Variation and Inheritance of Isozyme Loci in Controlled Crosses of *Salix alba* and *Salix fragilis*

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Summary

The expression of polymorphism in different enzyme systems was investigated in clones of *Salix alba* and *Salix fragilis.* Dormant buds and sprouting leaves were used but only few consistent enzyme patterns could be revealed and were retained for further testing. When considering all obtained enzyme profiles, a total of 43 genes could be scored, of which 15 may show polymorphism within *S. alba* or *S. fragilis.* Polymorphism was observed in aconitase, alcohol dehydrogenase, phosphoglucomutase, leucine aminopeptidase, glutamate oxaloacetate transaminase, β-esterase, isocitrate dehydrogenase, glutamate dehydrogenase, peroxidase, superoxide dismutase and xanthine dehydrogenase. The genetic basis of phosphoglucomutase, leucine aminopeptidase glutamate oxaloacetate transaminase and shikimate dehydrogenase could be inferred directly from intraspecific and interspecific controlled crosses. Full-sib progeny showed mendelian segregation for lap-1 and most of the pgm-2 alleles, whereas in other genes such as β-est-1 and adh-2, there was only fixed heterozygosity. Most of the isozymes can not be used directly as good markers at species level. Differences between *S. alba* and *S. fragilis* are mainly in the allele frequencies. The number of genes and alleles of the polyploid *S. alba* and *S. fragilis* were compared to those of earlier reports on diploid *Salix* species. The polyploids contain less genetic diversity in their soluble enzymes than the diploids. Duplicated genes did not led to a substantial increase in the heterozygosity levels in the *S. alba-S. fragilis* complex.

Key words: Salix alba, Salix fragilis, willow, isozymes, gene diversity, progeny testing, hybridization, polyploidy, evolution.

FDC: 165.3/.4; 176.1 *Salix alba;* 176.1 *Salix fragilis.*

Introduction

Biochemical genetic markers have become a valuable tool in the breeding of forest trees. Most of the applications are on conifers and there is less information on the genetics of woody angiosperms. Within Salicaceae most isozyme studies have been performed on Populus (e.g. RAJORA, 1990; MÜLLER-STARCK, 1992), whereas in Salix, the study of the inheritance of isozymes was limited to the diploid taxa (2x=38) such as *S. eriocephala* (ARAVANOPOULOS, 1992) and *S. exigua* NUTT. (ARAVANOPOULOS et al., 1993). Patterns of genetic variation in population studies were conducted on the section Longifoliae (BRUNSFELD et al., 1991; ARAVANOPOULOS, 1989, 1992), *S. silicicola* RAUP. and *S. alaxensis* (ANDERS.)COV. (PURDY and BAYER, 1995).

Isozyme studies based on full-sib families are important since such data are needed for a correct genetic interpretation of the phenotypic pattern. In the case of polyploids, the zymograms often are more complicated due to duplications and fixed heterozygosities. Enzyme variation that is physiologically induced at different developmental stages or that is environmentally induced should be excluded from the standard genetic variability analysis in populations. Inheritance studies are also required for the detection of segregation distortion (STRAUSS and CONKLE, 1986) and of linkage, which is especially useful in the case of polyploids. The amount and pattern of genetic variation within and among populations and closely related species further can be influenced by a wide range of factors, including mating system, natural selection, geographic distribution, and historical events (LOVELESS and HAMRICK, 1988).

This study focuses on the genetics of *Salix alba* and *Salix fragilis* which are closely related species with a widely sympatric distribution throughout Europe. The boundary between the two species is defined by a relatively poor amount of diagnostic features in their morphology. In many localities the two species coexist in mixed stands. The taxonomic identification as well as the determination of so-called functional population units is still uncertain. These willow species are dioecious and thus obligate outcrossers. Clonal growth however is very common as known for most polyploid species. The high chromosome number (2x=76) may indicate ancient auto-or allopolyploid origins. Isozyme studies on *Salix exigua* NUTT. showed that in spite of its relatively high chromosome number (2x=38), the willows seems to remain functionally diploidized. Some enzymes retain their duplicate control due to homologous genes, while others lose their diploid expression, possibly due to gene silencing or hybrid dysgenesis (ARAVANOPOULOS et al., 1993). The lack of clear-cut diagnostic characters, together with the fact that interspecific controlled crosses are successful and that intermediate morphological forms largely dominate on the field, support the hypothesis that *S. alba* and *S. fragilis* may hybridize frequently. Hybrids and introgressed hybrids seem to dominate when considering the morphology (DE BONDT, 1996). Studies on allozyme variation in other *Salix* species (e.g. *S. silicicola* RAUP., *S. alaxensis* (ANDERS.) COV.) revealed that differentiation between populations is low (PURDY and BAYER, 1995). This is consistent with the reviewed data on trees with a dioecious breeding system and wind-dispersed seeds (HAMRICK and GODT, 1989). Factors that promote high levels of genetic diversity within populations in *Salix* species include dioecism, high fecundity, wind-dispersed seeds and long-lived clonal growth. Characteristic for *Salix* thus might be the allelic evenness of allozyme distribution (BRUNSFELD et al., 1991) as well as the low probability for genetic drift (PURDY and BAYER, 1995).

In this study, we first investigated a large number of clones to elucidate the polymorphism in the different enzymes and thereafter selected the most reliable polymorphic enzymes such as GOT, LAP and PGM for studying their inheritance in controlled interspecific and intraspecific crosses between *S. alba* and *S. fragilis.*

Materials and Methods

Plant material: The *S. alba* and *S. fragilis* clones originated from Belgium and consisted of the parental types and progeny of 8 controlled crosses (*Table 1*) and of a basic collection of 239

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clones. The clones were collected in 1982, 1986, 1990 and 1996 and further cultivated at the Institute for Forestry and Game Management. The controlled crosses were performed in 1990 by the institute. The morphological identification was based on the UPOV guidelines (Union for Protection Of Varieties) and resulted in a classification of putative pure clones and of hybrid clones.

Table 1. – List of eight parental types and of 8 families resulting from intraspecific and interspecific crosses of *S. alba* and *S. fragilis* clones.

Controlled crosses Code	Species	Locality	Clone nr.
Female parental types			
A	S. alba	Oudenaarde	86.027
B	S. alba	Oudenaarde	86.061
C	S. fragilis	Lessen	82.042
D	S. fragilis	Geraardsbergen	90.004
I	S. alba	Oudenaarde	86.080
Male parental types			
E	S. alba	Oudenaarde	86.111
G	S. fragilis	Montignies-les-Lens	82.103
H	S. fragilis	Onkerzele	90.002
Progenies (F1) Code	Female x male	Individuals (n)	Family nr.
B x E	alba x alba	35	90.005
B x H	alba x fragilis	15	90.008
D x G	fragilis x fragilis	40	90.015
D x H	fragilis x fragilis	40	90.016
C x E	fragilis x alba	40	90.009
I x G	alba x fragilis	12	90.024
A x G	alba x fragilis	14	90.003
D x E	fragilis x alba	14	90.013

Extraction procedures, enzyme electrophoresis and staining procedures: Mature vegetative buds or sprouting leaves were used for enzyme analysis. The buffer conditions and the procedures for electrophoresis were as used in TRIEST (1989). The buds or leaves were used fresh or after freezing in liquid nitrogen and storage at –80 °C. Best results were obtained with fresh material. When using stored leaf material, the tissues were extracted immediately in cold conditions. Interference with secondary plant compounds (most likely phenols) could inhibit enzyme activities. Therefore the following buffers have been screened: A = 10 ml sample buffer, 100 µl β-mercapto-ethanol, Nonidet; A + 10 mg/ml PVP-10 ; A + 10 mM thioureum. Addition of PVP (polyvinyl pyrolidone) improved the staining activity. Different concentrations of PVP-10 (10 mg/ml, 20 mg/ml, 30 mg/ml) and Tris-Cl (0.062 M, 0.1 M, 0.125 M) then have been compared with the enzyme activities obtained for similar plant materials. The combination of 0.062 M Tris-Cl with 10 mg/ml PVP gave the best results.

A Tris-Cl buffer was used throughout the electrophoresis procedure. Poly-acrylamide gels of 7.5% were used for vertical slab gel electrophoresis. The staining of enzymes was according to the procedures mentioned in TRIEST (1989). Starch gel electrophoresis gave unsatisfactory results for most of the enzyme separations. Twenty-two enzyme systems had been tested, only the following were retained because of their activity : aconitase (ACO, EC 4.2.1.3), alcohol dehydrogenase (ADH, EC 1.1.1.1), aldolase (ALD, EC 4.1.2.13), acid phosphatase (APH, EC 3.1.3.2) β-esterase (β-EST, EC 3.1.1.1), diaphorase/menadione reductase (DIA/MNR, EC 1.6.99.2), esterase (EST, EC 3.1.1.2), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), glutamate dehydrogenase (GDH, EC 1.4.1.2), glutamate oxalo-acetate transaminase (GOT, EC 2.6.1.1), glucose phosphoisomerase (GPI, EC 5.3.1.9), isocitrate dehydrogenase (IDH, EC 1.1.1.42), leucine-amino-peptidase (LAP, EC 3.4.11.1), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), peptidase (PEP, EC 3.4.11.13), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), phosphoglucomutase (PGM, EC 2.7.5.1), peroxidase (POD, EC 1.11.1.7), shikimate dehydrogenase (SkDH, EC 1.1.1.25), superoxide dismutase (SOD, EC 1.15.1.1), xanthine dehydrogenase (XDH, EC 1.2.1.37). Only LAP, PGM and GOT showed reproducible patterns and may serve for the genetic analysis of the progeny.

Levels of allozyme variation were estimated at the level of the families by the mean number of alleles per locus (A) and percent polymorphic loci (P). The genetic variability and standard genetic identities were calculated with the BIOSYS program (SWOFFORD and SELANDER, 1981).

Results

Activity and use of the enzymes at different developmental stages

The expression of enzymes in buds and leaves may change according to the stage of development in buds and young leaves. The best results were obtained with extracts from buds and from young leaves that sprouted indoors. All leaf samples from outdoor collections revealed less clear activity for the majority of the enzymes, most probably due to the effect of increasing amounts of phenols in the extract. A severe loss of enzyme activity and consequently loss of information for further genetic interpretation was noted in developing leaves of three weeks old (*Table 2*). Major changes between young and older leaves were that β-EST and SOD kept activity but

Table 2. – Activity and potential use of the enzymes revealed at different developmental stages (++P : clear banding pattern and polymorphism; ++M : clear banding pattern but monomorphic; + : clear activity but unclear banding pattern; +/- : low activity and inconsistent; - : no activity; 0 : not tested).

	buds	young lvs 1-3 weeks indoor	young leaves < 3 weeks outdoor	older leaves > 4 weeks outdoor
ACO	0	+	+/–	+/–
ADH	+	++P	++P	–
ALD	0	–	0	0
APH	0	–	0	0
β-EST	++P	++P	++P	+/-P
EST	0	–	–	0
G6PD	+	+	–	0
GDH	++P?	+	+/–	+/–
GOT	++P	++P	+/–	–
GPI	0	+	–	0
IDH	++P	++M	0	+/–
LAP	++P	++P	++P	+/-P
MDH	+	+	+/–	0
ME	++M	+	0	–
MNR	+	+	+/–	0
PEP	0	+	+/–	0
6PGD	+	+	+P?	0
PGM	++P	+P	+	+
POD	++P	+	+/–	+/–
SkDH	++M	++M	++M	+/–
SOD	++P	++P	++M	+/–
XDH	0	++M	++P	+/–
USE				
++P	8	5	4	0
++M	2	3	2	0
+	5	11	2	1
+/-	–	–	7	10
–	–	3	3	2
0	7	–	4	9

became inconsistent since novel bands appeared while the diagnostic ones faded away. ADH showed a full zymogram in young leaves but became inactive at later stages. GOT, SkDH, POD, GDH, IDH, XDH, ACO and ME showed a clear loss of activity while LAP and PGM kept activity, however often as broad overlapping patches which are difficult to interprete in terms of genes and alleles.

Genetic interpretation of enzyme patterns

A total of 43 genes can be scored, of which 15 may show polymorphism within *S. alba* or *S. fragilis* (*Table 3*). Nevertheless, these 15 genes are to be considered as a maximum that can be revealed in the considered enzyme systems.

Table 3. – The maximum number of genes and alleles observed in enzymes of *Salix alba* and *Salix fragilis* buds and sprouting leaves.

Enzyme	Scored loci	Variable loci	Alleles
ACO	2	2	4
ADH	2	1	4
ALD	–	–	–
APH	–	–	–
β-EST	5	5	12
DIA	2	0	?
EST	–	–	–
G6PD	2	0	2
GDH	2	0	3
GOT	3	1	5
GPI	1	0	1
IDH	2	1	4
LAP	3	1	4
MDH	>2	0	?
ME	1	0	1
PEP	2	0	2
6PGD	2	0	2
PGM	3	1	3
POD	>2	1	6
SkDH	1	0	1
SOD	5	1	6
XDH	1	1	2
Total	43	15	>70

Monomorphic enzymes

No polymorphisms were detected for eight enzyme systems, namely shikimate dehydrogenase, glucose-6-P-dehydrogenase, 6-P-gluconate dehydrogenase, malic enzyme, glucose-P-isomerase, peptidase, malate dehydrogenase and diaphorase.

Diaphorase (DIA) = menadione reductase (MNR)

DIA showed two zones of activity and consisted of a slow one-banded but uniformic zone and of a fast but rather inconsistent zone of bands in three possible positions. The DIA pattern however coincided with the activity of NADH-DH. The isozymes of the fastest zone could be the expression of one locus dia-1 encoding for different monomeric enzymes. No definite genetic interpretation could be given here and no correlation between DIA and one of both species could be noted.

6-P-gluconate dehydrogenase (6PGDH)

6PGDH had two unclear zones of activity, corresponding to 6pg-1 and 6pg-2. When present, 6pg-1 always had a three-banded pattern, most probably indicating fixed heterozygosity of this dimeric enzyme.

Glucose-6-P-dehydrogenase (G6PDH)

G6PDH showed two zones of activity, however g6p-1 and g6p-2 were inconsistent in their expression and often very faint.

Glucose-P-isomerase (GPI)

GPI was difficult to reveal in buds as well as in young leaves and gpi-1 showed a single but monomorphic product.

Malate dehydrogenase (MDH)

MDH showed two zones of activity consisting of a slow zone with two bands of major activity (but no polymorphism) and of a faster zone with up to five, but unclear and patchy bands. At least two genes (probably more) are involved.

NADP-dependent malic enzyme (NADP-ME)

ME had one band and was uniform for *S. alba* and *S. fragilis.* Therefore, it could not be detected whether this enzyme is a dimer or a tetramer.

Peptidase (PEP)

PEP was difficult to reveal but showed two products of weak activity. Both pep-1 and pep-2 appeared monomorphic. PEP enzymes are most likely monomeric.

Shikimate dehydrogenase (SkDH)

SkDH shows one band of major activity, sometimes accompanied by up to three additional bands of minor activity which could be breakdown products. There is no allelic segregation observed of the monomers so it may be presumed that Skdh-1 is monomorph for all investigated *S. alba* and *S. fragilis* samples (*Fig. 1*).

Polymorphic enzymes

Aconitase (ACO)

ACO was difficult to reveal and showed a maximum of 2 bands in aco-1 of young leaves. Aco-1 has 3 alleles, but all different homozygotes and heterozygotes have not been observed yet. A slower system aco-2 sometimes was revealed in sprouting leaves, but it appeared to be monomorphic. No clear marker for *S. fragilis* or for *S. alba* could be noted (*Fig. 1*).

Alcohol dehydrogenase (ADH)

In buds, ADH showed no clear polymorphism and was only active as a single band eventually accompanied of minor bands. In young developing leaves, ADH became fully expressed as products of 2 genes. Adh-1 was monomorphic while adh-2 showed either 1, 2 or 3 bands. The 3 banded pattern could be interpreted as a fixed heterozygote, since no segregation was noted. The expression of less than 3 bands most probably was due to lowered activity of the heterodimer or one of the homodimers (*Fig. 1*).

β-esterase (b-EST)

β-EST showed 5 zones of activity (a to e). The slowest one was rather inconsistent, the second one remains one-banded and monomorphic, while the 3 fastest zones together showed rather consistently a 2-banded or a 3-banded pattern in buds. There were no intermediates observed, only presence-absence situations. The genetic background remained unknown. The 3-banded pattern was observed in several *S. fragilis*-like specimens. In sprouting leaves, β-EST becomes fully expressed in the 5 zones. The 3-banded pattern remained characteristic for those *S. fragilis*-like samples, while all other putative hybrid forms and *S. alba* exhibited additional products. Numerous combinations have been detected such that β-EST

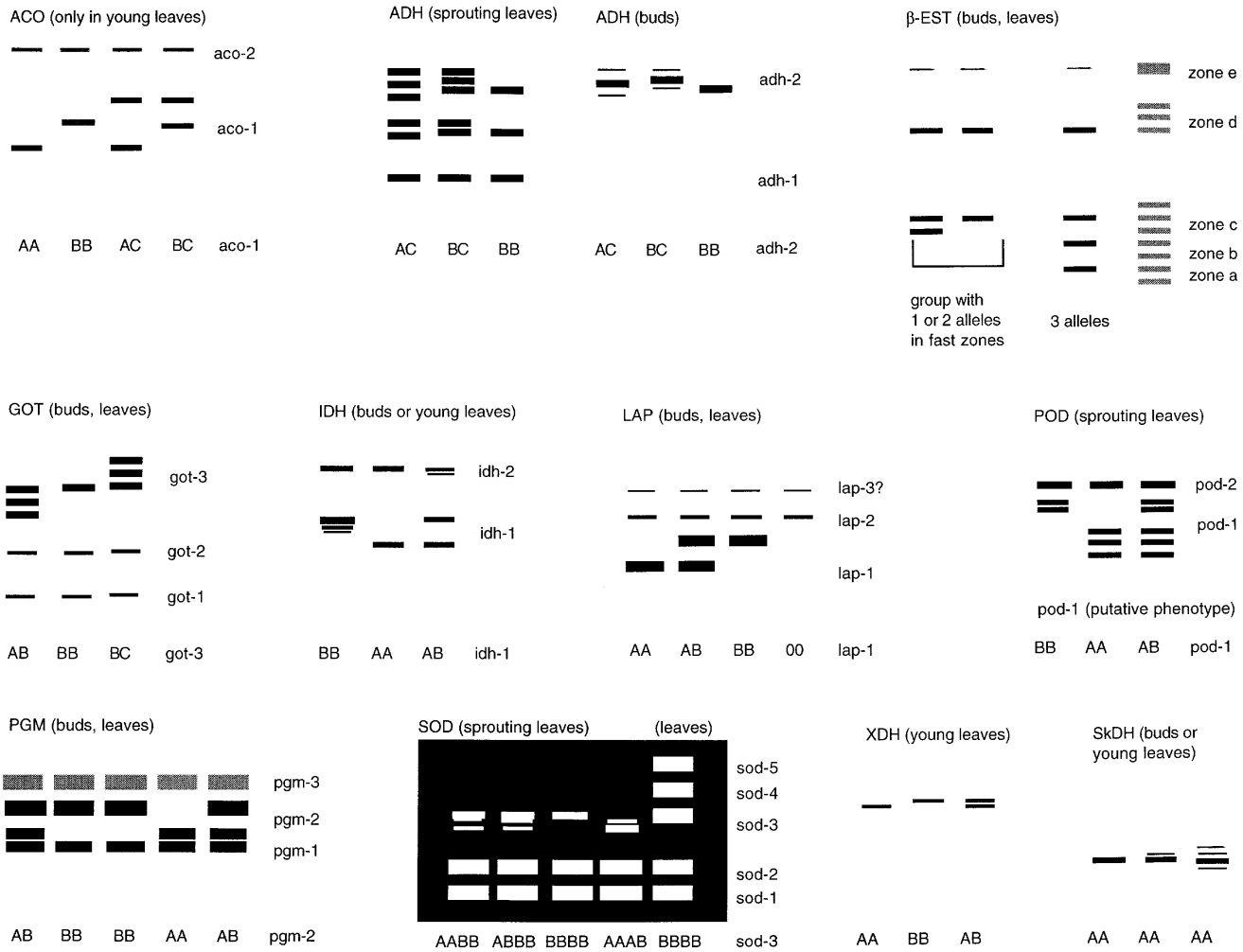

Fig. 1. – Representation of allozyme patterns for the polymorphic loci of ACO, ADH, β-EST, GOT, IDH, LAP, POD, PGM, SOD, XDH and SkDH considered in *S. alba* and *S. fragilis* with the corresponding genotype assessed (migration from top to bottom and alleles are designated alphabetically according to the mobility of the protein they encoded from the fastest to the slowest one). The codes AA, AB etc. refer to the putative genotype of the polymorphic locus of each enzyme. Most of the enzymes are similar when extracted from buds or leaves, unless indicated otherwise.

remained a promising but difficult marker to elucidate the possible degree of hybridization between individuals. Difficulties are certainly in comparing the alleles of a corresponding gene, between two or more samples, due to the complexity of presence-absence situations (*Fig. 1*).

Glutamate dehydrogenase (GDH)

The hexameric GDH rarely showed the typical 7-banded pattern with bands at equal distance, encoded by two genes. Several samples of *S. fragilis*-like specimens showed a pattern that is more skewed towards the fast migrating zone (most probably a faster allele for gdh-1 was involved) and some patterns exhibited more than 7 bands (it is unclear how many exactly because the patterns never were fully expressed). GDH is difficult to reveal and often remains patchy or appears as a single band in gdh-1.

Glutamate-oxaloacetate-transaminase (GOT)

GOT showed two zones of activity in buds and three in leaves. The fastest zones are one-banded and monomorphic, while the slowest zone can be 3-banded. However, the got-3 pattern appears to be fixed at the heterozygous stage. The slower band in got-3 is more indicative for *S. fragilis*-like clones than for *S. alba*-like clones (*Fig. 1*).

Isocitrate dehydrogenase (IDH)

IDH had two zones of activity. A slow (but not always appearing) zone with 1 or 2 bands and a fast zone with 1 or 2 bands. IDH is known as a dimeric enzyme and since no segregation or a three-banded pattern was observed, a duplication could be involved here (*Fig. 1*).

Leucine amino peptidase (LAP)

LAP was characterized by a fast zone of activity (lap-1) with 2 alleles and two slow zones (lap-2 and lap-3) with much lower activities. The same two alleles were found in both *S. alba* and *S. fragilis*. More rarely, a presumed null allele was observed. Because of their high frequencies, LAP alleles could be used to check the degree of clonality on the field and to detect genetic structuring of populations (*Fig. 1*).

Peroxidases (POD)

POD showed a complex pattern with three patterns of activity in the faster zone, namely a one-banded monomorph zone, a two-banded monomorph zone and a three-banded monomorph zone (the latter with only presence/absence as differential expression). The progeny of all intraspecific *S. alba* crosses lacked the three-banded zone, while offspring from all

intraspecific *S. fragilis* crosses had this three-banded zone (and in three cases out of the four, the offspring of the intraspecific *S. fragilis* crosses lack the two-banded zone). Thus POD could provide an additional marker when using sprouting leaves. Nevertheless, it can mainly be interpreted phenotypically (*Fig. 1*).

Phosphoglucomutase (PGM)

PGM presumably had three genes, of which the slowest one (pgm-3) might be more typical for *S. alba*. Segregation of 3 alleles was observed for pgm-2. As PGM is a monomer, the four-banded pattern can only be explained by the existence of 2 or 3 genes. The sometimes patchy nature of PGM banding patterns at pgm-3 made it difficult to reveal the separate alleles and assign them to a particular gene (*Fig. 1*).

Superoxide dismutase (SOD)

SOD had three zones of activity (in buds) but showed no polymorphism in the fastest zones (two thick bands designated as sod-1 and sod-2). The slower zone (sod-3) however gave differences in activity of the 1-3-banded pattern as known for the balanced and unbalanced heterozygotes (*Fig. 1*). This might reflect an autopolyploid nature. In leaves, this expression of balanced and unbalanced heterozygotes quickly fades away during development and is replaced by the activity of two additional slower gene products (sod-4 and sod-5).

Xanthine dehydrogenase (XDH)

XDH is a monomer and showed two alleles at close migration position. There was no obvious correlation with either one of the species or their hybrids. Xdh-1 could only be revealed during a short period of some developing leaves (*Fig. 1*).

Controlled crosses

For the parental types and their progeny, a complete data set was obtained for LAP, PGM, GOT and SkDH. Due to fast changes during development of the young leaves, several data were missing for ADH, IDH and β-EST. Very incomplete were ACO, SOD and POD, though it could be promising markers. All other enzyme systems were monomorphic or inconsistent for further use. We were confronted frequently with the fact that certain individuals never showed activity for particular enzymes, even when repeated under changed buffer conditions for the extraction and staining.

For the further genetic analysis of the inter- and intraspecific crosses, only the four most reliable and complete set of enzyme stainings in buds (GOT, LAP, PGM, SkDH) could be considered, representing 9 loci of which lap-1, pgm-2 and got-3 could be polymorphic among the parental types and their progeny (*Table 4*). The lap-1 gene contained 2 alleles. All progeny samples followed unambiguously the presumed segregation of alleles A and B as inferred from the parental types. There was no diagnostic difference between *S. alba* and *S. fragilis* in LAP alleles, except that the *S. fragilis* parents all were homozygous for allele B in lap-1 whereas the *S. alba* parental genotypes were either homozygous or heterozygous. The frequency of the rarer allele A was about 0.27 in the case of a single heterozygote parent and about 0.53 when both parental types were heterozygous. The pgm-2 gene contained 2 alleles occurring in both *S. alba* and *S. fragilis* individuals (*Table 4*). Apparently, there was no species specific allele for pgm-2. The segregation of alleles A and B into the progeny was as expected from the genetic analysis. The AA genotype was only observed in the parental type G and in the progeny of IxG, DxG and DxH (D and H were heterozygous for pgm-2 and were *S. fragilis* or *S. fragilis*-like). The GOT pattern was similar for all parental types and contained a 3-banded slow migrating zone. This could be interpreted as the result of a duplication (gene duplication or chromosome doubling). Consequently, all progeny contained the 3-banded pattern. The only exception was within the progeny of DxG and DxH that expressed the common 3-banded pattern in their offspring as well as a homozygous, 1-banded pattern in the slowest migrating zone. An interpretation thereof is difficult to give, only based on the present knowledge. Maybe got-3 has to be considered as two duplicated genes and that only the intraspecific crosses within *S. fragilis* allow such a segregation.

Basic electrophoretic data were used for combined family comparisons. The families each contain the available set of F1 genotypes, pooled together with both parental genotypes. The 9 loci (4 enzymes) contained 12 alleles in total. Each family consisted of 12 to 40 available individuals. Basic genetic variability measures showed low values for each family. The mean number of alleles (A) ranged between 1.1 to 1.3 and the percentage of polymorphic loci (P) between 11% to 33%. The genetic distances between the families ranged from 0.012-0.184 (ROGERS, 1972) or from 0 to 0.128 (NEI, 1978). Since lap-1 clearly followed the expected segregation ratio, it can be effectively used as a marker in clones from the field at different hierarchic levels. Pgm-2 alleles mostly followed the expected segregation, except for the progeny of DxH and IxG. The number of samples from IxG was too low, while the result of DxH still requires an explanation.

The β-EST patterns were not always consistent as a whole, but major differences could be found in the fastest migrating zone or the β-est-1 alleles of the parental types D and G that were previously identified morphologically as *S. fragilis*. As they genetically deviated largely from the presumed pure *S. fragilis* samples (TRIEST et al., 1997), this β-EST variation might be non typical for the species as a whole. The progeny of DxG followed the parental patterns, whereas the progeny of DxH might represent segregation of alleles in the β-est-1

Table 4. – Progeny testing for lap-1 and pgm-2 in inter- and intraspecific crosses of *Salix alba* and *Salix fragilis* (*) = significant at 0.05 level, NS = non significant).

Cross	Parental	Progeny			Chi-square	p	Significance
	Lap-1	AA	AB	BB			
B x E	AB x AB	8	19	6	1	0.61	*
B x H	AB x BB	0	8	7	0.067	0.80	*
D x G	BB x BB	0	0	40	–	–	–
D x H	BB x BB	0	0	40	–	–	–
C x E	BB x BB	0	0	40	–	–	–
I x G	BB x BB	0	0	12	–	–	–
A x G	BB x BB	0	0	14	–	–	–
D x E	BB x AB	0	5	5	0	1	*
	Pgm-2	AA	AC	CC			
B x E	CC x CC	0	0	35	–	–	–
B x H	CC x AC	0	7	8	0.067	0.80	*
D x G	AC x AA	24	15	0	2.077	0.15	*
D x H	AC x AC	17	21	2	11.35	0.003	NS
C x E	AC x CC	0	20	20	0	1	*
I x G	AA x AC	3	9	0	3	0.08	NS
A x G	AC x AA	14	10	0	0.667	0.41	*
D x E	AC x CC	0	7	6	0.077	0.78	*

system (e.g. the progeny individuals 16/3 and 16/6 are similar to H, while 16/19 and 16/20 had bands common with both D and H). From the RAPD data (TRIEST et al., 1997), it is recently known that the parental type H exhibits heterozygosity for a large number of its genes (amplification products). This could explain the different pattern of segregation in this family. The ADH allozymes were not always fully expressed in young developing leaves, but when active they showed diagnostic differences between *S. alba* and *S. fragilis.* Because of the unclear activity in leaves from the families, the data set on ADH was too small to include in the genetic variability analysis.

Discussion

Genetic basis of enzymatic variation

The genetic variability analysis based on the enzyme polymorphism was also limited by the lowered activity in buds for a number of enzymes. Therefore only GOT, LAP, PGM and SDH could be used effectively. ADH, β-EST, and IDH sometimes gave unclear banding patterns. The β-EST pattern in buds shows also less activity in two of its slowest genes when compared to young leaf extracts. Young leaves are more promising, but the desired enzyme activity could only be revealed during a period of about 3 weeks. Nevertheless, the electrophoretic data can be indicative for major trends in the polyploid *S. alba-S. fragilis* complex.

The genetic interpretation of enzymatic variation in tetraploid *S. alba* and *S. fragilis* (2X = 76) is more complicated than in e.g. similar studies of the American diploid (2X = 38) *S. exigua, S. silcicola, S. alaxensis* and *S. viminalis.* When comparing to *S. exigua,* much of the same enzymes were revealed in our study, except APH, ALP and PPO (ARAVANOPOULOS et al., 1993). *S. exigua* has zymograms with less loci for ADH and PGM, but as an average *S. exigua* revealed more polymorphic genes than *S. alba* or *S. fragilis* (e.g. 6PGD, SDH). The diploid *S. exigua* thus has more allelic variation per gene, whereas *S. alba* and *S. fragilis* have genes with reduced allelic variation however compensated by additional duplicated genes (e.g. PGM, GOT). The endemic *Salix silicifolia* was found less polymorphic than the more widespread *S. alaxensis* when using 10 polymorphic genes in 9 enzymes (PURDY and BAYER, 1995). Both species are even differentiated in several enzymes.

When compared to these diploid North American willows, the Belgian tetraploid *Salix alba* and *Salix fragilis* contain a comparable number of genes for ACO, ADH, 6PGD and POD and even less for GPI and SkDH. The polyploids only seem to have more genes for PGM since the diploids exhibit only one gene, comparable to the variation observed in pgm-2. The additional pgm-1 and pgm-3 genes in the polyploids however are monomorphic. Even when comparing the number of alleles, it becomes evident that the diploids contain more gene diversity. In e.g. *S. silicicola,* five alleles were observed in lap-1, skdh-1 and g6pd-2 and up to six alleles in idh-2.

This is somehow in contradiction with the hypothesis that polyploids are more polymorphic and contain more genetic diversity than diploids. In general, molecular data not only demonstrate numerous examples of recurrent formation of polyploid taxa, but also strongly suggest that multiple origin is the rule, rather than exception, in polyploid evolution (SOLTIS and SOLTIS, 1993). Polyploids may have formed numerous times in different geographical areas. In Draba species from Norway, such a proces of repeated allopolyploidization offers an explanation for the taxonomic complexity (BROCHMAN et al., 1992). In general, the process of multiple polyploidization would certainly enrich the total gene pool of the polyploid species. Once that genetic variation is introduced into polyploid species, the dynamic evolutionary processes continue to act at the polyploid level. Segregation among progeny from a heterozygous polyploid and subsequent fixation of new recombinants also may increase the array of genotypes present in polyploid species.

Since the diploids in *Salix,* investigated yet for allozyme diversity are genetically far more diverse and heterozygous, a hypothesis to test for the polyploid *S. alba* and *S. fragilis* thus might be that their process of polyploidization has led to a loss of variation (bottle-neck in the speciation proces) and that this genetic erosion is somehow compensated by duplications. Duplicated genes for PGM however code for monomorphic gene products in pgm-1 and pgm-3 and do not increase the heterozygosity level. Another hypothesis to test is whether *S. fragilis* is fixed for a larger portion of its genes than *S. alba.* A first indication in favour of this hypothesis can be found in the low frequency of allele A of lap-1.

Genetic interpretation inferred from controlled crosses

The electrophoretic data of parental types with their offspring from intra- and interspecific crosses illustrate, that they form a sufficient set of genetic markers for further application. The F1 progenies followed the segregation possibilities as expected from the parental types in lap-1 and pgm-2. Isozyme polymorphism has revealed that the hybrid F1 progeny from controlled crosses shows segregation for lap-1 and pgm-2. The monomeric lap-1 and pgm-2 with only two and three alleles, respectively, should be regarded as good markers for additional population studies. Interpretation of lap-1 and pgm-2 supports the hypothesis that in spite of the very high chromosome number (2n = 76), *S. alba* and *S. fragilis* seem to be functionally diploidized in the expression of these particular gene products. However, the progeny of DxH is an exception to this. Maybe the activation of a duplicated gene interfered at the position of one of the homozygotes.

On the other hand, gene duplication events have occurred in PGM, GOT, ADH, most likely also in β-EST, while slower migrating LAP genes are noted as well. This duplicated control can be due to homologous chromosome parts in the polyploid complex. Got-3 and also est-1 and adh-2, when revealed, mostly have fixed heterozygous enzyme patterns and do not show homozygotes. Hybrids thus are difficult to identify since the putative diagnostic enzymes ADH and β-EST exhibit 2 main patterns corresponding to either *S. alba* or *S. fragilis* instead of the expected intermediate patterns.

Most of the enzymes can not be used directly as good markers for *S. alba* and *S. fragilis* species or their hybrids because both species mainly contain similar alleles and differ only in their allele frequencies. However, this particular use depends entirely on the local source of the material. As such, the fact that the intraspecific families DxG and DxH are closely related has also to do with the particular genotypes of the parental types (e.g. homozygous for lap-1) than solely with the taxonomic identity of both parents. The highest genetic distances are observed between family IxG and family DxG (also between BxE and DxG) but this can again be explained by the characteristics of the parental types rather than on the basis of diagnostic enzymes per species. The use of enzymes for a qualitative analysis of many families is apparently too much influenced by initial parental allelic composition. This effect is even more pronounced in the case of lowered sample sizes of loci.

The goal of studying the offspring of several controlled crosses in *Salix* was to infer the genetic control of isozymes not

solely on the estimations of their multimeric structure before carrying out a survey on clones from the field. The isozyme polymorphism has provided the useful information. Full-sib progeny from controlled crosses shows mendelian segregation for lap-1 and pgm-2 (most probably, this can be expected as well for aco-1). Got-3 segregates as well but is only exceptionally observed in the intraspecific *S. fragilis* progeny of family DxG. Est-1 and adh-2 (most probably also 6pg-1) have fixed heterozygous enzyme patterns and do not show homozygotes. The hybrids are difficult to identify since the more or less diagnostic enzymes ADH and β-EST exhibit 2 main patterns corresponding to either *S. alba* and *S. fragilis.* Most *S. fragilis*-like samples are homozygous or nearly fixed for the allele B in lap-1 which might be a good marker for calculating the fixation index and hierarchical F-statistics in clones from the field (TRIEST et al., 1998).

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A Model for Infusion of Unrelated Material into a Breeding Population

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Abstract

A consequence of selection in a closed breeding population is an increased level of relatedness. One remedy to this may be infusion of unrelated genetic material into the breeding population. A model is established to study such infusion assuming that new plus-trees equivalent with the old are available. The model uses group merit as the criterion for balancing genetic gain and relatedness measured by group coancestry. Infusion is optimized by finding the maximum group merit. The model involves variables such as average breeding value, structure (family number and size), heritability, relatedness (group coancestry) and its importance (penalty coefficient), and inbreeding. The most important determinant for infusion is the breeding value of the bred material followed by the relatedness between the selected families. An example with considerable similarities to the Swedish breeding program of Norway spruce and Scots pine was given. For establishing the first generation breeding population, it seems optimal to add about 20% to 25% new plus-tree selections rather than to make all selections in the progenies of the existing untested plus-trees. If the plus-trees were progeny tested, about 5% to 10% new selection seems desirable. For more advanced generations, the desire of infusion depends on progress in breeding value and accumulation of relatedness and inbreeding in the breeding population.

Key words: Diversity, inbreeding, relatedness, group coancestry, group merit, selection, breeding population.

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