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Article

Genetic Segregation and Genomic Hybridization Patterns Support an Allotetraploid Structure and Disomic Inheritance for *Salix* Species

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Abstract: The *Salix alba* L. (white willow)—*Salix fragilis* L. (crack willow) complex includes closely related polyploid species, mainly tetraploid ($2n = 4x = 76$), which are dioecious and hence obligate allogamous. Because little is known about the genome constitution and chromosome behavior of these pure willow trees, genetic analysis of their naturally occurring interspecific polyploid hybrids is still very difficult. A two-way pseudo-testcross strategy was exploited using single-dose AFLP markers in order to assess the main inheritance patterns of tetraploid biotypes (disomy *vs.* tetrasomy) in segregating populations stemmed from *S. alba* × *S. fragilis* crosses and reciprocals. In addition, a genomic *in situ* hybridization (GISH) technology was implemented in willow to shed some light on the genome structure of *S. alba* and *S. fragilis* species, and their hybrids (allopolyploidy *vs.* autopolyploidy). The frequency of *S. alba*-specific molecular markers was almost double compared to that of *S. fragilis*-specific ones, suggesting the phylogenetic hypothesis of *S. fragilis* as derivative species from *S. alba*-like progenitors. Cytogenetic observations at pro-metaphase revealed about half of the chromosome complements being

less contracted than the remaining ones, supporting an allopolyploid origin of both *S. alba* and *S. fragilis*. Both genetic segregation and genomic hybridization data are consistent with an allotetraploid nature of the *Salix* species. In particular, the vast majority of the AFLP markers were inherited according to disomic patterns in *S. alba* × *S. fragilis* populations and reciprocals. Moreover, in all *S. alba* against *S. fragilis* hybridizations and reciprocals, GISH signals were observed only on the contracted chromosomes whereas the non-contracted chromosomes were never hybridized. In conclusion, half of the chromosomes of the pure species *S. alba* and *S. fragilis* are closely related and they could share a common diploid ancestor, while the rest of chromosomes are morphologically differentiated in either *S. alba* or *S. fragilis* and they should derive from distinct diploid ancestors.

Key words: *Salix* spp.; polyploidy; DNA markers; GISH analysis

1. Introduction

The genus *Salix* (family of *Salicaceae*) is one of the most important taxonomic entities of the world for the great number of species and varieties, with around 300 taxa of deciduous trees and shrubs widespread both in the boreal and austral hemisphere [1–3]. Almost half of these taxa are distributed in the Eurasian continent, one third are found in the American continent from Alaska to Patagonia, and the remaining are present in Europe and the Mediterranean basin [4,5]. The genus *Salix* shows all forms of development from tall trees (hot-temperate climates), to shrubs (cold-temperate climates) and to creeping and dwarf shrubs (cold and arctic zones). More clearly than in other genera of phanerophytes, each single species of willow is related to very specific environmental conditions, so that the corresponding plant associations show distinctive ecophysionomic aspects due to the climatic and edaphic characteristics of their own habitats [5]. Hybridization between *Salix* species is very common. In addition to the naturally occurring hybrids in various parts of the world, a considerable number of others have been artificially produced by controlled matings [3]. Spontaneous hybridization is determined by dioecism and is affected by variation of flowering times in different species. In the genus *Salix*, both balanced and introgressed hybrids seem to dominate when considering only morphological traits, but studies based on biochemical and molecular markers revealed that genetic differentiation within populations is low as well as genetic flow between populations [6,7]. This finding is consistent with historical data available for forest trees with a dioecious-type breeding system and wind-dispersed seeds [8]. Nevertheless, a striking absence of geographical effects in the haplotype distributions of *Salix* species was recently discovered by [9] and likely owed to recent and continual plastid capture events, aided by wide-spread hybridization and long-range seed dispersal, but primarily propelled by one or more trans-species selective sweeps. In addition, [10] reported that *S. alba*, *S. fragilis*, and their hybrid *S.* × *rubens* are genetically recognizable and that only few hybrids and any backcrosses to the parental species are present in mixed stands. Overall findings supported a clear separation of the pure species, *S. alba* and *S. fragilis*, and an intermediate position of *S.* × *rubens* individuals.

Morphology indicates that pure species of the *Salix alba* L. (white willow)—*Salix fragilis* L. (crack willow) complex and their natural hybrids form a polyploid complex of closely related willows [11–14].

The problem of the separation of the hybrid entities between *S. alba* and *S. fragilis* is one of the critical matters of systematics of the genus *Salix* [3]. This complex includes mostly tetraploid trees, which are dioecious and thus obligate outcrossers. As a consequence, natural populations of these willows can be represented by a mixture of highly heterozygous genotypes, sharing a common gene pool as a result of recurring hybridization [15]. Species like *S. babylonica* L., *S. pentandra* L. and *S. triandra* L. are the only ones that successfully intercross with both *S. alba* and *S. fragilis* [5,16]. Although they have similar ecological characteristics, *S. fragilis* and *S. alba* are distinguishable in terms of temperature requirements and areas of spontaneous occurrence, being the former native to Western Asia and naturalized in central and northern Europe, and the latter more spread in temperate regions and the Mediterranean basin [11,12]. On the basis of plant morphology, European populations seem to include mainly balanced and introgressed *S. alba* × *S. fragilis* hybrids [13,17]. The analysis of leaves, buds and twigs has indicated that many potential hybrids are present within natural populations [18]. This finding agrees with the observations of several botanists [11,19,20] that the vast majority of willows in the *S. alba—S. fragilis* complex could be hybrids or introgressants. Owing to their continuous variation, most of the phenotypic features have however a low diagnostic value for identifying interspecific hybrid constitutions, assessing introgression patterns, and defining genetic variation structure and relatedness at the population level. Consequently, the taxonomic classification of pure species as well as the identification of hybrid individuals is still today a matter of debate.

Molecular studies carried out using controlled crosses and field clones contradict traditional hypothesis on the extensive occurrence of hybrids in the *S. alba—S. fragilis* complex. In particular, molecular markers revealed that both species have kept their gene pools well separated and that interspecific hybridization actually does not seem to be a dominating process [13]. New biotechnological developments have expanded the range of plant DNA polymorphism assays for characterizing and investigating germplasm resources and genetic relatedness, as well as for linkage mapping, gene targeting and assisted breeding. The area of willow research showing the greatest development with respect to the use of molecular marker technology is that of population genetics [7,13,14,18,21–24], whereas information on structural genomics is scanty. In fact, nothing is known about willow genomic constitution and origin, and whether species are autopolyploid or allopolyploid remains an open question. Because of the many basic chromosomes ($x = 19$), willow species with $2n = 38$ may represent ancient polyploid derivations [25–27], that however behave like functionally diploidized genomes [28,29]. In fact, diploidization affects the chromosome behavior during meiosis along with gene dosages and inheritance models in polyploids. Cytological observations of the pairing modes of tetraploid plants with $2n = 76$, useful to reveal bivalent or multivalent formation, are difficult owing to the high chromosome number and small chromosome size of willow species. Moreover, genetic analysis in willow has been restricted not only because of genome architecture complexity, but also due to the lack of discriminant molecular markers and the need of experimental populations amenable to molecular analysis. As a result, a few genetic linkage maps have been constructed and data on segregation patterns or recombination estimates are scanty for tetraploid willow [14].

Molecular investigations of tetraploid species belonging to the *S. alba—S. fragilis* complex could provide valuable data about the genetic inheritance and genomic structure of willows. The genetic segregation assay of single-dose markers was used to assess the inheritance pattern of tetraploid types (disomy *vs.* tetrasomy), whereas genomic *in situ* hybridization technology was exploited to shed light

on the genome constitution of tetraploid willows (allotetraploidy *vs*. autotetraploidy). New findings supporting the genome organization and gene transmission patterns in tetraploid *Salix* spp. are presented and a working hypothesis that explains why the gene pools of *S. alba* and *S. fragilis* species have remained well separated is formulated and discussed.

2. Materials and Methods

2.1. Willow Plant Materials

For genomic DNA fingerprinting, we considered a total of 36 genotypes belonging to pure species of the *S. alba—S. fragilis* complex, including 16 of *S. alba* and 16 of *S. fragilis*. In particular, a comparable number of male and female trees for both *S. alba* and *S. fragilis* were collected from Italian (provinces of Alessandria, Udine, Ferrara and Nuoro in Northern regions and Sardinia island, respectively) and Spanish (different localities around Madrid) populations. Two putative *S. alba* × *S. fragilis* hybrids (from Val Venosta, Bolzano, Italy) were also evaluated, along with two genotypes of *S. pentandra* (from Caoria, Trento, Italy) adopted as reference outgroups (for details on plant materials, see also [15]. Each clonal genotype (*i.e*., accession) was represented by at least two replicated genomic DNA samples. For genetic segregation and recombination analyses, two experimental F_1 populations of 69 progeny plants each were produced by performing reciprocal crosses between *S. alba* and *S. fragilis* pure parents. Parental trees were chosen on the basis of their morphological traits (phenotype) and molecular fingerprints (genotype) supporting a reliable pure origin of single species and a remarkable genetic divergence between species in terms of marker polymorphisms. Both the male and female *S. fragilis* parental genotypes originated from Brno (Czech Republic), whereas the female and male *S. alba* parental genotypes belonged to natural populations from Scarperia (Florence, Italy) and Panfilia (Ferrara, Italy), respectively. Seeds were germinated in jiffy pots and plantlets were grown in the greenhouse. For GISH analysis, the chromosomes of actively cycling cells were prepared from root tip meristems of several *S. alba*, *S. fragilis* genotypes, one *S. alba* × *S. fragilis* and one *S. fragilis* × *S. alba* hybrids stemmed from experimental crosses [7,14].

2.2. Molecular Marker Analysis

2.2.1. Genomic DNA Isolation

For each willow plant, total genomic DNA was isolated from 0.5 g of whole young leaves without petiole, of about 3–4 cm in length, chosen among the last ones formed in the shoot apical meristem, using the Nucleon PhytoPure kit (Pharmacia Biotech) according to the manufacturing' instructions with some changes to adapt it to *Salix* species. The concentration of genomic DNA samples was determined by optical density reading (DU650 spectrophotometer, Beckman) at 260 nm (1 O.D. = 50 μg/mL) and the purity calculated by the $O.D._{260}/O.D._{280}$ ratio and by the $O.D._{210}$–$O.D._{310}$ pattern [30]. An aliquot of genomic DNA for all samples was also assayed by electrophoresis on 1% agarose gels in order to assess its integrity. Samples were used for generating DNA fingerprints and performing GISH experiments.

2.2.2. Genomic AFLP Fingerprinting

Genomic DNA fingerprints of the natural accessions and experimental populations were generated by AFLP markers using primer combinations already tested in willow species [7,14]. Genomic DNA of individual plants was restricted and ligated to adapters according to [31]. Briefly, about 500 ng of genomic DNA was digested and ligated for 4 h at 37 °C using 5 U each of *Eco*RI (or *Pst*I) and *Mse*I restriction endonucleases, 1 U of T4 DNA ligase, 50 pMol of *Mse*I-adaptor and 5 pMol of *Eco*RI-adaptor (or *Pst*I-adaptor) in 1× RL buffer (20 mM Tris-acetate, 20 mM magnesium acetate, 100 mM potassium acetate, 5 mM DTT, 2.5 mg BSA) added with ATP to a final concentration of 10 mM. Then, 5 μL of eight-fold diluted digested and ligated DNA was pre-amplified to select and bulk restriction fragments to the correct size and configuration, in 20 μL reaction mixture containing 75 ng of *Eco*RI+N (or *Pst*I+N) and *Mse*I+N primers, 1× PCR buffer (50 mM MgCl, 1.5 mM MgCl2, 10 mM Tris-HCl), 10 mM dNTPs and 1 U of *Taq* DNA polymerase. Hot PCR analysis was performed with a [^{33}P]-labeled *Eco*RI+3N (or *Pst*I+2N) primer and an unlabeled *Mse*I+3N primer. The conserved sequences of restriction site-related primers were as follows: *Eco*RI-specific primer: AGACTGCGTACCAATTC; *Pst*I-specific primer: GACTGCGTACATGCAG; and *Mse*I-specific primer: GACGATGAGTCCTGAGTAA. The PCR cycling conditions were as follows: 1 cycle of 45 sec at 94 °C, 30 s at 65 °C, 1 min at 72 °C and a touch down profile (13 cycles with −0.7 °C/cycle) for the annealing step followed by 18 cycles at 55.9 °C annealing temperature and finally by an extension cycle of 5 min at 72 °C. After PCR, 8 μL of loading buffer (98% formamide, 10 mM EDTA, 0.005% each of xylene-cyanol and bromophenol-blue) was added to each tube. Samples were denatured at 90 °C for 5 min and then immediately placed on ice. For each sample, 5 μL were loaded onto a 6% polyacrylamide gel using a standard electrophoretic system that was run for 2 h and 45 min at 80 W. Markers were visualized on autoradiograms after 18 h exposure at −80 °C with intensifying screens.

2.2.3. Genomic DNA Polymorphism Analysis

Molecular markers were scored as present (1) or absent (0) over all lanes, using the 1D software (Kodak) for image analysis, and recorded as a binary matrix. All genetic diversity statistics [32,33] were calculated using the software POPGENE version 1.21 [34]. Cluster analyses based on genetic similarity coefficients [35] were performed using the software NTSYS version 2.1 [36]. Different measures of genetic variation within and genetic differentiation between willow accessions were used to estimate the levels of polymorphisms within and between species, including interspecific hybrids.

2.2.4. Genetic Segregation Analysis

A molecular marker segregating from one willow parental genotype can be present as a single dose allele (SDM), double dose (DDM), triple dose (TDM) alleles. Whereas SDMs are expected to segregate in the same way regardless of the ploidy level and genome constitution, the expected segregation ratio of higher dose markers such as DDMs, TDMs, *etc.* varies according to the genome constitution (*i.e.*, autopolyploidy *vs.* allopolyploidy), the ploidy level, and the pairing behavior (*i.e.*, bivalents, trivalents, tetravalents). For instance, a DDM of an allotetraploid can be inherited in all or three out of four of the gametes depending on whether the two copies of the marker allele belong to homologous or

homeologous chromosomes [14]. The expected segregation ratio presence *vs.* absence in gametes of autopolyploids with bivalent pairing at meiosis is, instead, (3h-2):(h-2) where h represents the ploidy level [37]. Therefore, a DDM of an autotetraploid can be inherited in five out of the six possible gametes. In species such as willow with a tetraploid genome, it is therefore very important to distinguish SDMs from non-SDMs. The segregation patterns (*i.e.*, presence *vs.* absence) of marker alleles observed in the F_1 mapping populations were assayed by chi-square analysis to test the goodness of fit with the 1:1 and 3:1 ratios expected, respectively, for marker loci that were either polymorphic or shared between parents. In the case of autotetraploidy, parental marker loci genotypes would be *simplex* (*a*---) by *nulliplex* (----) and *simplex* (*a*---) by *simplex* (*a*---), whereas in case of allotetraploidy they would be heterozygous (*a*-) by homozygous (--) and heterozygous (*a*-) by heterozygous (*a*-). The observed number of AFLP species-specific polymorphisms and segregating parental (both male and female) SDMs at all mapped marker loci [14] were analyzed by 2 × 2 contingency tests in order to compare the genomic affinity between the two species and the efficiency of the two molecular marker systems. The F_1 progenies were scored for marker loci polymorphisms. All AFLP marker alleles polymorphic between parents that fitted a 1:1 segregation ratio were assayed by the Mather's χ^2 test to assess association [38]. *S. alba*-specific and *S. fragilis*-specific SDMs were ordered in maternal and paternal co-segregation groups. SDMs of each co-segregation group were then tested for the linkage phase on the basis of parental *vs.* recombinant patterns. The number of SDMs linked in coupling and the number of those linked in repulsion calculated over all pairwise comparisons were assayed to fit the 1:1 proportional ratio. When the difference between coupling and repulsion phase marker combinations was not statistically significant, chromosome pairing was considered preferential. However, if the difference was significant, chromosome pairing was considered to be random [39].

2.3. Genomic in situ Hybridization (GISH) Analysis

Slides were treated with RNAse (10 mg/mL, 1:100 in 2 × SSC) at 37 °C for 1 h, with pepsine (500 µg/mL, 1:100 in 0.1 M HCl) for 40 min and with formaline buffer for 10 min. The hybridization mixture (20 µL per slide) consisted of 50% formamide, 20% dextran sulphate, 2× SSC, 1% SDS and the DNA probes: 100 ng of *S. alba* or *S. fragilis* DNA, labeled with fluorescent high prime and detected with FITC. The chromosome slides (prepared from eight individuals each of *S. alba* and *S. fragilis* pure species, and two intermediate hybrids from *S. alba* × *S. fragilis* and *S. fragilis* × *S. alba* progenies) were hybridized with the total genomic DNA of pure species (*S. alba* or *S. fragilis*) adopted as templates. It is worth noting that *S. alba* and *S. fragilis* pure accessions derived from natural populations of Czech Republic (Brno), Italy (Northern regions and Sardinia island), Spain (Madrid) and Turkey. The parental accessions used to perform *S. alba* × *S. fragilis* crosses and reciprocals *S. fragilis* × *S. alba*, and characterized by AFLP markers, were also used for GISH analysis, along with two interspecific hybrids deriving from our experimental populations (assessed as F1 progenies on the basis of AFLP fingerprints).

2.3.1. Preparation of DNA Probes

DNA probes consisted of genomic DNA labeled with FITC—High Prime kit (Roche). A total of 1–2 μg of genomic DNA diluted in 16 μl of sterile water was denatured by heating in a boiling water bath for 10 min. After having cooled it quickly on ice, 4 μL of FITC—High Prime were added, mixed and spinned and incubated overnight at 37 °C. The reaction was stopped by adding 2 μL of 0.2 M EDTA pH = 8 and incubating at 65 °C for 10 min. Then 2.5 μL of 4 M of LiCl and 75 μL of cold 100% ethanol were added to the probe and stored at −20 °C for 2 h. The vials were centrifuged at 13,000 rpm for 20 min to get the pellet of the probe that was washed with 50 μL of cold 70% ethanol and dried at room temperature for only 3 min. Then the probe was dissolved in 20 μL of sterile water and incubated at 65 °C for about 2 h.

2.3.2. Preparation of Chromosome Slides

For the somatic chromosome preparation, root tips were collected in the early morning, treated in a 8-hydroxyquinolin solution (0.1%) for 3–4 h at room temperature and kept fixed in the Carnoy's solution (composed of three parts of absolute ethanol and one part of glacial acetic acid) and stored at +4 °C until use. Root tips were treated with citrate buffer (10 mM citric acid, 10 mM sodium citrate, pH = 4.5) for 15 min and incubated in a pectolytic enzyme mixture containing 1% pectolyase Y23, 1% cellulase RS and 1% cytohelicase in 10 mM citrate buffer at 37 °C for about 2 h and 30 min. Preparations were squashed in a drop of 60% acetic acid and the slides with root tips put on thermoblock at 45 °C for 2 min, washed with Carnoy. Slides were dehydrated in absolute ethanol for a few minutes, dried at room temperature and stored at −20 °C until use.

2.3.3. Genomic *in situ* Hybridization

Slides with chromosome complements were left overnight at 37 °C. The next day the slides were treated with 100 μg/mL RNase A in 2 × SSC at 37 °C for 60 min and washed three times with 2× SSC at room temperature for 5 min. The slides were then incubated with 10 mM HCl at 37 °C for 2 min, treated with 100 μL of a pepsin solution (5 μg/mL in 10 mM HCl) at 37 °C for 15–20 min, followed by washing twice with 2× SSC for 5 min, then with 4% paraformaldehyde solution for 10 min and finally three times with 2× SSC for 5 min each. Before air-drying, the slides were rapidly dehydrated in a graded alcohol solution series (70%, 90% and 100%) for 3 min each. Samples of 40 μL of the hybridization mixture containing 100 ng of the probe DNA, 2 mg of sheared herring sperm DNA (Gibco BRL), 50% deionised formamide, 10% (w/v) sodium dextran sulphate (Sigma), 2× SSC and 0.25% (w/v) SDS were denatured at 70 °C for 5 min and then directly put on ice for at least 5 min. Each slide with 40 μL of the hybridization mix was denatured at 80 °C for 2.5 min left overnight at 37 °C in a tightly closed humidified container. The slide was then washed in 2× SSC buffer for 15 min, transferred to 0.1× SSC buffer at 42 °C for 30 min and incubated at 37 °C for 60 min in blocking buffer (Boehringer Mannheim). Biotin-and/or digoxigenin labeled DNA probe was detected by a Cy3™ avidin-streptavidin detection system and a fluorescein isothiocyanate (FITC)—anti-digoxigenin detection system (Boehringer Mannheim). All slides were counterstained either with 10 mg/mL DAPI (42,6-diaminido-2-phenylindole) or with 5 mg/mL propidium iodide (PI). Photographs were taken using a Zeiss Axiophot photomicroscope

equipped with epi-fluorescence illumination and single-band filters for DAPI, FITC and Cy3-PI, using 400 ISO colour negative film. Films were scanned at 1200 dpi for digital processing with Adobe Photoshop® (Adobe Inc., San Jose, CA, USA).

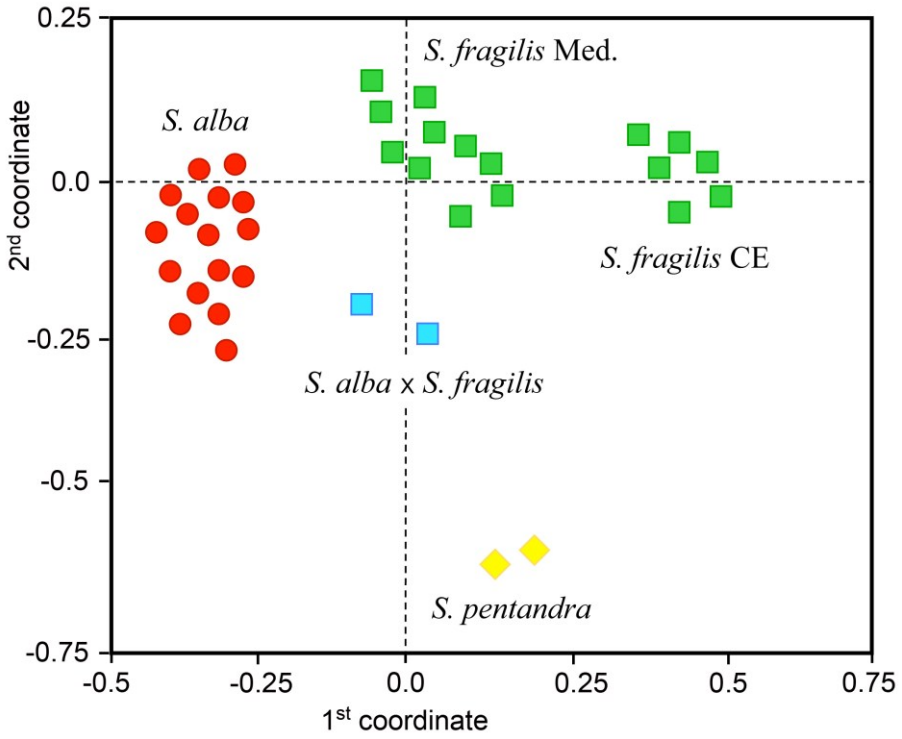
3. Results

3.1. Genomic DNA Fingerprint Analysis

A total of 305 amplicons were scored across all 36 genomic DNA fingerprints, including reproducible polymorphisms among the assayed willow plants. In fact, the adoption of replicated samples for each clonal genotype allowed us to verify a full reliability of the obtained molecular results. This characterization of *Salix* germplasm was based on three *Eco*RI/*Mse*I and 3 *Pst*I/*Mse*I primer combinations, selected during preliminary experiments for their polymorphism information content, which enabled a successful evaluation of the genetic relationships within as well as between species.

UPGMA grouping methods were useful to assess genetic variation within single species and genetic relationships among pure species (Figure 1).

Figure 1. Outcome from UPGMA (unweighted pair-group method with arithmetic mean) grouping analysis of *Salix* spp. samples on the basis of molecular amplified fragment length polymorphism (AFLP) fingerprints, depicting genetic relationships among *S. alba* and *S. fragilis* pure species, *S. alba* × *S. fragilis* hybrids, using *S. pentandra* as outgroup entities. Centroids of the 36 willow trees were obtained from the mean genetic similarity matrix and bi-dimensionally plotted according to the first two components that explain about 60% of the total genetic variation detected across all marker loci.



In particular, cluster analysis was useful to plot the centroids of willow accessions into four main subgroups according to the first two coordinates, which explained about 41% and 19% of the total genetic variation: pure *S. fragilis* individuals from the Mediterranean area formed a first subgroup, while a second subgroup included pure *S. fragilis* individuals from Central-Europe and a third subgroup was composed by pure *S. alba* individuals. The *S. alba* × *S. fragilis* hybrids were plotted separately from the pure species subgroups. In addition, the reference *S. pentandra* individuals were clustered apart forming a fourth well-separated subgroup. The first coordinate grouped separately the Central-European accessions of *S. fragilis* from the other accessions of the same species deriving from the Mediterranean area, and also from all the accessions of *S. alba*. The second coordinate enabled to distinguish clearly the accessions of *S. pentandra* from the *S. alba* accessions and from all *S. fragilis* accessions, irrespectively of their Mediterranean or Central-European origin, along with the *S. alba* × *S. fragilis* hybrids (see Figure 1).

The finding of genetically differentiated subgroups and the lack of genotypes with intermediary characteristics confirm that both species of the *S. alba—S. fragilis* complex have kept their gene pools well separated and that interspecific hybridization *S. alba* × *S. fragilis* does not seem to be a dominating process in natural populations. It is worth mentioning that the subgroup of Central-European accessions of *S. fragilis* resulted more similar to the Mediterranean accessions of *S. fragilis* than to the *S. alba* accessions (0.748 against 0.644, respectively). The greatest genetic differentiation was observed between Central-European accessions of *S. fragilis* and *S. pentandra* (0.558). However, the fact that accessions of Mediterranean *S. fragilis* were genetically more similar to accessions of *S. alba* (0.830) rather than to Central-European accessions of *S. fragilis* (0.748) underlines the importance in evolutionary terms of the pedo-climatic environment.

On the basis of molecular data, it is likely that the spatial isolation, together with the habitat characteristics, could play an important role in the process of differentiation of separate subgroups within the same species.

3.2. Segregation Pattern Analysis of Marker Alleles

The experimental F1 populations stemmed from *S. alba* × *S. fragilis* crosses and reciprocals were analyzed with 10 *Eco*RI/*Mse*I and 10 *Pst*I/*Mse*I primer combinations, yielding an average of 56 reliable markers per single AFLP fingerprint (ranging from a minimum of 48 up to a maximum of 72 markers).

Main statistics for marker alleles that were scored as polymorphic or shared between parents, and that were analyzed for segregation patterns are summarized in Table 1. A total of 1,122 AFLP marker loci were detected in the F_1 progenies using the selected primer combinations: 870 marker alleles (77.5%) were polymorphic between parental species, whereas 252 marker alleles (22.5%) were shared. Of the polymorphisms, 568 were *S. alba*-specific (51% of total marker alleles) and 320 were *S. fragilis*-specific (28% of total marker alleles). The difference between the amount of *S. alba*-specific and *S. fragilis*-specific markers, as assessed by a contingency test, was highly significant ($\chi^2 = 30.4$***, 1 d.f.). Within each species, the gender contribution in terms of maternal and paternal polymorphisms was comparable in both *S. alba* (282 *vs.* 268) and *S. fragilis* (132 *vs.* 142).

Chi-square analyses for various segregation ratios (from 1:1 up to 11:1, presence *vs.* absence) were performed to determine segregation patterns of *S. alba* and *S. fragilis* marker alleles according to

allo- and/or auto-tetraploidy (see Table 1). The most represented segregation ratios ranged from 0.75 to 1.25 as expected for SDMs, with the mean ratios of 0.99 for *S. alba* and 1.08 for *S. fragilis* markers. Low proportions of marker loci with skewed segregation patterns were observed in both species (6.0% in *S. alba* and 4.6% in *S. fragilis*).

Table 1. Information on the inheritance patterns observed for single-dose and multiple-dose marker alleles. Marker types refer to the parental individuals whereas segregating, distorted and monomorphic markers refer to the F1 progenies.

Marker Types		Segregating Markers ($p > 0.05$)*				Distorted	Monomorphic
		1:1 ratio	3:1 ratio	5:1 ratio	11:1 ratio	Markers	Markers
Polymorphic	Total	292 (33.6)	-	24 (2.8)	-	48 (5.5)	506 (58.1)
Species-specific	*S. alba*	194 (34.2)	-	11 (1.9)	-	34 (6.0)	329 (57.9)
	S. fragilis	98 (32.5)	-	13 (4.3)	-	14 (4.6)	177 (58.6)
Gender-specific	Female	149 (34.0)	-	11 (2.5)	-	19 (4.3)	259 (59.1)
	Male	143 (33.1)	-	13 (3.0)	-	29 (6.7)	247 (57.2)
Shared	Total		22 (8.7)		6 (2.7)	11 (4.4)	213 (84.5)

* For each marker type is reported the total number of loci and the relative percentage (within brackets).

A total of 316 (35.5%) marker alleles of those polymorphic between parents were shown to segregate in the F_1 progenies, with 292 (89.6%) that were inherited as SDMs. In particular, 194 *S. alba* (81 paternal and 113 maternal) and 98 *S. fragilis* (62 paternal and 36 maternal) SDMs polymorphic between parents segregated in a 1:1 Mendelian fashion ($p \leq 0.05$), whereas 22 SDMs shared between parents segregated in a 3:1 Mendelian fashion ($p \leq 0.05$). Of the remaining marker loci analyzed, only 29 paternal and 19 maternal were markedly distorted ($p < 0.01$). In particular, segregation distortion of marker alleles inherited from *S. alba* and *S. fragilis* did not differ significantly ($\chi^2 = 0.419$, d.f. = 1). While 1:1 and 3:1 are the only segregation ratios expected for allotetraploid genomes and detectable uniquely with SDMs, additional segregation patterns in autotetraploids may also be identified using DDMs. Of the 870 and 252 marker loci, respectively, polymorphic and shared between parents, only 24 (2.8%) plus 6 (2.7%) showed a segregation attributable to DDMs on the basis of chi-square values. Inheritance patterns observed for marker alleles of these loci correspond to segregation ratios, respectively, of 5:1 and 11:1 expected in duplex by nulliplex and duplex by simplex crosses (see Table 1).

In conclusion, segregation patterns of most markers support allotetraploidy for willows and only a few markers scored segregation patterns concordant with autotetraploidy.

3.3. Chromosome Pairing Behavior

The chromosome pairing was successfully assessed discriminating between preferential or random models. *S. alba*-specific and *S. fragilis*-specific SDMs were ordered in maternal and paternal co-segregation groups and then tested for the linkage phase (*i.e.*, coupling *vs.* repulsion) in all possible pairwise comparisons among marker loci.

The maternal and paternal SDMs were ordered in 55 co-segregation groups according to the Mather test. SDMs of each pair of co-segregation groups were then tested for the linkage phase on the basis of parental or recombinant patterns. For pairwise combinations of associated genomic loci, the number of

marker alleles linked in coupling and the number of marker alleles linked in repulsion were assayed to fit the 1 to 1 expected ratio. Of the *S. alba* co-segregation groups, 14 of the 19 maternal linkage groups and 13 of the 16 paternal linkage groups showed non-significant deviations ($p \leq 0.05$), suggesting preferential chromosome pairing. In *S. fragilis*, of the co-segregation groups detected, only 3 out of 20 maternal and paternal linkage groups scored significant deviations ($p \leq 0.01$), further supporting preferential chromosome pairing. The linkage phase of marker loci belonging to *S. alba* and *S. fragilis* co-segregational female and male marker loci groups is reported in Table 2.

Table 2. Main statistics about linkage phase (coupling *vs.* repulsion) of marker loci belonging to *S. alba* and *S. fragilis* co-segregational female and male linkage groups. A comparable number of markers linked in coupling and repulsion were found for most linkage groups, suggesting that basic chromosomes pair preferentially as occurs for allotetraploid species with disomic inheritance.

Species (Gender)	Linkage Groups *	Linkage Phase		Size (cM)	No. loci	Chromosome Pairing (%)
		Coupling	Repulsion			
S. alba ♀	19	929	728	709	73	Preferential (74)
S. alba ♂	16	424	374	340	48	Preferential (81)
S. fragilis ♀	6	36	43	98	13	Preferential (100)
S. fragilis ♂	14	156	186	321	33	Preferential (79)

***** Only 8 out of 55 of the co-segregational marker loci grpups scored a significant (P<1%) distortion from the 1:1 expected ratio between coupling and repulsion phase linked loci.

In conclusion, a comparable number of markers linked in coupling and repulsion for most co-segregation groups suggests that most basic chromosomes pair preferentially as it happens for allotetraploid species with disomic inheritance.

3.4. Cytological Observations of Chromosome Morphology

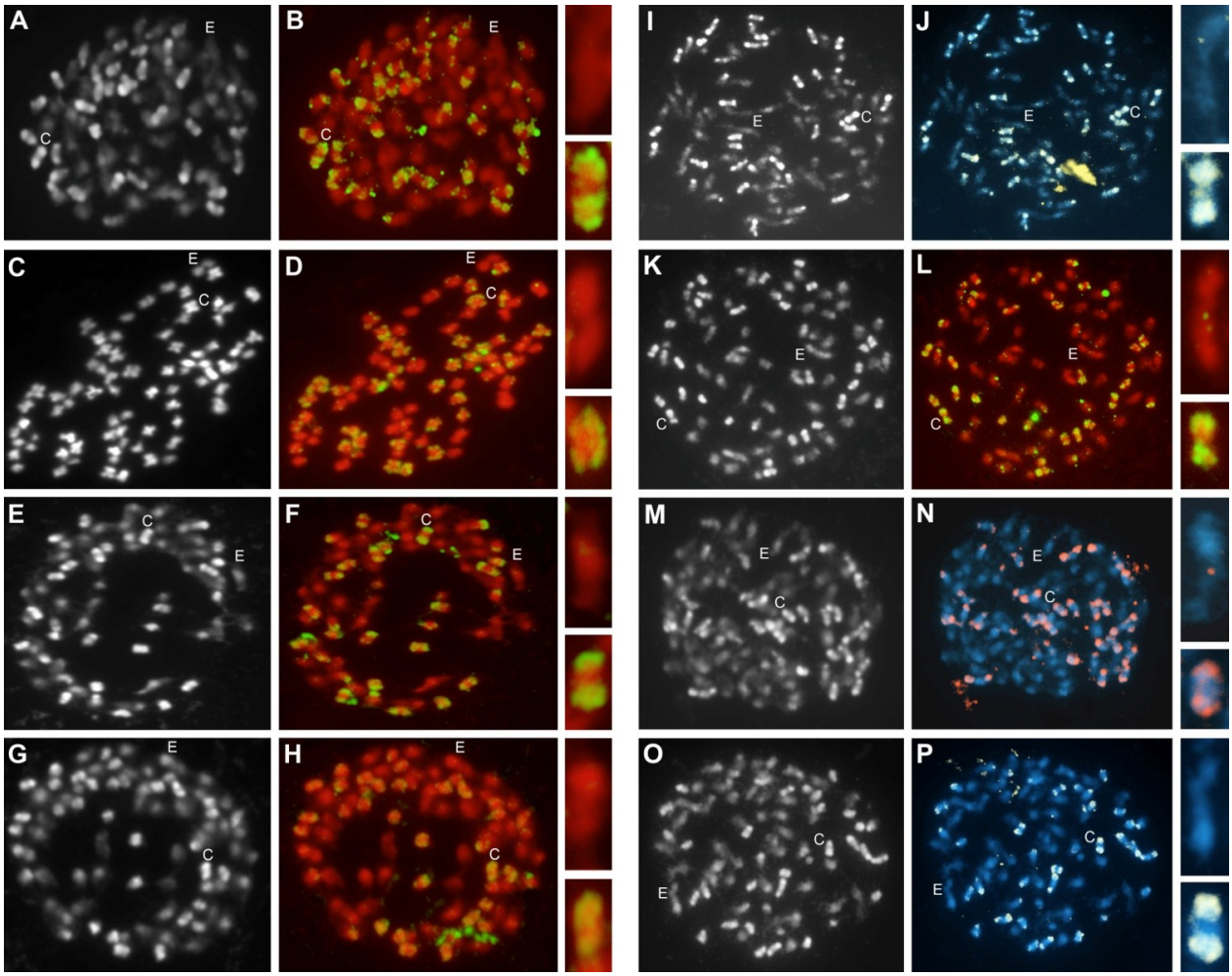
Cytological analysis carried out on chromosomes isolated from somatic cells belonging to root tip meristems proved that about half of the chromosome complements at pro-metaphase are actually much more extended and much less colorable than the remaining chromosomes, which appear almost completely contracted and fully colorable (for details, see Figure 2). The finding of two sets of chromosome complements of 38 elements each characterized by antagonist contraction ratios support the existence of two distinct diploid ancestral genomes in the tetraploid species of *S. alba* and *S. fragilis*.

A variable morphology for all basic chromosomes is in agreement with allotetraploidy, involving hybridization between divergent diploid species.

3.5. Genomic in situ Hybridization Analysis

Chromosome preparations of pure species and hybrids were hybridized using *S. alba* (or *S. fragilis*) total genomic DNA as probe labeled with FITC. The GISH experiments showed signals of both *S. fragilis* and *S. alba* labeled genomes prevalently on contracted chromosomes of the hybrids (Figure 2, panels A–H). When a pure species (*S. alba* or *S. fragilis*) was hybridized with the other, signals were still evident on the same set of contracted chromosomes (Figure 2, panels I–P).

Figure 2. Main results from genomic *in situ* hybridization (GISH) experiments. Hybridization signals and patterns obtained using *S. alba* as probe and *S. fragilis* as template (panels A-D) and *S. fragilis* as probe and *S. alba* as template (panels E-H). Hybridization signals and patterns obtained using *S. alba* as probe and interspecific hybrid as template (panels I-L) and *S. fragilis* as probe and interspecific hybrid as template (panels M-P). Both male and female *S. alba* and *S. fragilis* accessions are the parental genotypes used for *S. alba* × *S. fragilis* and *S. fragilis* × *S. alba* crosses, whereas interspecific hybrids refer to two intermediate F1 progeny genotypes. Note the great variation of genomic *in situ* hybridization signals and patterns in contracted (*i.e.* large spots) and extended (*i.e.* small dots) chromosomes across all experiments. Magnifications of specific contracted (C) and extended (E) chromosomes are reported for each GISH-derived karyotype.



It is worth emphasizing that, at the tetraploid level ($2n$ = 76 chromosomes), each of the species-specific genomic probes was found to hybridize the other target-species genomic templates across all technical and biological replicates, always involving the 38 contracted chromosomes. This finding strongly suggests that *S. alba* and *S. fragilis* share a common diploid ancestor species.

The absence of hybridization signals on extended chromosomes over all GISH experiments suggests that these chromosomes are not homeologous to the *S. alba*—*S. fragilis* contracted ones and that the genomes of pure species should have a quite different origin. In particular, hybridization signals on

contracted chromosomes were usually strong and uniform along one or both arms, whereas the extended chromosomes totally lacked fluorescent signals or revealed only small fluorescent dots (see Figure 2). The presence of hybridization spots on the extended chromosomes may indicate the occurrence of inter-genomic translocations and re-arrangements that could have taken place through genetic recombination among homeologous chromosomes.

4. Discussion

Polyploidy has long been recognized to be associated with novel morphologies and adaptations, but how genome duplication ultimately translates into novel evolutionary opportunity has remained obscure in many species. Understanding of evolution forces, mechanisms and outcomes at genomic and genetic levels is a prerequisite to developing a fuller appreciation of the role of polyploidy to morphological evolution and ecological adaption of willows.

In general, allotetraploids are those polyploids that have arisen through the processes of interspecific hybridization and chromosome doubling, whereas autotetraploids are those polyploids that have arisen from conspecific parents [40]. The former are characterized by fixed (*i.e.*, non-segregating) heterozygosity, resulting from the combination of divergent parental genomes, such that bivalent formation occurs at meiosis and disomic inheritance operates at each locus. In autopolyploids all homologous chromosomes can pair with each other, so they usually exhibit multivalent formation at meiosis and are characterized by polysomic inheritance, because more than two different alleles occur at each locus. Verification of segregation patterns and construction of linkage maps for polyploids can have great implications not only for marker-assisted selection, but also for plant genomics and population genetics in evolution studies. During the last decades, a few theoretical, simulation and explorative studies have been performed to estimate linkage in polyploids [37,39,41–45].

Here we report genetic segregation and genomic hybridization data, which help shedding light on the genomic structure and genic inheritance in tetraploid willows of the *S. alba—S. fragilis* complex.

Of the total molecular markers found to be polymorphic between parents, more than half did not segregate as a consequence of multiple-dose alleles at the loci being tested, whereas about one-third (33.6%) were inherited as SDMs. A total of 292 amplified fragment length polymorphism (AFLP) markers, segregating properly in the mapping populations were used in genetic linkage analysis. The fact that most loci scored only 1:1 and 3:1 segregation ratios, as expected for single-dose marker alleles, and very few showed 5:1 or 11:1 segregation ratios, as expected for double-dose marker alleles, is a first strong evidence supporting an allotetraploid nature and disomic inheritance in *Salix* spp. Moreover, in *S. alba*, 73 maternal and 48 paternal SDMs were mapped to 19 and 16 linkage groups, in *S. fragilis*, 13 maternal and 33 paternal SDMs were mapped in 6 and 14 linkage groups (see Table 2). A comparable number of markers linked in either coupling or repulsion was identified for most co-segregation groups. In polyploid species, chromosome pairing may be preferential, random, or of either type suggesting an allopolyploid, autopolyploid, or mixed genomic origin, respectively. Our findings clearly suggest that most of the chromosomes pair preferentially as occurs in species exhibiting an allotetraploid genome with disomic inheritance. In fact, the proportion of single-dose marker alleles linked in each group according to a coupling or repulsion phase was in agreement with the expected 1:1 ratio.

An important, still open issue concerns the genomic affinity and relationship between *S. alba* and *S. fragilis*. The fact that of the 1122 marker loci totally identified in the two male and female parents, the vast majority (77.5%) were proven to be polymorphic and only 22.5% were shared between parental species, highlights that *S. alba* and *S. fragilis* individuals have genotypes deeply differentiated. If it is true that derived species are more rare and contain less alleles, the highly significant difference between *S. alba*-specific and *S. fragilis*-specific markers found in both parental combinations (on average, 65.3% *vs.* 34.7%, respectively) supports the phylogenetic hypothesis primarily formulated by [24], that *S. fragilis* is a derivative of *S. alba*-like progenitors. Moreover, the pairwise analysis of co-segregation groups linked in repulsion indicated that most of the willow chromosomes pair preferentially in *S. alba—S. fragilis* interspecific hybrids. This finding strongly supports a low genomic affinity between these two species and consequently suggests that the recombination potential between the two species is limited.

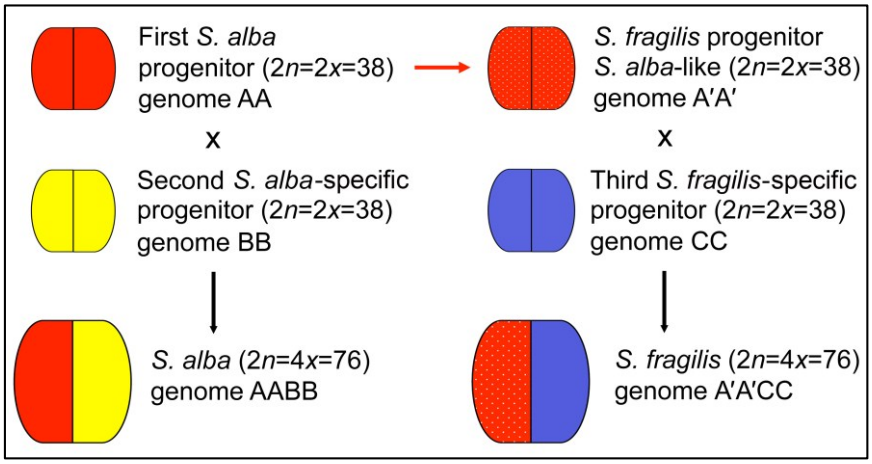
The analysis of DNA fingerprints in *S. alba* and *S. fragilis* natural populations showed the presence of diversified groups of willows and absence of genotypes with intermediary characteristics: this result confirms that both species have kept their gene pools well separated and that interspecific hybridization *S. alba* × *S. fragilis* does not seem to be a dominating process. A high genetic differentiation and a low gene flow among populations were found by [14], suggesting a low presence of spontaneous hybridization between these two willow species in natural populations. Recently, [10] reported that *S. alba*, *S. fragilis*, and their hybrid *S.* × *rubens* are distinctly separated and that only few hybrids and any backcrosses to the parental species are present in mixed stands. Furthermore, as a preliminary investigation, it is worth mentioning that all capsules collected from a subsample of plants belonging to either *S. alba* × *S. fragilis* or *S. fragilis* × *S. alba* hybrid progenies set seeds unable to germinate (Meneghetti, pers. comm.). If this finding will be confirmed by additional tests, it may be asserted not only that inter-specific plants deriving from both *S. alba* × *S. fragilis* crosses and reciprocals form unviable seeds, but also hypothesized that back-crosses of inter-specific hybrids with pure *S. alba* or *S. fragilis* species are impossible. As a consequence, the *S. alba—S. fragilis* complex would have a predominance of balanced hybrids, but it could not include fertile introgressed types within natural populations. It is worth mentioning that a massive non-random coalescence failure (*i.e.*, incomplete lineage sorting) along with a striking absence of geographical effects (*i.e.*, isolation by distance) in the haplotype distributions of many *Salix* species was recently discovered and it is likely owed to recent and continual plastid capture events, aided by wide-spread hybridization and long-range seed dispersal, but primarily propelled by one or more trans-species selective sweeps [9].

Although the analysis of pairing behavior of tetraploids in *Salix* spp. could help explaining the genomic organization of willow trees, cytological observations in this species complex are difficult to perform owing to the high number and small size of chromosomes. Nevertheless, our cytological observations of chromosomes isolated from root tip meristematic cells proved to be informative and revealed that about half of the chromosome complements at pro-metaphase are more extended than the remaining chromosomes, which are already almost completely contracted. This finding, which is supported by well-documented karyotypes, is very important because it further agrees with allotetraploidy in *Salix* spp.

In addition, the GISH experiments showed hybridization signals of both *S. fragilis* and *S. alba* labeled genomes prevalently on contracted chromosomes of the *S. alba* × *S. fragilis* hybrids used as template

genomes. Surprisingly, when the two pure species were hybridized one with the other on both ways (*i.e.*, using *S. alba* genome as template and *S. fragilis* chromosomes as probe and vice versa), signals were still evident on the same set of contracted chromosomes showing reproducible patterns. The absence of large hybridization spots on non-contracted chromosomes over all experiments, exception made for a few small hybridization dots, suggests that the extended chromosomes are not homeologous to the *S. alba—S. fragilis* contracted ones. If this is true, the genomes belonging to the diploid ancestors of the two pure species should have different origin and could share half of the chromosome complements at the tetraploid levels (Figure 3).

Figure 3. Working hypothesis relating to the genomic organization of *S. alba* and *S. fragilis* genomes, along with their origin and relationship with putative diploid ancestor species. Letters A and A′ refer to the shared or closely related diploid progenitor species of *S. alba* and *S. fragilis* whose genome(s) contributed with contracted chromosomes (*i.e.*, first common ancestor), whereas letters B and C indicate the two additional and different progenitor' genomes which transmitted the extended chromosomes to both *S. alba* and *S. fragilis* species (*i.e.*, *S. alba*-specific and *S. fragilis*-specific second and third progenitor, respectively).



On the whole, genomic analyses based on DNA fingerprints and GISH patterns revealed that *S. alba* and *S. fragilis* are much differentiated genotypically and that *S. fragilis* species may have derived from *S. alba*-like progenitors. The fact that both *S. alba* and *S. fragilis* show halved chromosome complements with different contraction ratios in tetraploid genomes and that hybridization signals are evident on the same set of contracted chromosomes and absent in the rest of extended chromosomes, allow us to hypothesize that two sets of non-contracted chromosomes found in both *S. alba* and *S. fragilis* belong to different diploid ancestors (see Figure 3). Such explanation of the genomic structure could also explain why interspecific *S. alba* × *S. fragilis* hybrids do not set viable seeds.

5. Conclusions

Our multidisciplinary investigations of the polyploid complex *S. alba—S. fragilis* based on DNA fingerprints and GISH patterns, along with robust data from genetic segregation of marker alleles and karyological observation of mitotic chromosomes, have contributed to document and collect new evidences on the intra-genomic organization and inter-specific introgression in tetraploid willows.

Overall findings converge towards an allopolyploid origin for *S. alba* and *S. fragilis* and also underline an additive effect resulting from the fusion of the two distinct ancestral genomes in both species. Karyology revealed tetraploid genomes sharing two clearly distinguishable subsets of chromosomes of equal number but different shape and size. Molecular markers proved that both species have kept their gene pools well separated and that interspecific hybridization is not as marked as expected. Genomic hybridizations between pure species and hybrids proved that species-derived probes successfully recognize only half of the somatic cell chromosomes. This finding strongly suggests that *S. alba* and *S. fragilis* share one common diploid ancestor species or two highly related ones (*i.e.*, contributing with contracted chromosomes), but differentiate each other for the second diploid ancestor species (*i.e.*, contributing with extended chromosomes). Nevertheless, the uncommon occurrence of molecular markers segregating as multiple-dose alleles and the sporadic appearance of hybridization spots on non-contracted chromosome complements may indicate the occurrence of few inter-genomic translocations and re-arrangements that could have taken place through genetic recombination among homeologous chromosomes.

All events involving inter-genomic DNA exchanges should have increased the combinatorial potentials of genetically different or divergent gametes produced by independent assortment, hence representing a key component of the evolutionary mechanisms occurred in polyploid willows. After all, authors [27] mentioned entire families of woody angiosperms, including Salicaceae, having high basic chromosome numbers that were hypothesized to represent ancient polysomic polyploids.

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Author Contributions

Gianni Barcaccia conceived the study, designed the experiments, and collaborated to data analysis, and preparation and revision of the manuscript. Stefano Meneghetti performed all the experiments, including AFLP and GISH analyses, and wrote the first draft of materials and methods. Gianni Barcaccia and Hans de Jong supervised the laboratory activities at the University of Padova and Wageningen, respectively. Margherita Lucchin devised the study, collaborated to the selection of plant materials, to the interpretation of results and to the preparation of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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