GENETIC MARKERS: TOOLS FOR IDENTIFYING AND CHARACTERISING SCOTS PINE POPULATIONS

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SUMMARY

Genetic diversity is the basis for ecological biodiversity; it can only be assessed by using markers which reflect the variation present within the genome. Biochemical plant constituents interact with other components of the ecosystem and are thus of greater value as adaptive markers than as truly genotypic characters; however, the terpene components of coniferous resin systems are under strong genetic control and are valuable population diversity indicators. Molecular markers are impervious to environmental modification and therefore represent the genotype most faithfully. A survey is presented of the applications of the major biochemical and molecular marker systems in the analysis of Scots pine populations, together with indications of the advantages and limitations of each category of marker. The unusually high phenotypic diversity evident within the species at the morphological and physiological levels, both within populations and between geographical regions, is further substantiated by the degree of genotypic diversity evident from biochemical and molecular analysis. Molecular markers currently available mostly measure selectively neutral variation; among the most important needs for the future is the development of molecular markers for adaptive traits.

KEY WORDS: Genetics
Markers
Variation
Diversity
Adaptation

INTRODUCTION

Scots pine *(Pinus sylvestris* L.) is the most widely distributed coniferous species. Its vast natural range encompasses a great diversity of ecological and physiographic conditions, which have caused the species to evolve and fragment into a large number of local variants or «races». Many of these are recognisably discrete, and have been afforded subspecific or varietal status. In addition to this extensive regional variation, the genetic diversity within races or populations of Sots pine is also known to be very high. It is this

genetic diversity which is the basis for the development of ecological biodiversity among all those animal, plant, and fungal species which are associated with, and depend upon, Scots pine dominated ecosystems. A high degree of genetic diversity at the morphological, physiological and biochemical levels between individuals within tree populations will stimulate the emergence of a concomitantly wide range of genotypes within coexisting species, as well as allowing opportunities for an increased biodiversity above the specific level.

Morphological features are inadequate for studying genetic diversity, owing to their limited representation of the total genome, and to the high degree of environmental modification to which they are susceptible. It is essential to use markers which usefully reflect the identity of the genetic material of the plant. In practice this has involved the analysis of the biochemical products of gene activity; the best biochemical compounds to use are the most direct, or primary, products of transcription of the genome, rather than derived secondary compounds whose biosynthesis can be subject to environmental pressures. In theory, the ultimate markers in terms of immunity from environmental modification are the base sequences of the genomes themselves. The history of marker technology has been one of a gradual approach to the genome. Early work involved the analysis of secondary gene products, which are easily separable and identifiable using relatively simple techniques. Later advances in technology led to the study of isozyme variants of the enzymes which initiate the formation of the secondary compounds, and directly mirror transcribed base sequences within the genome. Finally it has now become possible to identify polymorphisms within the nucleic acid base sequences themselves (Forrest, 1994).

Biochemical markers such as phenolic and terpenoid compounds are comparatively simple to analyse, but in addition to their susceptibility to a degree of environmental modification they have the disadvantage of representing only a very small and biased fraction of the total genome. The development of molecular markers based on DNA sequences has overcome this drawback, so that there is now no theoretical limit to total genomic analysis. The genomes of conifers, however, are very large even by human genome standards, and consist for the most part of non-coding sequences, many of which are highly repetitive. As a consequence, molecular markers are generally selectively neutral, in the sense that they predominantly sample parts of the genome which have no effect on the biological performance of the plant. Thus the great majority of molecular markers currently in use provide no information on variation in adaptive traits, which are of paramount importance to the survival of the plant and to the tree breeder who wishes to ensure the suitability of planting material to planting site and to maximise the development of traits of economic importance. Instead, these markers, being uninfluenced by selective pressures, are of value for providing a record of the evolutionary history of populations, for tracing their migration routes, and for indicating their former population sizes and degree of fragmentation (Ennos *et al.,* 1998), as well as for assessing general levels of genotypic variation within and between populations. The majority of enzymes studied in isozyme analysis also probably fall into this category of marker system.

Conversely, the secondary compounds commonly used as markers are in many cases believed to have some adaptive significance to the plant. They may have a direct influence on its ability to respond to the attacks of pests and diseases and to tolerate adverse climatic conditions; or they may be closely linked to genes which control such traits, and can thus be used as indirect markers for these traits. They may therefore be used as adaptive markers, and can identify individuals or populations showing adaptive features of im-

portance to the forester. Despite their comparative instability in the face of environmental variation, biochemical markers nevertheless have a valuable role to play in the study of those interactions between plants and their environment which determine their ecological fitness and contribute to the biodiversity of the ecosystem.

It is important to appreciate that patterns of adaptive variation are caused by natural selection, whereas patterns of selectively neutral variation originate as a result of migratory events, gene-flow within and between populations, and genetic drift. It follows that the spatial distribution of the genetic variation within and between populations derived from the analysis of biochemical marker systems is likely to differ substantially from that based on molecular markers. The two classes of marker systems serve to characterise different sets of attributes dispersed among Scots pine populations, and the data deriving from their distinctive natures should be interpreted accordingly. Within the context of the biodiversity of a wide-ranging species such as Scots pine, a variety of different processes will have contributed to the genetic diversity which we presently observe, ranging from interglacial population differentiation, the historical sequences of large-scale postglacial population migrations, to the many forms of selective pressures which have operated to varying degrees over time and space up to the present day.

PHENOLICS

Overview of phenolic compounds as biochemical markers

The phenolic compounds occuring in plants include a wide range of molecules, which are difficult to extract quantitatively from plant tissue and to analyse and quantify satisfactorily. The flavonoids are an important and diverse class of phenolic compounds which occur abundantly in conifers; they are of modest molecular size and fall into a number of well-defined natural and biosynthetically related groups. However, there are in addition several classes of larger molecular size such as the lignins and tannins, which are also of fundamental importance to the plant, and these may be of very large or indefinite molecular structure. Different extraction techniques lead to qualitative as well as quantitative differences in the phenolics obtained from a given tissue type, so that in quantitative work it is essential to optimise and then to standardise the protocol for the material to be analysed. There are several different techniques available for estimating «total phenolics» in a plant extract, but they in fact measure different attributes and inevitably give different results, which are not necessarily comparable even in relative terms. Phenolic constituents can be degraded during sampling and extraction by commonly occuring oxidases and other enzymes. Techniques used in subsequent handling and storage of extracted material can affect the phenolic concentrations, and treatments such as freeze-drying and oven-drying can cause profound alterations and interconversions of phenolics and their glycosides unless strict precautions are taken. As is the case with terpenoids, the phenolic components of plants are subject to environmental modification, and to a generally much higher degree. Their ubiquitous occurrence throughout the various tissues and organs of the plant, coupled with differential production of different components in different tissues, makes them rather less satisfactory as a class of genetic markers than the terpene components of discrete coniferous oleoresin systems. Despite these drawbacks, there can be little doubt that the

phenolics are of great significance in their involvement with resistance to many herbivores, both insects and higher animals, and to pathogenic fungal diseases.

Chemotypic and geographic variation

Relevant data for Scots pine are scanty, and highly biased towards a small representation of the more easily extractable and readily identifiable components of needles. In an early report, Thielges (1972) found only minor differences in paper chromatographic patterns of phenolics between 26 provenances of Scots pine from a wide range of British, European, and Russian origins growing in a provenance test in Michigan, and no correlation between morphological and phenolic variation. The existence of distinct chemotypes in European populations has been demonstrated (Lebreton *et al.,* 1990). Populations from low elevations are characterised by the presence in needles of the flavonoids taxifolin (dihydroquercetin), quercetin, and procyanidin, whereas high altitude or high latitude populations consist almost entirely of prodelphinidin-rich chemotypes. Differentiation among 12 populations within Poland was demonstrated by Szweykowski and Urbaniak (1982), and quantitative differences in phenolic composition of bark phloem were observed between two populations from France and Poland (Quencez, 1996). Young seedlings from nine seed origins within Finland and Estonia planted at three locations at different latitudes in these countries were studied by Nerg *et al.* (1994); concentrations of total phenolics were lowest in the most northerly planting site, but did not differ significantly between seed origins over this relatively restricted geographic range.

Association with biotic and abiotic factors

Fungi

The levels of taxifolin glucoside in the phloem of Scots pine stems may be indicative of susceptibility to attack by bark beetles and their associated fungi, whereas synthesis of other phenolics, including stilbenes and the flavonoid pinocembrin, appears to be an induced response (Lieutier *et al.,* 1996; Bois and Lieutier, 1997). However, these effects may be a generalised response to wounding rather than a specific reaction to particular aggressors. The growth *in vitro* of the fungi studied by these authors was inhibited by pinocembrin and the stilbene pinosylvin (Bois, 1996). Gibbs (1972) had previously shown from *in vitro* studies that pinosylvin was inhibitory to the growth of the butt-rot fungus *Heterobasidion annosum* (Fr.) Bref.; while Bonello *et al.* (1993) found that this fungus induced significant accumulations of stilbenes both locally and systemically in more susceptible seedlings. There are reports of an accumulation of pinosylvin in seedlings infected with *Botrytis cinerea* (Fr.) Pers. (Gehlert *et al.,* 1990) and of pinosylvin dimethyl ether in Scots pine infected with *Peridermium pini* (Pers.) Lév. (Westfelt, 1966). Bonello and Pearce (1993) challenged Scots pine seedlings with the root pathogen *Cylindrocarpon destructans* (Zinssmeister) Scholten, and reported an increase in the content of cell wall-bound phenolics with a concurrent decrease in soluble phenols.

Herbivores

Thielges (1968) reported an induced response of the polyphenol metabolism of Scots pine to pine sawfly *(Neodiprion sertifer* Geoff.) attack. Larsson *et al.* (1992) found that larvae of *N. sertifer* and *Diprion pini* L. reared on either of the two distinct flavonoid chemotypes of Scots pine (see previous Section) showed no large differences in survival or weight, suggesting that taxifolin and other associated flavonoids have no effects on insect performance. Auger *et al.* (1994) have related the relative toxicity of different clones of Scots pine to *Diprion pini* larvae to the relative proportions of taxifolin and its glucoside. However, improved prediction of levels of toxicity have been derived from relative rates of interconversion of the two compounds in the needles, although this reaction is not insect-specific and feeding bioassays with taxifolin did not show any direct effect on larval development (C. Bastien, *pers. comm.).*

Browsing of Scots pine by arctic hare *(Lepus timidus* L.) was discouraged by a high total phenolic content of bark (Rousi and Häggman, 1984). However, a later report (Rousi *et al.,* 1987) indicated that additional factors, such as the physical characteristics of the bark, can be important in influencing choice of browse material, so that phenolic concentration *per se* is not a reliable predictor of susceptibility. Damage by voles *(Microtus* spp.) in relation to total phenolic content of saplings has been studied in Finland by Rousi (1989), who found no correlation. Conversely, choice of food source by moose *(Alces alces* L.) was reported to be highly correlated with phenolic composition of the needles (Sunnerheim-Sjöberg and Hämäläinen, 1992); taxifolin glucoside from tissue extracts was shown to have a deterrent effect on palatibility (Sunnerheim-Sjöberg, 1992).

Abiotic stress

The levels of phenolics in needles have been studied in relation to industrial pollution, both under controlled conditions (e.g. Giertych and Karolewski, 1993) and in the field (Karolewski and Giertych, 1995); induced increases in total phenolics were reported in both cases. Similar effects were caused by the presence of toxic metal ions in the nutrient media of containerised seedlings (Karolewski and Giertych, 1994). A dose-dependent induction of stilbene synthesis by exposure of seedlings to ozone was found by Rosemann *et al.* (1991); there were also interactions of this effect with *Heterobasidion annosum* infection (Bonello *et al.,* 1993). Induction of pinocembrin and stilbene synthesis in stressed seedlings was studied at the enzymic and molecular levels by Fliegmann *et al.* (1992). Chalcone synthase (a key enzyme in the biosynthesis of flavonoids including pinocembrin) was already present in non-stressed seedlings, whereas stilbene synthase was detectable only after a lag period following induction; this suggests that stilbene formation is a reaction to stress, as found also as a result of exposure to ozone and to various fungal infections.

TERPENES

Overview of terpene analysis in conifers

Terpenes are the major components of the resin systems and of the leaf oils of conifers. A resin sample is a complex mixture of many terpenes, with the composition being under strong genetic control, and to a large extent independent of environmental conditions (e.g. Baradat and Yazdani, 1988). In many species the terpene composition is related to the origin of the plant material, so that terpenes can be used as a means of studying the genetic variation on a geographical or ecological basis, and of verifying the origin of material of disputed or unknown provenance. Examples for a wide range of North American conifers are given by von Rudloff (1975). Terpenes are also of direct ecological and selective significance, since they are associated with adaptive features such as resistance to pathogens and climatic tolerance. Analysis by capillary gas-chromatography is technically straightforward and can be automated. Each chromatogram yields a large amount of genetic information for a variety of terpenes of different classes (monoterpenes, sesquiterpenes, and diterpenes including resin acids). Moreover, the compositions of the different discrete resin systems within a given plant (vegetative bud, stem cortex, leaf oleoresin, etc.) are under separate genetic control and in many cases quite different, so that analysis of more than one resin system can enhance the quantity of genetic information obtained.

Geographic variation

A number of surveys of the population variation in Scots pine terpenes have been published, both on national and range-wide scales; these are summarised in Table 1. The first major report (Tobolski and Hanover, 1971) used material from a provenance collection in Michigan, and showed a high degree of variation in composition over the natural range and significant geographical trends in the relative concentrations of certain monoterpenes. These features were found by Ruby and Wright (1976) to be strongly correlated with genetic traits in adaptive characters; several of the recognised taxonomic varieties of Scots pine with proven genetic basis have characteristic terpene compositions.

Surveys of native populations in Finland have shown a cline in the concentrations of certain terpenes from north to south (particularly in the proportion of trees rich in 3-carene) (Juvonen and Hiltunen, 1972), while in Sweden the frequency of trees whose needles contain the diterpene alcohol isoabienol is greater in northern provenances (Gref, 1981). In general, monoterpenes show much greater population differentiation than resin acids or other diterpenes. Terpene analysis has identified the descendants of preglacial refugial populations in Spain (Pardos *et al.,* 1990), the French Massif Central (Weissmann and Lange, 1990), and north-west Scotland (Forrest, 1980; Kinloch *et al.,* 1986).

Within the UK, about 45 populations have been intensively sampled (Forrest, 1980, 1982); the north-western Scottish populations differed from all others in shoot cortical monoterpene composition, Shieldaig being the most highly differentiated woodland. These north-western woodlands are similar in terpene composition to certain French and Spanish populations, with which they may share a common ancestry. South-western populations formed a less distinctive regional group; while Barisdale, the most remote and isolated woodland in the far west, was distinct from other woodlands.

TABLE 1

POPULATION VARIATION IN SCOTS PINE TERPENES

Variación en terpenos de las poblaciones de Pino silvestre

Region	Number of populations	Resin system	Terpenes analysed	Population distinction	Authors
Eurasia	108	Stem cortex	Monoterpenes	High	Tobolski and Hanover, 1971
	188	Stem cortex	Monoterpenes	High	Ruby and Wright, 1976
	50	Main stem	Resin acids	High	Bridgen and Hanover, 1982a
	17	Stem cortex	Monoterpenes	High	Forrest, 1982 & unpubl.
Finland and Estonia	72	Needle oil	Monoterpenes	Moderate	Juvonen and Hiltunen, 1972
	73	Needle oil	Monoterpenes	Moderate	Hiltunen, 1975
	9	Shoots	Monoterpenes Resin acids	Moderate Nil	Nerg *et al.,* 1994
	4	Needle oil	Terpenes Resin acids	Moderate Moderate	Manninen *et al.,* 1998
France	2 1	Twig oil Stem xylem	Terpenes Terpenes	High	Chalcat *et al.,* 1985 Weissmann and Lange, 1990
Scotland	45	Stem cortex	Monoterpenes	Moderate	Forrest, 1980
Spain	7	Stem xylem	Monoterpenes Resin acids	Moderate Nil	Pardos *et al.,* 1990
Sweden	12	Needle oil	Isoabienol Resin acids	Moderate Nil	Gref, 1981
	26	Stem buds	Monoterpenes	Moderate	Yazdani *et al.,* 1985
	10	Stem buds	Monoterpenes	Moderate	Yazdani and Nilsson, 1986
USSR	126	Stem	Monoterpenes	Moderate	Chudnyi and Prokazin, 1973

Association with biotic factors

The terpenes of conifers may be directly involved biologically in host-pathogen interactions, in addition to being of possible use as indirect genotypic markers for resistance. In some cases induced changes in the terpene composition have been reported as a result of herbivore or fungal pathogen attacks. However, only a small fraction of the relevant literature is concerned with Scots pine.

Fungi

Various terpenes have been shown to be toxic to fungal growth *in vitro,* but few studies have dealt directly with pathogens of Scots pine. Gibbs (1972) showed that oleoresin collected from Scots pine caused substantially greater reduction in the growth rate of *Heterobasidion annosum* isolates *in vitro* than that collected from Corsican pine *[P. nigra* var. *maritima* (Ait.) Melville]. However, Ladejtschikova and Pasternak (1982) found no relation between monoterpene composition and the resistance of Scots pine to *H. annosum,* and Gref (1981, 1982) also found no relation between several diterpenoid resin acids and resistance to two fungal parasites. Inoculation of seedlings with the root pathogen *Cylindrocarpon destructans* caused an increase in mean content of antifungal resin acids in the roots (Bonello and Pearce, 1993). Delorme and Lieutier (1990) studied the effects of inoculation of Scots pine trees with fungi associated with bark beetles; the subsequent growth of the mycelium was limited by increased quantities of resin (and of total monoterpenes, which increased three-fold) rather than by qualitative changes in monoterpene composition.

Herbivores

Bridgen and Hanover (1982b) found a seasonal relationship between the levels of diterpenoid resin acids and the feeding intensity of several insect pests on Scots pine of Swedish origin. They further found correlations between the levels of specific individual resin acids and resistance to these insects in a study involving 50 seed origins. Larsson *et al.* (1986) and Björkman and Larsson (1991) also found that diterpenoid resin acids were involved in the feeding behaviour of sawfly *(Neodiprion sertifer)* larvae on Scots pine in Sweden; feeding on high resin acid shoots increased the resistance of the larvae to predatory ants, but reduced their growth and survival. Evaluation of clonal seed orchard material in the Latvian SSR for resistance to the shield bug *Aradus cinnamomeus* Panz. and pine shoot moth *Rhyacionia duplana* Hb. showed that progeny having the highest production of resin was most resistant (Baumanis *et al.,* 1982). Monoterpene composition may influence host selection by pine shoot beetles (Scolytidae) (Sjödin *et al.,* 1989; see also *New Scientist* 7 August 1986 p. 27). In contrast to these reports, Buratti *et al.* (1990) obtained no evidence for a direct relationship between the levels of diterpene resin acids in needles and the feeding behaviour of the sawfly *Diprion pini;* while Manninen *et al.* (1998) found no consistent relationships between needle terpene or resin acid concentrations and susceptibility to three aphid species. Sadof and Grant (1997) found that limonene was the only monoterpene component of volatile emissions from several distinct genotypes to show a consistent correlation with resistance to Zimmerman pine moth *(Dioryctria zimmermani* Grote).

Studies on the effects of secondary metabolites on animal browsing behaviour have concentrated mainly on phenolic compounds. However, Danell *et al.* (1990) found that while there was no correlation between monoterpene concentration in the needles of 30 Scots pine clones and the degree of moose browsing they suffered, there was a significant negative correlation with the concentration of the diterpene pinifolic acid. Sunnerheim-Sjöberg (1992) reported that the glucoside of the monoterpene angelicoidenol, found to occur in Scots pine twigs, acted as a major deterrent to moose feeding, more so than any phenol-containing fractions. Consumption of needles by capercaillie *(Tetrao urogallus* L.) was significantly reduced by artificially increasing the resin concentrations in the food, by spraying resin-rich ether extract on the needles (Sjöberg and Lindén, 1991).

Future development

Much published work has been based upon the analysis of the composition of only a single resin system within the plant, and frequently upon only a subset of the total terpenoid compounds present (commonly the monoterpenes or the resin acids). Parallel data from more than one resin system (e.g. foliar volatiles in conjunction with stem cortical oleoresin) are easily obtainable from the same shoot samples, and can provide enhanced genotypic information. The data can be further amplified by analysis of all classes of terpenes, which is generally possible in a single GC analysis. In population surveys of genetic diversity, the repertoire of statistical analyses employed should be optimised to derive maximal output from the data generated. Distinctions between populations can be graphically illustrated by various forms of cluster analysis, by canonical variate analysis, and by generating Andrews curves (Andrews, 1972).

ISOZYMES

Isozyme analysis: definitions and theoretical background

Isozymes are the distinct molecular forms of an enzyme which may be present in a given species; if they are alternative products of the same gene locus, they are called allozymes. The isozymes of an enzyme catalyse the same or similar biochemical processes inside the cell or organelle. After the mechanisms of genetic control had been elucidated, isozyme gene markers emerged as powerful tools in genetics and ecology. The distinct molecular forms can be separated by their differential mobilities in an electric field (electrophoresis); of the various electrophoretic techniques currently available (Rothe, 1994), horizontal starch gel electrophoresis is the most widely used. Isozymes are co-dominant markers, so that both alleles at a locus can be identified. In conifers the endosperm is haploid so that observations of segregation in seeds provide information on inheritance. The question of whether allozyme polymorphisms are selectively neutral or adaptive is controversial; interpretation will depend upon the species, ecophysiological conditions, and the roles of different enzyme systems in metabolism. Various statistical techniques are available for data processing, especially with respect to population differentiation and diversity estimates (Müller-Starck *et al.,* 1992; Goudet *et al.,* 1996).

The number of isozyme studies in tree species has increased greatly in recent years, with Scots pine being one of the most intensively studied species (Prus-Glowacki, 1991). Isozyme analysis has been used in botanical systematics, and has contributed towards an improved phylogeny of pine species as recently published by Price *et al.* (1998). Comparative isozyme surveys show that gymnosperms have high values of genetic diversity, species with wide distributions in the boreal-temperate zones having the highest values (Hamrick *et al.,* 1992).

Geographic variation, diversity and reproductive systems

Scots pine exhibits low levels of genetic differentiation among populations within regions, but greater differentiation between populations derived from different glacial refugia (Müller-Starck *et al.,* 1992). An early study of isozyme variation between eight Polish populations showed that those from northern Poland formed a separate group (Krzakowa, 1982). Initial studies involving populations from several European states showed that southern provenances (from Spain, Hungary, and Turkey) were clearly distinct from northern ones (Mejnartowicz, 1979; Kieliszewska-Rokicka, 1981). There are no complete range-wide studies available for Scots pine, but Table 2 lists genetic data for mean number of alleles per locus (A_L) and diversity (H_o, H_e, G_{ST}) as in Müller-Starck *et al.* (1992). Comparison of isozyme data sets is problematic because of variation in sampling methods, population characteristics, and experimental and statistical protocols; variation estimates decrease as sample size increases, so that only analyses involving more than five loci have been listed here. Heterozygosity is found to be close to 0.3 in northern Europe when sample size exceeds 30 individuals or when more than 10 loci are assayed (e.g. Kinloch *et al.* 1986; Muona and Szmidt 1985). Notably lower values were found in Lapland and in China (Wang *et al.,* 1991). Prus-Glowacki and Stephan (1994) showed that populations from Spain posses lower values of heterozygosity than populations from further north and east; the high values of around 0.35 for other northern areas could have been due to small sample sizes. Levels of genetic differentiation between populations are somewhat higher in southern and western areas than elsewhere. Decrease in genetic variation towards the margins of a species' range has also been found in other conifers (Guries and Ledig, 1982; Li and Adams 1989), but may be undetected in populations not growing in markedly marginal habitats and therefore not subject to directional selection or drift. Further bias may arise when the genetic variation of old stands is compared with that of younger stages, owing to the increase in heterozygosity with age in conifer populations (see Section below).

Isozymes have been extensively used in the study of the mating systems of Scots pine populations, both in natural stands and in seed orchards (Müller-Starck, 1991). Mating occurs preferentially between neighbouring trees, and inbreeding rates up to 33 % have been recorded (Müller, 1977; Rudin *et al.,* 1974), depending on seed tree density, climatic conditions, and flowering intensity. Early inbreeding depression, i.e. embryonic recessive lethals, eliminates a large proportion of selfed progenies during embryo development (Kärkkäinen and Savolainen, 1993). It has been found that only 25 % of the seedlings within 5 m distance from the seed tree had that tree as seed parent (Yazdani and Lindgren, 1992). Future research on the mating systems of forest trees and of the effects of forest management will be enhanced by computer modelling.

TABLE 2

GEOGRAPHIC VARIATION IN SCOTS PINE POPULATION DIVERSITY BASED ON ISOZYMES

Variación geográfica de la diversidad de poblaciones de Pino silvestre basándose en isoenzimas

Region	No. of pops.	No. of loci	A_L	H_o	H_e	G_{ST}	Authors
China	4	14	2.5	0.205	0.206		Wang *et al.,* 1991
NE Europe	16	7	2.74	0.361	0.363	2.5	Prus-Glowacki and Stephan, 1994
Spain	7	7	2.85	0.310	0.325	4.0	
Germany	9	10	3.1	0.268		2.0	Müller-Starck, 1987
Poland	9	9	2.6		0.385		Mejnartowicz and Bergmann, 1985
	6	8	3.3		0.376		Mejnartowicz and Palowski, 1989
Russia	18	21	4.1	0.286	0.282		Goncharenko *et al.,* 1994
	13	8	2.6	0.357	0.356	0.7	Prus-Glowacki and Bernard, 1994
Scotland	14	16			0.303	2.8	Kinloch *et al.,* 1986
Sweden	3	9	2.9	0.297	0.303	1.0	Gullberg *et al.,* 1982
	9	9	2.9			2.0	Gullberg *et al.,* 1985
	3	14	3.0		0.25-0.30	0.6	Muona and Szmidt, 1985
(Lapland)	3	14	2.9	0.193	0.219		Wang *et al.,* 1991

Abbreviations:
A_L - mean number of alleles per locus
H_o - observed heterozygosity
H_e - expected heterozygosity
G_{ST} - percentage of total diversity between populations

Anthropogenic influences on genetic structure

Seed orchards

Seed orchards may be set up for the regular provision of a supply of easily harvestable seed, for the improvement of seed quality (genetic gain), and for gene conservation. Seed orchard design should take account of variation in reproductive success between clones. Contamination by background pollination in Scots pine seed orchards has been estimated to reach levels of 60 percent (e.g. Harju and Muona, 1989), which causes severe losses in expected genetic gain. High rates of selfing will increase empty seed percentages, whereas high outcrossing rates will result in a good seed crop but with probable

contamination from non-orchard pollen; for northern Swedish provenances planted in southern Swedish seed orchards, this not only reduced the breeding value but also lowered the hardiness of planting stock (El-Kassaby *et al.,* 1989). Supplemental mass pollination was shown to increase seed yield and simultaneously to reduce the level of contamination (El-Kassaby and Ritland, 1986), but coordination with reproductive phenology is essential for success. Finally, the effect of seed grading on genetic structure should not be ignored, since embryos of heavier seeds have higher levels of heterozygosity than those from lighter seeds (Szmidt, 1987).

Stand management

The silvicultural regeneration of forest stands is one of the greatest influences on population genetic structure. Early studies showed that natural regeneration is well suited for gene conservation. To find differences in genetic structure between parental populations and their progeny, which have an excess of homozygotes, was initially surprising (e.g. Tigerstedt *et al.,* 1982), but analysis of different age classes showed that homozygotes are selectively eliminated during the early stages of stand development from the wide variety of genotypes resulting from mating (Muona *et al.,* 1987). Studies on the genetic effects of selective thinning in Scots pine stands demonstrated that genetic diversity is not compromised (Hertel and Kohlstock, 1994).

Atmospheric pollution

In common with other species, Scots pine subpopulations tolerant of heavy metal pollution or industrial emissions have been found to exhibit higher levels of genetic diversity than damaged ones (e.g. Geburek *et al.,* 1987). However, there are conflicting reports, which may have arisen from comparing healthy populations as a whole with damaged populations growing in different localities, rather than comparing groups of resistant and susceptible trees within populations.

Future development

Isozyme analysis has developed as a powerful tool for the study of natural genetic dynamics and of the genetic response of forest tree species to anthropogenic factors. There remain problems associated with comparative studies of Scots pine populations, which require concerted efforts to standardise laboratory protocols and to set up a database of allelic profiles and genetic diversity. This would indicate the genetic resources existing within the species, enable the monitoring of ecological influences, and provide the European Community with the means to regulate the transfer of seed.

DNA MARKERS

Introduction

Plant cells contain genetic information in three distinct organelles: nuclei, chloroplasts, and mitochondria, which in conifers show three different modes of inheritance. The chloroplast genome is paternally inherited (Birky, 1995), so that gene-flow by pollen dispersal can be analysed by the use of markers characterising this genome; in contrast, the mitochondrial genome is inherited maternally and can thus provide information on seed dispersal (Ennos, 1994). The nuclear genome shows biparental inheritance and undergoes recombination, and therefore shows the highest level of genetic variation. When measuring population parameters using DNA-based markers, we have the option to select markers which show either uniparental or Mendelian inheritance, and can therefore choose the most appropriate tools to solve particular problems. Taxonomic variations in several DNA marker systems (especially chloroplast-DNA polymorphisms) have increased our understanding of pine phylogeny (Price *et al.,* 1998; Wang *et al.,* 1999).

Nuclear markers

The haploid nuclear genome of *Pinus* consists of 13.8 x 10^9 base-pairs dispersed among 12 chromosomes. Several types of nuclear-DNA based marker systems have been established in pines, which have been used to analyse the genetic structure of populations and to construct linkage maps to locate genes responsible for important phenotypic characters.

Dominant markers

Techniques based on the polymerase chain reaction (PCR) have been developed (e.g. Random Amplified Polymorphic DNAs (RAPDs) and Amplified Fragment Length Polymorphisms (AFLPs)) which amplify a number of arbitrary segments of the genome. Since these techniques result in a multibanding gel pattern on separation of the PCR fragments, multiple loci can be analysed from a single reaction. Dominant marker systems do not provide unambiguous information on the genetic structure of individuals and populations, since the presence of a band can represent either a homozygous or heterozygous status; the recessive status is represented by the lack of the appropriate band. RAPD analysis has recently become a popular technique, providing a large number of independent genetic markers from a single analysis. The dominant Mendelian inheritance of RAPD fragments was shown in Scots pine by Lu *et al.* (1995); no fragments originating from the chloroplast and mitochondrial genomes were found, suggesting that RAPDs can be used as a specific nuclear genome marker system for pine populations. Szmidt *et al.* (1996) observed that the percentage of polymorphic loci and the heterozygosity were lower for allozymes than for RAPDs in two Scots pine populations; both marker systems showed that most of the variation resided within populations. RAPDs together with other molecular marker systems have been used for the construction of a linkage map of the pine genome (Devey *et al.,* 1996). AFLP markers have been developed for Scots pine (Lerceteau

and Szmidt, 1999), and tested for their use in taxonomic studies and in the construction of linkage maps.

Co-dominant markers

Co-dominant markers have the advantage that both alleles at a given locus can be identified, giving more information than dominant markers. Ribosomal-DNA polymorphisms are included here, although these are not clear co-dominant markers since the result of an analysis is always the sum of the amplification of several thousand loci of this gene family.

Nuclear genomes are interspersed with tandemly repeated oligonucleotide motifs; these regions are called microsatellites or simple sequence repeats (SSRs). The regions (loci) can be individually amplified by PCR, and show an extensive Mendelian polymorphism appearing as length variations due to different numbers of repeat units. Analysis of nuclear-SSR loci has proved to be a powerful tool in studying the genetic structure of populations (Terauchi and Konuma, 1994). Gupta *et al.* (1994) revealed the existence of such sequences in the pine genome, and several SSR loci have recently been described in Scots pine (Kostia *et al.,* 1995; Soranzo *et al.,* 1998). These loci reveal a greater genetic diversity than other molecular markers (e.g. Karhu *et al.,* 1996).

Ribosomal-DNA (rDNA)

Plant ribosomal genes are present as a tandemly repeated multigene family, with as many as 10,000 repeats at several chromosomal locations called nucleolar organiser (NOR) regions. Within each repeat unit, the genes coding for the ribosomal subunits are separated from each other by internal transcribed spacer regions (ITS1 and ITS2), and from the adjacent repeat unit by the non-transcribed intergenic spacer (IGS). These spacer regions are highly polymorphic and can be used in the genetic analysis of populations (Schaal and Learn, 1988). In Scots pine at least eight NOR regions have been identified (Karvonen *et al.,* 1998). A survey of the variability of the rDNA gene family in different populations revealed 13 rDNA phenotypes; most of the variation (86 %) was observed within populations (Karvonen and Savolainen, 1998).

Low repeat number DNA sequences

Very few analyses of Scots pine populations have been reported using polymorphisms of either isolated genes or anonymous sequences with low or zero repetition. However, the detection of polymorphisms in appropriate genes involved in adaptive processes could provide information on population adaptation. Karhu *et al.* (1996) analysed several Scots pine populations using a range of molecular marker systems, including Restriction Fragment Length Polymorphisms (RFLPs) of one anonymous gene and two anonymous genomic regions. The three regions showed high polymorphism, but differentiation among populations and correlations with an adaptive trait (date of bud set of seedlings) were low, indicating that these regions behaved like neutral markers.

Cytoplasmic markers

The chloroplast genome sequence is highly conserved, having a much lower mutation rate than the nuclear genome. The organellar genomes are usually maternally inherited, with the exception of the chloroplast genome in gymnosperms which is paternally transmitted (Birky, 1995).

Chloroplast-DNA (cpDNA)

In pines, cpDNA marker systems can provide information on paternal gene flow (pollen movement). Recently a powerful cpDNA SSR marker system was reported for pine species (Powell *et al.,* 1995), capable of detecting diversity both between and within populations more efficiently than isozyme or RFLP markers. In Scots pine, Provan *et al.* (1998) analysed seven Scottish and eight continental European populations with cpDNA SSRs, finding much higher diversity levels within populations compared with other marker systems including monoterpenes and isozymes. Small but significant genetic variation was detected between populations, but there was no significant difference between the Scottish and the European populations.

Mitochondrial-DNA (mtDNA)

Maternally inherited genetic markers are powerful tools for differentiating populations. These markers mostly show low levels of intrapopulation variation, while detecting significant levels of interpopulation variation, since genome dispersal is solely dependent on seed migration. In contrast to the structural stability of the chloroplast genome, the mitochondrial genome shows a comparatively high degree of structural evolution combined with a low rate of gene sequence mutations. These phenomena make marker identification difficult, and thus very few markers have been reported for mtDNA in *Pinus.* Most of the markers reported so far are RFLPs, which are laborious to use for the analysis of large numbers of individuals. Sinclair *et al.* (1998) analysed 20 Scots pine populations from Scotland using the cox I gene as an RFLP hybridisation probe, and identified three variants. Genetic differentiation of populations was 13 times higher with the mtDNA markers than with nuclear isozyme markers. They later expanded this work to include 18 populations from continental Europe (Sinclair *et al.,* 1999); the mitotype diversity was greatest in Spain, where the most southerly population was predominantly composed of an additional fourth mitotype. Their results indicated at least three different postglacial sources for the present Scots pine populations of western Europe. Recently a PCR-based polymorphic marker system has also been developed for *Pinus* by Soranzo *et al.* (1999), which detects variations in repeat number of an SSR region in the mitochondrial genome. Using this locus it was possible to distinguish several *Pinus* species, but no intraspecific variation within Scots pine has so far been detected.

Future development

Table 3 lists the genetic characteristics and major fields of application of the various types of DNA markers, together with those of the biochemical marker systems which have been described here. Both dominant and co-dominant marker systems have been used in the analysis of Scots pine populations. Highly developed marker systems such as RAPDs, nuclear SSRs, and rDNA ITS mainly represent repetitive regions of the nuclear genome, with the rDNA markers being further confined to the NOR regions. These neutral markers are ideal for assessing the genetic diversity of populations, since the distribution of their variation is not influenced by selective forces. For the analysis of population differentiation, potentially the most valuable neutral markers are those based on the maternally inherited mtDNA genome, but such systems are at present insufficiently well developed. Close linkage of any important genes controlling adaptive characters with molecular markers currently available can only be a fortuitous and rare occurrence. There is therefore an urgent need to develop additional DNA polymorphisms representing genes involved in adaptive processes; such non-neutral markers would indicate the differentiation of populations on the basis of selective and adaptive features.

TABLE 3

CHARACTERISTICS AND APPLICATIONS OF GENETIC MARKERS IN CONIFERS

Características y aplicaciones de los marcadores genéticos en las coníferas

Marker type		Genetics	Application				
			Diversity	Evolution	Pop. diff.	Pollen flow	Seed flow
NUCLEAR							
metabolic	Phenolics	Co-d, Ad	+	+	(+)		
	Terpenes	Co-d, Ad	+	(+)	+		
protein	Isozymes	Co-d, N(/Ad)	+		(+)		
DNA	RAPD	Dom, N	+	(+)	(+)		
	LRND	Co-d, N/Ad	+				
	SSR	Co-d, N	+				
	ITS	Co-d, N	+	+			
	AFLP	Dom, N	+		(+)		
CYTOPLASMIC							
mtDNA	RFLP	Maternal, N		+	+		+
cpDNA	SSR	Paternal, N	(+)	+	(+)	+	

Abbreviations:
mtDNA - mitochondrial DNA; cpDNA - chloroplast DNA;
RAPD - random amplified polymorphic DNA;
LRND - low repeat number DNA;
SSR - simple sequence repeats;
ITS - internal transcribed spacer of ribosomal DNA;
AFLP - amplified fragment length polymorphism;
RFLP - restriction fragment length polymorphism;
Co-d - co-dominant; Dom - dominant;
Ad - adaptive; N - neutral.

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RESUMEN

Marcadores genéticos: herramientas para identificar y caracterizar poblaciones de Pino silvestre

La diversidad genética es la base de la biodiversidad ecológica, que puede evaluarse únicamente utilizando marcadores que reflejen la variación presente dentro del genoma. Los constituyentes bioquímicos de las plantas interactúan con otros componentes del ecosistemas y tienen un mayor valor como marcadores adaptativos que como verdaderos caracteres genotípicos. Sin embargo, los componentes terpénicos de los sistemas resiníferos de las coníferas están bajo un control genético estrecho y son indicadores muy valiosos de la diversidad de las poblaciones. Los marcadores moleculares son insensibles a las modificaciones ambientales y por tanto representan el genotipo de forma más real. Se presenta un estudio sobre las aplicaciones de los principales sistemas de marcadores bioquímicos y moleculares en el análisis de las poblaciones de Pino silvestre, indicando las ventajas y limitaciones de cada categoría de marcador. La diversidad fenotípica es inusualmente alta dentro de las especies a los niveles morfológicos y fisiológicos, tanto dentro de poblaciones como entre regiones geográficas. También se justifica por el grado de diversidad genotípica evidente mediante análisis bioquímicos y moleculares. Los marcadores moleculares actualmente disponibles miden principalmente la variación neutral. Entre las principales necesidades para el futuro se encuentra el desarrollo de marcadores moleculares de caracteres adaptativos.

PALABRAS CLAVE: Genética
Marcadores
Variación
Diversidad
Adaptación

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