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Hybridization among *Epimedium* (Berberidaceae) species native to China

Maoyin Sheng[a,b], Qingfu Chen[c], Lingjiao Wang[d], Xingjun Tian[a,*]

[a] School of Life Science, Nanjing University, 210093 Nanjing, Jiangsu, China
[b] Institute of South China Karst, Guizhou Normal University, 550001 Guiyang, Guizhou, China
[c] School of Life Science, Guizhou Normal University, 550001 Guiyang, Guizhou, China
[d] School of Vocation and Technology, Guizhou Normal University, 550001 Guiyang, Guizhou, China

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ABSTRACT

The hybridization of fourteen plant populations belonging to seven *Epimedium* species native to China was studied by self-cross, infra-population cross, inter-population cross within species, and interspecific cross. Self-pollination studies on nine populations indicated high incompatibility; capsule-set rates were higher than zero in only three (4.61–6.76%). Interspecific cross-pollinations demonstrated high cross-abilities in most cross combination (15.38–92.44% capsule-set rate), and the F_1 seeds possessed high germination rates (>20%). The F_1 hybrids of three interspecific cross combinations were raised to maturity. The morphology, karyomorphology of somatic cells, and pollen mother cell (PMC) meiosis of these F_1 plants revealed that they were all highly fertile (>76.10% in pollen viability), that there were few structural differences in chromosomes among species, and that most PMCs had 6 bivalents at MI. Abnormal chromosomal behaviors occurred in a minority, including chromosome bridges, unequal segregation of chromosome number, lagging chromosomes, and micronuclei. A series of experimental crosses provided strong evidence for an outbreeding system and a weak internal barrier to hybridizations in the taxa studied.

1. Introduction

Barrenwort is a perennial plant of the genus *Epimedium* L. (Berberidaceae) and a traditional Chinese medicine named Yinyanghuo, Xianlinpi, or Yangheye (Zhang et al., 2002; Wu et al., 2003; Xie and Sun, 2006). It is effective in strengthening kidneys and curing rheumatism, is widely used for the treatment of osteoporosis, hypertension, and coronary heart disease (Guo and Xiao, 1999), and is also used to strengthen immunity and prevent dementia (Xie and Sun, 2006; Zhang et al., 2002; Wu et al., 2003). Icariin flavonoids from *Epimedium* have been shown to inhibit the growth of cancer cells in vitro (Guo and Xiao, 1999). In Japan, Europe, and America, barrenworts are also popular as garden plants.

Epimedium is a genus of the old world. There are more than 60 species worldwide, distributed from the Mediterranean region, through western Asia, and into China and Japan (Stearn, 2002). The center of genus diversity is China (Ying, 2002), and 52 taxa of *Epimedium* were reported native to China (Guo et al., 2008). Linnaeus first recorded this genus and its type species, *E. alpinum*, in 1753 (Linnaeus, 1753). Subsequently, Morren and Decaisne (1834), Franchet (1886), Komarov (1908), and Stearn (1938, 2002) performed systematic studies of *Epimedium*. Stearn (2002) has established the most comprehensive classification system for this genus. However, the classification was established wholly based on corolla characteristics, such as petal type, the form and relative size of the inner sepals and petals, and flowers dimensions. Thus, the botanical classification of *Epimedium* was based solely on morphological characteristics. Morphological and cytological analysis of intraspecific and interspecific hybrids will further define this botanical classification.

To date, many studies have attempted to define this genus and deduce its evolutionary history using pollen morphology (Zhang and Wang, 1983; Liang and Yan, 1991), cytology (Sheng and Chen, 2007), isozymology (Koga et al., 1991), molecular biology (Nakai et al., 1996; Wang et al., 2001; Sun, 2004; Sun et al., 2005), biogeography (Ying, 2002; Zhang et al., 2007), and chemical classification (Guo and Xiao, 1999; Koga et al., 1991; Guo et al., 2008). The boundaries and evolutionary history of Chinese *Epimedium* species is still unclear because many *Epimedium* species with multifarious petals are nested together in China, and the petal evolution is quite controversial (Guo et al., 2008). As an intractable genus in taxonomy, there still remain several problems with the classification of *Epimedium*, and with the phylogenetic relationships between Chinese *Epimedium* species. It is, therefore, essential to establish a natural arrangement for *Epimedium*.

Suzuki studied the hybridization and pollination of seven populations of *Epimedium* species native to Japan (Suzuki, 1983, 1984). Hybridization potential is very important for defining taxonomy

* Corresponding author. Tel.: +86 25 83686787.
E-mail address: shmoy@163.com (X. Tian).

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and phylogeny, and for uncovering the impact of genetic drift, introgression, and selection pressures on interspecific relationships and speciation (Suzuki, 1983). There are no reports on hybridization of Chinese *Epimedium* species, however, even through China is the diversity center. To more precisely define the taxonomy and physiology of this genus, hybridization studies are warranted, especially for Chinese *Epimedium* species.

With the rapid development and commercialization of traditional Chinese medicine, greater numbers of wild *Epimedium* plants are being harvested. Thus, breeding and cultivation is an important challenge for the further development of *Epimedium* in the pharmaceutical industry. Barrenwort is also a popular garden plant in Japan, Europe and America, so hybridization of Chinese *Epimedium* species may impact the ornamental cultivation of these plants.

In the present study, we collected fourteen populations belonging to seven species of *Epimedium* native to China, and conducted a series of experimental crosses. The aims were to clarify the classification and crossability of Chinese *Epimedium* species, both to advance methods for cultivation and to assess the ornamental characteristics of these new Chinese *Epimedium* crosses.

2. Materials and methods

2.1. Plant materials

Seventy individual plants from fourteen populations were collected from their major distribution area of Guizhou Province, China and identified as belonging to seven *Epimedium* species (Table 1). They were cultivated in pots in a greenhouse at the Institute of Plant Genetics and Breeding, Guizhou Normal University, Guiyang, China.

2.2. Hybridization

The hybridization experiments were of the following four types: (1) self-cross; (2) intra-population cross; (3) inter-population cross within species; (4) interspecific cross.

For self-crossing, an inflorescence was enclosed within a cellophane bag just before flowering and, using pollen from the same individual, manual pollination was applied until stigmas of all the flowers were fully pollinated. After pollination, the inflorescence was closed within the bag once again. In crossing, flowers emasculated before stamens dehisce were used as the female parent. The procedure was repeated for several days. When sufficient numbers of artificial crosses had been made, all other buds in the inflorescences were removed. These experiments were performed using cellophane bags in the greenhouse to prevent pollination from other sources. Twenty days later, the capsule-set of all crosses was evaluated. The capsule-set and the mean number of plump seeds per capsule were used to assess compatibilities. The mean number of ovules per flower was determined by examining several flowers from each cultivated plant (of each species) to evaluate the seeds/capsule ratio.

Hybrids were stored in a deep freeze at −18 °C for one year. One year later, hybrid seeds were sprouted on damp sand beds at room temperature and then transplanted into pots in a growth chamber. In the first year, the plants showed only vegetative growth. In the second year, the plants bloomed and fructified. Morphological characteristics of the leaves, flowers, and inflorescences were recorded. The fertility of pollen grains was determined from the percentage of pollen grains stained in 1% I_2–KI solution.

2.3. Cytological observation of meiosis and mitosis

For meiotic investigation of pollen mother cells (PMCs), young flowers of about one week age were fixed in Carnoy II solution and stained in 2% (w/v) aceto-carmine. The improved procedure for observation of mitotic chromosomes in root tips was employed as described by Sheng and Chen (2007). Briefly, the root tips were pretreated in saturated α-bromine naphthalin solution at room temperature for 5 h, fixed in ethanol:acetic acid (3:1) solution overnight, hydrolyzed in 1 M hydrochloric acid at 60 °C for 10 min, washed in distilled water for 5 min, and finally stained with the improved carbolfuchsin solution. Squashed slides were made using Balata as the mounting medium.

2.4. Karyotype analyses

Karyotype analyses were conducted as described by Ma and Hu (1996). The absolute length of the chromosome (AL, μm) = the length of the magnified chromosome (mm) × 1000/magnification. The relative length of the chromosome (RL, %) = (the length of the chromosome/total length of all chromosomes) × 100. The arm ratio (AR) = the length of long arm of the chromosome/the length of the short arm of the chromosome. The length ratio (L/S) = the length of the longest chromosome/the length of the shortest chromosome. The position of the centromere and the karyotype class followed Levan et al. (1964, see Ma and Hu, 1996) and Stebbins (1971, see Ma and Hu, 1996), respectively.

3. Results

3.1. Self-incompatibility

The data obtained from selfing experiments in the seven species *E. acuminatum*, *E. letorrhizum*, *E. luodianense*, *E. wushanense*, *E. chlorandrum*, *E. simplicifluom*, and *E. myrianthum* are presented in Table 2. Most of the selfed flowers did not show an enlargement of ovary or capsule-set, except three species, *E. acuminatum*, *E. wushanense* and *E. chlorandrum*, with average capsule-set rates of 6.76%, 5.61% and 4.61%, respectively. The average number of plump seeds per capsule of *E. acuminatum*, *E. wushanense* and *E. chlorandrum* were 1.78, 1.80 and 1.33, respectively. The numbers of ovules per ovary were much lower than wild types of these taxa (Table 3). The indices of self-incompatibility of *E. acuminatum*, *E. wushanense* and *E. chlorandrum* are 0.05, 0.04 and 0.02, respectively, while the indices of the rest were all 0. Based on the index categories from Miriam et al. (2009), the three species of *E. acuminatum*, *E. wushanense* and *E. chlorandrum* are self-incompatible and the other four species are strongly self-incompatible.

3.2. Infraspecific crossability

Intra-population pollinations of *E. acuminatum*, *E. letorrhizum*, *E. luodianense*, *E. wushanense*, *E. chlorandrum*, *E. simplicifluom*, or *E. myrianthum* resulted in capsule-set rates of more than 70.51% (Table 3). Also, the seed-sets per capsule were high (2.64) and the germination rates of seeds obtained from these cross-pollinations ranged from 33.01% in *E. acuminatum* (AC3) to 63.02% in *E. letorrhizum* (LE2).

Table 3 also shows the results of inter-population crosses within species. The capsule-set rates obtained from these crosses were more than 63.48%, as high as those from intra-population pollination. Seed-sets and seed germination rates were also high at more than 3.01 and 33.69%, respectively. Thus, crosses among populations that were located in either the same or different districts resulted in similar compatibilities.

3.3. Interspecific crossability

The capsule-sets and seed-sets of 42 interspecific crosses of the seven species were evaluated (Table 4). Hybridization of all