ISOZYME DIFFERENTIATION BETWEEN SYMPATRIC CLONES OF *SALIX ALBA* AND *SALIX FRAGILIS*

Ludwig Triest [1], Bart De Greef [1], Jos Van Slycken [2]& Els Coart [2]

[1] General Botany and Nature Management, Vrije Universiteit Brussel, Pleinlaan 2, B–1050 Brussels, Belgium
E-mail: ltriest@vub.ac.be
[2] Institute for Forestry and Game Management, Ministry of the Flemish Community, Gaverstraat 4, B–9500 Geraardsbergen, Belgium

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ABSTRACT

The allozyme variation in the progeny of controlled crosses within and between *Salix alba* and *Salix fragilis* was assessed for LAP, PGM, GOT and SkDH representing 12 alleles in 9 loci of which only 3 showed polymorphism. *Lap–1* and *Pgm–2* clearly followed the expected segregation and suggested that these polyploid willow species are functionally diploidized in the expression of these genes. Gene duplications have occured in PGM, GOT and ADH and most likely also in b–EST. Fixed heterozygosity was observed for *Got–3* in the progeny of an intraspecific cross of *S. fragilis*. Based on the inferred genetic control of these allozymes, a survey of 239 clones of the morphological *S. alba – S. fragilis* complex indicated that there is rather a differentiation in allozyme frequencies at the level of the putative species than at the level of geographical hierarchies. The fixation index for *Lap–1* indicated a lack of heterozygotes in all investigated *S. alba* locations. At the level of catchments, these values ranged from 0.457 to 0.617 and suggest that this might be a common situation. The lack of heterozygosity for *Lap–1* was even more pronounced in the *S. fragilis* clones that were mainly homozygous or monomorphic. The hierarchical structuring of heterozygosity indicated that most of the differentiation occured at the level of locations or tributaries and that there is no further differentiation between the catchment level considered for each species.

Key words: *Salix alba*, *Salix fragilis*, willows, isozymes, clones, genetic diversity, hybridization.

INTRODUCTION

The amount and pattern of genetic variation within and among populations of closely related species can be influenced by a wide range of factors, including mating system, natural selection, geographic distribution, and historical events (LOVELESS & HAMRICK 1988). Closely related plant species have been genetically compared with emphasis on the difference between widespread and restricted distributions (KARRON 1987; HAMRICK & GODT 1989, PURDY & BAYER 1995). There are however few studies on the comparison of related widespread species, of which one is very common and another is only sparsely distributed, *e.g. Salix alba* L. versus *Salix fragilis* L. on the European continent. Since it is observed that endemic species or species with a restricted distribution contain fewer loci polymorphic and less heterozygosity than do more widespread species (HAMRICK & GODT 1989), it would be interesting to investigate similar processes among sympatric species of which one is much more dominant than the other.

Salix alba and *Salix fragilis* 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. Consequently, large overlaps exist which make it difficult to unambiguously identify samples from the field. 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 ($2n = 76$) may indicate ancient auto-or allopolyploid origins. Isozyme studies on *Salix exigua* Nutt. (ARAVANOPOULOS *et al.* 1993) and *Salix eriocephala* Michx. (ARAVANOPOULOS *et al.* 1994) showed that in spite of its relatively high chromosome number ($2n = 38$), the willows seem 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). Also the allozyme variation in full-sib families of *Salix viminalis* L. indicated Mendelian segregation (THORSEN *et al.* 1997). 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 in nature. 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 & BAYER 1995). This is consistent with the reviewed data on trees with a dioecious breeding system and wind-dispersed seeds (HAMRICK & 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 & BAYER 1995).

This study focuses on the genetics of isozymes in *S. alba* and *S. fragilis*. As the designation of enzyme loci in many species currently is accomplished by extrapolation from few available studies, we also compared our enzyme patterns of $2n = 76$ polyploids with those from a $2n = 38$ polyploid (ARAVANOPOULOS *et al.* 1993; ARAVANOPOULOS *et al.* 1993; THORSEN *et al.* 1997). 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 (TRIEST *et al.* 1998).

The objective of this study was to further investigate the use of allozyme variation in *Salix* clones from the field in order to detect the amount and hierarchical distribution of the genetic diversity. Therefore the size of a 'population' at the level of a location, a tributary or a river catcment was estimated.

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 239 clones originating from 4 catchment zones (Table 2). The clones were collected in 1982, 1986,

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	Putative 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 (F_1)			
crosses	female × male	individuals (n)	family nr.
B × E	*alba* × *alba*	35	90.005
B × H	*alba* × *fragilis*	15	90.008
D × G	*fragilis* × *fragilis*	40	90.015
D × H	*fragilis* × *fragilis*	40	90.016
C × E	*fragilis* × *alba*	40	90.009
I × G	*alba* × *fragilis*	12	90.024
A × G	*alba* × *fragilis*	14	90.003
D × E	*fragilis* × *alba*	14	90.013

Table 2. List of 239 *S. alba* (A) and *S. fragilis* (F) clones investigated for isozyme polymorphisms. An analysis was done for each hierarchical arrangement (4 catchments, 9 tributaries and 14 localities).

Catchments (4)	Tributaries (9)	Localities (14)	Species	No of clones
1. Dender	1. Mark	1. Mark	1A	20
			1F	12
	2. C. Dender	2. Wodecq – Ghoy	2A	15
		3. Rebaix – Lessen	3A	20
			3F	5
		4. Silly – Gibecq	4A	16
			4F	4
	3. E. Dender	5. Gages	5A	5
			5F	3
		6. Herchies – Lens	6A	11
			6F	8
	4. Baudour	7. Baudour	7A	3
			7F	6
	5. W. Dender	8. Mainvault	8A	12
		9. Beloeil – Blicquy	9A	10
2. Haine	6. Haine	10. Haine	10A	28
			10F	9
3. Rhosnes	7. Rhosnes	11. Anvaing	11A	11
			11F	2
		12. Escanaffles	12A	8
			12F	2
4. Schelde	8. Dendermonde	13. Appeles –Vl assenbroek	13A	12
	9. Rupel – Durme	14. Hamme – Hingene	14A	15

1990 and 1996 and further cultivated at the Institute for Forestry and Game Management. The controlled crosses were performed in 1990. The morphological identification was based on the UPOV guidelines and resulted in a classification of presumably pure clones and of hybrid clones. Mature vegetative buds or sprouting leaves were used for enzyme analysis.

Extraction procedures, enzyme electrophoresis and staining procedures: 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 because interference with secundary plant compounds (most likely phenols) could inhibit enzyme activities. Therefore following buffers have been screened: A = 10 ml sample buffer, 100 µl β-mercapto-ethanol, Nonidet ; A + 10 mg·ml^{-1} PVP-10; A + 10 mM thioureum. Addition of PVP (polyvinyl pyrolidone) improved the staining activity. Different concentrations of PVP-10 (10 mg·ml^{-1}, 20 mg·ml^{-1}, 30 mg·ml^{-1}) 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^{-1} 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 enzyme systems had been tested, only the following were finally retained because of reproducible patterns and of possible genetic analysis of patterns in each individual : alcohol dehydrogenase (ADH, EC 1.1.1.1), β-ESTerase (β-EST, EC 3.1.1.2), glutamate oxalo-acetate transaminase (GOT, EC 2.6.1.1), isocitrate dehydrogenase (IDH, EC 1.1.1. 42), leucine-amino-peptidase (LAP, EC 3.4.11.1), phosphoglucomutase (PGM, EC 5.4.2.2), shikimate dehydrogenase (SkDH, EC 1.1.1.25).

Levels of allozyme variation were estimated at the level of the families and for the clones, pooled at different hierarchical levels. Genetic diversity was measured by four parameters: mean number of alleles per gene (*A*), percent polymorphic loci (*P*), the ob

served (H_o) and expected heterozygosity (H_e). Variability at higher hierarchical levels or of the species were calculated by treating the pooled clones as if they were one population. Fixation indices (F), which reflect deviations from Hardy-Weinberg expectations are calculated for each hierarchical level. The partitioning of genetic diversity within and among groups of clones was measured by the F-statistics of WRIGHT (1965). The genetic variability, fixation indices, F-statistics, standard genetic identities were calculated with the BIOSYS program (SWOFFORD & SELANDER 1981). UPGMA clustering (Genetic distance of NEI 1978) and principal coordinate analysis based on genetic distance (NEI 1972) was done with NTSYS-PC (Exeter software).

RESULTS

Controlled crosses

We considered 4 reliable enzyme stainings (GOT, LAP, PGM, SDH) in buds representing 8 loci of which *Lap–1*, *Pgm–2* and *Got–3* could be polymorphic among the parental types and their progeny (Fig. 1). SkDH remained monomorphic in all samples. The *Lap–1* gene contained 2 alleles. All progeny samples followed

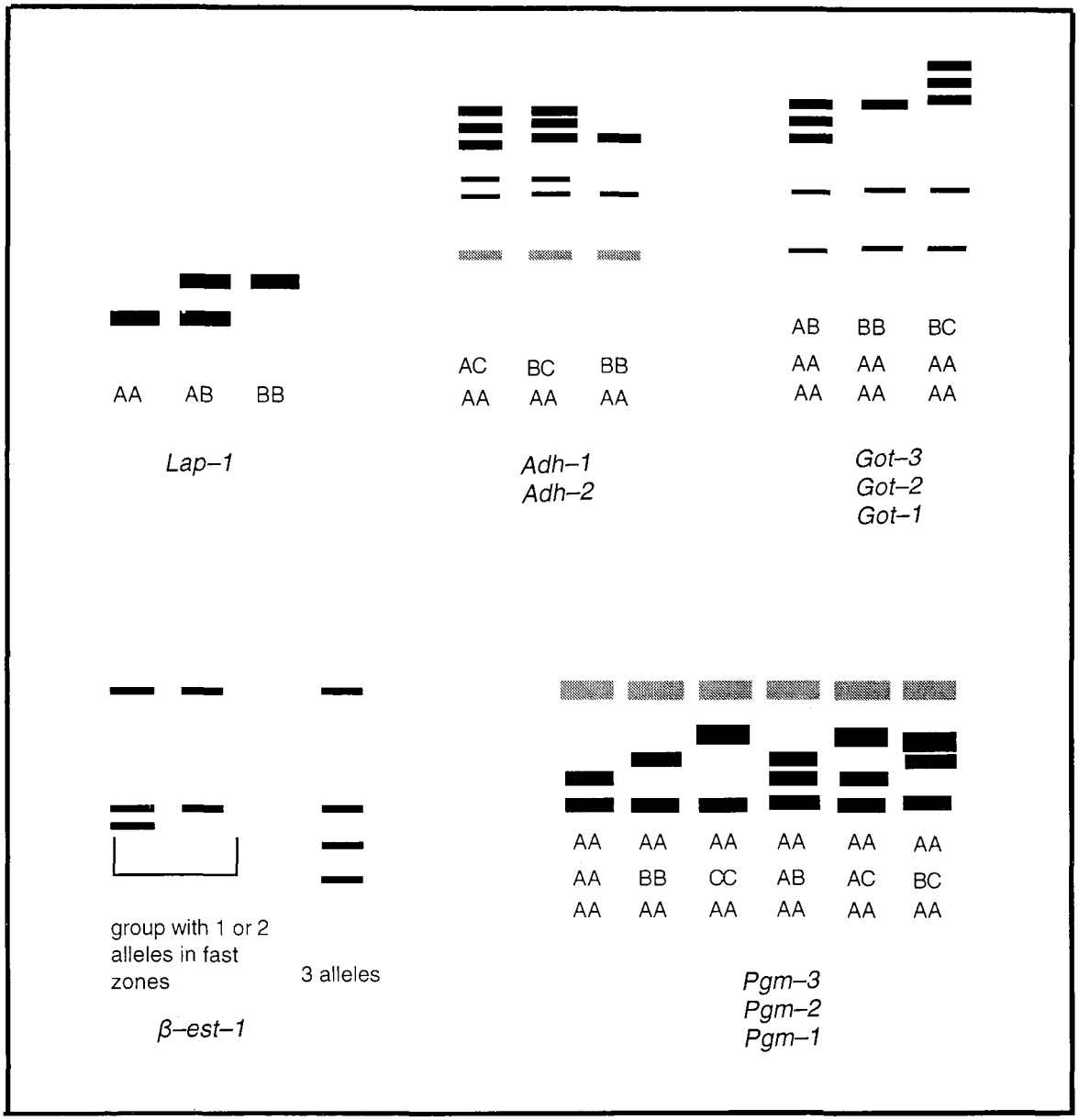

Fig. 1. Representation of allozyme patterns for the polymorphic loci of LAP, ADH, GOT, b–EST and PGM considered in *S. alba* and *S. fragilis* with the corresponding genotype assessed (migration from top to bottom and alleles are designated alphabetically according to the mobility of the protein they encoded from the fastest to the slowest one).

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 (Fig. 1). 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 I × G, D × G and D × H (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 contains a 3-banded slow migrating zone (Fig. 1). 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 D × G and D × H 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 was difficult to give, only based on the present knowledge. Maybe the intraspecific crosses within *S. fragilis* only allowed such a segregation. The crosses where *S. alba* is involved showed a 3-banded pattern.

Electrophoretic data were used for combined family comparisons. The families each contained the available set of F_1 genotypes, pooled together with both parental genotypes. The 8 loci (4 enzymes) contained 12 alleles in total. Each family consisted of 12–40 available individuals. Basic genetic variability measures showed low values for each family. The mean number of alleles (A) ranged between 1.1–1.3 and the percentage of polymorphic loci (P) between 11–33% (Table 3). The genetic distance between the families ranged from 0.012–0.184 (ROGERS 1972) or from 0–0.128 (NEI 1978). A cluster analysis (Fig. 2) revealed that family 15 (D × G) as well as family 16 (D × H), are clustered together, which were both intraspecific crosses of *S. fragilis*. The interspecific families were not clustered as a single group but are distributed all over the phenogram. As a whole, the clustering levels were low. Since *Lap–1* and *Pgm–2* clearly followed the expected segregation, both could be effectively used as markers in clones from the field at different hierarchic levels.

The b–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* (Fig. 1). As they genetically deviated largely from the presumed pure *S. fragilis* samples (unpublished RAPD data), the β-EST variation might be non typical for the species. The progeny of D × G followed the parental patterns, whereas the progeny of D × H might represent segregation of alleles in the *β-EST–1* system (only two F_1 specimens were similar to H, while another two to both D and H). 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* (TRIEST *et al.* 1998). 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.

Field collections

The results from the interspecific crosses demonstrated that hybrids between *S. alba* and *S. fragilis*, depending on the parental genotypes, were not necessarily clustered separately when based on allozymes of LAP, PGM, GOT and SDH. For the study of 239 clones of

Table 3. Summary of the allozyme variability for 9 putative loci within eight families of the controlled intra- and interspecific crosses (N = mean sample size per locus, A = mean number of alleles per locus, P = percentage of polymorphic loci, H_o = observed mean heterozygosity, H_e= expected mean heterozygosity).

Families	Codes	N	A	P	H_o	H_e
C × E	90.009	40	1.1	11	0.056	0.042
B × H	90.008	15	1.2	22	0.111	0.086
D × H	90.016	40	1.2	22	0.133	0.099
B × E	90.005	35	1.1	11	0.064	0.056
I × G	90.024	12	1.2	22	0.194	0.112
D × E	90.013	14	1.3	33	0.211	0.160
A × G	90.003	14	1.1	11	0.079	0.053
D × G	90.015	37	1.2	22	0.108	0.083

S. alba, *S. fragilis* and presumed morphological hybrids however, also β-EST and ADH could be included in the genetic variability analysis.

The putative *S. alba* and *S. fragilis* individuals were considered apart within each region. The latter identifications were based on morphological surveys and RAPD results (unpublished data). All samples were initially pooled into 14 localities (putative populations with clones at a distance of about 5–10 km). In a second analysis, the 14 localities were pooled into 9 larger geographical zones corresponding to tributaries (distances between clones are 10–25 km). In a third analysis the samples were pooled into 4 larger geographical zones corresponding to a river basin. The hierarchy (from localities to catchments) was used to calculate the F_{IS}, F_{IT} and F_{ST} values per species.

Localities

The putative *S. alba* – or *S. fragilis*-like individual trees were pooled according to their location among 14 localities. The mean number of alleles mostly was 1.2–1.3, more rarely 1.4–1.5 (only in four *S. alba* localities). Similarly, the percentage of polymorphic loci mostly was 15–23 %, more rarely 30 %. The mean heterozygosity ranged from 0.090–0.205 in the *S. alba*-like specimens and from 0.077–0.167 in the *S. fragilis*-like specimens (Table 4). The calculated fixation index varied enormously but had mostly positive values for *Lap–1* and negative values for *Pgm–2*. When considering the ten *S. fragilis* locations, six were fixed for *Lap–1*, whereas no fixation occurs for the *S. alba* locations (Table 5). The genetic distances (unbiased NEI 1978) ranged from 0–4 % in *S. alba* clones and from 0-11 % in *S. fragilis* clones. The maximum genetic distance between *S. alba* and *S. fragilis* may reach 21 %. In order to check whether the willows from these regions were intermediate forms, we subjected the matrix of genetic distances to a principal coordinate analysis. This ordination analysis revealed that there are two main groups, corresponding to either *S. alba* or *S. fragilis* (Fig. 3). The ordination biplot showed two main groups clearly separated along the first axis (Eigenvalue of 1.67). This separation was

Table 4. Summary of allozyme variability of *S. alba* (A) and *S. fragilis* (F) from 14 localities (*N* = mean sample size per locus, *A* = mean number of alleles per locus, *P* = percentage of polymorphic loci, H_o = observed mean heterozygosity, H_e = expected mean heterozygosity).

Localities	Codes	*N*	*A*	*P*	H_o	H_e
Salix alba						
Mark	1A	16.8	1.3	23	0.106	0.066
Wodecq – Ghoy	2A	12.5	1.3	23	0.145	0.105
Rebaix – Lessen	3A	15.6	1.4	23	0.111	0.078
Silly – Gibecq	4A	7.0	1.3	23	0.107	0.092
Gages	5A	2.0	1.2	15	0.092	0.100
Herchies – Lens	6A	5	1.3	31	0.129	0.149
Baudour	7A	3	1.3	23	0.205	0.149
Mainvault	8A	9	1.3	23	0.145	0.120
Beloeil – Blicquy	9A	8	1.4	31	0.090	0.129
Haine	10A	17	1.3	23	0.129	0.115
Anvaing	11A	9	1.3	23	0.166	0.103
Escanaffles	12A	4	1.2	23	0.148	0.123
Appels –	13A	12	1.4	23	0.150	0.137
Vlassenbroek	14A	15	1.5	31	0.143	0.134
Hamme – Hingene						
Salix fragilis	1F	12.0	1.2	23	0.160	0.098
Mark	3F	5.0	1.3	23	0.123	0118
Rebaix – Lessen	4F	4.0	1.2	15	0.154	0.088
Silly – Gibecq	5F	2.0	1.2	15	0.115	0.090
Gages	6F	7	1.3	23	0.092	0.123
Herchies – Lens	7F	6	1.3	23	0.167	0.103
Baudour	10F	8	1.3	23	0.154	0.177
Haine	11F	2	1.2	15	0.115	0.090
Anvaing	12F	2	1.2	23	0.077	0.128
Escanaffles						

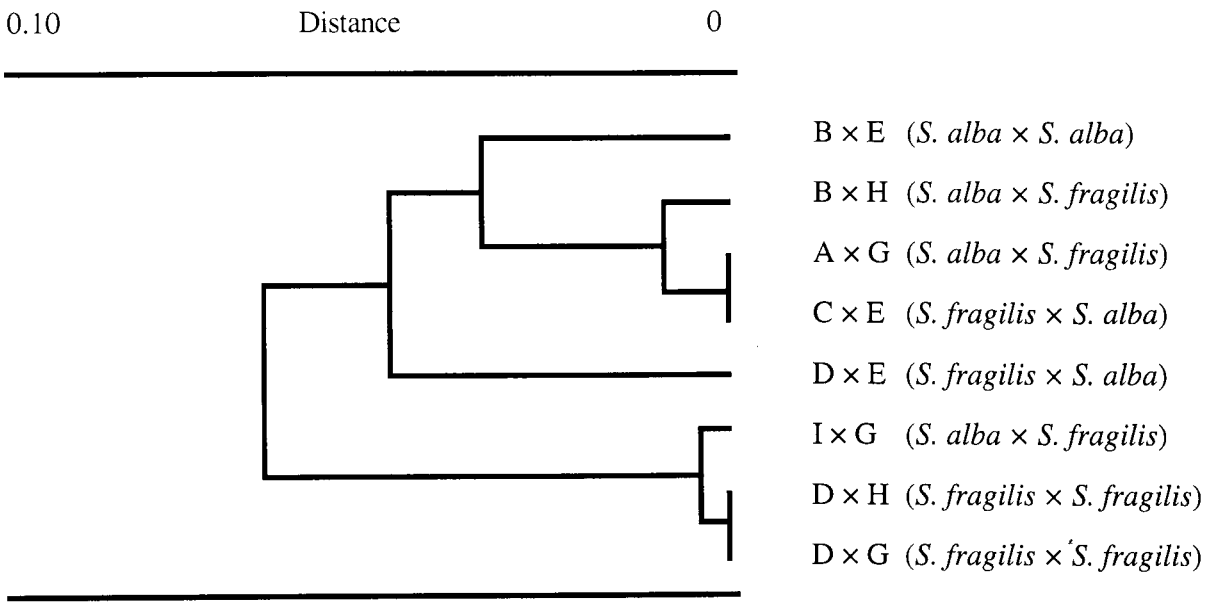

Fig. 2 UPGMA cluster analysis of isozyme differentiation between 8 families of intra- and interspecific crosses of *S. alba* and *S. fragilis* based on NEI (1978) genetic distance.

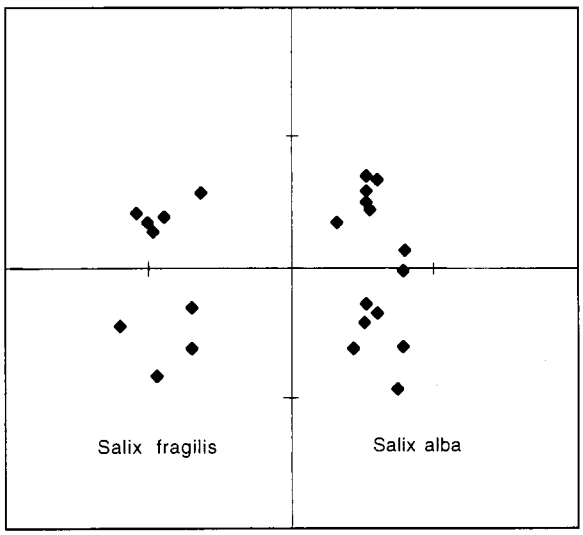

Fig. 3. Principal coordinate analysis of the isozyme differentiation between *S. alba* (A) and *S. fragilis* (F) in 239 clones from 14 localities.

primarily based on the different alleles of *Adh–2* and *Est–4*. Localities 11 and 6 (both *S. fragilis*) deviated highly towards opposite directions in the biplot. Maybe the variation on the second axis could be explained by the fact that within *S. fragilis* there was no fixation for *Lap–1* in regions 3, 6 and 12 (lower left corner of biplot), while the frequency of allele A in *Lap–1* was high for locality 11 (upper left corner), because of the lower sample size.

Table 5. Fixation indices for *Lap–1* and *Pgm–2* in 14 localities of *S. alba* and *S. fragilis*.

Localities	*S. alba*		*S. fragilis*	
	Lap-1	*Pgm-2*	*Lap-1*	*Pgm-2*
1	–0.081	–0.099	0.619	–1.000
2	0.583	0.510		
3	–0.081	0.141	1.000	–0.379
4	0.817	0.143	–	–1.000
5	0.524	–	–	–1.000
6	0.542	–0.429	0.746	0.082
7	–1.000	–0.091	–0.091	–0.756
8	0.314	–0.667		
9	0.583	–0.091		
10	0.603	0.059	–	–0.684
11	–0.158	–0.655	–	–0.333
12	0.746	–0.667	1.000	–0.333
13	0.500	–0.364		
14	0.659	–0.254		

Nine tributaries

In a second analysis, the isozyme data of each species from 14 localities were pooled into 9 larger areas at the level of tributaries (Table 2). *S. alba* was present throughout the entire region. *S. fragilis* was present in only 6 tributaries and sympatrically with *S. alba*. The mean number of alleles per locus was 1.3–1.5 (*S. alba*) and 1.2–1.4 (*S. fragilis*). The percentage of polymorphic loci was 23–31% for both species. The mean

Table 6. Fixation indices for *Lap-1, Pgm-2* and *Got-3* in *S. alba* and *S. fragilis* from nine tributaries.

Tributaries	*Lap–1*	*Pgm–2*	*Got–3*
S. alba			
Mark	–0.081	–0.099	–1.000
C. Dender	0.362	–0.269	–0.825
E. Dender	0.736	0.161	–0.823
Baudour	–1.000	–0.091	–1.000
W. Dender	0.455	–0.294	–0.337
Haine	0.603	0.059	–0.895
Rhosnes	0.457	–0.608	–1.000
Dendermonde	0.500	–0.364	–0.488
Rupel-Durme	0.659	–0.254	–0.758
S. fragilis			
Mark	0.619	–1.000	–1.000
C. Dender	1.000	–0.379	–0.667
E. Dender	0.814	–0.268	–0.474
Baudour	–0.091	–0.756	–1.000
Haine	mono	–0.684	–1.000
Rhosnes	1.000	0.000	–0.600

Table 7. Fixation indices for *Lap–1, Pgm–2* and *Got–3* in *S. alba* and *S. fragilis* from 4 catchments.

Catchment	*Lap–1*	*Pgm–2*	*Got–3*
S. alba			
Dender	0.498	–0.024	–0.709
Haine	0.603	0.059	–0.895
Rhosnes	0.457	–0.608	–1.000
Schelde	0.617	–0.355	–0.622
S. fragilis			
Dender	0.825	–0.556	–0.721
Haine	mono	–0.684	–1.000
Rhosnes	1.000	0.000	–0.600

heterozygosity ranged from 0.106–0.205 (*S. alba*) and from 0.096–0.160 (*S. fragilis*). The fixation index for *Lap–1* in *S. alba* mostly varied from 0.362–0.736 and only exceptionally was negative due to the smaller sample sizes (–0.081 and –1). The latter negative values should be regarded as less reliable and consequently were omitted from the discussion. The fixation index for *Lap–1* in *S. fragilis* varied mostly from 0.619–1.0 or was monomorph. Only in tributary 4 (Baudour) the value was much lower (–0.091), but this was again due to an extreme small sample size for this species. The fixation values for *Pgm–2* were mostly negative, showing no differentiation at this level between the two species. (Table 6). The genetic distances (NEI 1978) among the *S. alba* from the 9 tributaries were extremely low and varied from 0.1 –2.7%. For the *S. fragilis*, averaged as 6 tributaries, the genetic distances were equally low and varied from 0–2%

Table 8. Hierarchical F-statistics combined across all loci

	Localities	Tributaries	Catchments
Tributaries	0.243		
Catchments	0.141	–0.135	
Species	0.141	–0.135	0.000

Four catchment zones

When pooling the isozyme data of each species into 4 larger areas, corresponding to entire river catchments (Table 2), the mean number of alleles per locus was 1.3 –1.5 (*S. alba*) and 1.3–1.4 (*S. fragilis*). The percentage of polymorphic loci was 23–30.8 % for both species. The mean heterozygosity ranged from 0.116 –0.159 (*S. alba*) and from 0.096–0.154 (*S. fragilis*). In *S. fragilis*, the fixation index for *Lap–1* varied between 0.825 and 1 or *Lap–1* remained homozygous for allele B (Table 7), whereas a higher value of heterozygosity was observed in *S. alba* for *Lap–1*, however without reaching an equilibrium (0.457 to 0.617). For *Pgm-2*, the trend was not clear because the F-values varied between 0.059 and –0.608 (*S. alba*) and between 0 and –0.684 (*S. fragilis*). The genetic distances (NEI 1978) among *S. alba* from these 4 different catchments were close to zero. The same holds true for *S. fragilis*. Two clear groups at genetic distances of 9.2–11.4 % were formed corresponding to the species which was illustrated likewise in the cluster analysis (Fig. 4).

F-statistics

The hierarchical *F*-statistics were calculated and compared between the level of localities, tributaries, catchments and the total per putative species. The F-statistics combined across loci resulted in positive values (0.141–0.243) at the lowest hierarchical level and reached negative values (–0.135) for comparisons at the tributary level and higher categories. The F_{ST} (catchment-total) value was zero (Table 8). The hierarchical F-statistics at only one locus such as *Lap–1* indicated similar trends, namely positive values (0.108 –0.157) for comparisons between localities and the higher categories and slightly negative values (–0.026 to –0.057) above the tributary level. The F_{ST} (catchment-total) for *Lap–1* was nearly zero (Table 9).

Table 9. Single locus hierarchical F-statistics of *Lap-1*.

	Localities	Tributaries	Catchments
Tributaries	0.157		
Catchments	0.135	-0.026	
Species	0.108	-0.057	-0.031

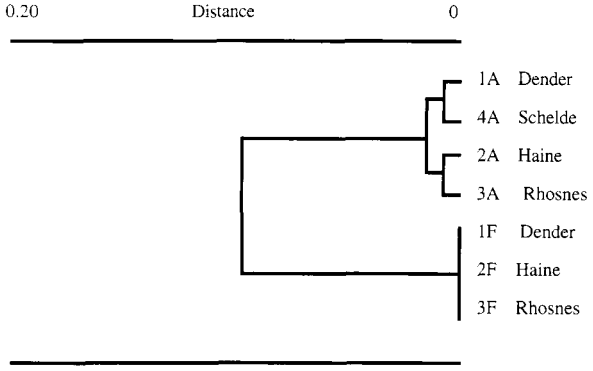

Fig. 4. UPGMA cluster analysis of the isozyme differentiation in 239 *S. alba* (A) and *S. fragilis* (F) clones pooled into 4 and 3 catchment zones respectively based on NEI (1978) genetic distance.

DISCUSSION

The genetic variability analysis based on the enzyme polymorphism was limited by the lowered activity in buds for a number of enzymes. Therefore only GOT, LAP, PGM and SDH could be used effectively. ADH and β-EST 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 *S. alba – S. fragilis* complex.

Genetic interpretation inferred from controlled crosses

Most of the enzymes can not be used directly as suitable 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 D × G and D × H 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 I × G and family D × G (also between B × E and D × G) 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 much influenced by initial parental allelic composition. This effect is even more pronounced in the case of reduced sample sizes of loci and when species-specific gene markers are not prevailing.

The electrophoretic data of parental types with their offspring from intra- and interspecific crosses illustrate, even at low sample sizes, that they form a sufficient set of genetic markers for further application. The F_1 progenies followed the segregation possibilities as expected from the parental types. The monomeric LAP with only two alleles has to be regarded as a suitable marker for population studies. The interpretation of LAP supports the hypothesis that in spite of the very high chromosome number ($2n$ = 76), *S. alba* and *S. fragilis* seem to be functionally diploidized (TRIEST *et al.* 1998).

On the other hand, gene duplication events have occurred in PGM, GOT, ADH, most likely also in β-EST, while additional slower LAP genes are noted as well (TRIEST *et al.* 1998). This duplicated control can be due to homologous chromosome parts in the polyploid complex. Segregation distortion is observed in family 15 for GOT, most probably due to fixed heterozygosity. Isozyme polymorphism has revealed that the hybrid F_1 progeny from controlled crosses shows segregation for *Lap–1* and *Pgm–2*. *Got–2* (also *Est–1* and *Adh–2* when revealed) have fixed heterozygous enzyme patterns and do not show homozygotes. Hybrids are difficult to identify since the putative diagnostic enzymes ADH and b-EST exhibit 2 main patterns corresponding to either *S. alba* and *S. fragilis*.

The genetic interpretation of enzymatic variation in tetraploid *S. alba* and *S. fragilis* ($2n$ = 76) is more complicated than in *e.g.* the diploid ($2n$ = 38) *S. exigua*. 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). ARAVANOPOULOS *et al.* (1994) observed 11 variable loci in 47 genes in *Salix eriocephala*. The endemic *Salix silicifolia* was found less polymorphic than the more widespread *S. alaxensis* when using 10 polymorphic genes in 9 enzymes (PURDY & BAYER 1995).

Both species are even differentiated in several enzymes. For *Salix viminalis*, 11 polymorphic loci were observed in 8 enzymes. In general, there is no high degree of allelic variation in willow species, when compared to for example most conifer species.

Clones from the field

When comparing the results obtained through pooling the data per putative species, it becomes evident that the *S. fragilis*-like individuals from region 6 (Locality Herchies – Lens) account for the largest deviation in genetic distances. This variation in the *S. fragilis* from the Dender East tributary is due to the presence of rare alleles in *Est–1* and *Got–2*.

When considering the samples pooled by the four catchment zones, the species show a high level of allelic richness (indicated by the values of *A* and *P*) as well as a high level of allelic evenness. This indicates that besides the fixation of rare alleles in *S. fragilis*-like individuals of region 6 (Dender East catchment area), all other patterns of variation occur in both species and in the different catchment areas. The question is whether this indication of genetic drift holds true at larger sample sizes of loci as well as of individuals (our unpublished RAPD results are concordant to isozymes with regard to the genetic deviation of the samples from the "Dender East" catchment). As such, the isozyme polymorphism can be used to detect local genetic drift and local patterns of genetic structuring. Isozymes thus can also be used as a first indication of the amount of genetic diversity within an area (quantitative information), but not necessarily in all cases as the kind of genetic diversity (qualitative information). Thus, for these species , the isozymes as a tool, will be most informative for processes within the historical and functional metapopulation structures, once that these entities are better defined and localised. Measures of fixation indices are valuable to illustrate patterns of heterozygosity levels. The hierarchy used in the treatment of the basic data for this species complex also demonstrates that the geographic extent to work with, most likely is at the tributary level as defined in this study (length of the main tributaries is about 10–25 km). The deterministic power to reveal allelic differentiation is much better when pooling the localities into smaller tributaries than when using the entire catchment zones. The cluster at catchment level clearly indicates that the *S. alba* from the four catchments considered here, are nearly similar. The *S. fragilis* from three catchments also are very similar because it is only at a certain tributary level among others, that a different multi-locus genotype can be observed. If the considered area is too large, then this kind of genetic structuring will not be detected.

F-indices and hierarchical analysis

The F-indices for *Lap–1* show that for both species there is a lack of heterozygotes in each catchment. The observation that for *S. alba*-like clones these F-values are ranging from 0.457–0.617 might indicate that this is a common and probably more widespread situation. The lack of heterozygosity for *Lap–1* is even more pronounced in the *S. fragilis*-like clones that are mainly monomorphic or totally homozygous. Only in the tributaries 1F (Mark), 3F (E. Dender) and 4F (Baudour) there are *Lap–1* heterozygotes observed.

Lap–1 in *S. alba* thus rarely reaches an equilibrium. This distortion can be explained in different ways. In the case of a lack of outcrossing and regeneration by seeds, it can be stated that clonal growth dominates on the field despite high fecundity rates. However, when considering large areas, there should be enough different clones in the sampling to obtain an equilibrium. Most likely there is a lack of exchange of genetic material within *S. alba* because of the absence of true dense populations. In the case when there is considerable outcrossing between the two taxa, it can be interpreted as the effect of introgressive hybridization with *S. fragilis*, resulting in skewed allele frequencies for the latter species since allele A has not yet been observed in the homozygous condition. Another explanation could be that the considered "putative" populations are not reflecting the real units, but became too much influenced by the introductions of clones.

The hierarchical structuring of heterozygosity indicated that most of the differentiation occurs at the lower levels (localities or tributaries) and that there is almost no further differentiation between catchments and the species. Thus the genetic structure of both species is reaching an "equilibrium" at the level of catchment zones and in many of the tributaries. Tributaries of 10–25 km in length are thus most likely the entities to further examine the putative hybridization process or events of allelic fixations.

Species with life history characteristics similar to *Salix* (an outcrossing breeding system, large geographic range, long-lived, high fecundity and wind-dispersed seeds) often exhibit high levels of genetic diversity but low differentiation. However, it can be stated that at present sexual reproduction is rare among *Salix* populations. The established stands encounter an infrequency of seedling recruitment and the use of willow clones dominate the rural landscape. Only the samples from the Schelde (Localities 13 & 14) were supposed to be at least spontaneous, not necessarily natural. It can be suggested that the actual distribution of willow trees

still may reflect possible patterns that were present on a regional scale. The choice of clones for further distribution along the agricultural land, could have originated from the available populations from alluvial plains and river margins. This way, diversity should be maintained within both *S. alba* and *S. fragilis* clones. The observation that the whole range of morphological forms is present in almost every region suggests that no thorough selection on that basis was made of the available resources. We expect that the genetic diversity has been maintained likewise within a region, however without revealing a reliable population genetic structuring. Deviations from Hardy-Weinberg equilibria and accompanying heterozygote deficiencies or excesses have been reported for other broad-leaved tree species such as *Robinia pseudoacacia* L. (SURLES *et al.* 1989), *Fagus sylvatica* L. (CUGUEN *et al.* 1988), *Populus tremuloides* Michx. (JELINSKI & CHELIAK 1992) and *Fagus grandiflora* Ehrh. (HOUSTON & HOUSTON 1994). In *Fagus sylvatica*, a heterozygote deficit was noted in two peroxidase loci. HOUSTON & HOUSTON (1994) related the heterozygote deficit of tree species to their reproductive strategies such as root-sprouting. Measurements of population genetic structure in a species where random mating among unique genets is assumed may lead to incorrect interpretation of results if a mixed mode of reproduction is operative (COOK 1983). Stand data therefore have to be adjusted by considering identical genotypes in close vicinity as ramets of the same clone. By recognition of clonal entities, HOUSTON and HOUSTON (1994) could generally increase the average values of F-statistics. The F-statistics give more evidence that the level of differentiation is between locations (5–10 km) and tributaries (10–25 km) and that the level to consider as a "putative" population is more likely the tributary level. Perhaps the relatively unmanaged freshwater tidal mudflats of the river Schelde may harbour nearly natural situations, however, still in terms of recent settlements since a few centuries.

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REFERENCES

ARAVANOPOULOS, F., ZSUFFA, L. & CHONG, K. 1993: The genetic basis of enzymatic variation in *Salix exigua*. *Hereditas* **119**: 77–88.

ARAVANOPOULOS, F., ZSUFFA, L. & CHONG, K. 1994: Inheritance of isozymes in *Salix eriocephala* Michx. *J. Heredity* **85**: 381–388.

BRUNSFELD, S., SOLTIS, D. & SOLTIS, P. 1991: Patterns of genetic variation in *Salix* section *Longifoliae* (*Salicaceae*). *Amer. J. Bot.* **78**: 855–869.

COOK, R. 1983: Clonal plant populations. *Amer. Sci.* **71**: 244–253.

CUGUEN, J., MERZEAU, D. & THIEBAUT, B. 1988: Genetic structure of the European beech stands (*Fagus sylvatica* L.): F-statistics and importance of mating systems characteristics in their evolution. *Heredity* **60**: 91–100.

DE BONDT, R. 1996: Role of genetic diversity and identity in replantations – case study on *Salix* sp. MSc. thesis, Vrije Universiteit Brussel, 82 p.

HAMRICK, J. L.& GODT, M. J. 1989: Allozyme diversity in plant species. *In*: Plant population genetics, breeding and genetic resources. (ed. Brown, A., Clegg, M., Kahler, A. and Weir, S.). pp. 43–63. Sinauer, Sunderland, MA.

HOUSTON, D. & HOUSTON, D. 1994: Variation in American beech (*Fagus grandifolia* Ehrh.): isozyme analysis of genetic structure in selected stands. *Silvae Genetica* **43**: 277–284.

JELENSKI, D. & CHELIAK, W. 1992: Genetic diversity and spatial subdivision of *Populus tremuloides* (*Salicaceae*) in a heterogeneous landscape. *Amer. J. Bot.* **79**: 728 –736.

KARRON, J. 1987: A comparison of levels of genetic polymorphism and self-compatibility in geographically restricted and widespread plant congeners. *Evolutionary Ecology* **1**: 47–58.

LOVELESS, M. & HAMRICK, J. 1988: Genetic organization and evolutionary history in two North American species of *Cirsium*. *Evolution* **42**: 254–265.

NEI, M. 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583–590.

NEI, M. 1972: Genetic distance between populations. *Amer. Naturalist* **106**: 283–292.

PURDY, B. & BAYER, R. 1995: Allozyme variation in the Athabasca sand dune endemic, *Salix silicola*, and the closely related widespread species, *S. alaxensis*. *Syst. Bot.* **20**: 179–190.

ROGERS, J. 1972: Measures of genetic similarity and genetic distance. *Studies in Genetics, Univ. Texas Publ.* **7213**: 145–153.

SURLES, S., HAMRICK, J. & BONGARTEN, B. 1989: Allozyme variation in black locust (*Robinia pseudoacacia*). *Can. J. For. Res.* **19**: 471–479.

SWOFFORD, D. & SELANDER, R. 1981: BIOSYS-1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Hered.* **72**: 28 –283.

THORSEN, J., JORDE, P., ARAVANOPOULOS, F., GULLBERG, U. & ZSUFFA, L. 1997: Inheritance and linkage of isozyme loci in the basket willow (*Salix viminalis* L.). *J. Heredity* **85**: 381–388.

TRIEST, L. 1989: Electrophoretic polymorphism and divergence in *Najas marina* L. (*Najadaceae*): molecular

markers for individuals, hybrids, cytodemes, lower taxa, ecodemes and conservation of genetic diversity. *Aquat. Bot.* **33**: 301–380.

TRIEST, L., DE GREEF, B., D'HAESELEER, M., ECHCHGADDA, G., VAN SLYCKEN, J & COART, E. 1998: Variation and inheritance of isozyme loci in controlled crosses of *Salix alba* and *Salix fragilis*. *Silvae Genetica* **47**: 88–94.

WRIGHT, S. 1965: The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution* **19**: 395–420.