Variation and Genetic Parameters of Leaf Morphological Traits of Eight Families from *Populus simonii* × *P. nigra*

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**Abstract:** Leaf morphology in *Populus* L. varies extensively among sections, species and clones under strong genetic control. *P. nigra* L. (section *Aigeiros*), with large and triangular leaves, is a commercial forest tree of economic importance for fast growth and high yield in Europe. *P. simonii* Carr. (section *Tacamahaca*) with small land rhomboid ovate leaves performs cold and dry resistance/tolerance in the semi-arid region of Northern China. Leaf morphological traits could be used as early indicators to improve the efficiency of selection. In order to investigate the genetic variation pattern of leaf morphology traits, estimate breeding values (combining ability), as well as evaluate crossing combinations of parents, 1872 intersectional progenies from eight families (*P. simonii* × *P. nigra*) and their parents were planted with cuttings for the clonal replicate field trial in Northern China. Four leaf size traits (area, perimeter, length, width) and roundness were measured with leaf samples from the 1-year-old clonal plantation. Significant differences regarding leaf traits were found between and among three female clones of *P. simonii* from Inner Mongolia, China and six male clones of *P. nigra* from Casale Monferrato, Italy. The genetic variation coefficient, heritability and genetic variance component of most traits in male parents were greater than these of female parents. Heritability estimates of male and female parents were above 0.56 and 0.17, respectively. Plentiful leaf variations with normal and continuous distributions exited in the hybrid progenies among and within families with the genetic variation coefficient and heritability above 28.49 and 0.24, respectively. Heritability estimates showed that leaf area was the most heritable trait, followed by leaf width. The breeding value ranking of parents allowed us to select the parental clones for new crosses and extend the mating design. Two male parental clones (N430 and N429) had greater breeding values (general combining ability, GCA) of leaf size traits than other clones. The special combining ability (SCA) of the crossing combination between *P. simonii* cl. ZL-3 and *P. nigra* cl. N430 was greater than that of others. Eight putatively superior genotypes, most combined with the female parental clone ZL-3, can be selected for future testing under near-commercial conditions. Significant genetic and phenotypic correlations were found between five leaf morphology traits with the coefficients above 0.9, except for leaf roundness. The results showed that leaf morphology traits were under strong genetic control and the parental clones with high GCA and SCA effects could be utilized in heterosis breeding, which will provide a starting point for devising a new selection strategy of parents and progenies.

**Keywords:** *Populus simonii* × *P. nigra*; leaf morphology traits; breeding values; genetic variation; cross combination; heterosis
1. Introduction

Plant leaf plays a major role in photosynthesis, transpiration and respiration. As the light capturing organ and the site of carbon and water exchange in plants, leaf reflects the resource utilization strategies and environmental adaptability [1–4]. Previous intensive studies on biochemical and physiological processes (e.g., photosynthesis, respiration or nutrient allocation) have contributed immensely to the understanding of the function and the importance of leaves as the energy source organ of plants [5]. However, comparatively little is known about how leaf size and shape is determined and contributes to leaf physiological functions [6]. Leaf size and shape as the result of the interactions of multiple genetic and environmental factors [7] indicate the significant phenotypic diversity of leaf morphology. Abundant genetic variations in leaf size and shape occurred among species and populations within the same species, which are controlled by strong heredity and carry genetic and environmental variation information [8–12]. Leaf size, morphology, structure and orientation directly affect the efficiency of photosynthesis and have an important impact on forest productivity [13]. Marron et al. [14] and Gebauer et al. [15] found that leaf shape and size being closely related to growth traits became an adaptive trait and early indicator of biomass yield. Furthermore, leaf morphology is widely used not only in forest species identification and taxonomic research, but also in a large number of studies on the relationship between leaf morphology, physiological characteristics and biomass yield. With the development and application of molecular biology technology and leaf morphology analysis software, more attention has been paid to the variation and genetics of leaf morphology among populations and individuals within the same species. Recently, the underlying genetic and molecular mechanisms controlling leaf morphological variation have been emphasized as a popular topic [16,17].

Poplar, as an important forest species in temperate regions, presents a wide geographic distribution and strong adaptability. Poplar trees are readily transformable, vegetative propagation and display rapid growth, making them widely used in timber and energy forest construction, village virescence and ecological protection [18–21]. Plentiful variations among sections, species, populations and clones indicate its high levels of ecological and inter/intraspecific diversity. Interspecific and intersectional hybrids exhibit strong growth vigor, superior adaptability, fecundity, stress resistance and other traits as compared with their parents, which achieved remarkable economic benefits. For example, P. × canadensis, the hybrid between P. deltoides and P. nigra, showed strong heterosis and produced large quantities of industrial wood in Europe, America and Asia [22,23]. P. xiaohei, the hybrid between P. simonii (section Tacamahaca) and P. nigra var. italica (section Aigeiros), performing with high cold resistance/tolerance, is widely planted for shelter forest and timber forest construction in cold and dry regions such as Inner Mongolia, Northern China [20]. Phenotypic diversity and multilevel genetic variation in the leaf morphology of poplars [24,25] made them ideal for population analyses and association studies with the aim to reveal the intrinsic relationship between trait genetic variation and environmental adaptability [26]. P. nigra (section Aigeiros) with large and triangular leaves is an important economic species for its fast growing and high-yield production in the Alluvial plain area of Europe [27]. P. simonii (section Tacamahaca) with small land rhomboid ovate leaves, the species characterized by the resistance of cold, drought and barren resistance, is widely used for shelterbelt and timber forest construction in barren land, cold and arid regions such as Inner Mongolia, Northern China [28]. Hybridization between P. simonii and P. nigra produced the superior clones with characteristics such as rapid growth and resistance to cold, drought and barren areas [29], in order to expand their cultivation area and save water and fertilizer resources for their fast and high-yield forest construction. Generally, the leaves with large areas, high chlorophyll and strong photosynthesis for plant [30] are conductive to the accumulation material and energy, making a large photosynthetic area and a physiological basis of vigorous growth of section Aigeiros clones [31]. On the other hand, increasing transpiration rate resulted in the increasing of leaf area and leaf size [32]. However, under drought stress, the single leaf area decreased significantly to reduce leaf water consumption [33,34]. Leaf anatomical studies found that there was a thick and loose spongy parenchyma layer in the leaves of P. trichocarpa (section Tacamahaca), resulting in the leaves being thick and large, and the stomata
having high densities and lengths at the abaxial leaf surface; cell expansion is the main reason for the increase in leaf area. On the other hand, the leaves of *P. deltoides* (section *Aigeiros*) were thin and small, and had stomata with low density and small stomatal length at the abaxial leaf surface; cell division is the main reason for the increase in leaf area [35]. Moreover, evolution of differential cellular polarity plays a significant role in leaf morphological variation observed in the subgenera of *Populus* [36]. The formation of leaf morphological characteristics is regulated by many functional genes’ expression, transcription regulators and microRNAs [37–39]. The mechanism of poplar heterosis was discussed widely in terms of growth variation, photosynthetic capacity, water use efficiency, hormone content, genetic mapping and allelic variation [40–44]. However, there are few reports on the genetic variation of leaf traits, genetic relationship between hybrid progenies and parents and heterosis of these leaf morphology traits of poplar [16,45].

Accurate estimation of genetic parameters of leaf traits is of great significance to poplar breeding and selection. With the aim to realize the estimated genetic variance components without bias, the key is to eliminate the influence of environmental factors on genetic variance. In breeding practice, a large amount of non-equilibrium data is generated due to the limitation of measuring the number of offspring or preservation conditions; it is difficult to obtain accurate genetic parameters by classical genetic methods. The genetic model and statistical analysis method based on the mixed linear model can easily solve this problem even with a large population size, a complex population structure and unbalanced observed data [46,47]. With the development of computational methods and technology, a mixed linear model approach has become the main method of genetic evaluation and has been widely used in crop [48,49], tree [50,51] and animal [52,53] breeding. Breeding value, also known as additive effects value, is an important parameter in forest selection and breeding. Improving the prediction accuracy of tree breeding values can improve the breeding efficiency and readily to estimate genetic gain, but its value cannot be directly measured. Statistical methods can be used to dissect genetic effects and environmental effect through phenotypic values and the genetic relationships between individuals [54]. The Best Linear Unbiased Prediction (BLUP) method [55,56] can predict the value of random effects, effectively reduce the error variance of prediction breeding value, improve the accuracy of selection and make it possible to predict the breeding value of different generations of individuals. An accurate BLUP prediction value and forward selection based on an individual breeding value will improve the selection efficiency and genetic gain in the process of genetic improvement [57,58]. With the rapid development of genomics in recent years, the combination of genomics and quantitative genetics has a significant impact on the genetic gain of traits. It can accurately predict the phenotypes of candidate individuals in the early stages, while the prediction of multiple traits also shows great advantages. The combination of genomics and quantitative genetics, based on the accurate prediction of BLUP breeding value, laid a solid foundation for the estimation of forest genetic parameters, early selection and accurate evaluation of parents. The BLUP breeding value has been widely used in animal breeding and selection research. In recent years, breeding value estimation and genome breeding value estimation have also been applied to *Eucalyptus nitens* [59–61], *Pinus elliottii* [62], *Paulownia* [63] and *Picea* [64,65] with reference to animal breeding experience, but there are few reports on BLUP applications in poplar [58].

In this study, 1872 intersectional progenies of eight families (*P. simonii* × *P. nigra*) and their parents were used and measured leaf morphological traits (area, perimeter, length, width, roundness) with leaf samples from the 1-year-old clonal plantation. The aim of the study was to analyze: (1) genetic variation on leaf traits among and within eight families and their parental clones, (2) prediction on BLUP of leaf traits based on mixed linear model and (3) GCA and SCA on leaf traits of eight crossing combinations with the different parental clones. Such analyses were expected to provide a theoretical basis for analyzing leaf variation of the F1 progenies between three *P. simonii* clones and six *P. nigra* clones, helping to explore the selection in early hybrid progeny and the combination of parental clones and breeding strategies.
2. Materials and Method

2.1. Experimental Materials

In the spring of 2014, $F_1$ progeny seedlings of 8 families were obtained from the intersectional hybridizations between $P. simonii$ and $P. nigra$ and raised in the greenhouse of CAF (Chinese Academy of Forestry), Beijing, China. Three female clones of $P. simonii$ (‘1-XY’, ‘XY-5’, ‘ZL-3’) were selected from the gene bank of $P. simonii$ in Inner Mongolia, Northern China. Six male clones of $P. nigra$ (‘N188’, ‘N020’, ‘N139’, ‘N151’, ‘N429’, ‘N430’) were selected and introduced from the gene pool of $P. nigra$ in Casale Monferrato, Italy. After this, the seedlings of 1872 genotypes from 8 families (Table 1) were planted at the Tongzhou Base of RIF (Research Institute of Forestry) in Beijing, China. In the spring of 2016, cuttings of $F_1$ progenies and their parents were planted for the clonal replicate field trial at Fengnan Base of CAF in Tangshan City, Hebei Province. Five plants for each genotype of $F_1$ progenies and each clone of parents were grown in a randomized complete block design, one plot per block with spacing of $30 \times 50$ cm and conventional management with field practices.

Table 1. The number of $F_1$ progenies from 8 families resulting from $P. simonii \times P. nigra$.

| Mother $P. simonii$ Clones | Father $P. nigra$ Clones |
|-----------------------------|----------------------------|
|                             | N020 | N139 | N151 | N188 | N429 | N430 |
| 1-XY                        | 149  | 355  | 273  | 280  | -    | -    |
| XY-5                        | -    | -    | -    | 82   | -    | -    |
| ZL-3                        | -    | -    | -    | 479  | 194  | 60   |

2.2. Experimental Site

The experimental area is located at the Fengnan Base of CAF in Tangshan City, Hebei Province, Northern China and lies between $39^\circ 29\arcmin$ N latitude and $118^\circ 16\arcmin$ E longitude. Due to the semi-humid continental monsoon climate, its annual average temperature is $12.5 ^\circ C$ with a minimum of $-9 ^\circ C$ in winter and a maximum of $32 ^\circ C$ in summer. The annual precipitation and the annual frost-free period are 596.4 mm and 190 days, respectively. The soil is sandy-loam type with a pH of 7.5.

2.3. Leaf Traits Measurements

In August 2017, three plants per genotype/clone performing with uniform growth and without disease and insect damage were selected as the average for subsequent measures for study use. One of the leaves from the sixth to the ninth from top to down of each plant were collected, then put into plastic bags with moistened filter paper and brought back to the laboratory. Each leaf sample was scanned with an HP ScanJet4010 scanner and saved as JPG (Joint Photographic Experts Group) files. Finally, Digimizer (MedCalc Software bvba) software was used to measure leaf morphologic traits (length, width, area and perimeter) and calculate leaf roundness [66].

2.4. Statistical Analysis

All summary statistics were analyzed using the R program. The variance components were estimated by restricted maximum likelihood, using the average information REML algorithm implemented in the ASREML program. The analysis of variance components were tested according to the following general mixed linear model:

- For parental clones: $Y_{ij} = \mu + C_i + e_{ij}$

  where $Y_{ij}$ is the phenotypic value of the $j$th individual in clone $i$, $\mu$ is the overall mean, $C_i$ is the effect of clone $i$ and $e_{ij}$ is the residual effect.

- For $F_1$ progenies: $Y_{ijklm} = \mu + T_k + F_{ij} + C_{l(T k)} + e_{ijklm}$
where \( Y_{ijklm} \) is the observation of the \( m \)th ramet of the \( l \)th clonal plant within \( k \)th genotype from the \( ij \)th full-sib family, \( \mu \) is the mean population and \( T_k \) is the random effect of the \( k \)th additive genetic \( \sim N(0, \sigma^2_A) \), \( F_{ij} \) is the random specific combining ability (SCA) effect of the \( ij \)th full-sib family \( \sim N(0, \sigma^2_{SCA}) \), \( C_l(T_k) \) is the fixed effect of the \( l \)th clonal plant within the \( k \)th genotype \( \sim N(0, \sigma^2_C(G)) \), and \( e_{ijklm} \) is the residual random error \( \sim N(0, \sigma^2_e) \).

Individual-tree narrow-sense heritability \( (h^2_s) \) was estimated for each leaf trait at each clone within each genotype using the individual animal models as follows:

\[
h^2_s = \frac{\sigma^2_a}{\sigma^2_p}
\]

where \( \sigma^2_a \) was the additive genetic variance and \( \sigma^2_p \) was the phenotypic variance.

The additive genetic coefficient of variation \( (CV_A) \) was calculated as follows:

\[
CV_A = \frac{\sigma_a}{\overline{X}} \times 100\%
\]

where \( \sigma_a \) was the square root of the additive genetic variance for a trait and \( \overline{X} \) was the trial mean for the trait.

The correlation estimates were obtained using the above model in the multivariate formulation \[59\]. The correlations related to genetic and environmental effects between traits 1 and 2 were calculated with the following formulas:

the additive genetic correlation: \( \rho_a = \frac{\text{cov}_a(1,2)}{\sigma_a(1)\sigma_a(2)} \);

the phenotypic correlation: \( \rho_p = \frac{\text{cov}_p(1,2)}{\sigma_p(1)\sigma_p(2)} \)

where \( \text{cov}(1,2) \) is the covariance between traits 1 and 2 and \( \sigma_1, \sigma_2 \) are the standard deviations of traits 1 and 2, respectively.

The best linear unbiased predictors (BLUPs) were computed by solving the mixed model equations using ASReml 3.5.3 software. Considering that the leaf shapes of these plants exhibited a large difference, Fisher’s Least Significant Difference (LSD) test was used to carry out multiple comparisons of 5 leaf morphology traits among the parental clones and the 8 families. The individual BLUP for the male and female parents is called GCA, and for the full-sib families is called SCA. Both parameters were calculated to estimate the combination ability of crossing combinations between different parental clones so that favorable genes or characters can transmit to their progenies.

3. Results

3.1. Genetic Parameters and Frequency Distribution of Leaf Traits in Parents and F\(_1\) Progenies

Five investigated leaf morphology traits (area, perimeter, length, width, roundness) differed significantly between three female parental clones of \( P. simonii \) (section Tacamahaca) and six male parental clones of \( P. nigra \) (section Aigeiros) (Table 2). Four leaf size traits of the male parents, \( P. nigra \), had greater means than those of the female parents, \( P. simonii \). The genetic effects were stronger in male parental clones, and the clonal variance components for leaf traits in the male parents were much higher than these of the female parents. The male parental clones varied from 56.4% of leaf roundness to 99% of leaf area, while the female parental clones varied from 2.4% of leaf roundness to 23.8% of leaf width. Leaf areas of the female parental clones had, significantly, the highest \( CV_A \) (92.69%), followed by those of the male parental clones (81.13%), while leaf perimeter, length and width obtained similar values around 40% and that of leaf roundness was around 11%. Heritability estimates on four leaf size traits of the male parental clones were all above 0.96 while that of the female parental clones ranged from 0.173 (leaf area) to 0.238 (leaf width) as leaf size traits were weakly controlled by additive effects.
Table 2. Genetic parameters on leaf morphology traits of female parent *P. simonii* and male parent *P. nigra*.

| Trait          | Mean ± SE | Range      | $\sigma^2_C$ | $\sigma^2_e$ | $CV_A/\%$ | $h^2$     |
|----------------|-----------|------------|--------------|--------------|-----------|-----------|
| Leaf area      | 3.39 ± 0.21 | 1.79–5.01  | 9.873 **     | 47.061       | 92.69     | 0.173 ± 0.143 |
| Leaf perimeter | 2.73 ± 0.09 | 1.90–3.23  | 0.831 **     | 3.186        | 33.39     | 0.207 ± 0.239  |
| Female         |           |            |              |              |           |           |
| Leaf length    | 9.01 ± 0.30 | 6.84–11.05 | 3.721 *      | 14.838       | 42.75     | 0.201 ± 0.562  |
| Leaf width     | 6.65 ± 0.30 | 4.31–8.44  | 4.958 **     | 15.908       | 33.48     | 0.238 ± 0.669  |
| Leaf roundness | 5.74 ± 0.18 | 4.40–6.71  | 0.456 **     | 0.292        | 11.76     | 0.610 ± 0.222  |
| Male           |           |            |              |              |           |           |
| Leaf area      | 11.43 ± 1.55 | 5.37–27.93 | 85.988 **    | 8.222        | 81.13     | 0.991 ± 0.006  |
| Leaf perimeter | 5.02 ± 0.39 | 3.33–9.29  | 5.779 **     | 0.101        | 47.89     | 0.983 ± 0.010  |
| Leaf length    | 13.73 ± 0.85 | 10.15–23.09 | 26.277 **   | 0.885        | 37.34     | 0.967 ± 0.021  |
| Leaf width     | 12.19 ± 0.78 | 8.04–20.14 | 28.003 **    | 0.774        | 43.41     | 0.973 ± 0.017  |
| Leaf roundness | 5.41 ± 0.12 | 4.07–6.20  | 0.329 **     | 0.254        | 10.60     | 0.564 ± 0.172  |

Note: *, ** indicate variance components significantly different from zero at $p \leq 0.05$ and $p \leq 0.01$, respectively. The approximate standard errors (SEs) for the genetic parameters are indicated for each leaf trait.

F$_1$ progenies of eight families resulting from interspecific crossing of *P. simonii × P. nigra* showed the intermediate means of four leaf size traits between those shown by their two parents (Table 3). In F$_1$ progenies, the $CV_A$ of four leaf size traits were above 25%—leaf perimeter had the largest (76%). The estimated heritability of all five leaf traits was above 0.2, and leaf area had the highest heritability (0.74). The genetic variance components ranged from 79.49% for leaf roundness to 83.08% for leaf perimeter, which indicated an obvious additive genetic effect and strong heredity.

Table 3. Genetic parameters on leaf morphology traits of F$_1$ progenies.

| Trait          | Mean ± SE | Range      | $\sigma^2_A$ | $\sigma^2_e$ | $CV_A/\%$ | $h^2$     |
|----------------|-----------|------------|--------------|--------------|-----------|-----------|
| Hybrid progeny |           |            |              |              |           |           |
| Leaf area      | 7.29 ± 0.02 | 0.62–26.83 | 8.219 **     | 1.857        | 39.31     | 0.742 ± 0.007 |
| Leaf perimeter | 3.76 ± 0.01 | 1.20–7.49  | 0.707 **     | 0.144        | 76.21     | 0.382 ± 0.009 |
| Leaf length    | 11.08 ± 0.02 | 3.86–21.87 | 4.237 **     | 0.982        | 25.87     | 0.681 ± 0.008 |
| Leaf width     | 10.06 ± 0.02 | 2.79–17.55 | 4.398 **     | 0.962        | 28.49     | 0.691 ± 0.008 |
| Leaf roundness | 6.34 ± 0.01 | 2.38–7.01  | 0.341 **     | 0.088        | 45.23     | 0.239 ± 0.007 |

Note: ** indicates variance components significantly different from zero at $p \leq 0.01$, respectively. The approximate standard errors (SEs) for the genetic parameters are indicated for each leaf trait.

Data from five leaf morphology traits measured in the F$_1$ progenies of eight families resulting from *P. simonii × P. nigra* exhibited a nearly normal distribution (Figure 1, the data not shown here) with a kurtosis from 0.40 to 3.38 and skewness from $-1.31$ to 0.48, while that of leaf roundness showed the highest kurtosis (3.38) and the lowest skewness ($-1.31$). Among eight families, the F$_1$ progenies from two families, ZL-3×N188 and 1-XY×N139, showed the most concentrated probability distribution of five leaf traits. The F$_1$ progenies of each family exhibited the distribution, which biased their parent with higher leaf traits than another parent. The distributions of leaf size traits were biased toward the fathers while the genotypes with longer and wider leaves than their parents were isolated from the offspring population. On the contrary, the distribution of leaf roundness was biased towards the mother and the genotypes with larger leaf roundness than their parents were isolated from the offspring population.
Figure 1. Frequency distribution on leaf morphology traits in F$_1$ progenies. Note: (A–E) in the figure represent the frequency distribution of leaf area, perimeter, length, width and roundness.
3.2. Genetic Variation of Leaf Traits in Parents and F<sub>1</sub> Progenies

The results of ANOVA on five leaf morphology traits of the parents showed that the variations were considered significant or extremely significant among three female parental clones of <em>P. simonii</em> and significant among six male parental clones of <em>P. nigra</em> when the <em>P</em> value of the ANOVA <em>F</em>-test was below 0.05 or 0.01 (Table 2). In the same way, the variations of five leaf morphology traits of their offspring were extremely significant among eight families and among the F<sub>1</sub> genotypes within families (<em>p</em> ≤ 0.01) (Table 3).

The multiple comparison Fisher’s Least Significant Difference (LSD) test assessed the differences among the three female parental clones, six male parental clones and eight families after parametric ANOVA (Table 4). Five leaf morphology traits were significantly different among three clones of <em>P. simonii</em> and among six clones of <em>P. nigra</em>. The differences of leaf traits among three female parental clones of <em>P. simonii</em> were found that 1-XY had the largest mean leaf area, width and roundness and ZL-3 had the largest mean leaf perimeter and length. Among six male parental clones of <em>P. nigra</em>, N429 and N020 had the highest means regarding four leaf size traits in correspondence with the lowest mean of roundness (4.33 and 4.54), while N151 and N188 had the smallest mean of four leaf size traits.

### Table 4. Mean value and Least Significant Difference (LSD) results on leaf morphology traits of parents and F<sub>1</sub> progenies.

|                | Leaf Area/cm<sup>2</sup> | Leaf Perimeter/cm | Leaf Length/cm | Leaf Width/cm | Leaf Roundness |
|----------------|--------------------------|-------------------|----------------|----------------|----------------|
| Female         |                          |                   |                |                |                |
| 1-XY           | 4.20 a                    | 2.84 a            | 8.50 b         | 7.83 a         | 6.50 a         |
| XY-5           | 2.54 c                    | 2.36 b            | 8.57 b         | 5.21 c         | 5.78 b         |
| ZL-3           | 3.44 b                    | 3.04 a            | 10.14 a        | 6.97 b         | 4.79 c         |
| Male           |                          |                   |                |                |                |
| N020           | 23.87 b                   | 8.13 b            | 20.44 b        | 18.59 a        | 4.54 d         |
| N139           | 7.30 c                    | 3.98 c            | 11.33 c        | 10.52 b        | 5.78 b         |
| N151           | 6.22 c                    | 3.80 cd           | 11.06 c        | 9.45 c         | 5.41 c         |
| N188           | 6.24 c                    | 3.58 d            | 10.79 c        | 8.82 c         | 6.12 a         |
| N429           | 26.21 a                   | 8.72 a            | 21.96 a        | 19.44 a        | 4.33 d         |
| N430           | 7.16 c                    | 3.98 c            | 11.41 c        | 10.25 b        | 5.69 b         |
| Hybrid combinations |                  |                   |                |                |                |
| 1-XY×N020      | 7.38 c                    | 3.72 bc           | 11.07 c        | 10.14 c        | 6.54 a         |
| 1-XY×N139      | 7.39 c                    | 3.74 b            | 11.07 c        | 10.20 c        | 6.54 a         |
| 1-XY×N151      | 7.05 e                    | 3.69 bc           | 10.88 bc       | 9.88 d         | 6.34 c         |
| XY-5×N188      | 6.85 f                    | 3.72 b            | 10.94 d        | 9.72 d         | 6.02 e         |
| ZL-3×N188      | 7.19 d                    | 3.68 c            | 10.91 d        | 9.99 d         | 6.51 a         |
| ZL-3×N429      | 8.06 b                    | 4.09 a            | 11.74 b        | 10.56 b        | 5.94 f         |
| ZL-3×N430      | 8.53 a                    | 4.14 a            | 12.05 a        | 10.80 a        | 6.12 d         |

Note: At the <em>p</em> < 0.05 level, there was no significant difference among the parental clones/hybrid families with the same letters, significant difference with different letters.

The difference of the F<sub>1</sub> progenies within eight families revealed that the progenies of two families, ZL-3×N429 and ZL-3×N430, had the largest means of leaf size traits in correspondence with the lowest means of roundness (5.94 and 6.12). The leaves of three families, 1-XY×N151, XY-5×N188 and 1-XY×N188, were significantly smaller than these of others and their leaf roundness showed the intermediate mean of the parents. The progenies of other three families, 1-XY×N020, 1-XY×N139 and ZL-3×N188, exhibited the highest means of roundness as well as the intermediate means of four leaf size traits.

3.3. Breeding Values and Combining Ability Analysis on Leaf Traits of Parents

The estimated results of breeding value (i.e., GCA) regarding leaf traits (Table 5) showed that the families with ZL-3 as their female parent had the largest GCA (0.45), and the families with 1-XY as their female parent had the smallest GCA (−0.36) regarding leaf area. GCAs regarding leaf roundness and...
width were the largest in the families of ZL-3 as their female parent (0.38 and 0.33, respectively). For the families with 1-XY as their female parent, the GCA regarding leaf roundness of 1-XY was the lowest (−0.53), followed by leaf area and width (−0.36 and −0.32). Among the families of six male parental clones of *P. nigra*, GCA regarding four leaf size traits (leaf area, perimeter, length and width) of N430 was the largest (1.36, 0.58, 1.43, 0.73), followed by these of N429 (0.52, 0.50, 0.87, 0.28). The families of the remaining four male clones had GCAs less than 0, while GCA regarding leaf roundness of the families with N020, N139 and N430 as their male parents was the largest (0.86, 0.87).

Table 5. General combining ability (GCA) and special combining ability (SCA) on leaf morphology traits of 8 families resulting from *P. simonii*×*P. nigra*.

| Traits            | Female                  | SCA                      | GCA       |
|-------------------|-------------------------|--------------------------|-----------|
|                   | N020       | N139       | N151       | N188       | N429       | N430       |           |
| **Leaf area**    | 1-XY       | −0.03      | −0.01      | −0.36      | −0.56      | -          | -          | −0.36     |
|                   | XY-5       | -          | -          | -          | −0.50      | -          | -          | −0.09     |
|                   | ZL-3       | -          | -          | -          | −0.22      | 0.63       | 1.06       | 0.45      |
|                   | GCA        | −0.03      | 0.05       | −0.71      | −1.18      | 0.52       | 1.36       |           |
| **Leaf perimeter**| 1-XY       | −0.08      | −0.06      | −0.11      | −0.08      | -          | -          | 0.03      |
|                   | XY-5       | -          | -          | -          | −0.15      | -          | -          | −0.04     |
|                   | ZL-3       | -          | -          | -          | −0.12      | 0.28       | 0.33       | 0.01      |
|                   | GCA        | −0.26      | −0.20      | −0.32      | −0.30      | 0.50       | 0.58       |           |
| **Leaf length**  | 1-XY       | −0.13      | −0.13      | −0.32      | −0.26      | -          | -          | −0.01     |
|                   | XY-5       | -          | -          | -          | −0.21      | -          | -          | 0.14      |
|                   | ZL-3       | -          | -          | -          | −0.29      | 0.52       | 0.81       | −0.03     |
|                   | GCA        | −0.40      | −0.36      | −0.79      | −0.75      | 0.87       | 1.43       |           |
| **Leaf width**   | 1-XY       | 0.01       | 0.07       | −0.25      | −0.41      | -          | -          | −0.32     |
|                   | XY-5       | -          | -          | -          | −0.32      | -          | -          | −0.01     |
|                   | ZL-3       | -          | -          | -          | −0.14      | 0.41       | 0.63       | 0.33      |
|                   | GCA        | 0.05       | 0.24       | −0.45      | −0.84      | 0.28       | 0.73       |           |
| **Leaf roundness**| 1-XY       | 0.24       | 0.24       | 0.04       | −0.28      | -          | -          | −0.53     |
|                   | XY-5       | -          | -          | -          | 0.09       | -          | -          | 0.15      |
|                   | ZL-3       | -          | -          | -          | 0.21       | −0.36      | −0.17      | 0.38      |
|                   | GCA        | 0.86       | 0.87       | 0.47       | −0.12      | −1.22      | 0.86       |           |

Note: The values in bold indicated that GCA or SCA regarding leaf traits were the maximum among the families.

Based on the mixed linear model, the breeding values of eight families (i.e., SCA) were obtained (Table 6). The results showed that the highest SCA observed with respect to leaf area of crossing combination ZL-3×N430 (1.06) among the eight families. Among the families of ZL-3 with high GCAs, ZL-3×N430 (1.06, 0.33, 0.63) and ZL-3×N429 (0.63, 0.28, 0.41) had higher SCAs regarding leaf area, perimeter and width. For the trait of leaf length, the highest SCA appeared in the crossing combination ZL-3×N430 (0.81), but the GCAs of the families with ZL-3 as female parent were negative values. The same went for the leaf roundness. The highest SCA appeared in two crossing combinations, 1-XY×N020 (0.24) and 1-XY×N139 (0.24), which fully reflected the heterosis and indicated the greater degree of family differentiation obtained in this experiment. Two crossing combinations, ZL-3×N430 and ZL-3×N429, had the top two breeding values above 0 with regard to leaf size traits, which indicated that in these two families' leaf size traits were strongly controlled by heredity.
Table 6. Ranking with breeding value (BV) based on mixed linear model for 8 families.

| No. | Leaf Area | Leaf Perimeter | Leaf Length | Leaf Width | Leaf Roundness |
|-----|-----------|---------------|-------------|------------|----------------|
|     | Family BV| Family BV     | Family BV   | Family BV  | Family BV      |
| 1   | ZL-3×N430| 1.06          | ZL-3×N430   | 0.33       | ZL-3×N430      | 0.63           | 1-XY×N139 | 0.24 |
| 2   | ZL-3×N429| 0.63          | ZL-3×N429   | 0.28       | ZL-3×N429      | 0.52           | 1-XY×N020 | 0.24 |
| 3   | 1-XY×N139| −0.01         | 1-XY×N139   | −0.06      | 1-XY×N139      | −0.13          | 1-XY×N139 | 0.07 |
| 4   | 1-XY×N020| −0.03         | 1-XY×N020   | −0.08      | 1-XY×N020      | −0.13          | 1-XY×N020 | 0.01 |
| 5   | ZL-3×N188| −0.22         | ZL-3×N188   | −0.08      | XY-5×N188      | −0.21          | ZL-3×N188 | 0.21 |
| 6   | 1-XY×N151| −0.36         | 1-XY×N151   | −0.11      | 1-XY×N151      | −0.26          | ZL-3×N430 | −0.17 |
| 7   | XY-5×N188| −0.50         | ZL-3×N188   | −0.12      | XY-5×N188      | −0.29          | XY-5×N188 | −0.32 |
| 8   | 1-XY×N188| −0.56         | XY-5×N188   | −0.15      | 1-XY×N151      | −0.32          | 1-XY×N188 | −0.41 |

Note: The values in bold indicated that GCA or SCA regarding leaf traits were the maximum among the families.

According to the ranking breeding values of different traits in the parental clones and the progenies (Table 7), the hybrid genotypes E4-40, E4-39 and E4-42 from family ZL-3×N188, E5-124 from family ZL-3×N429 and E6-14 from family ZL-3×N430, had ZL-3 as their female parent and appeared in the top 10 for the four leaf size traits, which indicated that the female parent ZL-3 had strong genetic superiority. In the breeding value ranking of leaf roundness, two male paternal clones, N139 and N020, were at the ranked 5th and 6th, respectively, indicating that these two male paternal clones have strong additive effect on leaf roundness.

Table 7. Ranking with breeding value (BV) based on mixed linear model for parent clones and F1 genotypes.

| No. | Leaf Area | Leaf Perimeter | Leaf Length | Leaf Width | Leaf Roundness |
|-----|-----------|---------------|-------------|------------|----------------|
|     | Genotype BV| Genotype BV   | Genotype BV | Genotype BV| Genotype BV   |
| 1   | E4-40     | 9.36          | E4-40       | 2.14       | E4-40         | 5.47           | E4-42         | 4.76          | C3-102 | 0.97 |
| 2   | E4-39     | 7.70          | E4-42       | 2.04       | E4-39         | 5.11           | E6-14         | 4.39          | E6-48  | 0.89 |
| 3   | E4-42     | 6.98          | C3-95       | 1.91       | E6-38         | 4.88           | E4-39         | 4.10          | C3-226 | 0.89 |
| 4   | E6-38     | 6.80          | E5-124      | 1.86       | E4-42         | 4.66           | C3-53         | 3.96          | C4-90  | 0.87 |
| 5   | C3-95     | 6.52          | E4-39       | 1.79       | E5-190        | 4.51           | C3-95         | 3.85          | N139   | 0.87 |
| 6   | C3-53     | 6.22          | E5-190      | 1.78       | C3-95         | 4.38           | E6-34         | 3.75          | C4-85  | 0.85 |
| 7   | E5-124    | 6.07          | C3-53       | 1.75       | E5-124        | 4.33           | E4-40         | 3.61          | C4-85  | 0.85 |
| 8   | E5-190    | 6.05          | E6-14       | 1.68       | D4-67         | 4.26           | E5-124        | 3.43          | C4-236 | 0.84 |
| 9   | E6-14     | 6.02          | D4-67       | 1.67       | E6-14         | 4.25           | C4-222        | 3.37          | E6-35  | 0.83 |
| 10  | E5-69     | 5.73          | E5-128      | 1.63       | C3-53         | 3.97           | E6-51         | 3.28          | C3-290 | 0.81 |

Note: C1, C2, C3, C4, D4, E4, E5 and E6 represented hybrid combinations 1-XY×N020, 1-XY×N139, 1-XY×N151, 1-XY×N188, XY-5×N188, ZL-3×N188, ZL-3×N429, ZL-3×N430, respectively. The values in bold indicated that GCA or SCA regarding leaf traits were the maximum among the families.

3.4. Phenotypic and Genetic Correlations between Leaf Morphology Traits

The results of correlation analysis on five leaf traits indicated that both genetic and phenotypic correlation coefficients between leaf size traits (leaf area, perimeter, length, width) of hybrid progenies were greater than 0.910 significantly (Figure 2). Leaf roundness exhibited strong negative correlations with four leaf size traits which ranged from −0.439 to −0.233 (p < 0.001).
1. Variation of Leaf Traits of the Parental Clones and F1 Progenies

The leaf morphology trait in *Populus* varies extensively and significantly among sections, species and clones under strong genetic control [24,25,36]. Overall, the performance results together with large variation in leaf parameters showed that considerably higher genetic gain is possible with larger leaf size and higher resistance. In the study, intraspecific variation of leaf traits was significant between the female parental clones of *P. simonii* and the male parental clones of *P. nigra*. *P. simonii* (section *Tacamahaca*) with distribution of arid and cold areas in Inner Mongolia revealed the clonal variation of smaller leaves, while *P. nigra* (section *Aigeiros*) naturally distributed in humid and warm regions of Europe showed the clonal variation of larger leaves. Heritability estimates of four leaf size traits of the male parental clones (*P. nigra*) were greater than these of the female parental clones (*P. simonii*), indicating that the measured traits were under low genetic control in the female parents which contained small additive genetic variance components and were largely influenced by environment. High variable environmental conditions such as climate and soil in different sites exert a large influence on the phenotypic variation and plasticity of the parental clones with an adaptive advantage. In addition, the F1 progenies of eight families from the crossing of three female parental clones of *P. simonii* and six male parental clones of *P. nigra* showed significant variation of leaf traits among families and among genotypes within families. Leaf size traits of the F1 progenies were moderate as compared with their female and male parental clones while some progenies were larger than their parents with regard to leaf traits, indicating that the phenomenon of super heterosis was controlled by strong heredity [4,35,67,68].

Remarkable phenotypic variation of leaf shape and size reflects the natural selection operating function, which is considered to be the result of adaptation to a particular environment [3]. Leaves play a major role in photosynthesis, as the light capturing organ and the site of photochemical reactions, and are responsible for most of the carbon fixation in a plant and therefore are critical factors influencing...
plant success. To absorb sufficient light energy, leaves must be as wide as possible, while leaves must be as flat and thin as possible to facilitate gas exchange [69]. Leaf shape is considered as a highly complex trait controlled by a large number of loci, each contributing only a small effect; these loci likely act via modulation of gene expression [16] and hence the substantiated obvious additive effect of leaf traits. Drost et al. [36] found in their study of backcross population (BC$_1$) between _P. deltoides_ and _P. trichocarpa_ that leaf width and aspect ratio represented the vast majority of information on leaf shape variation in BC$_1$, which was the main difference of leaf shapes between their parents. In addition, the range of variation with regard to leaf shape of hybrid progenies mostly depended on the difference between their parents [4], which probably provides the perspective of utilization in the heterosis of breeding. Moreover, the data presented by Tsarev et al. [70] found that for different genotypes, even for the same tree species, the results of hybridization were different, and the difference of hybrids with different hybridization variants was significant. This indicates that parental differences as well as variation took a significant effect on the genetic variation of leaf morphology traits in the F$_1$ progenies. An optimistic outlook was retained on the abundant variation among progeny populations which would stabilize the genetic properties, substantiate greater selection potential and improve the efficiency and effect of breeding.

4.2. Combining Ability and Parental Evaluation of Parent Cross Combinations

Based on the mixed linear model, the GCA of the leaf morphological traits of eight crossing combinations between _P. simonii_ and _P. nigra_ were obtained by using the BLUP method. The breeding values (GCA) regarding leaf area, width and roundness of the female parental clone ZL-3 (_P. simonii_) were the largest and above 0.33. Similarly, the female clone 1-XY could pass on a large leaf perimeter to their offspring. Among the male parental clones of _P. nigra_, the GCA of leaf size traits of N429 and N430 were above 0.28, while those of N188 and N151 were below -1.18. The male parental clones, _P. nigra_ N430 and N429, showed higher GCAs in cross combinations, ZL-3×N430 and ZL-3×N429. Interestingly, however, the female parental clones, such as ZL-3, showed lower GCAs but the highest SCAs of leaf length in the crossing combination with N430, which indicated a certain degree of heterosis and strong genetic control. By the breeding value ranking the families, two topcross combinations were selected, i.e., ZL-3×N430 and ZL-3×N429, whose leaf size traits had strong heritability. These two hybrid combinations can be used in poplar breeding and selection in the future.

We delightedly found widespread evidence of hybridization under natural conditions between the species of section _Aigeiros_ and section _Tacamahaca_ with high occurrence of hybrids characterized by rapid growth of section _Aigeiros_ and diversified resistance of section _Tacamahaca_. Typically, high crossing compatibility among the interspecific hybridization between the species of both sections made it therefore become an important direction to carry out resource-saving resistance breeding for the diversity of ecological conditions in poplar cultivation areas in China. In terms of combining ability, assessment of GCA and SCA from the crossings of superior parental clones is probably more important than their own characters. The families and the clones should be focused on rather than poplar hybrid types and parental species alone [71]. There has been cases where the combining ability of the superior parental clones was high; their hybrids probably showed heterosis on the superior characters of parents [72–74]. Therefore, as long as the parents were carefully selected, even though the GCA of the parents was not high, the best effect of hybridization was probably obtained by combining with breeding target and using the crossing combination of the parents with high SCA [70].

4.3. Correlation and Early Selection of Leaf Morphological Traits between Parents and F$_1$ Progenies

Both the genetic and phenotypic correlation coefficients among four leaf size traits (leaf area, perimeter, length, width) of hybrid progenies were all significant and above 0.9 in the study. The strongest relationship among all correlations was presented between leaf area and length, at an extremely significant level. Moreover, leaf area is known to be an important functional trait, the relative importance of which is significantly correlated with leaf length and width [75], and even
became a major determinant of leaf shape [28]. The proportional relationship between leaf area and the product of leaf length and width also generally remained stable during leaf evolution [75]. As the characters of early rapid growth, strong ability of photosynthetic and biosynthesis, and large production in a single growing season, poplar is extremely well suited to high-yield biomass production [35]. Strong positive correlations between leaf traits and plant growth indicated that leaf area development and leaf size were the robust indicator of biomass production in Populus [13,76–78], therefore the larger their leaf size the greater potential is for larger growth. It has been widely confirmed that biomass yield is dependent on leaf number and leaf area, whatever the growth conditions of P. deltoides and P. euramericana as well as their hybrids [77,79,80]. On the other hand, the leaf increment rate and the largest leaf area appeared to be robust indicators of biomass production which can readily promote poplar growth regardless of the conditions and species [77,80,81]. Leaf area therefore can be used as an early indirect selection index for high yield of future poplar breeding and improving [12,14,82]. For further correlation studies, different and various leaf traits, e.g., leaf dry weight, leaf number, leaf thickness, specific leaf area, petiole traits, leaf anatomy and stomatal characteristics, allowed us to comprehensively and systematically analyze the correlation between leaf shape and growth which made leaf traits an alternative to precise and direct selection for biomass production.

Based on the mixed linear model, using the BLUP method to estimate the breeding value makes the selection of the poplar breeding more accurate and provides a more reliable theoretical basis for the selection and mating of hybrid parents and the design of breeding strategies. The results of leaf morphological traits in the study will be used as important phenotypic traits for constructing genetic maps in poplar and association analysis (GWAS), together with a recent boom in high through-put technologies. These can lay a foundation for the potential genetic and molecular mechanism which controlled leaf morphological variation as well as further mapping quantitative trait loci (QTL) [83].

5. Conclusions

In this study, intraspecific and interspecific variations of leaf morphology traits were significant between and among the parental clones of P. simonii and P. nigra. The F1 progenies showed intermediate morphological characteristics between those of their parents, but they also displayed heterosis on leaf traits under strong genetic control. Leaf morphology traits were under strong genetic control. The heritability estimates showed that leaf area was the most heritable trait, followed by leaf width. The significant genetic and phenotypic correlations were found between five leaf morphology traits with coefficients above than 0.9, except for leaf roundness. Based on the mixed linear model, two male parental clones (N430 and N429) had the largest breeding value (GCA) for leaf size traits. The SCA of the crossing combination between P. simonii cl. ZL-3 and P. nigra cl. N430 was greater than that of other crossing combinations.

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