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Pre-pollination isolation by pollinator specificity: settling moth versus hawkmoth pollination in two sympatric *Habenaria* species (Orchidaceae)

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Abstract

Background *Habenaria* species typically produce green or white flowers, bear nectar spurs, emit crepuscular-nocturnal scents, and are usually pollinated by crepuscular/nocturnal moths. However, the roles of floral traits in pollinator differentiation contributing to reproductive success in sympatric *Habenaria* species require further definition. In this study, we investigated flowering phenology, floral traits, pollinator behavior, and reproductive success of two co-occurring and co-blooming *Habenaria* species (*H. ciliolaris* and *H. schindleri*) in southeastern China. We also conducted intraspecific and interspecific hand pollinations to determine their respective breeding systems and the extent of interspecific post-pollination isolation.

Results Both species are self-compatible but rely on pollinators for sexual reproduction. *Habenaria ciliolaris* was pollinated by two species of nocturnal, settling moths (*Thinopteryx nebulosa* and *Porsica* sp.). They foraged for nectar upside down or from one side, carrying pollinaria on their eyes because the curved lateral lobes of the labellum block the movements of settling moths on these flowers. *Habenaria schindleri* was pollinated by two crepuscular hawkmoths (*Eupanacra mydon* and *Hippotion rafflesi*), which carried pollinaria between their palpi while hovering in front of flowers while taking nectar. The proboscis lengths of pollinators of both *Habenaria* species matched the spur lengths of their corresponding flowers. *Habenaria ciliolaris* experienced a high level of inbreeding depression. Interspecific pollination by applying pollen grains from *H. schindleri* to the stigmas of *H. ciliolaris*, resulted in a low level of seed set.

Conclusions Differences in floral morphology and nectar volume/concentration appear to contribute to segregating members of the pollinator guild of some *Habenaria* species. These findings offer new insights to our understanding of the partitioning of pollinators between co-blooming congeners in the Orchidaceae.

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Keywords *Habenaria*, Hawkmoth, Settling moth, Pollination, Inbreeding depression, Reproductive isolation, Species coexistence

Background

Reproductive isolation in plants occurs through multiple barriers that operate sequentially, restricting gene flow between species [1, 2]. It plays a key role in the formation of new species and the maintenance of species integrity [3, 4]. Reproductive barriers are classified according to their timing in the life history, and include pre-pollination mechanisms such as ecogeographic, temporal, mechanical, and pollinator isolation; post-pollination to pre-zygotic mechanisms such as conspecific pollen precedence and gametic incompatibilities; and post-zygotic mechanisms such as hybrid inviability, hybrid sterility and F_2 breakdown [1, 2, 5, 6]. Pre-pollination barriers limit the transfer of pollen between species, which are often stronger and more effective in maintaining reproductive isolation than post-pollination barriers [2, 3, 7]. Floral isolation refers to a reduction in interspecific pollen transfer relative to intraspecific pollen transfer that is caused by floral traits and pollinator behavior [8, 9]. Divergent floral traits may attract specific pollinators, influencing their behavior and pollen deposition locations [9, 10]. Adaptation to different pollinators can drive divergence in floral traits, and pollinator-mediated selection of floral traits is a major driving force behind the diversification of flowering plants [9–15]. Floral trait differentiation and corresponding pollinator divergence act synergistically with other isolating mechanisms to promote coexistence among taxa within the same lineage [9, 16, 17]. Therefore, pollinator shifts within lineages appear to be crucial to the evolution and diversification of angiosperms [15, 18].

Previous studies have focused primarily on pollination systems involving shifts between closely related plant species in which there is a switch to pollinators in vastly different orders or classes, such as shifts from bee to hummingbird pollination in *Costus* (Costaceae) [19] or from hummingbird to bat pollination in centropogonid Campanulaceae [20]. Fewer studies have addressed more subtle shifts where congeneric plant species remain dependent on pollen vectors belonging to the same order, such as the crepuscular-nocturnal members of the Lepidoptera. In particular, the Orchidaceae is one of the largest families of flowering plants, comprising over 29,000 species and it accounts for nearly 10% of the species diversity of flowering plants [21–23]. As floral characters account for most of the long historical and excessive attention paid to orchid taxonomy, theorists have logically attributed orchid speciation to an adaptive radiation exploiting regional guilds of specific pollinators [24–27]. Orchids generally exhibit strong floral isolation and low levels of pollinator sharing among co-occurring and related species. In fact, each orchid species is usually pollinated by only one or a few pollinator species, regardless of the diversity inherent within local pollinator guilds [8, 28, 29].

The genus *Habenaria* Willd. currently consists of approximately 900 species distributed across temperate and tropical regions [21, 30, 31]. The flowers of this genus have spurs containing nectar, are typically green or white, and emit scents at night or dusk, consistent with pollination syndromes that exploit nocturnal moths. Hawkmoths and settling moths, representing several unrelated families, are the most frequently reported pollinators for *Habenaria* species [32–37]. A few exceptions document pollination by diurnal butterflies [38–40]. Although there is a growing number of studies on the pollination biology of *Habenaria* species, most focus on a single species, with only a few exceptions exploring multiple sympatric species [32, 36, 40, 41]. These studies suggest that both pollinator specificity and pollinator sharing exist in sympatric *Habenaria* species. For sympatric species sharing pollinators, their pollinaria are attached to different body parts of the same pollinators due to morphological differentiations between pairs of *Habenaria* species [36, 40, 41]. Recent experiments removing particular parts of flowers suggest that floral traits can influence the attachment locations of pollinaria on the pollinators and reproductive success in *Habenaria* species [38, 42]. Interlinked floral traits appear to mediate floral isolation as the flowers interact with pollinators. Therefore, we can expect that differentiation in floral morphology may lead to different pollination processes in this highly diverse plant genus.

To our knowledge, only two studies have tested the strength of both pre- and post-pollination isolation among sympatric *Habenaria* species, both conducted in Yunnan Province, southwestern China. Their results suggest that pre-pollination barriers, particularly pollinator specificity and pollinaria attachment site differentiation, play a key role in reproductive isolation, while the strength of post-pollination barriers varies across different species pairs [36, 41]. Naturally occurring populations in China often consist of multiple species of *Habenaria* that co-bloom along the same environmental gradient. However, the relative contributions of their diverging floral traits, incompatibility systems, and limited pollinator availability as factors promoting reproductive isolation among congeners require further elucidation [36, 41].

During our preliminary field observations, we found that two sympatric species, *Habenaria ciliolaris* Kraenzl. and *H. schindleri* Schltr., bloomed at the same time in the Jiulianshan National Natural Reserve, located on the border of Jiangxi and Guangdong Provinces in southeastern China. These two species occupied different microhabitats: *H. ciliolaris* in the dense subtropical evergreen broadleaved forest understory (Fig. 1 A), and *H. schindleri* in the open wet grassland (Fig. 1 J), both within three kilometers of each other. They exhibited different floral traits, including differences in petal morphology and color, the distance between viscidia, spur length, nectar volume, and floral scents detected by the human nose. Notably, the spur length of *H. ciliolaris* was about 2 cm, while that of *H. schindleri* was about 4 cm, twice as long. Additionally, the lateral lip lobes of *H. ciliolaris* curve forward to the same height of the stigma and column, whereas the lip of *H. schindleri* is pendulous (Fig. 1). We predict that the curved lobes of the lip in *H. ciliolaris* should block visitation by hovering hawkmoths. Consequently, these two orchid species are likely pollinated by different insects. Using these two sympatirc *Habenaria* species as model systems, we address the following questions: (1) What mechanisms determine the differentiation of pollination systems between these two sympatric *Habenaria* species? (2) What reproductive

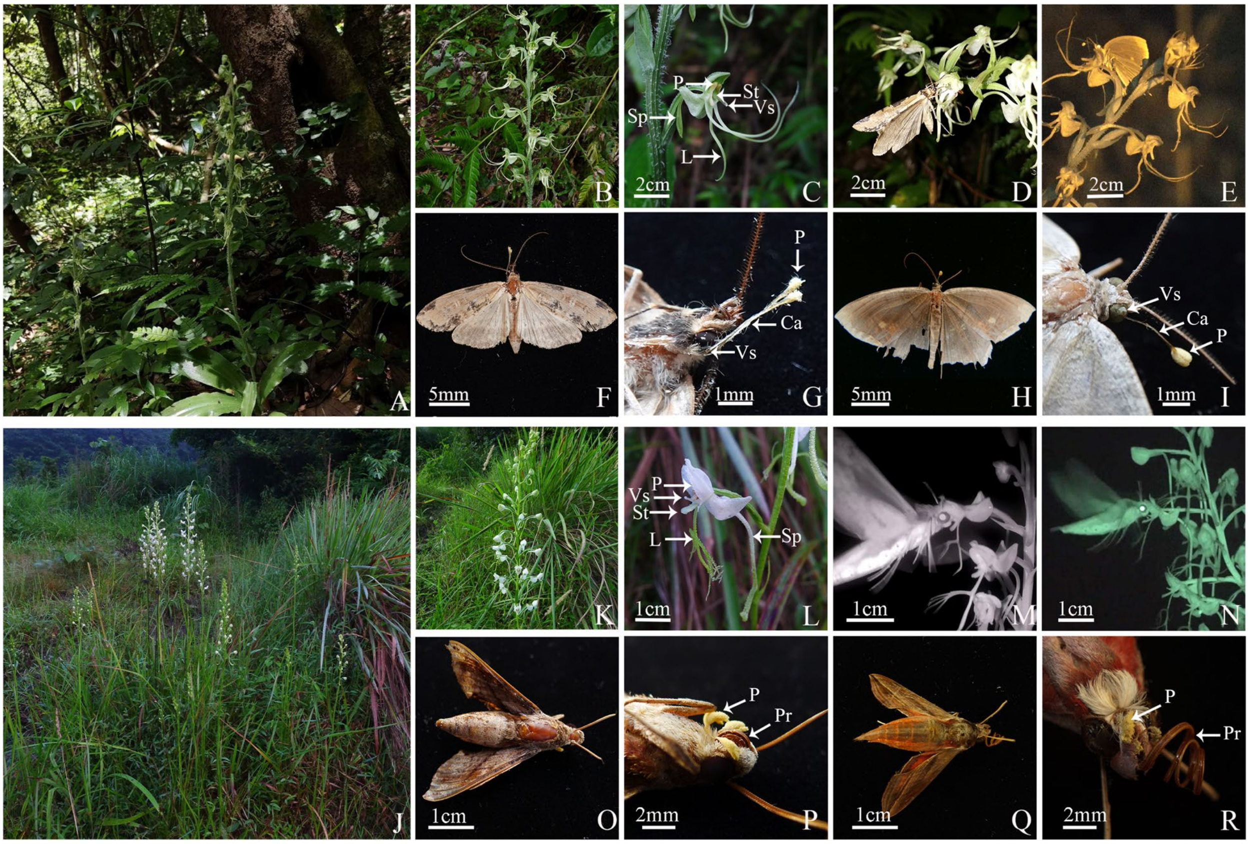

Fig. 1 Habitats, flowers, and pollinators of *Habenaria ciliolaris* (A-I) and *Habenaria schindleri* (J-R) in Jiulianshan National Nature Reserve, Jiangxi Province, southeastern China. **A** Blooming plants of *H. ciliolaris* growing under a subtropical evergreen forest. **B** An inflorescence of *H. ciliolaris.* **C** A flower of *H. ciliolaris*, showing the Pollinia (P), Viscidium disk (Vs), Stigma (St), Labellum (L), and Spur (Sp). **D** A *Porsica* sp. foraging on a *H. ciliolaris* flower. **E** A *Thinopteryx nebulosa* foraging on a *H. ciliolaris* flower. **F**-**G** *Porsica* sp. carrying a pollinarium of *H. ciliolaris* on its eye. P, Pollinia; Ca, caudicle; Vs, viscidium attached to the eyes of the moth. **H**-**I** *Thinopteryx nebulosa* carrying a pollinarium of *H. ciliolaris* on its eye. P, Pollinia; Ca, caudicle; Vs, viscidium attached to the eyes of the moth. **J** Blooming plants of *H. schindleri* growing on a wet grassland at the edge of a subtropical evergreen forest. **K** An inflorescence of *H. schindleri.* **L** A flower of *H. schindleri*, showing the Pollinia (P), Viscidium disk (Vs), Stigma (St); Labellum (L), and Spur (Sp). **M** A *Eupanacra mydon* foraging on a *H. schindleri* flower. **N** A *Hippotion rafflesi* foraging on a *H. schindleri* flower. **O**-**P** A *Eupanacra mydon* carrying pollinaria of *H. schindleri* between its palpi. P, Pollinia; Pr, Proboscis. **Q**-**R** A *Hippotion rafflesi* carrying pollinaria of *H. schindleri* between its palpi. P, Pollinia; Pr, Proboscis

isolation mechanisms prevent reproductive interference between them?

Methods

Study species and site

Habenaria ciliolaris is a terrestrial orchid primarily found in southern China, extending into northern Vietnam [43]. It typically grows in the shaded understory of forests found along valleys at elevations ranging from 100 to 1800 m. The racemose inflorescence, measuring 9–23 cm, bears 6–30 flowers that emit a pleasant scent to humans at night. The perianth segments are greenish-white. The lip petal is long, tri-lobed, and filiform at its base, with two reflexed lateral lobes and a pendulous mid-lobe (Fig. 1 A and B). *Habenaria schindleri* is also terrestrial, and primarily distributed through eastern China, extending into Korea and Japan [43]. It typically grows in wet grasslands at elevations ranging from 200 to 1700 m. The racemose inflorescence, measuring 5–18 cm, bears 5–25 flowers, which do not emit any noticeable scent detected by the human nose. Five of the perianth segments are white with greenish and spreading trilobed lip (Fig. 1 I and J).

This study was conducted during the flowering seasons of both species in 2018 and 2019 in the Jiulianshan National Nature Reserve, located on the border of Jiangxi and Guangdong Provinces in southeastern China. We selected two populations for fieldwork, each comprising more than 60 individuals. The *H. ciliolaris* population was situated in a subtropical evergreen broadleaved forest near a stream, with population center coordinates at 114°27′E, 24°33′N, and elevations ranging from 495 to 518 m. The *H. schindleri* population grew in a wet grassland at the edge of a subtropical evergreen broadleaved forest, with population center coordinates at 114°29′E, 24°33′N, and elevations ranging from 721 to 739 m. Full GPS information is not provided due to conservation considerations for these orchid species. Voucher specimens were deposited in the Herbarium of Nanchang University (JXU).

Floral phenology and morphology

We studied the floral phenology, morphology, and nectar secretions of *H. ciliolaris* and *H. schindleri* from mid-July to the end of September in 2019. Thirty individuals from each population were randomly selected and tagged to record phenological data, including the date of the first flower opening, the last flower withering, and the opening and withering dates of each flower within each inflorescence.

To measure floral traits, one flower was randomly selected from each of the 30 inflorescences. These flowers were measured using digital calipers (MNT-150, Shanghai, China) with an accuracy of 0.01 mm. The following floral traits were measured: 1) spur length, 2) the distance from the spur's opening (sinus) to the viscidium, 3) the distance from the spur's terminus to the viscidium, 4) the distance between the two stigmatic lobes, 5) the distance between the two viscidia on each stigmatic lobe (rostellum), 6) the length of one of the two caudicles, 7) the length of one of two pollinia, and 8) the width of one of the two pollinia (see Table 1).

Nectar measurements

We randomly selected 20 inflorescences in the bud stage and covered each inflorescence with an organza mesh bag to prevent insect visitation. Once all flowers on an inflorescence had opened, we randomly selected two flowers, one for nocturnal and one for diurnal nectar measurement, respectively. Nectar in *H. ciliolaris* was measured at approximately 00:00 (when the pollinators are most active) and 12:00, while nectar in *H. schindleri* was measured at approximately 5:30 (when the pollinators are most active) and 17:30. We measured the nectar level inside the spur using a digital caliper. Afterward, we cut off the spur tip with scissors and estimated the nectar volume using a glass microcapillary tube with an inner diameter of 0.5 mm. The concentration of dissolved nectar solutes was determined with a handheld sugar refractometer (LH-T10, 0–50%, Hangzhou, China).

Pollinator observations

We observed floral visitors of *H. ciliolaris* and *H. schindleri* from mid-July to the end of September over two consecutive years. Diurnal visitors were observed from 6:00 to 18:00 over 6 days (a total of 72 h) for both species. Crepuscular and nocturnal visitors were observed from 18:00 to 06:00 over 16 days (a total of 192 h) for both species.

The foraging behavior of pollinators was recorded using four digital cameras (model HDR-AC3, Ordro, Shenzhen, China). Pollinators foraging on the flowers were captured with butterfly nets. The position of pollinaria attachment was recorded, and the proboscis of the insects were carefully unrolled and measured using a digital caliper. To minimize disruption to pollination services, we measured the proboscis length of only one individual per pollinator species due to the low abundance of visitors. Voucher specimens of each visiting species were collected, pinned, and deposited in the Zoological Museum of Nanchang University. These specimens were identified by entomologist Xing-Ping Liu of Jiangxi Agricultural University, China.

Table 1 Floral morphology and nectar production of *H. ciliolaris* and *H. schindleri* in Jiulianshan National Nature Reserve, Jiangxi Province, southeastern China

Floral traits	Replica	*H. ciliolaris*		*H. schindleri*		U test results	
		range	mean ± SD	range	mean ± SD	W	*P* value
Floral morphology (mm)							
Length of spur	30	20.44–23.79	22.34 ± 0.99	37.86–40.75	39.09 ± 0.76	0	< 0.001
Distance between opening of spur to the viscidium	30	2.71–3.01	2.87 ± 0.09	3.82–4.05	3.94 ± 0.06	0	< 0.001
Distance between the terminus of the spur to the viscidium	30	23.3–26.59	25.19 ± 0.99	41.87–44.68	43.03 ± 0.75	0	< 0.001
Distance between the two stigma lobes	30	0.91–1.23	1.07 ± 0.10	0	0	900	< 0.001
Distance between two viscidia	30	7.23–9.09	8.14 ± 0.48	0	0	900	< 0.001
Length of caudicle	15	2.45–2.74	2.59 ± 0.10	1.43–1.59	1.53 ± 0.05	225	< 0.001
Length of pollinium	15	0.59–0.77	0.69 ± 0.06	0.71–0.89	0.81 ± 0.05	12	< 0.001
Width of pollinium	15	0.44–0.54	0.49 ± 0.03	0.31–0.41	0.36 ± 0.03	225	< 0.001
Nectar properties							
Nectar level (day, mm)	20	5.07–8.47	6.57 ± 0.83	16.69–25.16	20.45 ± 2.95	0	< 0.001
Nectar level (night, mm)	20	5.79–7.97	6.61 ± 0.69	22.46–39.79	35.09 ± 4.89	0	< 0.001
Nectar volume (day, μL)	20	2.50–5.93	4.39 ± 0.99	4.89–10.17	7.16 ± 1.47	22	< 0.001
Nectar volume (night, μL)	20	3.15–5.84	4.70 ± 0.85	7.08–16.37	12.34 ± 2.75	0	< 0.001
Nectar concentration (day, %)	20	13.5–26.0	19.53 ± 3.10	23.0–28.5	25.65 ± 1.48	12	< 0.001
Nectar concentration (night, %)	20	12.0–22.0	18.53 ± 2.38	12–25.5	21.85 ± 4.78	96	0.005

Pollination efficiency and pollen limitation

To estimate pollination efficiency (*PE*), we examined pollinarium removal and deposition in 30 flowers of *H. ciliolaris* and *H. schindleri* during their later flowering stages. *PE* was calculated following Scopece et al., (2010) [44]: $PE = F_p \,/\, F_r$, where F_p represents the percentage of pollinated flowers and F_r represents the percentage of flowers with one or both pollinaria removed. To measure the level of pollen limitation in *H. ciliolaris* and *H. schindleri*, we calculated the pollen limitation index (*PLI*) following Larson and Barrett (2000) [45]: $PLI = 1–(P_o/P_s)$, where P_o is the percentage of fruit set in open-pollinated controls (i.e., natural pollination, as shown below), and P_s is the percentage of fruit set in plants that received supplemental cross pollen (see hand cross-pollination below). *PLI* ranges from 0 (indicates no pollen limitation) to 1 (indicates extreme pollen limitation) [46].

Hand pollinations

In July 2019, 30 inflorescences in bud were bagged for both *H. ciliolaris* and *H. schindleri*. The following six treatments were initiated 3–5 days after the buds opened: (a) Natural pollination – all flowers were left intact and exposed to insect visitors throughout their respective floral lifespans; (b) Bagging (bagged intact flowers) – all flowers were left intact but covered with a mesh bag until they withered to test for possible autonomous selfing; (c) Emasculation – anthers were removed, and flowers were kept under the bag until they withered to test for apomixis; (d) Hand self-pollination – a pollinium from a flower was applied to its own two stigmatic lobes; (e) Hand cross-pollination – a pollinium from one flower were applied to the stigmatic lobes of another flower at least 10 m away; (f) Interspecific pollination – a pollinium from *H. ciliolaris* was applied to the stigmatic lobes of *H. schindleri* and vice versa. Fruit set and seed embryonic development for each treatment were recorded in October 2019.

Embryonic development

To detect the extent of inbreeding depression within each species and assess seed viability under interspecific pollination, ten dehiscent ovaries from each treatment of hand pollination (see above) were harvested to assess embryonic development. All seeds from each fruit were placed on a petri dish and examined under a light microscope (Olympus BX51, Tokyo, Japan). Seeds were categorized into four groups following the method of Ren et al. (2014) [47] and Tao et al. [48]: large embryo, small embryo (embryos that were half or less than half of large embryos), aborted embryo (collapsed, reduced, and incomplete development), and no embryo (transparent seed coat lacking an embryo). We checked 100 seeds in each capsule. The inbreeding depression index (δ) was calculated according to Suetsugu et al. (2015) [49] using the formula: $\delta = 1 -$ (proportion of well-developed seeds after self-pollination / proportion of well-developed seeds after cross-pollination). Only seeds with large embryos were considered well-developed.

Data analysis

Since most data did not follow a normal distribution, the Mann–Whitney U test was used to assess differences in floral morphological traits and nectar properties (nectar level, volume, and percentage of dissolved solutes) between *H. ciliolaris* and *H. schindleri*. The Kruskal–Wallis test, followed by pairwise Wilcoxon rank-sum tests, was conducted to evaluate differences in embryonic development among different pollination treatments. All analyses were performed using R statistical software (version 4.4.1) [50].

Results

Floral phenology, morphology, and nectar

The flowering periods of the two populations of *H. ciliolaris* and *H. schindleri* overlapped for 42 days. The *H. ciliolaris* population bloomed for 44 days, from July 25 to September 6. The flowering period of a single inflorescence in *H. ciliolaris* lasted 25–28 days ($N=30$), and the lifespan of a single flower lasted 18–20 days ($N=30$). In contrast, the *H. schindleri* population bloomed for 60 days, from July 27 to September 24. The flowering period of a single inflorescence in *H. schindleri* lasted 15–30 days (N = 30), and the life span of a single flower lasted 8–11 days ($N=30$).

Habenaria ciliolaris and *H. schindleri* exhibited significant differences in floral morphology (Table 1, Fig. 2). *Habenaria schindleri* exhibited greater spur length, distance from the sinus to the viscidium, distance from the spur terminus to viscidium, and pollinium length compared to *H. ciliolaris*. In contrast, *H. ciliolaris* showed a greater distance between the two stigma lobes, distance between the two viscidium, caudicle length, and pollinium width than *H. schindleri* (Table 1, Fig. 2).

The nectar level (the height of nectar solution in the spur), volume, and percentage of dissolved solutes of *H. schindleri* were significantly higher than those of *H. ciliolaris*, both during the day and at night (Table 1). During the day, the nectar level of *H. schindleri* was about three times that of *H. ciliolari*. At night, the nectar level of *H. schindleri* was about five times higher than that of *H. ciliolaris*. In *H. ciliolaris*, the nectar level ($W=198$, $p=0.96$), volume ($W=165$, $p=0.35$), and percentage of solutes ($W=257$, $p=0.13$) in the spur did not differ significantly between night and day. Conversely, in *H. schindleri*, the nectar level ($W=7$, $p<0.001$) and volume ($W=24$, $p<0.001$) were significantly higher at night compared to the day while the percentage of dissolved solutes was lower at night compared to the daytime ($W=319$, $p=0.001$).

Pollinator observations

Two species of settling moths and two species of hawkmoths were the effective pollinators of *H. ciliolaris* and *H. schindleri*, respectively. Pollinator visits to the flowers of both *H. ciliolaris* and *H. schindleri* are infrequent. During the daytime, no pollinators were observed visiting the flowers of either species.

Two species of settling moths, *Thinopteryx nebulosa* (Geometridae) and *Porsica* sp. (Notodontidae), were effective pollinators of *H. ciliolaris* (Fig. 1 C-H). These

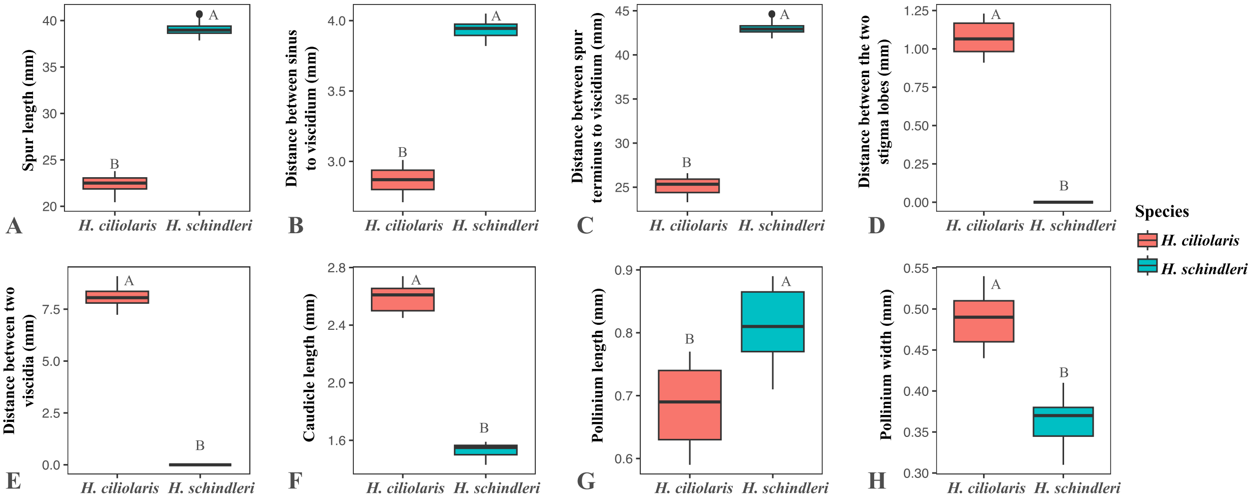

Fig. 2 Boxplot of eight floral morphological traits of *H. ciliolaris* and *H. schindleri*. **A** Spur length; **B** Distance from sinus to viscidium; **C** Distance from the spur terminus to the viscidium; **D** Distance between the two stigma lobes; **E** Distance between two viscidia; **F** Caudicle length; **G** Pollinium length; **H** Pollinium width. Different upper-case letters indicate significant differences ($P<0.01$)

moths foraged primarily from 22:00 to 03:00. During their visits, they first landed on the flowers of *H. ciliolaris* before probing for nectar from either side. The proboscis lengths of *Thinopteryx nebulosa* (17.35 mm, $N = 1$) and *Porsica* sp. (16.63 mm, $N = 1$) were slightly shorter than the spur length of *H. ciliolaris* (22.34 mm). Consequently, the moths had to force their proboscides deeply into the spur to access the nectar, often pressing their heads against the column and causing the viscidia of the pollinaria to attach to their eyes. A total of eight visits by *Porsica* sp. and two visits by *Thinopteryx nebulosa* to the flowers of *H. ciliolaris* were recorded.

Two species of hawkmoths, *Eupanacra mydon* and *Hippotion rafflesi* (Sphingidae), were the effective pollinators of *H. schindleri* (Fig. 1 K-P). These hawkmoths foraged primarily from 4:00 to 6:00. Their foraging behaviors were similar: they hovered above the flowers before inserting their proboscides into the spurs. The proboscis lengths of *Eupanacra mydon* (33.21 mm) and *Hippotion raffles* (38.35 mm) were both shorter than the spur length of *H. schindleri* (39.10 mm). As a result, these hawkmoths had to force their proboscides deep into the spur, pushing their head against the column until the flowers's viscidia adhered to their palpi. A total of four visits by *Eupanacra mydon* and two visits by *Hippotion raffles* to the flowers of *H. schindleri* were recorded. In addition to these two hawkmoth pollinators, two visits by the long-tongued hawkmoth *Rhyncholaba acteus* (proboscis length: 59.35 mm) to *H. schindleri* were also recorded. Although these insects were able to reach the nectar, they did not contact the reproductive parts of the orchid and therefore could not transport the pollinaria. Consequently, they can be considered nectar thieves.

Pollination efficiency and pollen limitation

We found that 53.33% of the flowers of *H. ciliolaris* had one or two pollinaria removed, and 66.67% had whole pollinia or fragments deposited on their receptive, stigmatic lobes. In contrast, 66.08% of the flowers of *H. schindleri* lacked one or two pollinaria, while 70.00% had pollinaria or fragments deposited on their stigmatic lobes (Table 2). The pollination efficiency (*PE*) of *H. ciliolaris* ($PE = 1.25$) was higher than that of *H. schindleri* ($PE = 1.06$). Both species exhibited pollination efficiencies slightly higher than 1, indicating that, on average, at least one flower was pollinated for each flower acting as a pollen donor. Additionally, *H. ciliolaris* ($PLI = 0.333$) exhibited greater pollen limitation than *H. schindleri* ($PLI = 0.25$). The *PLI* values were calculated based on fruit set data under natural and cross-pollination conditions, as shown in Table 3.

Breeding system, fruit set and embryonic development

Both bagged intact flowers and emasculated flowers (with anthers removed) failed to produce fruits in both species (Table 3). The fruit set of naturally pollinated flowers was 66.7% for *H. ciliolaris* and 70% for *H. schindleri*. For hand self-pollinated and cross-pollinated flowers, fruit set was higher: 96.7% and 100%, respectively, for *H. ciliolaris*, and 93.3% for both hand self- and cross-pollinated flowers of *H. schindleri*.

The fruits of *H. schindleri* had a higher proportion of large embryos compared to *H. ciliolaris* across all pollination treatments: natural, hand self-, and cross-pollination (Fig. 3A). In *H. ciliolaris*, the highest proportion of large embryos was observed in fruits resulting from hand cross-pollination, followed by natural pollination, with the lowest proportion found in hand self-pollination. In contrast, no significant differences in the proportion of large embryos were detected between the different treatments for *H. schindleri* (Fig. 3A). Hand self-pollinated fruits of *H. schindleri* and cross-pollinated fruits of *H. ciliolaris* had a lower proportion of small embryos compared to other treatments (Fig. 3B). Hand self-pollinated fruits of *H. ciliolaris* had the highest proportion of aborted embryos, while cross-pollinated fruits of *H.*

Table 2 Pollinators and pollination features of *H. ciliolaris* and *H. schindleri*

Species	Pollination time	Pollinators	Proboscis length(mm)	Pollinaria attach site	Pollination efficiency (*PE*)
H. ciliolaris	22:00–03:00	*Porsica* sp. (Notodontidae)	17.35 ($N = 1$)	On the eyes	1.25 (66.67 / 53.33)
		Thinopteryx nebulosa (Geometridae)	16.63 ($N = 1$)	On the eyes	
H. schindleri	04:00–06:00	*Eupanacra mydon* (Sphingidae)	33.21 ($N = 1$)	Between the palpi	1.06 (70.00 / 66.08)
		Hippotion rafflesi (Sphingidae)	38.35 ($N = 1$)	Between the palpi	

Pollination efficiency (*PE*) is calculated as $PE = F_p / F_r$, where F_p represents the percentage of pollinated flowers, and F_r represents the percentage of flowers with one or both pollinaria removed

Table 3 Fruit set and proportion of seeds with large embryos under different hand pollination treatments in *H. ciliolaris* and *H. schindleri*

Treatments		***H. ciliolaris***				***H. schindleri***		
	Flowers (N)	**Fruits (N)**	**Fruit set (%)**	**Large embryo (%)**	**Flowers (N)**	**Fruits (N)**	**Fruit set (%)**	**Large embryo (%)**
Bagging	30	0	0	0	30	0	0	0
Emasculation	30	0	0	0	30	0	0	0
Self-pollination	30	29	96.7	34.0	30	28	93.3	53.6
Cross-pollination	30	30	100	52.5	30	28	93.3	59.7
Natural pollination	204	136	66.7	45.0	227	159	70.0	56.6
Interspecific pollination	154	125	81.2	14.8	82	59	72.0	38.6

The Pollen Limitation Index (*PLI*) values for these two orchid species in the text were calculated based on the fruit set data under natural and cross-pollination. The *PLI* is calculated as follows: $PLI = 1-(P_o/P_s)$, where P_o is the percentage of fruit set in open-pollinated controls (i.e., natural pollination), and P_s is the percentage of fruit set in plants that received supplemental cross pollen (i.e., hand cross-pollination)

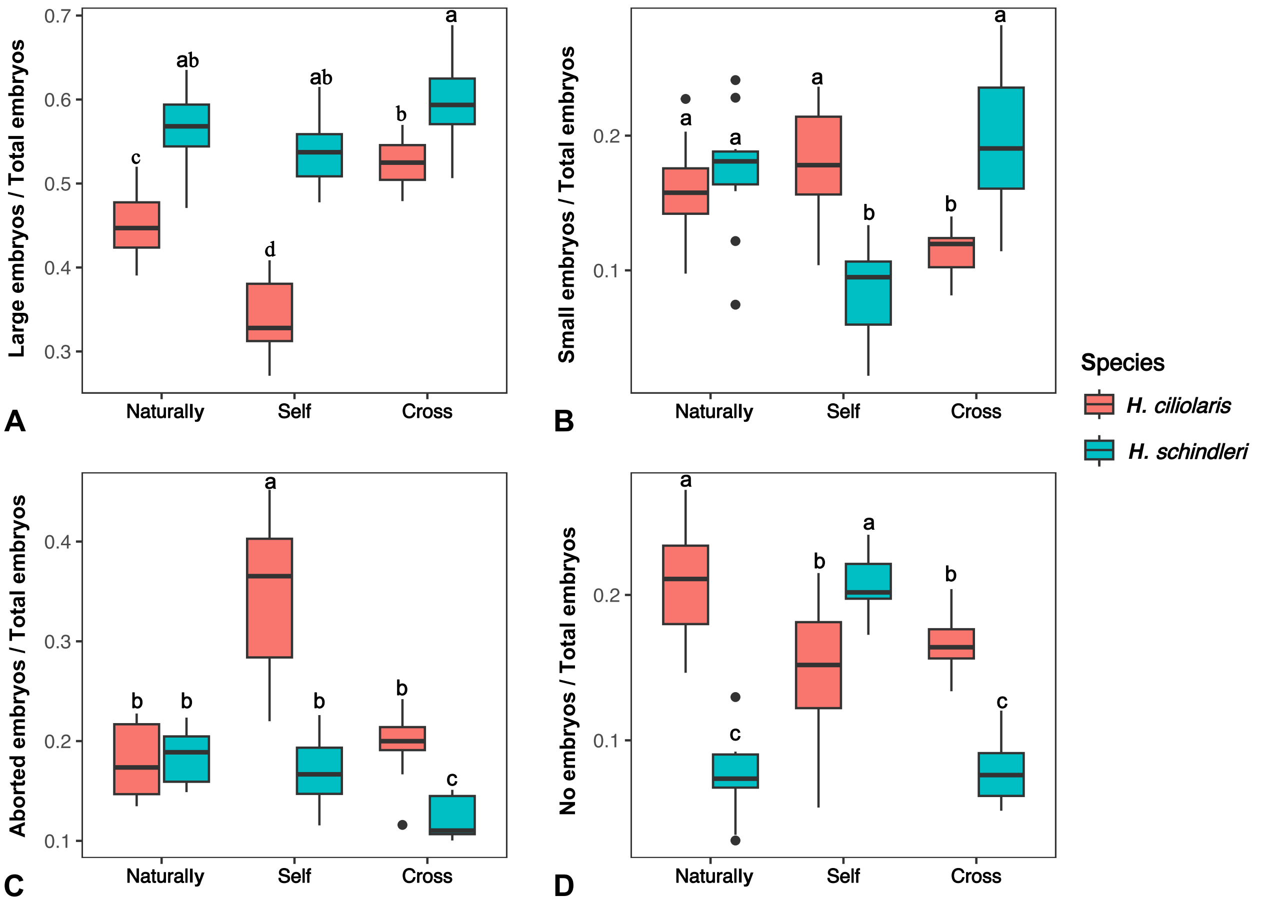

Fig. 3 Comparative embryonic development in *H. ciliolaris* and *H. schindleri* seeds produced by natural, hand-self and hand-cross pollination. Embryos were assigned to four categories: large (**A**), small (**B**), aborted (**C**), and no embryo (empty testa) (**D**). Different lower-case letters indicate significant differences ($P < 0.05$)

schindleri had the lowest proportion (Fig. 3C). Natural pollinated fruits of *H. ciliolaris* and self-pollinated fruits of *H. schindleri* exhibited the highest proportion of empty embryos (Fig. 3D). The inbreeding index (δ) for *H. ciliolaris* ($\delta = 0.352$) was higher than that for *H. schindleri* ($\delta = 0.102$).

In the hand interspecific pollination treatments, the fruit set for *H. ciliolaris* as the ovule donor was 81.2%, while fruit set fell to 72.0% when *H. schindleri* was the ovule donor. Both fruit set rates were lower than those observed under intraspecific hand self- and cross-pollination, but higher than the rates under natural pollination (Table 3). The proportion of large embryos in fruits resulting from interspecific pollination with *H. schindleri* as the ovule donor was 38.6%, which was not significantly different from those resulting from intraspecific pollination. In contrast, the proportion of large embryos in fruits resulting from interspecific pollination with *H. ciliolaris* as the ovule donor was 14.8%, which was significantly lower than those resulting from intraspecific natural ($W = 16$, $P = 0.001$), hand self- ($W = 29$, $P = 0.011$), and cross-pollination ($W = 10$, $P < 0.001$).

Discussion

This study provides a detailed investigation of the floral traits, pollination biology, and breeding systems of two sympatric *Habenaria* species. Our results indicate that, despite overlapping flowering periods of nearly one and a half months, *H. ciliolaris* and *H. schindleri* exhibit significant differences in habitat preferences, floral traits, and nectar rewards. These differences contribute to a suite of strong pre-pollination barriers between the two species, which are sympatric and co-blooming.

The importance of pre-pollination isolation for interspecific isolation

Multiple reproductive isolation barriers in angiosperms operate sequentially, with early barriers often been stronger and contributing more to maintaining reproductive isolation than later barriers [2, 3]. The flowering phenology of *H. ciliolaris* and *H. schindleri* largely overlapped, meaning there is no phenological isolation between these two species. *Habenaria ciliolaris* has a broader distribution compared to *H. schindleri*, and most of the range of *H. schindleri* overlaps with that *H. ciliolaris* [43, 51]. As a result, ecogeographical isolation at the species distribution range scale is also weak.

Floral isolation and pollinator specificity has long been considered crucial factors in the formation and maintenance of the extraordinary diversity of orchids [8, 26, 27]. Our findings suggest that floral trait divergence and pollinator specificity form a complete isolation barrier between *H. ciliolaris* and *H. schindleri*. The spur length of *H. schindleri* is approximately twice that of *H. ciliolaris*, both of which are well adapted to their respective pollinators: short-tongued settling moths and long-tongued hawkmoths. Similar adaptation to short-tongued and long-tongued pollinators have been observed in other orchid lineages, such as *Gymnadenia* [52] and *Disa* [15], as well as in other angiosperm lineages like *Mimulus* (Phrymaceae) [3] and *Aquilegia* (Ranunculaceae) [12, 53]. These results suggest that the adaptation to different pollinators with varying tongue lengths may be a major driving force behind the evolution of floral tube/spur length in flowering plants [12, 26]. However, further studies are needed to determine whether the differentiation in pollinators between these two orchids is driven by pollinator habitat preferences, floral scents, flower color, or floral morphology.

Orchids generally exhibit weaker post-pollination isolation compared to pre-pollination isolation, although strong post-pollination isolation has been found in some food-deceptive orchids [54]. Our field-based interspecific pollination experiments resulted in high fruit set and viable seed production. However, the proportion of large embryos in *H. ciliolaris* resulting from interspecific pollination was significantly lower than that from intraspecific pollination. This indicates that the post-pollination barrier is weak between *H. ciliolaris* and *H. schindleri*. Similar findings were reported for sympatric *H. limprichtii* and *H. davidii* in northwestern Yunnan Province, southwestern China [36]. In contrast, strong post-pollination isolation was observed between *H. delavayi* and *H. limprichtii*, as well as between *H. delavayi* and *H. davidii* at the same site. Furthermore, complete post-pollination isolation was found between *H. fordii* and *H. davidii* in southeastern Yunnan Province, where interspecific pollination resulted in no seed set [41]. These results suggest varying degrees of interspecific isolation among *Habenaria* species. More studies that thoroughly quantify various isolation barriers are needed to better understand the role of both pre- and post-pollination barriers in the diversification and species coexistence of this species rich orchid lineage.

Differentiation between hawkmoth and settling moth pollination

Hovering hawkmoths and a taxonomically diverse guild of settling moths are the most frequently reported pollinators of *Habenaria* [32–37, 55]. *Habenaria* species pollinated by hawkmoths typically exhibit specialized floral traits, such as white flowers, long spurs, upturned petal lobes, and white rostellum and stigma lobes, which serve as visual guides to the spur entrance [32, 39, 55]. These adaptations allow hovering hawkmoths to access nectar more easily and come into contact with the flower's sexual organs with greater efficiency. Additionally, hawkmoth-pollinated species often produce higher nectar volumes and concentrations compared to those pollinated by settling moths. This is important for maintaining mutualistic relationships with their pollinators, as hovering while foraging is energy-intensive [56]. In this study, *H. schindleri* displayed floral traits consistent with hawkmoth pollination, including white petals, spreading lateral sepals, pendulous lip lobes, a longer spur, and a generous nectar volume. In contrast, *H. ciliolaris* exhibited greenish-white petals, reflexed lateral sepals, a shorter spur, and a smaller nectar volume. Notably, the curved lateral lobes of the lip of *H. ciliolaris* (Fig. 1B-C) may restrict a forager's access to nectar when foraging directly in front of the flower. This may prevent typical hawkmoth hovering behavior and make the species more suited to settling moth pollinators. We observed no hawkmoth visits to the *H. ciliolaris* population in this study, likely due to the dense subtropical evergreen broadleaved forest understory where it grows, which is less accessible to the hawkmoth pollinators of *H. schindleri*. Further studies are needed to confirm the functional role of the curved lateral lobes in *H. ciliolaris*.

Plants often experience directional selection for tube or spur lengths that exceed the proboscis lengths of their pollinators, ensuring contact between the pollinator's head, thorax, or other body parts and the flower's reproductive organs [57–60]. *Habenaria* species exhibit considerable variation in spur length across regions, ranging from less than 1 cm to 25 cm in South America [32], and up to 9 cm in China [43]. In our study, the spurs of *H. ciliolaris* and *H. schindleri* were 5–22 mm longer than the proboscis length of their pollinators, requiring the pollinators to push their heads against the flowers to access nectar. This behavior ensures contact between the pollinators' heads and the flowers' reproductive structures. We have recorded a species of long-tongued hawkmoth acting as nectar thieves in *H. schindleri*. Similar instances of long-tongued hawkmoths acting as nectar thieves have been documented in only a few orchids, including *Habenaria* [55, 61, 62] and its close relative *Platanthera* [63, 64].

Interestingly, *H. schindleri* shares a pollinator, *Eupanacra mydon*, with the sympatric *H. dentata*, another species that produces white flowers but emits little discernible scent [39]. However, *H. dentata* blooms later (September 19 to October 26) than *H. schindleri*. Furthermore, the pollinaria of *H. schindleri* attach between the palpi of *Eupanacra mydon*, while those of *H. dentata* attach to the moth's eyes [39]. This difference in pollinaria attachment positions may be attributed to the greater viscidia distance in *H. dentata*. Similar differentiation has been observed in other sympatric *Habenaria* species. For instance, two sympatric *Habenaria* species in southwestern China, *H. limprichtti* and *H. delavayi*, share the hawkmoth *Trichoplusia intermixta*, with pollinaria attaching to the insect's thorax/legs and proboscis, respectively [36]. Similarly, two sympatric *Habenaria* species in southern Brazil, *H. macronectar* and *H. megapotamensis*, share the hawkmoth *Manduca* cf. *lucetius*, but their pollinaria attach between the palpi and on the eyes of the insect, respectively [40]. These findings suggest that variations in flowering phenology, pollinator types, and pollinaria attachment positions play a crucial role in pre-pollination isolation between sympatric *Habenaria* species.

Pollination efficiency and inbreeding depression

Both *H. ciliolaris* and *H. schindleri* are self-compatible but pollinator-dependent, consistent with other *Habenaria* species [38, 39, 48] and many other orchid lineages [28]. *Habenaria schindleri* and *H. ciliolaris*, along with two other sympatric congeners, *H. rhodocheila* [38] and *H. dentata* [39], displayed a pollination efficiency slightly greater than 1. This suggests that, on average, slightly more than one flower was pollinated per flower acting as the pollen donor. Similar results were observed in four sympatric *Habenaria* species in southern Brazil [40]. *Habenaria* species have two pollinaria per flower [43], with some flowers acting as pollen donors contribute only one pollinarium [40]. *Habenaria* species often exhibit moderate to high fruit set, which is attributed to their self-compatibility, specialized floral structures, nectariferous spurs, and scents that attract Lepidopteran pollinators [39, 48, 55]. However, in our study, the fruit sets of *H. ciliolaris* and *H. schindleri* were lower than those of the sympatric species *H. dentata* [39] and *H. rhodocheila* [38]. The lower fruit set in *H. ciliolaris* may result from its less conspicuous greenish-white flowers and the curved lateral lobes of its lip, which may hinder pollinator access. Its habitat in the complex and shaded subtropical evergreen forest understory makes it more challenging for pollinators to locate, probe for nectar, and contact the column. Plants in species-rich communities may experience greater pollen limitation due to intensified interspecific competition for pollinators that are polyphagic and generalist foragers [46, 65]. Our previous studies have indicated that *H. dentata* and *H. rhodocheila*, which typically grow well exposed in forest margins and along roadsides, rarely suffered from pollen limitation. In contrast, *H. ciliolaris* typically inhabiting the dense understory of subtropical forests and exhibits a higher level of pollen limitation than the other three sympatric congeners. Two *Habenaria* species distributed from the Atlantic tropical rainforest area of Brazil exhibited a much higher level of pollen limitation than our *H. ciliolaris* population [46]. More observations and analyses are needed to further investigate the relationship between habitat types and pollen limitation in *Habenaria* species. Finally, nectar thieves, which compete for nectar resources with legitimate pollinators, may negatively impact the pollination efficiency and fruit set of *H. schindleri*. The negative effects of nectar thieves on reproductive success have been well documented in other plants, such as *Corydalis ambigua* (Papaveraceae) [66] and *Metrosideros polymorpha* (Myrtaceae) [67].

Evidence from some orchid lineages indicates that nectar rewards increase the number of flowers probed by pollinators and the time they spend on an inflorescence, thereby enhancing pollination efficiency. However, this also leads to higher rates of geitonogamous pollination and inbreeding depression [68, 69]. Our hand pollination results suggest that the *H. ciliolaris* population has exhibited some degree of inbreeding depression. Similar findings have been reported for *H. limprichtii* [48], *H. rhodocheila* [38], and *H. dentata* [39]. These results suggest that while nectar rewards may enhance pollination efficiency and fruit set, they also increase the risk of inbreeding depression due to pollinator-mediated autogamy and geitonogamy in natural populations of *Habenaria* species. Although the pollination efficiency of *H. schindleri* was lower than that of the other three sympatric *Habenaria* species, its inbreeding depression index was the lowest among them [38, 39]. High levels of pollination efficiency and inbreeding depression were also observed in *H. limprichtii* from southwestern China [48]. Further studies are needed to elucidate the correlation between floral traits, pollination efficiency, the pollen limitation index, and inbreeding depression in *Habenaria*.

Above all, our findings suggest that both *H. ciliolaris* and *H. schindleri* exhibit a specialized pollination system characterized by a high degree of adaptation to specific pollinators (short-tongued settling moths and long-tongued hawkmoths, respectively) and involving intricate floral morphology and precise pollen placement mechanisms. Although pollinator visits are infrequent, they appear sufficient for fruit and seed production, suggesting a highly specialized pollination system. This specialization enhances pollination efficiency by ensuring that only specific pollinators can effectively transfer pollen, thereby reducing pollen waste, promoting reproductive success, and maintaining species integrity. However, this system also entails significant risks for the two orchids, as it makes them highly dependent on the presence and abundance of their specific pollinators. Environmental changes, human-induced disturbances, habitat loss, or declines in pollinator populations can severely disrupt this mutualistic relationship, potentially reducing reproductive success and increasing vulnerability to local extinction.

Conclusions

Both *H. ciliolaris* and *H. schindleri* are self-compatible, but they depend completely on settling moths (*Thinopteryx nebulosa* and *Porsica* sp.) and hawkmoths (*Eupanacra mydon* and *Hippotion rafflesi*), respectively, for fruit set. While their flowering periods overlap, they maintain divergent floral morphologies and nectar properties. The spur lengths of the two orchids match the proboscis lengths of their respective pollinators. Natural populations of both *H. ciliolaris* and *H. schindleri* show high levels of fruit set rates, with *H. ciliolaris* showing greater pollination efficiency (PE) value. However, *H. ciliolaris* suffers from significant inbreeding depression. When these two species are sympatric and co-blooming interspecific isolation at the pre-pollination level appears to be maintained primarily by pollinator specificity.

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Authors' contributions
BY, ZR, and ST designed the study. XC, ST, HL, WC, HX, LH, HL, and BY conducted the experiment and performed the analyses. ST wrote the original draft with revisions by ZR, CPN, and BY. All authors contributed to the article and approved the submitted version of the manuscript.

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Data availability
All data generated or analysed during this study are included in this published article.

Declarations

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