RAPD MARKERS LINKED TO MAJOR GENES BEHIND FIELD RESISTANCE AGAINST THE GREEN SPRUCE APHID *ELATOBIUM ABIETINUM* (WALKER) IN *PICEA SITCHENSIS* (BONG. (CARR.)).

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ABSTRACT

Within the open-pollinated offspring of two orchard clones which have been heavily attacked by the green spruce aphid in 1989, a large number of dominant RAPD-markers has been screened for co-segregation with recorded field resistance. Three of these markers - one in the first family and two in the second family – co-segregated. In the first family further checks of the zygosity of the mother performed on her haploid megagametophytes confirmed her heterozygosity at the marker locus. The allele frequency amongst the remaining orchard clones where unity for the recessive allele, i.e. a near-perfect test-cross situation was revealed for this particular marker. In the second family the two markers co-segregating with resistance segregated independent of each other. A highly significant interaction between these two markers concerning field resistance indicates epistatic gene effects between two non-linked resistance genes. Considering this evidence it is concluded, that three RAPD-markers may have been identified, each of which is linked to segregating resistance gene-loci coding for aphid resistance in the offspring from these two particular parent clones. The total number of detected resistance gene-loci may be two, possibly three. Average effects of each combination of the resistant gene and linked marker covered a range of 0.6-1.2 times the within-family phenotypic standard deviation in field resistance.

Keywords: *Elatobium abietinum*, *Picea sitchensis*, RAPD, resistance genes, linkage, QTL

INTRODUCTION

The host-aphid-environment interaction

The original host for the green spruce aphid *Elatobium abietinum* is believed to be Norway spruce. In Europe co-evolution between the host and the insect has developed into a stable balance, where Norway spruce is tolerating quite dense aphid populations without showing serious symptoms of needle loss. In contrast, for the introduced North-American *Picea sitchensis, glauca,* and *pungens,* the aphid is causing severe needle loss after mild winters in NW-Europe. In the mild climate prevailing here, the aphid develops a very effective asexual reproduction which exploits the mild early spring for vigorous reproduction (BEJER-PETERSEN 1962). In contrast, sexual reproduction is occurring in continental parts of Europe (KLOFT *et al.* 1964 and VON SCHELLER 1963) where overwintering eggs are able to resist severe winter frost prevailing here. General modeling of the sexual and asexual coexistence as a response to varying risk of winter frost in aphids has been performed by RISPE *et al.* (1998). In the aphid *Rhopalosiphum padi* (L.) with both types of reproduction, it has been demonstrated with isozymes that sexual generations follow Hardy-Weinberg expectations and show much more genetic diversity than the asexual generations (SIMON *et al.* 1996). In NW-Europe a certain genetic diversity in the asexual reproducing green spruce aphid populations has been detected with RAPD-markers but has not yet been compared with Central European populations (SIGURDSSON *et al.* 1998 – personal communication).

The importance of the pest is judged to be increasing due to climatic changes in the direction of milder winters in NW-Europe. The associated needle loss causes, in severe case's, mortality especially in combination with late spring frost - otherwise the damage is limited to loss of increment.

Quantitative genetic parameters of field resistance

Heritability of the analyzed field resistance or tolerance expressed as percentage needle loss has in the present investigation been estimated to 0.26 (WELLENDORF *et al.* 2000). The level of heritability over two successive years with aphid attacks (1989 and 1998) is reasonably stable (0.26 versus 0.21), whereas genetic correlation between the expressed resistance in the two years is estimated to moderate +0.51.

Objective and plan of the investigation

The object of the present investigation is identification of genetic markers in *Picea sitchensis* linked to major genes behind field tolerance to the green spruce aphid.

We have thereby started our search of QTLs in forest trees by looking for tolerance or resistance genes against a pest, in this case the green spruce aphid *Elatobium abietinum*. It is *a priori* expected that few major genes may be controlling field resistance against this aphid in the spruces, as this has been the experience in other much more extensively studied aphid-hosts such as apple (Roche *et al.* 1997), wheat (Zhang *et al.* 1998 and FRITZ & CALDWELL 1999) and barley (MOHARRAMIPOUR *et al.* 1997).

The plan of the investigation is presented in Fig 1.

Literature review of QTL-mapping in forest trees

QTL-mapping depends on available marker types, previous mapping, test organisms, pedigrees, and - on top of that - on the genetic background of the investigated more or less quantitative traits. In principle, any of the current approaches to linkage mapping of markers can be combined with QTL-mapping, if quantitative traits are observed on members of the mapping populations in appropriate diplophases. Three prerequisites are, (i) a restricted number of major genes affect the target trait, (ii) the target trait has a reasonably high heritability within the mapping population, and (iii) the sample size of the mapping population is sufficient. These prerequisites can to a certain degree substitute each other.

Types of two-generation pedigrees that have been used for mapping QTLs in forest trees are:

- One parent and open-pollinated assumed half-sib families
- Parent and selfed offspring
- Two parents and full-sib families (F_1) – in some cases extended to three generations.

Concerning the number of generations represented in the pedigrees, three, two, and even one generation pedigrees have been applied. A peculiarity of *Pinaceae* is that by observing on the haploid megagametophytes of the mother it is possible to look at her haploid gametes directly (WILCOX *et al.* 1996). This means that you may have "half generations" represented in the pedigree, e.g. OP-offspring with rescued megagametophytes in the seed sample represent a 1.5 generation pedigree.

In the following earlier published investigations on QTLs relevant for forest trees are put into a framework of specific situations:

Open-pollinated families – rescued megagametophytes

In *Pinaceae* co-segregation is pursued between target traits and mother-contributed markers observed in rescued haploid megagametophytes. The pollen cloud represents a broad, neutral population sample in assumed linkage equilibrium (O'MALLEY & MCKEAND 1994). Applying this approach using RAPD in search of major genes for resistance against the fusiform rust

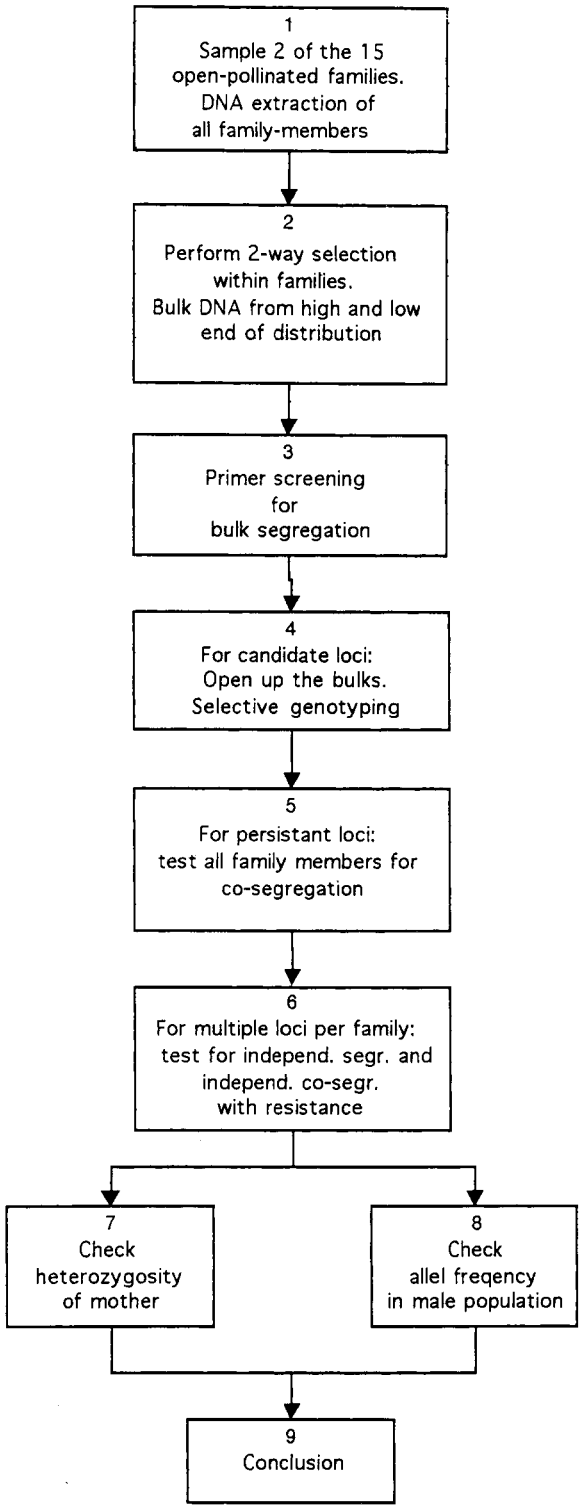

Figure 1. Plan of investigation.

disease in loblolly pine was performed by Wilcox *et al.* (1996). A recent presentation by Wu *et al.* (1999) extends QTL with the classic quantitative genetic framework of average effects, dominance and epistatic deviations (FALCONER & MACKAY 1996).

Selfed families – rescued megagametophytes

Seedling death after selfing caused by a recessive lethal allele has been found in *Pinus radiata* using dominant RAPD markers (KUANG *et al.* 1998). The lethal locus was localized by combining evidence from genotyping of saved megagametophytes of the germinating seedlings (including the mortalities), unsown seeds and the diploid tissue from the surviving seedlings.

Open-pollinated families – dominant markers in pseudo test-cross situations

In any open-pollinated half-sib family, co-segregation is pursued between quantitative traits and mother contributed dominant markers observed in pseudo test-cross situations. This approach has been pursued using RAPD in *Eucalyptus* (GRATTAPAGLIA *et al.* 1996) after inspiration from animal breeders. The target traits were in this case growth and wood quality traits.

Full-sib families – dominant markers

In large full-sib families, co-segregation between quantitative traits and separate mother and father contributions can be dissected. This is possible both with and without saved megagametophytes. In the last case, reciprocal pseudo test-cross situations must be pursued; such an investigation has been performed by GRATTAPAGLIA *et al.* (1995) within a F_1-hybrid FS-family after crossing the two *Eucalyptus* species *E. grandis* and *E. urophylla*. It was then possible to conclude, that variation within the F_1 hybrid FS-family for one trait originated from parent one, and for another trait originated from parent two. Target traits were in this case associated with vegetative propagation and the markers were RAPDs. This approach has further been pursued with AFLPs by MARQUES *et al.* (1999). Adding the dimension of stability of QTLs at successive ages has been dissected in *Eucalyptus* hybrids by VERHAGEN *et al.* (1997) and in *Pinus radiata* by KUMAR *et al.* (2000).

Three-generation outbred pedigrees

It is possible to detect multiple alleles of QTL if co-dominant markers are available with multiple alleles represented in a three-generation outbred pedigree, i.e. with 4 known grandparents. To promote a high degree of heterozygosity in the unrelated F_1-parents, grandparent pairs of contrasting QTL expression is the most ideal situation. It is further required that a sufficiently large F_2 family is available as a cross between members of the unrelated F_1 individuals. This option has been pursued in *Pinus teada* by GROOVER *et al.* (1994) using a map based on RFLP markers and subsequently followed-up by KNOTT *et al.* (1997) applying multiple markers on the same mapping population, both groups looking for QTL behind wood density. Both investigations indicate a moderate number of QTL behind this trait. A similar approach has been used in *Eucalyptus nitens* for seedling height and leaf area (BURNE *et al.* 1997).

Screening markers for co-segregation with target traits

A special problem in screening of numerous markers for co-segregating with the target traits is to economize the lab-effort. Bulking DNA from both extreme samples of the phenotypic distribution is one approach – "bulked segregant analysis" – advocated by MICHELMORE *et al.* (1991) in screening for markers linked to resistance genes. Another method is to keep members of the two extreme samples separate. That method "selective genotyping" is recommended for more polygenic traits. In any case, a three or two step procedure evolves: (i) bulked segregant analysis (pursuing one or two major gene-loci), (ii) selective genotyping followed by (iii) the ultimate test of co-segregation in a representative and large sample of the mapping population. Such procedures have in forest trees been demonstrated by GRATTAPAGLIA *et al.* (1996) for quantitative traits in *Eucalyptus* and by WILCOX *et al.* (1996) for resistance genes in *Pinus teada* .

Statistical methods of QTL detection are a vigorously developing field *of research*

The sophistication's range from simple statistical comparison of mean values for the quantitative traits of alternative genotypes of each marker without concern of further mapping (single marker analysis according to FALCONER & MACKAY (1996)) to interval mapping, where co-segregation between mapped markers and quantitative traits results in an indication of in which interval between mapped markers the QTL is residing (LANDER & BOTSTEIN 1989).

MATERIAL AND METHODS

Plant material and field observations

The investigation was carried out in two of 15 open-

pollinated families in which phenotypic variation in aphid attack has been recorded in February 1990 (JENSEN *et al.* 1997).

These families originate from the open pollinated mating in 1968 of 15 clones present in a seed orchard, FP611 on the location Vosnæs in Eastern Jutland. Abundant flowering was recorded in the orchard that year and seed was harvested on all clones for the first time. Based on morphological traits, a few percentage of the offspring showed up to be spontaneous hybrids with *Picea glauca*; these were avoided in the following analyses.

Back in 1958 the ortets behind the parent clones were selected for resistance against the green spruce aphid. The realized overall gain in 1990 of this early selection was demonstrated in the open-pollinated progeny tests F154C, at the plantation of Kåse in North western Jutland (JENSEN *et al.* 1997). Field resistance of open-pollinated progeny from all 15 clones in the orchard was investigated, and a significant variation between families remained.

The investigated progeny-test is laid out as a randomized block experiment with 16-tree square plots and 5 replications. Of the original 80 family members in the two sampled families, 58 and 68 Sitka spruce individuals respectively remained to be sampled in 1997, 25 years later.

The tolerance or resistance to the aphid attack was visually judged as percentage of needles lost in the whole tree. The original observations were stratified into upper and lower crown and on previous years' needles and older needles, a total of 4 observations per tree. An average of the 4 reasonably correlated fractions is used for all subsequent analyses.

The phenotypic value of individuals within families was derived as deviations from the local plot mean multiplied by a factor $(1-1/N)$ where N is the number of surviving trees in the plot. The factor is an adjustment for the restricted sample size in the plot.

DNA extraction and the RAPD assay

The applied procedure of DNA-extraction (CTAB method, BOUSQUET *et al.* 1990, CARLSON *et al.* 1991) and RAPD assay (WILLIAMS *et al.* 1990) are modified by SKOV (1998a; 1998b). RAPDs are highly polymorphic dominant DNA-markers obtained by PCR.

Two sets of 100 10-mer RAPD-primers each were obtained from John Carlson, University of British Columbia (one set was termed the "conifer set") and used for the extensive primer screening. Further 50 primers from Operon technologies (Alameda, California) series A, F, G, J and Y were used.

Co-segregation between RAPD-markers and field resistance

The principle behind detection of linkage relationships between markers and quantitative trait loci (QTLs) in open-pollinated families is described by O'MALLEY & MCKEAND (1994) and by WELLENDORF & SKOV (1998). Co-segregation of markers and closely linked QTLs is visualized in Table 1 from the latter publication.

Table 1. Inheritance of a marker and closely linked QTL in open-pollinated offspring from a double heterozygote mother tree.

Female individual			Male population in assumed linkage equilibrium	
Genotype ($2n$) ↓	gametes (n)	→	$\mathbf{A}_{(+/-)}$	$\mathbf{a}_{(+/-)}$
		freq	p	q
A+a-	**A+**	0.5	**AA** + 0.5 q	**Aa** + 0.5 q
	a–	0.5	**aA** – 0.5 p	**aa** – 0.5 q

Legend:
freq: frequency of gametes
A: dominant marker allele (RAPD or AFLP)
a: recessive marker allele
+: QTL-allele with positive effect
–: QTL-allele with negative effect
A+: Allele A combined with QTL-allele +
a–: Allele a combined with QTL-allele –
$\mathbf{A}_{(+/-)}$ and $\mathbf{a}_{(+/-)}$: Allele **A** or **a** not associated with QTL-alleles in population samples in linkage equilibrium.

The key point is that co-segregation in the open-pollinated offspring of a double heterozygote female parent reflects linkage relationships in the gametes of that female. The male population in assumed linkage equilibrium is a neutral background in which alleles from linked loci recombine freely.

With the dominant RAPD-markers it is not possible to discriminate between the three marker genotypes **AA**, **Aa** and **aA** in the offspring. Therefore, the test is most effective if the frequency **q** of the recessive allele in the pollen cloud is high; in that case we are close to the test-cross situation, where we test an unknown genotype by mating to a homozygote recessive. This is

termed a "pseudo test-cross situation" (GRATTAPAGLIA & SEDEROFF 1994).

Point's 2–3 in the plan of the investigation refer to the method developed by MICHELMORE *et al.* (1991), in which screening for markers co-segregating with resistance is performed on bulked DNA from individuals drawn from the two contrasting ends of the phenotypic distribution. For markers discriminating between these two bulks (in our samples represented by 5–8 individuals in each bulk corresponding to approximately the upper and lower 10% of the distribution), individual control is regained in the selective genotyping, which still only comprises the contrasting phenotypes (point 4). For loci that here reject a χ^2 test of independence between high and low level of resistance and occurrence of the RAPD band (**A_** versus **aa**), the whole family is tested for co-segregation between marker and individual phenotypic resistance in the field (point 5). This test is performed as a one-way analysis of variance in which the mean resistance of the two groups with and without the dominant marker (**A_** versus **aa**) are compared.

If more than one marker in each family is co-segregating with resistance, a test of independent segregation of these markers is performed. If independent segregation occurs it is further tested if the co-segregation between the individual markers and field resistance is independent, or alternatively, if interaction can be detected between the effect of pairs of assumed QTLs and field resistance. This test is performed by a two-way factorial ANOVA with interaction, where the two fixed main effects are presence / absence of RAPD bands of the pair of marker loci investigated and the dependent variable is aphid attack of individuals with the stated marker combination.

For those marker-loci that demonstrate a highly significant effect on resistance, two further tests are attempted. The heterozygosity of the marker in the female parent is checked in her haploid megagametophytes obtained in recently harvested seed (point 7). The frequency of the dominant marker allele in the fertilizing pollen cloud back in 1968, when seed was harvested for the investigated offspring, is estimated by typing all the 15 clones in the orchard (point 8). This check is made to record how close we are to the "pseudo test-cross" situation (Table 1). The actual differences in mean aphid attack between the two parts of the families carrying the alternative alleles are estimated and set in proportion to the phenotypic standard deviation within families. This tells us something about the magnitude of the average effect of the detected resistance genes (FALCONER 1989).

Based on the revealed conditions, conclusions are drawn about RAPD-markers linked to major genes behind field resistance.

Table 2. Results of the initial screening for bulk segregation of approximate 1000 loci distributed to 250 RAPD-primers followed-up by selective genotyping and ANOVA-tests of co-segregation among all available family members.

Loci passing the bulk-segregant analysis	Performed selective genotyping of individual bulk-members		Test of co-segregation among all available family-members	
Primer	family 42 (S 8053)	family 51 (S 8062)	performed	significant ($p \leq 0.005$)
1	x			
12		x		
18	x		x	
20	x			
29		x	x	
30		x	x	
32	x		x	x
50-1	x		x	
50-2	x		x	
54	x			
58	x			
60	x			
73		x	x	
90	x		x	
116		x	x	
116		x		
123		x		
153		x		
184	x		x	
193		x	x	
203		x		
213	x			
213	x			
225		x		
248		x		
266		x	x	
268		x	x	
269	x			
297		x		
320	x			
348		x		
362	x			
530		x		
530		x		
561		x		
564	x			
589		x	x	
600		x		
A11	x			
F07		x	x	x
F08		x	x	(x)
G12		x		
J01	x			
Y17		x	x	
Y17				
Σ 45	20	25	17	3

(x): significant at the *p*-level 0.0058.

RESULTS

The preliminary results of the extensive primer screening in the progenies are presented in Table 2 (plan point 3–5 in Fig 1). Approximately 1000 RAPD loci distributed to 250 primers have been screened by bulk segregant analyses. 45 of these, which looked promising, have been opened up by selective genotyping. Here only 14 rejected the χ^2-test of independence between occurrence of the RAPD band and field resistance. Amongst those 14, three (one in family S 8053 and two in family S 8062) passed the ANOVA-test of highly significant co-segregation in the whole family (P-level 0.005).

Table 3 presents a sample of performed tests of candidate RAPD-markers for co-segregation with field resistance in the two investigated families (plan point 4-5).

As can be seen here, one marker in family S 8053 and two markers in family S 8062, appear to co-segregate with field resistance.

In Table 4 a judgment of occurrence of "false positives" (LIU 1997) is shown.

From this table it is concluded that only one marker in family S8062 (F08) lies beyond any reasonable doubt about possible "false positives." On the other hand, the two other markers are retained as candidate markers for linkage with resistant genes, as the risk of rejecting real linkage relationships as "false negatives" must also be taking into account.

The question of independence between the two markers and their associated QTLs in the latter offspring-family is therefore relevant for further investigation (plan point 6). A test of independent segregation of the two markers G12 and F08 in family S 8062 has been performed by a standard χ^2-test (not shown). There is no sign of deviation from independent segregation, i.e. the two loci seem to be unlinked.

Table 5 shows a test for any detectable interaction between field resistance and the occurrence of the two markers G12 and F08 in family S 8062. The test has been performed as a two-way factorial ANOVA with interaction and replicated observation in each cell corresponding to defoliation of individuals with the specified marker combinations. A highly significant interaction indicates epistatic gene effect between the two resistance genes each linked to the two independent segregating RAPD-markers F08 and G12.

Fig. 2 shows a graphic presentation of the nature of the epistatic interaction. It seems as though the functional allele in the resistance-locus linked to G12 is partially suppressing the effect of the allele in the other resistance locus linked to F08; only in the case of absence of the functional allele linked to G12 is the effect of the resistance allele linked to F08 fully expressed.

Table 6 shows the performed checks of zygosity of the parent clone V 2708 of family S 8053 as well as estimates of the allele frequencies in the remaining orchard clones - assuming no surviving selfings. An adjustment of the expected allele frequency in the orchard-generated pollen-cloud is based on information from two sources, (i) the uneven ramet-reprensentation of clones in the orchard and (ii) counting of the uneven cone setting of individual clones in the orchard; it is here assumed that the contribution to the pollen-cloud

Table 3. Summary table of tests of candidate RAPD-markers for co-segregating with field resistance.

Family	RAPD locus	Selective genotyping				ANOVA test of co-segregation in the whole family				Within-fam variation aphid attack		Av diff in aphid attack between +/– genotypes	
		No in RES : SUS samples	χ^2	d.f.	P-level	Marker segregation +/–	F-test	d.f	P-level	n	ρ_P	%	/ρ_P
S 8053	18	5 : 6	5.24	1	0.0221	25 : 34	2.53	1.57	0.1169	63	18.44	–	–
	32	**6 : 8**	**10.50**	**1**	**0.0012**	**30 : 28**	**12.40**	**1.56**	**0.0009**	↓	↓	**16.0**	**0.80**
OP	50-1	6 : 8	2.86	1	0.0906	31 : 32	0.42	1.61	0.5217	↓	↓	–	–
offspring	50-2	6 : 8	7.88	1	0.0050	26 : 25	4.21	1.49	0.0457	↓	↓	11.3	0.61
of V 2708	90	6 : 8	5.83	1	0.0157	21 : 42	3.57	1.61	0.0637	↓	↓	(9.1)	(0.50)
	589	5 : 8	5.92	1	0.0149					↓	↓		
S 8062	30	8 : 7	0.58	1	0.4468	23 : 28	1.16	1.49	0.2873	66	22.00	–	–
	203	8 : 8	4.00	1	0.0455	25 : 28	1.61	1.51	0.2098	↓	↓	–	–
OP	**G12**	**8 : 7**	**9.60**	**1**	**0.0019**	**29 : 24**	**8.28**	**1.51**	**0.0058**	↓	↓	**13.5**	**0.61**
offspring	**F08**	**8 : 7**	**11.43**	**1**	**0.0007**	**34 : 14**	**31.44**	**1.46**	**0.0000**	↓	↓	**25.5**	**1.16**
of V 2717	73	8 : 8	2.62	1	0.1056	12 : 40	0.51	1.50	0.4803	↓	↓	–	–

OP-offspr.: open-pollinated offspring; RES; resistant; SUS: susceptible

Table 4. Judgement occurrence of false positive.

Number of candidate markers	Significance level for individual tests	Expected number of false positives amongst 1000 tests	Actual number of positive outcomes among 1000 individual tests	
			family S8053	family S8062
Ca 1000	0.05	50		
	0.01	10		
	0.005	5	1	2
	0.001	1	1	1
	0.0001	0.1	0	1

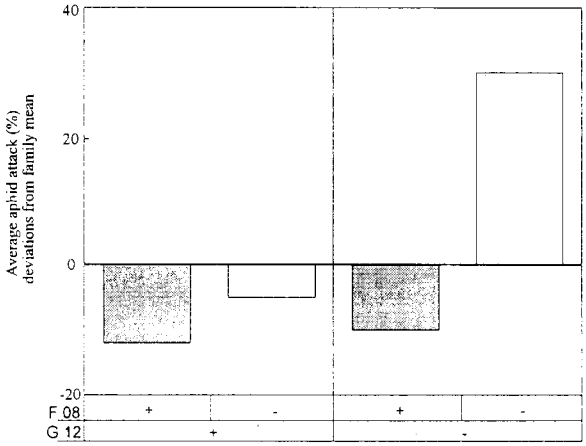

Figure 2. Demonstration of the recorded interaction between the two RAPD-markers on the average degree of aphid attack within the offspring family S 8062. The interaction is interpreted as epistatic gene action between two non-linked resistance genes, which is linked to the two RAPD-markers F08 and G12 respectively.

Table 5. Test of independent co-segregation between RADP-markers G12 and F08 and field resistance in offspring family S 8062.

2-way factorial ANOVA of aphid attack (%) – deviation from family-mean.

Source of variation	d.f.	Sum of Sq.	Mean Sq.	F-ratio	Prob>F
RAPD **G12**	1	3007.15	3007.15	23.94	0.0000
RAPD **F08**	1	5003.23	5003.15	39.84	0.0000
G12 * F08	1	2612.34	2612.34	20.80	0.0000
Within cell error	43	5400.66	125.60		

is proportional to cone setting of individual clones (plan point 7 and 8, referring to KJÆR & H. WELLENDORF (1997)). An alternative method of estimating the allele-frequencies of the fertilizing pollen cloud is to combine the genotype of the mother with the allele-frequencies in the progeny. The results of these estimations on both parent clones are presented in the last column of Table 6. Details of calculations behind the results presented in Table 6 are listed in Appendix A1 and A2.

DISCUSSION

Check of assumptions

Concerning the mating-pattern in the orchard, one of the RAPD-markers, 50-2 which is homozygote recessive in orchard clone V 2708, delivers the most direct evidence. Although not informative for co-segregation detection, as the parent is not a heterozygote, it delivers a direct estimate of the allele-frequency of the fertilizing pollen cloud (Appendix A1-1). As can be seen from Table 6, a significant deviation is apparent between the expected frequency of the dominant allele in the orchard-generated pollen cloud (0.15–0.16) and the effective, fertilizing pollen cloud (0.51). This indicates that background pollen may have contributed substantially in the flowering year in 1968, the year where seed was harvested for foundation of the analyzed progenies. This was the first abundant flowering year in the then 10 year old orchard; it is a well-known trend that young orchards may be slow to build-up a sufficient pollen-cloud to counter the proportion of background pollen. That background pollen was in operation in that particular year is also reflected in a distinct, but low proportion of species-hybrids with White spruce (*Picea glauca*) in the progenies. The frequency of these hybrids has in later harvesting years decreased to zero (BRANDT, personal communication). As mentioned in the introduction, these hybrids have been excluded from the analyses.

That a certain amount of background pollen has contributed to the offspring of the two analyzed families is not necessarily seriously disturbing the assumptions that the father population is a representative sample of a population in linkage equilibrium. Provided allele-frequencies are not much different between the two populations, the orchard clones and the immigrating pollen-cloud, the effective male-population might even be a better population sample than the 15 orchard clones alone. Numerous studies of neutral markers have shown minor differences between provenances concerning allele frequencies (NESBITT *et al.* 1995).

Table 6. Attempted checks of the mating situation in the orchard for candidate markers co-segregating with field resistance. RAPD locus 32 demonstrates a nearly pure test-cross situation.

Parent	Offspring family	RAPD locus	Parent genotype		Informative on co-segregation	Freq **p** of dominant allele in remaining orchard pop		Freq **p** of dominant allele in fertilizing pollen cloud
			Buds (2*n*)	MG (*n*)		Raw	Adjusted	
V 2708	S 8053	**32**	A_	Aa	yes	0.00	0.00	0.03
		50-2	aa		no	0.15	0.16	0.51
		90	A_		yes	0.08	0.04	–0.33*)
V 2717	S 8062	**G12**	A_		yes			0.09
		F08	A_		yes			0.42

Legend:
MG: Megagametophytes (haploid seed endosperm)
Remaining orchard pop: The orchard population excluding the actual parent clone.
Raw: p estimated under the assumption of all clones contributing equally to the gamete pool.
Adjusted: It is taken into account, that clones are unequal represented by ramets and ability to flower judged by there relative cone-setting.
*) Sampling error and /or distortion of segregation of the female parents gamets for this particular marker may be the reason for this negative frequency estimate.

Furthermore, the most widely used Sitka provenances in Denmark are of Washington origin complemented with small proportions of Queen Charlotte origin, i.e. origins other than Washington in widespread use in Denmark are restricted.

In the case of a massive 50% background pollination, the fertilizing pollen cloud must be composed of a mixture of two population samples of comparable proportions, i) the orchard generated pollen cloud and ii) the imigrating pollen cloud. These two pollen samples may have the same population genetic parameters concerning the neutral markers, whereas they must differ if QTL for resistance is included as a result of the realized genetic gain amongst the orchard clones for this trait. Such mixtures of divert populations are known to generate a certain amount of linkage disequilibrium in the initial generations (FALCONER & MACKAY 1996) and thereby violating our underlying assumption of linkage equilibrium in the fertilizing pollen cloud (Table 1). However, the amount of disequilibrium is not judged to be of the same magnitude as the direct gametes produced by the individual female parents. For these reasons, it is still judged valid, that the recorded trait's associations between markers and resistance within the investigated two half-sib families are mainly due to linkage and expressed in the progeny through recombinations in gametes of the female parents.

Co-segregation between markers and resistance genes

As can be seen in family S 8053, OP-progeny of parent clone V 2708, the co-segregating RAPD-marker 32 demonstrates a nearly pure (pseudo) test cross situation (Table 6 and Appendix A1-1). The megagametophyte segregation identifies the parent-clone V 2708 to be a heterozygote. All the 14 other clones in the orchard are recessive homozygotes, i.e. the fertilizing pollen-cloud is expected not to carry the dominant allele, provided no background-pollen has migrated into the orchard. For these reasons the observed 30 : 28 segregation of the two alleles of RAPD-marker 32 (Table 3) reflects the expected 1 : 1 segregation of the heterozygote mother V 2708. However, a small fraction in the effective pollen cloud might carry the "rare dominant allele" due to immigrating pollen.

The RAPD locus 50-2 which showed a modest 5% significance for co-segregation (Table 3) is not really informative, as the mother-clone is a homozygote recessive. The difference in resistance between the two fractions with and without the marker phenotype **A_** is either due to chance or a trait-association occurring in the fertilizing pollen sample, otherwise assumed to be in linkage equilibrium, i.e. with independent segregation of alleles in even linked loci.

To conclude, RAPD-locus 32 in family S 8053 – the open pollinated offspring of parent clone V 2708 –

satisfies all the check-points and the average effect of the combination of marker and resistance gene is able to change the phenotypic mean value by 0.80 phenotypic standard deviations within this particular family.

In the other analyzed family, S 8062 (OP-progeny of parent clone V 2717) two loci G12 and F08 both co-segregate with resistance by their own rank. According to Table 6, the G12-case again reflects a pseudo-test cross situation, as the effective pollen-cloud shows a derived estimate of the frequency of the dominant RAPD-allele of 0.09. In the F08-case, we are far from the pseudo-test cross situation as the frequency of the dominant allele now is estimated to 0.42; in other words, this linked resistance gene is able to penetrate the "foggy" situation shown in Table 1 with more intermediate marker-allele frequencies in the male population. From this point of view it must represent the most powerful resistance gene, also because – in spite of the far from ideal analytical situation – it is showing the highest level of significance of the co-segregation and the largest average effect (1.16 phenotypic standard deviation within the family).

Relationships between the three detected marker-resistance gene associations.

The question of how many resistance genes we have approached by the investigation may be answered by at least two, probably three. In the second family S 8062, the two markers each co-segregating with resistance segregated independent of each other, i.e. two unlinked resistance genes may be involved. Concerning the resistance gene in the first family (S 8053) co-segregating with marker 32, we do not know if it represents one of the same gene-loci or the same resistance-alleles either, as those detected in the second family.

The second question of interest is, are the two resistance genes in the second family S 8062 coding for different resistance mechanisms ? The apparent epistatic gene effects with the resistance gene linked to G12 hiding the effect of the resistance gene linked to F08 (Fig. 2), may reflect one very important mechanism linked to G12. The other mechanism may only be really important in case the first mechanism is not active.

Accordance with aphid resistance reported in other plants

To our knowledge, no other markers have been reported to be linked to aphid resistance in conifers. In deciduous trees, apple is a tree-crop of economic importance and its genome has been well mapped (HEMMAT *et al.* 1994 and MALIEPAARD *et al.* 1998). An early identified dominant resistance gene – Sd_1 – has been found (ALSTON & BRIGGS 1968) to be resistant to two of three biotypes of the rosy leaf curling aphid (ALSTON & BRIGGS 1977). Three RFLP- and four RAPD markers have been found to be linked to the Sd_1 gene (ROCHE *et al.* 1997). These findings open up for marker aided selection in apple breeding as a partial substitute for direct screening for resistance.

As mentioned in the Introduction, aphid resistance in annual crops has in many cases been traced back to Mendelian segregating genes. So it is not astonishing, that the same results occur in trees, in deciduous as well as in conifers.

Perspectives

In trees characterized by slow generation turn-over, the option of early marker aided selection for late expressed traits as aphid resistance and other economic important traits, is much more important than in annual crops. This is because in annual crops it is possible during the first year to select directly on your expressed target trait, whereas in trees this is normally not possible because it may last decades before the economic important traits are expressed.

Recommended follow-up investigations

Before marker aided resistance breeding for aphid resistance can be realized in operational scale, further investigations may be required to clarify the following questions:

a) Which resistance mechanisms are acting behind the recorded variation in field resistance judged by needle retention and aphid fecundity?
b) Is there an overlap in identity of resistance gene-loci detected in each of the two families?
c) Are further resistance gene-loci operative in a broader genetic base of host populations?
d) Is the detected resistance associated with a special strain of the aphid, or is the attacking aphid population of a broad genetic base?
e) Is the obtained genetic gain in resistance sustainable in the future?
f) Can the detected associations between markers and resistance be utilized in breeding programs?

Point a) requires specific resistance studies applying entomology and associated biological sciences, as chemistry (secondary gene-products), plant anatomy, etc.

Point b) requires further co-segregation studies of the involved 15 parents to the investigated progeny-test, especially identifying parents heterozygotes in co-segregating markers present in different families. This

point is further brought into perspective by placing the co-segregating markers on an accumulating consensus map for the host species *Picea sitchensis* or the whole genus *Picea.*

Point c) is partially covered by the measures recommended in point b); however, as all the orchard clones are selected for resistance and a good response has been realized in the bulked progeny from the orchard (JENSEN *et al* 1997), it is possible, that this population already is fixed for some resistance genes and therefore not representative for segregation within other non-selected populations. Therefore, it would be advisable to analyze a broader range of progeny tests.

Point d) requires genetic analyses of the aphid populations. Such studies are underway in a joint investigation in Iceland, Denmark, UK, France and Norway applying RAPD-markers (FAIR3 1998). In Iceland where the Aphid was first detected in 1959, RAPD-polymorphy has been recorded both within and between geographic separate sampling sites (SIGURDSSON *et al.* 1998 – personal communication), i.e. genetic diversity seems to occur here. The aphid populations in Iceland are assumed to originate from Denmark. The aphid is commonly showing a non-sexual reproduction in maritime climates in North-western Europe. In contrast, sexual reproduction is occurring in continental parts of Europe (KLOFT *et al.* 1964, VON SCHELLER 1963). However, occasional sexual reproduction may take place in Denmark. Oviparous females, males and eggs were first recorded in 1995/96 (HARDING & CARTER 1997). At the present stage, it is not clear if sexual reproduction is necessary for maintaining or generating genetic diversity in delineated parts of the overall distribution area of the aphid.

Concerning point e), the obtained genetic gain in resistance obtained by the applied selection of the mature parent trees back in 1958 has been expressed in their seed orchard progeny in 1990, 32 years later, i.e. the resistance seems to have sustained during a third of a century. If the aphid should be able to circumvent the obtained improved resistance by mutation followed-up by selection is doubtful as full immunity in large-scale plantations may not be possible to develop, partly because Sitka spruce is not the only host for the aphid; the other main plantation species in Denmark, Norway spruce is the original host for the aphid, and here a balance has been obtained after natural co-evolution during long periods of time. Lack of a sexual reproduction may also be a draw-back for the ability of the aphids to respond to even widespread use of resistant genotypes of Sitka spruce. However, factorial "crossing" experiments between an array of host and aphid genotypes are recommended to clarify if specific host-pest interactions are important.

Point f) may be checked by crossing the two investigated parents, V 2707 and V 2717 with each other and with other partners and follow-up with marker-aided selection in the progeny based on the three detected RAPD-markers. Rescuing the megagametophytes of the germinating seedlings (SKOV 1998b) will make it possible to dissect the mother from the father contribution and thereby perform the marker aided selection on the progeny (WELLENDORF & SKOV 1998). Later, resistance to actual aphid attacks of the marker-selections compared to a neutral control will deliver the ultimate check of the realized genetic gain.

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Appendix A1-1. Comparison of estimates of allele-frequencies in pollen clouds fertilizing orchard clone V 2708. Allele freq estimated by two methods: (1) Based on observations on parent's genotypes combined with ramet-representation and reproductive potential; (2) Based on observations on parent's and offspring's genotypes.

RAPD-marker **32**. Freq of A: p; freq of a: q; Parent determined to be heterozygte, based on segregation amongst haploid megagametophytes.

Female parent				Male population					
				Estimated allele freqencies based on parents				Estimated allele freqencies based on progeny and parent genotype	
				raw		adjusted for ramets and flowering			
Clone	Genotype	Gametes	Freqency	A p	a q	A p	a q	A p	a q
				0.000	**1.000**	**0.000**	**1.000**	0.034	0.966
V 2708	Aa	A	0.000	AA 0.000	Aa 0.500	AA 0.000	Aa 0.500	AA 0.017	Aa 0.483
		a	1.000	aA 0.000	aa 0.500	aA 0.000	aa 0.500	aA 0.017	aa **0.483**

↑ Expected genotype frequencies in the offspring

↑ Observed genotype frequencies of hom rec in the offspring

x.xxx: Primary determined observations and frequencies; x.xxx: Derived variables; hom rec: Homozygote recessive .

Appendix A1-2. RAPD-marker 50-2. Freq of A: p; freq of a: q; Parent assumed homozygote recessive, as the phenotype is "absence"of the dominant RAPD band.

Female parent				Male population					
				[1] Estimated allele freqencies based on parents				[2] Estimated allele frequencies based on progeny and parent genotype	
				raw		adjusted for ramets and flowering			
Clone	Genotype	Gametes	Frequency	A p	a q	A p	a q	A p	a q
				0.155	**0.845**	**0.156**	**0.844**	0.510	0.490
V 2708	aa	A	0.000	AA 0.000	Aa 0.000	AA 0.000	Aa 0.000	AA 0.000	Aa 0.000
		a	1.000	aA 0.155	aa 0.845	aA 0.156	aa 0.844	aA 0.510	aa **0.490**

↑ Expected genotype frequencies in the offspring

↑ Observed genotype frequencies of hom rec in the offspring

x.xxx: Primary determined observations and frequencies; x.xxx: Derived variables. hom rec: homozygote recessive

Appendix A1-3. RAPD-marker 90. Freq of A: *p*; freq of a: *q*; Parent determined to be a heterozygote, based on segregation amongst haploid megagametophytes.

Female parent				Male population					
				Estimated allele frequencies based on parents				Estimated allele frequencies based on progeny and parent genotype	
				raw		adjusted for ramets and flowering			
Clone	Genotype	Gametes	Frequency	A *p*	a *q*	A *p*	a *q*	A *p*	a *q*
				0.080	**0.920**	**0.040**	**0.960**	(–0.034*)	(1.334*)
V 2708	**Aa**	A	0.500	AA 0.040	Aa 0.460	AA 0.020	Aa 0.480	AA	Aa
		a	0.500	aA 0.040	aa 0.460	aA 0.020	aa 0.480	aA	aa **0.667***
						↑			↑
						Expected genotype frequencies in the offspring			Observed genotype frequencies of hom rec in the offspring

x.xxx: Primary determined observations and frequencies; x.xxx: Derived variables. hom rec: Homozygote recessive.

*) Sampling error and/or distortion of segregation of the female parents gametes for this particular marker may be the reason fro these "impossible" frequency estimates.

Appendix A2-1. Comparison of estimates of allele frequences in pollen clouds fertilizing orchard clone V 2717. Allele frequency estimates are based on observations on parent's and offspring's genotypes.

RAPD-marker **G12**. Freq of A: *p*; freq of a: *q*; Parent assumed heterozygote, as segregation occurs in the progeny.

Female parent				Male population					
				Estimated allele frequencies based on parents				Estimated allele frequencies based on progeny and parent genotype	
				raw		adjusted for ramets and flowering			
Clone	Genotype	Gametes	Frequency	A *p*	a *q*	A *p*	a *q*	A *p*	a *q*
								0.094	0.906
V 2717	Aa	A	0.500	AA	Aa	AA	Aa	AA 0.047	Aa 0.453
		a	0.500	aA	aa	aA	aa	aA 0.047	aa **0.453**
						↑			↑
						Expected genotype frequencies in the offspring not determined			Observed genotype frequencies of hom rec in the offspring

x.xxx: Primary determined observations and frequencies; x.xxx: Derived variables. hom rec: Homozygote recessive.

Appendix A2-2. Comparison of estimates of allele frequences in pollen clouds fertilizing individual clones. Allele frequency estimates are based on observations on parent's and offspring's genotypes.

RAPD-marker F08. Freq of A: p; freq of a: q; Parent assumed heterozygote, as segregation occurs in the progeny.

Female parent				Male population					
				Estimated allele freqency based on parents				Estimated allele freq based on prog and parent genotype	
				raw		adjusted for ramets and flowering			
Clone	Genotype	Gametes	Frequency	A p	a q	A p	a q	A p	a q
								0.416	0.584
V 2717	Aa	A	0.500	AA	Aa	AA	Aa	AA 0.208	Aa 0.292
		a	0.500	aA	aa	aA	aa	aA 0.208	aa **0.292**
					↑				↑
				Expected genotype frequencies in the offspring not determined				Observed genotype frequencies of hom rec in the offspring	

x.xxx: Primary determined observations and frequencies; x.xxx: Derived variables. hom rec: Homozygote recessive.