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Association Analysis of the Growth of Black Poplar (*Populus Nigra* L.) Under Contrasting Nitrogen Levels

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Abstract: The European black poplar (Populus nigra L.) has been used as a germplasm resource for the breeding of new poplar varieties around the world. The identification and screening of its high nitrogen use efficiency genotypes could enable the breeding of new resource-efficient poplar varieties. The accessions were screened using MALDI-TOF MS genotyping technology for ammonium transporter (AMT) and nitrate transporters (NRT) genes against phenotypic data for seedling height and ground diameter traits, in both low and high nitrogen environments. Allele re-sequencing of seven genes related to root development was carried out using the minisequencing method. By cluster analysis, 101 accessions of black poplar were divided into 4 populations, and it was concluded that Central Europe is the origin of the evolution of low-nitrogen and highefficiency populations of European black poplar. Association study between SNP typing and seedling height and ground diameter traits showed that there were significant correlations between four SNP loci and growth traits under the contrasting N levels. We found that SNP3 and SNP4 in the PttAMT1;3 gene were significantly associated with seedling height traits, and that SNP2 and SNP7 in the PttAMT1;2 and PttAMT1;5 genes, respectively, were significantly associated with ground diameter traits. Thus, considerable allelic diversity is present within the candidate genes studied and can be utilized to develop functional markers to select for poplars with improved growth under N stress conditions.

Keywords: Nitrogen; Populus nigra; SNP; association analysis

1 Introduction

European black poplar (*Populus nigra* L.) is a riparian tree species that has a wide geographic distribution, ranging from Western Europe to Central Asia and Northern Africa. It is not only ecologically important as an indicator species for biodiversity of riparian ecosystems [1], but is also one of the most commonly used species in poplar breeding programs, because of its adaptability to a broad range of ecological conditions, rapid growth, easy clonal propagation, and favorable crossing ability with many major poplar species [2,3]. It is currently utilized in many breeding programs as a 'parent pool' to achieve superior hybrids for biomass production [4,5].

Nitrogen (N) is a macronutrient crucial for maximizing the yield of numerous crops. The global demand for fertilizer nutrients N for 2017 was 113.6 million tons and it was expected to increase to 118.8 million tons in 2020; an annual growth rate of 1.5% [6]. To increase land yields, nitrogen fertilizer use has often been excessive, leading to environmental pollution and increased input costs for farmers [7]. Ideal plant species and clones should thus not only have high yield characteristics but also high nutrient utilization efficiencies, especially with regards to the frequently used N, Phosphorus (P), and Potassium (K) elements of most fertilizers. Genes are keys to controlling the expression of important agronomic traits, and they

usually have complex multiple quantitative trait loci that can be identified by genetic mapping and molecular identification of the functional loci. It has been reported that the family genes of Ammonium transporter (AMT) and (NRT) Nitrate transporters of poplar have an important influence on the efficiency of nitrogen uptake and utilization [8,9]. Molecular markers that were not affected in the genome by environmental and genetic variations were used to identify poplar varieties for use as genetic resources.

There are relatively few reports on the association analysis between SNP markers and European black poplar phenotypes, including focusing on the wood material traits and water and high light efficiency [10, 11]. Studies have shown that poplar growth is extremely sensitive to nitrogen [12], so it is important to fully exploit and mine the functional variation in the genes related to nitrogen uptake and phenotypic variation through association analysis in the germplasm resources of *P.nigra*.

2 Materials and Methods

2.1 Plant Material

Nitrogen use efficiency (NUE) was evaluated based on the growth traits of a heuristic black poplar set of 101 accessions. The black poplar cuttings were selected from countries in Europe and Asia, to represent the genetic diversity of the species (Tab. 1).

Nation	Latitude° N	Longitude° E	Ν
Belgium	04°20′	50°40′	1, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95
Netherlands	04°45′	52°20′	6, 7, 78, 79, 80, 81, 82, 83, 84
U.K	00°00′	51°30′	3
Russia	38° 00′	52° 00′	22, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51
Czech Republic	14° 30′	50° 00′	11, 12, 13, 65
Germany	13°20′	52° 30′	2, 17, 18, 19, 20, 21, 67, 68, 69, 70, 71, 72
			73, 74, 75, 76, 77
Hungary	19°00′	47° 30′	57, 58, 59, 60, 61, 62, 63, 64, 66
Slovakia	20°00′	49° 00′	8,9
Croatia	16°00′	45°45′	4, 5
Italy	10°13′	45°43′	23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36
Romania	26°10′	44°30′	14
Yugoslavia	20°30′	40°00′	10
Spain	03°40′	40°30′	15, 16
Turkey	37°37′	37°44 <i>'</i>	52, 53, 54, 55, 6
China	87°48′	48°00′	96, 97, 98, 99, 100, 101

Table 1: Source for *P. nigra* association mapping population

2.2 Evaluation of Growth Traits

The test site is located in Yuquanshan Nursery, Haidian District, Beijing. The N, P and K content of the test site were determined [13]. Black poplar seedlings were planted in sample plots with either high N (HN) levels, apply urea to each seedling of European black poplar twice, and apply 4 grams per plant, or low N (LN) levels, where nitrogen fertilizer was not applied; the other macro- and micro-nutrients applied were the same in both treatments [13]. The seedling heights and ground diameters in the different fertilization environments were measured and used to determine their Nitrogen use efficiency (NUE). The date of seedling height and ground diameter were surveyed before the growth of caps.

2.3 DNA Extraction and SNP Selection

The leaves from the black poplar were collected and stored at -20°C. The genomic DNA was extracted from the leaves according to the Plant Genomic DNA Kit instructions (Beijing TIANGEN). Tag SNPs were selected using a pairwise tagging algorithm and r^2 exceeded 0.8 for all SNP sites for the 7 genes. PCR primers for mass spectrometry were designed using iPLEX GOLD (Sequenom MassARRAYW) and as shown in Tab. 2 [14].

Table 2: Primer pars for amplification

SNP ID	Gene	Forward primer sequence	Reverse primer sequence	iPLEX® (Extension) primer sequence	
SNP1	PttAMT1;2	ACGTTGGATGTCTCTTT GGCTACGCCTTTG	ACGTTGGATGGTAGGAA AGTCACTCAAACC	TCACTCAAACCAA ATAAATGG	
SNP2	PttAMT1;2	ACGTTGGATGGAACTG GGAGCATAAGTAGG	ACGTTGGATGTCAGCCTC TGACCTAGCTC	GACCTAGCTCCTCT CCTCTCCA	
SNP3	PttAMT1;3	ACGTTGGATGCCTGGC TGCTCATTTGATAC	ACGTTGGATGCAATGGA CCCATAGTAGCAC	CCATAGTAGCACT AACCCATCCG	
SNP4	PttAMT1;3	ACGTTGGATGCTCATCT TCAGCTGAGATCC	ACGTTGGATGTGGGTTA GTGCTACTATGGG	CCATTGTTTTTTGT TCTTCATAA	
SNP5	PttAMT1;3	ACGTTGGATGGAAAGA ACCCAGTTTGTTGC	ACGTTGGATGGAACCAG TGAGACACAACAG	ACACAACAGGGTA AACGAA	
SNP6	PttAMT1;3	ACGTTGGATGATGACG ATGACGACGAGTCC	ACGTTGGATGTCCCTCTT ACGCATTTGCAC	GGGTTGTAGAAGT AGGCTCAATTT	
SNP7	PttAMT1;5	ACGTTGGATGCCAATA GCACTCCATTGTCC	ACGTTGGATGATGGATTC AATCCTGGTTCG	ATCCTGGTTCGTTT ATCAA	
SNP8	PttAMT1;5	ACGTTGGATGGAAGCT TTTGAGGATCTCAG	ACGTTGGATGTACACAT AAGCAAGCCCACC	CTTGTCAAGTCCAT TCCTGCC	
SNP9	PttNRT1;2	ACGTTGGATGGGTAGT TAATGGGCTACCTC	ACGTTGGATGTCGCAGT AGCAGTATTGCTC	TGCTCTGTGGGAC ACCG	
SNP10	PttNRT1;2	ACGTTGGATGGAGCTA GGCCTACCAAAATC	ACGTTGGATGCCCTGGTG CATATGATAAGG	GCATATGATAAGG AATTAGCAG	
SNP11	PttNRT2;1	ACGTTGGATGATGGGT GTCATGATTTGCGG	ACGTTGGATGACTACGTC TTTAGAAGGCGG	GCCGCCCCATTGTG GGAAGT	
SNP12	PttNRT2;3	ACGTTGGATGGGAGCA GCAAAAGTAGAGAC	ACGTTGGATGTTCCGGTT GTTCTCAGTAGC	AGCTGCCCCTCAC ATGC	
SNP13	PttNRT2;4	ACGTTGGATGACTACG TCTTTAGAAGGCGG	ACGTTGGATGATGGGTG TCATGATTTGCGG	CTCCCTGTGATGTT GGTC	
SNP14	PttNRT2;4	ACGTTGGATGTATGCA TCTTCACCCTGTCG	ACGTTGGATGCCTGCTGT GTGAAGCTTTAG	AGCTTTAGATCGA ACCTATTAAAT	
SNP15	PttNRT2;4	ACGTTGGATGAGAAAC GCACACCCATATCG	ACGTTGGATGGTCTCTGG AAGCATCTTCTC	AATGGGTGCAGTT TGTGACCTCTT	
SNP16	PttNRT2;4	ACGTTGGATGAATGTC AGCTGTTTCGAGCC	ACGTTGGATGGGGCATA AAGAGCGCATAGC	GAGCGCATAGCAG CCCA	

2.4 Population Structure and Association Analysis

A model-based program STRUCTURE 2.2.3 was used to infer subgroups with distinctive allele frequencies using a burn-in of 100,000 and run length of 100,000, and an individual was assigned to a group if more than 75% of its genome fraction value was derived from that group. Five runs of the structure program were performed and an average likelihood value, L (K), across all runs was calculated for each K. The model criterion for detecting the most probable value of K was DK, an ad hoc quantity related to the second-order change in the log probability of data with respect to the number of clusters inferred by the structure program [15]. The population structure matrix (Q) identified at K = 4, was used as the number of model-based populations.

2.5 Association Analysis

TASSEL 5 and SPSS were used to conduct association analysis for the growth habits of the black poplar accessions [16]. The following models were involved for association analysis: Single factor analysis of variance was performed using SPSS version 13.0, the mixed linear model pattern in TASSEL software was used to identify significant nucleotide variations associated with growth traits and contrasting levels of nitrate. A *p*-value less than 0.05 was considered significant.

3 Results

3.1 Population Structure

The population structure of the 101 black poplar accessions was initially inferred using STRUCTURE 2.3.4 and the peak of delta *K* was observed at K = 4 [17], indicating the presence of the four main populations (clusters, Q1-Q4) in the 101 black poplar accessions (Fig. 1). The five populations (group 1, group 2, group 3, group 4 and the admixed group) represented 15 (14.8%), 30 (29.7%), 20 (19.8%), 25 (24.8%) and 11 (10.9%) of the black poplar accessions (Fig. 2(a)), based on the standard q-value of 60%, respectively (Fig. 2(b)).



Figure 1: Relationship between K and ΔK based on STRUCTURE analysis of chickpea genotypes based on SNP marker data



b

Figure 2: (a) Inferred population structure of 101 black poplar accessions with different geographical origins. Each sample is marked by a single vertical line. Each color represents a genetic cluster; (b) *Vertical bars* represent individual black poplar lines. The area of different colors illustrates the proportion of either each subpopulation based on these SNPs markers

Among the introduced 101 black poplar accessions, the clonal sources from Central Europe are the most abundant, reaching 32 provenances, and the population represented by green and yellow accounts for 62.5% of the Central European population, as shown in Fig. 3. The clones introduced from Western Europe are 22 provenances, and the population represented by green and yellow accounts for 62.5% of the Western European population. It is inferred that Central Europe and Western Europe are the centers of evolutionary origin of the black poplar population.



Figure 3: Population clustering map of different provenances of black poplar accessions. Ungrouped: a population of no more than 60% in color

3.2 Association Analysis Using the Generalized Linear Model (GLM)

We attempted to discover the NUE through analyzing the genotyping data of the 16 SNPs and the two traits with GLM approaches, for selected genes from the Ammonium transporter (AMT) and Nitrate transporter (NRT) gene families. The results showed that only 4 SNP markers showed significant differences in seedling height and ground diameter as shown in Tab. 3.

Table 3: Association analysis between 4 SNP markers and value of seedling height and ground diameter in N-unfertilized conditions and N-fertilized condition

	<i>p</i> -value		Effect/100%		p-value		Effect/100%	
	ANOVA	GLM	ANOVA	GLM	ANOVA	GLM	ANOVA	GLM
	seedling height in N-unfertilized (L N)				seedling height in N-fertilized (H N)			
SNP3	0.0197*	0.0380*	7.70	7.70	0.0234*	0.1109	7.37	_
SNP4	0.0197*	0.0200*	7.70	7.70	0.0234*	0.0909	7.37	_
ground diameter in N-unfertilized (L N)				ground diameter in N-fertilized (H N)				
SNP2	0.0048**	0.0130*	5.82	_	0.0011**	0.0020**	9.80	9.80
SNP7	0.0006**	0.0010**	7.76	7.76	0.0011**	0.0020**	10.23	10.23

*, **Seedling height and ground diameter among accessions significant at p < 0.01 and p < 0.05, respectively

SNP 2, SNP 3 and SNP 4, SNP 7 were located in the coding region of the *PttAMT 1;2, PttAMT 1;3* and *PttAMT 1;5* gene. SNP 2, SNP 3 and SNP 4 were non-synonymous mutations, while SNP4 was synonymous mutations. The association study indicated that SNP3 was significantly associated ($p \le 0.05$) with the seedling height of black poplar and the contrasting levels of nitrate explained about 7.70% of the variation for seedling height in the LN field, and about 7.37% of the variation in the HN field. The seedling height in the HN and LN fields with the GG genotype was higher than that for the GT and TT genotypes, as shown in Fig. 4(a). The analysis result of SNP 4 is similar to SNP 3. The seedling height in the HN and LN fields with the TT genotype was higher than that for the GT and GG genotypes, as shown

in Fig. 4(b).

SNP 2 and SNP 7 had only two genotypes. The association study indicated that SNP2 was significantly associated with the ground diameter the of the black poplar and the contrasting levels of nitrate explained about 5.82% of the variation for ground diameter in the LN field, and about 9.80% in the HN field. Ground diameter in the HN and LN fields with the CT genotype was higher than that of the CC, as shown in Fig. 4(c).

During the evolution of black poplar, the nitrogen use efficiency of different provenances was different. We infer the population genetic structure by STRUCTURE, and classify black poplar into four categories. Through analysis, it is concluded that Central Europe and Western Europe STRUCTURE. Combined with the evaluation of nitrogen use efficiency of black poplar in Europe, the low-nitrogen high-efficiency type was inferred to infer that Western Europe was the main origin of evolution of European black populations.

The analysis result of SNP 7 is similar to SNP 2. The association study indicated that SNP 2 was significantly associated with the ground diameter the of the black poplar and the contrasting levels of nitrate explained about 7.76% of the variation for ground diameter in the LN field, and about 10.23 in the HN field. Ground diameter in the HN and LN fields with the GA genotype was higher than that of the AA, as shown in Fig. 4(d).



Figure 4: a Genetypic effect of SNP 3 on value of seedling height in *P.nigra*; b Genetypic effect of SNP4 on value of seedling height in *P.nigra*; c Genetypic effect of SNP2 on value of groud diameter in *P.nigra*; d Genetypic effect of SNP7 on value of groud diameter in *P.nigra*

4 Discussion

For most plants, ammonium and nitrate are essential sources of nitrogen required for growth. In well-aerated soil, the concentration of nitrate is usually 10-1000 times higher than that of ammonium. However, when nitrate and ammonium exist simultaneously, plants seem to be more likely to absorb

ammonium nitrogen [18]. The molecular basis of the low affinity transport system is unclear, but there is increasing evidence that the ammonium high affinity transporters could be members of the AMT1 family, as the *AMT* protein plays a major role in the absorption of soil nitrogen by plant roots. For example, in the root system of thale cress (*Arabidopsis thaliana* (L). Heynh.), all of the *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* genes were fully expressed [18]. Under the condition of nitrogen deficiency, plants enhance the expression of specific ammonium transporter genes, which is one of the most important internal mechanisms utilized by plants to adapt to nitrogen stress conditions [19].

Identification of candidate genes that are responsible for variation in continuous traits or quantitative traits has been a challenge in modern genetics [20]. The seedling height and ground diameter are the most direct growth traits for evaluating seedling production for trees. Therefore, the two trait values were correlated with SNP loci in this study and analyzed by of one-way ANOVA and a general linear model. The correlation analysis between seedling height and ground diameter showed that of the 16 successful SNP markers, 4 of them were significantly or extremely significantly correlated with the two measured traits during both the low and high nitrogen treatments. Therefore, it was preliminarily concluded that these four SNP markers are related to the Nitrogen use efficiency (NUE) of European black poplar.

The *AMT1;2* gene is one of the greatest contributors to plant ammonia uptake, from the *AMT* family of genes. Sonoda and Kumar et.al have found that *OsAMT1; 2* in *Oryza sativa* is expressed in the roots and induced by ammonium ions, the increase of the *OsAMT1;2* gene expression was 50% of the *OsAMT1;1* gene when treated with low concentrations of ammonium (10 μ M) in the ammonium nutrient solution for 2 d [21,22]. When nitrogen deficient conditions occur, the *OsAMT1;2* gene expression is enhanced to three times that found during normal nitrogen supply conditions. Our genotyping results indicated that there is a C- to T-transition at SNP2 in the coding region of the *PtAMT1;2* gene, which leads to an amino acid change from Thr to Ile. The different *PtAMT1;2* genotypes showed significant associations with the ground diameters of the black poplar. There are CC and CT genotypes, and the CC genotype has a frequency of 0.871, making it the dominant genotype. Therefore, it could be speculated that its correlation with nitrogen use efficiency may be because the mutation affects the transcriptional regulation levels of the *PttAMT1;2* gene in different individuals. It could thus be utilized as an identification marker for genotypes with high Nitrogen use efficiency (NUE).

The *AMT1;5* gene is expressed in the roots; but studies have shown that it is not expressed if there is sufficient nitrogen and its expression is significantly increased in nitrogen deficient conditions [23]. In the case of the deletion of the other AMT family genes, the *AtAMT1;5* gene will functionally replace them and absorb ammonium ions [23]. The presence of the *AtAMT1;5* gene will help to increase the ammonium ion uptake capacity of plants in the absence of nitrogen [23]. Our genotyping results indicated that there is an A- to G- transition at SNP7, a synonymous mutation, that is in the coding region of the *PtAMT1;5* gene, it could be speculated that its correlation with NUE may be due to the fact that the mutation affects the transcriptional regulation levels of the *PttAMT1;5* gene in different individuals, and significant association with the ground diameter in the HN and LN fields. The ground diameter of the GA genotype was higher than that of the AA genotype.

Nitrogen deficiency leads to enhanced expression of the *AMT1;3* gene. After 3 days of nitrogen deficiency in Arabidopsis, the expression of *AtAMT1;1* and *AtAMT1;3* genes in the roots was enhanced. After a long period of nitrogen starvation, there was a marked change in the mRNA expression profile of the *AtAMT1;3* gene [23]. The expression of *OsAMT1;3* was enhanced 4 folds by nitrogen starvation in comparison to when ammonium was the sole source of nitrogen [24]. Our genotyping results indicated that there were T- to G-transitions at SNP3 and SNP4 in the coding region of the *PtAMT1;3* gene, causing amino acid changes from Met to Arg and Lys to Asn. The different *PtAMT1;5* genotypes showed significant association with the seedling height of the black poplar. The seedling heights of the GG and TT genotypes were higher than GT genotype.

During the evolution of black poplar, the nitrogen use efficiency of different provenances was different. We infer the population genetic structure by STRUCTURE, and classify black poplar into four categories. It is inferred that Central Europe and Western Europe were the centers of evolutionary origin

of the black poplar population. Combined with nitrogen use efficiency evaluation of black poplar [13], it was inferred that Western Europe is the main origin of the evolution of low-nitrogen and high-efficiency black poplar populations.

In summary, we obtained 16 SNPs of 7 genes, and the association results revealed that SNP 3 and SNP 4 were significantly associated with seedling height and that SNP 2 and SNP 7 were significantly associated with ground diameter. Here, we provide the partial preliminary molecular information of the genes related to NUE for the first time. The association results might be useful and applicable in future poplar genetic marker-assisted selection processes and breeding programs.

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