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ARTICLE

Diversity Assessment of an Endemic *Carpinus oblongifolia* (Betulaceae) Using Specific-Locus Amplified Fragment Sequencing and Implications for Conservation

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ABSTRACT

Carpinus oblongifolia is an endemic species and the extant wild populations show a fragmentation distribution in the Baohua Mountain of Jiangsu Province in eastern China. Understanding of genetic diversity plays an important role in *C. oblongifolia* survival and sustainable development. The wild *C. oblongifolia* population was artificially divided into four subpopulations according to the microhabitats, and another two subpopulations were constructed by progeny seedlings cultivated with the mature seeds. Then, the leaf buds of 80 individuals from six subpopulations were sampled to develop single nucleotide polymorphisms (SNPs) using specific-locus amplified fragment sequencing (SLAF-seq). Based on these SNPs, we aimed to characterize the genetic diversity and population structure of *C. oblongifolia* and provide an illumination and reference for effective management of such a small endemic population. The level of genetic diversity was low at the species level, and the progeny subpopulations had a relatively higher genetic diversity than the wild subpopulations. This may be attributed to a high gene flow and an excess heterozygosity to reduce the threat of genetic drift-based hazards. Moreover, the progeny subpopulations had the ability to form new clusters and a great contribution to the genetic structure variation of *C. oblongifolia*. These results will assist with the development of conservation and management strategies, such as properly evacuating competitive trees to provide more chance for pollen and seed flow *in situ* conservation, and establishing sufficient seedling plantlets under laboratory conditions for reintroduction to enlarge the effective population size.

KEYWORDS

Carpinus oblongifolia; endemic species; genetic diversity; population structure; SLAF

1 Introduction

Carpinus species are very abundant worldwide and approximately 40 species have been reported, of which the majority are from Asia, especially China [1]. *Carpinus oblongifolia* (Hu) Hu & W. C. Cheng (Betulaceae) is an endemic species, which is only distributed in the Baohua Mountain of Jiangsu Province in eastern China (east longitude 118°58′–119°58′, north latitude 31°37′–32°19′, Fig. 1A according to the records from *Flora of China* [2]. Baohua Mountain is one of the preserved areas of subtropical vegetation with relatively complete natural ecosystems; the main vegetation types are

deciduous evergreen broadleaved mixed forests [3], which contain some endemic, wild rare plant resources such as *Yulania zenii* (W. C. Cheng) D. L. Fu (Magnoliaceae), an identified 'critically endangered' plant species by the China Expert Workshop (2014) in IUCN (https://dx.doi.org/10.2305/IUCN.UK.2014-1.RLTS.T32427A2818816.en). The highest peak of Baohua mountain is 437 m above sea level. According to our recent field investigations, the extant wild *C. oblongifolia* population distributes at an altitude of 170–300 m along roads, paths and ravines in the northeast hillsides, within an area of 3 km^2, with approximately 130 trees [3]. *Carpinus oblongifolia* is a monoecious deciduous tree (up to 12-m tall), blooms from end March to early April with wind-pollinated flowers and ripens in mid-October with wing-like seeds. Despite the scarcity, this species is not yet among the rare or endangered plants in China for the time being, and its genetic background (essential to create management strategies) has never been studied. The incomplete diameter class structure, especially with insufficient number of seedlings and saplings at the young stage, and the non-dominant importance values in the wood layer indicated that the population structure of *C.oblongifolia* is unstable [3]. This will affect its long-term survival and evolution in changing environments. Furthermore, only a few wild trees with a high diameter at the breast height were found to produce seeds during our field investigations. The regeneration ability of the progeny will directly affect the population maintenance and development.

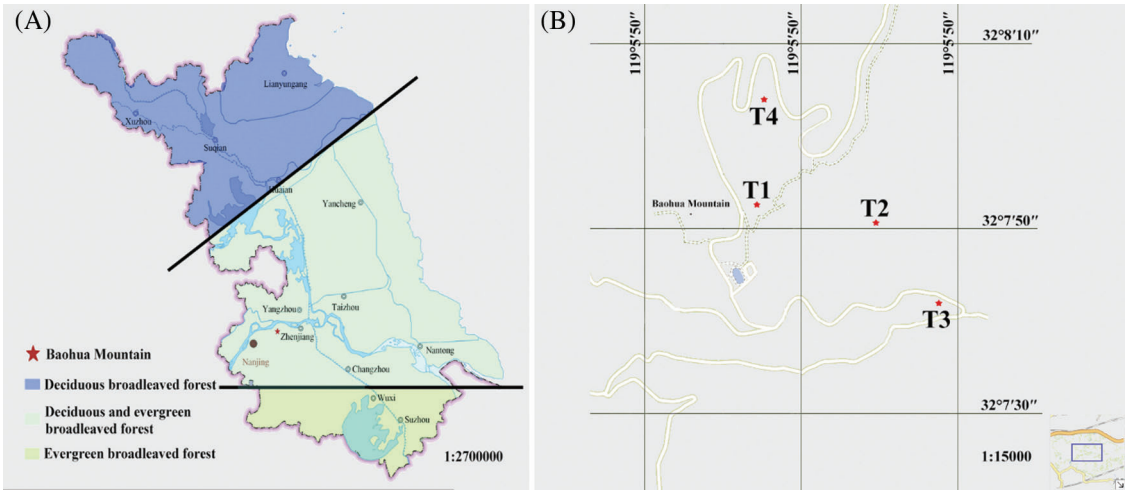

Figure 1: Location of study area. (A) Location of Baohua mountain in Jiangsu province. (B) Location of four wild subpopulations of *C. oblongifolia* in the core of its distribution

Genetic diversity is a fundamental component of biological diversity. Knowledge of the genetic background of a plant species plays an important role in its survival and sustainable development [4], especially for endemic species affected by habitat fragmentation. Habitat fragmentation has been generally recognized as a major threat to plant population survival, and a decreased genetic diversity may limit a species' ability to adapt to different environmental conditions [5]. Based on previous studies on endemic species, genetic divergences were expected between endemic populations even on small islands [6,7]. Small, isolated populations are particularly vulnerable to the effects of inbreeding, genetic drift and reduced genetic diversity. Genetic diversity is suggested to be the forefront of conservation policies and management [8]. Numerous previous studies have mainly focused on the genetic diversity of fragmented populations. For example, *Parrotia subaequalis* (H. T. Chang) R. M. Hao & H. T. Wei (Hamamelidaceae), an endemic species distributed in eastern China, is identified as a 'critically endangered' plant species in IUCN, and *in situ* and *ex situ* conservation plannings were implemented based on genetic data of the population [9,10]. Therefore, understanding the genetic background of

C. oblongifolia will help to evaluate and develop its conservation strategies for maintaining the biodiversity and ecosystems of the Baohua Mountain.

Single nucleotide polymorphisms (SNPs) are the most abundant type of genetic markers in most genomes, and are considered an ideal molecular marker for evaluating the genetic background [11]. Next-generation sequencing technology can generate high-density SNP datasets for most analyzed organisms and provide a viable new strategy for studying population genetics [12]. High-throughput specific-locus amplified fragment sequencing (SLAF-seq) is an accurate and cost-effective sequence-based SNP identification method [13,14] that has been conducted on a wide variety of plants since its first use in several varieties of *Thinopyrum elongatum* (Host) D.R. Dewey (Poaceae) [15]. SNPs can be used to more precisely address DNA variations at the population and individual levels than traditional methods, because SLAF-seq technology deepens sequencing depth and ensures genotyping accuracy [13,16]. This is especially suitable for species without reference genomes and can effectively overcome genomic complexity. In this study, we developed genome-wide SNP markers using SLAF-seq technology for a *C. oblongifolia* population with 80 individuals of four wild subpopulations and two progeny subpopulations. Genetic diversity and population structure were estimated by 1,267,011 newly developed genome-wide SNPs. Our study aimed to elucidate the genetic background of the *C.oblongifolia* population, which would provide an illumination and reference for effective management of such a small endemic population.

2 Methods

2.1 Plant Materials

The extant wild *C. oblongifolia* population was artificially divided into four subpopulations based on the microhabitats in the core of its distribution (Fig. 1B). Then, bulked healthy leaf buds were collected from 10 wild trees (diameter at breast height > 2.5 cm to avoid obvious progeny plants as much as possible) in each subpopulation. A total of 40 wild trees were sampled, which represented nearly one-third of the extant population and these samples were dried in silica gel and stored at −80°C before DNA extraction. On the other hand, mature seeds were collected from one individual wild tree on each T3 and T4 subpopulations in mid-Oct, respectively. Then, these seeds were cultivated at the greenhouse during one year to obtain true progeny seedlings. After 8 months, 20 progeny seedlings from each of the T3 and T4 subpopulations were arbitrarily selected, which were correspondingly named T6 and T5 subpopulations. The leaf buds of these seedlings were sampled separately. Finally, 80 *C. oblongifolia* individuals including 40 wild trees and 40 progeny seedlings were used for DNA extraction and SLAF-seq analysis. Detailed information on the sampling site is listed in Table 1.

Table 1: Detailed information of six *Carpinus oblongifolia* subpopulations

Subpopulation	Longitude	Latitude	Altitude (m)	Slope (°)	Microhabitats	Sample size (N)	Note
T1	E119°05′24″	N32°07′57″	222	26.9	Path edge	10	Wild tree
T2	E119°05′33″	N32°07′52″	174	25.3	In the forest	10	Wild tree
T3	E119°05′23″	N32°07′59″	210	31.4	In the forest	10	Wild tree
T4	E119°05′45″	N32°07′41″	293	20.3	Roadside	10	Wild tree
T5	–	–	–	–	–	20	T4 progeny
T6	–	–	–	–	–	20	T3 progeny

2.2 DNA Extraction and SLAF-Seq

The DNA was isolated *via* the CTAB protocol [17]. The DNA concentration and quality of all samples were examined using a NanoDrop-2000 UV-Vis spectrophotometer (Thermo, USA), and DNA samples were diluted to 100 ng/μl for SLAF-seq. The isolated genomic DNA was then digested with the restriction enzyme RsaI + EcoRV-HF®, which was used for *Juglans regia* L. (Juglandaceae) [18], a closest relative-related genus in an all known genomes. The enzyme digestion efficiency was 93.49% using the known genome (*Oryza sativa* subsp. *japonica*) as the control. The paired-end mapping of the control genome showed 97.40% of double-end comparison using SOAP (version 2.1.7) [19]. The obtained SLAF tags were linked to Dual-index sequencing adapters and a SLAF sequencing library was constructed according to procedures described by Sun et al. [13] with a few modifications. The paired-end sequencing was performed on the Illumina HiSeq 2500 sequencing platform (Illumina Inc., USA) at Beijing Biomarker Technologies Corporation (Beijing, China).

2.3 Data Processing and SNP Markers Development

The raw output produced by Illumina HiSeq 2500 was further analyzed for each sample using Dual-Index [20]. After adapter sequences were filtered by Fastx–toolkit (version 0.0.13) [21], the data quality was estimated by calculating GC content and Q30 ($Q = -10 \times \log10e$; this indicates a 0.1% chance of an error). SLAF tag number and depth of each sample were evaluated. The sample with the most tags was used as the reference. Polymorphic SLAF tags were determined by comparing the sequence variation between different individuals, and were mapped to the reference sequence using the Burrows–Wheeler Alignment (BWA, version 2.1) tool [22]. Alleles were defined in each SLAF by minor allele frequencies > 0.05. SNP markers were identified from polymorphic SLAF tags using GATK (version 4.0) [23] and SAMTOOLS (version 2.6.2) [24]. Only SNP markers called by both methods were considered to have a high consistence in the sequencing population.

2.4 Statistical Analyses

After SNP pretreatment, genetic diversity parameters including the number of observed alleles (Ao) and expected alleles (Ae), observed heterozygosity (Ho), expected heterozygosity (He), genetic diversity (Ne), Shannon's diversity index (I), polymorphism information content (PIC) and inbreeding coefficient (Fis) were calculated for species and each subpopulation using calculation scripts developed by Biomarker Technologies Corporation (Beijing, China). The hierarchical analysis of molecular variance (AMOVA) was estimated and used to partition the genetic variance among subpopulations and within individuals in ARLEQUIN (version 3.5.2.2) [25]. The fixation index (Fst), as a measure of population divergence, was also estimated in ARLEQUIN.

Bayesian clustering was applied to analyze the population structure of the 80 individuals of *C. oblongifolia* using ADMIXTURE (version 1.3.0) [26]. Based on the same set of SNPs, the number of subgroups (K) was predicted from 1 to 10, and the number of ancestors was determined according to the position of the minimum value, with an error rate obtained from cross-validation (CV). Then, a clustering tree was constructed in MEGA X (version 10.0.2) [27] using the neighbor-joining (NJ) algorithm (Kimura 2-parameter model, 1000 bootstrap replicates) [28]. Principal component analysis (PCA) was also carried out to identify the genetic similarity of the populations using EIGENSOFT (version 5.0.1) [29].

3 Results

3.1 SLAF-Seq Summary

A total of 474.64 Mb of reads were obtained after filtering and removing adaptor sequences from SLAF-seq of the 80 *C. oblongifolia* individuals. Among these reads, 94.74% of sequencing bases were high-quality with quality scores of 30 (Q30, which indicates a 0.1% error rate), and the average

guanine–cytosine (GC) content was 38.09%. The distribution of SLAF tag lengths was shown in Supplementary Fig. 1, and most tags were between 314–364 bp. In the wild subpopulations T1–T4, the number of SLAFs for each individual ranged from 131,315 to 323,600 (average, 187,553), and the SLAF sequencing depth ranged from 18.1-to 68.8-fold (average, 32.8-fold). In the progeny subpopulations T5 and T6, the number of SLAFs for each progeny ranged from 135,042 to 273,972 (average, 175,442), and the SLAF sequencing depth ranged from 16.0-to 36.0-fold (average, 26.0-fold; Supplementary Fig. 2). Altogether, 1,124,547 SLAFs were obtained, of which 222,092 were polymorphic SLAFs (19.7%) with an average sequencing depth of 29.45-fold. SLAF-seq alignment and clustering by both GATK and SAMTOOLS generated a total of 1,267,011 SNPs. Detailed information on the SLAF sequencing and SNP detection results for the 80 *C. oblongifolia* individuals is shown in Supplementary Table 1.

3.2 Genetic Diversity

Based on these high-quality SNPs, genetic parameters were separately estimated to evaluate *C. oblongifolia* genetic diversity. At the species level, the He and PIC were 0.287 and 0.237, respectively (Table 2). The He and PIC of 40 wild individuals were 0.285 and 0.229, and that of 40 progeny seedlings were 0.317 and 0.257, respectively. At the subpopulation level, T5 (He = 0.341, PIC = 0.275) had the highest genetic diversity, whereas T3 (He = 0.297, PIC = 0.244) had the lowest genetic diversity. The genetic diversity of the wild subpopulations T3 (He = 0.297, PIC = 0.244) and T4 (He = 0.317, PIC = 0.257) was lower than that of their progeny subpopulations T6 (He = 0.332, PIC = 0.268) and T5 (He = 0.341, PIC = 0.275), respectively. Moreover, the Ho was always higher than its He, and all values of Fis were negative in six subpopulations (Table 2).

Table 2: Genetic diversity parameters estimated from SNP markers of *C. oblongifolia* subpopulations

Subpopulation	N[a]	Ao	Ae	Ho	He	Ne	I	PIC	Fis
T1	10	2	1.554	0.408	0.330	0.348	0.500	0.266	−0.409
T2	10	2	1.547	0.418	0.324	0.342	0.492	0.261	−0.446
T3	10	2	1.482	0.341	0.297	0.314	0.461	0.244	−0.331
T4	10	2	1.524	0.365	0.317	0.334	0.484	0.257	−0.350
Wild subpopulation	40		1.448	0.293	0.285	0.302	0.468	0.229	−0.382
T5	20	2	1.572	0.407	0.341	0.350	0.514	0.275	−0.403
T6	20	2	1.557	0.397	0.332	0.341	0.503	0.268	−0.402
Progeny subpopulation	40	2	1.509	0.367	0.317	0.335	0.484	0.257	−0.397
Species level	80	2	1.454	0.305	0.287	0.289	0.450	0.237	−0.393

Note: N[a] = sample size. Ao = number of observed alleles. Ae = number of expected alleles. Ho = observed heterozygosity. He = expected heterozygosity. Ne = genetic diversity. I = Shannon's diversity index. PIC = polymorphism information content. Fis = inbreeding coefficient.

The hierarchical AMOVA revealed that 5.7% of the total molecular variation was attributed to among subpopulations, and 94.3% to within individuals ($P < 0.001$, Table 3). In addition, the Fst value was 0.057. Based on the Fst value, the number of migrants per generation (Nm) was 4.114.

3.3 Population Structure

Three methods were used to analyze the molecular variation of individual plants to assess the genetic structure of six subpopulations. Firstly, the membership fractions of the 80 *C. oblongifolia* individuals were estimated (K ranged from 1 to 10), and the results showed an optimum value of $K = 3$ according to

cross-validation (CV) errors. Comparison of population structures of the 80 individuals when K = 3 showed that most individuals from T1–T3 subpopulations clustered together, most individuals from T4 and T5 subpopulations clustered together, and the individuals of the T6 subpopulation formed another cluster (Fig. 2). Groups 1–3 included 35, 31 and 14 individuals, respectively (Table 4). Secondly, the NJ clustering tree showed two major groups (Fig. 3A). Group 1 included T1, T2, T3 and T6 subpopulations, and Group 2 contained T4 and T5 subpopulations. Finally, PCA separated the 80 individuals into two clusters (Fig. 3B). One cluster was made up of T1–T3 and T6 subpopulations, and the other cluster was mainly composed of T4 and T5 subpopulations.

Table 3: Distribution of genetic variation in six *C. oblongifolia* subpopulations based on analysis of molecular variance

Source of variation	df	Variance components	Percentage of variation(%)	Fst
Among subpopulations	5	338.1	5.7	0.057***
Within individuals	80	5802.9	94.3	
Total	85	6141.0	100	

Note: Fst = fixation index. ***$P < 0.001$.

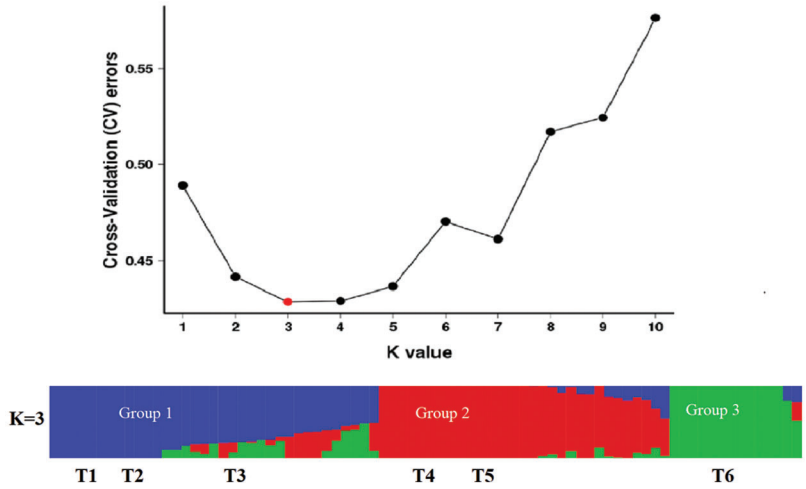

Figure 2: Analysis results of population structure. The estimated number of groups through cross-validation (CV) errors (*K* from 1 to 10) and the variation pattern of six subpopulations when *K* = 3. Different colors indicated different clustered groups

Table 4: Group distributions of different subpopulations

Subpopulations	Group 1	Group 2	Group 3
T1	10	0	0
T2	10	0	0
T3	6	3	1
T4	2	8	0
T5	0	20	0
T6	7	0	13
Total	35	31	14

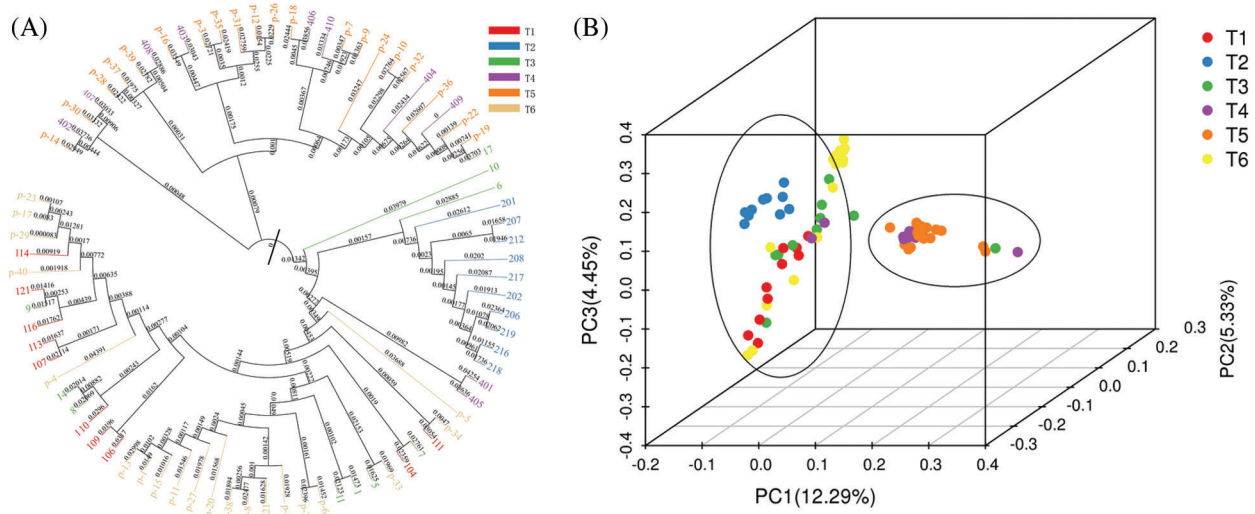

Figure 3: Characterization of the genetic structure of 80 *C. oblongifolia* individuals. (A) Phylogenetic tree of the 80 individuals based on analysis of the 1,267,011 SNPs. The number in the figure represent the branch length. (B) PCA plot of the six subpopulations based on analysis of 1,267,011 SNPs

Further analysis found that the Group 3 identified by ADMIXTURE analysis was integrated into the Group 1 in the NJ clustering tree and PCA analysis. Moreover, the group 3 by ADMIXTURE analysis were mostly from the T6 and T3 progeny subpopulations (Table 4). Therefore, the genetic structure of T1–T4 subpopulations, T3 and its progeny T6 subpopulations, T4 and its progeny T5 subpopulations, was separately analyzed. Four wild subpopulations showed an optimum value of $K = 1$ and one group of PCA plot (Fig. 4), T3 and its progeny T6 subpopulations displayed an optimum value of $K = 2$ and two clusters of PCA plot (Fig. 5), and T4 and its progeny T5 subpopulations showed an optimum value of K = 1 and one group of PCA plot (Fig. 6).

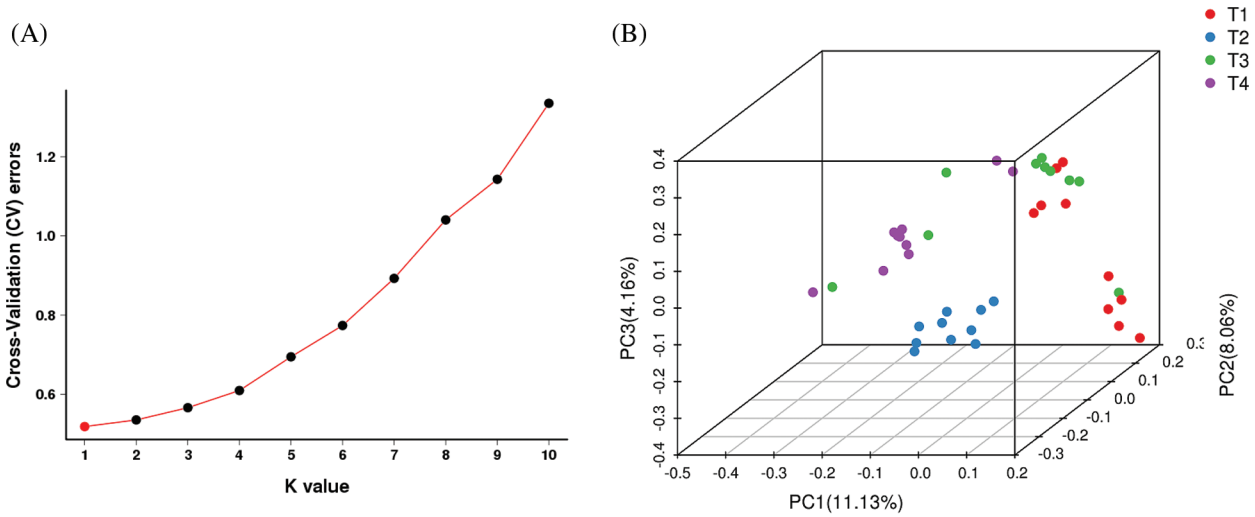

Figure 4: Variation in the genetic structure of four wild *C. oblongifolia* subpopulations. (A) Estimated *K* value based on cross-validation errors. (B) PCA plot of the four subpopulations

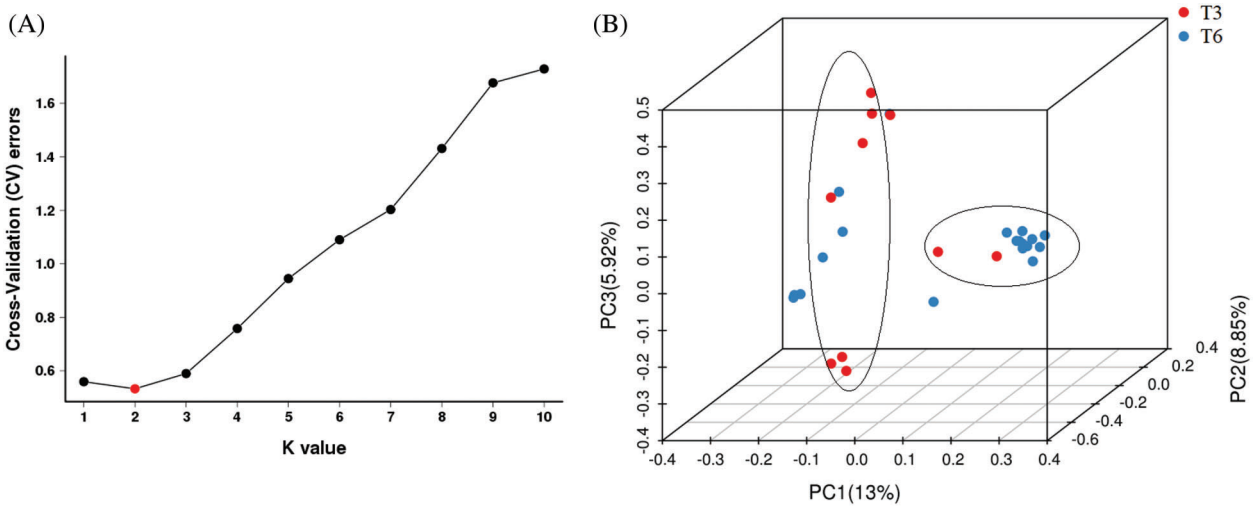

Figure 5: Variation in the genetic structure of T3 and its progeny T6 subpopulations. (A) The estimated K value ranged from 1 to 10 based on cross-validation errors. (B) PCA plot of all individuals in the T3 and its progeny (T6) subpopulations

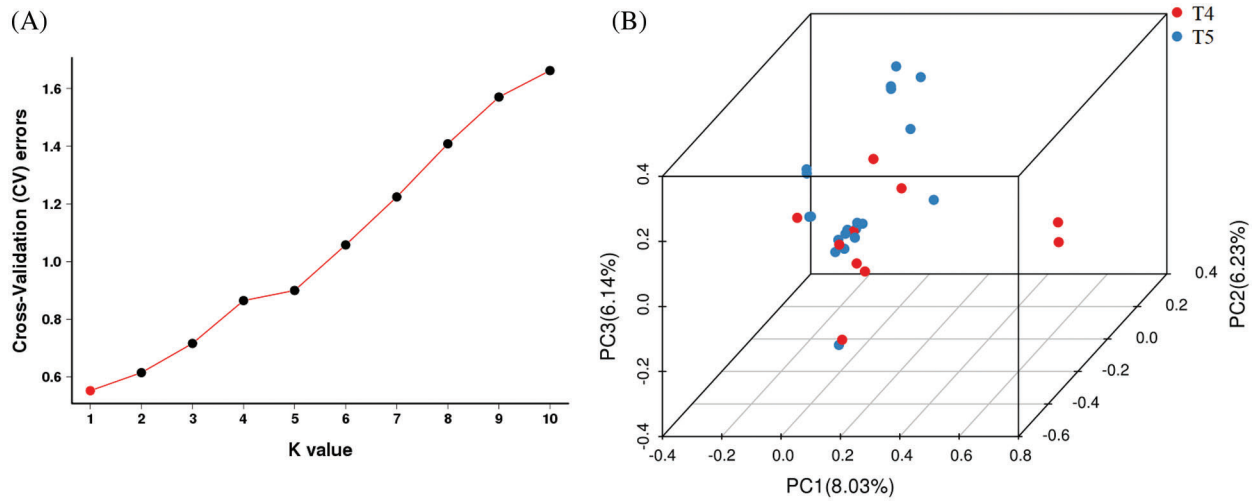

Figure 6: Variation in the genetic structure of T4 and its progeny T5 subpopulations. (A) The estimated K value ranged from 1 to 10 based on cross-validation errors. (B) PCA plot of all individuals in the T4 and its progeny (T5) subpopulations

4 Discussion

4.1 SNP-Marker Development Using SLAF-Seq

In this study, 80 *C. oblongifolia* individuals were analyzed by SLAF-seq with an average sequencing depth of 29.45-fold and Q30 value of 94.74%. This is the first application of SLAF-seq in *Carpinus*, a genus with no reference genome. Finally, a total of 1,267,011 SNPs were identified from the 222,092 polymorphic SLAFs. The number of SNPs identified from genome-wide range far exceeded the number of polymorphisms detected using traditional methods in *Carpinus*, such as fewer than 200 polymorphic bands in AFLP analyzing the genetic diversity of *Carpinus laxiflora* (Siebold & Zucc.) Blume (Betulaceae) [30] and *Carpinus betulus* L. (Betulaceae) [31] populations. The molecular markers

density of SLAF-seq indicates its superiority in analyzing genetic backgrounds, which satisfied the need in assessment of genetic diversity and population structure.

4.2 Genetic Diversity

Genetic diversity can reflect the potential of plants to adapt to environmental changes, and reveal the biodiversity and maintenance mechanisms of ecosystem functions. At the species level, the genetic diversity of 80 examined *C. oblongifolia* (He = 0.287) was lower than that of *C. betulus* using SSR technology (He = 0.309) [32] and AFLP markers (Hj = 0.333) [31] , and that of *C. laxiflora* based on microsatellite markers (He = 0.780) [33]. Moreover, compared with other endemic species, the He value of *C. oblongifolia* (He = 0.287) was lower than that of *Parrotia subaequalis* (He = 0.464) [10] and *Sinojackia rehderiana* Hu (Styracaceae) (He = 0.782) [34], and also lower than that of perennial (He = 0.650) and wind-dispersed (He = 0.610) endemic species [35]. Genetic diversity of a species is correlated with its geographic distribution, life form, breeding system, seed dispersal, gene flow, natural selection, and habitat fragmentation [36]. The relatively low level of genetic diversity in *C. oblongifolia* is probably attributed to its extremely narrow distribution, because the genetic diversity of species in small populations is lower than in large populations under influence of genetic drift and inbreeding [37,38].

However, the progeny subpopulations of *C. oblongifolia* had a relatively higher genetic diversity than the wild subpopulations (Table 2), even though the progeny seedlings were clearly derived from only one wild tree. It is possible that gene flow is responsible for the diversity within populations, because wind-pollinated plants have evolved a higher outcrossing rate than animal- or insect-pollinated species [39]. Pollen dispersal ability of wind-pollinated *C. laxiflora* is higher than that of insect-pollinated *Magnolia obovata* Thunberg (Magnoliaceae), although they have common migration histories in the same area [30]. Previous studies have indicated that species of Betulaceae are predominately outcrossing [40]. Moreover, the significantly negative values for Fis in wild trees and progeny seedlings demonstrated a higher excess heterozygosity than expected (Table 2). This was obviously different from that of *C. laxiflora* (Fis = 0.618), another species of the same genus [30]. Heterozygotes produced by frequent gene exchange might increase the genetic diversity in a population and reduce the incidence of deleterious gene homozygosity from inbreeding, because the breeding system has been shown to be a major determinant of plant genetic diversity [41]. Therefore, high gene flow and excess heterozygosity might be the features of *C. oblongifolia*, which help maintain the genetic diversity and reduce the threat of genetic drift-based hazards, such as the depletion of genetic variation and inbreeding depression.

4.3 Population Structure

Outcrossing system can guarantee that a few migrants per generation are sufficient to counter genetic differentiation [42]. These outcrossing species are generally characterized by low genetic differentiation between populations, as *Carpinus*. The Fst value reflecting genetic differentiation was 0.057 among *C. oblongifolia* subpopulations, which was even lower than that in *C. laxiflora* (Fst = 0.060) [30], *C. betulus* (Fst = 0.074) , and *Carpinus orientalis* Mill (Betulaceae) (Fst = 0.086) [31]. This was supported by the fact that genetic variation was mainly attributed to individual differences (Table 2). The difference among individuals was consistent with the high levels of gene flow among populations (Nm = 4.114). A high Nm may facilitate gene exchange between populations, which effectively inhibits genetic differentiation caused by genetic drift. Gene flow is a critical determinant of population genetic structure. In the analysis of genetic structure of three different methods, T4 subpopulation and its progeny T5 subpopulation always clustered together to form an independent group. The number of groups in ADMIXTURE analysis were one more than that in NJ clustering tree and PCA analysis, but the new cluster was mainly composed of the T6 subpopulation. Further analysis confirmed that the progeny T6 subpopulation had the ability to form new cluster. That is, the progeny seedlings have a great

contribution to the genetic structure variation of *C. oblongifolia*. Moreover, Ho was always higher than He, which indicated that *C. oblongifolia* could have experienced genetic bottlenecks [43]. The relatively narrow genetic background of *C. oblongifolia*, a limit number of parents, and the founder effect could lead to chain unevenness imbalance. Thus, it is feasible to enlarge the effective population of *C. oblongifolia* through more progeny seedlings to maintain health development and genetic diversity of the population.

4.4 Implications for Conservation and Management

Baohua Mountain was designated as a provincial nature reserve by the government of Jiangsu Province, which provides a certain amount of protection to these plants and their habitats; however, it is clearly insufficient, because threats are still present due to human activities. In addition, not much action has been taken yet, which creates uncertainty regarding the future viability of this endemic species. This indicates a need to construct a management plan.

Undoubtedly, *in situ* conservation is the most effective method to conserve species resources [44,45]. According to investigations, *C. oblongifolia* distributes fragmentated in mixed evergreen and deciduous broadleaved forests with a non-dominant importance value in the wood layer [3]. This makes it difficult for the pollen and seeds of *C. oblongifolia* to disperse over long distances in the forests, even though its seeds are small with wing-like structures that theoretically allow them to be dispersed over long distances to increase effective population size. However, the features of high gene flow and excess heterozygosity in *C. oblongifolia* population would help maintain the genetic diversity. The progeny subpopulations had a relatively higher genetic diversity than the wild subpopulations and had a great contribution to the genetic structure variation. However, the supply of sapling and seedlings in the extant wild population is really insufficient [3] and only few wild trees with higher diameter at breast height can grow seeds. Therefore, we suggest that the competitive trees should be properly evacuated to provide more chance for pollen and seed flow of *C. oblongifolia* to improve effective gene exchange. This consideration is especially important for *in situ* conservation because gene flow by pollen and seeds has considerable potential as an evolutionary force. Moreover, a detailed investigation that should be made is the further analysis of the environmental conditions that allow seedling and sapling establishment to provide an opportunity for the natural regeneration of the *C. oblongifolia* population.

In *ex situ* measures now underway, seeds are stored and living plants are grown at Nanjing Botanical Garden Mem. Sun Yat-sen, where is near Baohua Mountain. The relatively higher genetic diversity in the progeny subpopulations indicated that the replenishment of more seedling plants could enlarge the effective population size. Considering that there are only few saplings and seedlings in the extant wild population, seed quality and germination tests were conducted in the same research institute, which showed that the viability of seeds was very low. Therefore, we recommend that seeds should be collected as much as possible to cultivate sufficient seedling under laboratory conditions. Then these plantlets could be reintroduced to Baohua Mountain or located in neighboring nature reserves to enlarge the effective population size. Additional, different biotechnological approaches could be applied for conservation of endemic species [46]. The genetic diversity within the reintroduction population might be higher than that in its source population as reported in previous studies [47,48].

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Availability of Data and Materials: All raw data files are available from the NCBI database (SRA Accession No. PRJNA650558).

Authors' Contributions Conception and Design: YL, QW, SL; experiments: PW, SW, RY; data analysis: SL, PW and QW; writing: SL; revisions: all authors.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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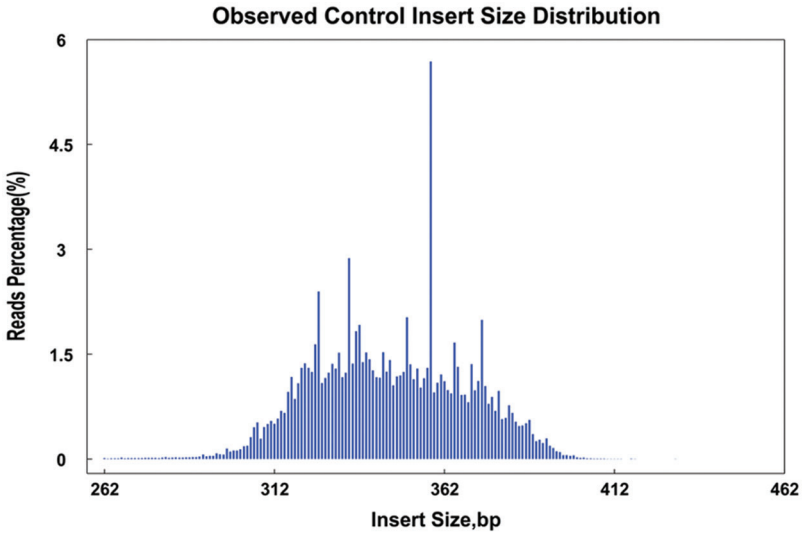
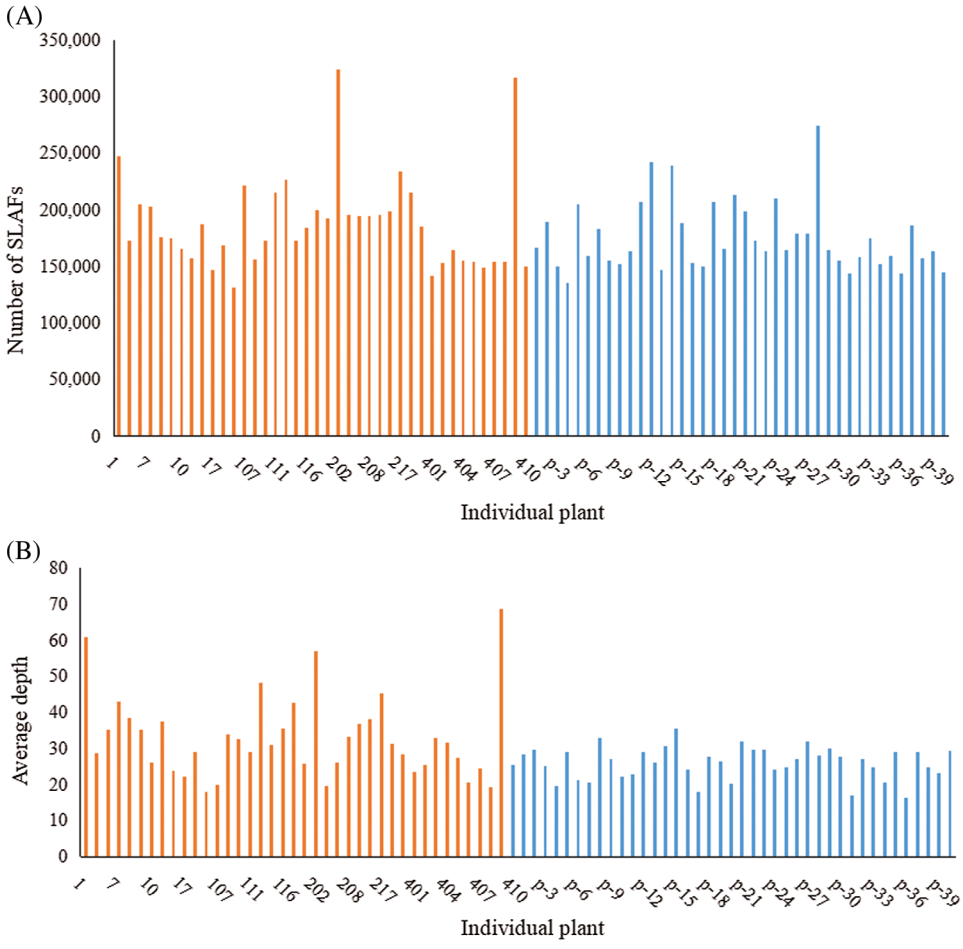
Supplementary Materials



Supplementary Figure 1: The length distribution of the SLAF tags. The abscissa represents the insertion fragment with different length. The ordinate represents the reads percentage



Supplementary Figure 2: Number and depth of SLAFs in wild and progeny populations. Horizontal axes in A and B indicate individual plants represented by different colors: orange, 40 wild population individuals; blue, 40 progeny population individuals. The vertical axes indicate the number and average depth of SLAFs in A and B, respectively

Supplementary Table 1: Summary of the sequencing and SNP detection results for 80 *Carpinus oblongifolia* individuals

Sample ID	Sub-population	Total reads	SLAF number	Total depth	Average depth	GC percentage (%)	Q30 percentage (%)	Total SNP	SNP number	Hetloci ratio (%)	Integrity ratio (%)
104	T1	5,225,947	168,626	4,891,335	29.01	38.28	93.15	1,267,011	508,158	14.6	40.1
106	T1	2,500,482	131,315	2,375,878	18.09	37.67	95.33	1,267,011	385,036	11.68	30.38
107	T1	4,824,269	221,479	4,441,112	20.05	40.09	95.12	1,267,011	707,121	16.06	55.81
109	T1	5,595,758	156,086	5,306,915	34	36.86	95.27	1,267,011	450,264	12.66	35.53
110	T1	5,943,910	172,725	5,608,147	32.47	37.48	94.84	1,267,011	519,857	13.22	41.03
111	T1	6,671,011	214,899	6,225,310	28.97	39.2	95.24	1,267,011	672,247	15.18	53.05
113	T1	11,709,661	226,075	10,928,485	48.34	37.71	94.4	1,267,011	654,977	15.44	51.69
114	T1	5,728,296	173,015	5,382,708	31.11	37.67	94.27	1,267,011	509,178	14.38	40.18
116	T1	6,909,769	184,090	6,533,284	35.49	38.27	95.3	1,267,011	524,283	13.44	41.37
121	T1	8,977,247	199,595	8,490,701	42.54	37.45	95.45	1,267,011	575,115	13.57	45.39
201	T2	5,255,229	192,405	4,951,557	25.74	38.58	95.33	1,267,011	595,204	14.03	46.97
202	T2	20,008,755	323,600	18,473,556	57.09	38.17	94.15	1,267,011	818,027	19.46	64.56
206	T2	4,102,371	195,118	3,810,140	19.53	39.32	95.24	1,267,011	621,848	13.86	49.07
207	T2	5,464,758	194,268	5,085,356	26.18	38.4	94.08	1,267,011	598,514	15.5	47.23
208	T2	7,095,976	194,444	6,481,423	33.33	38.36	91.8	1,267,011	596,958	15.55	47.11
212	T2	7,664,809	195,465	7,183,760	36.75	37.94	94.31	1,267,011	584,730	15.25	46.15
216	T2	8,085,118	198,524	7,583,057	38.2	37.76	94.38	1,267,011	586,567	15.26	46.29
217	T2	11,217,038	233,327	10,566,610	45.29	38.64	95.49	1,267,011	669,917	15.08	52.87
218	T2	7,217,725	215,334	6,753,472	31.36	38.85	95.28	1,267,011	668,109	15.32	52.73
219	T2	5,643,113	184,919	5,270,920	28.5	38.07	94.44	1,267,011	554,449	13.36	43.76
1	T3	16,016,050	246,795	15,051,514	60.99	39.49	94.64	1,267,011	779,869	19.69	61.55
5	T3	5,270,313	172,780	4,972,271	28.78	38.53	94.69	1,267,011	536,361	13.06	42.33
6	T3	7,762,542	204,371	7,197,598	35.22	38.28	94.12	1,267,011	640,205	15.35	50.52
7	T3	9,159,438	202,313	8,700,267	43	37.72	95.5	1,267,011	591,806	14.18	46.7
8	T3	7,131,615	175,397	6,736,010	38.4	37.29	94.56	1,267,011	528,942	13.35	41.74
9	T3	6,476,634	174,906	6,126,545	35.03	38.35	94.72	1,267,011	537,631	13.27	42.43
10	T3	4,731,026	165,166	4,307,972	26.08	38.19	91.51	1,267,011	511,828	14.09	40.39
11	T3	6,163,544	157,722	5,893,309	37.37	37.73	95.46	1,267,011	464,438	11.96	36.65
14	T3	4,748,450	187,755	4,463,540	23.77	38.8	95.15	1,267,011	586,362	13.33	46.27
17	T3	3,415,974	146,856	3,258,632	22.19	37.55	95.29	1,267,011	425,531	13.46	33.58
401	T4	3,522,939	141,338	3,320,600	23.49	37.38	95.29	1,267,011	417,255	10.76	32.93
402	T4	4,120,903	152,634	3,887,455	25.47	37.21	94.81	1,267,011	456,340	12.35	36.01
403	T4	5,730,061	164,729	5,440,233	33.03	36.72	95.39	1,267,011	470,990	13.5	37.17
404	T4	5,189,907	155,284	4,912,928	31.64	36.89	95.39	1,267,011	442,767	12.31	34.94
405	T4	4,467,710	154,095	4,210,085	27.32	37.3	95.23	1,267,011	447,866	12.98	35.34
406	T4	3,220,572	149,064	3,051,627	20.47	37.24	95.24	1,267,011	429,124	11.97	33.86
407	T4	4,016,632	154,612	3,800,263	24.58	36.63	95.32	1,267,011	446,376	12.74	35.23
408	T4	3,180,978	154,605	2,978,116	19.26	38.03	94.24	1,267,011	467,454	14.19	36.89
409	T4	23,328,454	316,637	21,774,780	68.77	38.4	95.12	1,267,011	840,551	19.52	66.34
410	T4	4,029,475	149,756	3,815,317	25.48	37.14	95.48	1,267,011	417,531	12.68	32.95

(Continued)

Supplementary Table 1 (continued)

Sample ID	Sub-population	Total reads	SLAF number	Total depth	Average depth	GC percentage (%)	Q30 percentage (%)	Total SNP	SNP number	Hetloci ratio (%)	Integrity ratio (%)
p-3	T5	4,020,533	150,478	3,798,210	25.24	37.25	95.16	1,267,011	440,906	12.22	34.79
p-7	T5	4,011,496	182,613	3,767,302	20.63	38.89	95.34	1,267,011	560,986	13.56	44.27
p-9	T5	4,368,487	152,312	4,144,389	27.21	37.42	95.18	1,267,011	439,844	12.02	34.71
p-10	T5	3,856,598	163,418	3,616,793	22.13	37.69	94.39	1,267,011	495,570	14.23	39.11
p-12	T5	7,571,605	241,898	7,030,995	29.07	38.32	94.93	1,267,011	694,487	16.52	54.81
p-14	T5	7,874,643	238,500	7,271,842	30.49	38.59	95.24	1,267,011	730,700	16.88	57.67
p-16	T5	3,934,211	153,518	3,718,889	24.22	37.43	95.27	1,267,011	439,646	13.32	34.69
p-18	T5	6,209,785	207,423	5,778,588	27.86	39.35	93.73	1,267,011	641,109	16.21	50.6
p-19	T5	4,741,220	165,928	4,400,203	26.52	38.67	92.04	1,267,011	525,975	12.28	41.51
p-22	T5	5,395,064	172,665	5,114,134	29.62	37.64	94.86	1,267,011	534,548	11.78	42.18
p-24	T5	5,471,591	210,513	5,083,792	24.15	38.77	95.2	1,267,011	670,486	15.53	52.91
p-26	T5	5,204,453	179,337	4,839,099	26.98	38.6	92.97	1,267,011	529,205	15.01	41.76
p-28	T5	8,612,029	273,972	7,707,038	28.13	37.56	94.38	1,267,011	678,099	14.82	53.51
p-30	T5	4,522,839	155,302	4,318,726	27.81	37.84	95.55	1,267,011	454,605	12.64	35.88
p-31	T5	2,559,280	143,683	2,424,602	16.87	37.84	95.27	1,267,011	422,434	12.8	33.34
p-32	T5	4,572,871	158,246	4,306,448	27.21	37.72	94.58	1,267,011	484,459	13.02	38.23
p-35	T5	4,863,693	159,546	4,614,420	28.92	37.46	94.88	1,267,011	478,061	13.43	37.73
p-36	T5	2,494,836	144,252	2,335,255	16.19	38.17	94.91	1,267,011	425,174	12.24	33.55
p-37	T5	5,801,782	186,006	5,418,993	29.13	38.68	93.67	1,267,011	586,287	15.55	46.27
p-39	T5	3,996,905	163,824	3,773,551	23.03	37.68	95.32	1,267,011	505,677	13.24	39.91
p-1	T6	5,093,908	166,718	4,753,777	28.51	38.02	93.24	1,267,011	505,604	13.49	39.9
p-2	T6	5,933,257	189,592	5,619,698	29.64	38.26	95.12	1,267,011	588,013	14	46.4
p-4	T6	2,796,124	135,042	2,636,807	19.53	37.95	95.22	1,267,011	398,288	12.64	31.43
p-5	T6	6,413,781	205,143	5,961,162	29.06	39.43	95.33	1,267,011	657,029	14.75	51.85
p-6	T6	3,575,565	158,857	3,360,640	21.16	38.02	94.32	1,267,011	486,861	14.01	38.42
p-8	T6	5,378,937	154,778	5,115,312	33.05	37.27	94.99	1,267,011	447,720	12.15	35.33
p-11	T6	5,087,838	206,446	4,737,023	22.95	39.34	95.18	1,267,011	655,007	14.31	51.69
p-13	T6	4,077,615	146,852	3,842,703	26.17	36.69	94.72	1,267,011	430,270	10.58	33.95
p-15	T6	7,072,086	188,539	6,699,447	35.53	37.52	95.06	1,267,011	557,438	13.98	43.99
p-17	T6	2,857,107	150,355	2,713,246	18.05	38.81	95.44	1,267,011	449,985	12.09	35.51
p-20	T6	4,665,865	212,836	4,337,269	20.38	40.06	95.47	1,267,011	691,807	15.41	54.6
p-23	T6	5,149,687	162,984	4,855,367	29.79	37.78	94.32	1,267,011	494,966	13.31	39.06
p-21	T6	6,718,486	198,424	6,306,218	31.78	39.43	94.62	1,267,011	612,920	13.53	48.37
p-25	T6	4,320,315	164,844	4,103,808	24.9	38.24	95.67	1,267,011	481,316	13.07	37.98
p-27	T6	6,071,493	178,916	5,719,928	31.97	39.03	93.31	1,267,011	561,619	13.63	44.32
p-29	T6	5,220,544	164,650	4,942,492	30.02	38.45	94.4	1,267,011	498,916	13.73	39.37
p-33	T6	4,737,806	175,070	4,361,159	24.91	38.63	94.53	1,267,011	543,500	12.05	42.89
p-34	T6	3,290,469	152,147	3,112,532	20.46	37.31	95.36	1,267,011	455,255	12.11	35.93
p-38	T6	4,132,267	157,238	3,915,946	24.9	38.2	95.04	1,267,011	458,872	13.29	36.21
p-40	T6	4,448,376	144,825	4,226,039	29.18	37.2	95.3	1,267,011	434,621	11.5	34.3