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RESEARCH ARTICLE

Genetic decline and inbreeding depression in an extremely rare tree

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Abstracts Many endangered species have small population sizes, with less than 10 remaining individuals in some extreme situations. Although the consequences of a small population size have received considerable research attention, few studies have examined the fate of extremely rare plants. *Ostrya rehderiana* is one such species, with only 5 naturally-regenerated surviving individuals and less than 150 artificially-regenerated progeny. Using amplified fragment length polymorphisms (AFLPs), we found that there was a low percentage of polymorphic loci but moderate heterozygosity in the 5 wild individuals. A severe decline in genetic diversity was observed in the progeny, with a decrease of 36.7% in heterozygosity and of 12% in the number of markers that were amplified per individual compared with the parental generation, a result which was caused by genetic drift and inbreeding. The effective population size was estimated to be 1. A significant positive relationship between parental genetic dissimilarity and the number of surviving offspring was observed, which indicated that inbreeding depression might have purged more inbred offspring. Implications for protection and recovery of the genetic variation of extremely rare plants, such as *O. rehderiana*, are proposed.

Keywords Genetic diversity · Inbreeding depression · Bottleneck · Parentage analysis · AFLPs · Endangered species · *Ostrya rehderiana*

Introduction

Due to natural or anthropogenic disturbances, many species may experience a serious demographic bottleneck (Lu et al. 2006). In extreme situations, only one or several individuals remain across the entire distribution range. For example, China has 12 plant species that have only between one and ten naturally-regenerated surviving individuals in the field (Gu 2003). These species are on the brink of extinction. However, little attention has been paid to these extremely rare plants, although the consequences of a small population size have received considerable research attention in the past decades (Ouborg et al. 2006).

In small populations, genetic drift and inbreeding can play essential roles in genetic variation and persistence. Both genetic drift and inbreeding will reduce within-population genetic diversity, a potential threat for the persistence of small populations (Willi et al. 2006). Inbreeding depression, which decreases the fitness of inbred offspring compared with outbred offspring, is particularly important for threatened species (Frankham 2005), because these species generally have small population sizes and thus inbreeding is unavoidable (Leimu et al. 2006; Reed 2005). However, the consequences of inbreeding on fitness have been scarcely detected in long-lived trees under field conditions, partly due to the difficulty in tracking the fate of offspring of biparental inbreeding. Highly polymorphic molecular markers, such as amplified fragment length polymorphisms (AFLPs) and microsatellites, have proven to be effective in the identification of the parents of an

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individual survivor in the field (Ahmed et al. 2009). When certain assumptions are made (e.g., an equal chance for parental individuals to mate) surviving offspring from each mating pair in the cohorts can serve as an indicator of offspring fitness and thus the relationship between parental relatedness and offspring fitness can be inferred in the field.

Ostrya rehderiana Chun ($2n = 16$) (Betulaceae) (Meng et al. 2004) is an extremely rare and endangered deciduous tree endemic to China, with only five remaining naturally-regenerated individuals. When it was first recorded in 1927, *O. rehderiana* was found fairly commonly in the open woods in Tianmushan of Zhejiang Province (Chun 1927). The rapid reduction in its population size was most probably due to deforestation and the planting of bamboos. *O. rehderiana* is wind-pollinated and, in general, bears abundant but very light seeds, which show inferior viability. Due to limited natural recruitment, seeds have been collected and germinated in common gardens and since 1980s, approximately 1,000 seedlings have been transplanted to the field or distributed to plant gardens (Guan and Tao 1988; Zhang et al. 1988). However, during our survey in 2008, we found that fewer than 150 individuals had survived in the field and in gardens, which indicated a high mortality rate for this tree.

In this study, we evaluated the genetic diversity of *O. rehderiana* and identified the parentage of the surviving offspring with the following aims: (1) to compare the genetic difference between naturally-regenerated individuals and their offspring; (2) to test whether the genetic relatedness between mates influenced progeny survival in the field; and (3) to provide some recommendations for the conservation of extremely rare plants such as *O. rehderiana*.

Materials and methods

Sample collection

The remaining five naturally-regenerated individuals of *O. rehderiana* and most of their offspring are located in Tianmushan, in the northwestern Zhejiang Province, China. Of the five wild remaining individuals, the largest survivor was about 0.9 m in diameter at breast height (DBH) and was estimated to be more than 200 years old. Another four parental individuals were about 0.6 m at DBH and were estimated to be more than 100 years old. These trees are believed to be the remnants of the previous large population. In Tianmushan, most of the offspring were more than 10 m in height and about 0.15 m at DBH, and were at the pre-mature stage. These trees had been germinated in the common gardens from seeds that had been collected from the five mature individuals, and transplanted to the field after 1 or 2 years of growth. The five naturally regenerated individuals and their 128 progeny individuals were sampled. Healthy leaves were taken from each individual and dried with 50 g silica gels in a sealable bag.

DNA extraction and genotyping

Total DNA was extracted from the dried leaves with the modified CTAB method (Fan et al. 2004). First, we tried to develop microsatellites to analyze the genetic structure of *O. rehderiana*. However, we failed to obtain polymorphic simple sequence repeat (SSR) markers and, therefore, we used AFLP markers. The AFLPs using of six primer pairs (E-AAG/M-CAC, E-ACT/M-CAG, E-ACC/M-CAG, E-AAG/M-CTA, E-ACA/M-CAA and E-ACT/M-CAT) were detected using fluorescently labeled primers. DNA profiles were resolved on an ABI Prism 377 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). Using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA), we scored the AFLP profiles for the presence or absence of fragments of between 70 and 500 bp in length.

Data analysis

Genetic variation was determined in parental and progeny generations by using the total number of markers scored, as well as the number and proportion of polymorphic markers. The expected heterozygosity was calculated using the equation $H_E = 2pq$ and averaged over all loci, where q was measured by the Bayesian estimate (Eq. 15, Zhivotovsky 1999). Previous studies have found that species of Betulaceae are generally outcrossing (Xie et al. 2002) and F_{IS} is close to 0. However, the current population size of *O. rehderiana* is very small, and some biparental inbreeding might have occurred. Because similar values were obtained by setting the F_{IS} value of 0 to 0.5, the fixation index was set as 0.3 in the estimate of allelic frequencies. Difference in heterozygosity between parents and their offspring was tested by t test, with assumed inequality in variance (R Development Core Team 2008). To compare difference in the number of bands between parents and their progeny, we tested the differences between the 5 parental individuals and five offspring that had been re-sampled randomly from the offspring pool, and 1,000 re-samplings were conducted using R (R Development Core Team 2008).

We estimated the effective population size (N_e) using the method described by Krauss et al.'s (2002): $N_e = \frac{H_0}{2(H_0 - H_1)}$, where H_0 and H_1 are Bayesian estimates of mean heterozygosity in the parental and progeny generations, respectively.

Although AFLPs are less efficient in parentage studies than co-dominant microsatellites, the loss of information could be counterbalanced by a high number of polymorphic loci (Gerber et al. 2000; Wang and Santure 2009). In the present study, parentage of each progeny was assigned manually using genetic exclusion analysis, and exclusion probabilities were calculated using the FaMoz software (Gerber et al. 2003). When non-sole parentage was obtained, a full-pedigree likelihood approach (Wang and Santure 2009) was used to assign the progeny to the potential parent using the COLONY v.2.0.1.1 software (Jones and Wang 2010). We defined the polygamous mating systems with an inbreeding model. Allelic dropout rate was 0 and genotyping errors of the markers were 0.01%. The allele frequencies were estimated from the current samples by the program.

We constructed a similarity matrix for all pairs of samples using the Jaccard coefficient of similarity (Keil and Griffin 1994). This value was converted into a measurement of dissimilarity (D), where $D = 1 - S$. Average similarity across all pairs of individuals was calculated for parents, progeny, half sibs, and full sibs (following parentage assignment). To infer the potential impacts of inbreeding depression, we checked the relationship between parental dissimilarity and their offspring and tested their significance using analysis of variance (ANOVA) (R Development Core Team 2008).

Results

Six AFLP primer pairs generated a total of 204 bands in the parental generation. A decrease of 12% in the number of markers amplified per individual was found in the offspring when compared with the parental generation (Table 1). Only one band was absent in the progeny generation. Percentages of polymorphic bands in the parental and progeny individuals were 29.9 and 40.4%, respectively (Table 1). However, the average number of bands per individual of the parental generation was 181.0, significantly higher than that of the progeny (159.3) ($P < 0.001$). The Bayesian estimate of heterozygosity of the parental generation was 0.406, which was also significantly higher than that of the progeny generation (0.257) (t test, $P < 0.001$).

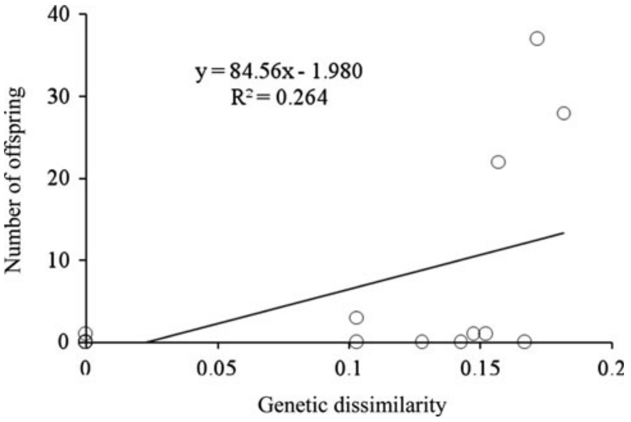

Fig. 1 The relationship between genetic dissimilarity of a pair of parents and number of their surviving offspring in *O. rehderiana*

The sole parent pair was identified for 40 of 128 offspring using the genetic exclusion method. Exclusion probabilities were 0.94273 for single parents and 0.99237 for parent pairs. Combined with the likelihood approach, a total of 93 offspring were assigned to the potential parent pair. All the progeny, except for one individual, were outcrossed. Among the 15 possible parent combinations, parentage was dominated by two combinations: P1 × P3 and P2 × P5, with 37 and 28 progeny, respectively. A significant positive relationship was found between genetic dissimilarity and the number of progeny of mates (Fig. 1) ($P < 0.05$).

Mean genetic dissimilarity (D) of the progeny (0.136) was smaller, but not significantly, than that of the parents (0.145) (Table 1). However, genetic dissimilarity of full sibs (0.134) was significantly smaller than that of the half sibs (0.150) ($P < 0.001$). According to the heterozygosity values estimated for the parental (0.406) and progeny generations (0.257), effective population size was estimated to be 1.

Discussion

As expected, low levels of genetic diversities were found in the five extant, naturally-regenerated individuals of the extremely rare tree *O. rehderiana*. The percentage of

Table 1 Genetic statistics generated by six amplified fragment-length polymorphism primer pairs for parental and progeny generations in *O. rehderiana*

	N	*Nb*	*Npb*	*Nb-ind* (SEM)	PPB (%)	H_E (SEM)	*D* (SEM)
Parents	5	204	61	181.0 (2.80)	29.90	0.406 (0.003)	0.145 (0.0095)
Progeny	128	203	82	159.3 (0.57)	40.39	0.257 (0.007)	0.136 (0.0003)

N number of individuals, *Nb* Number of bands, *Npb* number of polymorphic bands, *Nb-ind* mean number of bands per individual, *PPB* Percentage of polymorphic bands, H_E expected heterozygosity estimated by a Bayesian method (see text), *D* average genetic dissimilarity between all pairs of individuals, and *SEM* standard error of the mean polymorphic loci (29.9%) was lower than that of other extremely rare plants, such as *Metrosideros bartlettii* (44%) (Drummond et al. 2000) and *Grevilla scapigera* (75.5%) (Krauss et al. 2002), but higher than that of the extremely rare *Metrosideros boninensis* (12.9%) (Kaneko et al. 2008). However, expected heterozygosity ($H_E = 0.406$) was higher than that of the above three species. Relatively high heterozygosity hinted that the population has not experienced a long-term bottleneck, as heterozygosity reduced more slowly than number of allele (Luikart and Cornuet 1998). This conclusion was also supported by previously recorded data. When this species was first reported, it was found fairly commonly in open woods (Chun 1927), and the extant parental individuals are the remnants of the previously large population, as determined by their age. Therefore, low genetic variation was the direct result of a loss of individuals, and genetic drift did not play a major role in the genetic composition of the parental individuals.

Despite the low level of genetic variations in the parental individuals, we found a substantial decline in genetic variation in the offspring. Heterozygosity of the progeny had decreased 36.7% compared with their parents' levels (Table 1). Krauss et al. (2002) found a similar rapid decline in a translocated population of the endangered plant *Grevillea scapigera*. Such a decline in genetic variation was due to genetic drift and biparental inbreeding, the most influential factors for extremely small populations (Nei et al. 1975), e.g., those in the present study ($N_e = 1$). Inbreeding was also supported by the significant decrease in number of bands per individual in the offspring generation, which was most likely to be caused by homogeneity of recessive alleles at heterozygous loci in parents. Furthermore, the progeny of *O. rehderiana* were, on average, 6.2% more inbred than their parents (Table 1), a common consequence of biparental inbreeding (Chen et al. 2003).

Previous studies have indicated that species of Betulaceae are predominately outcrossed (Xie et al. 2002), and inbreeding depression has been observed in self-bred plants compared with outcrossed families (Wang et al. 1999). In this study, only one surviving offspring was selfed as revealed by parentage analysis, which indicated the presence of outcrossing in *O. rehderiana*. A significant positive relationship between the parental genetic dissimilarity and the number of surviving offspring indicated that inbreeding depression had probably played an influential role in offspring survival. Inbred offspring would not have survived during either stage of the life cycle, such as at seed production, germination, seedling growth stages, and so on, whereas outcrossed progeny having relatively higher fitness have a higher probability of survival to the present time. Such a positive relationship between the genetic relatedness of parents and offspring fitness has also been observed in other plants (Hirao 2010; Seltmann et al. 2009; Wagenius et al. 2010). Our results were inferred from dominant markers, which have lower powers than microsatellites in sibship and parentage assignments (Gerber et al. 2000). However, the loss of information could be counterbalanced by a high number of polymorphic loci (Gerber et al. 2000), such as found in the present study.

Implications for conservation

It is obvious that all five naturally-regenerated individuals of *O. rehderiana* have a high conservation priority, and that their surviving offspring also need protection. The major causes of mortality of *O. rehderiana* in the field are human disturbance and competition by other trees or bamboos. Hence, fencing these mature individuals to avoid direct disturbance is needed. The cutting of bamboos and trees that compete with *O. rehderiana* for light may also be considered in habitats that contain both mature individuals and their offspring, and this consideration is especially important for the growth of seedlings and samplings because *O. rehderiana* is a shade-intolerant tree.

To reduce the risk of extinction of *O. rehderiana*, it is necessary to increase the effective population size to protect and accumulate genetic variation for long-term adaptive potential. A direct approach would be to reproduce each parental individual via asexual reproduction, at least for several copies, because the death of any of the five parental individuals will lead to a potentially permanent loss of genetic variation. However, massive asexual reproduction should be avoided because this option may increase inbreeding and have deleterious impacts on the ability of this plant species to adapt to the changing environments (Li et al. 2005). In addition to protection of the native habitat and to allow seeds to fall and germinate in the field, large scale reseeding in neighboring nature reserves could help to maintain genetic diversity (Rottenberg and Parker 2003). That the offspring contained nearly all bands in wild trees and had a higher percentage of polymorphic loci than that of the wild individuals indicated that establishment of translocated populations may be a possible approach. However, genetic variation at the individual level was lower in the offspring than that of the parents. Therefore, the translocated populations should contain many more individuals to attain a similar level of genetic diversity than the surviving wild individuals.

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