Analysis of Genetic Linkage in *Salix exigua* NUTT.

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Abstract

Genetic linkage was investigated in thirteen isoenzymic loci in *Salix exigua* NUTT. progenies that conformed to a 2x2 balanced factorial mating design. An additional putative sex determination locus system was also included in the analysis. Joint segregation was tested in all of the 91 possible two-locus combinations across different families. Linkage investigations involved both contingency table χ^2 tests and likelihood ratio tests (LOD-scores). The recombination fraction (θ), standard errors and confidence intervals were estimated with maximum likelihood methods. One tightly linked group including loci *Acp-2* and *Alp-1* and another possible group involving *Aco-2*, *Cto-1* and *Ppo-1* were found. An overall positive interference was detected. There was no indication for non-random associations between sexual phenotypes and electrophoretic loci. Results are discussed in light of the potential application of linkage associations in breeding.

Key words: isoenzymes, *Salix exigua,* linkage, LOD scores, recombination fraction, mapping distance.

FDC: 165.3; 165.41; 176.1 *Salix exigua.*

1. Introduction

Studies of genetic linkage have progressed in many organisms including plants especially with the advent of biochemical (isoenzyme) and molecular (RFLP and RAPD) markers. Nevertheless, information regarding linkage relationships in forest angiosperms is still scarce, in spite of the broad implications that knowledge of linkage groups could have for tree genetics and breeding. Such information assists in answering questions pertaining to the organization and establishment of genetic variation in natural populations, permits the assessment of the distribution of available loci in the genome, and provides references to locate unmapped genes on chromosomes leading to gene mapping. Attempts by conventional forest genetics to address the above questions are laborious and very complicated (WRIGHT, 1976).

Many authors believe that studies of the genetic structure of any population should include linkage analysis (e.g. RUDIN and EKBERG, 1978; ECKERT *et al.,* 1981) and estimates of the recombination frequency and map distance between linked loci. The understanding of linkage relationships is essential for population models dealing with the maintenance of balanced polymorphism through epistatic selection. An expected response to selection includes functions of the linkage between pairs of involved loci. Model simulations involving two or more loci showed that the behavior of multilocus systems cannot be predicted from single locus theory (LEWONTIN, 1974).

The usefulness of identifying instances of joint segregation is also paramount in breeding. Knowledge of the positions of marker loci can permit the study and manipulation of important genes or of QTLs within or among species. Apart from such applications which have encountered success in crop plants (TANKSLEY, 1993), linkage maps have proved to be of importance for taxonomy and evolution (MITCHELL-OLDS, 1995). Several instances of synteny have been identified and although not as dramatic as in *Animalia* (MONZOT, 1983), they do permit a synergistic interaction of genetic research among related plant groups (WEEDEN and WENDEL, 1989).

Linkage studies in forest trees have focused almost exclusively on conifers. Today the genomes of *Abies, Pinus* and *Picea* are becoming relatively adequately mapped (TULSIERAM *et al.,* 1992; YAZDANI *et al.,* 1995). The level of organization cannot be compared however with the densely mapped genomes of maize, barley, tomato and other crops. On the other hand, information on forest woody angiosperms is limited. Few linkage groups have been identified for example in *Betula pendula* (HATTEMER, *et al.,* 1990), in *Prunus avium* (SANTI and LEMOINE, 1990), in *Liriodendron* spp. (PARKS *et al.,* 1990), and in *Liquidambar* spp. (HOEY and PARKS, 1990). *Eucalyptus* spp. present an exception (GRATTAPAGLIA and SEDEROFF, 1994). In poplars, only *Populus tremuloides* (LIU and FURNIER, 1993) and the hybrid *P. trichocarpa x deltoides* (BRADSHAW *et al.,* 1994) have been adequately mapped. In another report, regarding poplars of the *Tacamahaca* section, three linkage groups were detected (MULLER-STARK, 1992). Analyses of joint segregation by MALVOLTI *et al.* (1991) in *Populus deltoides* and RAJORA (1990) in *P. nigra* and *P. maximoviczii,* did not lead to the identification of linkage groups. Limited information exists in *Salix.* THORSEN *et al.* (1997) recently reported the presence of four possible linkage groups in *Salix viminalis.*

This study investigates the linkage relationships among isoenzymic loci in *Salix exigua* NUTT. (section *Longifoliae,* subgenus *Salix*), which is a diploid (n = 19) North American willow. *S. exigua*, one of the most widespread willow species in North America, is important in hybridization and breeding programs for biomass production (ZSUFFA and ARAVANOPOULOS, 1989). The taxonomical classification of *S. exigua* is still a matter of debate (CHONG *et al.,* 1995b) posing an additional theoretical interest in genetic studies of this species. The genetic basis of enzymatic variation in this species has already been reported (ARAVANOPOULOS *et al.,* 1993).

2. Materials and Methods

2.1. Loci investigated

Inheritance analysis in *S. exigua* revealed 13 segregating loci: *Acp-2, Aco-1, Aco-2, Adh-2, Alp-1, Cto-1, Per-1, Per-3, Pgm-1, Pgd-1, Pgi-2, Ppo-1,* and *Sdh-2,* in 11 enzyme systems: acid phosphatase (ACP), aconitase (ACO), alcohol dehydrogenase (ADH), alkaline phosphatase (ALP), cytochrome oxidase (CTO), peroxidase (PER), 6-phosphogluconate dehydrogenase (6-PGD), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), polyphenol oxidase (PPO) and shikimate dehydrogenase (SDH) (ARAVANOPOULOS *et al.,* 1993). This study employed the parental clones and full-sib progeny of a 2x2 balanced factorial mating design, involving female clones I61 and I62, and male clones I66 and I293. These clones were originally selected as "plus-trees" from unrelated populations of south-eastern Ontario (MOSSELER and ZSUFFA, 1989).

In addition, sex was recorded in the progeny individuals. Although the existence of a heteromorphic pair of chromosomes in the dioecious *Salicaceae* has not yet been confirmed (GRANT and MITTON, 1979), MOSSELER and ZSUFFA (1989) provide evidence that such a sex determination system may exist in Salix with the male being the heterogametic sex. Sexual phenotype was also included in the data set as a segregating additional putative sex-determining locus system (denoted *Sdl-1*). The distribution of the observed progeny genotypes with respect to sex was compared with the expected distribution of 1:1. Mendelian inheritance was examined by testing the "goodness of fit" to the expected ratios (χ^2 test). The consistency of the results over different crosses was tested by computing χ^2 heterogeneity values.

2.2. Estimation of linkage and recombination fractions

The 13 biochemical loci and the postulated sex-determining locus were investigated for both autosomal and sex linkage. The statistical procedures used to test for linkage and to estimate the recombination fraction are described below. A doubly heterozygous tree produces four kinds of gametes: A_1B_1, A_1B_2, A_2B_1, A_2B_2. Parental and recombination types of gametes are associated with the numbers of gametes in coupling (those in categories A_1B_1 and A_2B_2) and repulsion (those in A_2B_1 and A_1B_2). However, because the breeding history of a certain parental clone is unknown, it is not clear whether the parental category is associated with the coupling or the repulsion phase. This complicates the estimation of the recombination frequency and is disregarded in a maximum likelihood estimation of the recombination fraction (θ), if θ is estimated as the quotient of the number of the putative recombinants to the number of all analytical gametes (GEBUREK and von WUEHLISCH, 1989). For this reason the use of the maximum likelihood procedure is essential, since it considers the fact that the putative recombinants are not necessarily the *de facto* recombinants. This formal approach was sometimes overlooked in the past, in favor of linkage estimation with the ad hoc "natural" procedure of the binomial estimator k/n, where k = number of observations in the smaller class (unknown if in coupling or in repulsion) and n = sample size. This approach may generate reliable results only when the observed categories are considerably dispersed and sample sizes are large (n>200), a situation uncommon in forest genetics research.

Non-random joint segregation of pairs of loci was estimated by employing the LINKAGE-1 version 3.50 (SUITER *et al.,* 1983) software. This program assesses linkage employing contingency table χ^2 tests to compare observed two-locus segregation data with those expected from actual single-locus segregation ratios. When significant deviation from independent assortment is observed, LINKAGE-1 estimates linkage values, as well as recombination fractions (θ) and corresponding standard errors (s), by using the maximum likelihood formulae developed by ALLARD (1956). Suspect linkage groups were further investigated with the LINKEM (VOWDEN *et al.,* 1995) software. This program, employs the likelihood ratio test (LOD-score) for linkage, as well as contingency table χ^2 tests. In double heterozygotes, unbalanced segregation in one pair of alleles does not affect the χ^2 analysis of joint segregation (BAILEY, 1961), but does not permit the use of the likelihood ratio linkage test. Therefore in such cases linkage were investigated only by the contingency table χ^2 test approach. Homogeneity of the recombination fractions from different families was estimated according to BAILEY (1961).

Locus pairs that could be tested in one family only, were not considered. When a pair of loci segregated in more than one family and produced additive offspring genotypic classes across families, then the test for linkage and the estimation of recombination frequencies was conducted from individual families, as well as jointly from all available families. Linkage tests in these cases were based both on the total χ^2 value across families, and the χ^2 and LOD estimates of the pooled data set. If it was not possible to pool the data, a weighted average recombination frequency from individually informative families, was computed according to COLQUHOUN (1971), with a standard error estimated according to BAILEY (1961). Pooling data from different families produces more robust tests when compared to estimates of the total χ^2 value across families due to the reduction in the degrees of freedom, however since parental phase is unknown there is a small chance that pooling may mask true association among locus pairs.

2.3. Estimation of mapping distances

In gene mapping, methods of statistical analysis are employed to deduce the relationship among the physical phenomena of chiasma formation, crossing over, and the actual distance between two genes. If n loci belong to the same linkage group the $n(n\text{-}1)/2$ recombination fractions θ can be reduced to n-1 map intervals w by a suitable mapping function $w = f(\theta)$, which is the *sine qua non* for a linkage map. The function suggested by KOSAMBI (1944) was employed. Mapping distance was calculated according to KOSAMBI (1944), and the associated standard error was calculated according to OWEN (1950). In order to estimate the impact of chiasma interference, the coefficient of coincidence (C) and its variance were calculated according to BAILEY (1961).

3. Results and Discussion

3.1. Inheritance of sex expression

Sex expression in progeny individuals was initially scored when 3 years old by MOSSELER and ZSUFFA (1989), who observed a considerable proportion of non-flowering plants, as well as a number of hermaphrodites. For the purpose of this research these progenies were re-scored when approximately 8 years old. At this age, most of the *S. exigua* progeny presented well developed flowers. No hermaphrodite plants were observed and some non-flowering plants were confined to only one family (I61 x I293). This family was not included in the subsequent investigation for sex linkage, since the total number of individuals that bore flowers (8 female and 4 male) was small for statistical testing. In the other families sex expression was total. Segregation analysis showed that the distribution of males and females in the progenies did not deviate significantly from the expected distribution of 1:1, while results over different families were statistically homogeneous (*Table 1*). Despite a general tendency in *Salix* towards female-biased sex ratios (which may be explained by hypothesizing meiotic ratio distorters in presumed sex chromosomes), other *S. exigua* intraspecific families were not reported to deviate from the 1:1 sex ratio (MOSSELER and ZSUFFA, 1989), in agreement with our results. These findings may suggest the notion of a putative

Table 1. – Segregation in the progenies of controlled crosses of *S. exigua* of a putative sex determining locus system and results of χ^2 tests of "goodness of fit" to expected ratios for 1:1 inheritance.

Family	Offspring genotypes		χ^2 (df)	$P>\chi^2$
	XX	XY	0.273 (1)	0.602
I62XI66	18	15	0.030 (1)	0.862
I62xI293	17	16	3.667 (1)	0.056
I61xI293	11	22	0.495 (1)	0.475
Pooled	46	53	3.790 (2)	>0.100
Heterogeneity				

Table 2. – The number of families (below the diagonal) and the total number of trees (above the diagonal) examined in each particular pair of 14 polymorphic loci.

Locus	Aco-1	Aco-2	Acp-2	Adh-2	Alp-1	Cto-1	Per-1	Per-3	Pgd-1	Pgi-2	Pgm-1	Ppo-1	Sdh-2	Sdl-1
Aco-1		66	97	99	33	98	99	33	66	64	65	96	99	98
Aco-2	2		95	97	64	96	99	64	64	31	63	94	98	66
Acp-2	3	3		130	64	129	130	64	97	64	96	127	131	97
Adh-2	4	3	4		64	129	130	64	97	64	96	127	131	99
Alp-1	1	2	2	2		65	64	64	31	31	31	62	65	33
Cto-1	3	3	4	4	2		130	64	97	64	96	127	131	98
Per-1	3	3	4	4	2	4		64	130	64	96	127	131	99
Per-3	1	2	2	2	2	2	2		33	31	31	62	65	33
Pgd-1	2	2	3	3	1	3	4	1		33	96	96	98	33
Pgi-2	2	1	2	2	1	2	2	1	1		33	64	66	66
Pgm-1	2	2	3	3	1	3	3	1	3	1		96	98	65
Ppo-1	3	3	4	4	2	4	4	2	3	2	3		131	98
Sdh-2	3	3	4	4	2	4	4	2	3	2	3	4		99
Sdl-1	3	2	3	3	1	3	3	1	3	2	2	3	3	

Table 3. – Potential linkage groups, their recombination fractions (θ), standard errors (s) and associated 95% confidence intervals (I) within and across informative families. χ^2 tests, LOD scores and associated probabilities are also presented.

Locus pair	Cross	χ^2 (df)	P	LOD	P_{LOD}	θ	s	I
Acp-2:Alp-1	I61xI66	20.401 (1)	<0.0001	5.31	<0.0001	0.094	0.051	0.02-0.28
	I61xI293	16.433 (1)	<0.0001	2.72	<0.0002	0.193	0.060	0.08-0.35
	Pooled	32.156 (1)	<0.0001	7.74	<0.0001	0.143	0.044	0.07-0.24
	Total	31.269 (2)	<0.0010					
Aco-2:Cto-1	I62xI293	8.214 (2)	0.0160	1.67	0.003	0.200	0.100	0.07-0.41
	I61xI66	3.391 (3)	0.3350	0.06	0.300	0.450	0.087	0.29-0.50
	I61xI293	16.976 (6)	0.0090	1.63	0.003	0.230	0.063	0.12-0.40
	Total	28.582 (11)	<0.005			0.273	0.045	–
Cto-1:Ppo-1	I62xI66	6.077 (4)	0.1930	[1]	–	0.342	0.076	0.22-0.47
	I62xI293	8.689 (4)	0.0690	–	–	0.319	0.074	0.21-0.45
	Pooled	8.436 (4)	0.0770	–	–	0.330	0.053	0.24-0.43
	I61xI66	24.817 (9)	0.0030	–	–	0.230	0.053	0.13-0.34
	I61xI293	27.097 (9)	<0.001	–	–	0.370	0.061	0.26-0.48
	Pooled	36.517 (9)	<0.001	–	–	0.300	0.041	0.22-0.38
	Total	44.953(13)	<0.001			0.311	0.057	
Adh-2:Pgm-1	I62xI66	0.308 (1)	0.5790	0.06	0.300	0.455	0.087	0.29-0.50
	I62xI293	15.252 (6)	0.0180	0.66	0.040	0.350	0.079	0.22-0.50
	I61xI293	2.691 (2)	0.2600	0.57	0.050	0.316	0.120	0.14-0.50
	Total	18.252 (9)	<0.050			0.325	0.048	

[1]) LOD scores are not presented since their validity may have been influenced by the segregation distortion in *Ppo-1*

sex determination locus system for *S. exigua.* If such a system is present, it would appear to present Mendelian inheritance in the families of this research.

3.2. Estimation of linkage parameters

All of the 91 possible two-locus combinations which can be formed from the 14 loci used, could be tested. Significant segregation distortion was observed for both loci of a tested pair in a single case only, in particular in the *Pgi-2:Ppo-1* pair of cross I62xI66 (ARAVANOPOULOS *et al.,* 1993). This combination was excluded from the analysis. The number of families employed and the total number of genotypes used in the linkage analysis are presented in *table 2.* It should be noted that 84.4% of the two-locus combinations could be investigated in two or more families (with the number of progeny individuals employed ranging from 63 to 132). There were 221 two-locus tests that were initially investigated over the four full-sib families.

To evaluate linkage from different families both the χ^2 test and the LOD score were investigated. In the former case the rejection of the null hypothesis H_0:θ=0.5 was associated with a first-type error level of 0.05 for the total χ^2 value across all informative families. In the latter case the predetermined threshold level for LOD was set to 1.50 for two reasons: (a) higher levels appear to provide a very conservative test (GERBER and RODOLFE, 1994), and (b) due to the high chromosome number of *S. exigua,* any locus pair has an elevated *a priori* probability for linkage.

Sex linkage was not detected in *S. exigua.* This lack of non-random associations between isoenzyme loci and sexual phenotype is prevalent in plants. Although sex-linked enzyme loci have been commonly detected in animal species (MCPHERSON and BERLOCKER, 1985), this phenomenon is very rare in plants. Few reports exist, for example the associations between enzyme loci and sex in *Gleditsia triacanthos* (SCHNABEL and HAMRICK, 1990) and *Plantago lanceolata* (WOLFF, 1987), and between a RAPD marker and sex determination in *Pistacia vera* (HORMAZA *et al.,* 1994).

The potential linkage groups detected in this study involved autosomal loci. These are presented in *table 3.* As it can be judged from this *table Acp-2* and *Alp-1* formed a closely linked group. Two other pairs (*Aco-2:Cto-1* and *Cto-1:Ppo-1*) were considered as possibly linked, while the *Adh-2:Pgm-1* pair was regarded as not linked.

Table 4. – Linkage groups, parental phases, associated statistics and mapping parameters: the χ^2 test (χ_r^2) for the homogeneity of recombination fractions among families and associated probabilities, the Kosambi mapping distances (D) and associated standard errors (s_D) in cM (ns: not significant).

Linkage group	Cross	Parental phase	χ_r^2 (df)	P	D	s_D
Acp-2:Alp-1	I61xI66	AA/BB x AA/AA				
	I61xI293	AA/BB x AA/AA				
			1.337 (1)	ns	14.71	4.79
Aco-2:Cto-1	I62xI293	AB/AC x AB/BC				
	I61xI66	AA/BB x AB/AC				
	I61xI293	AA/BB x AB/BC				
			6.371 (2)	0.05<p<0.025	30.63	6.41
Cto-1:Ppo-1	I62xI66	BB/CC x BB/CC				
	I62xI293	BB/CC x BB/CC				
	I61xI66	AA/BB x BB/CC				
	I61xI293	AA/BB x BB/CC				
			0.144 (1)	ns	36.41	9.30

The results of the most likely parental phase for the alleles of the presumed linked groups are summarized in *table 4.* Since information about parental phase was spread across different families depending on the linkage group, it was possible to investigate if the different crosses conferred a consistent view of the unknown parental phase. In the *Acp-2:Alp-1* group parental phase could be investigated in the doubly heterozygous clone I61 in two independent crosses. In both crosses associated with the greatest likelihood function was the same coupling phase:

Acp-2A_____Alp-1A
Acp-2B_____Alp-1B

In the *Aco-2:Cto-1* group the doubly heterozygous I61 clone could be investigated in two crosses. In both of these crosses the same arrangement of parental phase possessed the highest likelihood function. The same parental phase was also observed in two crosses with regards to parental clone I293. The most likely parental phase of parental clone I66 resulted from the analysis a single cross. The parental phases in these parental clones were:

I61: Aco-2A_____Cto-1A Aco-2B_____Cto-1B
I66: Aco-2A_____Cto-1B Aco-2A_____Cto-1C
I293: Aco-2A_____Cto-1B Aco-2B_____Cto-1C

In the *Cto-1:Ppo-1* group, parental phase could be tested in four different crosses and due to the factorial mating design employed, each parental clone could be tested twice. In all parental clones a consistent view of parental phases emerged within all clones. Individual alleles were found to be in coupling. The parental phases which consistently presented the highest likelihood function were:

I61: Cto-1A_____Ppo-1A Cto-1B_____Ppo-1B
I62: Cto-1B_____Ppo-1B Cto-1C_____Ppo-1C
I66: Cto-1B_____Ppo-1B Cto-1C_____Ppo-1C
I293: Cto-1B_____Ppo-1B Cto-1C_____Ppo-1C

An inspection of parental phases in the above group provided further evidence of the parental phase of *Cto-1* alleles in parental clones I61, I66, I293. This phase was consistent in the analysis of the *Aco-1:Cto-1* group as well as in the analysis of the *Cto-1:Ppo-1* group. In general results showed a consistent view of the parental phase of individual alleles in different crosses where parental clones were shared. This result can be regarded as in favor of the presence of the linkage groups as discussed above.

Homogeneity of recombination fractions among families was observed in two groups while in the other group some heterogeneity was detected ($0.025<p<0.05$; *Table 4*). In general it was observed that recombination rates varied among families. Similar observations have been demonstrated in many taxa and have been mainly attributed to sampling error, but also to genetic (inversion polymorphisms, pre- or post-zygotic selection) or environmental factors (GRELL, 1966; RUDIN and EKBERG, 1978).

The KOSAMBI mapping distances associated with the inferred linkage groups are depicted in *table 4.* The three locus pairs that were considered as linked formed two separate linkage groups, one tightly linked, and a possible one rather loosely linked. The relative localization of these loci into linkage groups is:

Acp-2-----$^{14.71\ cM}$-----Alp-1
Aco-2----------$^{30.63\ cM}$----------Cto-1----------$^{36.41\ cM}$----------Ppo-1

These groups presumably mark two different chromosomes.

The presence of a group with three consecutively linked loci permitted the estimation of interference parameters. The estimate of the coefficient of coincidence for the *Aco-2:Cto-1:Ppo-1* was $C=0.830$ ($s_C=0.817$) and reflected an overall positive interference. Positive interference would mean that the occurrence of one exchange between homologous chromosomes in *S. exigua* would reduce the likelihood of another in its vicinity. Similar interference levels have been reported in conifers (RUDIN and EKBERG, 1978; STRAUSS and CONKLE, 1986) and in various other plant and animal species (SWANSON *et al.,* 1981).

3.3. General conclusions

As it has been reported for many conifer and crop plant species, in general linkage conservation exists above the species level (WENDEL and WEEDEN, 1989). In agreement with the results of this study THORSEN *et al.,* (1997), found no linkage between *Acp-2:Pgm-1* and *Acp-2:Sdh-2* in *Salix viminalis.* Joint segregation in *Aco-1, Aco-2, Per-1, Pgm-1, Pgi-2* and *Sdh-2,* was also investigated by LIU and FURNIER (1993) in *Populus*

tremuloides. In concordance with the results of this study these 13 locus pairs were also found not linked in *P. tremuloides*. Furthermore, in accordance with the above results, PER and 6-PGD loci were also found not to be linked in *Populus* spp. (HYUN *et al.,* 1987; RAJORA 1990).

The establishment of linkage groups in *S. exigua* provides a basis for further gene mapping using biochemical (for example the 38 enzyme loci that were invariable in the families of this study; ARAVANOPOULOS *et al.,* 1993), and molecular markers (such as those developed by CHONG *et al.,* 1995a). Furthermore, the assignment of particular genes and quantitative trait loci in linkage groups could be facilitated. Detailed gene mapping is executed today almost exclusively with the aid of DNA markers such as RFLPs and RAPDs. However, most of the RFLP and RAPD maps that exist today in higher organisms, including plants, are well anchored in stable isoenzyme markers that have been mapped in advance.

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