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Microsatellite Variation of *Quercus aquifolioides* Populations at Varying Altitudes in the Wolong Natural Reserve of China

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Genetic variation and differentiation were investigated among five natural populations of *Quercus aquifolioides* occurring along an altitudinal gradient that varied from 2000 to 3600 m above sea level in the Wolong Natural Reserve of China, by analyzing variation at six microsatellite loci. The results showed that the populations were characterized by relatively high intra-population variation with the average number of alleles equaling 11.33 per locus and the average expected heterozygosity (H_E) being 0.779. The amount of genetic variation varied only little among populations, which suggests that the influence of altitude factors on microsatellite variation is limited. However, there is a significantly positive correlation between altitude and the number of low-frequency alleles ($R^2=0.97$, $P<0.01$), which indicates that *Q. aquifolioides* from high altitudes has more unique variation, possibly enabling adaptation to severe conditions. F statistics showed the presence of a slight deficiency of heterozygosity ($F_{IS}=0.136$) and a low level of differentiation among populations ($F_{ST}=0.066$). The result of the cluster analysis demonstrated that the grouping of populations does not correspond to the altitude of the populations. Based on the available data, it is likely that the selective forces related to altitude are not strong enough to significantly differentiate the populations of *Q. aquifolioides* in terms of microsatellite variation.

Keywords altitudinal gradients, genetic variation, genetic differentiation, microsatellites, *Quercus aquifolioides*
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1 Introduction

At any given time, the distribution and genetic diversity of a species are strongly influenced by environmental heterogeneity (Hanski and Ovaskainen 2003, Currat et al. 2004). For example, altitudinal gradients impose heterogeneous environmental conditions, such as rugged topography, a complex pattern of vegetation and flowering delay, and they are likely to markedly affect the genetic variation pattern of plants. Some studies have shown that the level of genetic variation within populations decreases and genetic differentiation between populations increases along altitudinal gradients due to severe conditions at high altitudes (Taira et al. 1997, Premoli 2003). However, there are also investigations indicating opposite results (Oyama et al. 1993, Wen and Hsiao 2001, Gämperle and Schneller 2003) and studies showing that genetic variation and differentiation do not correlate with altitude (Macdonald et al. 2001, Saenz-Romero and Tapia-Olivares 2003). So far, the genetic processes underlying adaptation to high-altitude conditions are not fully understood. To explore the influence of altitude factors on the genetic processes of plants, we examined five natural populations of *Quercus aquifolioides* occurring along an altitude gradient.

Q. aquifolioides, an endemic woody plant species, is widely distributed in the Yunnan and Sichuan provinces in Southwest China. It is a component of mixed forests and is characterized by small, thick, coarse leaves and a good ability to adapt to cold and dry habitats (Wu and Raven 1994). Although it is mainly restricted to sunny, south-facing slopes of middle altitudes, it occupies a large range of habitats across varying altitudes. Due to long and cold winters, high-altitude populations are expected to undergo more fluctuations and extinction-colonization events than low-altitude populations. At the tree line of high altitudes, *Q. aquifolioides* becomes stunted and resembles brushwood (Xu and Guan 1992, Zhou and Guan 1992). Within its habitats it plays a very important role in preventing soil erosion and water loss, in regulating microclimate, and also in maintaining ecological stability in general. In the Wolong Nature Reserve, *Q. aquifolioides* forms an evergreen, monospecific forest. It has been shown that growth, and morphological and physiological traits of *Q. aquifolioides* are closely related to altitude (Li et al. 2006). However, the pattern of genetic variation present in the natural populations in *Q. aquifolioides* along altitudinal gradients is not known.

Recently, due to their hyper-variability and co-dominant inheritance pattern, microsatellites have been successfully employed to detect variation and fine-scale genetic structure of the *Quercus* species, e.g., *Q. robur* and *Q. petraea* (Mariette et al. 2002, Bruschi et al. 2003, Cottrell et al. 2003, Muir et al. 2004). Altitudinal gradients allow the study of plant genetic responses to ecological gradients, e.g., to variation in light, temperature, nutrients or water availability. In the present study, we investigate the level and pattern of genetic variation at six microsatellite loci in five natural populations of *Q. aquifolioides* along an altitude gradient ranging from 2000 to 3600 m above sea level in the Wolong Nature Reserve. This work provides new, useful information for better understanding of the adaptation and ecological role of *Q. aquifolioides*.

2 Materials and Methods

2.1 Plant Material and Sampling

During 2004, a total of 159 *Q. aquifolioides* individuals were collected from 5 different altitudes in the Wolong Nature Reserve of China (E 102°25´~103°24´, N 30°45´~31°25´). Sampling localities were selected along a vertical transect that spanned approximately 1600 m over a linear distance of about 50 km. Each sampling interval was about 400 m (populations 1–5 correspond to altitudes 2000, 2400, 2800, 3200 and 3600 m, respectively). Geographic and altitude distances were determined using the Global Positioning System (GPS). In each population, twenty-five to thirty-five individuals were randomly sampled among adult trees, each separated by a distance of at least 50 m to prevent collecting ramets from the same clone patch. For molecular analyses, buds or young leaves from each tree were collected at the time of the lowest tannin content (Feeny 1970), then frozen quickly and stored at –86 °C until DNA extraction.

Table 1. Microsatellite variation across five natural populations of *Q. aquifolioides*.

Locus	Reference	T_A (°C)	Size range (bp)	A_O	H_O	H_E
*QpZAG*1/5	Steinkellner et al. 1997	56	142–198	25	0.975	0.942
*QpZAG*9	Steinkellner et al. 1997	51	204–286	26	0.736	0.939
*QpZAG*16	Steinkellner et al. 1997	55	112–206	26	0.887	0.920
*QpZAG*110	Steinkellner et al. 1997	50	204–268	26	0.598	0.950
*MSQ*4	Dow et al. 1995	50	203–227	4	0.717	0.519
*MSQ*13	Dow et al. 1995	50	206–246	10	0.881	0.691

T_A, annealing temperature; A_O, number of alleles detected; H_O, observed heterozygosity; H_E, expected heterozygosity.

2.2 DNA Extraction and Microsatellite Analysis

Total DNA was extracted from buds or young leaves, as described by Dow et al. (1995). After screening microsatellite loci using primers developed by Dow et al. (1995) and Steinkellner et al. (1997), the following six loci were chosen for the present study, based on the clarity of resolution and the degree of polymorphism: *QpzAG*1/5, *QpzAG*9, *QpzAG*16, *QpzAG*110, *MSQ4* and *MSQ*13 (Table 1). PCRs were carried out in 25-µl volumes of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.8 mM dNTPs, and 1.0 unit of *Taq* polymerase in Perkin-Elmer Gene Amp PCR System 9700 thermocyclers using a basic cycle profile of: one cycle (94 °C for 4 min), 35 cycles (94 °C for 45 s, T_A for 30 s, 72 °C for 1 min), and one cycle (72 °C for 5 min), where T_A is the annealing temperature (Table 1). PCR products were electrophoresed on 8% w/v non-denaturing polyacrylamide gels. The electrophoresed gels were silver-stained to visualize the DNA bands. Alleles were scored on the basis of size in comparison with external standards, a pbR322 sequencing ladder, using the Gel Doc 1000™ image analysis system (Biorad).

2.3 Data Analysis

The following analyses were performed using GENEPOP ver. 3.4 (Raymond and Rousset 1995): Tests for deviations from the Hardy-Weinberg equilibrium for each locus-population combination using an exact test in which P values were estimated using a Markov chain method, genotypic linkage disequilibrium assessment for all combinations of locus pairs within populations also using a Markov chain method with GENEPOP default values, and tests for population differentiation between all possible pairs of populations for each locus and over all loci using log-likelihood (G)-based exact tests (Goudet et al. 1996) with default values.

Basic descriptive statistics (H_E, H_O, genetic distances, etc.) were compiled using TFPGA ver. 3.2 (Miller 1997). The same program was also used to test population differentiation using a contingency table and Markov chain approach with 10 batches of 1000 dememorizations and 2000 permutations per batch. For pair-wise comparisons between populations, F_{ST}-values were calculated using ARLEQUIN version 2.0 (Schneider et al. 1997) with significances based on a permutation process. To determine the partitioning of genetic variation at different altitudes, the analysis of molecular variance (AMOVA) approach of Excoffier et al. (1992) was performed to estimate the hierarchical structure of genetic diversity using the program ARLEQUIN version 2.0.

3 Results

3.1 Microsatellite Variability

Five individuals from different populations were used in an initial microsatellite primer test including 15 primer pairs, of which six primer pairs that resulted in clear, polymorphic profiles were selected for the full analysis. The six loci displayed considerable polymorphism among the

five populations sampled, with a total of 117 alleles identified and the number of alleles per locus ranging from four to twenty-six (Table 1). The most variable loci, *QpZAG*1/5, *QpZAG*9, *QpZAG*16, and *QpZAG*110, each possessed 25 or 26 alleles. The least variable locus, *MSQ*4, had 4 alleles across the populations. All six microsatellite loci were polymorphic in all sampled populations, and a high level of expected heterozygosity was detected, the values across populations ranging from 0.519 (*MSQ*4) to 0.950 (*QpZAG*110) with the average value equaling 0.820 (Table 1).

3.2 Genetic Structure

Over the six loci examined, the mean observed heterozygosity (H_O) was lower than the expected heterozygosity (H_E), causing the overall heterozygote deficiency (F_{IS}) and inbreeding coefficient or fixation index (F_{IT}) to be positive (Table 2). However, the levels of inbreeding within individual populations and among all sampled individuals were not significant, as indicated by jackknifed F_{IS} and F_{IT} estimates (0.136 and 0.193, respectively; Table 2). The F_{IS} values varied a great deal across loci (–0.548 to +0.767). The results of the tests for Hardy-Weinberg equilibrium indicated slight deficits of heterozygosity in *Q. aquifolioides*, both for the whole set of populations and for each population. However, global tests for the deviations from the Hardy-Weinberg expectations revealed significant deficiencies of heterozygosity at three loci, *QpZAG*9, *QpZAG*16, and *QpZAG*110 ($P < 0.001$) (Table 2).

Table 2. *F* statistics (Wright 1965) following the method of Weir and Cockerham (1984) for six polymorphic loci across five natural populations of *Q. aquifolioides*.

Locus	F_{IS}	F_{IT}	F_{ST}	*Nm*
*QpZAG*1/5	–0.066	–0.029	0.035	5.861
*QpZAG*9	0.183	0.226	0.053	4.027
*QpZAG*16	0.767	0.790	0.100	2.136
*QpZAG*110	0.353	0.375	0.033	5.283
*MSQ*4	–0.548	–0.346	0.131	2.020
*MSQ*13	–0.373	–0.265	0.079	3.168
Mean	0.136	0.193	0.066	3.749
± SE	0.176	0.167	0.015	
95%CI	(0.414, –0.218)	(0.462, –0.125)	(0.095, 0.043)	

F_{IS}, deficiency of heterozygosity relative to the Hardy-Weinberg expectation; F_{IT}, the overall inbreeding coefficient; F_{ST}, differentiation among populations; *Nm*, gene flow.

Table 3. Genetic variation within population based on six microsatellite loci. The altitudes of the population sites are given in parentheses.

Population	N	A_O	A_E	H_O	H_E	N_r
1 (2000 m)	25	7.500	5.322	0.687	0.759	6
2 (2400 m)	33	13.000	7.404	0.763	0.798	9
3 (2800 m)	35	11.500	6.761	0.662	0.786	12
4 (3200 m)	34	11.833	7.213	0.721	0.796	17
5 (3600 m)	32	12.833	7.321	0.578	0.758	23
Across populations	159	11.333	6.804	0.682	0.779	47
Species level	159	21.000	11.353	0.682	0.827	47

N, sample size; A_O, mean number of alleles per locus; A_E, effective number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; N_r, the number of low-frequency alleles.

3.3 Within-population Variation

Genetic parameters based on allele data at the six microsatellite loci in the five populations are shown in Table 3. The total amount of genetic variation for the whole species was high, equaling 0.827. In individual populations, the mean observed number of alleles per locus varied from 7.50 to 12.83, with a mean of 11.33 (Table 3), while the effective number of alleles varied from 5.32 to 7.40, with an average of 6.80. The observed heterozygosities (H_O) ranged from 0.578 to 0.763 (mean 0.682), and the expected heterozygosity (H_E) varied from 0.758 to 0.798 (mean 0.779). A comparison of the genetic diversity of *Q. aquifolioides* was performed among the five populations. The range of variation within populations was very small, the expected heterozygosity across the six loci varying from 0.758 (population 5) to 0.798 (population 2). At the same time, all populations followed the pattern that the number of low-frequency alleles (N_r) within populations increased from the mountain foot to the top, which is shown in Table 3. The correlation coefficient between the altitude and the number of low-frequency alleles was significantly positive ($R^2 = 0.97$, $P < 0.01$) (Fig. 1).

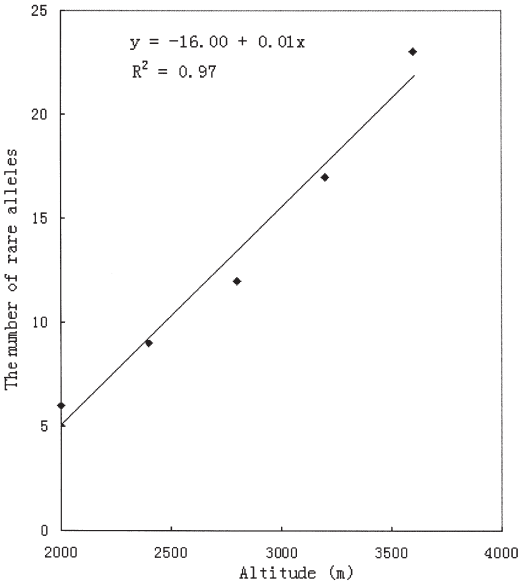

Fig. 1. Correlation analysis between altitude and the number of rare alleles in five *Q. aquifolioides* populations.

3.4 Inter-population Differentiation

The genetic differentiation among populations was measured by F_{ST} and analyzed by a hierarchical analysis of genetic diversity (AMOVA). Relatively little microsatellite variation was found among populations. The pair-wise F_{ST} estimates ranged from 0.040 to 0.107, with an average of 0.066, which shows that only 6.6% ($P < 0.001$) of the genetic variation was present among populations and 93.4% ($P < 0.001$) within populations (Table 4). The highest-altitude population (population 5) was highly divergent when compared to the other populations. The overall gene flow (*Nm*), which is the estimate of the average number of migrants between all studied populations per generation, equaled 3.749.

The UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) was used to cluster the five populations according to their genetic distances and to further show the genetic relationships among the populations (Fig. 2). As shown in the dendrogram, population 3 and population 4 clustered first, and population 5 was distinctly separated from the other populations.

Table 4. Pairwise genetic distances (Nei 1972) between the five *Q. aquifolioides* populations (below) and the F_{ST} values between the populations (above) based on six microsatellite loci .

Population	1	2	3	4	5
1	0.000	0.071	0.053	0.047	0.107
2	0.308	0.000	0.048	0.046	0.082
3	0.213	0.220	0.000	0.040	0.094
4	0.184	0.214	0.178	0.000	0.088
5	0.473	0.384	0.443	0.410	0.000

4 Discussion

In the present study, the microsatellites examined were highly variable possessing a great number

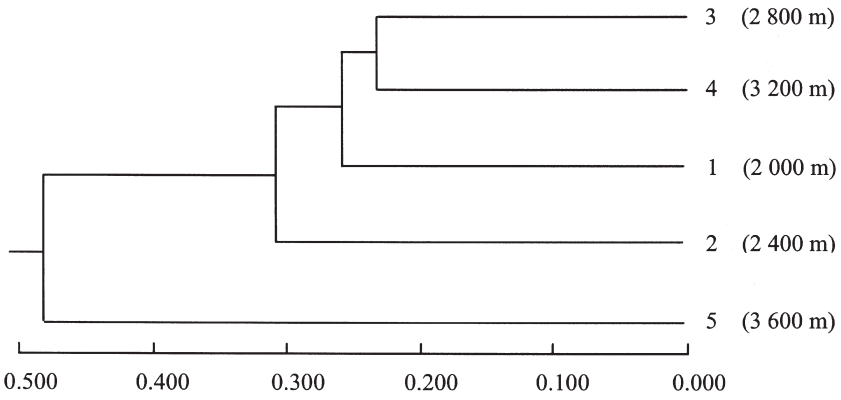

Fig. 2. Dendrogram for the populations originating from the Wolong Nature Reserve. Calculations were performed using the unweighted pair-group arithmetic mean-method for the genetic distance values given in Table 4.

of alleles, except for the locus *MSQ*4 (4 alleles). At all altitudes, similar numbers of alleles and high values of heterozygosity were observed. The level of polymorphism detected (an average of 20 alleles per locus and heterozygosity of 0.820 for the whole species) is almost equal to the values observed in other *Quercus* species based on microsatellite loci and similar sample sizes, e.g., *Q. petraea* (Streiff et al. 1998), *Q. petraea* (Cottrell et al. 2003), and *Q. lobata* (Dutech et al. 2005). The discovery of the average observed heterozygosity being slightly, although not significantly, lower than expected heterozygosity (mean F_{IS}=0.136) within natural populations of *Q. aquifolioides*, is similar to that detected in other oak species (Guttman and Weigt 1989, Schnabel and Hamrick 1990, Berg and Hamrick 1993, 1994, Chung et al. 2002). However, the F_{IS} values were found to vary a great deal across loci (–0.548 to +0.76). Heterozygosity deficiencies were observed at the microsatellite loci *QpZAG*9, *QpZAG*16 and *QpZAG*110, while a significant excess of heterozygotes was present at *MSQ*4 and *MSQ*13. Perhaps, natural selection has a role in controlling the pattern of genetic variation at some microsatellite loci. Altitudinal gradients impose highly heterogeneous environmental conditions, which are likely to markedly affect the neutral sites closely linked to the site under selection (Strobeck 1983, Hudson and Kaplan 1988). Therefore, a surplus or deficiency of heterozygous individuals and *F* values deviating from zero may indicate that selective forces are acting at these loci within the population (Lewontin and Cockerham 1959).

Quercus species are generally self-incompatible and wind-pollinated. Therefore, inbreeding and associative mating are unlikely factors contributing to the genetic structure of *Q. aquifolioides*. In addition, it occupies a large range of habitats across different altitudes (from 2000 to 3600 m above sea level) and shows a good ability to adapt to cold and dry habitats. As Hamrick et al. (1992) have proposed, widespread, long-lived, and wind-pollinated woody plants may maintain high levels of genetic variation. Here, all sampled populations of *Q. aquifolioides* possessed high levels of genetic variability. However, the expected heterozygosity ranged from 0.758 (population 5 at 3600 m) to 0.798 (population 2 at 2400 m), which indicated the influence of altitude factors on microsatellite variation was not obvious. Although the level of genetic diversity was quite similar at altitudes ranging from 2000 to 3600 m, a greater number of rare alleles, perhaps linked to non-neutral loci, were present at high altitudes. Since the high elevation sites are more remote and the trees are much smaller, the populations may have been much less subject to human disturbance when compared to lower-altitude populations.

Wood cutting and timber harvesting at the lower elevations could have reduced effective population sizes in the past (i.e. a genetic bottleneck), which may have led to the loss of the rare alleles. A similar tendency has also been found in *Arabis serrata*, in which the mean number of alleles per polymorphic locus was greater at high altitudes (Oyama et al. 1993).

Comparably, AMOVA and the cluster analysis did not reveal a distinct subdivision among populations. Although the high-altitude population 5 possessed a great genetic distance with low-altitude populations, the organization of the nodes of the UPGMA phenogram indicated no particular altitudinal pattern. It appears that at least four (populations 1–4) of the five studied populations can be considered to form a single panmictic unit. At the same time, the pattern of altitudinal divergence observed among the six microsatellite loci indicated that diversifying selection has not driven adaptive differentiation in the face of extremely high levels of gene flow (by wind, and by animal and human vectors). All microsatellite loci exhibited uniformly low F_{ST} values. Using Wright's (1943) infinite-island approximation, $F_{ST} = 1/(1+4Nm)$, the weighted mean values of F_{ST} translated into an estimate of $Nm \approx 3.749$, which is close to 4. Kimura and Weiss (1964) have shown that when $Nm \geq 4$, the homogenizing effect of gene flow is sufficient to prevent stochastic differentiation of allele frequencies. Under such conditions, local adaptation may be constrained by high levels of gene flow that produces a spatial averaging of fitness variation among different altitudes. Also considering the overall lack of association between microsatellite variation and adaptive trait variation in *Q. aquifolioides* (Li et al. 2006), it is likely that the selective forces due to altitude are not strong enough to significantly differentiate the studied populations in terms of microsatellite loci.

All available evidence indicates that the genetic structure of *Q. aquifolioides* is even across the altitudinal gradient of 2000–3600 m, covering the same mountain, the same valley, the same slope (south-facing), and a narrow area (a linear distance of about 50 km), that was surveyed. Since no apparent altitudinal population structure was detected, except for the presence of more frequent rare alleles at high altitudes, it appears that the level and pattern of genetic variation in *Q. aquifolioides* is not notably related to altitude.

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