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Do molecular markers reflect patterns of differentiation in adaptive traits of conifers?

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Abstract We have examined patterns of variation of several kinds of molecular markers (isozymes, RFLPs of ribosomal DNA and anonymous low-copy number DNA, RAPDs and microsatellites) and an adaptive trait [date of bud set in Scots pine (*Pinus sylvestris* L.)]. The study included Finnish Scots pine populations (from latitude 60°N to 70°N) which experience a steep climatic gradient. Common garden experiments show that these populations are adapted to the location of their origin and genetically differentiated in adaptive quantitative traits, e.g. the date of bud set in first-year seedlings. In the northernmost population, bud set took place about 21 days earlier than in the southernmost population. Of the total variation in bud set, 36.4% was found among the populations. All molecular markers showed high levels of within-population variation, while differentiation among populations was low. Among all the studied markers, microsatellites were the most variable ($\bar{H}_e$=0.77). Differences between populations were small, G_{ST} was less than 0.02. Our study suggests that molecular markers may be poor predictors of the population differentiation of quantitative traits in Scots pine, as exemplified here by bud-set date.

Key words Scots pine · Molecular markers · Isozymes · Population structure · Adaptive genetic variation

Introduction

Genetic markers have many different uses in plant breeding, evolutionary, and conservation studies (Adams et al. 1992). One of the goals of population genetic studies of forest trees based on markers is inferring the amount and distribution of variation in economically and/or adaptively important traits. This is important for seed zone designation and for planning conservation strategies.

It is often assumed that molecular markers can be used to predict the amount of quantitatively inherited variation. However, the amount of variation may well differ between quantitative traits and marker loci (Milligan et al. 1994). In quantitative traits, the level of genetic variation depends on a balance between mutation and selection, or between different selective pressures (Barton and Turelli 1989). Variation at isozyme, and other marker, loci may be governed mostly by mutation and drift (Kimura 1983).

Further, the level of differentiation between populations at neutral loci depends on a balance between migration and genetic drift (see, for example, Hartl and Clark 1989). Even low levels of migration will equalize gene frequencies between populations at such loci. When there is diversifying selection, the balance between selection and migration can result in considerable genetic differences between populations. While migration rates are equal for all genes, selection acts differently on different parts of the genome. Hence, neutral loci do not necessarily predict patterns of variation in traits subject to differential selection.

There is an extensive literature on variation of growth and survival between populations of conifers, based on provenance studies (Rehfeldt 1990). There are also many published reports of population variation and differentiation based on isozymes (Muona 1990; Hamrick et al. 1992). So far, there are very few reports on population variation based on nuclear-encoded DNA-markers (Neale et al. 1992).

Studies of quantitative and marker variation have rarely been combined. In the present study, we examine patterns of variation of several kinds of molecular markers (isozymes, ribosomal DNA, RFLPs, RAPDs and microsatellites) as well as an adaptive trait (date of bud set of first-year seedlings) in Scots pine. Our study area spans Finland, from latitude 60°N to 70°N, where there is a steep climatic gradient. The mean annual temperature between

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the northernmost and southernmost areas differs by 4.6°C, and the length of the growing season differs by 46 days. Transfer of seedlings between latitudes results in large effects on survival (Eiche 1966; Eriksson et al. 1980). Common garden experiments have also shown that populations are genetically differentiated and adapted to the location of their origin (Mikola 1982).

The objective of our paper is to compare levels of variability in these populations using the different classes of markers. Additionally, we want to test whether molecular markers reflect the differentiation between populations found in adaptive traits.

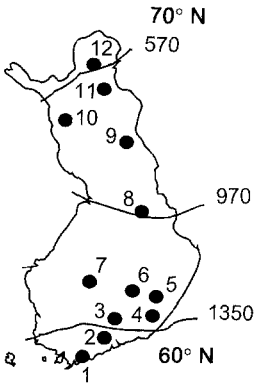

Fig. 1 Locations of Scots pine populations used in this study (*1*=Bromarv, *2*=Hausjärvi, *3*=Padasjoki, *4*=Punkaharju, *5*=Kerimäki, *6*=Viitaselkä, *7*=Saarijärvi, *8*=Sotkamo, *9*=Salla, *10*=Ylläs, *11*=Kaamanen and *12*=Alalompolo) and the temperature sums of growing seasons

Materials and methods

Plant material

Eleven populations of Scots pine from throughout Finland were sampled for the purpose of estimating levels of molecular-marker and quantitative trait variability and for examining the pattern variations across a south-north gradient. The same populations were not sampled for all markers. The origin of the samples and sample sizes are shown in Table 1 and Fig. 1.

Bud-set date

Samples of four populations of Scots pine from different latitudes (Table 1) were grown in a common garden experiment at the Punkaharju Forest Research Station. Seeds were bulked from several trees in each population. From each population, 450 seeds were sown on June 1st, 1994. Seedlings were grown under natural daylength in ten randomized blocks and the temperature followed the ambient temperature. The timing of the terminal bud set was scored twice a week from the beginning of August until the end of October. The bud was regarded as formed when it was seen clearly between the needles from above the seedling. Differences between the populations were tested by ANOVA (Sokal and Rohlf 1981) with populations being a random effect. Analyses were done with the SAS/STAT computer software (SAS Institute Inc. 1987).

Allozymes

Allozyme analyses, either from single-tree collections or bulked samples, were based on assays of embryos of germinating seeds (see Muona et al. 1988 for further details). We studied eight enzyme systems which yielded ten polymorphic loci: glutamate dehydrogenase (*Gdh*), glutamate-oxaloacetate transaminase (*Got-2*), fluorescent esterase (*Fest*), leucine amino peptidase (*Lap-2*), malate dehydrogenase (*Mdh-1*, *Mdh-3*), 6-phosphogluconate dehydrogenase (*6-Pgd-1*, *6-Pgd-2*), shikimate dehydrogenase (*Shdh-1*), and aconitase (*Aco*). Electrophoretic procedures are described in Muona et al. (1988).

Genotypic frequencies were used for calculating allelic frequencies and expected heterozygosities (H_e=1-Σpi^2). The proportion of variation between populations was estimated with G_{ST} (Nei 1973).

Restriction fragment length polymorphism

RFLPs were analysed in population samples and in a cross to verify the inheritance of the variation. Genomic DNA was prepared from needle samples of each tree by a modification of the CTAB method (Wagner et al. 1987). Needles (10 g) were frozen with liquid nitrogen and quickly homogenized into crude powder. The powder was mixed with cold extraction buffer and further homogenized (2×15 s, full speed). After these steps DNA extraction followed the procedure of Wagner et al. (1987). The same method of DNA isolation was also used in the rDNA and microsatellite studies.

RFLPs were studied by using one complementary DNA (cDNA) probe (PtIFG739) and two genomic DNA probes (PtIFG1D8, PtIFG1D11) from loblolly pine (Devey et al. 1991). The probes were hybridized to Southern blots containing Scots pine DNA cut with the restriction enzyme *Hin*dIII. Labelling of the probes, restriction digests, Southern blotting, and probe hybridization were conducted as described by Devey et al. (1991), with the exception of the final wash which was done under less stringent conditions (2×30 min in 0.5×SSPE, 0.1% SDS). The inheritance of RFLP bands revealed by the probes was determined from a sample of 20 progeny of the Scots pine full-sib cross E636C×E702 at the Finnish Forest Research Institute. Statistics were calculated as for allozymes.

RFLP analysis of ribosomal DNA variation

rDNA variation was studied in a sample of 97 individuals from four populations (Table 1). RFLPs were detected and analysed as described by Karvonen and Savolainen (1993). Shannon's index of phenotypic diversity (Hutcheson 1970) was used to quantify the levels of rDNA variation and to partition it between and within populations.

Random amplified polymorphic DNA (RAPD)

The expected heterozygosity can be regarded as the probability that an individual of a population is heterozygous at a random locus. This is usually estimated by considering a small set (about 10–20) of loci in many individuals. However, it is also possible to estimate average heterozygosity by examining a large number of loci in a single individual. This entails the assumption that the loci are independent, i.e. there is no correlation between the heterozygosity of different loci. In a large random mating population this assumption is likely to hold (see, for example, Savolainen and Hedrick 1995). Further, in random mating populations there is probably no linkage disequilibrium even between fairly closely linked loci (Hartl and Clark 1989). We have used this background in estimating variability and population divergence using RAPDs rather than combining data over individuals. The latter assumes homology of RAPD bands between different individuals, which is not always the case (Lynch and Milligan 1994). For each tree, one can obtain an estimate of the number of segregating and non-segregating bands. This can be regarded as an estimate of heterozygosity in the genome. This statistic (observed segregating bands/total number of bands) is binomially distributed. The estimate can be compared across trees representing different populations, even if we can not be sure that exactly the same genomic regions have been amplified in different individuals. This allows a comparison of heterozygosity between populations.

The same approach can be used for examining divergence between populations, in the following way. If two populations are not

Table 1 Populations of *P. sylvestris* and sample sizes for the different characters studied

Population	Growth rhythm	Allozymes	RFLP			rDNA	Microsatellites	
			1D8[a]	1D11[a]	c739[a]		9.3[b]	4.6[b]
1. Bromarv	291		23	20	22		25	26
2. Hausjärvi					22	22		
3. Padasjoki		300						
4. Kerimäki	323							
5. Viitaselkä			17	22	21	18		
6. Saarijärvi		450						
7. Sotkamo	186							
8. Salla	220							
9. Ylläs		100	22	19	17	30	25	20
10. Kaamanen			21	21	22	27		
11. Alalompolo		150						

[a] Probes (PtIFG–),
[b] Primers

genetically differentiated, an offspring between two individuals from the different populations should have the same average heterozygosity as the parents. If the populations are highly diverged, the hybrid between them should have increased heterozygosity. For instance, assume just one locus and two alleles with an average population frequency of alleles=0.5. If the populations were completely differentiated, G_{ST} would be 1.0 and the hybrid would be heterozygous at all loci. The heterozygosity of the hybrid is estimated with the proportion of segregating RAPD bands, as above. Again, the examined bands do not have to be from the same loci as in the parents, as we require a general estimate of heterozygosity.

We studied RAPD variation and divergence between northern and southern Finnish Scots pine populations with this approach. The trees were the plus trees E1101 from Punkaharju (Fig. 1) and P304 from the northern population in Salla. DNA was isolated from the haploid megagametophytic tissue of the seeds according to a modification of the method of Doyle and Doyle (1990). To estimate the segregating proportion of scorable RAPD bands, DNA from seven megagametophytes of each tree was tested with 120 10-base oligonucleotide primers (Operon). In the RAPD amplifications, the reaction volume was 12 µl, which consisted of 10 ng of template DNA, 0.8 µM of primer, 0.2 mM of each dNTP, 1.25 µl of 10×Taq buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, Promega), 2.6 mM of $MgCl_2$ (Promega), and 0.5 U of *Taq* DNA polymerase (Promega), overlaid with 50 µl of mineral oil. Reactions were carried out in microtitre plates (Hybaid) in a OmniGene temperature cycler (Hybaid). The amplification profile was 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 35 s at 35°C, 1 min at 72°C, followed by 10 min at 72°C. RAPD products were electrophoresed in 2% agarose gels with ethidium bromide in 0.5 M TBE-buffer at 3.75 V/cm, and photographed with a Polaroid camera under UV light.

Microsatellites

Microsatellite variation was studied in the Bromarv and Ylläs populations (Table 1). PCR reactions were done using primers from *Pinus radiata*. The sequences of the primers were 5′GAAAAAAAGGCAAAAAAAAGGAG3′/5′ACCCAAGGCTACATAACTCG3′ (P.R 4.6) and 5′GAAATTTAACACCACACCGTTG 3′/5′TGGGGCTTAAAGTGAAATGG3′ (P.R 9.3) (Smith and Devey 1995).

The PCR volume was 25 µl, containing 50 ng of genomic DNA template, 0.2 µM of each primer, 0.2 mM of each dNTP, 2.5 µl of 10×*Taq* buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, Promega) 2 mM of $MgCl_2$ (Promega) and 2.5 units of *Taq* polymerase (Promega). Samples were overlaid with 50 µl of mineral oil. Amplifications were performed in an OmniGene temperature cycler (Hybaid) with the following amplification profile: 2 min at 94°C; followed by 2 cycles of 1 min at 94°C, 30 s at 55°C and 1 min at 72°C; followed by 2 cycles of 1 min at 95°C, 30 s at 53°C and 1 min 72°C; 30 cycles of 1 min at 94°C, 30 s at 52°C, 1 min 72°C; and finally one cycle of 10 min at 72°C. PCR reactions were done using touchdown and secondary PCR to get maximum amplification. Amplified samples were electrophoresed in 4% agarose gels with ethidium bromide in 1×TBE buffer, and photographed with a Polaroid camera under UV-light.

Results

Bud set date

The mean number of days to terminal bud set in each population are shown in Table 2a. In the northernmost population, Salla, the bud set occurred about 21 days earlier than in the southernmost population, Bromarv. Statistically significant differentiation in the date of bud set between the populations was found with the analysis of variance (Table 2b). The between population component was 36.4%.

Table 2 **A** Mean days from sowing to bud set, number of observations (N) and standard deviations (SD) and **B** results of analysis of terminal bud-set formation between populations of *P. sylvestris*

A

Population	Mean	*n*	SD
Bromarv	113.6	291	13.0
Kerimäki	108.3	323	10.4
Sotkamo	101.8	186	8.8
Salla	92.3	220	13.1

B

Source of variation	*df*	Mean square	*F*
Population	3	20728.9	84.40***
Block	9	209.7	0.85
Population × block	27	245.6	1.90**
Error	980	129.6	

Significance level: ***, 0.001; **, 0.01

Table 3 Mendelian inheritance of RFLP variants in a cross between *P. sylvestris* plus trees E636C×E702. Genotypes of parents and progeny, number of progeny in each class, and χ^2-test for expected Mendelian ratios

Probe		E702	E636C	Offspring				χ^2
PtIFG1D8	Genotype	1–2	3–3	1–3	2–3			0.89
	Number of progeny			11	7			
PtIFG739	Genotype	1–4	1–3	1–1	1–3	1–4	3–4	3.78
	Number of progeny			3	6	7	2	
PtIFG1D11	Genotype	1–2	1–3	1–1	1–2	1–3	2–3	0.65
	Number of progeny			3	4	5	5	

Table 4 Restriction fragment length polymorphism. Allelic frequencies and expected heterozygosities (H_e) for three RFLP loci in populations of *P. sylvestris*

Probe	Allele	Populations			
		Bromarv	Viitaselkä	Ylläs	Kaamanen
PtIFG1D8	1	0.09	0.06	0.14	0.10
	2	0.28	0.35	0.18	0.26
	3	0.63	0.59	0.68	0.64
	H_e	0.52	0.53	0.49	0.51
PtIFG739	1	0.57	0.45	0.38	0.50
	2	0.09	0.05	0.21	0.02
	3	0.25	0.29	0.26	0.39
	4	0.09	0.21	0.15	0.09
	H_e	0.60	0.67	0.72	0.59
PtIFG1D11	1	0.88	0.82	0.71	0.62
	2	0.12	0.18	0.29	0.38
	H_e	0.21	0.30	0.41	0.47

Isozyme analysis

Eight enzyme systems provided ten polymorphic loci (frequency of the most common allele less than 0.95). Allele frequencies are not given. The average within-population heterozygosity ($\bar{H}_e$) for the populations was 0.34. The genetic differentiation between populations was very low with an average G_{ST} of 0.02 (see Table 7), and a range across loci from 0.001 to 0.046.

RFLP variation

The Mendelian inheritance at three RFLP loci was confirmed in a testcross (Table 3). The data obtained from the populations are presented in Table 4. Only one χ^2-value (Bromarv) indicated deviation from Hardy-Weinberg genotype frequencies (data not shown). There was a high level of variation ($\bar{H}_e$=0.49) due to the many frequent alleles, and low differentiation among populations (G_{ST}=0.02, see Table 7).

rDNA variation

Thirteen variable rDNA phenotypic patterns were scored among the 97 studied individuals, each of which represents the combined genotype for the eight rDNA loci that are present in the Scots pine genome (Karvonen et al. 1993). The frequencies of the rDNA patterns in northern and southern Finnish populations have been presented by Karvonen and Savolainen (1993). Differentiation between populations from northern and southern Finland accounts for 14% of the rDNA variation (see Table 7).

RAPD variation

The southern and northern plus trees E1101 and P304 had the same proportion of segregating loci (29%) among the large total number of bands (about 300). Thus, the general level of heterozygosity was similar in both populations. Further, the F_1 progeny (P315×E1101) had slightly fewer segregating loci (25.4%) (Table 5). However, the difference was not statistically significant. These results support the assumption that there is little differentiation between the southern and northern trees with respect to the RAPD markers, even though we cannot quantify this with a G_{ST} estimate.

Table 5 Proportion of segregating RAPD loci of all scorable (monomorphic + segregating) loci (and binomial standard errors) in *P. sylvestris* plus trees E1101, P304 and in the F_1 tree from a cross P315×E1101. Ambiguously amplifying bands were excluded

Tree	Number of scorable loci	Number of segregating loci	Proportion (%) of segregating loci
E1101	295	86	29.2 (0.026)
P304	290	84	29.0 (0.027)
P315 × E1101	339	86	25.4 (0.02)

Table 6 Microsatellite polymorphism of *P. sylvestris*. Allelic frequencies and expected heterozygosities (H_e) in two populations

Primer	Allele	Populations	
		Bromarv	Ylläs
P.R 4.6	1	0.02	0
	2	0.12	0.08
	3	0.04	0.13
	4	0.08	0.1
	5	0.06	0.08
	6	0.25	0.20
	7	0.05	0
	8	0.38	0.25
	9	0	0.05
	10	0	0.08
	11	0	0.03
	H_e	0.76	0.85
P.R 9.3	1	0.08	0.04
	2	0.46	0.36
	3	0.12	0.16
	4	0.22	0.20
	5	0.10	0.24
	6	0.02	0
	H_e	0.71	0.75

Table 7 Proportion of variation between *Pinus sylvestris* populations and total variation, when applicable. For allozymes, RFLPs and microsatellites, variation between populations was estimated as G_{ST} values. For rDNA, the proportion of variation between populations was estimated by using Shannon's diversity index. See text for details

Item	Variation between populations (%)	Total variation (H_T)
Bud set	36.4	–
Allozymes	2.0	0.34
RFLP	2.0	0.54
rDNA	14.0	–
Microsatellites	1.4	0.74

Microsatellite variation

Frequencies of the microsatellite alleles and expected heterozygosities are given in Table 6. Among all tests, there was only one significant deviation from H-W expectation (Bromarv P.R 4.6.) (data not shown).

We found 11 and six different alleles at the microsatellite loci. This resulted in very high levels of expected heterozygosity ($\bar{H}_e$=0.77). Genetic differentiation among populations was low with an average G_{ST} of 0.014 (Table 7).

Discussion

Amount of variation

This study allows a comparison of variation between allozymes and different types of DNA markers. The relationship of marker variation to the variation of quantitative traits can also be examined.

Single-locus marker types differ greatly in the level of variation (Tables 2–7). Isozymes are highly variable in *P. sylvestris*, compared to many other plant species (Hamrick et al. 1992), but RFLPs and especially the two microsatellite markers show even higher variability. The three specific RFLPs were chosen because they could be interpreted both in loblolly pine and Scots pine. They may not be a representative sample, but they do demonstrate that highly variable RFLPs can be found. We do not know of comparative population RFLP data for other conifers.

The microsatellite loci were chosen because their primer sequences were available from *P. radiata* (Smith and Devey 1995). They proved to be highly variable, the highest within-population expected heterozygosity was 0.85. While we have too few loci to estimate average microsatellite variability reliably, even a few loci with this level of variability can provide substantial information for many purposes. Smith and Devey (1995) found an expected heterozygosity of 0.60 for those same microsatellites in *P. radiata*.

For RAPDs, our estimate of proportion of segregating loci in an individual tree is about 30%. If this can be regarded as an estimate of heterozygosity, then this value agrees with the isozyme estimates for *P. sylvestris*. However, there can be problems in the identification of loci (see, for example, Lynch and Milligan 1994), so a direct comparison may not be appropriate. A higher estimate has been obtained for *Pinus attenuata* (Wu et al. 1996). The phenotypically scored rDNA RFLPs were also quite variable, but their usefulness is limited because of the lack of direct genetic interpretation. Among all these markers, microsatellites are clearly the markers of choice for QTL mapping or neutral marker studies because of their high variability. The only drawback is the difficulty and cost involved in finding them (Smith and Devey 1995). The different levels of variability in different single-locus markers are most easily explained in terms of mutation rates. It is well known that repetitive DNA sequences have high mutation rates (Jeffreys et al. 1988).

In our data set, populations were equally variable for all markers, and thus they all give the same information regarding relative levels of variability. As for the quantitative trait, our measurements were at the phenotypic level, but in a common garden environment. Phenotypic coefficients of variation of bud-set date in individual populations ranged from 0.09 to 0.14, but there was no trend for less variation in the north.

Other studies have also shown that, while the level of isozyme variability is similar in all populations (Hamrick et al. 1992), the level of quantitative variation among traits may differ (see, for example, Cornelius 1994). Often quantitative trait variation is expressed as heritability. However, in the long run, the adaptability of a trait is governed by the amount of genetic variation, and not by the ratio of additive genetic variation to the total phenotypic variation (Houle 1992). Thus, the desirable measure will be the additive coefficient of variation.

Undomesticated forest tree populations are so variable that measuring the amount of variability has not been a major concern. As suggested above, molecular markers may not be accurate predictors of variability in all quantitative traits. A complete lack of marker variation may predict lack of morphological variation, as in red pine (Fowler and Morris 1977; Mosseler et al. 1992). However, in *Acacia mangium* some growth variation is present even if markers are monomorphic (Moran et al. 1989). It is likely that differences in the strength of selection are responsible for the different patterns.

Differentiation between populations

All DNA markers suggest that much, or most, of the Scots pine nuclear genome is not differentiated between populations. For all single-locus marker groups, G_{ST} was less than 0.02. Low levels of differentiation have been found earlier for isozymes of Scots pine (Gullberg et al. 1985; Muona and Harju 1989) and also for most other species of conifers (see Hamrick et al. 1992 for a review). However, maternally inherited DNA markers in the mitochondrial genome of several pine species are highly differentiated between populations (Strauss et al. 1993).

More than a third of the variability in the date of bud set of first-year seedlings in a common environment was between populations. This result is in accordance with the earlier work of Mikola (1982) on the bud set, and of Aho (1994) on the frost resistance, of seedlings of Scots pine. Natural selection in the form of mortality of seedlings due to lack of frost tolerance is efficient (Eiche 1966), and accounts for the differentiation. Similar results of high differentiation have been found for many quantitative traits in trees, most often related to patterns of growth or phenology (Campbell 1979; Rehfeldt 1990, 1992; Li and Adams 1994; Yang et al. 1996). The degree of differentiation varies across species and traits. However, not all quantitative traits are differentiated, morphological characteristics of cone scales, for example, are similar across wide areas, presumably because they are not related to local survival (Koski 1970).

The phenotypic rDNA results were not directly comparable. However, if the genotypic microsatellite data of locus 9.3. are analysed with Shannon-Weaver statistics, the estimate is 10% for divergence. Thus rDNA data are in a range similar to other markers.

Marker genes are thus poor predictors of population differentiation of the quantitative traits of conifers. The contrasting patterns of variability can be understood as the results of a balance between mutation, migration and selection (Hedrick 1985). The efficient pollen flow (Koski 1970; Harju and Muona 1989) and lack of differential selection are responsible for the uniformity of populations in respect of most of the genome. Strong differential selection diversifies the loci that are responsible for local adaptation.

Even if molecular markers do not carry much information on quantitative variation, we emphasize that they are extremely useful tools in many applications requiring information about breeding systems, in the identification of parents of crosses, or monitoring changes of variability due to drift (Adams et al. 1992). Data on neutral markers now have to be interpreted as evidence of processes influencing the whole genome. Thus, marker studies can be no substitute for direct measures of quantitative variation influenced by differing spatially varying selection. However, recent advances in molecular methods allow the finding of markers very close to, or even at, loci influencing quantitative traits (Groover et al. 1994). Such markers would provide direct information about the traits. It may also be possible, soon, to have numerous DNA markers for structural genes which are adaptive and show a high degree of differentiation.

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