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# Heterodichogamy, Pollen Viability, and Seed Set in a Population of Polyploidy *Cyclocarya paliurus* (Batal) Iljinskaja (Juglandaceae)

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**Abstract:** *Research Highlights:* *Cyclocarya paliurus*, native to the subtropical region of China, is a monoecious species with a heterodichogamous mating system. Its flowering phenology and low seed success characteristics differ from other typical heterodichogamous Juglandaceae species. This could be caused by the existence of polyploidy in the population. *Background and Objectives:* *C. paliurus* has been attracting more attention as a result of its medicinal value. To meet the needs for leaf harvest, cultivation expansion is required, but this is limited by a shortage of seeds. This study aims to profile the flowering phenology and the efficacy of pollen dispersal as well as elucidate on the mechanism of low seed success in the population. *Materials and Methods:* The flowering phenology pattern of *C. paliurus* was observed in a juvenile plantation containing 835 individuals of 53 families from 8 provenances at the individual (protandry, PA and protogyny, PG) and population levels for 5 consecutive years (2014–2018). Slides with a culture medium of 10% sucrose and 0.01% boric acid were used to estimate pollen density and viability in the population, and seeds were collected from 20 randomly selected PA and PG individuals to assess seed success during 2017–2018. *Results:* Four flowering phenotypes and strongly skewed ratios of PA/PG and male/female occurred in the juvenile population. Sexual type and ratio changed significantly with the growth of the population over the years, showing an increasing monoecious group (11.1% to 57.2%) and a decreasing unisexual group (33.6% to 16.3%), as well as a tendency for the sexual ratio to move towards equilibrium (5.42:1 to 1.39:1 for PG:PA). Two flowering phases and bimodality in gender were displayed, as in other heterodichogamous species. However, the high overlap of inter-phases and within individuals was quite different from many previous reports. Owing to the low pollen viability of *C. paliurus* (~30%), low seed success was monitored in the plantation, as well as in the investigated natural populations. *Conclusions:* Female-bias (PG and F) and a skewed ratio of mating types corresponded to nutrient accumulation in the juvenile population. Heterodichogamy in *C. paliurus* was verified, but was shown to be different from other documented species in Juglandaceae. The latest finding of major tetraploidy in a natural population could explain the characteristics of the flowering phenology and seed set of *C. paliurus* and also give rise to more questions to be answered.

**Keywords:** protogyny (PG); protandry (PA); pollen viability; seed success; polyploidy

## 1. Introduction

Heterodichogamy, a transitional type from dichogamy to dioecy, is a polymorphic phenologic sexual system [1,2]. It is defined as two complementary morphs, protogyny (PG, female function before male) and protandry (PA, male function before male), that function synchronously and reciprocally to

one another at a population level [3]. Generally, disassortative mating between two morphs, regarded as the main pattern for heterodichogamous species, promotes proficient inter-morph cross-pollination, avoids selfing, and reduces inbreeding of intra-morph through temporal separation of the male and female functions in flowers [1,4]. To avoid frequency-dependent selection, the population has evolved to have a 1:1 morph ratio [5]. Based on this equilibrium ratio, genetic theory suggests that heterodichogamy was simply inherited with a single diallelic Mendelian locus [1], which has been confirmed in both *Juglans* and *Carya* genera from the Juglandaceae family [6,7].

Hitherto, plants with heterodichogamy have been documented in 13 families and 21 genera of flowering plants [8,9]. Heterodichogamy is particular found in Juglandaceae genera, including *Juglans*, *Carya*, *Pterocarya*, *Platycarya*, and *Cyclocarya* [1,9,10]. Renner [1] reported that about half of the heterodichogamous taxa are self-incompatible, but the Juglandaceae family is completely self-compatible [6,7]. However, Bai [11] demonstrated that the mating system of *J. mandshurica* is completely out-crossing.

Walnut and pecan species are regarded as a group of economically useful plants whose heterodichogamous features have gained extensive attention. This includes *J. regia*, *J. mandshurica*, *J. ailanthifolia*, *J. nigra*, *J. hindsii*, *J. cinerea*, and *J. cordiformis* in *Juglans*; *C. illinoensis*, *C. ovata*, *C. tomentosa*, and *C. laciniosa* in *Carya*; and some inter-specific hybrids in this family [7,8]. Different from nut- and/or timber-use juglandaceous plants, *C. paliurus* is important for its leaf-use value. In the past two decades, an extraordinary amount of progress has been made in determining the medicinal functions of this species. Publications have mostly stressed the actions of bioactive components, including cyclocariosides, cyclocaric acids, flavonoids, and steroids. These leaf metabolites have been verified to exert important medicinal and health functions on conditions such as hypoglycemia, hypolipidemia, and diabetes mellitus, as well as carrying out antioxidant activities [12–16]. However, leaf resources only from natural stands limited its wide applications. Therefore, the cultivation of *C. paliurus* with improved accumulation of bioactive metabolites for leaf harvest is concerned [17,18]. To date, sexual rather than vegetative propagation has been successful for extensive cultivation. Importantly, the release of deep seed dormancy has greatly driven the development of plantations [19]. However, seed shortages resulting from low plumpness is now the main issue for large-scale plantations. Therefore, the following questions need to be answered: Why does such a low seed plumpness occur in populations of *C. paliurus*? Is this affected by genetic heterodichogamy or yearly changing climatic factors? Is the flowering phenology in *C. paliurus* similar to that of other recorded heterodichogamous Juglandaceae species? Therefore, the aims of this study are the following: (1) To profile the flowering patterns of the two mating types of *C. paliurus* (PA and PG) and the population as a whole using consecutive 5-year observations on the juvenile plantation; (2) to monitor the density and viability of pollen at the population level during the flowering season; (3) to analyze and hypothesize the reasons for the low seed success in the population using the chromosomal ploidy level of *C. paliurus*.

## 2. Materials and Methods

### 2.1. Study Species

*Cyclocarya paliurus*, a species of monotypic genus in Juglandaceae, is endemic in subtropical region of China (from 24°16'12" N to 33°22'12" N and from 103°28'12" E to 119°22'48" E). Often, small populations (<25 individuals) grow in moist valleys in mountain regions at an altitude of 390–1836 m, and mature trees reach a height of 10–30 (40) m because of their heliophilous characteristic [20].

A single female inflorescence of *C. paliurus* is found at the apex of the growing shoot (Figure 1A); rarely, a single male instead of a female inflorescence is observed at this position (Figure 1C). A cluster containing 2–4 catkins is found at the lateral short branch (Figure 1A). Besides unisexual inflorescences, a small number of female inflorescences mixed with male flower(s) are observed in some individuals at certain years (Figure 1D). However, similar to maples, the male flowers in female inflorescence are

abortive [21]. Previous studies revealed that *C. paliurus* is a typically heterodichogamous species that undergoes wind-pollination [10,22].



**Figure 1.** Expressions of male and female inflorescences in *Cyclocarya paliurus*. (A) Male and female inflorescences of the protogyny (PG) type. The female flowers are mature, while the male flowers are still enclosed. (B) Protandry (PA) inflorescences at the elongation stage, showing the developmental differences between the two sexual inflorescences. Abnormal inflorescences include (C) a single male instead of a female inflorescence at the apex of growing shoot and (D) a female inflorescence mixed with a male flower.

## 2.2. Plantation of *C. paliurus*

A plantation of *C. paliurus* used in this study is located on Hongya Mountain and in Chuzhou City and Anhui Province, China (32°21' N, 117°58' E), where the climate is classified as northern subtropical humid monsoon, with an annual mean temperature of 15.5 °C, an annual rainfall of 1038 mm, and a frost-free period of 210 days. The site is dominated by a gravel mountain with a slight slope (5°–6°).

The plantation was established in 2008, containing 835 individuals of 53 families from 8 provenances (Lushan, Jiangxi; Hefeng Hubei; Shucheng, Anhui; Jianhe, Guizhou). The plant spacing was 3 m × 4 m, and the stand was not closed until 2018. A small number of flowering plants (mostly only one sexual inflorescence occurred) were observed in 2012; subsequently, an increasing number of plants exhibited sexual polymorphism.

### 2.3. Investigation of Flowering Phenology

Phenological monitoring was performed for a 5-year consecutive period (2014–2018), and observations were recorded from 2015, when at least one sexual flower (male/female) occurred in 3/8 individuals in the population. Characteristics recorded included flowering phenotype, flowering progress, and sex expression for all individuals. We also observed whether the flowering phenotype involved flowering or not, as well as the dates of onset, duration, and termination for male and female flowering each year.

The female flowering period was determined by two feathery stigmas with an angle of 120° and the change of the stigma color from green to brown (withering); the period of male flowering was from pollen shedding to shedding completion. The full-bloom stage for the population was defined as 50%–75% of flowers being in each flowering phase for monoecious individuals. Observations were made on male and female inflorescences tagged in the middle section of each plant.

### 2.4. Pollen Dispersal

To monitor the density and viability of pollen within the flowering season, pollen was collected from 5 trapping sites on stocks at a height of 1.5 m along a line of “S” in the stand. Six slides covered with a culture medium of 10% sucrose and 0.01% boric acid were placed on each stock. Three slides collected every two days, each for 24 h, were used for pollen density counting, while the remaining 3 slides were collected from 9:00–11:00 (when the highest pollen viability is observed) each day (except for rainy days) during flowering duration and used for pollen viability testing.

The slides were examined by (1) counting the number of pollen grains adhering to the surface of the culture medium under a microscope at ×50 magnification, and (2) culturing pollen in an incubator at 25 °C for 8 h. The pollen grain was considered viable when the pollen tube grew to the length of itself. The mean and standard error of the pollen density and viability were calculated over ten visual fields for each side.

### 2.5. Seed Collection

Respectively, 20 PA and 20 PG plants were randomly selected in 2017 and 2018, a total of 300–500 seeds were collected from the middle of each selected tree. Seed plumpness was judged by cutting seed along the hilum linear direction.

Values of pollen density and viability and seed plumpness are presented as mean ± SE, and an ANOVA analysis was conducted to determine differences among years and between mating types using SAS 18.1.

## 3. Results

### 3.1. Sex Expression of the Juvenile Population

As reported in the previous document, the flowering season of *C. paliurus* lasted about one month, from mid-April to the end of May. Two separated flowering phases occurred in the population: one during late-April to mid-May, while the other happened across all of May.

#### 3.1.1. Sexual Diversity

A total of five phenotypes from three groups were recognized for individuals in the population: The monoecious group (MO) containing individuals with both male and female inflorescences, including protogyny (PG) and protandry (PA); the unisexual group (UN) including individuals with only one sexual inflorescence, either male (M) or female (F); and unflowering trees (UF). MO and UN were both classified as flowering plants (FP).

### 3.1.2. Expression Features of Various Flowering Phenotypes

In a natural population, initial flowering usually occurs at the age of 10–15 years. However, the onset of flowering in a plantation is significantly earlier than in a natural population. In the 5-year-old plantation (in 2013), the F type was predominant among FP plants, up to 75.7% and 53.8% in 2014 and 2015, respectively; the second most dominant type was the PG type. Comparatively, few plants were of the PA type, and this minority was assigned to the M type (Table 1).

**Table 1.** Annual expression of various flowering phenotypes in the juvenile plantation of *C. paliurus*.

Flowering Group (FP)	Mating Type	Observed Year				
		2014	2015	2016	2017	2018
Monoecious group (MO)	number of PA	12	40	127	151	185
	number of PG	65	86	173	200	257
	PA:PG	5.42:1	2.15:1	1.36:1	1.32:1	1.39:1
	PG/MO (%)	84.4	68.3	57.7	57.0	58.1
	PG/FP (%)	18.8	28.8	37.5	37.0	45.2
	MO/T (%)	11.1	15.8	38.4	45.1	57.2
Unisexual group (UN)	number of F	262	161	147	169	98
	number of M	7	12	14	20	28
	F:M	37.4:1	13.4:1	10.5:1	8.5:1	3.5:1
	F/UN (%)	97.4	93.1	91.3	89.4	77.8
	F/FP (%)	75.7	53.8	39.0	31.3	17.3
	UN/T (%)	33.6	21.6	20.6	24.3	16.3
	FP/T (%)	43.2	37.4	59.0	69.4	73.5
	Total plants in the population (T) *	801	800	781	778	773

\* Data changed annually because of the death of individuals. PA: protandry, PG: protogyny; MO: monoecious group, FP: flowering plants, UN: Unisexual group; F: females, M: males; T: total individuals in population.

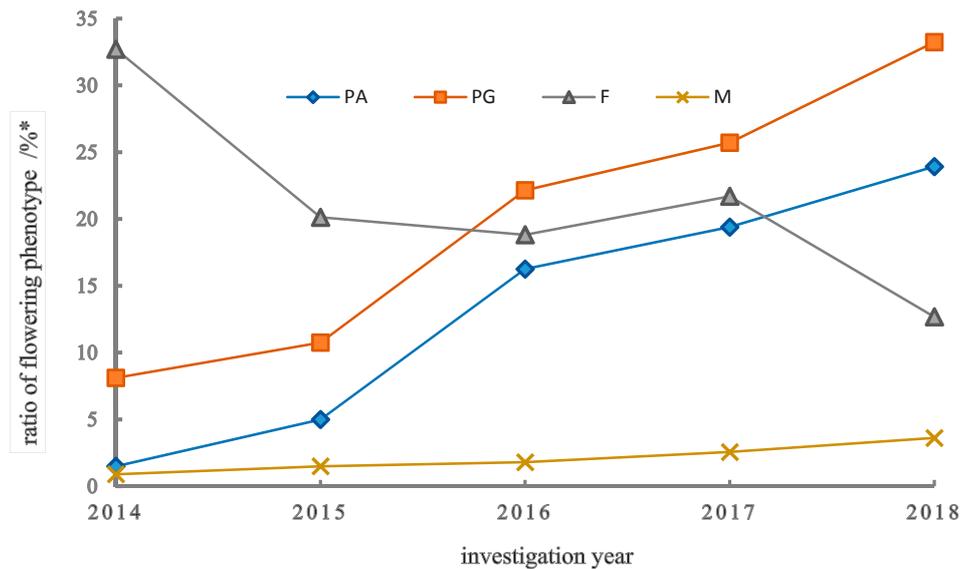
With the growth of the population, more individuals flowered. The FP/T showed a tendency to increase, from 43.2% in 2014 to 73.5% in 2018 (Table 1). More significantly, the promotion of MO/T increased rapidly from 11.1% (in 2014) to 57.2% (in 2018), whereas there was a descent in UN/T, from 33.6% (2014) to 16.3% (2018).

This data suggests that the ages of 6–10 years (defined as the juvenile population) could be regarded as the transitional stage towards maturation for *C. paliurus*.

### 3.1.3. Changing Patterns of Mating Types across Years

Although there was a tendency for the number of flowering individuals to increase (Table 1), the changing trends were divergent for various flowering phenotypes (Figure 2). In 2016, there were rapid increments of PA and PG morphs, accompanied by a rapid decrease in individuals of the F type. Of the four flowering phenotypes, similar increasing tendencies were observed in PA and PG types, but the proportion of PA was always less than that of PG. In addition, only a slight increment was observed in the M type, rising from 0.9% to 3.2%. On the contrary, the proportion of F type individuals dropped significantly from 32.71% to 12.68% across the five-year period (Figure 2).

Meanwhile, strongly skewed ratios of mating types, namely, PG/PA or F/M, always existed in the juvenile population (Table 1). Significantly, the ratio of PA/PG fell obviously from 5.42:1 in 2014 to 1.39:1 in 2018. Further the F/M ratio fell from 37.43:1 in 2014 to 3.5:1 in 2018. An  $X^2$  test indicated the protogynous-biased ratio in the population during 2014 to 2018 (PG/PA: 65/12,  $x^2 = 36.5$ , d.f = 1,  $p < 0.01$  in 2014; 86/40,  $x^2 = 16.8$ , d.f = 1,  $p < 0.01$  in 2015; 173/127,  $x^2 = 7.2$ , d.f = 1,  $p < 0.01$  in 2016; 200/151,  $x^2 = 7.7$ , d.f = 1,  $p < 0.01$  in 2017; and 257/185,  $x^2 = 11.7$ , d.f = 1,  $p < 0.01$  in 2018) significantly deviated from the equilibrium ratio of 1:1, but showed a tendency towards relative equilibrium.



**Figure 2.** Changing trends of flowering phenotypes with the growth of the *C. paliurus* population.\* Number of each flowering phenotypes/total plants in the population. F: females, M: males.

### 3.1.4. Reciprocal Transitions between Mating Types

Owing to the immaturity of the juvenile population, plants were sensitive to nutrient shortage and unstable environmental factors. Therefore, reciprocal transitions among five phenotypes happened frequently between adjacent years (Table 2). As a whole, with the growth of population, the ratio of transition from UF to FP showed a decreasing trend from 68.8% to 51.2%, whereas there was an opposite tendency for the transition from UN to MO (Table 2).

It seems that F and PG types played the core roles for all transitions. As shown in Table 2, reciprocal transitions happened more often between UF↔F, UF↔/PG, and F↔PG/PA, and scarcely between M↔F and PA↔PG. Remarkably, observed transitions between PA and PG were usually ambiguous and mainly happened in individuals with a high overlap of male and female flowering (Figure 3).

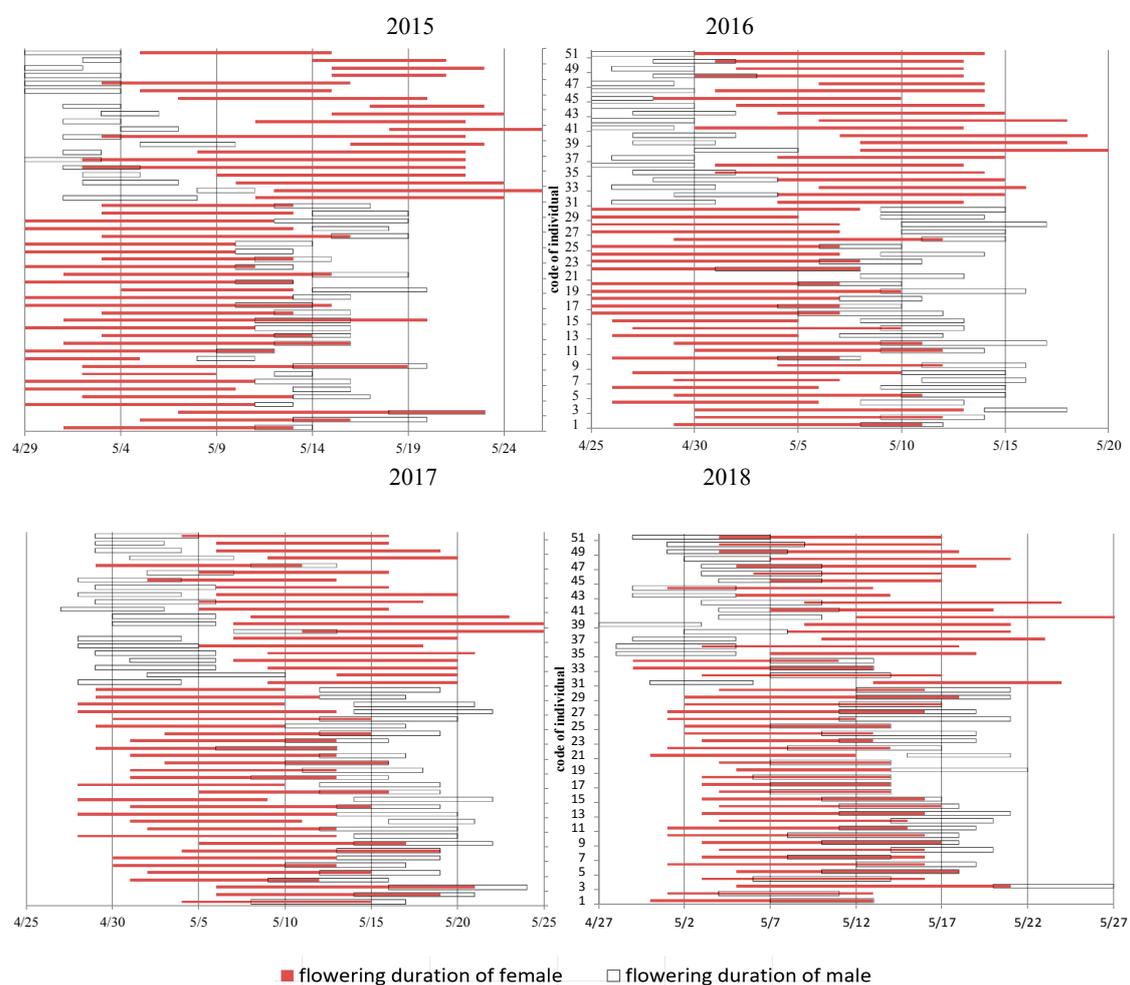
**Table 2.** Transitions between flowering phenotypes in the juvenile plantation of *C. paliurus*.

Transition Pattern	Number of Transitions between Phenotypes/Total Number of Transitions (%)				
	2014–2015	2015–2016	2016–2017	2017–2018	
UF→F, F→UF	53/20.2, 101/38.4	91/30.3, 3/1.0	85/31.7, 30/11.2	38/13.3, 32/11.2	
UF↔FP	UF→M, M→UF	5/1.9, 4/1.5	9/3.0, –	3/1.1, 4/1.5	5/1.8, 2/0.7
	UF→PG, PG→UF	6/2.3, 8/3.0	42/14.0, –	15/5.6, 3/1.1	24/8.4, 10/3.5
UF→PA, PA→UF	3/1.1, 1/0.4	38/12.7, –	11/4.1, 4/1.5	23/8.1, 12/4.2	
	Subtotal of UF	181/68.8	183/61.0	155/57.8	146/51.2
F→PG, PG→F	36/13.7, 8/3.0	46/15.3, 5/1.7	36/13.4, 14/5.2	48/16.8, 2/0.7	
	F→PA, PA→F	28/10.6, 4/1.5	43/14.3, 5/1.7	18/6.7, 18/6.7	39/13.7, 10/3.5
UN↔MO	M→PG, PG→M	2/0.8, 2/0.8	4/1.3, 2/0.7	9/3.4, 3/1.1	3/1.1, 11/3.9
	M→PA, PA→M	–, –	8/2.7, 1/0.3	7/2.6, 1/0.4	6/2.1, 2/0.7
subtotal	80/30.1	114/38.0	106/39.6	121/42.5	
MO↔MO	PG→PA, PA→PG	–, –	1/0.3	–, –	5/1.8, 3/1.1
UN↔UN	F→M, M→F	1/0.4, 1/0.4	2/0.7, –	5/1.7, 2/0.7	9/3.2, 1/0.4
total	263	300	268	285	

### 3.2. Flowering Phenology of PG and PA Types across Years

Over the five-year period, 21 PA and 30 PG plants were screened via consecutive observations. As shown in Figure 3, for all marked individuals, a longer flowering duration was observed in females than in males; phenology also coincided with the sequence of flowering expression, but differed in terms of the separation of male and female functions across years. Such labile phenological characters could be mainly affected by environmental factors (e.g., temperature, rain, and wind).

Within each PA individual, the separation between two sexual functions gradually shortened and tended to overlap over the years. In 2015, separation occurred in 17 PA trees; however, it only occurred in four trees in 2018. Although overlap was common within PG individuals, it varied across years. This is in accordance with the statistical data for all monoecious plants, which displayed more overlap in the PG type (50%–90%) than in the PA type (14.3%–47.6%). Therefore, the potential mating probability within PA/PG individuals seems to be dependent on the overlap degree of two sexual functions.



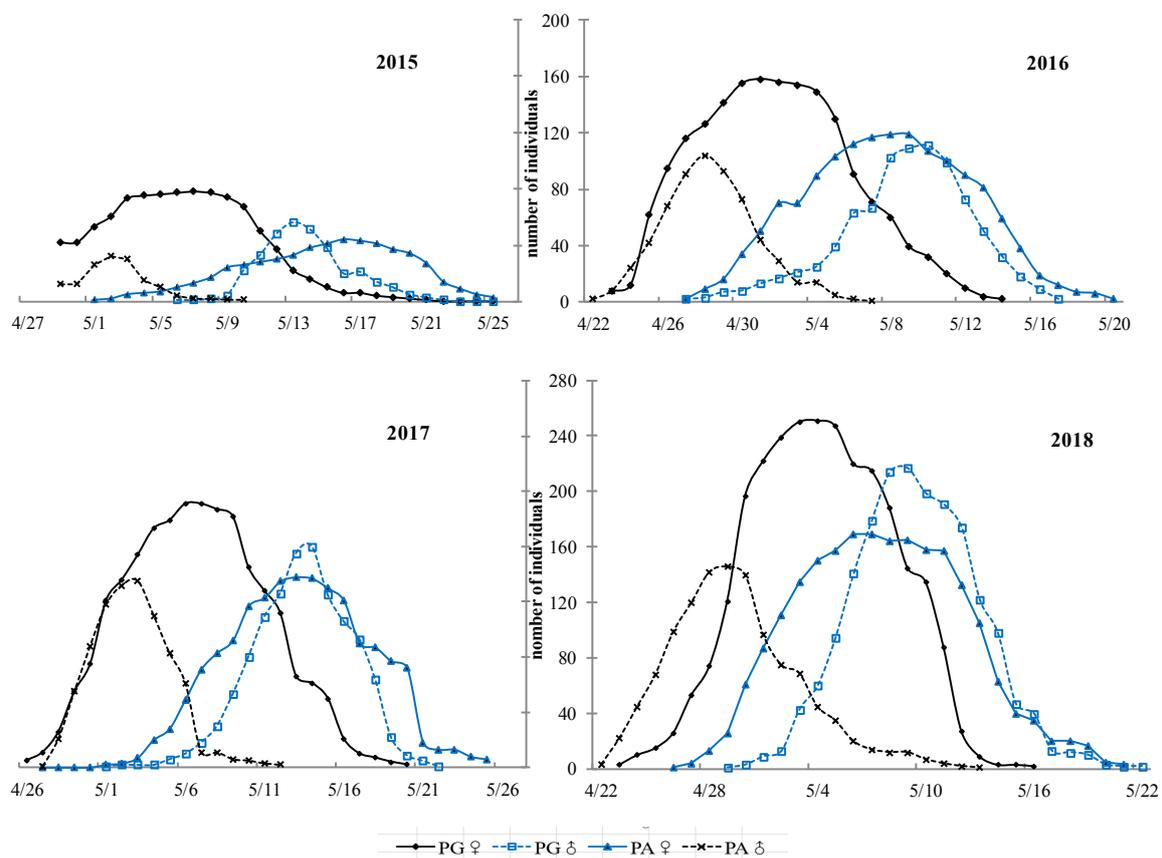
**Figure 3.** Flowering phenology of males and females within individuals based on 21PA and 30PG trees of *C. paliurus* during 2015–2018.

### 3.3. Phenological Characteristics of the Population

PA and PG morphs are the two main types of heterodichogamous species. However, in the juvenile population of *C. paliurus*, monoecious plants were not more than half of the total (57.2%) until 2018 (Table 1). Moreover, some monoecious plants could transfer to other phenotypes, such as F or unflowering ones (Table 2). Here, all individuals of PA or PG morphs were clustered together to describe the flowering characteristics of the population.

Figure 4 illustrates that two incomplete separation flowering phases existed in the population. In the first phase, from 22–26 April to 12–18 May, PG females and PA males were in bloom, while during the second phase, from 28 April 28–1 May to 20–26 May, PG males and PA females were in bloom. Each phase lasted 20–25 days and the obvious overlap (7–10 days) of inter-phases varied across years. Generally, the female flowering duration (>25% individuals in the population in bloom) for two morphs was significantly longer than that of the males (one week).

Comparatively, the male flowering duration was shorter by 4–5 days in the first phase than in the second one. In the first phase, the peak of male flowering was reached earlier than that of female flowering, which lowered the opportunity for inter-morph pollination. In contrast, the flowering peaks of the complementary sexes were rather well synchronized in the second phase (Figure 4). This difference in inter-morph synchronism for each phase could result in higher seed success in PA (pollinated in the second phase) than in PG (pollinated in the first phase) and a lower chance for pollen from PA males than PG males as contributors to pollinate the PG females. In addition, the overlap of inter-phases mainly happened between PG males and PG females, increasing the intra-morph inbreeding probability (Figure 4).



**Figure 4.** Number dynamic patterns of PA and PG flowering individuals in the population of *C. paliurus* during 2015–2018. The back lines describe the first flowering phase, while the blue lines describe the second phase. High overlap was obvious between the two phases and the two sexual functions within each phase.

We found that the flowering expression of population was more complicated than the assembly of PA/PG individuals. Separation of the two sexual functions mainly existed within individuals rather than in the population (Figures 3 and 4). Rather than selfing within individuals, inter-morph and intra-morph pollination were dominant at the population level.

### 3.4. Pollen Dispersal Characteristics of the Population

A sufficient quantity and high viability of pollen guarantee for seed success. To illustrate the effect of pollen on seed bearing, pollen density and viability during flowering in the population were surveyed for three consecutive years.

Generally, pollen density displayed an obviously rising trend with the maturation of the population. In the first phase, the maximum pollen density in 2018 was up to  $305 \pm 56$  grain/cm<sup>2</sup>, far above that of 2016 ( $113 \pm 19.7$  grain/cm<sup>2</sup>) and 2017, in the second phase, a similar maximum was observed over three years. The pollen density among inter-phases showed significant differences in 2016 and 2017 but was similar in 2018. This indicates that pollen dispersal is affected not only by nutrient conditions, but also by annual climate change.

In addition, the pollen viability kept rising with the growth of the population, showing maximums of 27.6% and 31.1% in 2018, higher than that of 16.4% and 28.6% in 2017 for the two flowering phases, respectively. Overall, the low pollen viability (30% or so) did not show a positive correlation with pollen density; moreover, irregular dynamics were monitored in the population over the three years (Table 3).

**Table 3.** Pollen density and viability during the flowering period in 2017 and 2018.

Flowering Stage	Observation Date (Month/Date)	Pollen Density (Grain/cm <sup>2</sup> )		Pollen Viability (%)	
		2017	2018	2017	2018
First stage	4/30	136 ± 25	305 ± 56	20.5 ± 3.9	18.3 ± 3.9
	5/01	/	262 ± 31	16.4 ± 4.2	27.6 ± 6.5
	5/03	178 ± 34	/	6.0 ± 3.2	11.0 ± 5.0
	5/04	/	47 ± 12	/	6.5 ± 2.6
	5/06	105 ± 12	/	13.9 ± 2.9	/
Second stage	5/08	151 ± 16	/	20.2 ± 6.8	26.4 ± 7.1
	5/10	247 ± 33	281 ± 29	28.6 ± 4.7	31.1 ± 5.9
	5/12	216 ± 26	/	20.0 ± 4.4	28.8 ± 3.4
	5/14	128 ± 23	33 ± 7	20.3 ± 5.7	13.9 ± 4.3
	5/16	23 ± 5	9 ± 4	5.6 ± 5.6	16.6 ± 12.6
	5/18	12 ± 5	0	0	0

Note: data was listed as average ± SE; '/' means no data offered because of rainfall; data in red was not from the same day but instead was from the previous/next day to show the changing tendency.

## 4. Discussion

Usually, mature trees (flowering individuals) of *C. paliurus* are up to 10–30 (40) m tall, and both male and female inflorescences cover the outer crown above the canopy of the forest. It is difficult to find natural populations of a sufficient size to monitor flowering phenology; thus, plantation could be a better option for phenological observation [20]. Plants in our plantation included 53 families from eight provenances, representing the majority of the natural germplasm.

### 4.1. Sexual Polymorphism and Bias Associated with Nutrient Conditions and Climatic Factors

In natural populations, several heterodichogamous species (e.g., *A. japonicum*) were reported to include not only monoecious trees (PA/PG), but also unisexual trees (M/F) [23–25]. Similar findings were also observed in the *C. paliurus* population. For the first time, we showed that the F type is overwhelmingly superior in juvenile stands (2014), followed by the PG type. Opposite changing trends—an increment in PG and a reduction in F type—were then monitored over 5 years. Meanwhile, the number of PA trees tended to increase, though this was at a slower rate than the PG trees (Figure 2).

Levy and Dean [26] pointed out that most woody plants can flower only when they reach a certain age or size; flowering expression can be linked to factors such as nutrient supply and adversity. A low nutrient supply may support the formation of female flowers for *C. paliurus*. Therefore, the female superiority (F/PG) could be associated with the nutrient conditions in the juvenile population. This hypothesis is supported by our previous findings that flowering expressions are significantly

related to the plant size of *C. paliurus* [10]. Alternatively, adaptive interpretation considers that female-bias favors the production of more seeds for species survival under adverse conditions, like nutrient shortage and changing climatic factors (dry, cold, etc.). However, more extensive studies for longer periods need to be conducted to confirm this.

#### 4.2. Morph Ratio and Transition

Generally, an equal morph ratio (PA:PG = 1:1) should occur in a heterodichogamous population, as shown in *Juglans* species, such as *J. regia*, *J. ailanthifolia*, and *J. mandshurica*, as well as in some *Acer* species (e.g., *A. opalus* and *A. japonicum*) (reviewed by Liu [8]). However, derivations from this equilibrium have been found in some species (e.g., *A. pseudoplatanus*) [24] and under certain situations (e.g., small population or human disturbance, like intentional selection) [27]. Similarly, a distinctive bias was found in our study. We speculate that the “juvenile” population is the main reason for this deviation, and the change in the morph ratio (PG/PA) from 5.42:1 in 2014 to 1.39:1 in 2018 predicts a state of equilibrium in the mature population.

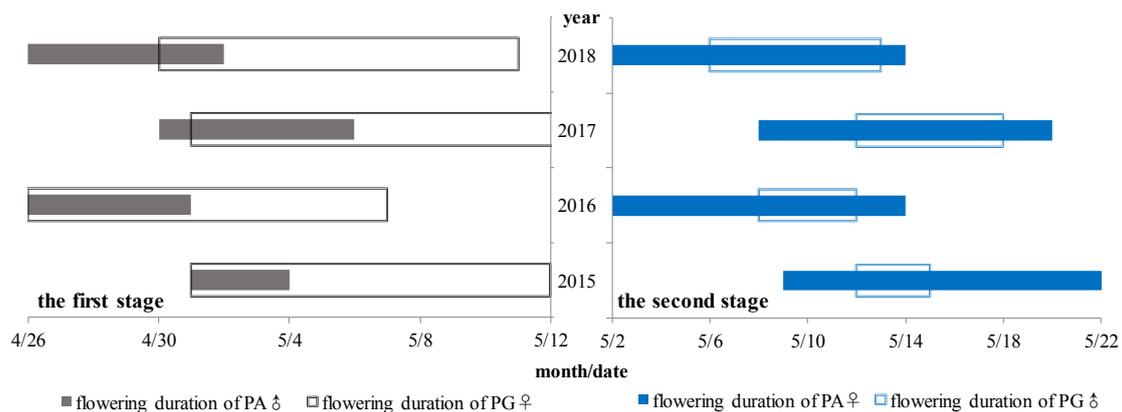
Labile sex expression was documented in some heterodichogamous trees across years, like in *Acer pseudoplatanus* [24,28]. It is reported that the sexual phenotype changed mainly from unisexual to the monoecious type, with few or no changes between PA and PG [8,9]. We found that unisexuality is a temporary trait based on the reduction of the ratio of M and F types with the maturation of the stand (Table 2). Similar findings were reported in *J. ailanthifolia* and *J. mandshurica* [9,29]. Researchers believe that the unisexual trait (F and/or M) is an environmental and/or nutrient (young age) response phenotype rather than a separate morph [9,24,25].

Morphs of PA and PG are expected to be stable according to the theory that sex morphs are genetically determined [6,7], which has been demonstrated by some observations in *J. regia* and *P. strobilacea*. [9,29]. However, documents involving transitions between two morphs in *C. ovata*, *C. tomentosa*, and *A. japonica* have been published [24,25,30]. Such a transition in *C. paliurus* was also recorded in several individuals. On these plants, highly overlapping flowering durations of two sexual flowers led to the ambiguous judgment of mating type in 2018 (Figure 3). Thus, further observation needs to be made to confirm such transitions.

#### 4.3. Flowering Phenology of the Individual and Population

Heterodichogamous populations showed two flowering phases and bimodality in gender for two distinct sexual morphs. This has been described in *J. regia*, *J. hindii*, *J. ailanthifolia*, and *J. mandshurica*, and *Platycarya strobilacea* [4,6,9,29,31]. Moreover, sufficiently long separation between individuals can avoid selfing but does not prevent intra-morph inbreeding [4]. Therefore, flowering separation and the overlap of two sex inter-phases as well as within-phase and within-individual are the key factors affecting mating patterns. As described by numerous publications, both features are divergent among heterodichogamous species, varying from complete separation to high overlap. In contrast to the pattern of no overlap within individuals and inter-phases in *J. mandshurica* [4] and a short period of overlap in the PG type of *J. ailanthifolia* [29], high overlap occurs extensively, not only inter-phase but also intra-morph and within individuals in *C. paliurus*. High overlap within individuals and intra-morphs seems to favor selfing and inbreeding but is contrary to the mechanism of avoiding assortative mating for heterodichogamous plants [4,32].

Mating fitness also depends on the synchronism of each sexual flower—very early/or late individuals would suffer low reproductive success. Bai et al. [4] observed that female flowering peaks were earlier than male peaks for two periods in *J. mandshurica*, and the PG morphs had a higher level of assortative mating than PA ones. We found that the flourish flowering period of the male preceded that of the female in the first stage, but the female flowering period covered the complete duration in the second stage for four consecutive years (Figure 5), suggesting that a higher seed set could be more successful in PA than in PG. This prediction was further supported by the result that seed plumpness in PA ( $22\% \pm 8.2\%$ ) was significantly higher than in PG ( $12\% \pm 6.8\%$ ) ( $p < 0.05$ ).



**Figure 5.** Flourish flowering duration (>50% individuals of PA and PG in bloom) of two stages in the population of *C. paliurus*.

#### 4.4. Reasons for Low Seed Success in *C. paliurus*

Heterodichogamy is recognized as a key factor affecting seed production, and high fruit production has been reported in many species, such as *J. regia* and *C. illinoensis* [33,34]. Strangely, seeds with low plumpness of 10%–30%, and rarely 50%, were collected from natural populations of *C. paliurus* for nearly ten years. The low seed success (0–30%) in our plantation during 2016–2018 was also in accordance with the natural situation.

Low seed success in a small population can result from the bias of two mating types, like in *A. pseudoplatanus* [28]. Limited by the habitat of *C. paliurus* or by human disturbance, the size of more than two-thirds of the natural populations investigated was less than 25 individuals [20]. This might be one of the reasons for the low seed set in the natural population; however, it cannot be the explanation for our plantation. Furthermore, the overlap of the flowering phenology in *C. paliurus* seems to provide a better chance of pollination, including crossing, inbreeding, and even selfing, and a high seed success should be expected, according to the 100% rate of self-compatibility in *Juglandaceae* species [1]. However, the investigated data did not support these speculations.

#### 4.5. Polyploid *C. Paliurus* Relating to Characters of Flowering and Seed Success

Different from documented heterodichogamous species in *Juglandaceae*, observations over five years demonstrated a high overlap between the two flowering phases and within individuals, low pollen viability rather than pollen density in the population (Table 3), and low seed plumpness not only in the natural stands and plantation, but also in the control pollination (12%–38%, data unpublished). This infers that unknown reason(s) could be responsible for the previously mentioned phenomenon.

Unexpectedly, data based on a genomic survey found the existence of a tetraploid individual. Subsequently, by screening 1087 individuals collected in our germplasm from 42 provenances from 13 provinces using a cell flow meter, we discovered that tetraploid plants occupied about 95%, and diploid and triploid (uncertain) ones accounted for about 5%. In a word, diploid-tetraploid plants coexist in a natural population. *C. paliurus* is the first species found to have both polyploidy and heterodichogamy characteristics.

Based on these findings, many differences observed in *C. paliurus* from other heterodichogamous species can be elucidated. The dominance of multi-allele rather than two-allele in polyploid *C. paliurus* controlled heterodichogamy might trigger the inter-phase overlap of flowering characteristics as well as within individuals. As we know, low pollen viability from polyploidy [35] could result in low seed success. However, based on the theory that heterodichogamy is simply inherited with a single diallelic Mendelian locus in diploid species [1], it is unclear how heterodichogamy is determined in polyploid *C. paliurus*. Further, it is unknown how *C. paliurus* with its low seed success rate ensures its succession. Thus, further research should be done in *C. paliurus* to disclose these secrets.

## 5. Conclusions

The female-bias (PG and F) and skewed ratio of mating types that occur in *C. paliurus* correspond to the nutrient accumulation in the juvenile population. Heterodichogamy in *C. paliurus* was demonstrated, but with a substantial difference from other recorded species. Low seed success in the population mainly results from low pollen viability. However, the latest finding of a polyploidy majority in the natural population could explain the flowering phenology and seed set characteristics of *C. paliurus*, but also raises more questions that need to be answered.

**Author Contributions:** This work was designed, directed, and coordinated by X.-X.F., who provided technical guidance for all aspects of the project and wrote the manuscript. X.M. was the principal investigator, contributed to the fieldwork and data analysis, performed the literature search, and helped with the writing of the manuscript. P.H. monitored the density and viability of pollens. X.-L.C. and Y.-Q.Q. assisted with the fieldwork and analysis of polyploidy for all individuals.

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## References

1. Renner, S.S. How common is heterodichogamy? *Trends Ecol. Evol.* **2002**, *16*, 595–597. [[CrossRef](#)]
2. Barrett, S.C. The evolution of plant sexual diversity. *Nat. Rev. Genet.* **2002**, *3*, 274–284. [[CrossRef](#)] [[PubMed](#)]
3. Webb, D.J.; Lloyd, D.G. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *N. Z. J. Bot.* **1986**, *24*, 16. [[CrossRef](#)]
4. Bai, W.N.; Zeng, Y.F.; Liao, W.J.; Zhang, D.Y. Flowering phenology and wind-pollination efficacy of heterodichogamous *Juglans mandshurica* (Juglandaceae). *Ann. Bot.* **2006**, *98*, 397–402. [[CrossRef](#)] [[PubMed](#)]
5. Clark, B.C.; Partridge, L.; Robertson, A. *Frequency-Dependent Selection*; Cambridge University Press: New York, NY, USA, 1988.
6. Gleeson, S.K. Heterodichogamy in walnuts: Inheritance and s ratios. *Evolution* **1982**, *36*, 892–902. [[CrossRef](#)] [[PubMed](#)]
7. Thompson, T.E.; Romberg, L.D. Inheritance of heterodichogamy in pecan. *Heredity* **1985**, *76*, 456–458. [[CrossRef](#)]
8. Liu, J.J.; Mao, X.; Li, X.C. A review on flowering mechanism in heterodichogamous plants. *J. Nanjing For. Univ.* **2016**, *40*, 147–154. (In Chinese) [[CrossRef](#)]
9. Fukuhara, T.; Tokumaru, S. Inflorescence dimorphism, heterodichogamy and thrips pollination in *Platycarya strobilacea* (Juglandaceae). *Ann. Bot.* **2014**, *113*, 467–476. [[CrossRef](#)]
10. Mao, X.; Liu, J.J.; Li, X.C.; Qin, J.; Fu, X.X. Flowering biological characteristics and mating system in immature plantations of heterodichogamous *Cyclocarya paliurus*. *J. Nanjing For. Univ.* **2016**, *40*, 47–55. (In Chinese) [[CrossRef](#)]
11. Bai, W.N.; Zeng, Y.F.; Zhang, D.Y. Mating patterns and pollen dispersal in a heterodichogamous tree, *Juglans mandshurica* (Juglandaceae). *New Phytol.* **2007**, *176*, 699–707. [[CrossRef](#)] [[PubMed](#)]
12. Shu, R.G.; Xu, C.R.; Li, L.N.; Zhi, L.Y. Cyclocariosides II and III: Two secodamarane triterpenoid saponins from *Cyclocarya paliurus*. *Planta Med.* **1995**, *61*, 551–553. [[CrossRef](#)] [[PubMed](#)]
13. Xie, M.Y.; Wang, Y.X.; Wen, H.L.; Yi, X. Determination of flavonoid compounds and vitamins in the leaves of *Cyclocarya paliurus* (Batal.) Iljinsk. *Food Sci.* **2001**, *22*, 66–68. (In Chinese) [[CrossRef](#)]
14. Wu, Z.F.; Meng, F.C.; Jiang, C.H.; Zhao, M.G.; Shang, X.L.; Fang, S.Z.; Ye, W.C.; Zhang, Q.W.; Zhang, J.; Yin, Z.Q. Triterpenoids from *Cyclocarya paliurus* and their inhibitory effect on the secretion of apolipoprotein B48 in Caco-2 cells. *Phytochemistry* **2017**, *142*, 76–84. [[CrossRef](#)]
15. Yang, H.M.; Yin, Z.Q.; Zhao, M.G.; Jiang, C.H.; Zhang, J.; Pan, K. Pentacyclic triterpenoids from *Cyclocarya paliurus* and their antioxidant activities in FFA-induced HepG2 steatosis cells. *Phytochemistry* **2018**, *151*, 119–127. [[CrossRef](#)] [[PubMed](#)]

16. Liu, Y.; Wang, T.L.; Fang, S.Z.; Zhou, M.M.; Qin, J. Responses of morphology, gas Exchange, photochemical activity of photosystem II, and antioxidant balance in *Cyclocarya paliurus* to light spectra. *Front. Plant Sci.* **2018**, *23*, 1042. [[CrossRef](#)] [[PubMed](#)]
17. Liu, Y.; Qian, C.Y.; Ding, S.H.; Shang, X.L.; Yang, W.X.; Fang, S.Z. Effect of light regime and provenance on leaf characteristics, growth and flavonoid accumulation in *Cyclocarya paliurus* (Batal) Iljinskaja coppices. *Bot. Stud.* **2016**, *57*, 28–41. [[CrossRef](#)]
18. Liu, Y.; Liu, Q.L.; Wang, T.L.; Fang, S.Z. Leaf nitrogen and phosphorus stoichiometry of *Cyclocarya paliurus* across China. *Forests* **2018**, *9*, 771. [[CrossRef](#)]
19. Fang, S.Z.; Wang, J.Y.; Wei, Z.Y.; Zhu, Z.X. Methods to break seed dormancy in *Cyclocarya paliurus* (Batal). Iljinskaja. *Sci. Hortic.* **2006**, *110*, 305–309. [[CrossRef](#)]
20. Li, X.C.; Fu, X.X.; Shang, X.L.; Yang, W.X.; Fang, S.Z. Natural population structure and genetic differentiation for heterodichogamous plant: *Cyclocarya paliurus* (Batal.) Iljinskaja (Juglandaceae). *Tree Genet. Genomes* **2017**, *13*, 80. [[CrossRef](#)]
21. De Jong, P.C. *Flowering and Sex Expression in Acer L.: A Biosystematic Study*; Mededelingen Land Bouwboege School: Wageningen, The Netherlands, 1976; Volume 76, pp. 1094–1098.
22. Mao, X.; Li, X.C.; Liu, J.J.; Fu, X.X. Scanning electron microscope observation on pollen morphology of six tree species in Juglandaceae. *J. Plant Resour. Environ.* **2016**, *25*, 104–106. (In Chinese) [[CrossRef](#)]
23. Dommée, B.; Bompar, J.L.; Denelle, N. Sexual tetra-morphism in *Thymelaea hirsuta* (Thymelaeaceae): Evidence of the pathway from heterodichogamy to dioecy at the infra-specific level. *Am. J. Bot.* **1990**, *77*, 1449–1462. [[CrossRef](#)]
24. Asai, T. *Dichogamy in Full Moon Maple (Acer japonicum Thunb.)*; Bulletin of the Hokkaido Forestry Research Institute: Tokyo, Japan, 2000; Volume 37, pp. 27–40.
25. Sato, T. Phenology of sex expression and gender variation in a heterodichogamous maple, *Acer japonicum*. *Ecology* **2002**, *83*, 1226–1238. [[CrossRef](#)]
26. Levy, Y.Y.; Dean, C. The transition to flowering. *Plant Cell* **1998**, *10*, 1973–1990. [[CrossRef](#)] [[PubMed](#)]
27. Pollegioni, P.; Olimpieri, I.; De Simoni, G.; Gras, M.; Malvolti, M. Barriers to interspecific hybridization between *Juglans nigra* L. and *J. regia* L. species. *Tree Genet. Genomes* **2013**, *9*, 291–305. [[CrossRef](#)]
28. Tal, O. *Acer pseudoplatanus* (Sapindaceae): Heterodichogamy and thrips pollination. *Plant Syst. Evol.* **2009**, *278*, 211–221. [[CrossRef](#)]
29. Kimura, M.; Seiwa, K.; Suyama, Y.; Ueno, N. Flowering system of heterodichogamous *Juglans ailanthifolia*. *Plant Species Biol.* **2003**, *18*, 75–84. [[CrossRef](#)]
30. McCarthy, B.C.; Quinn, J.A. Reproductive ecology of system of two sympatric tree species. *Am. J. Bot.* **1990**, *77*, 261–273. [[CrossRef](#)]
31. Knuth, P. *Handbook of Flower Pollination*; Clarendon Press: Oxford, UK, 1906.
32. Darwin, C. *The Different Forms of Flowers on Plants of the Same Species*; John Murray: London, UK, 1877.
33. Polito, V.S.; Pinney, K. The relationship between phenology of pistillate flower organogenesis and mode of heterodichogamy in *Juglans regia* L. (Juglandaceae). *Sex. Plant Reprod.* **1997**, *10*, 36–39. [[CrossRef](#)]
34. Zhang, R.; Peng, F.R.; Li, Y. Pecan production in China. *Sci. Hortic.* **2015**, *197*, 719–727. [[CrossRef](#)]
35. Solis Neffa, V.G.; Fernández, A. Chromosome studies in *Turnera* (Turneraceae). *Genet. Mol. Biol.* **2000**, *23*, 925–930. [[CrossRef](#)]

