Genetic diversity and stand structure of neighboring white willow (*Salix alba* L.) populations along fragmented riparian corridors: a case study

T. Sitzia[1*], G. Barcaccia[2], M. Lucchin[2]

[1] Università degli Studi di Padova, Department of Land, Environment, Agriculture and Forestry, Viale dell'Università 16, I-35020 Legnaro (PD)

[2] Università degli Studi di Padova, Department of Agronomy, Food, Natural Resources, Animals and the Environment, Viale dell'Università 16, I-35020 Legnaro (PD)

Authors' emails: tommaso.sitzia@unipd.it, gianni.barcaccia@unipd.it, margherita.lucchin@unipd.it

* Corresponding author: tommaso.sitzia@unipd.it

Abstract

Remnant riparian woodlands have an important landscape function, due to their ability to act as ecological corridors. In this study we used molecular markers to assess the genetic variation occurring within and between spontaneous white willow (*Salix alba* L.) riparian woodlands. Our main goal was to evaluate the extent to which the fragmentation of a woodland corridor along a heavily impacted river in northeastern Italy and stand structural conditions may have affected the population genetics. Although having different structures, the three examined white willow stands showed high estimates of genetic similarity, as well as low genetic differentiation between them, indicating that they shared a similar gene pool and that the stands could result from a common set of individual genotypes, and should be regarded as metapopulations. The magnitude of genetic diversity within each of the stands and genetic differentiation between them, despite their high sexual reproductive capacity associated with a highly marked gene flow, suggest that these stands are dynamic and capable of adaptive responses to possible changes in their fluvial environment. However, the factors influencing genetic diversity should be interpreted from a long-term perspective. Fluvial geomorphic patterns in regulated rivers may be modified to a degree that could lead to changes in dispersal processes, sexual reproduction vs. asexual propagation, and hence genetic diversity.

Keywords: *: ecological corridor; biodiversity conservation; fluvial ecosystem; genetic resources; metapopulation; amplified fragment length polymorphisms; Natura 2000*

Introduction

Remnant riparian woodlands have an important landscape function, due to their ability to act as ecological corridors, which offer important biodiversity benefits (Naiman, et al. 1993, Sabo, et al. 2005). Riparian corridors are complex ecosystems affected by variable flood regimes, geomorphic channel processes, altitudinal climate shifts, and upland influences. These factors put the plant community under a wide array of competitive pressures, resulting in a high level of biodiversity (Naiman, et al. 1993, Steiger, et al. 2005, Tabacchi, et al. 1998).

The natural lateral expansion of a riparian corridor reaches from the river channel to that portion of the land where vegetation may be influenced by elevated water tables or flooding (Naiman and Decamps 1997). However, changing water regimes (Pinay, et al. 2002) and direct human impacts may result in a reduction of riparian vegetation width (Gergel, et al. 2002, Richardson, et al. 2007, Sitzia, et al. 2016) or the extinction of plant communities (Andersson, et al. 2000, Nilsson and Jansson 1995). Management of the tree component in riparian woodlands is another influential factor controlling stand structure and hence the quality of the freshwater environment (Broadmeadow and Nisbet 2004). Nevertheless, plant communities may sometimes display a remarkable resistance to alterations and indirect disturbances (Ferreira, et al. 2005).

Genetic analysis provides a method of evaluating the degree of connectivity loss that has occurred (Frankham, et al. 2010). Recent studies have drawn attention to the risk of losing diversity in small, remnant , and genetically isolated populations (Sork and Smouse 2006, Young, et al. 1996). Several studies have been conducted in riparian woodlands, mainly on black poplar (*Populus nigra* L.) (Chenault, et al. 2011, Imbert and

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Lefevre 2003, Vanden Broeck, et al. 2004). However, less is known about the other members of the *Salicaceae* family, which includes many of the most important species in shaping the active zone of floodplains (Karrenberg, et al. 2002). There is therefore the need for a better understanding of the functional connectivity between woodlands dominated by *Salicaceae*, to improve both *in situ* and *ex situ* conservation strategies in regulated rivers (Rajora and Mosseler 2001).

Of particular importance for European floodplain forests is white willow (*Salix alba* L.), due to its distribution across a range of climatic and geomorphological variation, which is often coupled with a high level of urbanization (Klimo and Hager 2001).

Here we present a study on the Brenta River in northern Italy, at a site that has received much attention from fluvial geomorphologists due to its characteristic of a gravel-bed river strongly affected by human impacts. It also provides a rich source of knowledge about the potential effects of genetic variation, which is in turn of great interest for planners and conservationists (Martini, et al. 2004, Surian 2009, Surian and Cisotto 2007, Surian and Rinaldi 2003).

Given that forestry practices may have an influence on genetic diversity (Savolainen and Kärkkäinen 1992), we selected three stands under natural regeneration conditions, with different stand structures, to account for variability in treatments of the riverbank vegetation. Then, to assess genetic diversity and differentiation, along with gene flow, we used amplified fragment length polymorphism (AFLP) markers for genomic DNA fingerprinting, a molecular technique well established and commonly employed in population genetics, and in ecological and evolutionary research (Bensch and Akesson 2005). This method is particularly useful with *Salix* species (Barcaccia, et al. 2003, Barcaccia, et al. 2014, Meneghetti, et al. 2007), which are often difficult to distinguish using morphological characteristics (Beismann, et al. 1997).

Our study aims to answer a main research question: are the *S. alba* riparian stands meta-populations? The ultimate goal is to test the hypothesis that, despite the disturbances that may have occurred in their fluvial environment, these spatially separate populations in the three selected sites interact and are still interconnected as a demographic whole, i.e. metapopulations (Merriam 1984).

Materials and Methods

Study area

The study area is located on the Po Plain (northern Italy), along the middle course of the Brenta River, which crosses the northern part of Padova Province and lies inside the Natura 2000 site IT3260018 (Figure 1). A 16 km long river reach was selected where *S. alba* woodlands (*Salicetea purpureae*), belonging to the priority European Union habitat 91E0, were growing. The mean fluvial slope is 2.8 ‰, altitude ranges from 10 to 40 m a.s.l., and the channel width is often narrowed by human impacts. Rainfall is 850-1200 mm y^{-1} and mean annual temperature is 12.5 °C (Autorità di Bacino 2012).

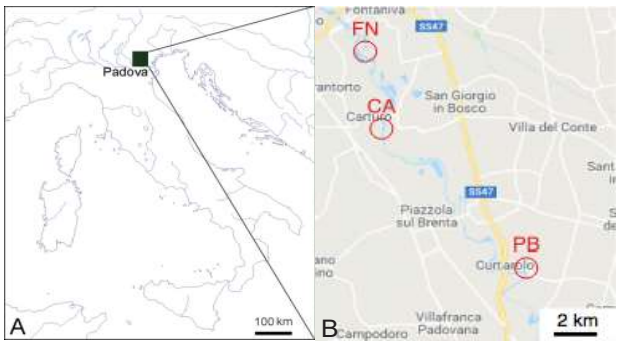

Figure 1
Study area in the province of Padova, Italy (A) and the Brenta River with the locations of the three stands: Fontaniva (FN), Carturo (CA) and Piazzola (PB), and the main roads (B).

Three sites were surveyed from top to bottom: Fontaniva (FN), Carturo (CA) and Piazzola (PB). They are 4 km and 9.6 km apart linearly and 4.6 and 15.3 km along the river course, respectively.

Three stands were chosen to be representative of the middle course of the river along a gradient of tree density, as found from a preliminary estimation of basal area. In the first site the Brenta River has a faster water flow, wider river bed and a more braided aspect, while the other two have a very slow water flow, because of a continuous series of curves, a narrow channel and minimal altitudinal-length ratio (30 m / 46 km). Mean annual discharge is around 70 m^3s^{-1} with wide variations during flooding and between the minimal (August/February) and the maximal peaks (May/November). A destructive flood, with a discharge peak of 2730 m^3s^{-1}, occurred on 3-4 November 1966, which swept away all trees along the riverbanks. After this event the tree vegetation gradually recolonized the gravel at a rate of approximately 1.31 ha y^{-1} (Costantini, et al. 2002). During the same period, the river bed increased its mean depth between 1.2-8.8 m and decreased its width and gravel surface, mainly due to gravel quarrying, and it is also at a very high risk of flooding (Autorità di Bacino 2012).

Data collection

Between October 2012 and March 2013, a 60x30 m transect was surveyed in both FN and PB, while in CA a 90x40 m one was used to account for its lower tree density. Each standing tree with diameter at breast height (DBH) ≥ 1 cm within transects was surveyed for topographical position, height, DBH, species, and dead or alive status. Topographical position was assessed through trilateration. The ratio between dead and living trees and the number of big (DBH ≥ 10 cm) dead trees were assessed to compare the naturalness of the stands.

To get an average estimation of the tree age we cored a subsample of 39 *S. alba* trees, whereas to perform molecular analyses we used 19, 21 and 20 *S. alba* trees randomly chosen within the FN, CA and PB stands, respectively, among those trees with DBH ≥ 10 cm.

Stand structural data analysis

After calculating the dendrometric descriptive parameters for each stand, the structural investigation first involved estimation of the distribution pattern with the nearest neighbor technique, adopting the aggregation index of Clark and Evans (1954), as modified by Donnelly (1978), to take into account edge effects (Sinclair 1985). Secondly, we assessed the existence of spatial clustering of stem DBHs with correlograms of the standard normal deviates of Moran's I(d) index (Moran 1950), against the critical value for a standard normal distribution.

The Donnelly index was calculated as:

$$E(\bar{y}) = 0.5\sqrt{A/n} + (0.051 + 0.041/\sqrt{n})L/n$$

$$SE(\bar{y}) = \sqrt{0.07A + 0.037L\sqrt{A/n}}\,/\,n$$

$$z = \frac{\bar{y} - E(\bar{y})}{SE(\bar{y})}$$

where n = number of stems
A = study area size
L = perimeter study area
$E(\bar{y})$ = expected value of mean nearest neighbor distance,
$\bar{y}$ = observed value of mean nearest neighbor distance.

The first expression presents a normal standard distribution for cases of total spatial randomness. The z-score permits the evaluation of the test's statistical significance. *SE*($\bar{y}$) is the standard error. The ratio R between expected and observed value ($\bar{y} / E(\bar{y})$ equals 1 with casual distribution, *R* > 1.0 suggests regular distribution and *R* < 1.0 clustered distribution.

The Moran's index I(d) (Moran 1950) of spatial autocorrelation was calculated as follows:

$$\mathrm{I(d)} = \frac{N\sum_{i=1}^{N}\sum_{j=1}^{N} w_{ij}(x_i - \bar{x})(x_j - \bar{x})}{W\sum_{i=1}^{N}(x_i - \bar{x})^2}$$

where:
N = number of sampled stems
w_{ij} = 1 when the stems i and j belong to the same distance class, 0 in the other cases
W = sum of the w_{ij},
x_i and x_j = value of DBH with $i = 1 \div n$; $j = 1 \div n$ and $i \neq j$,
$\bar{x}$ = mean value of DBH

Negative (positive) values indicate negative (positive) spatial autocorrelation. Values range from −1 (indicating perfect dispersion) to +1 (perfect correlation). A zero value indicates a random spatial pattern.

The spatial autocorrelation coefficients were calculated using only the *S. alba* trees. We tested several lag distances from a minimum of 30 to a maximum of 200 cm, and from 20 to 30 lags (i.e. distance classes). It was thus possible to interpret the correlograms in order to identify a generally intelligible trend. To test the global significance of the correlograms, we checked whether at least one value was significant at the $a = a$ / number of distance classes. In our case, the corresponding z values of a standard normal distribution were ±2.8070 and ±2.9346, with 20 or 30 distance classes, respectively. The significance of each single value was then checked for significant deviations from the expected value under the null hypothesis of random spatial distribution, adopting a *p*=0.05 threshold value (Cliff and Ord 1981). The calculations were performed with the MS Excel VB add-in Rookcase (Sawada 1999).

Molecular marker analysis

A subsample of 60 *S. alba* trees were randomly selected from the stands and used for DNA fingerprinting with AFLP markers according to experimental protocols and primer combinations already available for willow species (Barcaccia, et al. 2003, Barcaccia, et al. 2014, Meneghetti, et al. 2007). In addition, 12 crack willow (*Salix fragilis* L.) trees were analyzed for comparison as outgroup samples given that *S. alba* and *S. fragilis* are closely related species which likely share a common diploid ancestor, as reported by Barcaccia et al. (2014).

Total genomic DNA was isolated from 0.5 g of whole young leaves, chosen among the last ones formed on the shoot apical meristem of each plant, using the Nucleon Phyto-Pure kit (Pharmacia Biotech) according to the manufacturer's instructions. The concentration of genomic DNA samples was determined by optical density reading with a spectrophotometer (DU650, Beckman) and an aliquot of each genomic DNA samples was also assayed by electrophoresis on 1 % agarose gels in order to assess its integrity.

For generating DNA fingerprints by AFLP markers, the single steps of the molecular protocol, including restriction, ligation to adapters and amplification were performed according to Barcaccia et al. (2003), with some changes as described by Meneghetti et al. (2007) using primer combinations already tested in willow species (Barcaccia, et al. 2014). In particular, the analysis of DNA fingerprints was based on the detection of *Eco*-RI/*Mse*-I genomic restriction fragments by PCR amplification with eight different primer combinations having three selective nucleotides (Table 1S of the Supplementary materials). The core sequence of primers for the specific restriction site/oligonucleotide adapter combinations are as follows: *Eco*-RI primer (E): AGACTGCGTACCAATTC and *Mse*-I primer (M): GACGATGAGTCCTGAGTAA. Over all the PCR experiments for each primer combination, an individual genomic DNA for each of the three stands was replicated twice to assess the reproducibility of AFLP fingerprints.

Genetic data 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. Marker loci related to faint bands were removed from the dataset as the best amplicons were chosen according to reliability, intensity and clearness of the autoradiogram bands. Different measures of genetic variation within and genetic differentiation between stands were taken into account in order to compute genetic diversity statistics, along with gene flow estimates.

The heterozygosity (H) was computed for all marker loci in each of the three stands (H_S) and for the population of the study area as a whole (H_T) as follows:

$$H = 1 - \sum_{i=1}^{N} p_i^2 / N$$

where p_i is the frequency of the *i*-th marker allele in the given stand and *N* is the total number of marker loci.

The extent of genetic diversity between stands was calculated with the fixation index as $G_{ST} = (H_T - H_S) / H_T$ (Wright 1984), a measure of the genetic effect of total population subdivision as the proportional reduction in overall heterozygosity owing to variation in marker allele frequencies among different stands. The rate of gene flow (Nm) among stands was quantified from the fixation index as follows: $Nm = c\,(1 - G_{ST}) / G_{ST}$ where c=0.5 when the index is calculated from dominant molecular markers (McDermott and McDonald 1993). This index is <1 when there is no gene flow, i.e. when populations are differentiated only because of their locations, while values >1 indicate the presence of gene flow related to the exchange of marker alleles between populations.

Descriptive genetic diversity and differentiation statistics, as well as inbreeding coefficients, were calculated using the POPGENE software package (Yeh, et al. 1997).

Since genetic segregation and genomic hybridization patterns support an allotetraploid structure and disomic inheritance for *Salix* species (Barcaccia, et al. 2014), estimates of pairwise linkage disequilibrium (LD) between marker alleles were also computed over all genomic loci. In particular, significant associations between frequencies for marker alleles were investigated for each pair of loci for the whole population dataset and for single stands separately. The degree of LD between pair-wise combinations of marker alleles at different genomic loci was estimated by maximum likelihood from the frequency of all willow genotypes, according to Hill (1974). Only significant values, for $p < 0.05$ corrected according to Bonferroni's method, at a likelihood ratio test were shown. All calculations and analyses were conducted using the software Genetic Data Analysis (GDA) version 1.0 (Lewis and Zaykin 2001).

The population structure of the *S. alba* stands was investigated using the model-based (Bayesian) clustering algorithm implemented in the STRUCTURE software (Falush, et al. 2003), which groups and orders plant genotypes according to marker allele combination and distribution. The AFLP dataset was formatted following the tutorial of this software and then used to analyze molecular marker data collected from single stands and the population as a whole. All simulations were executed assuming the admixture model, with no a priori population information. Calculations were performed with 500000 iterations and 500000 burn-ins by assuming the allele frequencies among stands to be correlated (Falush, et al. 2003). Ten replicate runs were performed, with each run exploring a range of K spanning from 1 to 10. The most likely value of K was estimated using ΔK, as reported in other studies (Evanno, et al. 2005). Individuals with membership coefficients $q_i > 0.7$ were assigned to a specific group, whereas individuals with $q_i < 0.7$ were identified as admixed.

An ordination analysis was performed for monomorphic and polymorphic AFLP markers according to the unweighted pair-group arithmetic average (UPGMA) clustering algorithm, and the centroids of all accessions were bidimentionally plotted from the symmetrical genetic similarity matrix. In particular, the coefficient of Dice (1945) was applied to calculate the proportion of genetic similarity in all pair-wise comparisons of *S. alba* plants. A principal coordinates analysis technique was then applied to compute the first two principal components from the qualitative data matrix. The calculations and analyses were conducted using the appropriate routines in the NTSYS software package (Rohlf 2008).

Results

Structure of the stands

The main dendrometric parameters and nearest neighbor analysis are reported in Table 1 and Table 2, respectively. The most informative spatial correlograms are reported in the Supplementary materials (Figure 1S).

Table 1

Stand structural attributes of the study transect. The table reports both the data for the total CA transect (CA_t) and the two sub-transects (CA_1 and CA_2). DBH mean (±95 % conf. limits), G: basal area per hectare, H/D: mean ratio between H and DBH, bd: number of big dead trees (DBH ≥ 10cm), d/a %: ratio between dead and alive trees, d/ha: number of dead trees per hectare.

Study transect	DBH mean (cm)	DBH max (cm)	H mean (m)	G (m^2/ha)	willows/ha	trees/ha	H/D	bd	d/a %	d/ha
CAt	17.1±1.15	50	16.8	19	813.9	866.7	0.98	0	5.3	47.2
CA_1	14.7±1.04	37	16.3	19	1,100.0	1,305.6	1.11	0	5.5	72.2
CA_2	22.7±2.69	50	18.4	18	433.3	488.9	0.81	0	6.8	33.3
PB	16.7±1.01	39	19.6	35	1,605.6	1,894.4	1.17	6	12.9	250.0
FN	19.0±1.74	51	20.2	29	1,027.8	1,283.3	1.06	17	19.5	244.4

Table 2
Results of the nearest neighbor analysis. n = number of stems, A = study area size, L = study area perimeter, E($\bar{y}$) = expected value of mean nearest neighbor distance, $\bar{y}$ = observed value of mean nearest neighbor distance, z = z-score, R = ($\bar{y}$ /E($\bar{y}$)).

Study transect	A (m^2)	n	L (cm)	$\bar{y}$ (cm)	$E(\bar{y})$ (cm)	z	R
CAt	3600	275	26,000	108.14	185.96	-12.64	0.58
CA_1	1800	249	18,000	78.08	138.31	-12.50	0.56
CA_2	1800	91	18,000	141.63	233.31	-6.69	0.61
PB	1800	303	18,000	58.79	125.04	-16.83	0.47
FN	1800	171	18,000	127.48	167.92	-5.69	0.76

As a main finding, the spatial distribution of trees was aggregated in clusters and the correlograms showed positive autocorrelation at short distances. This suggests that the stand derives from a few centers of dispersion and its structure is determined by groups, which are internally homogeneous.

The Fontaniva (FN) stand showed the least aggregated structure (R = 0.75) with intermediate values of density (1023 trees/ha) and groups with high autocorrelation in group size 0.8 m and 2-3 m. Moreover, a high degree of naturalness was found, with 244 dead trees/ha (19.5 % total) and 17 big dead standing trees.

Trees in the Carturo (CA) stand revealed a medium aggregated pattern (0.56 ≤ R ≤ 0.61) with low values of tree density (814 trees/ha). Groups with high correlation (heights and diameters) with size of 0.8, 2, 8, and 12 m were documented. This stand had the lowest degree of naturalness in terms of number of dead trees.

Trees in the Piazzola (PB) stand scored the highest degree of aggregation (R = 0.47) and tree density (1606 trees/ha). Groups with high autocorrelation with sizes of 3, 6, and 8 m were detected. The degree of naturalness was shown to be high; with as many as 250 trees being dead (13 % total) and 6 big dead standing trees.

Representative photos of the stand interiors, distribution and size of the trees are reported in Figure 2.

As a general conclusion, we found a gradient of clustering increase from FN-CA-PB, with FN having the tallest mean tree sizes and the greatest amount of deadwood, with PB having the most dense stand, but with a high H/D mean ratio, and CA having a low density stand, with a relatively short height and very small amount of deadwood. In addition, we identified a gradient of increasing naturalness from CA-PB-FN as determined by density of dead trees.

Mean tree age was 14.5 years, ranging from a minimum of 6 to a maximum of 22.

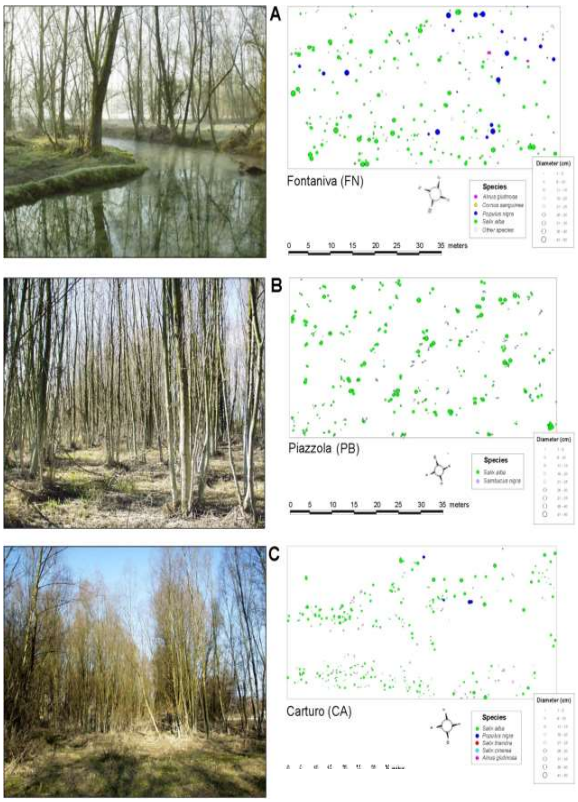

Figure 2
The stand interiors, distribution and size of the trees in Fontaniva (A), Piazzola (B) and Carturo (C) transects (size: 60x30 m, 60x30 m and 90x40 m, respectively). The perimeter of each transect is shown by the grey line and the colored dots represent trees.

Genetic variation

The analysis of AFLP fingerprints revealed 276 markers, of which 178 (64.5 %) were polymorphic among trees of the population as a whole (Table 3). In particular, in the PB, CA, and FN stands a total of 251, 252, and 261 markers were detected, respectively, of which 127 (50.6 %), 135 (53.6 %), and 149 (57.1 %) were polymorphic among trees within each of the three stands, respectively.

Total genetic diversity (H_T) was 0.159 and the mean genetic diversity within stands (H_S) was 0.151, with a minimum value of 0.146 in PB and a maximum of 0.155 in CA. The fixation index, as a measure of the proportion of genetic diversity attributable to DNA polymorphisms among the three stands (G_{ST}), was 0.050, indicating that 95 % of the observed genetic variation is due to markers polymorphic within stands, whereas only 5 % is due to markers polymorphic between stands. Consequently, the low genetic differentiation between stands is also proved by the high levels of gene flow (N_m), which was greater than 1, ranging between 5.61 in PB and 20.41 in CA, with an average value of 9.50 (Table 3). This finding is consistent with an intensive exchange of genetic material between stands by means of crosses among trees in different stands.

Table 3

Main genetic diversity statistics related to the three Salix alba stands along the Brenta River, including number of sampled individuals (n_i), markers (nm) and polymorphic markers (npm), allele frequencies (p_i), genetic diversity within stands (H_S), degree of genetic diversity between stands (G_{ST}) or fixation index, gene flow (Nm) and total genetic diversity (H_T)

Stand	n_i	nm	npm	p_i	H_S	G_{ST}	N_m
PB	20	251	127	0.6460	0.146	0.082	5.61
CA	21	252	135	0.6289	0.155	0.024	20.41
FN	19	261	149	0.6020	0.152	0.045	10.53
Total	60	276	178	0.5676	H_T=0.159	0.050	9.50

The genetic similarity of the whole population was 0.887, with estimates for individual stands varying between 0.887 and 0.894 in CA and PB, respectively. All stands were therefore shown to have high genetic within-stand uniformity.

We also compared the three stands in terms of genetic similarities and distances, finding that they are very similar to one another. In fact, genetic similarity estimates were 0.887, 0.883, and 0.884 for the three comparisons PB-CA, PB-FN, and CA-FN, and genetic distances ranged from 0.120 to 0.124 for the three comparisons. On the whole, both genetic diversity and genetic similarity estimates clearly demonstrated that the three *S. alba* stands have comparable within-stand genetic variation as well as genetic differentiation between them (Table 4).

Table 4

Results of the ANOVA using the estimates of genetic similarity calculated within and between *Salix alba* stands (FN, Fontaniva; CA, Carturo; PB, Piazzola). Statistics, which were computed after a significant overall ANOVA test, include the mean genetic similarity (MGS), variance and F values.

	PB			CA			FN		
	MGS	Variance	F	MGS	Variance	F	MGS	Variance	F
PB	0.89392a	0.00021	-						
CA	0.88708	0.00024	21.9989**	0.88666c	0.00023	-			
FN	0.88334	0.00043	0.688201 ns	0.88425	0.00042	6.692184*	0.89211b	0.00050	-

The cluster analysis performed using the whole set of AFLP markers allowed the definition of a UPGMA dendrogram from which it was evident that no discrimination was possible between trees belonging to the three stands was not possible. Genetically identical trees, i.e. deriving from vegetative propagation, were not found in any of the stands.

On the whole, the high coefficients of genetic similarity within stands as well as the low estimates of genetic diversity between stands suggest that the willow trees of the whole population share a common gene pool and confirm little genetic differentiation among them. This finding was also supported by the principal coordinates analysis (PCoA) based on the same set of AFLP markers which enabled definition of the centroids of the 60 analyzed trees (Figure 3). Ordination of the centroids, while confirming a main aggregation of trees belonging to the different stands, highlighted a distribution along the second axis that seems to reflect the geographic location of the three sampled sites along the river, in a north-south direction. The first two components were able to explain 59.4 % of the total variation, accounting for 32.6 % and 26.8 % of the total, respectively.

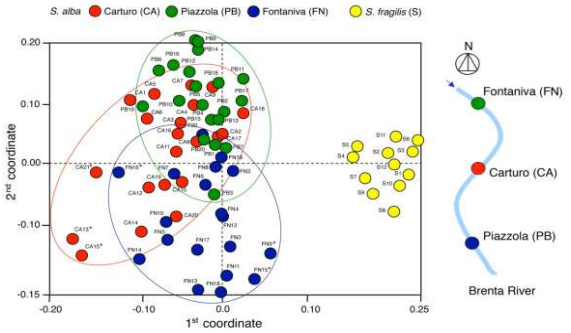

Figure 3

Principal coordinate analysis (PCoA) using AFLP markers where the centroids of the 60 analyzed *S. alba* trees are reported, with other 12 *S. fragilis* trees for comparison (individuals with a diverted or admixed haplotype are indicated by an asterisk).

In addition, of the 178 polymorphic markers identified, 51 (28.7 %) scored a frequency gradient among the three stands, including 29 and 22 marker alleles with an increasing and decreasing frequency, respectively, moving from FN to PB.

Investigation into the structure of the single stands and the whole population by estimation of ΔK (Figure 2S) suggested that these *S. alba* trees belong to a single group (K = 1), sharing the same haplotype, even if some individuals are genetically distinguishable and attributable to three subgroups (K = 3), as shown in Figure 4.

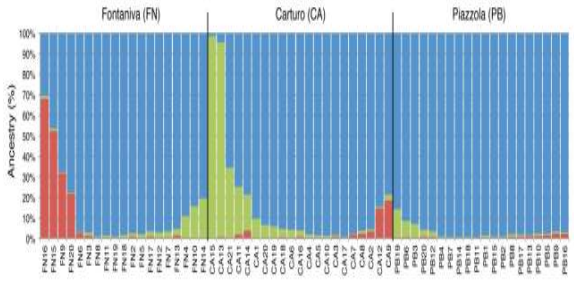

Figure 4

Analysis of the genetic structure of the three stands and the population as a whole. The x-axis reports individual trees over the three stands (FN, Fontaniva; PB, Piazzola; CA, Carturo), while the y-axis shows the proportion of ancestry (% of membership).

The PB stand was found to be the most genetically uniform, whereas some individuals in CA and FN stands, about 15 %, proved to have diverted or admixed haplotypes (with ancestry <70 %). For instance, individuals FN16, FN15 and CA13, CA15 were characterized by haplotypes strongly deviating from the rest of individuals in the same stand, most likely because of the presence of rare and/or private marker alleles at the investigated genomic loci which could be inherited from individuals out of the local gene pool (Figure 4).

It is worth mentioning that significant ($p < 0.05$) pair-wise linkage disequilibria between AFLP marker alleles of different genomic loci were found in the whole population. In particular, a total of 194 marker combinations showing strong LD were scored out of all comparisons, equal to 1.23 %.

Discussion

Previous work has documented the risk of losing genetic diversity in small isolated remnant riparian willow and poplar communities. For example, in black poplar woodlands, Rathmacher et al. (2010) suggested the occurrence of small-scale isolation by distance due to short-distance gene flow. Very few studies have been conducted on population-level genetic variation in *S. alba* communities, which are as threatened as those dominated by other riparian softwood species in Europe. In all these cases, molecular markers proved to be useful for estimating isolation by distance and spatial genetic structuring. In particular, a pattern of genetic structure that corresponds to the model of isolation by distance was found for *S. alba* at both intra- and inter-population levels along Alpine upstream rivers in Alto Adige and the Upper Rhine (Van Puyvelde and Triest 2007). Isolation and poor gene flow was also documented among tea-leaved willow (*S. phylicifolia* L.) populations, suggesting risks of genetic drift and inbreeding (Egelund, et al. 2012). Within the genus *Salix*, genetic diversity, divergence and gene flow among populations, as well as linkage disequilibrium were also investigated in several species, including *S. viminalis* L., *S. schwerinii* E. Wolf, *S. herbacea* L. and *S. myrsinifolia* Salisb. (Alsos, et al. 2009, Berlin, et al. 2011, Mirski, et al. 2017). However, owing to the different environments and willow species, within-stand features and between-stand distances, and willow species, this information is not comparable with that emerging from our case study.

The stand spatial structure of naturally regenerated *S. alba* stands has not been particularly studied in the past, nor the relationships between this and genetic diversity, which is in contrast with other forest types, like conifer and beech woodlands (Paffetti, et al. 2012).

In this research, we applied molecular analyses to assess the genetic variation occurring within and between *S. alba* woodlands along the Brenta River (North-East Italy) using AFLP markers. Our main goal was to evaluate the extent to which fragmentation of the woodland patches and their isolation along the river and stand structural conditions may be reflected on their genetic conditions, and to verify any possibilities of gene flow between the three stands.

Molecular-genetic information reported in the literature on the genus *Salix* is scanty, but the values of genetic diversity observed here are similar to those detected by Barcaccia et al. (2003) on the provenances of different origin for both *S. alba* and *S. fragilis*, with mean values of expected heterozygosity equal to 0.214 and 0.141, respectively. In addition, the mean genetic similarity of the whole population, 0.887, is also very similar to that calculated by Meneghetti et al. (2007) for a different population of the same *Salix* species, which was 0.914.

The sampled sites lie along a river sub-reach heavily impacted by several human modifications of the banks, channels and floodplains, which are frequently altered for cropping, recreation, quarrying and building purposes. Moreover, variable selection processes should have been related to the observed differences in stand structural attributes, management and ecological conditions.

We have no data regarding spatial scale patterns of *S. alba* trees for comparison. However, existing data show that mature trees can reach 30 m in height and 1 m in diameter (FAO, 1980), the average height at maturity is 10-15 m, DBH of mature trees (age 20 to 30 years) is 50-60 cm, although it may exceed 100 cm (CABI 2018). This means that the surveyed stands include mature trees, with a quite high h/d ratio, meaning that the *S. alba* trees attain greater heights than usual for their diameters in our study area. The three stands are somewhat different in terms of the spatial arrangement of individuals and their sizes, although they all showed an aggregated pattern of trees, which is in accordance with the general and established evidence that tree distribution is not uniform in naturally regenerated woodlands (Watt 1947). Given that they all had a chance to develop since the destructive 1966 flood, the observed stand structural differences must be due to management practices, like coppicing, or by intermittent disturbance from river flooding, or a combination of both. These disturbances, by altering the primary regeneration pathway, might alter the genetic diversity within populations (Inza, et al. 2012, Sjölund and Jump 2015).

Based on the aforementioned conditions, we would not be surprised to verify that the *S. alba* stands were isolated and gene flow impeded. However, we found a poorly structured population as a whole, with high degrees of genetic similarity both within and between stands, as well as low genetic differentiation among them, indicating that they share the same gene pool and stands could result from a common set of genotypes, with a few exceptions represented by deviant or admixed genotypes. These observations, together with those relating to the intense gene flow both within and between stands, suggest that they may be considered as belonging to a single metapopulation. The three stands of *S. alba*, in fact, although spatially separated and showing not uniform stand structures, belong to populations connected to each other.

These findings extend those of Smulders et al. (2008) on black poplar and of Mosner et al. (2012) on osier (*Salix viminalis* L.), confirming that also in *S. alba*, gene flow and dispersal can

take place across large distances, allowing the population to survive from a genetic point of view in altered floodplain conditions. In addition, the genetic diversity was not influenced by stand structural diversity, and we did not find any sign of asexual reproduction, which is another indication of seedling recruitment at the metapopulation level and the absence of frequent, high magnitude flooding (Barsoum 2002). This finding agrees with results of Pogorzelec et al. (2014) in relict species of downy willow (*S. lapponum* L.), Sochor et al. (2013) in natural populations of European violet willow (*S. daphnoides* Vill.) and Kikuchi et al. (2011) in Japanese populations of *S. hukaona* Kimura. The absence of a clear relationship between stand structure and genetic diversity agrees with studies conducted in other forest types, such as beech (Sjölund and Jump 2015) or chestnut woodlands (Aravanopoulos, et al. 2001).

The magnitude of genetic variability within each stand examined, together with their high sexual reproductive capacity and the extent of gene flow, suggest the *S. alba* populations are viable and capable of adaptive responses to possible changes in the environment, at least in the short term. The different management conditions should exert some selection pressure, which is potentially capable of determining differentiation of the three populations, but it is limited by an efficient gene flow and a likely common origin of the founder genotypes of the population as a whole.

In synthesis, our results indicate that in the very impacted Po Plain, gene flow among *S. alba* stands counteracts the loss of their habitats, which is in agreement with the observations by Sochor et al. (2013) of populations of European *S. alba* in the Czech Republic.

The sampled trees had a maximum age of 22 years. In the period since tree establishment, the active channel along the sub-reaches of the sampling sites, and those immediately upstream, underwent alternating periods of widening and narrowing, which resulted in a net average width reduction of 40 m. Several over-bankfull floods occurred during this period, but none capable of restructuring riparian vegetation since 1966. The longitudinal control works in the reach of the river where the sampling sites lie are the reason for the current aggradation or equilibrium tendency which nonetheless followed a long phase of incision, while the upstream reaches are dominated by erosion and a very reduced sediment supply from upstream (Moretto, et al. 2014).

The observed morphological pattern and dynamics suggest that seed redistribution by water determines the spatial patterns of seedling recruitment (Nilsson, et al. 2010). At the same time, we expect that seedling emergence was not limited, given the reduced peak flows (Barsoum 2002) and the availability of sites safe from flooding. This supports the hypothesis that the genetic diversity of the studied *S. alba* communities might be dependent on the balance of erosive and depositional processes (Richards, et al. 2002).

While this condition may have positive conservation impacts, it should be noted that the artificially prolonged geomorphic persistence has also changed the natural vegetation transversal succession process (Picco, et al. 2015) and leads to an over aging of the stands, without a proper regeneration process. Artificial regeneration through coppicing is a practice generally applied along channels. However, it is not integrated with an understanding of fluvial dynamics and not always justified by hydraulic safety or biomass needs. For example, a relatively large amount of logs that could serve as another source of vegetative recruitment was also observed at this site (Ravazzolo, et al. 2015). By contrast, the reconnection of floodplain water bodies with the main channel would prevent the aggradation processes that are now observed in the study sub-reaches, would provide habitats for wildlife (Muhar, et al. 2008) and facilitate migration of *S. alba* (Smulders, et al. 2009).

Conclusions

The reported case study demonstrates the significance of ecological networks in terms of conservation of intraspecific genetic variability: the ecological corridor represented by the Brenta River plays an important role in overcoming the limitations imposed by habitat fragmentation, allowing *S. alba* populations to overcome isolation and the consequent loss of genetic diversity. This is also true for the effects of forest management, as related to stand structure, which did not influence genetic diversity. However, water discharge regimes, sedimentation and erosion process in regulated rivers are not at equilibrium, which might lead to changes in dispersal processes, sexual reproduction vs. asexual propagation, and hence genetic diversity. The factors influencing genetic diversity should therefore also be considered from a long-term perspective. In this paper, we examine a case study whose basic features are limited in time and in space. To expand upon this, further research should deal with the relationships between genetic diversity, its spatial structure, and variable rates of channel change, floodplain turnover and habitat diversity (Richards, et al. 2002) across a sample of sub-reaches and rivers subject to a gradient of human pressure, physical, and biological constraints. This is particularly needed for the study of genetic diversity in *S. alba*, which has received very little previous attention.

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work and stand structural analysis, G.B. and M.L. performed the molecular and genetic analysis, T.S. wrote the first draft of the paper and all the authors contributed to the final version. M.L. coordinated the working group within a research project financed by the University of Padova.

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