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New resources for genetic studies in *Populus nigra*: genome wide SNP discovery and development of a 12k Infinium array

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27 genetics.

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29 **Running tittle**: *Populus nigra*'s SNP: Discovery and validation

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Abstract

Whole genome resequencing of 51 *Populus nigra* (L.) individuals from across Western Europe was performed on Illumina platforms. A total number of 1,878,727 SNPs distributed along a *P. nigra* reference sequence were identified. The SNP calling accuracy was validated by comparison with Sanger sequencing data. SNPs were selected within 14 previously identified QTL regions, 2916 expressional candidate genes related to rust resistance, wood properties, water-use efficiency and bud phenology, and 1732 genes randomly spread across the genome. Over 10,000 SNPs were filtered for the construction of a 12k Infinium BeadChip array dedicated to association mapping. The SNPs genotyping assay was performed with 888 *P. nigra* individuals. The genotyping success rate was 91%. Our high success rate was due to the discovery panel design and the stringent parameters applied for SNP calling and selection. In the same set of *P. nigra* genotypes, linkage disequilibrium throughout the genome decayed on average within 5 to 7 kb to half of its maximum value. As application test, ADMIXTURE analysis was performed with a selection of 600 SNPs spread out on the genome and 706 individuals collected along 12 river basins. The admixture pattern was consistent with genetic diversity revealed by neutral markers and geographical distribution of the populations.

These newly developed SNP resources and genotyping array provide a valuable tool for population genetic studies and identification of QTLs through natural-population based genetic association in *P. nigra*.

Introduction

Black poplar **(***Populus nigra* L., Salicaceae) is an Eurasian native species distributed within riparian corridors in lowland, piedmont and mountainous zones from Morocco and Ireland at the western limit of its natural range to Russia and China in the East (Dickmann and Kuzovkina, 2013). As a pioneer species, *P. nigra* plays an important role in the establishment of riparian ecosystems (Imbert and Lefèvre, 2003), where it can be found as isolated trees and in pure or mixed stands. Considered as threatened throughout its natural range by anthropogenic disturbances of the river bank and gene introgression from cultivars (*P. deltoides* x *P. nigra*) and from the worldwide spread out fastigiated form *P. nigra* var *italica*, (Cagelli and Lefèvre 1997; Vanden Broeck *et al.*, 2005), black poplar deserves special attention in terms of conservation at national and European levels (Lefèvre *et al.*, 2001). Microsatellite genetic variation analyses showed high genetic diversity within populations and weak but significant genetic differentiation across river basins suggesting high levels of gene flow (Smulders *et al.*, 2008; DeWoody *et al.*, 2015).

Ease of vegetative propagation, good coppice ability, resistance and tolerance to several bio-aggressors (Benetka *et al.*, 2012), a long growing season (Rohde *et al.*, 2011) and high plasticity in response to environmental changes (Chamaillard *et al.*, 2011) are important adaptive characteristics that have promoted black poplar as a parental pool in interspecific breeding programs world-wide (Stanton *et al.*, 2013). The first common garden experiments performed with natural populations of black poplar have revealed locally adapted populations for bud set phenology (Rohde *et al.*, 2011), and leaf traits (DeWoody *et al.*, 2015, Guet *et al.*, 2015). Local adaptation was also reported in other poplar species (Ingvarsson *et al.*, 2006, Keller *et al.*, 2010, Viger *et al.*, 2013) and also in other temperate widespread forest trees (Savolainen *et al.*, 2007). Past adaptation processes have most likely

generated wide reservoirs of standing genetic variation for many other adaptive traits in black poplar.

One main challenge is to identify loci/genes that underlie this phenotypic variation. Such information can then be used to access and manage genetic diversity and develop adapted marker-assisted selection schemes (Harfouche *et al.*, 2012). Association genetics is a promising method of achieving this goal in woody species with a long life cycle, late expression of important traits and considerable population genetic diversity (Neale and Savolainen, 2004; Neale and Kremer, 2011). The development of High Throughput (HT) genotyping tools is undoubtedly a prerequisite for such an approach. Single nucleotide polymorphisms (SNPs) are a suitable and very attractive genetic marker for this purpose. It is now well established that HT DNA sequencing technologies are powerful tools enabling the rapid discovery of large numbers of SNPs. Different options have been deployed in plants including tree species, including RNA sequencing *i.e.* HT-sequencing at the transcriptome level (Parchman *et al.*, 2010; Geraldes *et al.*, 2011; Howe *et al.*, 2013; Mantello *et al.*, 2014), and targeted sequencing, *i.e.* HT-sequencing of particular (captured) genomic regions such as the gene-enriched portion (Zhou and Holiday, 2012) and restricted genomic DNA (Grattapaglia *et al.*, 2011, Schilling *et al.*, 2014). For species with a relatively small genome, like *Populus* sp. (500Mb), whole genome HT-sequencing can be sensibly achieved (Slavov *et al.*, 2012; Evans *et al.*, 2014). Recently, studies have demonstrated the usefulness of both HT-sequencing and SNP arrays to assess candidate gene association genetics in natural populations of *P. trichocarpa* (Porth *et al.*, 2014; Mc Krown *et al.*, 2014). The success of association studies mainly depends on the availability of SNPs, the extent of linkage disequilibrium (LD), the extent of phenotypic variation of interest and the genetic structure in the association population. In *P. nigra*, these determinants are poorly documented. Indeed, studies were limited to relatively few SNPs identified within 2 to 39 genes, and LD was

reported to decay within 300 to 1000 bp (Chu *et al.*, 2009; Marroni *et al.*, 2012; Guerra *et al.*, 2013; Chu *et al.*, 2014).

In order to perform association studies in *P. nigra*, our aims were to identify SNP at whole-genome scale and to develop a SNP bead chip array. Due to the expected rapid decay of LD in most undomesticated tree species, we opted for a candidate-genomic-region approach that focused for leaf rust resistance, bud phenology, water-use efficiency and wood chemistry on both QTL intervals identified in *P.nigra* mapping pedigrees (Rohde *et al.*, 2011, Fabbrini *et al.*, 2012, Elmalki, 2013, Guet *et al.*, 2015) and candidate genes underlying QTLs in other poplar species (Novaes *et al.*, 2009; Rajan *et al.*, 2010; Rae *et al.*, 2008; Monclus *et al.*, 2012; Viger *et al*., 2013). SNP outside the candidates were also selected to provide genomic control tools to characterize neutral diversity and detect population structure. To reach this objective, we first created a *P. nigra* reference sequence using the *P. trichocarpa* genome sequence as a template (Tuskan *et al*. 2006) and identified a large set of SNPs at the whole genome scale by HT-resequencing of 51 *P. nigra* genomes. Second, we defined a SNP selection strategy in order to design a useful SNP array for candidate-based association studies in natural populations. Third, the usefulness of the array was evaluated by genotyping 888 *P. nigra* individuals. Data analysis focused on LD decay with distance and on the genetic structure of a large *P. nigra* association population sampled in 12 river basins over Western Europe.

Material and methods

SNP discovery and selection

Discovery panel and whole genome re-sequencing

A SNP discovery panel of 51 individuals selected as representative of the genetic diversity of an association population covering the range of the black poplar in Western Europe was used for HT-genome sequencing (Table S1).

Nuclear DNA was isolated from young leaves as described by Zhang *et al*. (1995) and Chalhoub *et al*. (2004). Whole-genome re-sequencing was performed at the Institute of Applied Genomics (IGA, Udine, Italy) and the INRA-EPGV/CEA-IG/CNG (Institut National de la recherche Agronomique-Etude du Polymorphisme des Génomes Végétaux/Commissariat à l'Energie Atomique-Institut de Génomique/Centre National de Génotypage, Evry, France) facilities using either a GAII analyzer or Hiseq 2000 Illumina platforms (Inc. San Diego, CA, USA). Paired-end sequencing libraries were prepared following the "Illumina Paired-End Sample Preparation" protocol, using an insert size spanning from 300 to 600 bp. Paired-end runs were performed for 75, 100, 110 or 114 cycles following Illumina instructions (Table 1). Illumina sequencer analyzer provided a quality score (Qscore) for each base, an average Qscore value was assigned to each read. Reads with Qscore values >30 were considered as good sequences.

Four individuals covering the wide Western latitudinal range of *P. nigra*, Poli (South-Italy), BEN3 (Spain), Blanc de Garonne (BDG) (South-West-France) and 71077-308 (East-France) were sequenced at coverage >25x (Tables 1, S1). Our objective was twofold; to maximize the genetic variation among individuals and to identify reliable SNPs. Forty-seven individuals covering the European latitudinal range were selected and sequenced at lower coverage (Tables 1, S1) in order to maximize the discovery of informative SNPs.

P. nigra reference sequence

To avoid confusion between interspecific polymorphisms between *P. trichocarpa* and *P. nigra* species and prompt the detection of intraspecific polymorphisms within *P. nigra*

(Isabel *et al*., 2013), we created a *P. nigra* reference sequence using short reads of the genotype 71077-308 (27x). This genotype was chosen for its read Qscore > 32. Paired-end reads were aligned onto the *P. trichocarpa* genome V2.0 (Tuskan *et al*., 2006). Indeed, pilot analyses on Sanger-sequenced BAC inserts showed the feasibility of using the *P. trichocarpa* genome sequence as a template for *P. nigra* (Zaina, unpublished data). The mapping of raw short reads was performed with the CLC Genomics Workbench v.4 (CLC Bio, Aarhus, Denmark). Mapping parameters were given in figure 1. Only paired-end reads that aligned to a unique location of the genome were considered. Duplications and repetitions were identified with RepeatScout using default parameters (Price *et al*., 2005). Due to computing constraints, only the first 40 scaffolds were extracted as part of the *P. nigra* reference sequence to be used in the SNP calling.

Strategy of SNP detection for designing the array

A multi-step strategy was designed to recover variants for the Illumina Infinium iSelect HD Custom BeadChip technology. The paired-end raw sequences of the 4 genotypes >25x were mapped separately onto the *P. nigra* reference sequence using the same procedure adopted above to create the *P. nigra* reference itself, with the exception of similarity set to 0.95. Reads for the 47 remaining accessions were aligned similarly but as a joint set. SNP detection was then performed on each of the 5 alignments, with the parameters detailed in figure 1. To evaluate the accuracy of the SNPs calling a comparison with the SNPs detected using ABI3730 Sanger sequencing was performed (Table S2, Methods S1 and S2).

Deletion-Insertion Polymorphisms (DIPs) were also detected to optimize SNP selection for the array design. DIPs were detected using the CLC software v.4 (Fig. 1).

Given the objective of the SNP array, candidate genomic regions (14) were considered on the basis of QTL for rust resistance, bud phenology in *P. nigra* and water-use

efficiency, wood properties in other *Populus* species (Fig. 3, Table S3). Candidate genes (2916) for the same traits were also considered on the basis of transcriptome studies and the literature (Fig. 3, Table S3). SNPs belonging to those candidate regions or genes were considered for the subsequent selection. Additional SNPs were retained within gene models (1732) spread across the poplar genome.

A pipeline written in Bash and Perl was set up to extract useful SNPs with 60-bp flanking sequences. The pipeline rescued only loci whose flanking sequences did not contain any SNP and/or DIP. If this was not possible, the pipeline was set to select SNPs with no SNPs and/or DIPs within ± 10 bp of the target SNP. The pipeline also discarded the SNPs within duplicated or repetitive regions.

A collection of SNPs detected by Sanger re-sequencing of full-length genes and gene fragments obtained previously by University of Udine and INRA teams in the framework of Popyomics and National projects were also considered (Method S1, Table S2).

The whole set of extracted SNPs was subjected to the Assay Design Tool by Illumina (https://iCom.illumina.com) in order to score and validate the SNPs in terms of the bead-chip performance. Final selection was performed to reach the desired 11,999 beads. This final selection was based on the SNP location in the genome (Table S3): i. 80 SNPs/Mb were retrieved from the QTL area showing a considerable effect (the phenotypic variance explained by the QTL was set at > 10%) ii. 20 SNPs/Mb were retrieved from the QTL area showing a low or moderate effect, iii. 5 SNPs/Mb were retrieved from non-QTL regions. SNPs requiring a single bead type (Infinium II) were also preferred to maximize the number of loci on the chip. In a few regions, the final target could not be reached with the current criteria, which were thus gradually relaxed to meet the targets. Moreover, for functional candidate genes for rust resistance and bud phenology, more than one SNP were selected per gene with the same criteria.

Genotyping assay

Plant material

A set of 888 individuals comprising 838 native *P. nigra* individuals (originating from 12 river basins and collected in the western part of Europe (Tables 2 and S1), of which most belonged to the Europop (Cotterell *et al.*, 2004) and the French National collections, and 50 full sib progenies were used in this study (Table S1). Among the 838 native *P. nigra*, 814 were part of the European association population established in the framework of the EU projects Popyomics, Evoltree, NovelTree and EnergyPoplar, and had already been genotyped with SSR markers (Storme *et al.*, 2004, DeWoody *et al.*, 2015; Jorge unpublished data). Within the total set, 11 individuals were used as parents in 9 different crosses and 2 to 6 progenies per cross were genotyped to facilitate and validate SNP clustering.

SNP genotyping

One sample (BDG) was repeated 14 times and used for technical control. DNA samples from 24 individuals were included twice to assess the repeatability of allele calls. SNP genotyping was conducted on the Illumina Platform at CEA-IG/CNG by INRA-EPGV according to the standard protocol of Illumina. Genotypes were recovered with Genotyping module v 1.9.4 (Genome Studio software v 2011.1, Illumina Inc.). Clusters were generated using a GenCall score cut-off of 0.15 as recommended by Illumina. The GenCall score, estimated for each data point (SNP × individual sample), implemented by the Genome Studio software reflected the position of the data point within the genotype cluster. Genotypes with lower GenCall scores are located further from the center of the genotype cluster and had lower reliability. Only those individuals with > 95% call rates were selected (*i.e.* the proportion of individual samples successfully genotyped in a locus). SNP clusters

were automatically generated and then the quality of the 3 expected clusters of each SNP was inspected visually. Subsequent adjustment of the cluster calling was performed if needed.

Linkage disequilibrium and population structure

To estimate LD decay and analyze population structure on neutral genetic diversity, SNPs and individuals were filtered according to several criteria. First, SNPs and individuals with missing data above 10% were discarded. Then, segregation and linkage conformity was checked within a 3x3 factorial mating design (Method S3). Finally, SNPs showing a significant departure from the Hardy-Weinberg equilibrium within more than 6 populations were discarded. LD between all pairs of SNPs was estimated as the square of the allelic correlation in R (R Core Team, 2014).

Population structure was investigated using the software ADMIXTURE (Alexander *et al.*, 2009), with K ancestral population ranging from 1 to 15. Since we used a candidate-based approach, the selected SNPs were not evenly spread throughout the genome. To account for such variation in SNP density across the genome, we sampled several subsets of SNPs. These subsets were sampled by chromosome, taking into account physical chromosome length and the desired final number of SNPs using different approaches:

- 2000-LD: 2000 SNPs minimizing the LD between SNPs by applying the Kennard and Stone algorithm (Kennard and Stone, 1969) to the LD matrix by chromosome,
- 600-LD: same as above but with a total target of 600 SNPs,
- 600-dist: 600 SNPs well scattered by applying the Kennard and Stone algorithm to the physical distance matrix by chromosome,
- 600-random: 600 SNPs randomly sampled by chromosome.

These 4 subsets were compared together and to the total set of high-quality SNPs to evaluate population structure by cross-validation in ADMIXTURE. The set that minimized the cross-validation error was selected to analyze population structure. The optimal number of groups was also determined by cross-validation for this set. The optimal set of SNPs according to the cross-validation in ADMIXTURE was used to carry out Principal Component Analysis (PCA) in R (R Core Team, 2014) as a complementary analysis of population structure. We used the optimal set of SNPs to estimate a measure of LD corrected for the bias attributed to population structure and cryptic relatedness as proposed by Mangin *et al.*, (2012). Briefly, we used the optimal set of SNPs to compute a genomic relationship matrix between individuals (Van Raden, 2008), and used this matrix to estimate a corrected measure of LD defined as the squared partial allelic correlation between SNPs (Lin *et al.*, 2012). The relationship between LD and physical distance was assessed following the model of Hill and Weir (1988) in order to determine the distance where LD decays to half its maximum value.

Results

Illumina next generation DNA sequencing technology was used to re-sequence 4 *P. nigra* genotypes (71077-308, BDG, BEN3 and Poli) at coverage >25x and 47 other genotypes at lower coverage. The read data and relative raw coverage obtained for each genotype are reported in Table 1.

SNP detection

P. nigra reference sequence

The sequence data obtained from the clone 71077-308 were selected due to their good quality to produce a reference sequence for *P. nigra* species, exploiting a mapping approach *versus* the *P. trichocarpa* genome sequence v2.0. We previously proved the

feasibility of this approach by mapping the short reads of another *P. nigra* genotype (the Spanish clone BEN3) *versus* two *P. nigra* BAC-clone sequences and *versus* the *P. trichocarpa* sequence portions corresponding to the BAC inserts. In the intraspecific alignment, the BAC sequences were covered for 98% of their length, as expected, and in the interspecific alignment, 75% of the corresponding *P. trichocarpa* regions were covered (Zaina, unpublished data). In the present work, the 71077-308's short reads covered 79% of the *P. trichocarpa* genome sequence V2.0. After mapping, we considered only the *consensus* specific to the first forty scaffolds, which resulted in a sequence 388,572,533 bp long (gaps included), representing the sequence used hereafter as the *P. nigra* reference sequence.

SNP calling

We used the *P. nigra* reference sequence obtained to map the paired-end reads of 71077-308, BDG, BEN3 and Poli (>25x). Approximately 60% input reads of 71077-308, BDG and Poli were mapped to a unique position in the reference sequence. The exception of BEN3 with a lower amount of mapped reads (42%) was explained by the lower quality score (reads average Qscore < 26) of its reads compared to the others (Table S4). In addition to the four alignments produced above, the reads derived from the re-sequencing of the 47 individuals (<25x) were mapped as a whole against the *P. nigra* reference sequence to obtain a fifth alignment.

These alignments were used for SNP discovery at the whole genome scale following the procedure summarized in figure 1. The total number of SNPs detected in each alignment along the *P. nigra* reference sequence is shown in Table 3, and referred to as input SNPs. The figure 2 shows the distribution of the input SNPs detected through the 5 alignments across the main 19 chromosomes of the reference *P. nigra*. Out of 388,572,533 bp of the *P.*

nigra reference sequence 110,098,472 bp were covered by the 4 genotypes and provided a total of 1,878,727 SNPs. The SNP frequency resulted to be 1 polymorphism every 58.6 bp.

To estimate SNP calling accuracy, we compared the SNPs identified within the 18 candidate genes for light signaling pathway (Table S2) resulting from the re-sequencing, using both Sanger and Illumina methods. A total of 96,164 sites were analyzed, including 1186 polymorphic sites from the Sanger SNP detection. The Illumina SNP detection resulted in 92.9% Sensitivity, 99.8% Specificity and 99.7% Accuracy, and provided 141 false positives (*i.e.* SNPs identified in Illumina data but not in Sanger data), corresponding to a 10.6% False Discovery rate (Method S2).

Development of the 12k Infinium BeadChip array

A total of 296,964 SNPs were retrieved from the 47 genotypes in the candidate regions while the other 4 genotypes provided 344,709 (Poli), 112,262 (BEN3), 174,035 (BDG) and 155,846 (71077-308) SNPs within the same regions (Table 3). The differences in the number of loci between the 5 alignments were consistent with the depth-coverage and read quality of the different genotypes. A map was created by using the IUPAC codes to group all the SNPs belonging to the different genotypes within the candidate loci. The map was integrated with the DIPs identified in the same five alignments (data not shown), to improve the further selection of SNPs for an efficient bead-chip array design (*i.e.* no polymorphisms within the SNP flanking sequences). Eventually, 189,616 SNPs, which correspond to 1 SNP every 1159 bp in the candidate regions and genes, were retained. This last set of 189,616 SNPs was subjected to the Illumina Assay Design Tool (ADT) to test for suitability with the bead-chip design. 133,821 SNPs passed the test, showing an ADT score $\geq$ 0.6 (*i.e.* the score threshold recommended by Illumina) (Table S5). A set of 669 SNP distributed onto the non-candidate regions were selected with the same criteria (Table S5).

In addition to the SNPs identified by the Illumina HTre-sequencing, 4691 SNPs from the Sanger re-sequencing of candidate genes in *P. nigra* were considered (Fig. 1, Table S2). After filtering selection detailed in figure 1, 2690 Sanger SNPs were available. Thus, the very last pool of SNPs consisted of 137,180 loci. To get the desired number of 11,999 beads required for the Illumina bead-chip array, the SNPs were reduced to 10,331 loci according to the stringent criteria detailed in Material and Methods (Tables S6, S7). Among them, 6311were located in QTL intervals.

Infinium BeadChip array performance

Of the 10,331 SNPs, 9127 included in the bead pool (88%) remained in the array after Illumina technical dropout. Eight samples were excluded for technical errors and 19 were excluded due to low call rate. The selection finally revealed 861 genotypes with a call rate $\geq$ 0.95. Each cluster was then inspected manually. SNPs were classified into different classes: polymorphic, monomorphic and failed (Table S8). Our validation showed 8322 well clustered SNPs leading to a chip success rate estimated at 91%; 8259 of them were polymorphic (90%). The reproducibility rate was 100% when we compared the 12 inter-plate controls. The same rate was obtained from the comparison of i. biological replicates of BDG and 1 inter-plate control, ii. duplicates of 24 genotypes. Heritability-based SNP validation was estimated to assess SNP assay quality. This was defined as the number of offspring genotypes that agreed with the expected inheritance over the total number of possible genotype calls. In 9 families, there were 608 Mendelian transmission inconsistencies out of the 411,877 allelic transmissions assayed, *i.e.* a genotyping miscall rate of 0.15% (ranging from 0.08% to 0.21%). We observed that 1.65% of SNPs had segregating errors.

A set of 259 SNPs from Sanger data was used to validate the efficiency of SNP genotyping in 10 individuals for which both Infinium and Sanger sequence data were available. We observed a very high rate of concordance (96%-99%) (Table S9). For 71077-308, BDG, BEN3 and Poli, we then compared genotype calls from NGS re-sequencing data to genotype calls from the chip. The concordance observed varied between 80% and 100% (Table S10). Of the 8259 SNPs, 7186 were located within 4903 genes; and 1132 genes harbored more than 2 SNPs (Table S11).

Application of the array

Identification of clonal duplication

Polymorphic sites (8259) were used to compute pair-wise similarity between all pairs of individuals. This analysis identified 35 duplets, 9 triplets, 4 quadruplets, 2 septuplets, and one duodeciduplet (Table S12). With the exception of 5 groups (3 duplets, one triplet and one quadruplet), all the individuals belonging to the same group came from the same population. Genotyping work performed with SSR markers was used to trace the origin of these results (Method S3, Table S12). Redundant individuals were removed from the individual data set for further analyses.

Population structure

We applied additional filters on SNPs and individuals for genetic analyses. Data Filtering on missing data (> 10%) resulted in discarding 13 SNPs and 26 individuals. Additional SNPs were discarded: 216 SNPs due to segregation problems (missing or not-expected genotyping class, segregation distortion and non-expected linkage, Fig S1) in factorial mating design (data not shown) and 98 SNPs due to significant deviations from Hardy-Weinberg equilibrium within at least 6 populations In the resulting set of individuals,

36 SNPs were monomorphic and were thus discarded from further genetic analyses. The final data matrix included 7896 high-quality polymorphic SNPs genotyped in 706 individuals. Due to our biased sampling of SNPs within candidate regions (Fig. 3, Table S13), we further selected several subsets of 600 and 2000 SNPs as being potentially better distributed throughout the genome. The optimal number of ancestral clusters K=7, corresponding to the lowest cross-validation error, was obtained with the set of 600 SNPs selected (Fig. 4a). The corresponding admixture results are shown in Figures 4b. Basento and Paglia populations from South and middle Italy emerged as distinct groups. For the other populations a clear admixture pattern was revealed, although individuals from the same populations still tended to cluster together. A principal component analysis on the same optimal set of 600 SNPs confirmed the results from ADMIXTURE. Indeed a relatively clear clustering of individuals according to their geographical origin was observed (Fig. S1).

linkage disequilibrium

As expected by the MAF (Minimum Allele Frequency) threshold (>0.2) applied to select SNPs in our discovery panel, the MAF of 92% of the high-quality genotyped SNPs is higher than 0.2 in the 7 admixture clusters. The frequency distribution of SNPs was more or less even across different MAF classes and across ADMIXTURE clusters with the exception of Italian clusters (Fig. S3). We calculated both LD and LD corrected for population structure confounding between all pairs of SNPs. The relationship between LD and physical distances was plotted and modeled (Fig. 5). As expected, the corrected LD decayed slightly faster than the uncorrected LD with physical distance: the r^2 and corrected r^2 dropped to half their maximum value within 5 and 7 kb, respectively.

Discussion

We reported the development of a high-quality SNP array in *P. nigra*. To our knowledge, this is the first significant SNP resource that has been reported for black poplar. As poplar has a relatively small genome (500 Mb), we decided to re-sequence the whole genome instead of using a genome reduction procedure developed by Stölsting *et al.*, 2013. In poplar, SNPs are mostly species specific (Isabel *et al.*, 2013), thus the available genome of *P. trichocarpa* could not be used directly as a reference to detect SNPs. Nevertheless, we were able to use it as a template to map the short reads of *P. nigra* to obtain a reference sequence of the black poplar genome. Indeed, the alignment of paired-end reads allowed us to obtain 389 $x10^6$ bp of *P. nigra* specific sequences (approximately 79% of the *P. trichocarpa* genome). The excluded regions generally corresponded to variations between the genomes of *P. trichocarpa* and *P. nigra*, which we expected to be mostly repetitive regions as observed by Ma *et al.*, (2013), between the genomes of *P. euphratica* and *P. trichocarpa*, or large insertion/deletions due to transposable elements as observed by Zaina and Morgante, (unpublished results) among BAC insert sequences belonging to *P. nigra*, *P. deltoides* and *P. trichocarpa*.

The comparison between the *P. nigra* reference sequence and 71077-308, BDG, BEN3 and Poli genotypes provided the first *P. nigra* whole genome SNP collection. The Italian genotype, Poli, contained more SNPs than the French and Spanish genotypes. This result was consistent with their genetic distances to the 71077-308 used to build the *P. nigra* genome reference (Jorge and Villar, unpublished results). Our procedure used to identify SNPs from resequencing of 4 genotypes >25x and 47 genotypes <25x proved to be reliable, reducing false discovery rate.

During our SNP selection process, most of the SNPs were lost during the final step, *i.e.* the selection of SNPs with no polymorphisms in their 60-bp flanking sequences. This can be explained by the high level of SNP frequency and heterozygosity in *P. nigra*. Hence

a huge collection of SNPs originating from complete genome coverage and a large SNP discovery panel was required to reach our final target of 12k beads. According to Groenen *et al.*, (2011), the number of SNPs should be at least 10 times higher than the number targeted for the final chip. The good genotyping results demonstrated that the strategy developed to detect and select SNPs was very effective, despite the lack of reference sequence for *P. nigra*. The high rate of concordant data between genotyping and SNP calling from Sanger sequencing and NGS genome sequencing, revealed the robustness of our selection criteria. Our genotyping success rate (91%) exceeded those recorded for other plant species with the same Infinium technology and in the same genotyping throughput range (6k-10k) (Chagné *et al.*, 2012; Verde *et al.*, 2012; Bachlava *et al*., 2012; Peace *et al*., 2012; Sim *et al.*, 2012; Delourme *et al.*, 2013; Li *et al.*, 2014; Dalton-Morgan, 2014; Lepoittevin *et al*., 2015; Livingstone *et al*., 2015). The success of the SNP array was due to the composition of the SNP discovery panel reflecting the genetic diversity of the populations under study. The choice of a high MAF threshold contributed to the high reliability of our genotyping work (Chen *et al*., 2014); However, the resulting genotypic data are biased toward intermediate frequencies and we may therefore have missed rare alleles potentially affecting some phenotypes of interest, as has previously been reported for wood composition in *P. nigra* (Vanholme *et al*., 2013).

As a first application of the array, in the present work we performed the largest study undertaken to characterize the genetic structure of the Western range of *P. nigra*. We found unexpected replicated genotypes, most replications were found within German populations and could be explained by duplication in nature due to vegetative propagation. The results are comparable to the earlier published data (Storme *et al.*, 2004; Smulders *et al.*, 2008; Chenault *et al.*, 2011), suggesting that in nature *P. nigra* is highly clonal along long tracts of riparian river basins that may stretch for several kilometers. As for other temperate riparian

447 species (*Populus* spp., *Salix* ssp., *Ulmus* ssp; Stuefer *et al.*, 2002; Santos del Blanco *et al.*,
448 2013; Lin *et al.*, 2009; Fuentes-Utrilla *et al.*, 2014), the rate of clonality observed could
449 enable persistence of local populations under unfavorable conditions (Storme *et al.*, 2004;
450 Smulders *et al.*, 2008; Chenault *et al.*, 2011). ADMIXTURE analysis agreed with the PCA
451 results indicating high level of admixture and low level of genetic differentiation between
452 populations. This finding was supported by the low Jost's D values. Important gene flow
453 usually observed in riparian populations such as poplars could explain our results (Imbert
454 and Lefevre, 2003). Individuals belonging to the same river basin clustered together and
455 cluster proximity reflected the close geographical proximity of the river basins within the
456 same drainage system. This general structure is in accordance with previous *P. nigra*
457 population genetic studies, although the sets of populations used only partially overlapped
458 and marker systems were different (Storme *et al.*, 2004; Smulders *et al.*, 2008; DeWoody *et*
459 *al.*, 2015). Besides a high level of admixture, a clear pattern of genetic differentiation
460 remains between populations belonging to different drainage systems. This structure could
461 also be explained by major geographical barriers limiting gene flow. The Alps are a strong
462 factor which separates Italian populations from the rest of Northern Europe populations. In
463 France, this structure is governed by the major watersheds, namely the Rhine, Rhône and
464 Loire/Allier, although some admixture exists between them. The most original data concerns
465 the Dranse population located along a mountain stream of the Alps, which appears admixed
466 mainly from Rhine F and Ticino populations. The Italian populations are also structured
467 along a latitudinal gradient and, by contrast with Northern European and French populations,
468 present a low level of admixture. The Apennines, the contrasted environments of such
469 Mediterranean gradient (max and min temperature, duration of daylight, global radiation)
470 and longer geographical distances act as strong barriers to gene flow between Northern and
471 Southern Italian populations.

In the 7 ancestral clusters identified using ADMIXTURE, the purple one is clearly admixed in all predefined populations, and do not follow a particular geographical pattern although the admixture appears more important in French populations (Fig. 4). Admixture could be due to introgression from cultivated poplars (Vanden Broek *et al.*, 2012) i. *P. nigra* and cultivated stands occupy the same habitat; ii. cultivated clones potentially can hybridize with *P. nigra* as most of them are *P. x canadensis* interspecific hybrids involving different *P. nigra* European genetic pools and iii. these clones are very few, highly related and widely deployed in whole Europe. This last reason probably could explain the strong differentiation of the 7th ancestral cluster.

Due to the high level of admixture, the 12 populations could be considered together to increase significantly the detection power of association tests, thanks to a large association population size and appropriate association methods which explicitly take into account its specific structure. The extent of LD revealed in this study is probably overestimated due to the selection of SNPs showing a moderate to high MAF, but it was in the same range as that found in *P. trichocarpa* (Slavov *et al.*, 2012). This information is important to develop whole genome association in *P. nigra*. The number of SNPs required to tag the entire *Populus* genome was estimated between 67K and 134K (Slavov *et al.*, 2012; Geraldes *et al.*, 2013). Based on the size of the genome used for these calculations (403 Mb), this means that we need densities between 166 SNPs/Mb and 332 SNPs/Mb. The presence and distribution of polymorphisms seems to be not a limiting factor in the black poplar genome, given the high values of SNP frequency (1 SNP/ 58.6 b). The SNP frequency from this study resulted to be higher than those found in previous studies (Marroni *et al.*, 2012; Chu *et al.*, 2014) since the analysis was targeted to the whole genome, including intergenic regions and pseudogenes.

Today either GBS or HT-genotyping array technologies can be proposed to perform Genome-wide association studies (GWAS) in poplar. GBS is a cost-effective method but the

high level of missing data and the lack of reproducibility can result on a huge loss of data (Elshire *et al*., 2011). In case of GWAS performing with large populations, the HT-genotyping array techniques could be more efficient if an international consortium designs an optimal SNP array for all the poplar species.

In conclusion, we have described the first genome-wide re-sequencing study in an extensive collection of the European native black poplar, *P. nigra* (L.), providing significant new genomic resources for this tree species of conservation and breeding significance throughout Europe and Eurasia. Our analysis has quantified LD decay and population structure providing essential keys to further population genetics in *P. nigra*.

We now have the resources in place to refine location of already known QTLs in *P. nigra* through multi-pedigrees genetic mapping (Giraud *et al.*, 2014), or association studies based on these natural populations for which phenotypes are available (Rohde *et al.*, 2011, DeWoody *et al.*, 2015, Guet *et al.*, 2015). We demonstrated that the bead-chip could be used for characterization of genetic diversity present in native populations of *P. nigra* or exploited in interspecific breeding pools, enabling development of landscape-scale and genomic-based conservation strategies in the face of climate change.

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Data accessibility

Collections of SNPs within the candidate regions and genes and outside are given in supporting information. Primer of Sanger Sequencing project are listed in supporting information

The *P. nigra* reference and the raw sequencing data will be available at http://services.appliedgenomics.org/gbrowse/populus/ hosted by Applied Genomic Institute in Udine (Italy).

The genotyping data will be available at https://urgi.versailles.inra.fr/Tools/GnpIS and http://www.evoltree.eu/index.php/e-recources/portals.

Author contributions

PFR, GZ, VJ, SG, VS, VG, AB -Sanger re-sequencing and SNP identification

PFR, GZ, VJ, SG, VS, AB, MM -NGS re-sequencing and SNP identification

PFR, VJ, VS, GZ, MV, AP, GT -Design of the SNP array

MVil -Collecting of *P. nigra* samples

CB, GT -Design of the population sampling

PFR, MCL, FC, MM -Coordination of NGS re-sequencing work

PFR, MCL -Coordination of the genotyping work

CA, SS, EDP -Bioinformatics, data basing

PFR, PP, VG -Analysis of genotypic data

VJ, VS, CB -Population genetics analysis

PFR, GZ, VJ, VS, CB -Writing of the manuscript

MVil, GT, MRA -Revision of the manuscript

Table 1: Raw sequence data used for SNP detection. *Vert de Garonne and Cazebonne 25 were subsequently found identical genotypes after HT genotyping. (A) Adour.

Genotype	Origin	River basin	Read length (b)	Total bp produced	Raw coverage (X)
Poli	Italy	Sinni River	100	34,031,232,782	81.6
BEN3	Spain	Ebro	100	21,882,737,550	52.5
71077-308	France	Rhône	76, 114	11,614,046,643	27.8
Blanc_de_Garonne	France	Garonne	100	10,499,784,562	25.1
92538	France	Creuse (Loire)	100	8,874,612,395	21.3
72145-7	France	Gard (Rhône)	100	8,279,967,553	19.8
6-A06	France	Drôme (Rhône)	100	8,124,691,652	19.5
1-A10	France	Drôme (Rhône)	100	7,616,642,138	18.3
92525-25	France	Loire	100	7,379,085,905	17.7
92520-6	France	Loire	100	7,100,652,141	17
92510-3	France	Loire	100	6,599,547,430	15.8
Sarrazin	France	Garonne	100	6,545,172,797	15.7
Vert_de_Garonne*	France	Garonne	100	5,865,971,615	14
6-A23	France	Drôme (Rhône)	100	5,733,143,633	13.7
NVHOF2/19	Germany	Rhine-D (Rhine)	100	5,638,954,091	13.5
6-A31	France	Drôme (Rhône)	100	4,957,635,050	11.9
99582-1	France	Loire	100	4,749,535,204	11.4
Cazebonne_25*	France	Garonne	100	3,885,764,113	9.3
PG-22	Italy	Paglia (Tibre)	100	3,542,852,254	8.5
SN-21	Italy	Ticino (Pô)	100	3,183,780,277	7.6
Ginsheim3	Germany	Rhine-D (Rhine)	100	3,114,417,000	7.5
NL-1238	Netherlands	Rhine_Ijssel	100	3,095,875,836	7.4
98568-1	France	Rhine F (Rhine)	100	2,811,019,907	6.7
SN-11	Italy	Ticino (Pô)	100	2,791,982,335	6.7
NL-1217	Netherlands	Rhine_Ijssel	100	2,543,452,219	6.1
NVHOF3/17	Germany	Rhine D (Rhine)	100	2,475,035,580	5.9
FTNY19	Hungary	Tisa	100	2,419,647,905	5.8
Ginsheim1	Germany	Rhine D (Rhine)	100	2,351,224,600	5.6
C2	Spain	Ebro	100	2,160,560,966	5.2
SN-26	Italy	Ticino (Pô)	100	2,174,897,241	5.2
C1	Spain	Ebro	100	2,116,880,335	5
NL-1329	Netherlands	Rhine_Ijssel	100	2,067,806,626	5
NL-1682	Netherlands	Rhine_Waal/Maas	100	2,046,322,170	4.9
PG-05	Italy	Paglia (Tibre)	100	2,055,865,151	4.9
cart5	Spain	Ebro	100	1,936,051,399	4.6
NL-2051	Netherlands	Individual clone	100	1,826,967,332	4.4
73193-25	France	Gave_de_Pau (A)	100	1,647,799,444	4
N-11	Italy	Ticino (Pô)	100	1,676,606,505	4
PG-13	Italy	Paglia (Tibre)	100	1,665,449,401	4
N-38	Italy	Ticino (Pô)	100	1,540,547,636	3.7
C6	Spain	Ebro	100	1,460,806,904	3.5
58-861	Italy	Cenischia (Pô)	100	1,425,822,523	3.4
FTNY18	Hungary	Tisa	100	1,336,413,883	3.2

BDX-06	France	Gave_de_Pau (A)	100	1,199,931,013	2.9
RIN4	Spain	Ebro	100	1,224,325,600	2.9
SN-40	Italy	Ticino (Pô)	100	1,195,698,229	2.9
C12	Spain	Ebro	100	1,026,605,990	2.5
71072-501	France	Rhône	100	1,020,158,073	2.4
NL-1797	Netherlands	Rhine_Waal/Maas	100	910,082,000	2.2
NVHOF3/5	Germany	Rhine D (Rhine)	100	878,908,000	2.1
N-47	Italy	Ticino (Pô)	100	691,873,200	1.7

872

Table 2: Summary of the number of *P. nigra* genotypes per river basin in the European *P.nigra* association populations.

River Basins	Country	No. individuals genotyped
Dranse (Rhône)	France	40
Durance (Rhône)	France	13
Drôme (Rhône)	France	155
Loire	France	180
Rhine F	France	62
Allier	France	113
Basento	Italy	14
Paglia	Italy	22
Ticino	Italy	103
Rhine D	Germany	54
Netherlands NL	Netherlands	48
All stands-Ebro	Spain	9

878 Table 3: Numbers of SNPs identified for the development of the bead-chip array.

SNPs	**47 accessions**	**POLI**	**BEN3**	**BDG**	**71077-308**
Input	758,043	937,79	282,299	491,85	460,047
Whithin candidate loci	296,964	344,709	112,262	174,035	155,846
After DIP removal	279,813	314,457	105,212	157,061	143,312
Supported by 5 accessions			278,330		
Supported by at least one >25x genotype clone			189,616		

879

880 **Figure legends**
881
882 Figure 1: Workflow of SNP detection and selection.
883
884 Figure 2: Genomic distribution of SNPs detected for the development of the 12k bead-chip
885 array. Around the plot colored bars represent the 19 *Populus* chromosomes (unit used is 2
886 Mb). Within the plot the traces represent the SNP distribution (calculated in windows of 100
887 kb) of BDG (red) BEN3 (light-blue) Poli (light-green) 71077-308 (yellow) 47 genotypes
888 (violet). The grey ovals tag the putative centromeric regions. The grey arrows tag the putative
889 centromeric regions. The red arrows highlight homozygous regions for the 71077-308 clone,
890 they represent homozygous genomic regions. Such homozygous areas have already been
891 observed in previous studies based on genetic mapping in *P. nigra* (El-Malki, 2013). The
892 plot was computed using the Circos software (Krzywinski *et al*. 2009).
893
894 Figure 3: Chromosomal distribution of SNP densities and summary of QTL locations for
895 wood composition, bud phenology, water-use efficiency and rust resistance in the poplar
896 genome. Numbers of SNP were calculated for all 500kb windows across all 19
897 chromosomes. Black vertical bars indicated low priority QTL intervals -1: bud phenology -
898 4: rust resistance -6: bud phenology, wood composition and wood density -8: bud phenology
899 and wood composition -10: bud phenology, wood composition and water-use efficiency -
900 11: rust resistance -12: rust resistance -13: bud phenology. Red vertical bars indicated high
901 priority QTL intervals -2: wood composition -3: rust resistance and bud phenology -5: wood
902 composition, wood density and bud phenology -7: wood composition and bud phenology -
903 12: bud phenology and water-use efficiency -13: wood composition -14: rust resistance.
904 Details on QTL position and references are given in table S3.
905
906 Figure 4: Population structure analysis estimated for 600 SNP distributed throughout the *P.*
907 *nigra* genome in validated genotypes – 4a: Estimation of the best value of K determined by
908 the cross validation error implemented in ADMIXTURE software. K was tested for different
909 sets of SNP detailed in the Material and Methods section.
910 – 4b: Admixture results from 706 individuals and 600 SNP K=6, K=7, K=8. Each color
911 represents a different ancestral cluster. Each individual was represented as a thin vertical bar
912 which was divided into color segments that were proportional to its memberships in the
913 ancestral clusters. At K=8, individuals collected along the Rhône river basin were divided
914 into 2 subpopulations, one is located on the upper part and the other one on the lower part of
915 the river. – 4c: Geographical distribution of the populations and the genetic structure
916 revealed by ADMIXTURE
917
918
919 Figure 5: Linkage disequilibrium *vs* physical distances. -5a: The decay of LD was
920 investigated by plotting all pairwise r^2 values against physical distance windows of 100kb. -
921 5b: r^2 values were corrected according the populations structure. -5c: The decay of LD was
922 investigated by plotting 600 pairwise r^2 values against physical distance windows of 100kb.
923

List of supplemental data

Methods S1: DNA extraction and Sanger sequencing of gene amplicons.

Methods S2: Calculation of Illumina sequencing accuracy.

Methods S3: Validation and Origin of replicates data with SSR genotyping.

Figure S1: Test of SNP segregation conformity within 8 progenies belonging to a 3x3 factorial mating design.
We genotyped the 6 parents and 290 progenies belonging to 8 families including in a 3 x 3 factorial mating design. The segregating markers were classified in 5 groups according to the expected segregation pattern deduced from genotype of the parents: BC1 (AB x AA), F1 (AA x BB), F2 (AB x AB), Mono. (AA x AA) and Miss. (missing data in at least one parent). Numbers in black are the total number of markers in each class. Conformity of the segregation pattern with the parental genotype has been checked in each family (numbers in red, numbers with * are number of marker for which a F2 segregating class is missing). Approximately 98 % of the markers analyzed in the progeny fit the expected Mendelian segregation ratios in each family. $\chi 2$ tests for segregation distortion were performed pooling half-sib families (lines and columns from the factorial mating design) at thresholds of P = 0.01. Among the SNP, 216 showed segregation distortion.

Figure S2: Principal component analysis: The first second and third axes explain 2.39%, 1.89%, 1.71% of the total variance respectively. Each dot represents one individual. Individuals used in the SNP discovery panel are indicated by black dots. The first axis differentiates South France populations from the East France populations and Northern Italy population. The second axis, revealed the separation of the Italian populations. The distribution of the discovery panel along the axes reflects the variation of the populations studied.

Figure S3: Distribution of Minor Allele Frequencies (MAF) for 7.896 SNPs in 7 clusters and the association population (706 individuals). Clusters are constituted based on Admixture analyses with 600 SNPs (see Fig. 4b).

Table S1: SNP-panel discovery and list of genotyped *P. nigra* individuals.
[1-9] progenies derived from controlled crosses between [1] SRZ and VGN [2] 71077-308 and VGN [3] SRZ and BDG [4] 71041-302 and BDG [5] 71072-501 and BDG [6] 71072_501 and SRZ [7] 71072-501 and SRZ [8] 71077-308 and L150-089 (*P. deltoides*) [9] 58-861 and Poli.

Table S2: Primer pairs developed within genes for Sanger re-sequencing and SNP collections. -Collection 1: Light signaling pathway -Collection 2: Rust resistance, wood properties, drought stress, randomly distributed along the genome

Table S3: List of candidate regions and candidate genes based on location of QTL hot spots for rust resistance drought stress, bud phenology, wood composition and transcriptome studies. Number in brackets were the QTL numbering in figure 3, QTL region and traits written in italic were inherited *P. deltoides* or *P. trichocarpa* species.

Table S4: Alignment results of the Poli, BEN3, BDG and 71077-308 short reads onto the *P. nigra* reference (389 Mb).

Table S5: List of SNPs extracted from HT-sequencing data. The SNP are denoted by SNP_IGA followed by the chromosome or scaffold number (V2.0) and the base position within the scaffold.

Table S6: Origin and number of SNPs included in the 12 000 BeadChip array.

Table S7: List of SNP included in the 12 000 BeadChip array.

Table S8: Performance of the BeadChip array.

Table S9: Comparison of genotyping data and Sanger data.

Table S10: Comparison of genotyping data and NGS data.

Table S11: Genomic position and gene assignation of the 8259 useful SNP.

Table S12: List and origin of unexpected replicates.

Table S13: Chromosomal distribution of SNP numbers, SNP distances and SNP densities. As expected from our selection strategy, the number of high quality SNPs per chromosome was highly variable (from 72 on chromosome 9 to 1870 on chromosome 6) (Table 4). Chromosome 6 had the highest density of SNPs (67 SNPs/Mb), and chromosome 18 the lowest density (4.3 SNPs/Mb).The largest physical region with no SNP was found on chromosome 17.

P. nigra reference sequence

71077-308 PE reads
Mapping of PE reads vs *P.trichocarpa* v2.0
CLC Genomics Workbench v.4

- Length fraction : 0.9
- Similarity : 0.9
- Min PE distance 250 b
- Max PE distance 800 b
- Unique matches retained

Masked for duplications and repetitions

- RepeatScout, default parameters

→

P. nigra variant calling

71077-308, BEN3, BDG, Poli, pool of 47 individuals PE reads
Mapping of PE reads vs *P. nigra* reference sequence
CLC Genomics Workbench v.4

- Similarity : 0.95
- Min coverage : 0.1 to 0.5 the average coverage
- Max coverage : 1.5 the average coverage
- Min variant frequency
  - SNP: 0,35 for 71077-308, BEN3, BDG, Poli; 0,15 for the pool of 47 individuals
  - DIP: 0,1
- Second allele frequency
  - >0,1 for 71077-308, BEN3, BDG, Poli
  - >0,05 for the pool of 47 individuals

↓

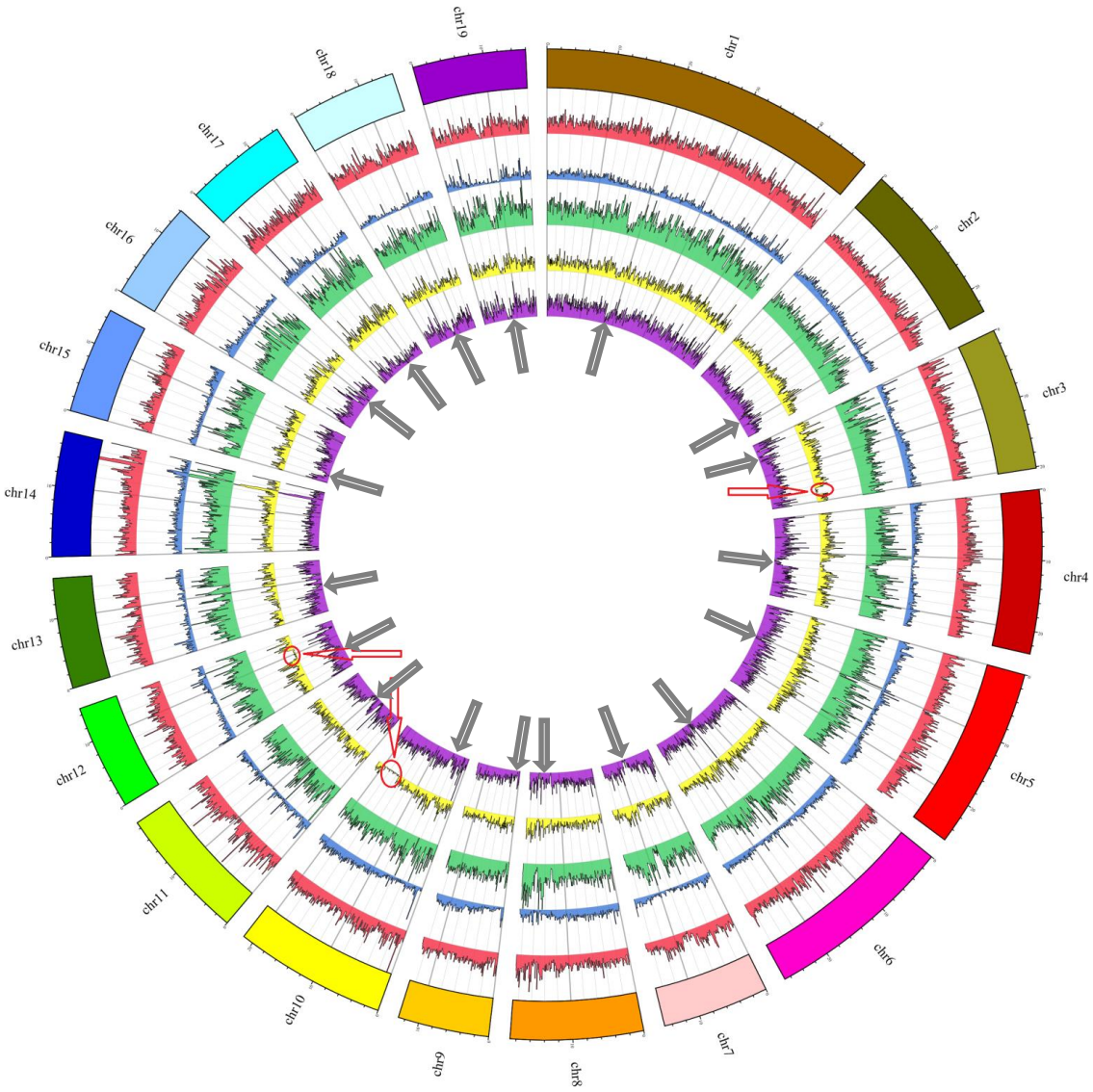
Extraction of SNPs for candidate regions and genes

60 b flanking sequences with no SNPs/DIPs
Remove duplicated / repetitive 121 b sequences

↓

Final SNPs included in the chip

	189 616 SNPs	+	***4 691 Sanger SNPs***
ADT score	≥ 0,85		>0,6
BLASTn identity	> 0,97		>0,9
Second allele frequency	≥ 0,2		>0,05
	↓		↓
	9443 SNPs		**888 SNPs**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chr2
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chr3
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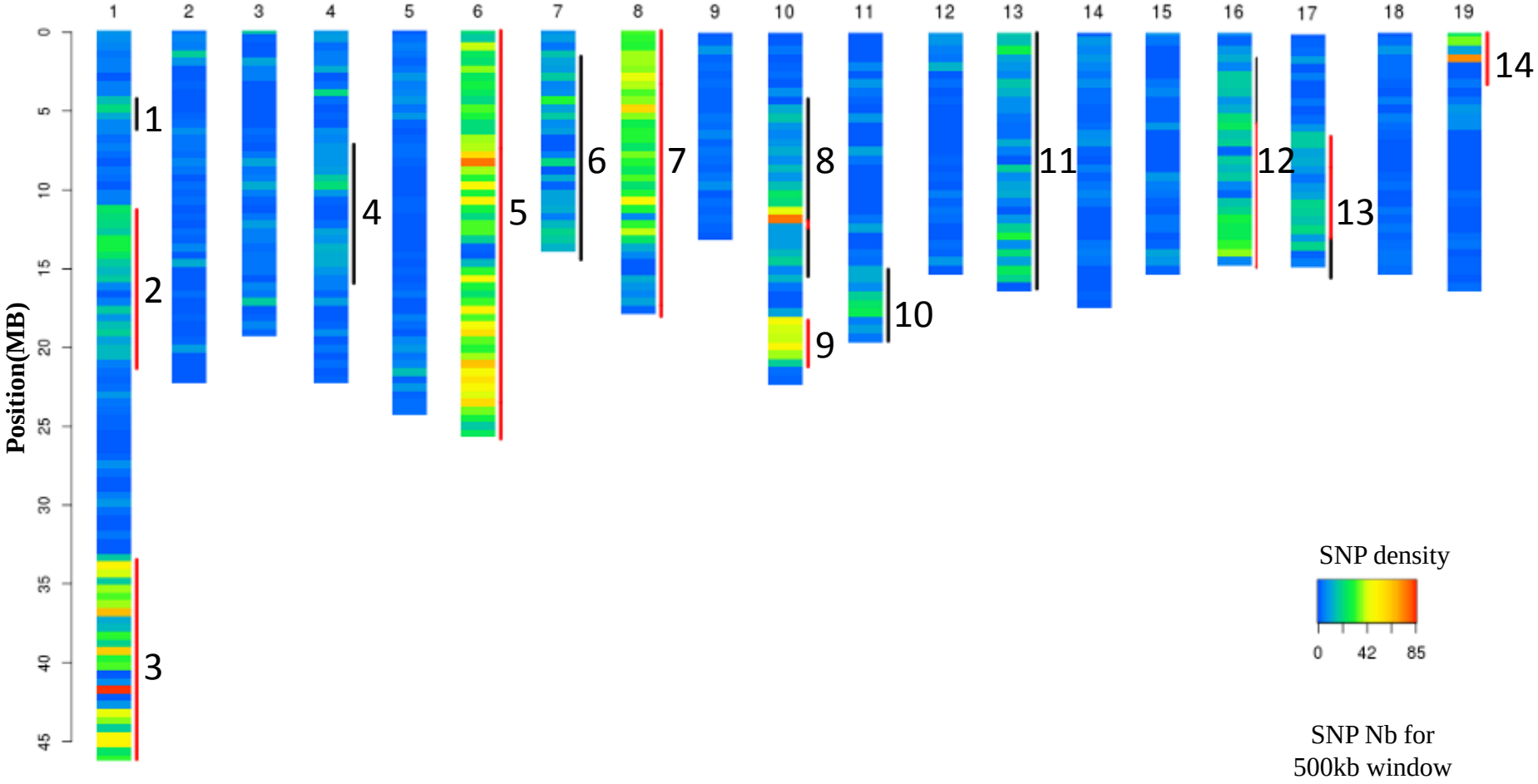
chr9
chr10


1
2
3
4
5
6
7
8
9
10
11
12
13
14
Position(MB)
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5
10
15
20
25
30
35
40
45
SNP density
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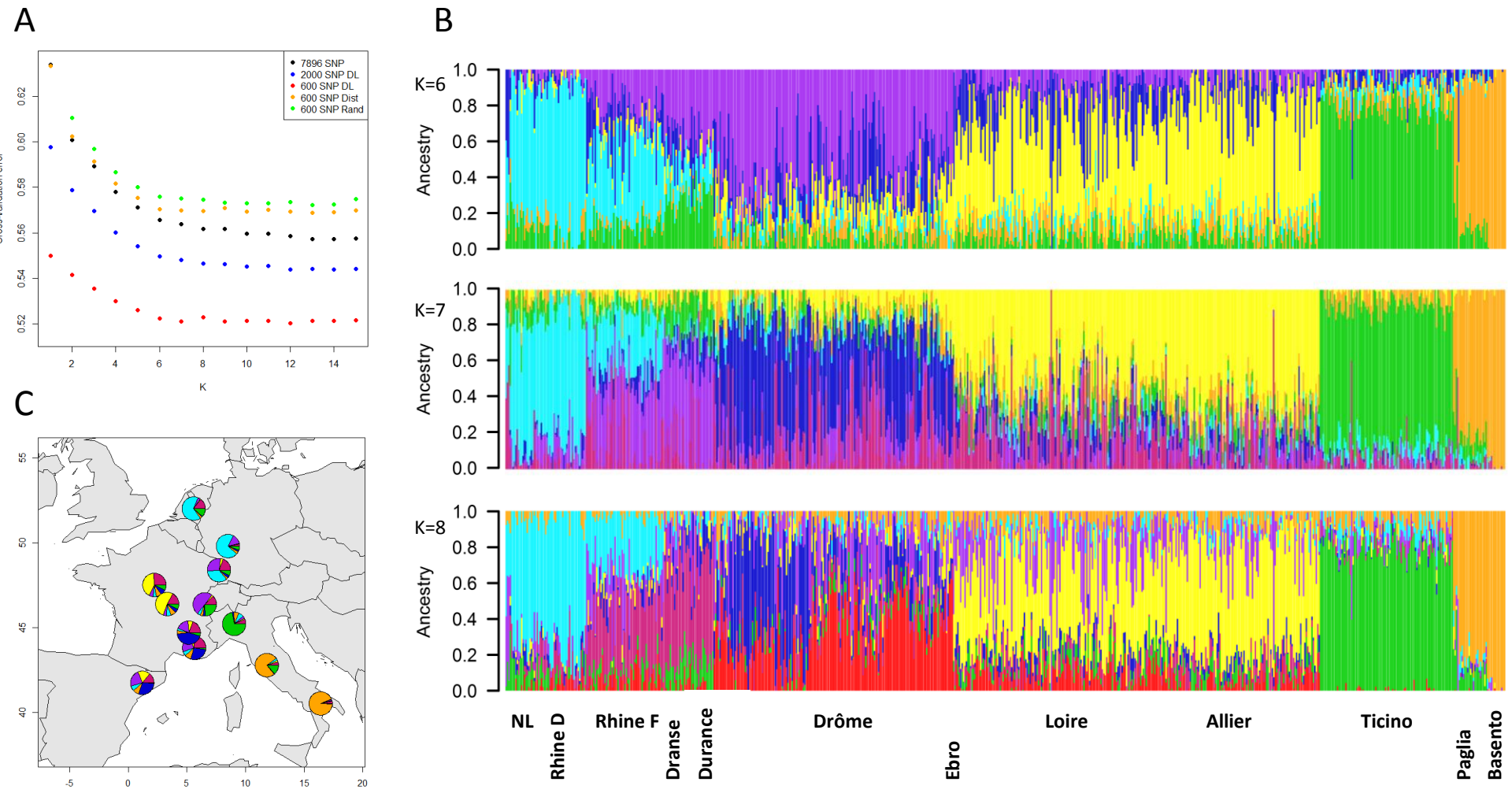
42
85
SNP Nb for
500kb window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2000 SNP DL
600 SNP DL
600 SNP Dist
600 SNP Rand
K
B
K=6
K=7
K=8
Ancestry
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Allier
Ticino
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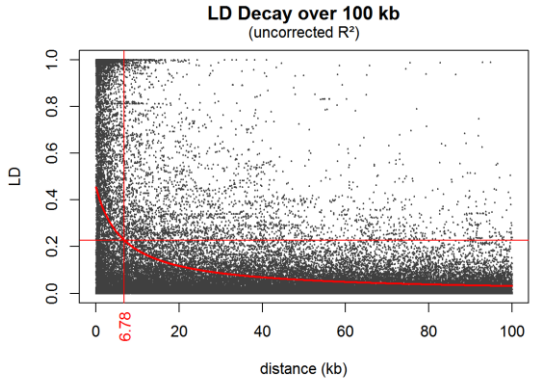
Basento
C

a


LD Decay over 100 kb
(uncorrected R²)
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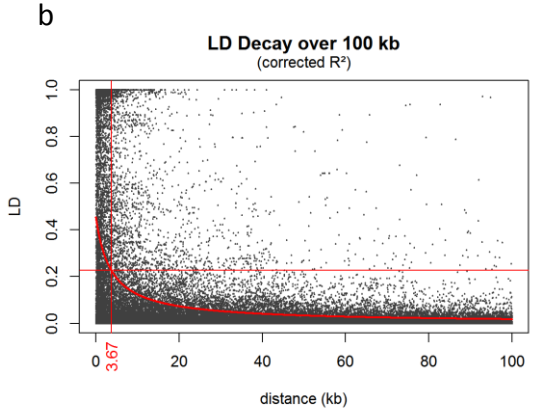
20
40
60
80
100
distance (kb)

b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(corrected R²)
LD
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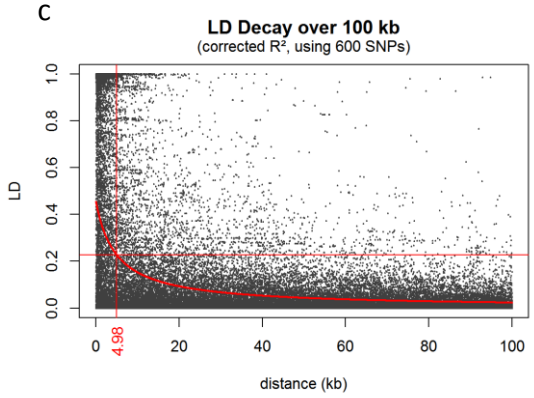
0.4
0.6
0.8
1.0
0
3.67
20
40
60
80
100
distance (kb)

c


LD Decay over 100 kb
(corrected R², using 600 SNPs)
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1.0
0
4.98
20
40
60
80
100
distance (kb)