

## RESEARCH PAPER

# Reduced recombination in gynodioecious populations of a facultative apomictic orchid

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Apomixis; character compatibility analysis; genotypic diversity; gynodioecy; Orchidaceae; pollinia receipt; *Satyrium ciliatum*.

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**ABSTRACT**

Many plants combine sexual reproduction with some form of asexual reproduction to different degrees, and lower genetic diversity is expected with asexuality. Moreover, the ratios of sexual morphs in species with gender dimorphism are expected to vary in proportion to the reproductive success of the sexual process. Hence, sex ratios can directly influence the genetic structure and diversity of a population. We investigated genotypic diversity in 23 populations of a facultative, apomictic gynodioecious orchid, *Satyrium ciliatum*, to examine the effect on genotypic diversity of variation in the frequency of females and in the amount of sexual reproduction. The study involved one pure female, seven gynodioecious (both females and hermaphrodites present) and 15 hermaphroditic populations. Pollinia receipt was higher in hermaphroditic than in gynodioecious populations. Analyses of variation in ISSRs demonstrated that genotypic diversity was high in all populations and was not significantly different between hermaphroditic and gynodioecious populations. We used character compatibility analysis to determine the extent to which recombination by sexual reproduction contributed to genotypic diversity. The results indicate that the contribution of recombination to genotypic diversity is higher in hermaphroditic than in gynodioecious populations, consistent with the finding that hermaphroditic populations received higher amounts of pollinia. Our finding of reduced recombination in gynodioecious populations suggests that maintenance of sex in hermaphrodites plays an important role in generating genotypic diversity in this apomictic orchid.

**INTRODUCTION**

Reproductive modes vary widely among flowering plant species, ranging from 100% outcrossing to clonality or pure apomixis. These varying modes are expected to influence genetic variation within and among populations (Richards 1986). Investigation of the patterns of genotypic diversity associated with variation in sexual reproduction would improve our understanding of the genetic consequences of facultative asexuality (Hamrick & Godt 1990; Eckert & Barrett 1993; Eckert *et al.* 2003). Asexual reproduction produces offspring that are genetically identical, both to maternal plants and to each other, so highly asexual populations are expected to show low genotypic diversity within populations (Silander 1985). However, based on isozyme data, reviews of genotypic diversity in clonal plants have revealed high levels of diversity in some predominantly asexually reproducing populations (Ellstrand & Roose 1987; Hamrick & Godt 1990). Furthermore, studies using more sensitive DNA markers have demonstrated that populations with the capacity to reproduce asexually have considerable genetic variation when compared to sexual populations (Diggle *et al.* 1998). One common explanation for this phenomenon is a small amount of

residual sexual reproduction, which produces variation *via* recombination (Vrijenhoek 1990).

In gynodioecious species, females are expected to be more pollen-limited than hermaphrodites (Lloyd 1974; Maurice & Fleming 1995). For example, in gynodioecious species such as *Stellaria longipes*, *Iris douglasiana* and *Geranium richardsonii*, females receive less pollen than hermaphrodites in natural populations (Philipp 1980; Uno 1982; Williams *et al.* 2000). Furthermore, for species such as *Silene vulgaris*, female sexual reproductive success correlates negatively with the frequency of females in the population (McCauley & Brock 1998). The frequency of females may greatly influence the total amount of sexual reproduction among populations. If the gynodioecious species reproduce by facultative asexuality, the sex ratio, resulting in variation in sexual reproduction, may affect genotypic diversity among populations.

Here, we examine an orchid with a rare combination of reproductive modes to address the question of how the presence and frequency of females affects genetic diversity within and among populations. The alpine orchid *Satyrium ciliatum* from southwestern Asia is the only orchid known to be gynodioecious, with populations that contain both females and hermaphrodites (Chen 1979). In addition, we recently discov-

ered populations containing only females, which nevertheless produce fruit, raising the possibility of asexual reproduction *via* apomixis, which has been confirmed by embryological observations (Huang *et al.* 2009). This orchid has gametophytic apomixis (Huang *et al.* 2009) rather than sporophytic apomixis (adventitious embryony), which is common in Orchidaceae (Whitton *et al.* 2008). We observed that hand-pollinated flowers set up to 42–58% more seed than unpollinated flowers that were emasculated and bagged. Furthermore, both hermaphrodites and females reproduced predominantly by apomixis (apomicitic seed set ranges from 20% to 40%), and females had a higher capacity for apomixis than hermaphrodites in both pollinated and unpollinated treatments (Huang *et al.* 2009). This combination of reproductive modes provides us with an opportunity to examine the relationship between genetic diversity and variation in both sex ratio and potential sexual reproduction.

The above system has several characteristics that are useful for addressing how the degree of sexual reproduction impacts genetic diversity. First, pollen is packaged into pollinia, which are membrane-bound sacs containing numerous massulae from an anther locule; pollinia and massulae are easy to see, and by merely observing a flower, one can tell whether or not a massula has been deposited on the stigmatic surface. Hence, we can estimate variation in the relative amount of pollination among populations. Second, successful pollination has been reported to be rare in other orchid species (Tremblay *et al.* 2005). Populations of *S. ciliatum* may vary greatly in the extent to which they reproduce sexually. This, in turn, may increase the chances of detecting such variation, if its genetic consequences are similarly large. Another important attribute is that the sex ratio of populations varies, ranging from those that contain only hermaphrodites to those that contain mostly females.

In order to determine whether genetic variation is related to the sex ratio and the degree of sexual reproduction within populations, we investigated (i) massulae receipt in multiple populations that varied in their sex ratio; (ii) genotypic diversity of populations by means of ISSR (inter-simple sequence repeat) markers; and (iii) whether the potential for sexual reproduction and the frequency of females affect genetic diversity within and among populations. Character compatibility analysis can estimate the relative contribution of recombination and mutation to genotypic variation (Mes 1998; van der Hulst *et al.* 2000). We used the analysis to estimate the relative contribution of sexual reproduction to genotypic diversity among the 23 populations investigated.

## MATERIALS AND METHODS

### Study population

*Satyrium ciliatum* Lindl. (Orchidaceae) is a perennial terrestrial orchid, predominantly distributed in southwestern China and neighbouring countries, including Sikkim, Bhutan and Nepal. Flowering plants have a corm, a single stem, two annual leaves (occasionally one leaf) and an inflorescence spike with 10–35 flowers, which open gradually from the bottom upward. It is self-compatible (Huang *et al.* 2009). Flowers are pink and have a twin-spurred labellum. Flowers on female plants have either aborted pollinia or no pollinia,

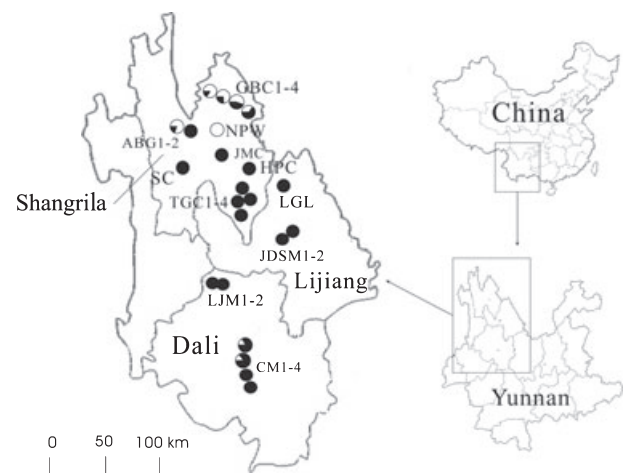
whereas hermaphrodites have fully-formed pollinia. This characteristic permits easy identification of sexual morphs in the field. *S. ciliatum* is diploid (unpublished data). We sampled 23 populations in three regions of Yunnan Province, China (Fig. 1), Dali, Lijiang and Shangri-La, which constitute most of the distribution range of the species in China, based on herbarium records (Chen 1979). These populations were classified as hermaphroditic (only hermaphrodites), gynodioecious (both hermaphrodites and females) or female (only females). The frequencies of hermaphrodites in each population and population size are given in Table S1.

### Potential amount of sexual reproduction in populations

We estimated the potential amount of sexual reproduction by counting the percentage of stigmas on which massulae had been deposited at a late stage of each flower, when the flower was close to wilting (late August and September 2007). We randomly sampled 20 hermaphrodites and 20 females in gynodioecious populations and 30 hermaphrodites in hermaphroditic populations. The percentage of flowers with massulae deposition was defined as the sum of the number of the two flower morphs with massulae deposition multiplied by the respective frequency and then divided by the total number of flowers.

### DNA extraction and ISSR amplification

To detect genotype frequencies, we randomly sampled at least 17 individuals in each population (see Table S1). DNA was extracted from silica gel-dried young leaves of sampled individuals following the method of Chapman *et al.* (2000). ISSR amplification was conducted in volumes of 25  $\mu$ l containing 0.25 mM each of dNTP, 2.5  $\mu$ l of 10  $\times$  Taq buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl), 1 mM primer, 1 U Taq polymerase (Tian Yuan Biotech, Wuchang District, Wuhan, China) and 60 ng of DNA template. Reactions were amplified in a PTC-100™ thermocycler (MJ Research, Inc., Waltham, MA, USA) using the protocol: 2 min at



**Fig. 1.** Locations of the 23 studied populations of *Satyrium ciliatum* in southwestern Yunnan Province, China. Open parts of circles represent the populations that include females; closed circles represent non-female (*i.e.* hermaphrodites only) populations.

94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C, and ending with 7 min at 72 °C. Amplification products were separated on 1.5% agarose gels, stained with ethidium bromide, run at 90 V in 0.5 × TBE (Tris–boric acid–EDTA) buffer, and then photographed under UV illumination. Seventy primers (SBS Genetech Co. Ltd., Haidian District, Beijing, China) were screened twice from 12 samples of *S. ciliatum* for unambiguous, clear and 100% reproducible band patterns. Nine primers (Table S2) were selected for amplification of each sample, and produced a total of 92 clear and repeatable bands. The bands were repeatable in two independent experiments.

### Measurements of genotypic diversity

ISSR bands were scored as present (1) or absent (0) for each sample. We calculated genotypic diversity for each population and also for all hermaphrodite and gynodioecious populations as a group. Four standard measures of genotypic diversity were calculated: (i) G, number of distinct genotypes observed (we regarded samples with the same banding pattern in all loci as the same genotype); (ii) genotypic richness (Dorken & Eckert 2001),  $R = (G-1)/(N-1)$ , where N = number of samples; (iii) genotypic diversity (Pielou 1969),  $D = 1 - \sum [N_i(N_i-1)/(N-1)]$ , where  $N_i$  is the number of samples observed with the *i*th genotype; (iv) genotypic evenness (Fager 1972),  $E = (D-D_{\min})/(D_{\max}-D_{\min})$ , where  $D_{\min} = (G-1)(2N-G)/N(N-1)$  and  $D_{\max} = N(G-1)/G(N-1)$ .

### Character compatibility analysis

We used character compatibility analysis to investigate the relative contribution of sexual reproduction to genotypic diversity among populations (Meacham 1981; Mes 1998). There are four pair-wise combinations of character states of unordered binary characters (11, 01, 10, 00). The probability of all four combinations being present through mutation alone in a clonal lineage is very small. If all four possible combinations are present, this is regarded as incompatibility and implies the existence of genetic recombination *via* sexual reproduction (Mes 1998; van der Hulst *et al.* 2000; Eckert *et al.* 2003). The number of combinations of incompatible characters of an individual with other individuals is the incompatibility count. The sum of all incompatibilities in an entire matrix (matrix incompatibility, MI) can be used as a measure of recombination; the higher the MI, the greater the contribution of recombination to genotypic variation. The relative contribution of recombination and mutation to genotypic variation was estimated by sequentially removing the genotype with the highest incompatibility until MI = 0. The higher the percentage of genotypes deleted to achieve MI = 0, the higher the contribution of recombination incurred by sexual reproduction to genotypic diversity. MI and the number of genotypes deleted to achieve MI = 0 for each population were calculated using the JACTAX routine in PICA version 4.0 (Wilkinson 2001) following the method of van der Hulst *et al.* (2000).

### Data analyses

One-way ANOVAs were used to compare the amount of sexual reproduction between seven gynodioecious and 15 hermaphroditic

populations. We performed a regression analysis to examine the relation between sex ratio and massulae receipt, and to examine the association between massulae receipt and the percentage of individuals that haplotypes have to remove to achieve MI = 0 cross populations. The nonparametric Kruskal–Wallis test was used to compare genotypic diversity and the percentage of deleted genotypes among female, gynodioecious and hermaphroditic populations. Mann–Whitney U tests were used for pair-wise comparison. All analyses were performed using SPSS (software SPSS, version 13.0, Chicago, IL, USA).

## RESULTS

### Massulae receipt

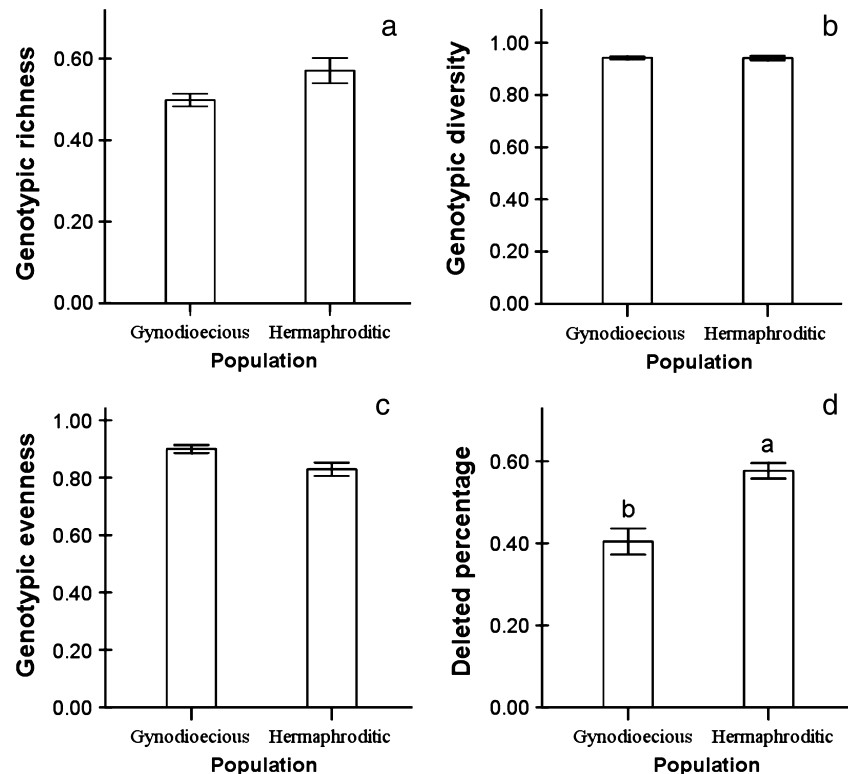
No massulae were observed on the stigmas of any individual in the female population, NPW. Overall, the average percentage of flowers that received massulae was  $1.0 \pm 0.22\%$  (mean  $\pm$  SE) in the other 22 studied populations. The percentage of flowers that received pollinia was higher in 15 hermaphroditic populations ( $1.4 \pm 0.3\%$ ) than in seven gynodioecious populations ( $0.3 \pm 0.1\%$ ;  $F_{1,20} = 6.31$ ,  $P = 0.021$ ). Flowers in one gynodioecious (GBC1) and four hermaphroditic populations (LJM1, LJM2, JMC and ABG2) did not receive any pollen massulae. The percentage of flowers that received massulae was from 0.1% to 3.1% in the other 17 populations, indicating that all populations experienced severe pollination shortage. Massulae receipt was associated with the sex ratio among populations ( $R^2 = 0.20$ ,  $F_{1,21} = 5.31$ ,  $P = 0.031$ ), indicating that the amount of sexual reproduction is probably higher in populations with more hermaphrodites.

### Genotypic diversity

We detected 312 distinct genotypes among 577 samples from the 23 populations. All populations had genotypes that were represented by only one sampled individual, as well as genotypes that were represented by more than one individual. No one genotype was predominant in any population and no genotypes were found to be shared among populations. All the measures of genotypic diversity, such as genotypic richness, genotypic diversity and genotypic evenness measures, were unexpectedly high in all populations (Table S1). Genotypic diversity ( $\chi^2 = 44.5$ ,  $df = 1$ ,  $P = 0.564$ ) and genotypic evenness ( $\chi^2 = 28.0$ ,  $df = 1$ ,  $P = 0.083$ ) were not significantly different between gynodioecious and hermaphroditic populations, but genotypic richness was marginally significantly higher in hermaphroditic populations than gynodioecious populations ( $\chi^2 = 25.0$ ,  $df = 1$ ,  $P = 0.052$ ; Fig. 2).

### Character compatibility analysis

The matrix incompatibility (MI) measure differed considerably among populations, and ranged from 17 in the hermaphroditic SC population to 279 in the hermaphroditic HPC population. The percentage of genotypes that had to be deleted to achieve MI = 0 varied from 29% in gynodioecious population GBC4 to 66.7% in hermaphroditic population TGC2 (Table S1). The percentage of deleted genotypes was



**Fig. 2.** Comparisons of genotypic richness (a), genotypic diversity (b), genotypic evenness (c), and percentage of genotypes deleted to achieve Matrix Incompatibility = 0 (d) between gynodioecious and hermaphroditic populations of *Satyrium ciliatum*. A significant difference was observed only in (d).

significantly higher in hermaphroditic than gynodioecious populations ( $\chi^2 = 6.5$ ,  $df = 1$ ,  $P = 0.001$ ; Fig. 2), indicating that recombination through sexual reproduction was higher in hermaphroditic than gynodioecious populations. The association between massulae receipt and the percentage of deleted genotypes was marginally significant ( $R^2 = 0.13$ ,  $F_{1,21} = 3.11$ ,  $P = 0.091$ ), suggesting that higher recombination may occur in populations with higher massulae receipt.

## DISCUSSION

We demonstrated that genotypic diversity was high in all *Satyrium ciliatum* populations, including the population containing only females. Genotypic diversity was not significantly different between 17 hermaphrodite and seven gynodioecious populations. The potential for sexual reproduction was low in all populations, with gynodioecious populations having a smaller amount of massulae receipt than hermaphrodites. However, character compatibility analysis indicated that the contribution of recombination to genotypic diversity *via* sexual reproduction was higher in hermaphroditic populations, which is consistent with the finding that massulae receipt was higher in hermaphroditic populations.

Compared to pollinia receipt measured in other *Satyrium* species in South Africa, which ranged from about 10% to 99% (Johnson 1997; Ellis & Johnson 1999), pollinia receipt by both female and hermaphroditic plants was extremely low in *S. ciliatum*. Our observation is consistent with the findings of previous studies that pollen limitation increased as the frequency of females increased, and limited the seed production of females (McCauley & Brock 1998; Ashman 1999; reviewed by Ashman 2006). During 3 years of field observation in four large populations (CM4, SC, ABG1 and LGL), we only occa-

sionally observed a bumblebee pollinating our studied species (Huang *et al.* 2009). A supplemental pollination experiment in two gynodioecious populations showed that the females had a lower amount of sexual reproduction but a higher capacity for apomixis than hermaphrodites (Huang *et al.* 2009). It must be noted that the quality of the pollen received by females may be higher, given that females were, by definition, outcrossed. Either selfing or outcrossing can generate new genotypes, but asexual reproduction cannot.

High genotypic diversity was observed in all populations. Such unexpectedly high genotypic diversity is not unusual in species with asexual reproduction. For example, in some apomictic species even conservative markers such as isozymes reveal high genotypic diversity, with a unique genotype for almost every individual within a population (Gornall 1999). In the facultative apomict *Hieracium pilosella* (Asteraceae), no genotypes were shared among three populations, as detected by ISSRs (Houliston & Chapman 2004). Such phenomena are in accord with model 2 of Bengtsson's (2003) hypothesis, which states that even a small number of sexual individuals in each generation are sufficient to make a predominantly asexual population show high levels of genotypic diversity. Moreover, a population founded by sexual offspring may thus retain its initial genotypic variation for a very long period, even if it later reproduces almost exclusively asexually. Thus, it is not surprising that population NPW, with only females, also had considerable genotype diversity (0.88). In some populations of an obligate apomictic dandelion, over 70% of the genotypes had to be removed to achieve a clonal structure (van der Hulst *et al.* 2000; Mes *et al.* 2002), suggesting that recombination substantially contributed to genetic variation in that species.

Common explanations for high levels of genotypic variation in facultatively apomictic populations include both



recombination through residual sexual reproduction and somatic mutation (Vrijenhoek 1990; Gornall 1999; Houliston & Chapman 2004; Paun *et al.* 2006). However, in facultatively apomictic species, previously it was difficult to determine which mechanism was the dominant factor, until compatibility analysis was developed as a reliable method to explore this question (Mes 1998). Although populations with a high capacity for sexual reproduction are generally expected to contain more genotypic diversity than those in which apomixis occurs more frequently, the amount of sexual reproduction has rarely been measured in genotypic analysis in facultative apomicts (but see Houliston & Chapman 2004). In the present study, the percentage of genotypes that had to be removed to achieve a clonal structure was less in gynodioecious than in hermaphroditic populations (Fig. 2). This indicates that recombination *via* sexual reproduction generated more genotypic variation in populations containing only hermaphrodites as compared to those containing females. This conclusion is in accord with the finding that the potential for sexual reproduction is higher in hermaphroditic than gynodioecious populations, indicating that residual sex indeed plays an important role in maintaining genotypic diversity of *S. ciliatum*.

Studies that combine compatibility analysis with the accurate measurement of relative amounts of sexual reproduction among populations could improve our understanding of plant reproductive success. The higher the amount of sexual reproduction, the greater is the contribution from recombination to genotypic diversity among populations with different sex ratios. This suggests that sexual reproduction plays an important role in maintaining genotypic diversity (Asker & Jerling 1992; Mogie 1992), and is a step forward from mere speculation about sex being a factor in these populations. Recent studies using highly sensitive molecular markers have also indicated that the accumulation of mutations might have an important effect on levels of genotypic variation identified in apomictic taxa (Mes *et al.* 2002; van der Hulst *et al.* 2003; Hörandl 2004; Houliston & Chapman 2004). In conclusion, our analysis of the relationship between the potential for sexual reproduction and genotypic diversity in a facultatively apomictic orchid suggests that sex is not the sole factor contributing to genotypic diversity, but plays an important role in generating genotypic diversity in apomictic species.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Attributes of populations of *Satyrium ciliatum*.

**Table S2.** Name and sequence of the nine primers used. Y = (C, T).

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