

Outcrossing mating system in natural populations of the endangered aquatic fern *Ceratopteris thalictroides* in China as revealed by microsatellite markers: implications for conservation

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Abstract

In this study, the mating system of the endangered aquatic fern *Ceratopteris thalictroides* in China was investigated using microsatellite markers. The estimates of the multilocus outcrossing rate (t_m) and single-locus outcrossing rate (t_s) were high at the species level ($t_m = 0.701$ and $t_s = 0.595$, respectively), indicating that *C. thalictroides* is a predominantly outcrossing species. Negative F (inbreeding coefficient of the maternal parents) values (-0.137) at the species level indicated an excess of heterozygotes and low inbreeding in the populations analyzed. Our investigation revealed that some of the natural and *ex situ* conservation populations of *C. thalictroides* have a prolific capacity for vegetative growth. The results showed that the homosporous pteridophyte *C. thalictroides* in China predominantly favors gametophytic crossing with a certain level of inbreeding along with vegetative growth. Ten populations have been established for *ex situ* conservation in Wuhan, China, with a survival rate of more than 90%, and mature spores were collected from them. Several factors, including high dispersal of spores and gametophytes by flowing water, high gene flow, and the wide distribution of the species in China, with the occurrence of some large populations, may have resulted in a high outcrossing rate in Chinese *C. thalictroides*. The high outcrossing rate of *C. thalictroides* showed that *ex situ* plantings are most suitable for enhancing possible mixing of the different populations, thus minimizing inbreeding.

Keywords: *Ceratopteris thalictroides*; endangered fern; high outcrossing rate; outcrossing species; SSR

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Introduction

Plant mating systems play a key role in structuring genetic variation both within and between species (Pickup *et al.*, 2019; Pelosi and Sessa, 2021). Studies of mating systems, genetic diversity, and population structure, as well as the spatial distribution of genotypes within populations of rare and endangered plant species, are necessary to establish effective and efficient conservation management strategies for endangered species (Pometti *et al.*, 2013). An accurate characterization of mating systems is important for the conservation of the evolutionary potential of natural populations because altered patterns of mating, such as increased inbreeding, may result in future genetic decline and population extinction (Saccheri *et al.*, 1998).

Ceratopteris is an aquatic, annual, circumtropical homosporous fern genus (Lloyd, 1974a; Hickok *et al.*, 1995). *Ceratopteris* has been used as a model plant for many years in the study of genetics, gametophyte biology, biochemistry, cell biology, developmental biology, and molecular biology (Hickok *et al.*, 1995; Bartz and Gola, 2018; Conway and Di Stilio, 2019; Kinoshian *et al.*, 2020; Geng *et al.*, 2021; Bora and Sarma, 2021). Taxonomic treatments of *Ceratopteris* by Lloyd (1974a) recognized four species: *C. thalictroides*, *C. cornuta*, *C. pteridoides*, and *C. richardii*. Recent studies suggest that *Ceratopteris* consists of eight species, three of which are cryptic (PPGI, 2016; Zhang *et al.*, 2020; Masuyama and Watano, 2010; Kinoshian *et al.*, 2020). Species exhibit one of three mating systems: automixis (gametophytic selfing), autogamy (sporophytic selfing), and xenogamy (sporophytic outcrossing) (Lloyd, 1974a; Hickok *et al.*, 1995; Haufler *et al.*, 2016). Self-fertilization of a single gametophyte is termed intragametophytic selfing (automixis, gametophytic selfing); crosses between gametophytes produced from spores of the same sporophyte have been termed intergametophytic selfing (autogamy, sporophytic selfing) and are equivalent to selfing in angiosperms. The equivalent to crossing in higher plants involves crosses between gametophytes (nonsib gametophytes) produced from spores of different sporophytes, termed intergametophytic crossing (xenogamy, sporophytic outcrossing). Homosporous pteridophytes are unique in having the extreme form of inbreeding known as intragametophytic selfing (gametophytic selfing), owing to free-living gametophytes that are potentially bisexual (Masuyama and Watano, 1990; Soltis and Soltis, 1990a; Haufler *et al.*, 2016).

Ceratopteris thalictroides (L.) Brongn. is a semiaquatic, annual, homosporous fern with a wide geographical distribution in tropical and subtropical regions of the world (Watano and Masuyama, 1994; Hickok *et al.*, 1995; Adjie *et al.*, 2007). In China, it mainly grows in agricultural fields, marshes, lakes, ditches, ponds, and wetlands in tropical forests (Dong *et al.*, 2008; Liao *et al.*, 2011). Although *C. thalictroides* was widely distributed in China prior to the 1960s, in recent decades, the number and size of *C. thalictroides* populations have rapidly declined mainly due to the degeneration of primary habitats and a continuous decline in the area of wetland coverage (Dong *et al.*, 2005, 2008, 2013; Liao *et al.*, 2011). The species is now endangered and listed in the second category of the Key Protected Wild Plants in China (Yu, 1999; National Forestry and Grassland Administration PRC, 2021). In several other countries, including neighboring Vietnam and India, the species is also listed as endangered (Raju, 1983). Earlier studies on *C. thalictroides* mainly dealt with its distribution, taxonomy, evolution, morphology, ecology, reproductive development, conservation genetics, and molecular biology (Lloyd, 1974a; Hickok *et al.*, 1995; Masuyama *et al.*, 2002; Adjie *et al.*, 2007; Liao *et al.*, 2011; Carey *et al.*, 2018; Chen *et al.*, 2019; Guan *et al.*, 2019). Research on molecular biology has mainly evaluated the genetic diversity, genetic structure, cryptic species status, molecular systematics and phylogeography, and adaptive evolution of *C. thalictroides* using Simple Sequence Repeat (SSR), RAD-seq, RNA-seq, nrDNA, hemoglobin genes, the MADS-box gene family, and cpDNA *rbcL* (Adjie *et al.*, 2007; Dong *et al.*, 2008; Masuyama and Watano, 2010; Yang *et al.*, 2016; Qi *et al.*, 2016; Zhang *et al.*, 2020; Kinoshian *et al.*, 2020). Although Watano and Masuyama (1991) inferred the mating system of *C. thalictroides* from Japan based on isozymes and selfing tests, little is known about the mating system of natural populations of *C. thalictroides* in China.

Mating systems of plants are commonly characterized by outcrossing rates (Ritland, 2002; Dauphin *et al.*, 2020). These estimates can be generated by examining allelic variation over many loci in progeny arrays from plants allowed to pollinate naturally, using a maximum-likelihood model to exclude progeny resulting from self-fertilization (Ritland and Jain, 1981). Inferences about plant mating systems increasingly use highly informative genetic markers and investigate finer facets of the mating system (Ritland, 2002). In recent studies, although several dominant markers, such as RAPD (Gaiotto *et al.*, 1997; Dong *et al.*, 2006), ISSR (Han *et al.*, 2009; Fernando *et al.*, 2015; Saki *et al.*, 2016), and AFLP (Gaiotto *et al.*, 1997; Pometti *et al.*, 2013; Zhao *et al.*, 2014; Dong *et al.*, 2018 b) markers, have been used to estimate outcrossing rates in plant species, outcrossing rates in plant populations have often been determined using codominant markers, such as isozymes and microsatellites, due to their high allelic polymorphism (Chaix *et al.*, 2003; Chen *et al.*, 2009; Adugna *et al.*, 2013; Sinha *et al.*, 2015; Sharma *et al.*, 2017; Suwarni *et al.*, 2018; Zhang *et al.*, 2019; Dauphin *et al.*, 2020; Sun *et al.*, 2020; Torres *et al.*, 2020; Calevo *et al.*, 2021; Heike and Dagmar, 2022; Lu *et al.*, 2022).

The principal aims of this study were (1) to determine the mating system in natural populations of *C. thalictroides* in China using microsatellites and to provide information for both *in situ* and *ex situ* conservation and management programs as well as (2) to study *ex situ* conservation methods of *C. thalictroides* and provide technical support for the protection of germplasm resources and population restoration.

Materials and Methods

SSR analysis

Plant material and total DNA extraction

The plant material used in this study was obtained from seven populations across the natural distribution range of *C. thalictroides* in China during July and August 2017 (Figure 1). Four of the populations occur in Jiangsu Province (SQS, QSC, XS, and TH). The other three populations (HN, LA, and QS) are located in Hainan, Zhejiang, and Hubei provinces, respectively (Figure 1). Most of the populations are small and diminishing. Approximately 5-10 g of young sterile fronds was harvested from each plant and immediately dried in a sealed zip lock plastic bag containing approximately 50 g of silica gel. A total of 50 individuals (ramets) from the seven populations were included in the SSR study. Details on the collection sites, population size, and sampling size are given in Table 1. Total DNA was extracted from 0.5 g of silica-dried leaf tissue following the procedure described by Doyle and Doyle (1987). DNA products were resolved electrophoretically on 1.5% agarose gels, visualized by staining with ethidium bromide and photographed under ultraviolet light. The concentration and purity of DNA were estimated by an Eppendorf BioPhotometer Plus (Eppendorf China Ltd.)

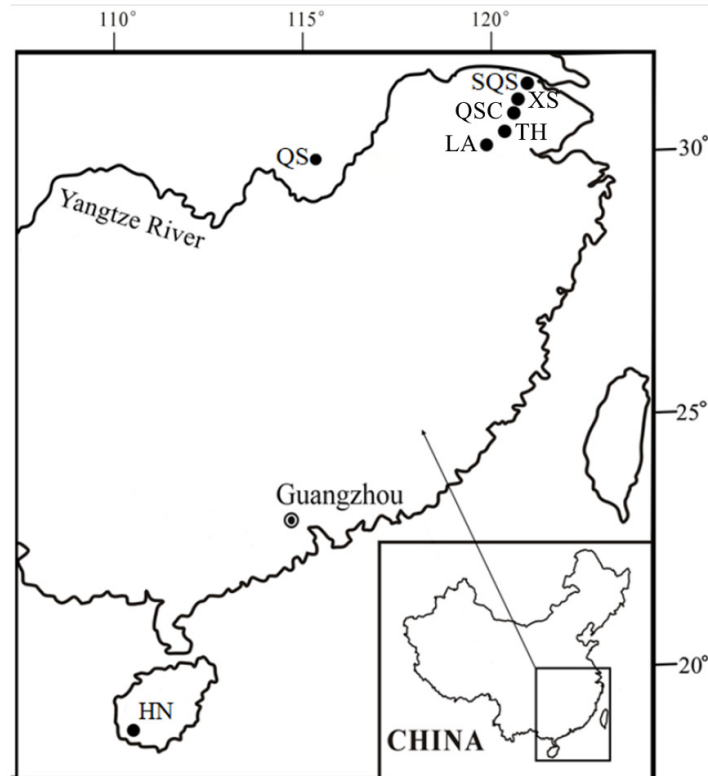


Figure 1. Distribution of *Ceratopteris thalictroides* populations sampled in the present study in China. Codes correspond to the populations in Table 1.

Table 1. Location, habitat, and sample size of *Ceratopteris thalictroides* populations studied in China

Population code	Locality	Latitude/Longitude (N/E)	Altitude (m)	Habitats	Population size	Population area (m ²)	Sample size
SQS	Suzhou, Jiangsu	31°14'/120°26'	34	Vegetation	50-60	6-8	4
HN	Lingshui, Hainan	18°40'/109°50'	139	Wetland	5-10	4-5	2
LA	Linan, Zhejiang	30°12'/119°43'	40	Rice field and ditch	50-60	150-200	4
QSC	Suzhou, Jiangsu	31°14'/120°25'	7	Ditch	100-120	8-10	7
XS	Suzhou, Jiangsu	31°14'/120°26'	12	Wetland and ditch	100-120	50-60	9
QS	Qichun, Hubei	30°04'/115°12'	28	Pond	20-25	70-80	12
TH	Taihu Lake, Jiangsu	30°34'/120°13'	13	Lake	370-500	25-30	12

SSR-PCR amplification

A total of 30 microsatellite markers (Cer1-Cer30; Yang *et al.*, 2016) that have already been mapped in *C. thalictroides* were tested on four randomly selected individuals for SSR-PCR. Thirty SSR primers were synthesized by Shanghai Jierui Bioengineering Co., Ltd. (Shanghai, China); 10 that produced reproducible, clear, polymorphic electrophoretic loci were selected (Table 2). PCR was carried out according to Yang *et al.* (2016). The reaction mixture consisted of 1 μ L of genomic DNA (50 ng/ μ L), 2.0 μ L of 10 \times Taq buffer (TianGen, Beijing, China), 1.5 mM MgCl₂, 0.5 μ L of dNTPs, 1 μ L (20 μ M) of each primer (0.5 μ L of each forward and reverse primer) and 0.25 μ L of Taq-Polymerase (2.5 U/ μ L), for a total of 20 μ L. The PCR amplifications were performed on an EDC-810 (Dongshenglong Generation) Thermal Cycler (Dongsheng Company) and carried out according to the following program: an initial denaturation phase of 5 min at 94 °C, followed by 35 cycles each comprising a denaturation step at 94 °C for 30 s, then hybridization for 30 s at a temperature between 58 °C and 60 °C, an extension step at 72 °C for 30 s, and finally an elongation phase of 5 min at 72 °C. A total of 5.0 μ L of loading buffer (98% formamide, 0.5 M EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol) was denatured at 94 °C for 5 min, and electrophoresis was run in

a 6% denaturing polyacrylamide gel on a DY CZ-30 DNA Sequencing System (Beijing Liuyi Biotechnology Co., Ltd.). Silver staining was conducted as described by Bassam *et al.* (1991). The sizes of the selective amplification products were estimated using a 50-bp DNA ladder (TianGen, Beijing, China).

Table 2. Nucleotide sequence of SSR primers used in this study

Primer	SSR primer sequences (5'-3')	
SSR2	Cer2-F:ACAGGGCCAAAGCTAGTCAA	Cer2-R:TACACACACACACGCACACA
SSR3	Cer3-F:GCCTATGGGTTTGGTGTGTC	Cer3-R:CCGTGTGTGTGTGTGCACAAG
SSR4	Cer4-F:AGGAAGGTGAGCAGTCTGAC	Cer4-R:GGTGTGTTGTTGTTGTTGTGCC
SSR5	Cer5-F:GGGGCAAGTCTCGTAAAAGA	Cer5-R:CAAGCACTCTGTTTCGCTCTG
SSR9	Cer9-F:AGTCAAGAAGGCTACAGCGG	Cer9-R:CGCTTAACCGTTTACCTATCG
SSR12	Cer12-F:TGAGACTCCACGCTACATGC	Cer12-R:ATCCTCCGTTGGTCTCCAGT
SSR13	Cer13-F:AACCCTGGAATCTTAGAGGAAAA	Cer13-R:TGAGGCTTCCTTACCTTGCT
SSR14	Cer14-F:GCAGCCCACACTCTACATC	Cer14-R:AGAGAGAGGGAGTTGTGCCA
SSR16	Cer16-F:CGAGGCTTGGACTCTTCATC	Cer16-R:CTACCAGAGGATTGGGAGCA
SSR21	Cer21-F:TGCAGAGATAGCCACACCAC	Cer21-R:TGAGTCAAAATTGCACCCAC

Mating system analyses

The MLTR 3.4 (Ritland, 2009) program is based on a multilocus mixed-mating model and the estimation procedure of Ritland and Jain (1981), which assumes that progeny are derived from either random mating (outcrossing) or self-fertilization. Using MLTR 3.4 software (Ritland, 2009), we calculated the following mating system parameters: estimates of multilocus (t_m) and single-locus (t_s) out-crossing rates, multilocus correlation of paternity (rpm), single-locus correlation of paternity (rps), correlation of t_m among progeny arrays (rt), and the fixation index of maternal parents (F). Biparental inbreeding was also estimated following Ritland (1990) as $t_m - t_s$. The standard errors for these parameters were calculated from 1,000 bootstraps with resampling of individuals within families using expectation-maximization (EM). The standard error was used to determine whether mating parameters were significantly lower than one or greater than zero.

To evaluate the effect of SSR primer numbers on mating system parameters in *C. thalictroides*, we calculated the parameters of the mating system with SSR primer pairs from three to 10 using the MLTR 3.4 program.

Observation of gametophytes

Spores of *C. thalictroides* were collected and refrigerated at 4 °C. Spores were disinfected for 1 minute in 5% NaOCl. Sterilized spores (approximately 500-600) were sown on agar (Knop's nutrient solution with 1% agar) medium in Petri dishes measuring 6 cm in diameter. The preparation method for Knop's nutrient solution with 1% agar was as follows: 1% agar was added to Knop's nutrient solution, and the mixture was heated until the agar was completely melted, and sterilized for 20 min (121 °C). The final packing medium in each Petri dish was approximately 30 ml, which was subjected to cooling. All experiments were carried out with replicate plates at 25 ± 1 °C and 80-85% humidity. All plates were illuminated with growth light at an intensity of 6000 LX, with 12 h light and 12 h dark in an SPRX - 600B intelligent artificial climate box (Shanghai Lang Gan Laboratory Equipment Co., Ltd.). Eight days following the sowing of spores, rates of spore germination were recorded under a Jiangnan BM2000 digital microscope (Nanjing Jiangnan Yongxin Optical Co., Ltd.). The emergence of rhizoids denotes spore germination in *C. thalictroides* (Warne and Hickok, 1987). Twenty days after sowing, hermaphroditic gametophytes of *C. thalictroides* (i.e., those that were large and cordate and had antheridia as well as archegonia) in each culture plate were recorded.

Vegetative growth and ex situ conservation

The vegetative growth of *C. thalictroides* was investigated by two methods. One was observing the vegetative growth of natural plants through field investigation from June to December, and the other was studying the clonal growth of plants through *ex situ* conservation at Jiangnan University (N 30° 30'/E 114° 9', altitude: 25 m), which is located in Wuhan, Hubei Province, China. Wuhan is situated in the central part of China and has a typical humid subtropical climate, with abundant rainfall, sufficient heat, winter cold and summer heat, and four distinct seasons. The annual mean temperature in Wuhan is 15.8 °C-17.5 °C, the extreme maximum temperature is 41.3 °C, and the annual rainfall is 1150-1450 mm. The average temperature in Wuhan in July is 28.8 °C. The *ex situ* conservation method was as follows: First, the plants from ten natural populations collected in the field during the period of sterile leaf growth (during July) were transplanted into flowerpots with soil (peat soil:loamy soil = 3:1) and then placed in an artificial pool of microflow water. The water temperature in July is between 23 °C and 26 °C. The depth of the submerged part of the seedling of *C. thalictroides* was approximately 2 cm during the growth of the seedling (during the period of sterile leaf growth), and the clonal growth of *C. thalictroides* was observed after confirmation of survival. Finally, the mature sporophytes and spores of *C. thalictroides* were collected from November to December.

Results

The estimates of the multilocus outcrossing rate (t_m) and single-locus outcrossing rate (t_s) were high at the species level in *C. thalictroides* ($t_m = 0.701$, $t_s = 0.595$, respectively) (Table 3). The t_m values ranged from 0.551 to 1.200 whereas the t_s values ranged from 0.602 to 1.200 among the seven populations (Table 3). The difference between the multilocus and single-locus estimates ($t_m - t_s$) ranged from -0.067 to 0.106 at the species level and within populations. At the species level, $rp(m)$, $rp(s)$ and rt were 0.067, -0.045 and -0.054, respectively. The difference ($rps - rpm$) between the estimates was -0.112. The value of the single-locus inbreeding coefficient of maternal parents (F) was -0.137 at the species level (Table 3).

Table 3. Mating system parameters of *Ceratopteris thalictroides* populations studied in China

Parameter	SQS	HN	LA	QSC	XS	QS	TH	Species level
t_m	1.200(0.163)	1.200(0.489)	0.551(0.351)	1.200(0.011)	1.200(0.071)	0.847(0.210)	1.200(0.019)	0.701 (0.062)
t_s	1.200(0.140)	1.200(0.489)	0.602(0.355)	1.200(0.004)	1.200(0.041)	0.914(0.189)	1.175(0.018)	0.595 (0.080)
$t_m - t_s$	0.000(0.048)	0.000(0.000)	0.051(0.078)	0.000(0.008)	0.000(0.043)	0.067(0.062)	0.025(0.022)	0.106 (0.039)
$rp(m)$	0.999(0.119)	0.999(0.498)	0.999(0.834)	0.477(0.177)	0.234(0.356)	0.033(0.418)	0.051(0.360)	0.067 (0.171)
$rp(s)$	0.999(0.117)	0.999(0.498)	0.999(0.856)	0.577(0.151)	0.653(0.211)	0.362(0.201)	0.289(0.249)	0.045(0.202)
$rp(s) - rp(m)$	0.000(0.023)	0.000(0.000)	0.000(0.266)	0.100(0.150)	0.420(0.371)	0.395(0.371)	0.238(0.302)	0.112(0.195)
rt	0.999(0.499)	0.999(0.498)	0.385(0.685)	0.999(0.056)	0.999(0.319)	0.200(0.356)	0.999(0.394)	-0.054 (0.193)
F	0.200(0.047)	0.999(0.000)	0.200(0.197)	0.200(0.003)	0.200(0.085)	0.200(0.048)	0.200(0.046)	0.137(0.045)

t_m , Multilocus outcrossing rate; t_s , Single-locus outcrossing rate; $t_m - t_s$, Difference in the outcrossing rate or biparental inbreeding; $rp(m)$, The multilocus correlation of paternity; $rp(s)$, The single-locus correlation of paternity; $rp(s) - rp(m)$, Parent correlation; Correlation of t (or s) estimate; F , Inbreeding coefficient of the maternal parents. Numbers in parentheses are standard deviations (SDs). Codes correspond to the populations in Table 1.

As the number of SSR primers increased from three to 10, the multilocus outcrossing rate (t_m) and the biparental inbreeding rate ($t_m - t_s$) increased accordingly (Table 4, Figure 2). Except for three pairs of primers, the single-locus outcrossing rate (t_s) also increased with the increase in the number of primers (Table 4). The data for nine pairs of primers and 10 pairs of primers were similar (t_m : 0.687 and 0.701, respectively; t_s : 0.581 and 0.595, respectively; $t_m - t_s$: 0.106 and 0.106, respectively) (Table 4, Figure 2). Ten pairs of SSR primers were suitable for evaluating the mating system of *C. thalictroides* in this study.

Table 4. Influence of SSR primer number on mating system parameters in *C. thalictroides*

Primer number	Mating system parameters						
	t_m	t_s	$t_m - t_s$	rp(m)	rp(s)	rp(s) - rp(m)	F
3	0.600(0.231)	0.596(0.233)	0.003(0.059)	-0.107(0.362)	-0.099(0.441)	-0.206(0.419)	-0.200(0.117)
4	0.356(0.149)	0.309(0.137)	0.047(0.035)	-0.016(0.438)	0.058(0.556)	0.042(0.624)	-0.099(0.099)
5	0.407(0.110)	0.373(0.105)	0.034(0.033)	0.149(0.370)	-0.209(0.434)	-0.060(0.464)	0.055(0.085)
6	0.558(0.085)	0.513(0.083)	0.045(0.029)	-0.130(0.341)	0.011(0.395)	-0.120(0.292)	-0.033(0.090)
7	0.550(0.081)	0.490(0.087)	0.060(0.044)	-0.144(0.333)	-0.002(0.357)	-0.146(0.285)	-0.057(0.063)
8	0.673(0.105)	0.588(0.139)	0.085(0.058)	-0.061(0.264)	0.000(0.284)	-0.061(0.234)	0.270(0.127)
9	0.687(0.060)	0.581(0.083)	0.106(0.041)	-0.148(0.203)	0.043(0.193)	-0.105(0.188)	-0.058(0.071)
10	0.701(0.062)	0.595(0.080)	0.106(0.039)	-0.112(0.195)	0.067(0.171)	-0.045(0.202)	-0.137(0.045)

The meaning of each mating system parameter is provided in Table 2. Numbers in parentheses are standard deviations (SDs).

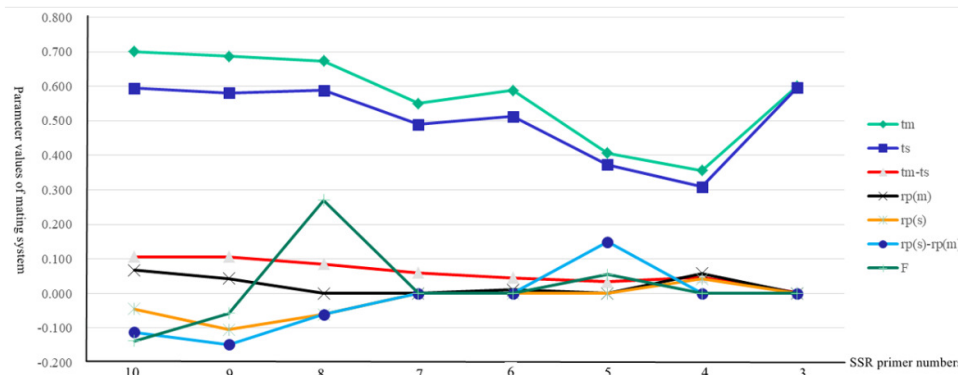


Figure 2. Influence of SSR primer number on the mating system parameters in *C. thalictroides*. Except for 3 pairs of primers, the multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s) and biparental inbreeding rate ($t_m - t_s$) increased with an increase in the number of primers.

In all cultures, only hermaphroditic gametophytes were observed. The gametophytes were cordate and had mature antheridia and archegonia (Figure 3).



Figure 3. Photograph of gametophytes of *C. thalictroides* under a digital microscope ($\times 40$)

Two types of vegetative reproduction were observed in some of the natural populations and *ex situ* conservation populations of *C. thalictroides* in China (Figure 4). Vegetative growth originated from either sterile or fertile fronds, giving rise to ramets (Figure 4). Of the 21 extant populations found in the field survey in 2021, only the population at Xishuangbanna in Yunnan Province showed vegetative growth. The 21 extant populations are located in Zhejiang, Jiangxi, Fujian, Jiangsu, Shanghai, Yunnan, Anhui, Henan and Guangxi provinces, China. Among ten *ex situ* conservation populations in Wuhan, China, only the population from Yihuang, Jiangxi Province showed clonal growth. The phenomenon in which numerous marginal leaf buds of sterile fronds develop into many sporophytes containing fertile fronds was also observed in September 2021, and mature spores were collected (Figure 4).



Figure 4. Vegetative growth of some natural and *ex situ* conservation populations of *C. thalictroides* in China (indicated by arrows). A-B: Vegetative growth of some natural populations (A: Sterile fronds gave rise to ramets; B: Fertile fronds gave rise to ramets); C-I: Vegetative growth of the *ex situ* conservation populations (C: The plants collected from the natural populations were transplanted into flowerpots; D: The habitat where vegetative growth of *C. thalictroides* was observed; E: Sterile fronds gave rise to ramets; F: Fertile fronds gave rise to ramets; G: Numerous marginal leaf buds of sterile fronds develop into many sporophytes containing fertile fronds; H: Mature sporophytes; I: Spores)

In Wuhan, China, ten populations from Zhejiang, Jiangsu and Fujian provinces have been established with *ex situ* conservation (Figure 5). The survival rate of plants is more than 90%, and mature sporophytes and spores were collected.



Figure 5. Main periods of *ex situ* conservation of *C. thalictroides* at Jiangnan University in Wuhan. A: The plants from the natural populations collected in the field were transplanted into flowerpots and artificial pools; B: The period of sterile leaf growth; C: The period of fertile frond growth; D: The period of mature sporophytes and spores.

Discussion

Plant mating systems are often described by the ‘mixed mating model’, where a fraction of progeny are derived from self-fertilization and the remainder are derived from outcrossing (Ritland, 2002). The outcrossing rate of angiosperms shows a large degree of variation, with many species showing outcrossing rates close to 0 and 1. The outcrossing rate of gymnosperms is relatively high, generally greater than 0.6 (Barrett and Eckert, 1990). In this study, the estimates of multilocus outcrossing rates (t_m) and single-locus outcrossing rates (t_s) were high at the species level ($t_m = 0.701$, $t_s = 0.595$, respectively) and in all populations (t_m , 0.551-1.200; t_s , 0.602-1.200, respectively) (Table 3), indicating that *C. thalictroides* is a predominantly outcrossing species. The high outcrossing rates in *C. thalictroides* based on SSR markers in this present study were similar to those reported in previous studies, including in *Pteroceltis tatarinowii* (SSR, $t_m = 0.987$) (Fan *et al.*, 2018), *Camellia tunghinensis* (SSR, $t_m = 0.938$ -1.002) (Tang *et al.*, 2014), *Camellia oleifera* (SSR, $t_m = 0.996$) (Zhang *et al.*, 2018), *Larix principis-rupprechtii* (SSR, $t_m = 0.829$) (Dai *et al.*, 2020), and *Saccharum arundinaceum* (SSR, $t_m = 0.86$) (An *et al.*, 2021). Similarly, Dong *et al.* (2018), using AFLP markers, reported high outcrossing rates ($t_m = 0.999$) at the species level in the endangered aquatic fern *Ceratopteris pteridoides* in China. A high degree of outcrossing was also reported in the endangered endemic aquatic lycophyte *Isoetes yunguiensis* in China based on AFLP ($t_m = 1.200$, Dong *et al.*, 2018b) and ISSR markers ($t_m = 0.955$). The outcrossing rates of *C. thalictroides* in China obtained in this study were higher than those obtained for other species. For instance, Santos-Garcia *et al.* (2010), using SSR data, revealed a low level of outcrossing in *Stylosanthes capitata* ($t_m = 0.31$) and *S. guianensis* ($t_m = 0.264$). The t_m - t_s ranged from -0.067 to 0.106 in all populations and at the species

level, indicating a low tendency for mating between relatives. The mating system can be inferred from F , which measures the deviation of observed genotypic frequencies from those expected at Hardy-Weinberg equilibrium (Wright, 1965). Negative F values indicate heterozygote excesses relative to Hardy-Weinberg expectations; a value of 0 signifies Hardy-Weinberg equilibrium (and random mating); and positive values indicate heterozygote deficiencies, probably reflecting high rates of inbreeding (Soltis and Soltis, 1990b). In this study, F was negative (-0.137), indicating an excess of heterozygotes and low inbreeding in the populations analyzed. The correlation of t_m within progeny arrays (rt) was low in all populations, showing that there was no difference in outcrossing rates among mother plants (Table 3). It is thus clear that *C. thalictroides* in China predominantly favors outcrossing.

Two different types of gametophytes (small, spatulate or amorphous males and large, cordate hermaphrodites) were observed in composite cultures of gametophytes of *C. thalictroides* (Schedlbauer and Klekowski, 1972; Watano and Masuyama, 1991). It has been assumed that fern species with an antheridiogen system may be predisposed to gametophyte crossing because antheridiogen generally leads to the formation of unisexual gametophytes (Watano and Masuyama, 1991). Outcrossing has previously been recognized in the sexual reproduction of *C. thalictroides* (Watano and Masuyama, 1991). A single herbarium specimen of *C. thalictroides* from Hong Kong (Hance, s.n., BM) exhibits sporangia with 16 and 32 spores, some of which are very small and shriveled but some of which are extremely large and rounded, indicating hybridization also occurs naturally in this species (Lloyd, 1974a). Dong *et al.* (2008) revealed low genetic differentiation among populations and high gene flow in *C. thalictroides* in China based on RAPD and ISSR markers, further indicating that Chinese *C. thalictroides* may possess a higher outcrossing rate. A number of causes, including high dispersal of spores and free-living gametophytes, sperm dispersed by flowing water, high gene flow, and the wide distribution of the species in China, with the occurrence of some large populations, may have resulted in a higher outcrossing rate in Chinese *C. thalictroides* than in Japanese *C. thalictroides* (Dong *et al.*, 2008).

Based on allozyme and selfing tests, Watano and Masuyama (1991) revealed that *C. thalictroides* in Japan is a predominant inbreeder. This result seems to contradict our results of high outcrossing rates in Chinese *C. thalictroides* based on SSR molecular markers (Table 3). However, we think that the results of the two evaluation methods are not contradictory. It is worth noting that the research methods and materials used for allozyme and selfing test experiments revealed a high level of gametophytic selfing in *C. thalictroides* from Japan. The materials (for enzyme extraction, 0.5 g of gametophyte per spore source) used for allozyme analysis were derived only from five subpopulations of a single zone of paddy field in Nagareyama-shi, Japan (Watano and Masuyama, 1991). The selfing test experiment is based on isolated gametophyte culture (Watano and Masuyama, 1991). Gametophyte morphology and sexual status typically vary between individuals and depend on both genetic factors and environmental conditions (Ranker and Houston, 2002). The possibility of producing hermaphroditic gametophytes allows for self-fertilization (intragametophytic selfing) of a single gametophyte (Hickok *et al.*, 1995). The potential for intragametophytic self-fertilization (automixis) renders homosporous pteridophytes unique among plants (Hickok *et al.*, 1995). Intragametophytic selfing facilitates the establishment of a sporophyte from a single spore (Lloyd, 1974b). With a homosporous hermaphroditic gametophyte, *C. thalictroides* has the potential for self-fertilization (Watano and Masuyama, 1991; Hickok *et al.*, 1995). In present study, the characteristics of hermaphrodites of *C. thalictroides*, with mature antheridia and archegonia promoting gametophytic selfing, were also observed (Figure 3). Isolated gametophyte culture from a single spore may make the species appear to have a preference for intragametophytic self-fertilization (automixis). A single population of *C. thalictroides* may reduce the level of gametophytic outcrossing and promote selfing (automixis, autogamy). In addition, if the mating system of only one population was evaluated, the results of Watano and Masuyama (1991) are not necessarily comprehensive. Data on mating systems from other populations could be more persuasive. That is, isolated gametophyte culture may make the species appear to have a preference for intragametophytic selfing (self-fertilization), and gametophytes formed by spore

culture from a single population may improve the inbreeding rate for allozyme evaluation of the mating system. Therefore, although *C. thalictroides* in China preferentially outcrosses, it may reproduce through this extreme form of selfing when mates are limited in order to adapt to changes in the living environment and maintain the continuity of offspring. Moreover, the mating system of a species can convert from outcrossing to selfing when a few closely related individuals' mate and show clonal growth (Innes and Hermanutz, 1998). An example occurs in *Solidago sempervirens*, which can change its mating system from outcrossing to selfing (Qian *et al.*, 2001). De Groot *et al.* (2012) suggest that intraspecific variation in the mating system may be common, at least among four rare and calcicole ferns (*Asplenium scolopendrium*, *Asplenium trichomanes* subsp. *quadrivalens*, *Polystichum setiferum* and *Polystichum aculeatum*). Wubs *et al.* (2010) reported that although *Asplenium scolopendrium*, a homosporous fern species, is preferentially outcrossing, when mates are limited, it can reproduce through this extreme form of selfing. Similarly, although some species have mixed mating systems, mating strategies in fern species vary mainly from self-fertilization to obligate intergametophytic crossing (Soltis and Soltis, 1990a, 1992). Intragametophytic selfing has also been reported as a trait that could be linked to invasiveness and successful colonization of new habitats (Lott *et al.*, 2003). Several fern species exhibit variation in selfing rates among populations, and they may rely on temporarily increased selfing rates for their colonization potential (Wubs *et al.*, 2010). *C. thalictroides* is a homosporous fern with a wide geographical distribution that annually colonizes aquatic environments, such as paddy fields and marshes (Watano and Masuyama, 1991; Hickok *et al.*, 1995; Kinoshian *et al.*, 2020). This implies that a high selfing capacity as a strategy for long-distance dispersal and colonization may be present in *C. thalictroides*. Consequently, we suggest that *C. thalictroides* may vary its mating strategy for population establishment after long-distance dispersal and colonization.

Vegetative propagation is nearly universal among aquatic plants (Cook, 1990). Clonal growth and mating systems exert a strong influence on the growth and genetic diversity of aquatic plant populations (Qian *et al.*, 2001). *C. thalictroides* has a prolific capacity for vegetative reproduction by means of numerous marginal leaf buds that rapidly develop into plantlets (Diao, 1990; Dong *et al.*, 2010). Diao (1990) reported that the vegetative growth of *C. thalictroides* was inferior to sexual reproduction in China. In previous studies, vegetative growth was observed in some natural populations of *C. thalictroides*, such as GD-2 and YN-3 (see Dong *et al.*, 2008). In a field survey, the phenomenon was also observed in the population (N 21° 55'38"/E 101° 15'16") of Xishuangbanna in Yunnan Province in October 2021. However, due to the wide distribution of *C. thalictroides* in China, it is difficult to continuously follow and investigate the whole life history of each plant of each natural population in field surveys. Therefore, detailed vegetative growth data for wild natural populations in China are lacking. Among the 10 *ex situ* conservation populations at Jiangnan University (N 30° 30'/E 114° 9') in Wuhan, only the population from Yihuang (N 27° 47'/E 116° 10'), Jiangxi Province, shows vegetative growth (Figure 4). Among the seven strains (sporophytes) protected *ex situ* at Jiangnan University, three strains showed vegetative growth by means of numerous marginal leaf buds that rapidly developed into plantlets in the population of Yihuang County. Numerous marginal leaf buds of sterile fronds developing into many sporophytes containing fertile fronds were also observed in September 2021, showing that spores can be produced before winter (Figure 4). Extensive cloning can lead to reduced reproductive potential in outcrossing species and increased inbreeding rates in self-compatible plants (Frankham *et al.*, 2002). It is therefore probable that vegetative growth also increased inbreeding rates within populations of *C. thalictroides*. However, without detailed vegetative growth data, the importance of vegetative growth in the reproductive system is difficult to assess. Thus, selfing capacity in *C. thalictroides* may be explained by several factors, including hermaphrodite characteristics, colonization potential, and vegetative growth.

In summary, although *C. thalictroides* is a predominantly outcrossing species, we suggest that the mating system in this species may change to promote the selfing rate when a few individuals or single spores are used to establish populations and under extreme conditions, such as isolated gametophyte culture and colonization

events along with vegetative growth. This result is similar to that of a previous study on *Ceratopteris pteridoides*. For example, Dong *et al.* (2018) reported that *C. pteridoides* predominantly favors gametophytic crossing (AFLP, $t_m = 0.999$) with a certain level of inbreeding along with vegetative growth.

Conservation strategies

A good knowledge of the mating system of *C. thalictroides* will provide critical baseline information for developing sustainable management strategies. Considering that the outcrossing rates of *C. thalictroides* in China are high, it would be advisable to establish as many *ex situ* conservation sites as possible, encourage mixing of more individuals from different populations, minimize inbreeding, promote outcrossing and enhance gene flow to preserve the greatest extent of genetic resources within the species. In addition, spores from different populations could be mixed and cultured in gametophyte culture to improve the outcrossing rate.

Ex situ conservation and *in situ* conservation are the two most effective measures for species conservation (Primack, 1993). In this study, ten populations of *C. thalictroides* from Zhejiang, Jiangsu and Fujian provinces were employed for *ex situ* conservation at Jiangnan University in Wuhan, China (Figure 5). In recent decades, botanical gardens have played an important role in the *ex situ* conservation of rare and endangered plants (Maunder, 1994). Wuhan Botanical Garden and Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Sciences play an important role in the conservation of species that once showed a wide distribution in tropical and subtropical regions of China. Both Wuhan Botanical Garden and Jiangnan University are located in Wuhan, and we will continue to work with the botanical gardens on *ex situ* conservation of endangered species such as *C. thalictroides*. One of the 21 extant populations of *C. thalictroides* found in 2021 was derived from *ex situ* conservation at Xishuangbanna Tropical Botanical Garden. Dong *et al.* (2008) reported that the YN-2 population (coordinates: 21°55'N/101°15'E, location: Mengna, Yunnan Province) is also an *ex situ* conservation population from the botanical garden.

In October 2021, the China National Botanical Garden System was officially launched in Beijing and Guangzhou. In January 2022, the State Council approved a plan to establish a national botanical garden in Beijing. The *ex situ* conservation of plants should be emphasized in the National Botanical Garden. It is believed that with the development of the National Botanical Garden System and the establishment of more national botanical gardens, an increasing number of rare and endangered plants, including *C. thalictroides*, will be effectively protected.

Habitat matching and the use of local propagules are considered to be the most successful factors in the long-term survival of a species (Krauss *et al.*, 2000). Loss of habitat is the single most important cause of species extinction (Primack, 1993). Dong *et al.* (2005) reported that ten previously recorded populations of *C. thalictroides* have been extirpated in China, and human activity was identified as the most important cause of destruction and loss of the primary habitat of this species. Because of uncontrolled real estate development, we found that the ZJ-1 and GX-1 populations (see Dong *et al.*, 2005, 2008) had been extirpated in the field survey in 2021. Dong (2012) reported that the population at West Lake in Hangzhou had also gone extinct due to tourism. Compared with those in 2008, the area and size (number of individuals) of the HN population determined in this study (the HN population is equivalent to the HL1 population in 2008, see Dong *et al.* (2008) have decreased due to excessive aquaculture (Table 1). Therefore, in attempts aimed at recovering populations of rare and endangered species, including *C. thalictroides*, habitat conservation that allows a large number of individuals to survive will be of utmost importance.

Conclusions

Our results showed that *C. thalictroides* in China predominantly favors crossing with a certain level of gametophytic selfing along with vegetative growth. A number of causes, including high dispersal of spores and free-living gametophytes by flowing water, high gene flow, and the wide distribution of the species in China, with the occurrence of some large populations, may have led to a high outcrossing rate in Chinese *C. thalictroides*. The species may vary its mating strategy to some extent. Factors may serve to promote the selfing rate in *C. thalictroides*, including the characteristics of hermaphroditism and colonization events and clonal growth. In Wuhan, China, ten populations have been established via *ex situ* conservation, and mature sporophytes and spores were collected. As a result of the high outcrossing rate of *C. thalictroides*, we recommend that *ex situ* plantings would be most suitable for enhancing possible mixing of the different populations, thus minimizing inbreeding.

Authors' Contributions

Song, C.Y., Bai, X.Y., Wei, J., Dong, Y.H., Ma, M., and Zeng, C.L. carried out the experiments. Dong, Y.H., Ning, T.T., Xu, G.Q., and Lan, H. contributed to the experimental design. Dong, Y.H., Song, C.Y., Bai, X.Y., Wei, J., Liao, K., Liu, H., Ma, M., and Zeng, C.L. analyzed the data and wrote the paper. All the authors have read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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