Research article

**Post-pollination barriers contribute to coexistence of partially pollinator-sharing *Arisaema* species (Araceae)**

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**Running title:** Reproductive isolation in sympatric *Arisaema* species

**ABSTRACT**

Reproductive isolation plays an important role in maintaining the species integrity of sympatric close relatives. For sympatric *Arisaema* species, interspecific gene flow is expected to be effectively prevented by pre-pollination barriers, particularly strong pollinator isolation mediated by fungus gnats. However, due to the lack of quantitative studies combining multiple pre- and post-pollination barriers, it is not known whether pre-pollination isolation is complete, and whether post-pollination barriers also contribute to reproductive isolation among some *Arisaema* species.We studied five pre- and post-pollination barriers (geographic isolation, phenological isolation, pollinator isolation, hybrid fruit production, and hybrid seed formation) among three sympatric *Arisaema* species (*A. bockii*, *A. lobatum*, and *A. erubescens*). The strength of individual barriers and their contribution to total isolation were quantified.The habitat elevations of the three *Arisaema* species mostly overlapped. Although phenological isolation and pollinator isolation reduced the frequencies of interspecific pollen transfer among these species, the partial overlap of flowering times and pollinator assemblages resulted in pre-pollination isolation that does not adequately prevent interspecific hybridization. Post-pollination barriers also contributed to reproductive isolation at the hybrid fruit and seed formation stages.We propose that, although pre-pollination barriers are expected to contribute more to total isolation than post-pollination barriers in *Arisaema*, pre-pollination barriers may not completely prevent interspecific pollen transfer among some *Arisaema* species. Post-pollination barriers, which are generally ignored, may also have contributed significantly to reproductive isolation in *Arisaema*.

**Key words:** reproductive isolation, *Arisaema*, species coexistence, pre- and post-pollination barriers, fungus gnat.

**INTRODUCTION**

When close relatives come into contact, species integrity may be disrupted due to frequent interspecific gene exchange (Gilman & Behm, 2011; Todesco et al., 2016). Mechanisms of reproductive isolation play an important role in maintaining the species integrity of sympatric close relatives by reducing interspecific gene flow (Christie & Strauss, 2019; Rieseberg, Wood, & Baack, 2006; Weber & Strauss, 2016). In plants, mechanisms promoting reproductive isolation can be classified into pre- and post-pollination barriers (Baack, Melo, Rieseberg, & Ortiz-Barrientos, 2015). Pre-pollination barriers are associated with adaptive ecological divergence, including geographic isolation, phenology isolation, and pollinator isolation; post-pollination barriers are associated with the accumulation of genetic incompatibilities, including gametic incompatibilities, conspecific pollen precedence, hybrid inviability, hybrid sterility and hybrid breakdown (Baack et al., 2015; Rieseberg & Willis, 2007). The reproductive barriers function sequentially in life history, and early-acting barriers can weaken the contribution of late-acting barriers to total reproductive isolation (Coyne & Orr, 1989). Pre-pollination barriers are stronger than post-pollination barriers and contributed more to the total reproductive isolation for many species pairs (Christie, Fraser, & Lowry, 2022; Lowry, Modliszewski, Wright, Wu, & Willis, 2008). However, for some species pairs, post-pollination barriers contributed more to the total reproductive isolation when pre-pollination isolation barriers were weak or absent (Liang et al., 2018; Pellegrino, Bellusci, & Musacchio, 2010). The pattern of reproductive isolation varies among species pairs. Therefore, it is important to quantify multiple pre- and post-pollination barriers and their contribution to total reproductive isolation for understanding the coexistence of sympatric related species (Sobel & Chen, 2014).

*Arisaema* Martius is a large genus in Araceae, containing about 200 deciduous or evergreen perennial herbs (Ohi-Toma, Wu, Murata, & Murata, 2016). Most species are distributed in subtropical to cool temperate regions of Asia and several species are endemic to North America and tropical East Africa (Gusman & Gusman, 2006; Murata, 2011). In many regions, *Arisaema* species often overlapped in their distribution (Matsumoto, Sakuwa, & Murata, 2018; Murata, 1995; Serizawa, 1988, 1997). It is generally accepted that interspecific gene flow among sympatric *Arisaema* species is effectively prevented by pre-pollination isolation (Matsumoto, Hirobe, Sueyoshi, & Miyazaki, 2021; Matsumoto et al., 2019; Murata, Ohno, Kobayashi, & Ohi-Toma, 2018). Divergence of habitat and flowering time reduced interspecific gene flow to some extent among sympatric *Arisaema* species (Matsumoto et al., 2021; Matsumoto et al., 2019; Murata & Ohno, 1989). In contrast to relatively weak geographic isolation and phenological isolation, selective fungus gnat visitation resulted in strong pollinator isolation among some *Arisaema* species (Kakishima, Sueyoshi, & Okuyama, 2020; Kakishima et al., 2019; Matsumoto et al., 2021; Matsumoto et al., 2019; Suetsugu, Sato, Kakishima, Okuyama, & Sueyoshi, 2021), and the strong and stable pollinator isolation mediated by fungus gnats guaranteed the prevention of interspecific hybridization as an alternative reproductive isolation (Matsumoto et al., 2021).

However, some natural hybridization occurred (Hayakawa et al., 2011; Hayakawa et al., 2013; Kobayashi, Murata, & Watanabe, 2005; Lee et al., 2011; Maki & Murata, 2001; Murata & Ohno, 1989; Sanders & Burk, 1992), suggesting that pre-pollination isolation was incomplete among some *Arisaema* species. In artificial crossing experiments among various Japanese *Arisaema* species, F1 hybrids had high germination rates and high pollen fertility (Murata & Ohno, 1989; Murata et al., 2018).Thus, subsequent studies treated post-pollination isolation as weak or absent among these species (Matsumoto et al., 2019; Suetsugu, 2022; Suetsugu et al., 2021). However, since the mechanism of post-pollination isolation at other stages have not been studied, we do not know whether there are strong post-pollination barriers among *Arisaema* species. The mechanisms of coexistence among sympatric *Arisaema* species are not well understood due to the lack of quantitative studies combining multiple pre- and post-pollination barriers.

In this study, we focused on three sympatric congeners in the genus *Arisaema*: *A. bockii*, *A. lobatum* and *A. erubescens*. We used a combination of approaches including field observations and experiments to clarify the mechanism of reducing interspecific gene flow, and then to quantify the strength of multiple pre- and post-pollination barriers and their contribution to total isolation. In doing so, we hope to solve the following questions: (1) what are the strengths of different isolating barriers, and how do these barriers act to maintain species boundaries when close relatives come into contact? (2) What is the contribution of pre- versus post-pollination barriers to the maintenance of species integrity? Our assessment of reproductive isolation barriers among these sympatric species will provide new insights towards a better mechanistic understanding of speciation and diversification in *Arisaema*.

**MATERIALS AND METHODS**

**Plant materials**

In this study, we focused on three dioecious *Arisaema* species: *A. bockii* and *A. lobatum* from the section *Pistillata*, and *A. erubescens* from the section *Sinarisaema*. The chromosome number of *A. bockii* is 2n = 26 (Li, Zhu, & Murata, 2010), while the chromosome numbers of *A. lobatum* and *A. erubescens* are 2n = 28 (Petersen, 1989; Watanabe, Kobayashi, & Murata, 2008). Although the shape and color of inflorescence are significantly different among the three species (Fig. 1A, B, C), the structure of inflorescence is similar. Inflorescence consists of a spadix with fertile flowers at the base and a well-developed sterile appendix above, and a spathe wrapping around the spadix and forming a hood covering the spadix tip (Fig. 1D, E). Like other dioecious *Arisaema* species, these species possess a pitcher-trap pollination system (Barnes, 1935; Vogel & Martens, 2000). In this pollination system, the odor emitted from the spadix appendage and/or the spathe attracts fungus gnats (Mycetophilidae and Sciaridae) into the tubular spathe (Barnes, 1935; Kakishima et al., 2019; Suetsugu et al., 2022; Suetsugu et al., 2021; Vogel & Martens, 2000). Attracted fungus gnats slip down to the bottom of spathe because the spadix appendage and inner surface of the spathe are covered with wax (Vogel & Martens, 2000). The male spathe has an exit hole at the bottom, through which the captured fungus gnats can escape with pollen grains (Fig. 1F, H). However, the female spathe has no exit hole, and the captured fungus gnats move around the female spadix, deposit pollen grains on the stigma, then die (Vogel & Martens, 2000) (Fig. 1G, I).

**Study site**

The study was conducted in Badagongshan National Nature Reserve, Hunan Province, China (29°47′06″N, 110°05′33″; 1369 m a.s.l.). At the study site, *A. bockii*, *A. lobatum* and *A. erubescens* had a sympatric distribution, from 1150 to 1410 m a.s.l. (1410 m a.s.l. is almost the highest elevation of the mountain where the study was conducted) and heterospecific individuals often co-occurred less than 5 m apart. In the survey, we found three putative hybrids with an intermediate morphology between *A. bockii* and *A. lobatum* (Fig. S1). We also found that all three species prefer to grow along the roadsides, only very few individuals occurred more than 5 m from the roadside.

**Geographic isolation**

To assess habitat differences among three *Arisaema* species growing along the roadsides, the habitat elevation of these species was recorded using a portable GPS (eTrex 309x; Garmin International, Olathe, KS, USA) from 2 May 2022 to 18 May 2022. We recorded all flowering individuals of these species (462 *A. bockii*, 117 *A. lobatum*, 251 *A. erubescens*) growing within 5 m of both sides of the road, ranging from 1150 to 1410 m a.s.l.

**Phenological isolation**

To assess the difference in flowering time among our three *Arisaema* species, their flowering phenology was recorded in two quadrats (5 × 50 m) with a mixed community of the three species. 60 *A. bockii* (25 female, 35 male), 64 *A. lobatum* (18 female, 46 male) and 79 *A. erubescens* (21 female, 58 male) in these quadrats were observed every day from 10 April 2022 to 29 May 2022. The flowering period began when the spathe opened above and ended when it faded and/or withered. Fewer females were observed than males because the sex ratio of *Arisaema* populations is usually biased towards males (Richardson & Clay, 2001).

**Pollinator isolation**

To investigate the differences in floral visitor assemblages among the three *Arisaema* species, floral visitors of these species were collected daily using a hand-made aspirator in our flowering phenology quadrats. The exit holes of the male spathes were plugged with cotton before the male spathes opened to prevent the escape of the attracted floral visitors. In 2022, we collected 588 arthropods from *A. bockii*, 315 from *A. lobatum* and 697 from *A. erubescens* (Table S1). The collected arthropods were fixed in a freezer overnight and dried at room temperature (~10–30 °C). The samples were observed under a stereomicroscope (M205C; Leica Microsystems, Cambridge, UK) and identified to family for dipterans and to genus for fungus gnats, using identification manuals (McAlpine et al., 1981, 1987), Other arthropods were identified to order rank only.

**Post-pollination barriers: hybrid fruit and seed formation**

To test for the effect of pollen source (intra- vs. inter-specific) on fruit and seed formation, interspecific and intraspecific crosses conducted in the mixed community of three *Arisaema* species in 2022. For each *Arisaema* species, 30 female inflorescences were bagged. With the spathe opening above, we cut the base of spathe tube partially horizontally from the junction with a knife to expose female inflorescence. The inflorescences were sandwiched between two pieces of stiff paper, so that the inflorescences, between the paper, formed two relatively enclosed areas. One area was brushed with conspecific pollen using a small brush, and the other was brushed with heterospecific pollen in 15 inflorescences. In another 15 inflorescences, one area was brushed with conspecific pollen and the other was brushed with another heterospecific pollen. Pollinated areas were marked at the base of the inflorescence. The spathes were restored to its original state with tape and bagged until it withered. Approximately 3 months after pollination, we collected the middle two rows of fruit in the pollinated region. More than 150 fruits were collected for each treatment. Large and plump fruits were recorded as setting fruit, while small and wrinkled fruits were not. Large and aborted embryos were counted per setting fruit under a stereomicroscope. The rates of setting fruit and large embryos were calculated to assess fruit and seed formation.

**Quantifying reproductive isolation**

To determine the strength of individual barriers and their contribution to the total isolation, reproductive isolation was quantified by a unified approach directly related to gene flow proposed by Sobel and Chen (2014). We did not calculate the strength of geographic isolation among these species, as all three *Arisaema* species grew in the highest altitude area of the study site, and the elevation we recorded are not representative of the differences in altitude adaptation among the three species.

The strength of phenological isolation was calculated following the equation 4A of Sobel and Chen (2014):

where and represent the mean number of flowering days in which the female plant of each *Arisaema* species overlapped with the heterogenous and conspecific male plant, respectively.

The strength of pollinator isolation was calculated with reference to the equation *RI*4A in Sobel and Chen (2014) as follows:

where and represent the mean number of flower visitors for taxa *i* per day captured by the female plant of each *Arisaema* species and the mean number of all flower visitors per day captured by the female plant of each *Arisaema* species, respectively.andrepresent the mean number of flower visitors for taxa i captured per day by the heterogenous and conspecific male plant, respectively. Arthropods taxon that could not be identified were not included in the calculations.

The strength of the isolation barrier at hybrid fruit and seed production was calculated following the equation 1, For the isolation barrier at hybrid fruit production, *H* and *C* represent the fruit set of interspecific and intraspecific outcrosses, respectively. For the isolation barrier at hybrid seed production, *H* and *C* represent the seed set of interspecific and intraspecific outcrosses, respectively.

The cumulative strength of multiple sympatric barriers was calculated based on the equation *RI*4S3 in Sobel and Chen (2014) as follows:

where *n* is the number of barriers under consideration, and and are the probabilities of heterospecific and conspecific gene flow respectively for each barrier, *i*.

The absolute contribution of each individual barrier to the total reproductive isolation were calculated by equation from Sobel and Chen (2014) as follows:

where denotes the combined isolation calculated by including all barriers from the first to act (1) through the focal barrier (*i*), and denotes the same calculation omitting the focal barrier.

**Statistical analysis**

Interspecific differences in altitudinal distribution range were tested by generalized linear models (GLMs) (normal distribution, identity link function). Interspecific differences in flowering [begin](javascript:;) and end dates were tested for by GLMs (Poisson distribution, log link function). The interspecific differences in the floral visitor assemblage were examined by permutational multivariate analysis of variance (PERMANOVA) (Bray-Curtis dissimilarity index, 999 permutations) with the package vegan in R 4.3.1. (R Core Team, 2021). The effect of pollen source (intra- vs. inter-specific) on fruit set and seed set were tested for by GLMs (binomial distribution, logit link function).

**RESULTS**

**Geographic isolation**

Habitat elevation varied significantly among the three *Arisaema* species (*χ*2 = 10.30, *P* < 0.05). The mean habitat elevation of *A. bockii* (1325.31 ± 56.88 m a.s.l.) was significantly lower than that of *A. erubescens* (1337.30 ± 29.09 m a.s.l.) (*χ*2 = 9.77, *P* < 0.05), and the mean habitat elevation of *A. lobatum* (1327.73 ± 41.68 m a.s.l.) was intermediate between them, with no significant differences (both *P* > 0.05). There was a large overlap in altitudinal distribution range among the three species (1197–1403 m a.s.l.), and all three species were concentrated in 1275–1400 m a.s.l. (Fig. 2).

**Phenological isolation**

The dates of initial flowering differed significantly among the three *Arisaema* species in the Badagongshan Reserve (*χ*2 = 802.33, *P* < 0.001). The mean date of initial flowering of *A. bockii* (day of year: 109.27 ± 4.16) was 2 days earlier than that of *A. lobatum* (111.50 ± 3.80), and they were 18 and 16 days earlier than that of *A. erubescens* (127.37 ± 4.32), respectively (Fig. S2). The end dates of flowering also differed significantly among the three Arisaema species (*χ*2 = 381.04, *P* < 0.001). The end date of flowering of *A. bockii* (128.98 ± 3.19) was 2 days later than that of *A. lobatum* (126.67 ± 4.35), and they were 18 and 16 days earlier than that of *A. erubescens* (139.43 ± 4.68), respectively (Appendix S3). Flowering phenology overlap of the three *Arisaema* species varied among different species pairs. Flowering phenology of *A. bockii* (41 days) overlapped substantially with that of *A. lobatum* (40 days) for 40 days, and the pinks were close (Fig. 3). Flowering phenology of *A. erubescens* (30 days) partially overlapped that of the former two species for 23 days, and the pinks differed (Fig. 3).

**Pollinator isolation**

Floral visitor assemblage differed significantly among the three *Arisaema* species (*R*2 = 0.20, *P* < 0.001). Although for all three *Arisaema* species the most frequent insect visitors were Mycetophilidae and Sciaridae fungus gnats (49.8488.09%), the assemblage of fungus gnats differed somewhat among the three *Arisaema* species. Both *A. bockii* and *A. lobatum* attracted various genera of fungus gnats. Some genera of fungus gnats were attracted almost exclusively to *A. bockii* (*Epicypta* spp. and *Sciara* spp.) or *A. lobatum* (*Boletina* spp. and *Brevicornu* spp.), while others were attracted to both *Arisaema* species (*Mycetophila* spp. and *Bradysia* spp.). *A. erubescens* attracted almost exclusively *Mycetophila* spp., which were also attracted to the other two *Arisaema* species (Fig. 4). By observing the morphological characteristics of fungus gnats, we found that each genus was dominated by one species (Fig. 5), and that if a genus visited the inflorescences of more than one *Arisaema* species, the main species was the same.

**Hybrid fruit and seed formation**

Fruit set ranged between 85.56 and 93.26% for intraspecific crosses and between 0 and 40.23% for interspecific crosses (Fig. 6). Interspecific fruit set in both crosses was significantly lower than intraspecific fruit set when *A. bockii* (*χ*2 = 2685.11, *P* < 0.001), *A. lobatum* (*χ*2 = 159.55, *P* < 0.001) and *A. erubescens* (*χ*2 = 116.03, *P* < 0.001) were the pollen recipient.

Seed set ranged between 37.61 and 50.44% for intraspecific crosses and between 21.93 and 40.23% for interspecific crosses (Fig. 6). Interspecific seed set in both crosses was significantly lower than intraspecific fruit set when *A. bockii* (*χ*2 = 18.95, *P* < 0.001) and *A. lobatum* (*χ*2 = 17.88, *P* < 0.001). When *A. erubescens* was the pollen recipient, Interspecific fruit set was significantly lower than intraspecific fruit set only when crossed with *A. bockii* (*χ*2 = 10.04, *P* < 0.001).

**Strength of pre- and post-pollination barriers and its contribution to total isolation**

The strength of phenological isolation (mean ± s.d., 0.41 ± 0.36) varied from 0.12 to 0.83 among the three *Arisaema* species. Reproductive isolation was weak between *A. bockii* and *A. lobatum* (0.12 and 0.12) but strong in the other two species pairs (0.39–0.83). The absolute contribution of phenological isolation was equal to its strength, since we assumed that it occurred before other isolation barriers quantified (Fig. 7).

The strength of pollinator isolation (0.49 ± 0.17) was relatively strong in all pairs of the three *Arisaema* species, ranging from 0.22 to 0.73. The absolute contribution of pollinator isolation (0.28 ± 0.14) varied from 0.14 to 0.51 (Fig. 7).

The strength of reproductive barrier at hybrid fruit formation was strong (mean ± s.d. 0.73 ± 0.26), ranging from 0.39 to 1. The absolute contribution of isolation barrier at hybrid fruit formation (0.21 ± 0.14) varied from 0.02 to 0.37 (Fig. 7).

The strength of reproductive barrier at hybrid seed formation (mean ± s.d. 0.16 ± 0.16) varied from 0.07 to 0.39. The absolute contribution of isolation barrier at hybrid seed formation (0.02 ± 0.04) varied from 0.002 to 0.08 (Fig. 7).

The cumulative absolute contribution to total RI varied from 0.11 to 0.97 for pre-pollination barriers and from 0.01 to 0.45 for post-pollination barriers. The contribution of pre-pollination isolation (0.69 ± 0.31) to total isolation was significantly higher than that of post-pollination isolation barriers (0.23 ± 0.16) (*χ*2 = 10.57, *P* < 0.01) (Fig. 8).

The strength of total reproductive isolation (mean ± s.d., 0.99 ± 0.01) exceeded 0.97 in most species pairs among the three *Arisaema* species, except for species pair lob♀boc♂ (RItotal = 0.56) (Fig. 7).

**DISCUSSION**

**Pre‐ and post‐pollination mechanisms of** **reproductive isolation**

Differential adaptation to habitat is an important isolation mechanism that reduces interspecific gene flow by reducing the chance of interspecies encounters when close relatives come into contact (Glennon, Rissler, & Church, 2012; Schemske, 2010). For example, differences in habitat elevation led to strong reproductive isolation among *A. ehimense*, *A. serratum* and *A. tosaense* (Murata & Ohno, 1989). However, habitat elevations of the three *Arisaema* species mostly overlapped in this study. Although we have not quantified geographic isolation here conclusively, we suggest that the weak isolation was caused by the similar habitats of the three species. Habitat overlapped partly among other *Arisaema* species (Matsumoto et al., 2021; Matsumoto et al., 2019). In this study, all three *Arisaema* species preferred to grow on forest margins caused by road construction. Human factors might increase the chances of interspecies encounters and weakened habitat isolation among three *Arisaema* species.

Differences in flowering time hinder temporally interspecific pollen transfer when close relatives encounter (Liu et al., 2020; Xu et al., 2020). In this study, the contribution of flowering phenology to reproductive isolation varied in different species pairs. Substantially overlapping flowering phenology led to weak phenological isolation between *A. bockii* and *A. lobatum*, but partially overlapping flowering phenology led to effective phenological isolation between the late-flowering *A. erubescens* and the former two. Flowering phenology overlapped partly among other *Arisaema* species (Matsumoto et al., 2021; Matsumoto et al., 2019; Murata & Ohno, 1989). The overlapping flowering time of the study species may be due to similar flowering phenology between close relatives (Du et al., 2015). For example, 63 of the 70 *Arisaema* species recorded in China bloom between April and July (Li et al., 2010). On the other hand, long flowering periods need to be maintained for *Arisaema* species living at high altitudes with highly variable weather conditions to ensure successful pollination (Barriault, Gibernau, & Barabe, 2009), which makes flowering times easier to overlap.

Pollinator isolation is divided into mechanical isolation due to the structural contrivances of the flower and behavioral isolation due to the constancy of the pollinating animals to one kind of flower (Grant, 1949). In this study, similar trap flower structure of the three *Arisaema* species can not cause mechanical isolation, but a degree of differential floral visitor assemblages suggests behavioral isolation. Selective fungus gnat visitation is an important contributor to reproductive isolation in *Arisaema*. Some other *Arisaema* species have strong pollinators isolation caused by nearly completely different fungus gnat visitation (Matsumoto et al., 2021; Suetsugu et al., 2021). However, since some genera of fungus gnats in this study were attracted to two or more *Arisaema* species at the same time, the interspecific pollinator isolation remained incomplete. Chemical studies of the key pollinator-attracting compounds derived from *Arisaema* inflorescences will provide insights into the mechanism that underlie pollinator isolation.

Intrinsic isolation mechanisms may reduce hybrid fruit and seed formation when interspecific pollen transfer is present (Baack et al., 2015; Christie & Strauss, 2019). In this study, the fruit set and seed set of interspecific crosses were significantly lower than those of intraspecific crosses in the artificial crosses experiment, suggesting that the intrinsic post-pollination barriers at hybrid fruit and seed formation may effectively reduce the production of hybrids among the three *Arisaema* species. Although natural hybridization has occurred among some *Arisaema* species (Hayakawa et al., 2011; Hayakawa et al., 2013; Kobayashi et al., 2005; Lee et al., 2011; Maki and Murata, 2001; Murata and Ohno, 1989; Sanders and Burk, 1992), and F1 hybrids had high germination rates and high pollen fertility among some *Arisaema* species (Murata and Ohno, 1989; Murata et al., 2018), we suggest that strong post-pollination barriers, especially barriers at hybrid fruit and seed formation, may still exist among some *Arisaema* species.

**Contribution of pre- and post-pollination isolation to total isolation**

Pre-pollination barriers associated with adaptive ecological divergence were important, while intrinsic post-pollination barriers associated with the accumulation of genetic incompatibilities contributed little to total reproductive isolation in many case studies of reproductive isolation in plants (Christie et al., 2022; Lowry et al., 2008). In this study, the early-acting pre-pollination barriers contributed more to reproductive isolation than the post-pollination barriers in most species pairs of the three *Arisaema* species, although the reproductive barrier at the fruit formation stage was strong in these species pairs. These results are consistent with other case studies of reproductive isolation in plants (Christie & Strauss, 2019; Karrenberg et al., 2019), and support the idea that ecological divergence as primary drivers of angiosperm divergence and/or the maintenance of contemporary species boundaries (Christie et al., 2022; Lowry et al., 2008). However, ecological divergence does not always contribute more to the maintenance of species integrity. For species pairs *lob*♀*boc*♂, pre-pollination barriers were weak and early post-pollination barriers at the fruit and seed formation stage were important contributors to reproductive isolation. Despite incomplete cumulative isolation of species pairs *lob*♀*boc*♂ (RItotal = 0.56) in this study, we found only a few of the morphologically intermediate putative hybrids. We suspect that intrinsic and extrinsic late post-pollination barriers such as hybrid sterility may play a role in maintaining their species boundaries. The difference in chromosome numbers between *A. bockii* (2n = 26) and *A. lobatum* (2n = 28) may reduce the fertility of hybrids, as has been reported for *A. limbatum* (2n = 26) and *A. ringens* (2n = 28) (Kobayashi et al., 2005).

Effective pre-pollination barriers are expected to prevent interspecific gene flow among sympatric *Arisaema* species (Matsumoto et al., 2021; Matsumoto et al., 2019; Murata et al., 2018; Suetsugu et al., 2021). For example, in 18 of the 20 species pairs among five *Arisaema* species examined, the strength of total pre-pollination reproductive isolation exceeded 0.96 (Matsumoto et al., 2021). In contrast, post-pollination barriers are considered to be weak or absent among *Arisaema* species (Matsumoto et al., 2019; Murata et al., 2018). However, in this study, pre-pollination barriers were not able to completely prevent interspecific gene flow among the three *Arisaema* species, although phenological isolation and pollinator isolation effectively reduced interspecific pollen transfer. Strong post-pollination isolation also played an important role in preventing interspecific gene flow among the three *Arisaema* species, particularly in the reproductive isolation between *A. bockii* and *A. lobatum*. Thus, both pre- and post-pollination barriers are required to effectively prevent hybridization and maintain the integrity of the three sympatric *Arisaema* species.

Intrinsic post-pollination barriers may enforce species boundaries when pre-pollination isolation does not completely prevent interspecific pollen transfer. However, as post-pollination barriers may result in the production of hybrids with low viability or fertility, or from mating costs associated with hybridization, such as wasted gametes and stigma clogging, selection would favor the evolution of pre-pollination barriers (Ortiz-Barrientos, Grealy, & Nosil, 2009; Servedio & Noor, 2003). This process is referred to as reinforcement (Hopkins, 2013). Investigation of the process of reinforcement will reveal whether the post-pollination barriers has facilitated the evolution of the pre-pollination barriers among the three sympatric *Arisaema* species.

**CONCLUSION**

In this study, we quantified multiple pre- and post-pollination barriers and their absolute contribution to total reproductive isolation among three sympatric *Arisaema* species in the Badagongshan Reserve. Although phenological isolation and pollinator isolation significantly reduced frequencies of interspecific pollen transfer when geographic isolation was weak, pre-pollination reproductive barriers may be insufficient to prevent interspecific hybridization among the three *Arisaema* species. Our study is the first to show the [effective](javascript:;) reproductive barriers at the hybrid fruit and seed formation stages between *Arisaema* species. Our study confirms that reproductive isolation among three sympatric *Arisaema* species is a result of a combination of pre-and post-pollination barriers. Future studies of reproductive isolation in *Arisaema* should include multiple pre- and post-pollination reproductive barriers of a broader range of species to determine if post-pollination reproductive barriers play an important role in the maintenance of contemporary species boundaries in this large genus.

**ACKNOWLEDGEMENTS**

We thank the Office of Badagongshan National Nature Reserve for providing permision to perform our fieldwork; Zhi-Rong Gu, Bi-Wu Qin and Ju-Ying Yuan for help in the field survey; Mr. Denis Filer for meaningful advice on improving this manuscript. This work was supported by the National Natural Science Foundation of China (Grant No. 81760695).

**DATA AVAILABILITY STATEMENT**

Data are archived with Dryad: <https://doi.org/10.5061/dryad.08kprr56g>

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**Figure legends**

**Fig. 1** Three examined *Arisaema* species in the Badagongshan Reserve. (A) *A. bockii*. (B) *A. lobatum*. (C) *A. erubescens*. (D–I) Inflorescences of *A. bockii*. (D) and (E) Vertical section of male and female inflorescence, respectively. (F) An exit hole in bottom of the male spathe. (G) the closed bottom of the female spathe. (H) Male spadix. (I) Female spadix.

**Fig. 2** Habitat elevation of the three *Arisaema* species growing along a roadside in the Badagongshan Reserve. Black circles are mean habitat elevation. Error bars represent the 5th and 95th percentiles. Small grey rhombuses indicate raw values. Different letters mean significant differences at P ≤ 0.05. Species abbreviations: *boc*, *bockii*; *lob*, *lobatum*; *eru*, *erubescens*.

**Fig. 3** Flowering phenology of *Arisaema bockii* (A), *A. lobatum* (B) and *A. erubescens* (C) in the Badagongshan Reserve. Species abbreviations as in Fig. 2 legend.

**Fig. 4** Floral visitor assemblages of the three *Arisaema* species in the Badagongshan Reserve. ‘Other arthropods’ includes arthropods that we were unable to identify. Species abbreviations as in Fig. 2 legend.

**Fig. 5** Main fungus gnats visiting the inflorescences of the three *Arisaema* species in the Badagongshan Reserve. (A) *Mycetophila* sp.1. (B) *Boletina* sp.1. (C) *Brevicornu* sp.1. (D) *Epicypta* sp.1. (E) *Sciara* sp.1 (F) *Bradysia* sp.1. Bar = 5mm.

**Fig. 6** Mean fruit set (A) and seed set (B) (± SEM) obtained from intra‐ and interspecific crosses between the three *Arisaema* species in the Badagongshan Reserve. Letters indicate species and shapes indicate maternal species for each cross: B/square = *A. bockii*, L/ circle = *A. lobatum*, E/ triangle = *A. erubescens*. Letters over symbols indicate significant differences between treatments within each maternal species. Species abbreviations as in Fig. 2 legend.

**Fig. 7** Strength of multiple pre-pollination and post-pollination barriers among the three *Arisaema* species and their absolute contribution to total isolation. The bar graphs represent the strength of individual barrier. The line graph represents the cumulative contribution to reproductive isolation of a mechanism after accounting for each of the investigated previous mechanisms. Species abbreviations as in Fig. 2 legend.

**SUPPORTING INFORMATION**

**Table S1.** Visitation frequency of arthropods to the three sympatric *Arisaema* species in the Badagongshan Reserve in 2022.

**Fig. S1.** Two examined *Arisaema* species and putative hybrid in the Badagongshan Reserve.

**Fig. S2.** Flowering start (A) and end dates (B) of the three examined species in the mixed *Arisaema* population of the Badagongshan Reserve.