Evol Ecol (2009) 23:447–462
DOI 10.1007/s10682-008-9244-6

ORIGINAL PAPER

Empirical assessment of the reproductive fitness components of the hybrid pine *Pinus densata* on the Tibetan Plateau

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Received: 13 June 2007 / Accepted: 3 January 2008 / Published online: 19 January 2008

Abstract *Pinus densata* is distributed on the Tibetan Plateau, where it forms extensive forests at high elevations. Genetic studies have provided evidence that *P. densata* originated through hybridization between *P. yunnanensis* and *P. tabuliformis*. To clarify the relationships among these pines, and assess their reproductive fitness in their respective habitats, we conducted a comparative analysis of eight cone and seed morphometric traits and six reproductive traits in them. Among the eight morphometric traits examined, six appeared to be intermediate in *P. densata* between those of *P. yunnanensis* and *P. tabuliformis*. There were significant differences among the three pines in all of the morphometric traits, and *P. densata* showed greater variability in these traits than the other two pines. In contrast to the morphometric traits, the reproductive traits (including the proportions of filled and empty seeds, ovule abortion rate, seed efficiency, meiotic abnormalities during microsporogenesis and pollen viability) differed little among the three pines, indicating that they have similar overall rates of effective pollination and fertilization in their respective natural environments. Despite their location on the high plateau, natural populations of *P. densata* appeared to have normal levels of reproductive success, comparable to those of the two parental species in their natural habitats. This study provides empirical data characterizing the reproductive success and adaptation of a

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Electronic supplementary material The online version of this article (doi:10.1007/s10682-008-9244-6) contains supplementary material, which is available to authorized users.

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stabilized homoploid hybrid in a novel habitat that is ecologically and spatially inaccessible to its parental species.

Keywords Cone and seed traits · Fertilization · Hybrid speciation · Microsporogenesis · Ovule abortion · Reproductive success

Introduction

Natural hybridization in plants serves as a source of novel adaptive genetic variation and can result in speciation (Anderson and Stebbins 1954; Grant 1981; Arnold 1997; Rieseberg 1997). Furthermore, the elimination of some hybrid genotypes and promotion of others via selection (endogenous and/or exogenous) can lead to the colonization of parental or novel habitats by fit hybrid lineages (Arnold 1997; Burke and Arnold 2001). Thus, to assess the role of hybridization in evolution, the reproductive fitness of hybrid lineages should be measured and characterized (Arnold and Hodges 1995; Burke and Arnold 2001; Miglia et al. 2005). To do this, viability and fertility traits (which are essential components of fitness) should be examined, such as fruit and seed set, ovule abortion, seed germination, growth potential and flowering ratios. In addition, observations of microsporogenesis, meiotic abnormalities and pollen viability can provide useful information regarding chromosomal rearrangements, genomic heterozygosity, fertility, genomic compatibility and the evolutionary history of putative parental and hybrid species (Chandler et al. 1986; Aparicio and Albaladejo 2003).

The evolutionary consequences of hybridization are best observed in stabilized later-generation hybrids (Grant 1981). To date, most experimental studies concerned with hybrid fitness have been restricted to early generation hybrids or backcrosses of annual and perennial herbs; fewer investigations have focused on the fitness of stabilized natural hybrid lineages. Furthermore, although the fitness of plant hybrids appears to be habitat dependent (Emms and Arnold 1997; Arnold et al. 2001; Gross and Rieseberg 2005), there have been very few measurements of hybrid fitness under natural conditions. Estimating fitness in natural populations is generally challenging. Thus, very few studies have integrated analyses of survival and reproduction in attempts to estimate the fitness of hybrids in natural populations (Arnold and Hodges 1995); probably because studies concerned with reproduction require long-term observations, especially for perennial woody plants. The scarcity of studies regarding stabilized hybrid fitness hinders our understanding of the processes and mechanisms of hybrid speciation and the maintenance of hybrid lineages.

Pinus densata forms extensive forests that regenerate well in the southeastern region of the Tibetan Plateau at high elevations, ranging from 2700 to 4200 m asl (Guan 1981; Wu 1956). Genetic investigations using allozyme, chloroplast (cp) DNA and mitochondrial (mt) DNA markers have provided evidence suggesting that *P. densata* originates from hybridization between *P. tabuliformis* and *P. yunnanensis* without any alteration in the ploidy level. Individual populations of *P. densata* have diverse genetic composition, with varying degrees of genomic contribution from each parental species (Wang and Szmidt 1994; Wang et al. 2001; Liu et al. 2003; Song et al. 2003). *Pinus tabuliformis* is widely distributed from northern to central China, while *P. yunnanensis* has a relatively limited range in southwestern China (Wu 1956). The geographic distribution of the three pines forms a succession, with *P. tabuliformis*, *P. densata* and *P. yunnanensis* in the north, middle and south of China, respectively. The high elevation habitats occupied by *P. densata* are inaccessible to any of the other pine species growing in the region (Wu 1956; Guan 1981). *Pinus tabuliformis* differs distinctively from *P. yunnanensis* in many morphological traits, and the distributions of the two species are non-overlapping. *Pinus densata* is morphologically intermediate between the other two pine species, it slightly overlaps the peripheral distributions of the other two pines (Wu 1956). The classification of pine species in the field is based on both morphological traits and geographical location.

The fact that *P. densata* grows and regenerates in habitats that are inaccessible to either of its parental species demonstrates its unique adaptation to the plateau environment. However, its reproductive fitness components had not previously been characterized. Therefore, to clarify the reproductive status of *P. densata*, the relationships among these pines, and their reproductive success in their respective habitats, we conducted a comparative analysis of cone and seed morphometric traits, mating traits, microsporogenesis and pollen viability in *P. densata*, *P. yunnanensis* and *P. tabuliformis*. Cone and seed traits in *Pinus*, including cone size, seed size and seed mass, have moderate-to-high heritability (Singh and Chaudhary 1993; Matziris 1998). Reproductive traits derived from cone and seed analysis, such as ovule abortion rate, the ratio of empty and filled seeds per cone and seed efficiency, are important indicators of the mating system and reproductive success in conifers (Sarvas 1962; Mosseler et al. 2000; Rajora et al. 2000, 2002). Outcrossing and selfing estimates, based on the ratio of filled and empty seeds per cone, accurately portray the extent of inbreeding depression on fecundity (Rajora et al. 2000). Poor pollination causes high ovule abortion rates in pines (Owens et al. 1981, 1982; Sarvas 1962). These mating system parameters provide valuable information on the reproductive and genetic status of natural conifer populations. The aim of the present study was to obtain empirical data on cone and seed morphometric traits and mating efficiency in natural populations of *P. densata*, relative to its parental species, in order to evaluate its reproductive fitness in the plateau environment. The characterization of the reproductive status in this pine complex provides information on the adaptation and stabilization of *P. densata* on the Tibetan Plateau.

Materials and methods

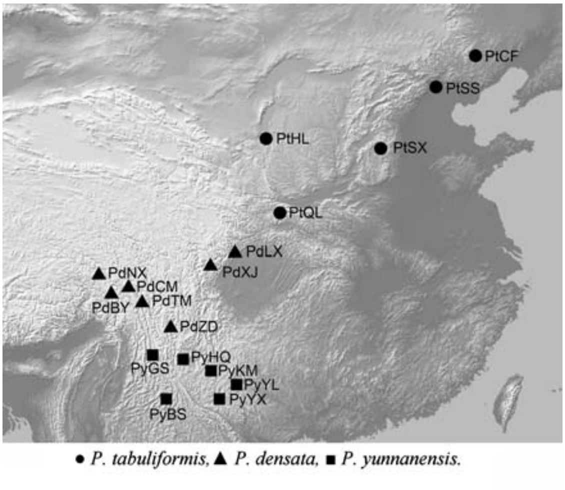
Population sampling of cones

Five, six and seven populations of *P. tabuliformis*, *P. yunnanensis* and *P. densata* were selected for cone collections, respectively, covering the central and marginal distributions of each of the three pine species (Fig. 1 and Supplemental Table 1). From each sampled population (which consisted of mature pure pine stand) 3–5 fully developed, healthy, mature cones were collected from the middle part of the crown of each of 10–20 randomly selected trees older than 35 years, spaced at least 100 m apart. Cones from the same population were mixed and individual cones were stored in separate cloth bags at room temperature, to allow them to dry and open. Cones that were difficult to open by ambient drying were soaked in warm water, dried in an oven at 40°C for 24 h and then the seeds were manually extracted from the opened cones. The cones were collected during their natural ripening seasons during 2003 and 2004.

Cone and seed analyses

We measured eight morphometric (cone length, no. of scales/cone, no. of fertile scales/cone, ratio of fertile scales, no. of full-sized seeds/cone, seed length, seed wing length and

Fig. 1 Geographic distribution of sampled populations



weight of 100 seeds) and four reproductive traits (ratio of filled seeds/cone, ratio of empty seeds/cone, ovule abortion rate and seed efficiency) (Table 1). In this study morphometric traits refer to those determined solely by an individual tree's genotype and/or genotype-environmental interactions, while reproductive traits are also influenced by mating parameters. According to Bramlett et al. (1977), the scales in a pine cone can be divided into fertile and infertile scales; fertile scales are located in the middle-to-upper part of the cone, and each scale contains two ovules at its base. The number and ratio of fertile scales in a cone determines its maximum potential seed production, which is twice the number of fertile scales. During cone development, some ovules will be aborted and some will develop into full-sized seeds. The proportion of full-sized seeds in a cone, which consists of both filled and empty seeds, directly reflects pollination success. In conifers, empty seeds are mostly caused by selfing (Sarvas 1962; Bramlett and Popham 1971) and the aborted ovules are results of unsuccessful pollination and other environmental factors (Sarvas 1962; Owens et al. 1981, 1982).

The seeds extracted from each cone were floated in 100% ethanol to separate the filled seeds from the empty seeds, and the number of filled seeds was determined and weighed. Seed and seed wing length were measured by randomly sampling 10 seeds from each cone; three replicates were taken and averaged. The ratios of filled and empty seeds to the total number of full-sized seeds were calculated for each cone. Seed efficiency was defined as the number of filled seeds relative to seed potential of the cone (i.e., 2 × no. of fertile cone scales). The number of aborted ovules was determined by subtracting the number of full-sized seeds from the seed potential, and the ovule abortion rate was calculated in relation to the seed potential.

Microsporogenesis and pollen viability

We investigated meiotic abnormalities during microsporogenesis and pollen viability in each of the three pine species as follows. One representative population from the center of the distribution range of each species was selected: population PdNX for *P. densata*, population PyKM for *P. yunnanensis* and population PtSS for *P. tabuliformis*. Young male

Table 1 Morphometric and reproductive traits of the three pine species

Trait	Species	Mean	Standard deviation	Coefficient of variation (%)
Cone length (cm)	*P. yunnanensis*	5.81^a	0.97	16.75
	P. densata	5.50^b	0.99	17.96
	P. tabuliformis	4.12^c	0.56	13.68
No. of scales/cone	*P. yunnanensis*	131.13^a	16.09	12.27
	P. densata	111.10^b	19.21	17.29
	P. tabuliformis	80.26^c	11.85	14.76
No. of fertile scales/cone	*P. yunnanensis*	48.83^a	11.15	22.84
	P. densata	33.67^b	11.17	33.17
	P. tabuliformis	19.46^c	4.71	24.22
Ratio of fertile scales (%)	*P. yunnanensis*	37.39^a	7.84	20.97
	P. densata	30.39^b	9.87	32.47
	P. tabuliformis	24.40^c	5.42	22.22
No. of full-sized seeds/cone	*P. yunnanensis*	71.99^a	17.57	24.41
	P. densata	51.00^b	20.57	40.33
	P. tabuliformis	32.52^c	9.73	29.91
Seed length (cm)	*P. yunnanensis*	0.52^a	0.07	13.82
	P. densata	0.59^b	0.10	16.92
	P. tabuliformis	0.69^c	0.08	11.06
Seed wing length (cm)	*P. yunnanensis*	1.32^a	0.21	16.23
	P. densata	1.46^b	0.24	16.27
	P. tabuliformis	1.05^c	0.14	13.02
Weight of 100 seeds (g)	*P. yunnanensis*	1.28^a	0.33	25.59
	P. densata	1.23^a	0.37	30.51
	P. tabuliformis	3.12^b	0.69	22.27
Ratio of filled seeds (%)	*P. yunnanensis*	71.00	19.03	26.80
	P. densata	67.65	20.45	30.23
	P. tabuliformis	67.71	26.42	39.02
Ratio of empty seeds (%)	*P. yunnanensis*	29.01	19.05	65.64
	P. densata	32.38	20.47	63.22
	P. tabuliformis	32.30	26.42	81.81
Ovule abortion rate (%)	*P. yunnanensis*	24.96^a	15.48	62.02
	P. densata	24.66^a	14.02	56.87
	P. tabuliformis	17.68^b	10.81	61.14
Seed efficiency (%)	*P. yunnanensis*	53.00^a	18.89	35.63
	P. densata	50.50^b	18.56	36.75
	P. tabuliformis	53.85^a	23.67	43.96

Means with different superscript letters (a, b, c) are significantly different (at $P = 0.05$) as determined by LSD multiple range tests

strobili and mature pollen were collected from 3 to 5 mature trees from each of these populations between March and May 2005. Young male strobili were collected daily in the field during meiosis and were longitudinally sliced in half and immediately fixed in Carnoy's fixative (ethanol:acetic acid = 3:1, v/v) for 24 h. They were then transferred to

90% ethanol for 12 h, and subsequently stored in 70% ethanol at −20°C until examination. Chromosome spreads were prepared by conventional squashing. Pollen mother cells were stained with aceto-carmine, observed under a light microscope, in phase contrast, and chromosomal abnormalities in any phase of meiosis at the time of fixation were recorded to assess the frequency of irregularities in microsporogenesis in each of the species at the cytological level.

Pollen was extracted from mature male strobili and subjected to germination tests using the ‘hanging drop’ method (Snyder and Clausen 1974), in which pollen grains were spread on a semisolid medium of 1% bacteriological agar, 15% w/v sucrose and 0.001% w/v boric acid then incubated in darkness at 28°C for five days. A pollen grain was scored as having germinated if it had produced a pollen tube that was longer than the width of the grain. At least 500 pollen grains of each tree were examined to calculate their pollen viability parameters.

Statistical analyses

Means, standard deviations and the coefficients of variation for each trait in each species were calculated. Significant mean differences between species were determined with a Least Significant Difference (LSD) multiple range test. The distribution of each trait within each population was characterized using the medians, quartiles and ranges of the observations. To assess the partitioning of the variation among populations and species, data on each trait were analyzed using a nested Analysis of Variance (ANOVA) model with population and species as independent variables. Data expressed as percentages were arcsin-transformed (Sokal and Rohlf 1981) prior to ANOVA and LSD analyses.

To evaluate the underlying dimensionality of the data and obtain an overview of the dominant patterns, Principle Component Analysis (PCA) was performed on a standardized matrix containing data on all eight morphometric traits. Unweighted pair group method using arithmetic averages (UPGMA) clustering analysis was performed for the 18 sampled populations using data on the eight morphometric characters. The Euclidean distances between populations were calculated from a z-score (numbers of standard deviations from the mean) transformed data matrix (Mendenhall 1967). The UPGMA clustering analysis, ANOVA and PCA were performed using the software packages NTSYS (Rohlf 1997), SAS version 8.0e and SPSS version 10.0, respectively.

Results

Cone and seed morphometric traits

The means, standard deviations and coefficients of variation for each trait in the three pine species are summarized in Table 1. *Pinus densata* appeared to be intermediate between *P. yunnanensis* and *P. tabuliformis* with respect to six of the eight morphometric traits (Table 1). The seed wing length was significantly longer in *P. densata* than in both of the other species. The 100-seed weight in *P. densata* did not differ considerably from that in *P. yunnanensis*, but was significantly lower than that in *P. tabuliformis*. With the exception of the seed weight, the other seven morphometric traits differed significantly between the three pine species (Table 2), and *P. densata* had higher coefficients of variation for all eight morphometric traits than the other two pines (Table 1).

Table 2 Results of ANOVA for the analyzed traits in the three pine species

Trait	Mean square (df)			% of variance		
	Between species	Populations within species	Residual	Between species	Populations within species	Residual
Cone Length (cm)	195.63 (2)	13.93 (15)	0.52 (810)	47.28***	19.05***	33.67
No. of scales/cone	171018.31 (2)	2729.37 (15)	0.52 (810)	70.07***	6.04***	23.85
No. of fertile scales/cone	55553.18 (2)	869.03 (15)	77.53 (810)	68.58***	5.74***	25.69
Ratio of fertile scales (%)	1.30 (2)	0.02 (15)	0.01 (809)	45.01***	3.18***	51.81
No. of full-sized seeds/cone	103036.76 (2)	2511.85 (15)	243.05 (810)	56.72***	7.34***	35.94
Seed length (cm)	1.99 (2)	0.12 (15)	0.01 (810)	49.83***	16.22***	33.95
Seed wing length (cm)	10.11 (2)	0.52 (15)	0.03 (810)	46.56***	13.10***	40.34
Weight of 100 seeds (g)	131.21 (2)	2.18 (15)	0.15 (505)	80.82***	6.31***	12.88
Ratio of filled seeds (%)	0.36 (2)	0.26 (15)	0.05 (530)	3.68 n.s.	12.07***	84.25
Ratio of empty seeds (%)	0.36 (2)	0.26 (15)	0.05 (530)	3.65 n.s.	12.10***	84.25
Ovule abortion rate (%)	0.57 (2)	0.24 (15)	0.03 (809)	6.29***	14.36***	79.35
Seed efficiency (%)	0.62 (2)	0.33 (15)	0.03 (529)	9.12*	22.31***	68.57

n.s.—not significant; * $P < 0.05$, *** $P < 0.001$

The medians, quartiles and ranges of the morphometric traits in each of the sampled populations are summarized in the box-plot shown in Fig. 2. The intermediacy of *P. densata* for most of the traits was evident from the plots. In addition, the ranges of most of the traits were larger within and among populations of *P. densata* than in the parental species; in fact, they almost spanned the complete numerical ranges of the traits in the two parental species.

ANOVA revealed that there were significant differences in all eight morphometric traits both among species and among populations (Table 2). Estimates of the distribution of variance were strongly trait-dependent. Most of the variation (69–81%) in numbers of scales and fertile scales per cone, and 100-seed weights was distributed among species, and only ca. 6% among populations within species, while 45–57% of the variation in the other five traits partitioned among species, and 3–19% among populations within species.

The Principal Component Analysis of the data obtained from the three pine species identified three components with eigen values greater than 1.0, which described almost 80% of the variation observed in the eight morphometric traits (Supplemental Table 2). The cone length, no. of fertile scales/cone, ratio of fertile scales and no. of full-sized seeds/cone, all of which are related to cone size, had high loadings on the first PCA axis. Seed traits including seed length, seed wing length, and the 100-seed weight had high loadings on the second PCA axis. The first two components explained 63% of the variation. The no. of scales/cone contributed most to the third component, which described 14.6% of the

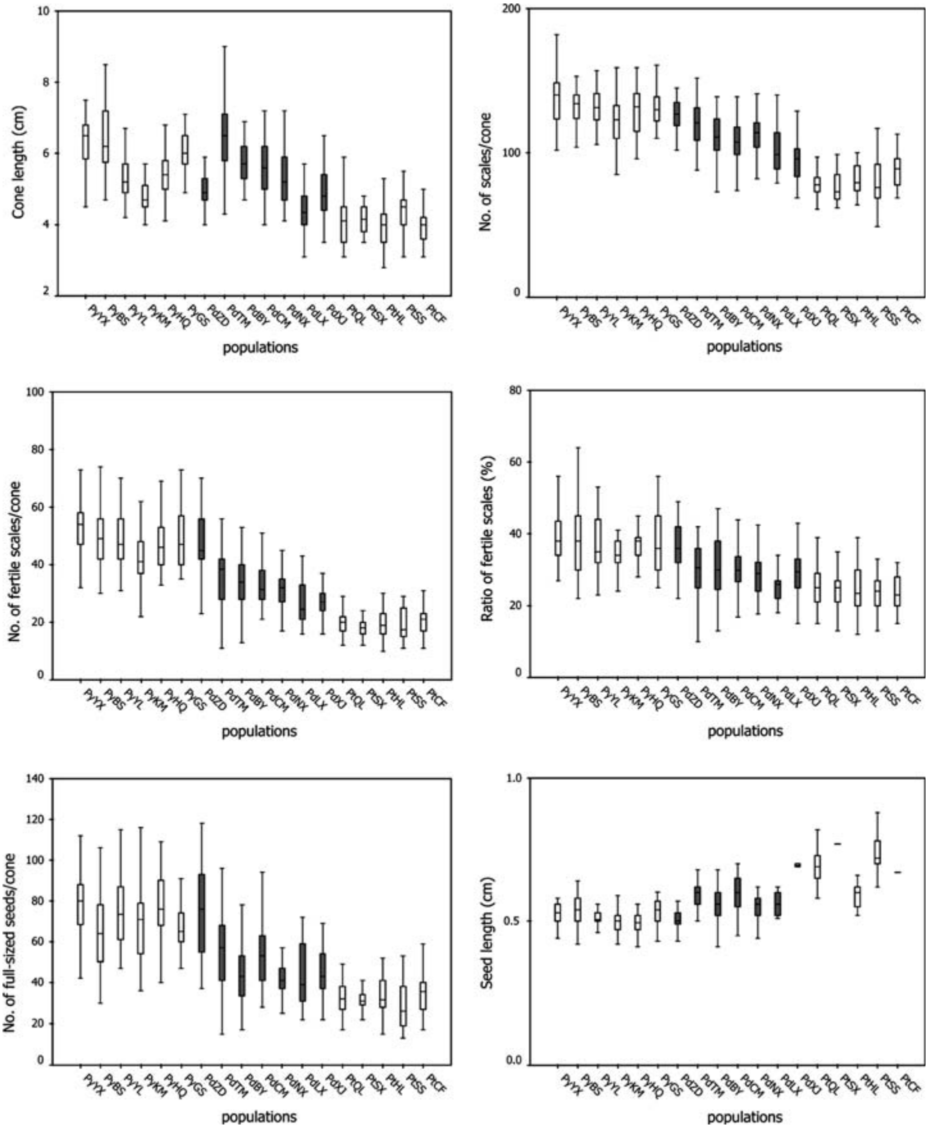


Fig. 2 Box-plots of the traits in each population. The boxes represent the interquartile range encompassing 50% of the values. The bar within each box indicates the median. *Pinus yunnanensis* and *P. tabuliformis* are positioned to the left and right of *P. densata* (in grey boxes), respectively

variation in the data set (Supplemental Table 2). A scatter diagram of the first two components in the three species is shown in Fig. 3. *Pinus tabuliformis* and *P. yunnanensis* were clearly separated. *Pinus densata* had a broad distribution that overlapped with the core spaces of the two parental species, and covered the space between them. Our results illustrate the intermediacy and large variability in cone and seed traits of *P. densata*, relative to its two parental species, in accordance with the results of the LSD multiple range test and box-plot analysis.

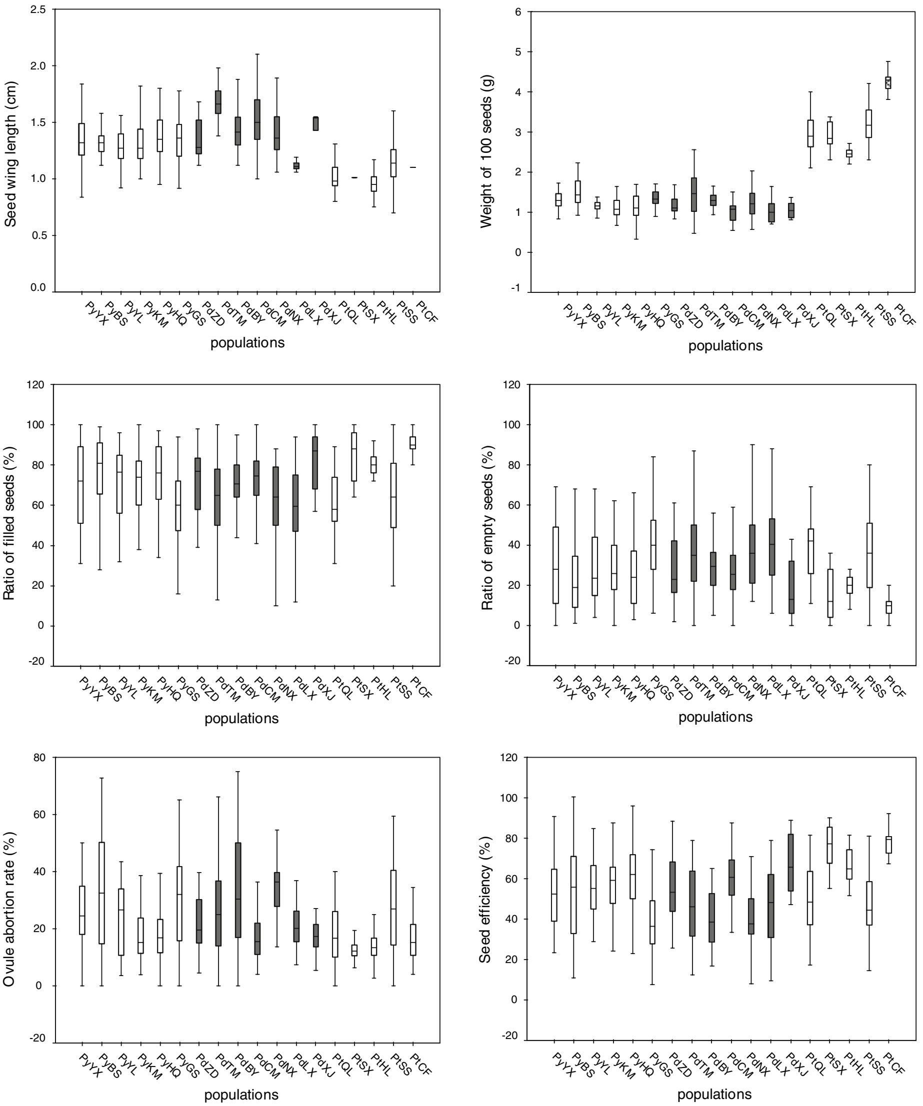
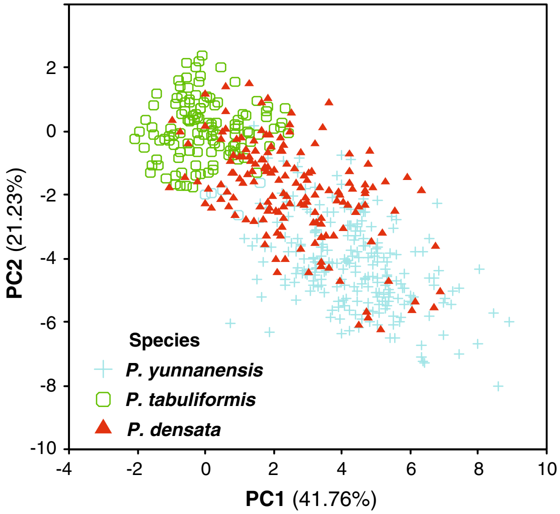


Fig. 2 continued

Based on the eight morphometric characters, the UPGMA clustering of the 18 populations produced three clearly defined groups (Fig. 4). All of the *P. tabuliformis* populations formed one cluster and *P. yunnanensis* another. All *P. densata* populations, except population PdZD, formed a sister group to the *P. yunnanensis* group. Morphologically, population PdZD showed little differentiation from *P. yunnanensis* in all eight morphometric traits (Fig. 2), therefore it was grouped with the *P. yunnanensis* cluster (Fig. 4). Population PdZD is located in the southeastern-most part of *P. densata*'s range, which overlaps with the range of *P. yunnanensis*.

Fig. 3 Scatter diagram of the first two principal coordinates (PC1 and PC2) from PCA for the three pine species based on cone and seed morphometric traits



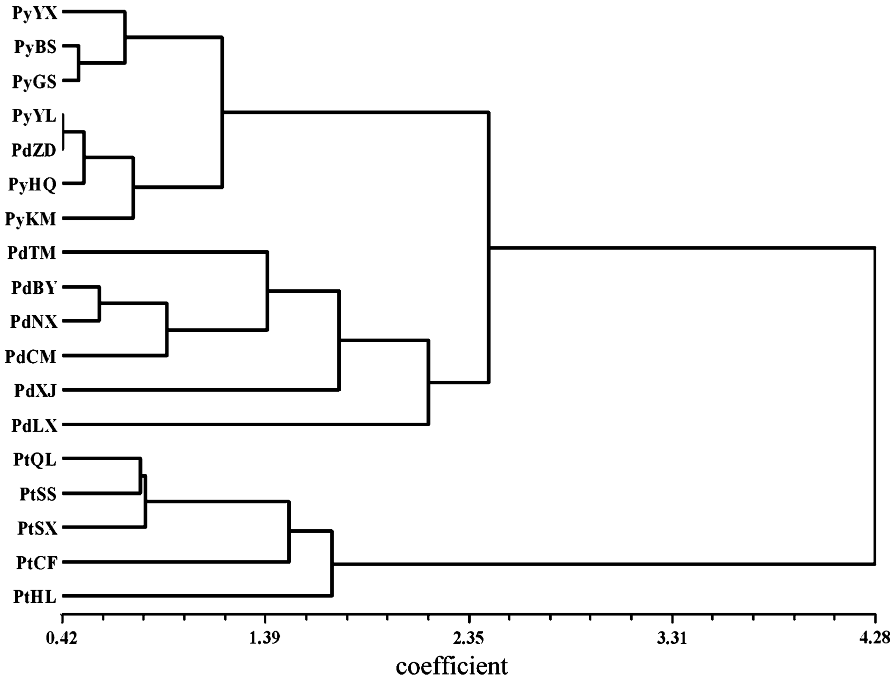


Fig. 4 UPGMA clustering of the sampled populations based on cone and seed morphometric traits

Reproductive fitness traits

The four traits examined in the cone and seed analyses (ratio of filled seeds, ratio of empty seeds, ovule abortion rate and seed efficiency) were regarded as reproductive fitness traits, since they reflected the mating and reproductive success of the pines. Among the three pine species, the ratio of filled seeds ranged from 68% to 71%, the ratio of empty seeds from 29% to 32%, the ovule abortion rate from 18% to 25%, and the seed efficiency from 51% to 54% (Table 1). Interestingly, two of the four traits (ratios of filled and empty seeds) showed insignificant differences among the three species, in contrast to the patterns of variation found for the morphometric traits (Tables 1 and 2). The ovule abortion rates in *P. densata* and *P. yunnanensis* were very similar (ca. 25%), but significantly differed from that in *P. tabuliformis* (18%). Seed efficiencies in *P. yunnanensis* and *P. tabuliformis* (ca. 53%) were slightly, but significantly, higher than in *P. densata* (51%) (Tables 1 and 2). At the population level, however, all these traits differed significantly. Partitioning of the variance components revealed another contrasting pattern to the morphometric data, since most of the variation in the four reproductive traits resided within populations (69–84%, Table 2), with only 4–9% among species. Box-plots also showed large ranges in these traits within each population (Fig 2).

Features of meiosis, especially frequencies of meiotic irregularities, in the three pine species were assessed by examining large numbers of pollen mother cells (Supplemental Table 3). The chromosome number was determined at metaphase I, and all samples had 24 (2n). The most frequent abnormalities in the two meiotic divisions were chromosome bridges and fragments, and irregularities related to chromosome segregation, such as precocious disjunction during metaphase (Supplemental Table 3). The highest frequencies of abnormalities (5–12%) were observed in metaphase I. In the final tetrad phase the presence of micronuclei was detected at very low frequencies (0.14–0.19%) in the three pines (Supplemental Table 3). *Pinus densata* did not show higher rates of meiotic abnormality than either *P. yunnanensis* or *P. tabuliformis*, and the meiotic behavior observed in the three species appeared to be very similar. However, abnormalities in chromosome association, such as the occurrence of univalents and multivalents, were not recorded in this study due to the difficulty in timing fixation in the field, and the diakinesis stage was not always possible to score in our samples, making the precise distinction of different chromosomal configurations difficult.

The pollen germination potential and rate were very similar in the three pine species ($P = 0.689$). The average germination rates were 93% ± 6%, 93% ± 7% and 89% ± 6% for *P. yunnanensis*, *P. densata* and *P. tabuliformis*, respectively.

Discussion

The long-term field experiments that are needed for rigorously testing fitness (even for annual plants) are costly in time and resources. Common garden experiments involving reciprocal transplantation are useful for evaluating the fitness of hybrids (Arnold et al. 2001; Kirk et al. 2005; Miglia et al. 2005) and assessing the evolutionary importance of hybridization in a given species complex. However, for woody plants with long generation times, like pines, the common garden approach requires life-long engagement. Furthermore, in some cases, such as the pine complex examined here, reciprocal transplantation experiments are not applicable because the parental species (in this case *P. yunnanensis* and *P. tabuliformis*) cannot normally grow and reproduce in the same locations, especially in the central part of the geographical range occupied by *P. densata*. According to our (unpublished) observations, mortality rates of *P. yunnanensis* and *P. tabuliformis* seedlings are very high in this region. Whether *P. densata* can survive in the parental territories is still unknown and requires further investigation. Therefore, in our study, we examined the reproductive fitness components in natural populations of *P. densata* and compared them to those of its parental species in their own habitats to evaluate the reproductive success of *P. densata* on the Tibetan Plateau.

Variation patterns in cone and seed morphometric traits

Two general patterns of morphological variation in *P. densata* were detected. Firstly, the species was intermediate between *P. yunnanensis* and *P. tabuliformis* with respect to six of the eight investigated traits, the exceptions being that seed weight in *P. densata* did not differ significantly from that in *P. yunnanensis*, but was significantly lower than that in *P. tabuliformis*, and the seed wings of *P. densata* were longer than those of both parental species (which may increase its potential for seed dispersal). Secondly, the range of most of the traits was larger in *P. densata* than in the other two pine species. The high variability of *P. densata* may have resulted from two sets of factors, one of which consists of the local ecological conditions. Topographically, the southeastern Tibetan Plateau consists of high mountains and deep valleys. The complex plateau geography creates widely differing microecological environments among locations and elevations. Thus, local adaptations may have had a major impact on the morphometric trait development of *P. densata*. The second set of factors could be related to the complex genetic background of *P. densata* populations. Investigations using allozyme, cpDNA and mtDNA markers have revealed high levels of diversity in genetic composition among *P. densata* populations, with varying degrees of apparent genomic contribution from each parental species (Wang and Szmidt 1994; Wang et al. 2001 Song et al. 2003), which may have affected the patterns of morphological variation among the hybrid populations. Taking into account all of these factors, we would expect the range of variation to be greater in *P. densata* than in either parental species, and such a variation pattern can be taken as further evidence of hybridity.

The morphology and ecological requirements of *P. tabuliformis* and *P. yunnanensis* are distinctly different. Assigning pine populations to species can be problematic, but only in the most peripheral regions of *P. densata*. Population PdZD was morphologically grouped with *P. yunnanensis*. Genetic analyses have indicated that *P. yunnanensis* and *P. tabuliformis* made 62% and 38% maternal contributions to the genetic composition of this population and that *P. yunnanensis* also made the major paternal contribution (Song et al. 2003). The large genomic component of *P. yunnanensis* in PdZD suggests that further gene exchange has occurred between the two species in this region since the hybridization event(s), which has had a strong morphological impact. Genetic classification is necessary to further clarify the population assignment in the periphery of the geographic ranges of these pine species.

Extreme or ‘transgressive’ phenotypes may make substantial contributions to the divergence in ecological niches that is essential for hybrid lineages to survive in and adapt to new habitats (Rieseberg et al. 1999; Schwarzbach et al. 2001). Thus, morphological characters in hybrids may be a mosaic of parental, intermediate and novel types. Extreme or novel characters have been observed in high proportions of later generation hybrids. In a survey of 171 studies that reported phenotypic variation in segregating hybrid populations, Rieseberg et al. (1999) found that 91% reported at least one transgressive trait, and 44% of 1229 traits examined were transgressive. Therefore, the cited authors concluded that transgression is the rule rather than the exception during the colonization and adaptation of hybrid lineages to new habitats. The differences in character expression in hybrid lineages are partially affected by the investigators’ choice of characters. The eight morphometric traits examined in this study may not necessarily respond strongly to adaptive constraints, and thus are not sufficient to evaluate thoroughly the contribution of transgressive segregation in *P. densata* to its niche divergence. Since *P. densata* grows at high elevations, where none of the other closely related pine species can normally grow, novel ecophysiological traits must have evolved in *P. densata* to facilitate its development in this new habitat. Investigations of the differences in resistance to cold, drought and UV radiation, water use efficiency, seed germination and seedling establishment among the three pine species would help to identify important traits that are unique to *P. densata*. Nevertheless, the present study provided basic information on patterns of variation in highly inheritable cone and seed morphometric traits in the three pine species, which is valuable for characterizing the identity of this hybrid.

Reproductive fitness and adaptation of *P. densata*

The reproductive traits, including ovule abortion rate, seed efficiency, and ratios of filled and empty seeds per cone, are important mating system and reproductive status parameters in conifers. For instance, the ratio of empty seeds is regarded as an inbreeding measure (Koski 1973; Mosseler et al. 2000; Rajora et al. 2002). In conifers, self-fertilization adversely affects embryo development, resulting in high levels of empty seeds, ranging from 80% to 100% (Bingham and Squillace 1955; Sarvas 1962; Bramlett and Popham 1971; Smith et al. 1988). On average, 86% of seeds produced in controlled self-pollination experiments with *P. tabuliformis* were empty (Wang and Shen 1989). Due to the high outcrossing rate at the seed stage, e.g., >90% in Scots pine (Kärkkäinen et al. 1996), the ratios of empty and filled seeds per cone are informative surrogates for selfing and outcrossing rates in *Pinus* (Rajora et al. 2000). Unpollinated ovules in *Pinus* gradually collapse and abort (Owens et al. 1981; Sarvas 1962). Therefore, all filled seeds that are formed result from successful fertilization. In some studies, seed efficiency is measured as the ratio of filled seed weight to cone weight (Mosseler et al. 2000). In our study we defined seed efficiency as the ratio of filled seeds to seed potential of a cone (i.e., 2 × the no. of fertile scales); therefore this parameter directly reflects the pollination and fertilization efficiency and fecundity.

Despite its distribution on the high plateau, natural populations of *P. densata* appeared to have normal levels of reproductive success, comparable to those of its two parental species in their natural habitats. The four derived reproductive parameters differed little among the three pines, indicating that they have similar overall rates of effective pollination and fertilization in their respective natural environments. The ovule abortion rate (18–25%) and seed efficiency (51–54%) observed in these three pine species were similar to those reported in *P. strobus* by Rajora et al. (2002), although the ratios of empty seeds (29–32%) in our three study species were slightly higher than those found in Scots pine (26%) by Kärkkäinen et al. (1996). Climatic conditions have a considerable impact on the coordinated sequence of developmental events leading to successful pollination, fertilization and embryo development. The absence of a noticeably higher ovule abortion rate, or lower seed efficiency in *P. densata*, indicate that this species has close to optimal pollination and fertilization success. The large variations in the reproductive traits found among and within populations of each species indicate that there are highly variable pollination environments among trees and stands, which could be affected by the stand density and the distribution of neighboring mature trees (Farris and Mitton 1984; Smith et al. 1988; Rajora et al. 2002).

Meiosis is a critical step in reproduction, and both genetic and environmental factors can affect the meiotic behavior in plants (McCormick 1993; Dorion et al. 1996). In interspecific hybrids, the combination of two distinct genomes frequently results in aberrant mitotic and meiotic divisions, since the cell cycles of the two genomes are not fully synchronous, and irregularities occur in chromosome pairing (Chandler et al. 1986; Adamowski et al. 1998; Atlagic and Skoric 1999; Rieseberg 2001). Prominent meiotic abnormalities can lead to the formation of micronuclei at the end of the first and second divisions, which strongly affect the final meiotic outcome. In addition to genetic factors, meiosis can also be disrupted by unfavorable environmental conditions, leading to limited pollen development and ovule abortion. Indeed, even a brief stress episode during meiosis in microspore mother cells can disrupt subsequent male sporogenesis, causing pollen sterility and reductions in seed set (Brooking 1976; Saini 1997). Thus, the high pollen viability and normal seed efficiency in *P. densata* demonstrate that it is reproductively adapted to the plateau environment. Populations of *P. densata* are in advanced generations, since their origin likely dates back to the uplift of the Tibetan Plateau (Wang and Szmidt 1994; Wang et al. 2001; Ma et al. 2006). Meiotic abnormalities that are evident in diploid F_1 hybrids, due to structural heterozygosity, should have been eliminated through successive generations since (*inter alia*) Hall (1955) showed that selection for fertility in diploids was highly effective in just three generations in *Bromus*, and rapid responses to selection for vigor and fertility have been observed in the five generations from F_2 to F_6 in *Gilia* (Grant 1966). These studies indicate that selection against the various non-viability and sterility factors is strong during the critical early generations of hybrid establishment. The inevitable natural selection for these traits in *P. densata*, even allowing for a degree of genome incompatibility, would have brought it go through the bottleneck and drift of the early generations and emerge with full vigor and fertility in its new ecological niche.

Several models of chromosomal speciation have been developed (see review by Rieseberg 2001), one of which (the recombinational model), suggests that rearrangements induced by hybridization may lead to rapid karyotype evolution and the rise of reproductive isolation from parental species. The ability of extant *P. densata* to cross with *P. yunnanensis* and *P. tabuliformis* is not clear and is currently under investigation. However, from the general results of previous pine hybridization experiments (Duffield 1952) and information on the genetic composition (Wang et al. 2001; Song et al. 2003) and morphological traits of marginal populations of *P. densata*, we hypothesize that *P. densata* and its parents are genetically compatible. Thus, the isolation among these pines is likely to be mainly prezygotic and maintained by divergent ecological requirements. Changes in the ecological attributes of populations, and ecological selection and spatial isolation, are the major factors promoting homoploid hybrid speciation in plants (Rieseberg 1997; Buerkle et al. 2000; Gross and Rieseberg 2005). Our study has provided empirical data on the reproductive success and adaptation of a stabilized homoploid hybrid in a novel habitat that is ecologically and spatially inaccessible to either of its parental species. It is important to note that strong temporal variation in female reproduction is frequently observed at the level of single trees and populations of conifers. Studies based on only a few seasons have limited potential to predict the lifetime reproductive success of any tree species. Reproductive success is usually described by the number of successful gametes transferred to the next generation throughout lifetime. A comprehensive evaluation of the fitness of the hybrid pine will emerge through continued monitoring of reproductive and physiological traits at different population developmental stages.

Acknowledgements We thank Dr. Y. J. Liu (Beijing Forestry University), H. Liu and Q. Duo (Tibet University) and S. G. Zhang (Yunnan Yinmore Biotech. Lab.) for their assistance during field work, and Prof. K. X. Xu (Inst. Botany, CAS) for statistical advice. This study was supported by grants from the Natural Science Foundation of China (NSFC 30325006 & 30121003).

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