

Specific Features of Pollination in the Manchurian Birthwort, *Aristolochia manshuriensis*

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Abstract—An analysis of pollination system in *Aristolochia manshuriensis* has shown that flower structure in this species is strictly adapted to cross-pollination, but the possibility of an autogamous or geitonogamous type of self-pollination with the involvement of insects is not excluded. The flowers of *A. manshuriensis* are most frequently visited by flies of the family Anthomyiidae, which markedly contribute to their pollination. Males account for 65% of pollinator insects collected from the flowers.

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INTRODUCTION

Many plants have various adaptations for successful pollination. Both external characters (odor, color, flower structure, etc.) and special genetic mechanisms, such as allele combinations determining self-compatibility or self-incompatibility, are aimed to make the pollen of a flower arrive, or not arrive, to the stigma of the same flower. In every plant family, the number of adaptations to a certain (most favorable) pollination type increases in the course of evolution. Narrow specialization of flower structures to pollination by a certain insect group has often both positive and negative effects. On the one hand, adaptation to pollination by a certain group of insects ensures pollination success at smaller numbers of pollen grains and ovules, allowing the plant to reduce expenditures for this process; on the other hand, the range of potential pollinators narrows, and the risk of their absence and, therefore, pollination failure increases.

The birthwort family (Aristolochiaceae) comprises plants with narrow specialization of flower structures for pollination by insects. Species of the genus *Aristolochia*, the largest in this family, are connected with two large insect groups, Lepidoptera and Diptera. The larvae of lepidopterans, mainly from the family Papilionidae, feed on *Aristolochia* leaves, but adult butterflies do not contribute to pollination. Adult dipterans feed on pollen, nectar, and perianth tissues and, in this course transfer pollen grains to the stigma.

Flowers in all representatives of the genus have common features due to which the flower is a perfect trap for insects: the perianth limb is connected to the inflated perianth region with a straight funnel-like, slightly bent, or U-shaped narrow tube (Figs. 1, 2). The

perianth tube often has a “signal trail.” In some species, the inflated perianth region has a semitransparent “window” in its upper wall, which lets the light in. The inner tube surface usually carries hairs and papillae (Hou, 1983). All these adaptations have the same purpose, to retain the pollinator. The flowers of *Aristolochia* species are pollinated by dipterans, with some of them being adapted to certain pollinators.

In Russia, the genus is represented by seven species, with two species occurring only in southern Primorye. One of them is the Manchurian birthwort (*Aristolochia manshuriensis* Kom.), a woody liana endemic to the Manchurian floristic region. This plant has a limited and disjointed range (Kurentsova, 1968) and is on the Red Data List of the Russian Federation as an endangered species (*Krasnaya kniga...*, 1988).

As in other species of the genus, the flower of the Manchurian birthwort has some adaptations to cross-pollination, which are manifested primarily in its shape, color, and structure. The perianth tube is U-shaped, up to 9 cm long, inflated around the gynostemium to 15 mm in diameter; the tube then constricts, distends at the bend to 16–18 mm, constricts again to 8 mm, and finally distends at the flower throat (Fig. 2). The perianth inner side has a deep purple ring around the gynostemium, with mottles of the same color spreading higher along the veins. In the narrowest region, the perianth has another wine-colored ring, with dense purple mottles spreading from it toward the flower throat. In the upper one-third of this ring, the inner perianth surface is papillose and carries numerous branching hairs. The limb is purple-brown or greenish yellow, 22–24 (28) mm in diameter, slightly trilobate (Golovach, 1973; Denisov, 2003).

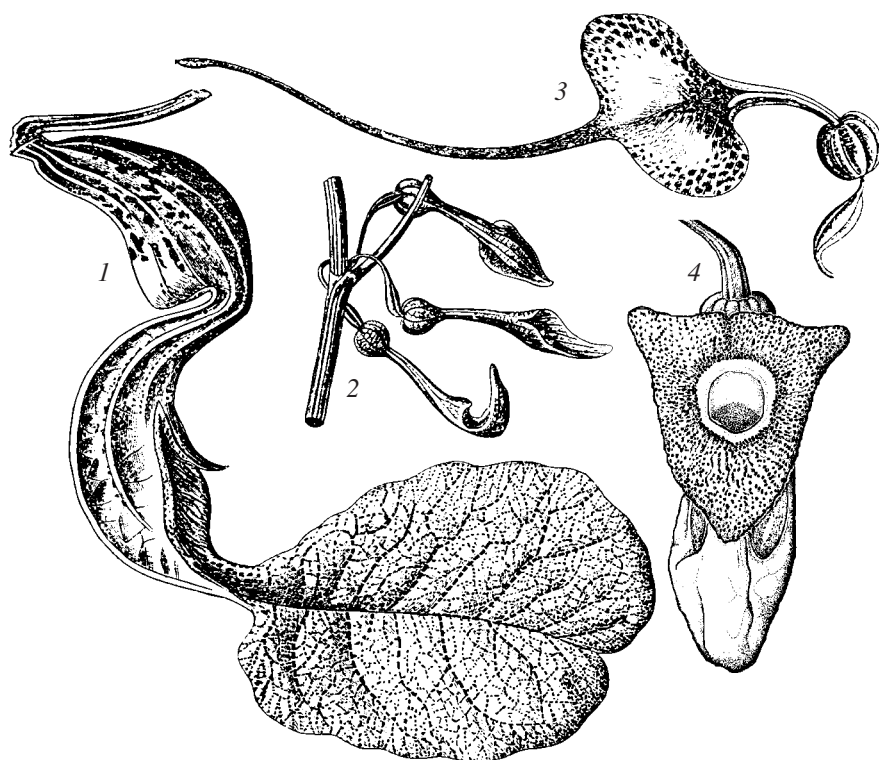


Fig. 1. Flowers of some representatives of the genus *Aristolochia*: (1) *A. labiosa*, (2) *A. clematitis*, (3) *A. cordata* (Kerner, 1902), and (4) *A. macrophylla* (Cronquist, 1981).

Our purpose was to study specific features of pollination in this rare relict plant. An analysis of the species composition of potential pollinator insects visiting *A. manshuriensis* flowers is of interest for elucidating plant adaptive mechanisms contributing to cross-pollination success. However, no such studies have yet been performed on this species.

MATERIALS AND METHODS

The study was performed with plants growing in the Botanical Garden–Institute of the Far East Division of the Russian Academy of Sciences and in a private plant collection maintained in the suburb of Vladivostok, in the periods of *A. manshuriensis* flowering (April 25–June 5) in the years 2002–2005.

Cytological analysis of reproductive organs. The structure of the androecium and gynoecium was studied at different stages of flowering. To estimate the rate of pollen tube growth, pistils with pollen grains on the stigma were collected. The material was fixed in the formalin–acetic acid–ethyl alcohol mixture (FAA; 7 : 7 : 100 ml) at 5-min intervals. The ovaries, styles, and anthers were embedded in paraffin by a routine method (Pausheva, 1970). Serial sections (8–10 μ m) were cut with an HI 430 microtome (Carl Zeiss, Germany) and stained with hematoxylin and alcyan blue as

described (Kamelina et al., 1992), with some modifications.

The period of stigma receptivity to pollen was determined according to Robinsohn (1924). Briefly, stigmas at different developmental stages, from the flower bud stage (10 days after emergence from the leaf axil) to flower withering, were placed in 1–2% KMnO_4 solution for 1–2 min, washed with flowing water for 5–10 min, and examined under a microscope. Receptive stigmas (producing a secretion) stained brownish red, while unreceptive stigmas remained unstained.

Pollen grains were counted in temporary preparations of gynostemium with mature anthers fixed in the FAA mixture. All pollen grains were removed with preparation needles and transferred to a drop of water on a glass slide. The preparation was covered with a coverslip and examined under a microscope. Ovules were removed from ovaries, placed in Petri dishes, and counted under a binocular microscope.

Analysis of flowering and fruiting dynamics. To determine the type of flower pollination, we studied the efficiency of this process under experimental conditions in the following variants: (1) natural self-pollination, (2) artificial self-pollination, (3) artificial cross-pollination, and (4) free pollination (control). In the natural self-fertilization experiment, 100 flowers at the bud stage were isolated in fine-mesh bags (10 \times 10) and remained so without any further treatment. In the artifi-

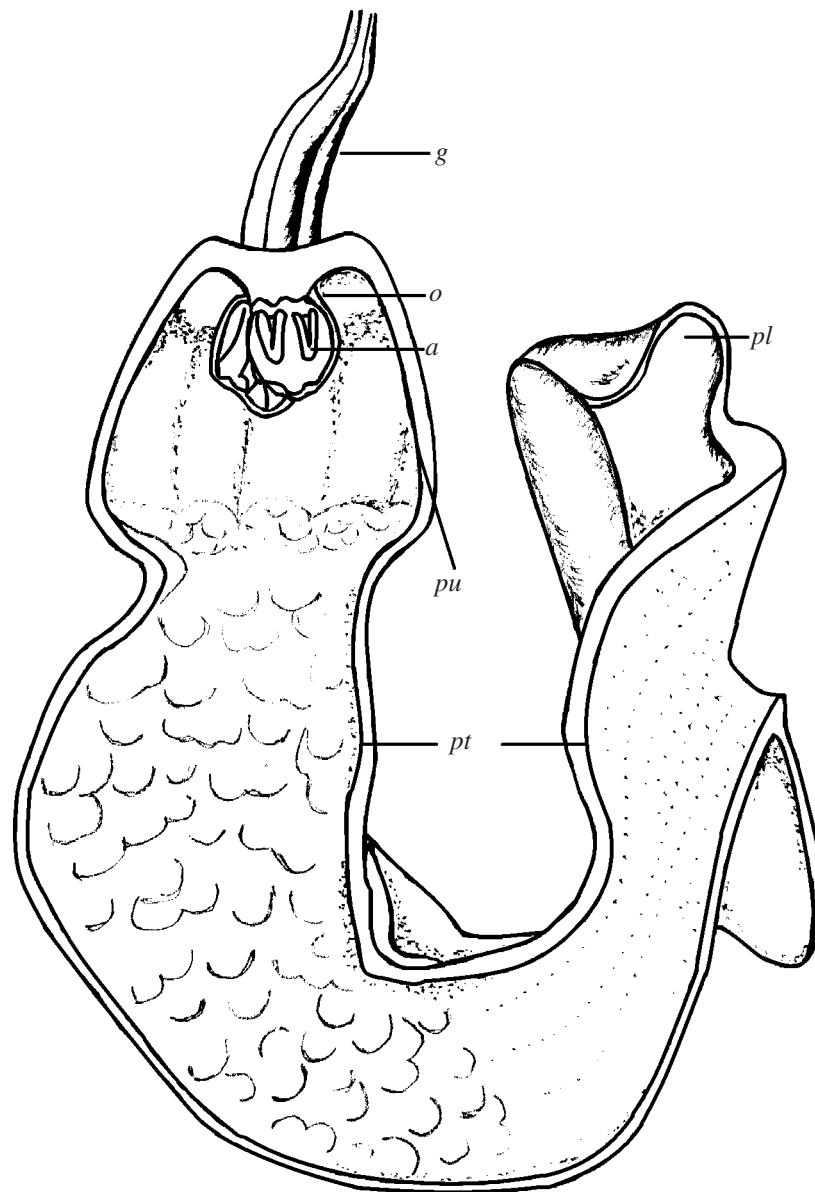


Fig. 2. Flower structure in *A. manshuriensis*: (g) gynostemium, (o) ovary, (pu) perianth utricle, (pl) perianth limb, (a) anthers, and (pt) perianth tube.

cial self-fertilization experiment with 25 flowers, the bags were removed on day 2 after flower opening, half of the perianth in each flower was cut off, mature pollen grains were transferred with a brush from the anthers to the stigma of the same flower, and all flowers were isolated again. The artificial cross-fertilization experiment was performed in the same way except that pollen was taken from a different plant. In the control, 100 flowers were individually marked with color threads and kept under observation until fruit formation.

To study pollen tube growth, the perianth of a blooming flower bud was cut off and mature pollen

grains were transferred to the stigma with a small brush. During the following 6 h, the pistils and ovaries were fixed in FAA at 5-min intervals, beginning from the fifth minute after pollination.

Collection of insects visiting the flowers. All these insects were taken directly from the flowers and placed in 70% ethyl alcohol or on cotton layers.

RESULTS

Structure of reproductive organs. The style and stamen threads in *A. manshuriensis* flowers grow

Table 1. Total numbers of insects collected from *Aristolochia manshuriensis* flowers

Taxon (order/family)	2002	2003	2005	Total
Diptera	16	78	41	135
Sciaridae	–	1	–	1
Phoridae	–	2	–	2
Syrphidae	–	1	–	1
Lonchaeidae	–	9	–	9
Lauxaniidae	–	1	–	1
Sepsidae	–	1	–	1
Chloropidae	1	24	–	25
Drosophilidae	–	5	–	5
Anthomyiidae	12	20	41	73
Muscidae	–	11	–	11
Calliphoridae	–	1	–	1
Sarcophagidae	1	1	–	2
Tachinidae	2	1	–	3
Hymenoptera	0	1	0	1
Braconidae	–	1	–	1
Coleoptera	0	0	2	2
Lathridiidae	–	–	2	2
Total	16	79	43	138

Note: (–) no insects found.

together to form the gynostemium. The stigma is trilobate; the tapering tips of lobes are bent down and cover the anthers located under them. The anthers are bisporangiate, located in groups of four on each of the three sides of the style. Bitegmal anatropic ovules have well-developed funiculi.

The flowers of *A. manshuriensis* are protogynous. A secretion appears on the stigma one week before the corolla opening. When the perianth lobes bend out, the anthers remain tightly closed. According to our observations, they open two to three days later, when the stigma is still receptive. The average ratio of pollen grains to ovules is 110 : 1.

Pollinators Most insects found on the *A. manshuriensis* flowers were of the families Anthomyiidae (51%) and Chloropidae (17%) (Diptera) (Table 1). Representatives of the Anthomyiidae were of nine genera and ten species: *Adia cinerella* (Fallén), *Anthomyia avisignata* Suwa, *Botanophila striolata* (Fallén), *Egla ciliate* (Fallén), *Delia linearis* (Stein), *D. tenuiformis* Suwa, *Paregle audacula* (Harris), *Pegomya geniculata* (Bouche), *Pegoplata virginea* (Meigen), and *Zaphne ambigua* (Fallén). The proportion of males among them

reached 65%. The Chloropidae were represented by two species of the genus *Elachiptera* Macquart: *E. tuberculifera* Corti and *E. sibirica* Loew. The majority of these insects were also males.

Experiments on pollination. To analyze the mating system in *A. manshuriensis*, we performed experiments on artificial cross-fertilization and self-fertilization and also on isolation of flower buds at the stage immediately preceding blooming. In any experimental series, not a single fruit was set in either isolated or manually pollinated flowers, whereas free pollination consistently resulted in fruit setting.

DISCUSSION

Some specific features of *A. manshuriensis* flowers—the bent perianth tube, odor, color, traplike structure, and proterogyny—are indicative of their strict specialization for cross-pollination by flower flies. Furthermore, the presence of a structure such as the gynostemium theoretically reduces the probability of self-pollination to a minimum (Nakonechnaya et al., 2006). However, we observed fruit setting after free pollination in a liana growing in the private collection solely, in the absence of conspecific plants within a radius of more than 400 m. This is evidence for the possibility of autogamous or geitonogamous self-fertilization in this species, since the insects participating in its pollination are unlikely to cover such a distance. Although the Anthonomiidae (the main birthwort pollinators) have not been studied in this respect, representatives of this family appear to be incapable of long-distance flight. This can be inferred from data on the spatial distribution of blow flies (Calliphoridae), which belong to the same group of calyprate dipterans. According to Smith and Wall (1998) the daily dispersal distance of these flies averages 135 ± 26 m; with regard to their life span and the effect of weather conditions, this means that the maximal distance of their dispersal per generation is less than 800 m.

The probability of self-pollination can also be indirectly inferred from the ratio between the numbers of ovules and pollen grains in the anthers. This ratio is individual for each species and indicates the prevailing type of pollination (Lloyd, 1965; Baker, 1967; Gibbs et al., 1975). Cruden (1977), having studied 96 plant species from different taxa, identified five pollination types, each characterized by a certain value of this ratio. The Manchurian birthwort has a ratio of 110 : 1 (see above); according to Cruden, this corresponds to an intermediate position between obligate and facultative autogamy (Table 2).

The genus *Aristolochia* is known to include both self-compatible and self-incompatible species. Self-pollination was observed in *A. littoralis* (Petch, 1924), *A. barbata*, *A. brasiliensis* (Burck, 1890, 1892), *A. bracteolata* (Razzak et al., 1992) and *A. inflata* (Sakai, 2002); cleistogamy, in *A. serpentaria* (Pfeifer,

1966). It is considered that some cross-pollinating species at the periphery of their ranges can adapt to a shortage of pollinators by using self-pollination as a reserve mechanism of reproduction (Elle and Carney, 2003). This was indeed observed in several species with low outcrossing rates in marginal populations (Schoen, 1982; Holtsford and Ellstrand, 1992). The aforementioned fact of fruit setting on a solitary *A. manshuriensis* liana, in the absence of cross-pollination, can also be a consequence of species adaptation to unfavorable conditions, particularly to the shortage of pollinators, since Primorye is at the northern boundary of its range.

What is the degree of cross-pollination success in the Primorye populations of *A. manshuriensis*? The absence of fruit setting in experiments on artificial cross-fertilization does not prove that outcrossing in this species is impossible, because fruiting failure was observed in all our experiments on artificial fertilization. This result may be explained by the fact that, in order to transfer pollen to the stigma, half of the U-bent perianth had to be removed, since the gynoceum was otherwise inaccessible. Such damage of the flower and consequent disturbances in its internal microenvironment may be harmful for pollen reception and germination on the stigma surface. However, complete fruiting failure was also observed in experiments with isolation of flower buds, in which they suffered no damage, whereas the control flowers set fruit regularly. This may be evidence that the probability of accidental (without a pollinator) pollen transfer to the stigma is negligible or the amount of this pollen is insufficient for fertilization. Indeed, the flower of *A. manshuriensis* is sufficiently large, and the gynostemium is arranged with the stigma lobes facing down (Fig. 2); hence, most pollen grains remain on the walls of perianth utricle and only a few of them arrive to the stigma. Therefore, pollinators are necessary for effective pollen transfer and fertilization.

As shown previously, no more than 2.3% of *A. manshuriensis* flowers set fruit when flowering in this species is abundant (Nakonechnaya et al., 2005), which is apparently due to the absence of effective pollination. In addition to the flower structure that makes pollen-stigma contact hardly probable without a pollinator, the lack of fertilization may be explained by partial incompatibility, i.e., the situation where fertilization fails if the pollen and stigma are of the same flower but can be successful if the pollen is from a different flower, even that of the same plant (Frenkel' and Galun, 1982). This phenomenon has been observed in some representatives of the genus *Aristolochia*, e.g., in *A. maxima*. Sakai (2002) inferred the presence of self-incompatibility in birthworts from (1) fruiting failure in self-pollination experiments and (2) successful fruiting upon cross-fertilization. She revealed this phenomenon in some other species of the genus, such as *A. gigas* and *A. ridiculata*, and explained a higher index of fruit setting upon free pollination in *A. inflata* (18.7%) than in *A. maxima* (2.4%) by differences in the level of self-incompatibil-

Table 2. Numerical ratios of pollen grains to ovules in flowers of plants with different mating systems*

Pollination type/number of species studied	Pollen/ovule ratio
Cleistogamy/6	4.7
Obligate autogamy/7	27.7
Facultative autogamy/20	168.5
Facultative outcrossing/38	796.6
Obligate outcrossing/25	5859.2

* According to Cruden, 1977.

ity between them. She concludes that partial self-incompatibility is an important factor determining cross-pollination and fruiting success in each of the two species.

In general, the results of our study show that, although the *A. manshuriensis* flower is morphologically adapted to strict cross-pollination, the plant is capable of autogamous or geitonogamous self-pollination if other reproductive individuals are absent. Pollinator insects are required for both cross- and self-pollination, since only they can transfer sufficient amounts of pollen to the stigma.

Dipterans of the families Anthomyiidae and Chloropidae were the most numerous visitors to *A. manshuriensis* flowers (73 and 25 ind., respectively). Small fruit flies (Chloropidae) can hardly be major pollinators of the plant: although they freely enter the flower, their small body size and, especially, chaetotactic characteristics do not allow them to transfer more than ten pollen grains, whereas at least 100 pollen grains are necessary for successful fertilization (Sakai, 2002). The role of larger flies (Anthomyiidae, Calliphoridae, Tachinidae, etc.) appears to be more important, since a single visit of such a pollinator coming from a conspecific plant can provide for more successful cross-pollination than several visits of small insects. However, we could not reveal any specialized pollinator of *A. manshuriensis* flowers among insects collected from them. Hymenoptera and beetles can also contribute to pollination, but their contribution is insignificant.

Flies are attracted to birthwort flowers mainly by their odor, which is species-specific in different representatives of the genus *Aristolochia* (Hou, 1983), whereas the role of flower coloration appears to be insignificant.

It has been noted that pollinators entering the flower remain inside for several hours, since getting out of the "trap" within a short time is not always possible. Analyzing flower morphology in *A. esperanzae*, *A. cymbifera*, and *A. brasiliensis*, Brantjes (1980) revealed a correlation between flower size (the diameter and length of the perianth tube and utricle) and thorax

height in the main pollinator insects. In our case, such a correlation holds only for large flies, which are the main pollinators of *A. manshuriensis*.

It is noteworthy that more than a half of insects collected from *A. manshuriensis* flowers were males. A similar fact was noted previously for *A. littoralis*: among 334 *Megaselia* spp. flies visiting the flowers of this species, the proportion of males reached 96% (Hall and Brown, 1993). On the other hand, only females were collected from the flowers of *A. pilosa*, *A. macrophylla*, and *A. labiata* (Carr, 1924; Hame and Costa, 1985; Wolda and Sabrosky, 1986). Female flies laid eggs into the perianth, where the larvae subsequently developed. We also noted *Scaptomyza pallida* (Zett.) (Drosophilidae) laying eggs into the perianth of *A. manshuriensis* flowers. The larvae of these flies develop on the inner perianth surface, reaching the adult stage within one week; therefore, adult flies of a new generation emerge prior to perianth abscission and become spattered with pollen before leaving the flower. Some flies fail to emerge before perianth abscission and complete their development in plant tissues decomposing on the ground. Flies of two *Elachiptera* species (Chloropidae) develop in this way.

What does attract male flies to birthwort flowers? Odor apparently plays an important role. For example, the odor of *A. littoralis* flowers has components similar to female pheromones of *Megaselia* spp., and male flies visit these flowers in search of a mate (Hall and Brown, 1993). In the case of *A. manshuriensis*, however, pollen appears to be the main attractor: the main pollinators of this species are widely polyphagous, and pollen is a major food resource for them (Minami and Enomoto, 1983; Suwa and Darvas, 1998). Entering the flower in search of food, insects fall in a kind of trap and spontaneously participate in pollination.

Thus, the Manchurian birthwort is adapted to pollination by insects, and the presence of adequate pollinators is a factor determining fruiting success. The actual numbers of developing fruits and seeds appear to be insufficient for maintaining the species range within its present-day boundaries, not to mention its expansion. This partly accounts for the fact that the area still occupied by this rare species is gradually decreasing.

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