. 2020

Acceptance date: 9 Oct. 2020

Publication date: 23 Mar. 2021