Genetics of Cunninghamia lanceolata Hook.

2. Genetic Variation Within and Between Two Provenance Samples

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Abstract

Genetic variation in seed samples of *Cunninghamia lanceolata* from two provenances of the western part of the People's Republic of China were studied. Genotypes of endosperm and corresponding embryo were scored simultaneously at ten enzyme gene loci.

Intrapopulational variation is described by means of the actual and conditional heterozygosities and the genetic diversities. These are given separately for the pool of the female and the male gametic contributions to the embryos as well as the genotypes. The characterization of interpopulational variation is based on a statistical comparison, on the genetic distances for alleles and genotypes, and on the measurement of population differentiation. The results clearly indicate considerable amounts of genetic variation within and between the studied provenances. Possible consequences for tree breeding are outlined briefly.

Key words: Enzyme gene marker, heterozygosity, genetic diversity, genetic distance, seed provenance, *Cunninghamia lanceolata*.

Zusammenfassung

Anhand von Saatgutstichproben wurde die genetische Variation zweier Herkünfte von *Cunninghamia lanceolata* aus dem westlichen Teil der Volksrepublik China untersucht. Genotypen von Endosperm und dazugehörigem Embryo wurden simultan an zehn Enzym-Genorten identifiziert.

Die Variation innerhalb der Populationen wurde anhand der aktuellen und bedingten Heterozygotiegrade sowie der genetischen Diversität beschrieben. Diese Maße wurden getrennt für die weiblichen und männlichen Gametenbeiträge zu den Embryonen sowie für diploide Genotypen berechnet. Die Charakterisierung der Variation zwischen den Populationen basiert auf einem statistischen Vergleich, auf den genetischen Abständen für Allele und Genotypen und auf der Erfassung der Populationsdifferenzierung. Die Ergebnisse zeigen übereinstimmend eine beträchtliche genetische Variation innerhalb und zwischen den untersuchten Provenienzen. Mögliche Konsequenzen für die züchterische Nutzung werden kurz angesprochen.

Schlagwörter: Enzym-Genmarker, Heterozygotie, genetische Diversität, genetischer Abstand, Saatgut-Provenienz, *Cunninghamia lanceolata*.

Introduction

The maintenance of the capacity of populations to adapt to changing environments is considered to be the most important criterion in the determination of long term survival ability. Such adaptability should in large part be a consequence of genetic variability, which in turn is a function of the genetic variation realized in an actual population and the ability to create genetic variation in subsequent generations (e.g. Gregorius *et al.*, 1985). In this way, genetic variation becomes more important the longer populations are exposed to varying environments and the more heterogeneous the spatial environments are. This

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holds for *Cunninghamia lanceolata* which is a long-lived coniferous species in a widespread habitat in 16 provinces in the southern part of China. To our knowledge, no information about the genetic variation in natural stands or seed samples has previously been available. Only very few studies have been concerned with other species in the family *Taxodiaceae* (e.g. Fins and Libby, 1982).

Inventories of genetic variation in *Cunninghamia lanceolata* populations are required in order to describe geographic variation and to better understand principles of adaptation. Generally, information about genetic variation in natural populations should be considered as an essential prerequisite for long term breeding programs, which necessarily should be based on population genetic principles.

Materials and Methods

Seed Samples

The seeds originate from the district of Hungya in west-central China and the district of De-Chang in south-western China. The distance between the two locations is approximately 700 km. Sample size is 151 seeds for the first provenance and 114 seeds for the second. Both seed samples were drawn randomly from the respective mixtures of crops from several stands of *Cunninghamia lanceolata*. The material was supplied by the Forestry Department of the Sichuan Agricultural University, Yaan, as off cial provenance samples from Hungya and De-Chang. All available seeds were used in this study.

Genotyping

The genotype of each seed was determined by separate analysis of its haploid endosperm and its diploid embryo. By this means, the female and the male contribution to the embryo is detectable as an ordered pair (Müller-Starck, 1976). Ten enzyme gene loci were scored simultaneously by horizontal starch gel electrophoresis, namely GDH-A, GOT-A, -B, -C, IDH-B, 6PGDH-A, B, PGI-A, SKDH-A, B (GDH = glutamate dehydrogenase, E.C. 1.4.1.2; GOT = glutamate oxalacetate transaminase, E.C. 2.6.1.1; IDH = isocitrate dehydrogenase, E.C. 1.1.1.42; 6PGDH = 6-phosphogluconate dehydrogenase, E.C. 1.1.1.44; PGI = phosphoglucose isomerase, E.C. 5.3.1.9; SKDH = shikimate dehydrogenase, E.C. 1.1.1.25). For genetic control, inheritance, linkage relationships and details on electrophoretic methods see part 1 (Müller-Starck and Liu, 1988).

Genetic Parameters

Computations were performed by means of the GSED program ("Genetic Structures from Electrophoretic Data", Gillet Gregorius *et al.*, in prep.). This included the compilation of allelic and genotypic structures, statistical comparisons by the log likelihood ratio test (G-test) of heterogeneity in contingency tables (model II: $k \times 2$ table, e.g. Weber, 1978, chap. 6.7.5; also Elandt-Johnson, 1971, chap. 13.10, for general concepts), and the calculations of measurements for intrapopulational variation such as heterozygosities and genetic diversities (Gregorius, 1978) as well as for interpopulational variation, i.e. the genetic distance (Gregorius, 1974) between the two studied provenances.

Results and Discussion

Intrapopulational Variation

The genetic variation within each provenance seed sample is described at the individual level and the population level. The first is considered by calculating average degrees of heterozygosity, and the second is based on the number of genetic types by taking into account their frequency distribution within the population ("genetic diversity"); for concepts and nomenclature see Müller-Starck and Gregorius (1986).

Heterozygosities

Two measurements of the average degree of heterozygosity were applied: The commonly calculated proportion of heterozygous individuals in the population ("actual heterozygosity", H_A) and a recently introduced measure which takes into consideration that heterozygosities are not independent of the underlying gene frequencies ("conditional heterozygosity", H_C, Gregorius *et al.*, 1986). The latter measure normalizes values of H_A by the maximum attainable heterozygosity, which amounts to $2 \cdot (1 - p)$, if one allele has a frequency p greater than 0.5, and to the value 1 in all other cases.

The average H_A- and H_C-values of each of the ten studied gene loci are listed for both provenances in *Table 1*, where the given values are the arithmetic mean for H_A and the ratio of the summed H_A-values to the summed maximum attainable heterozygosities for H_C. The actual heterozygosities show considerable variation among the gene loci for both provenances: The lowest values are obtained for GOT-A (0.026 and 0.060) and the highest for 6PGDH-A (0.535 and 0.523) and SKDH-A (0.523 for provenance 1). The average value over all gene loci is greater for provenance 1 than for 2 (0.322 vs. 0.275), but both are still within the range of heterozygosities found in coniferous species (0.270 as the average over 11 species — cf. Mitton, 1983). These values are much greater than those of angiospermous species, which on the average amount to 0.165 for 28 monocotyledonic species and to 0.113 for 74 dicotyledonic species (Mitton, 1983). However, single angiospermous tree species, such as *Fagus sylvatica* L., were shown to share the range of the conifers (H_A = 0.294, Müller-Starck, 1985).

The range of variation of the H_C-values is larger than that of H_A (0.328—0.999 for provenance (1), 0.300—0.830 for (2)). This indicates considerable variation among the gene loci with respect to the underlying allele frequencies. The advantage of H_C for monitoring heterozygosity becomes particularly evident in the case of GOT-A: The impression of an outstandingly low average heterozygosity (H_A-values of 0.026 and 0.060) is not realistic in the sense that these values already represent more than half of that which is attainable due to the specific allele frequencies (H_C-values of 0.590 and 0.600). This trend is even more pronounced in the case of 6PGDH-B (see *Table 1*). At both loci, the dominating presence of a single allele (frequency greater than 90%; see Appendix) is evidently responsible for such disparities between H_A and H_C. Nevertheless, the calculated average 10-locus values of both measurements of heterozygosity do not show conflicting trends, so that H_A may still function as a multilocus indicator of genetic variation, even though it is ambiguous as a single locus measurement, especially in the case of frequency distributions with one dominant allele.

Deviations between the H_C-values of the two provenances are pronounced in the case of single loci (e.g. GDH-A with 0.537 vs. 0.300, SKDH-B with 0.328 vs. 0.592) but not at the multilocus level.

Genetic Diversity

In both provenances, substantial genetic variation is realized at the population level. This already becomes

Table 1. — Actual and conditional heterozygosities for ten gene loci for each of the two provenances Hungya (1) and De-Chang (2). Sample size is 151 seeds for (1) and 114 seeds for (2).

Gene Locus	Heterozygosity			
	Actual		Conditional	
	(1)	(2)	(1)	(2)
GDH-A	.185	.158	.537	.300
GOT-A	.060	.026	.600	.590
GOT-B	.351	.281	.557	.616
GOT-C	.510	.430	.631	.544
IDH-B	.272	.237	.839	.635
6PGDH-A	.523	.535	.537	.539
6PGDH-B	.126	.088	.599	.830
PGI-A	.391	.465	.710	.655
SKDH-A	.523	.219	.652	.693
SKDH-B	.285	.307	.328	.592
Mean	.322	.275	.582[1)]	.569[1)]

[1)] Non-arithmetic mean (see article)

evident when the number of different genetic types is considered: For instance, the average number of alleles per locus is 4.3 for provenance (1) and 4.0 for provenance (2). If the studied loci were considered to constitute the entire genome, this would suffice to form a maximum possible number of 14, 288 · 10^9 genotypically different individuals for the first provenance and 6,804 · 10^9 for the second ($\Pi^{10}_{l=1}(n_l(n_l+1)/2)$ with n_l = number of alleles at locus l).

To take into account the frequencies of the observed genetic types, the genetic diversity (Gregorius, 1978) was calculated separately for the successful female and male gametes and the genotypes of each provenance. For each locus, the applied measure of genetic diversity ($v = (\Sigma^{n_l}_{i=1} p_i^2)^{-1}$ with p_i = frequency of the genetic type i) can be interpreted as the size of a hypothetical population having the same frequency of genetic types as the actual one but which is completely differentiated. Consequently, the given diversities are equal to the "differentiation effective number" of genetic types (Gregorius, 1987). The multilocus average of the respective diversities is calculated as the harmonic mean. In addition, the genic (allelic) multilocus diversity is given in *Table 2* ($(\Pi^{n_l}_{l=1} v_l) - 1$, with v_l = genic diversity at locus l), i.e. the number of genetically different gametic types which can be produced by each of the studied groups of individuals (seeds of the provenances) at the 10-locus level.

Due to the larger number of genetic types, the genotypic diversities generally should be greater than the allelic. Their ratio depends on the particular type of frequency distribution: For a given number of genetic types, more uniform frequency distributions should result in greater diversities than less uniform ones. This also explains why, in some cases, the ratio between allelic and genotypic diversities deviates substantially between the two provenances (e.g. SKDH-A). It is evident that certain loci reflect large diversities for all studied genetic types (e.g. GOT-C, 6PGDH-A). As mentioned above, these loci do not belong to those with a maximum number of genetic types, so that the obtained large diversities are mainly a consequence of an above average uniformity of frequency distributions.

Table 2. — Genetic diversities for ten gene loci for each of two provenances (1), (2) based on the frequencies of the alleles in the female and the male gametes and of the embryonic genotypes.

Gene Locus	Genetic Diversity					
	Allelic (1)		Allelic (2)		Genotypic	
	♀	♂	♀	♂	(1)	(2)
GDH-A	1.399	1.399	1.668	1.633	1.773	2.104
GOT-A	1.113	1.098	1.054	1.036	1.178	1.074
GOT-B	1.742	1.809	1.553	1.553	2.961	2.249
GOT-C	2.095	2.257	2.212	2.248	4.704	4.463
IDH-B	1.368	1.427	1.570	1.521	1.920	2.086
6PGDH-A	2.137	2.335	2.031	2.071	4.801	4.115
6PGDH-B	1.098	1.175	1.154	1.074	1.299	1.221
PGI-A	1.715	1.853	1.917	1.990	3.033	3.646
SKDH-A	2.673	2.276	1.370	1.427	5.407	1.819
SKDH-B	2.254	2.037	1.824	1.521	3.578	2.520
Mean	1.619	1.647	1.553	1.515	2.349	2.067
Genic multilocus diversity	187.48	209.95	105.46	85.30		

Comparing the mean diversities of the female and the male gametes, all loci indicate very small disparities between the two. This is a remarkable phenomenon, since seed collections usually focus on a limited number of trees with a heavy seed crop, a procedure which should result in a larger genetic variation among the successful male gametes than among the female. Consequently, both seed samples seem to originate from so many seed parents trees that differences between the diversities among the females and the males are compensated. These results also allow for the possibility of a substantially limited gene flow due to restricted pollen dispersal in *Cunninghamia lanceolata* in the sense that each seed parent has so few mating partners (including itself) that the diversities among the females and the males differ less than in the case of highly effective pollen dispersal.

For all genetic types, the mean diversities in *Table 2* agree in the indication of slightly greater values for provenance (1) than for (2). These differences are particularly pronounced in the genic multilocus diversities, as a consequence of the multiplier effect of this measure. Averaging this type of multilocus diversity for the females and the males of each provenance, the resulting number of genotypically different gametes which can be produced by the studied individuals of provenance (1) is more than twice that of provenance (2).

It has to be kept in mind that genetic diversities are not independent of the sample size, particularly in the case of highly differentiated populations. Both available seed samples were small, so that the calculated diversities have to be considered as lower limiting values. Furthermore, it cannot be ruled out that the comparison between the diversities of the two samples may be affected by the differences in their sizes (151 vs. 114 seeds). To monitor possible consequences for the ratio between the mean diversities of the provenances, the recently proposed measure of population differentiation δ_t (Gregorius, 1987) was utilized: $\delta_t = \frac{N}{N-1}(1 - \Sigma_i p_i^2) = \frac{N}{N-1}(1 - v^{-1})$. This measure considers the population (sample) size N and approaches a one-to-one relationship with the diversity v for large populations. Contrary to v, the measure δ_t ranges between zero and one. Based on the mean values over the loci, the following ratios between the two provenances are obtained on the level of the successful female and male gametes (see *Table 3*).

Table 3. — Comparison of the mean values, averaged over the ten loci, of genetic diversity and the measure of population differentiation (δ_t) for female and male allele frequencies of the provenances (1) and (2).

	$\varphi_{(1)}$	$\delta_{(1)}$	$\varphi_{(2)}$	$\delta_{(2)}$	Ratio $\varphi_{(1)} : \varphi_{(2)}$	Ratio $\delta_{(1)} : \delta_{(2)}$
$\bar{v}$	1.619	1.647	1.553	1.515	1.042	1.087
$\bar{\delta}_t$	0.385	0.395	0.359	0.343	1.072	1.152

The given values indicate that the ratio between provenances (1) and (2) with respect to mean genic diversity are not diminished if the normalized population differentiation is calculated instead. This fact supports the assumption that the different sizes of the wo provenance samples cannot interfere considerably in the obtained diversities. Furthermore, the ratio $\frac{N}{N-1}$ in the formula for δ_t clearly shows that these sample sizes are sufficiently large to prevent a severe biasing.

Interpopulational Variation

The genetic comparison between the two provenance samples is based on the results of statistical testing and the calculation of the genetic distance. The presence of interpopulat onal variation is already indicated by the fact that a large proportion of the genetic types found in one sample are not found in the other. In the case of the total number of genes (alleles) at all studied loci in the combined female and male gamete pool, out of a total of 49 genes, 9 (= 18.4%) are found only in provenance (1) and 6 genes (= 12.2%) only in (2), while 34 genes are common to both. The respective figures for the embryonic genotypes are: 36 (= 32.4%) appear probe-specific only in provenance (1), 15 (= 13.5%) only in (2), and 60 genotypes are represented in both provenances.

This information is limited in the sense that these sample-specific genetic types usually occur in small frequencies (for details see allelic frequencies in the Appendix). Biases due to sample size affect the genotypes more than the alleles because of the greater number of genetic types per sample. The chances for the occurrence of such biases should be at a minimum in comparisons between the frequencies among the male successful gametes. A complete survey of the frequencies of all observed genetic types (80 single locus distributions) is not possible here. To demonstrate the observed genetic differences between the two provenance samples, the combined frequencies among the female and male gametes are given in the Appendix.

Statistical Comparison

In *Table 4*, the results of a statistical (log likelihood ratio or G-) test of heterogeneity betwen the two provenances at each locus with respect to the combined female and male allele frequencies and those of the embryonic genotypes are given. As mentioned above, the frequency distribution of these genetic types should differ, at least as far as the manner of distribution of rare types over frequency classes is concerned. In spite of this, the resulting G-values of the two genetic types are quite similar at each locus. This fact provides evidence that consideration of the frequency distributions of one studied genetic type as opposed to the other results in very similar conclusions, but this is no indication that biases due to weak occupancy of frequency classes are necessarily absent.

The results in *Table 4* obviously demonstrate substantial interpopulational variation between the two provenance samples. Under the given conditions, seven out of ten loci reveal significant differences between the two samples for at least one of the studied genetic types. Outstandingly high G-values are obtained for GDH-A as well as SKDH-A and B. The opposite holds for 6 PGDH-B, GOT-A and B. In summary, the significant deviations which are revealed by a remarkably large proportion of gene loci support the conclusion that there is substantial genetic differentiation among populations of *Cunninghamia lanceolata*.

Table 4. — Statistical comparison between the two studie rovenances and a calculation of their genetic distance. Data refer to the combined female and male frequencies as compared to the embryonic genotype frequencies at each of the ten loci.

Gene Locus	G-Values from Heterog. Tests(+)		Genetic Distance	
	Alleles	Genotypes	Alleles	Genotypes
GDH-A	161.09***	111.35***	.263	.342
GOT-A	4.86ns	4.97ns	.030	.047
GOT-B	6.14ns	5.72ns	.089	.124
GOT-C	10.96*	27.86**	.089	.238
IDH-B	11.16*	15.86ns	.085	.120
6PGDH-A	20.18**	18.08ns	.064	.125
6PGDH-B	5.41ns	7.11ns	.023	.058
PGI-A	16.82**	33.35**	.128	.242
SKDH-A	46.74***	58.29***	.244	.355
SKDH-B	23.00***	33.06***	.184	.277

(+) Significance levels 0.05 (*), 0.01 (**), 0.001 (***); ns = not significant

Genetic Distance

To overcome the dependency of test statistics on the manner in which a sample is distributed in sample classes, genetic distances were calculated. The applied measure ($D = 1/2 \Sigma_i \mid p_i - q_i \mid$ with p_i and q_i as frequencies of genetic type i in two different samples — Gregorius, 1974) quantifies the heterogeneity between two frequency distributions on a continuous scale: Values of zero indicate two identical samples, while values of one designate the absense of common genetic types. The genetic distances given in *table 4* refer to the same genetic types as the statistical tests.

The calculated genetic distances and heterogeneity G-values reflect similar tendencies but also show some remarkable differences in the ranking among the loci and the genetic types. The high G-values at GDH-A, SKDH-A, B are confirmed, but the maximum at GDH-A is considerably less pronounced. Very low values are obtained only for GOT-A and 6PGDH-B but not for GOT-C. It is evident that the genetic distances are generally greater for the genotype as compared to the allele frequencies, which is a consequence of the higher number of genotypes than alleles. This trend is not revealed in the G-values — it is even reversed in the case of GDH-A. Differences between the two measures of interpopulational variation are also evident for GOT-A and GOT-B, which have similar G-values for both alleles and genotypes but differ considerably in the respective genetic distances. The opposite holds for GOT-B and GOT-C, which have the same allelic distances but G-values which indicate significant differences only for GOT-C.

Genic (allelic) distances which approach 0.2 or more and the correspondingly large genotypic distances suggest considerable interpopulational differentiation between the two samples and support the statements tentatively derived from the statistical comparison.

Conclusions

As a firs step in the characterization of genetic variation in *Cunninghamia lanceolata*, two provenance samples were analysed systematically with respect to the intra- and interpopulational variation. The relatively small sample size may affect the estimates on diversities and statistical comparison of the samples. Consequently, the diversity was focussed on alleles and considered separately for the female and the male gametic contributions

and additionally related to the less biased measurement of subpopulational differentiation. The statistical test only functioned as a supplement to the genetic distances, which are less dependent on the particular type of frequency distribution. Main emphasis was put on the interpretation of allele frequencies, because they are less influenced by the actual mating system than are the genotype frequencies.

The oberved estimates on the actual heterozygosities fit in the range of those from other coniferous species. Any estimates on heterozygosities are ambiguous in the sense that they may not be representative of the whole genome and may depend on the applied enzyme systems and possible also on the applied methods (e.g. WARD, 1977, 1978; GILL, 1978; BROWN and LANGLEY, 1979). It is self-evident that this is not a problem peculiar to the present study. The actual 10-locus estimation may suffice to prevent severe biases but still has to be considered as preliminary. In any case, biases due to the dependence of the actual heterozygosity on the underlying allele frequencies were avoided by calculating the recently introduced conditional heterozygosities, which clearly indicate that more than 50% of the maximum attainable heterozygosity is realized in both samples.

The diversities in both samples appear to be above average and show only minor differences between the female and the male gametic contributions. The comparison of these two diversities can serve as a useful tool in characterizing the mode of harvesting cones, as well as, to a certain extent, in developing general ideas on the effectivity of the gene flow by pollen dispersal.

Considering the interpopulational variation, the genetic distances reveal a trend similar to that of the applied statistical tests, but they involve fewer ambiguities and show a more pronounced differentiation among the gene loci. In general, the trend in the variation between the samples appeared very clearly: Compared to studies with similar sample sizes and ten gene loci (including 3 GOT- and 2 SKDH-loci) in various provenances of Scots pine (MÜLLER-STARCK, 1987), the monitored interpopulational variation in *Cunninghamia lanceolata* is outstandingly high.

If these results are also representative for other provenances, they have consequences for the establishment of breeding populations. The outstandingly large number of alleles per locus in both provenances (4.0 to 4.3) seems to reflect a strategy of maintenance of great genetic variability. It was demonstrated that a tremendous number of genotypically different individuals can already be formed by the ten gene loci. In order to avoid a decline in adaptability in breeding populations as compared to natural populations, their sizes should not be limited to only a few hundred clones (for examples of minimum numbers of clones with respect to gene locus see HATTEMER *et al.*, 1982). The occurence of geographic specific genetic information also suggests the establishment of variety of such breeding populations.

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Appendix

Survey of the allele frequencies at each of ten gene loci among the combined female and male gametes in seeds of two provenance samples of *Cunninghamia lanceolata* Sample size is 302 for the provenance Hungya (1) and 228 for De-Chang (2). The number of seeds is equal to one-half of these values. In this combined mode of presentation frequencies of alleles which are unique in the female or the male set are halved.

Gene Locus	Provenance	Frequency of Alleles Nos. 0-6 among Two Provenances						
		0	1	2	3	4	5	6
GDH-A	(1)			.172		.828		
	(2)	.009	.004		.250	.737		
GOT-A	(1)	.007	.040	.950	.003			
	(2)	.009	.013	.978				
GOT-B	(1)	.007	.305	.003	.685			
	(2)	.009	.219		.772			
GOT-C	(1)		.003	.335	.553	.096		
	(2)			.219	.605	.175		
IDH-B	(1)	.007	.073	.838	.079	.003		
	(2)	.009	.044	.785	.162			
6PGDH-A	(1)		.026	.427	.010	.513	.023	
	(2)	.009		.482		.504	.004	
6PGDH-B	(1)		.013	.937	.050			
	(2)	.009	.018	.947	.026			
PGI-A	(1)	.023	.046	.003	.725	.179	.020	.003
	(2)	.018	.026		.645	.307	.004	
SKDH-A	(1)	.030		.017	.599	.129	.149	.075
	(2)	.031		.013	.842	.035	.035	.044
SKDH-B	(1)	.056	.563		.377			
	(2)	.044	.741	.009	.206			

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