Genetic Diversity and Population Structure of *Dendrobium Nobile* in Southwest of China Based On Genotyping-by-Sequencing

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Abstract

Dendrobium nobile Lindl. is one of the most important Orchid plants worldwide. The genotype-by-sequencing (GBS) method has now been widely used to access genetic diversity because of its high-throughput and cost-effective in molecular markers. The goal of this study was to employ the GBS technique for diversity evaluation of *D. nobile* and determine genetic differences between populations. A total of 129 accessions of *D. nobile* collected originally between 2019 and 2020 from 10 imitation-wild cultivated populations growing in Sichuan, Guizhou and Yunnan of southwestern China were sequenced, a total of 135G clean reads and a total of 836,786 SNPs of high quality data was yielded and were used for final analysis of genetic diversity and population structure. The quality value 20(Q20) $\geq 92.61\%$, the quality value 30(Q30) $\geq 82.38\%$. The GC contents distributed between 37.58% and 38.82%. It was also found that more transitions than transversions, and the ratio of transition/transversion varied from 1.804 to 1.911. By the methods of STRUCTURE, the most appropriate number was found to be $k=3$, all accessions of *D. nobile* were classified into three groups, excepts for 14 accessions belonging to admixed group. Phylogenetic tree and principal component analysis (PCA) were consistent with the result. The first two principal components explained a total of 23.25% of the variation by PCA. The genetic diversity of ML population showed the lower genetic diversity as indicated by the effective number of alleles (N_e) = 1.287, polymorphism information content (PIC) = 0.141, and Shannon's information index (H) = 0.205, while WT population showed slightly higher genetic diversity by the N_e = 1.512, PIC = 0.256, and H = 0.360. ML population and other nine populations (FB, FM, FX, LJ, SJ, SP, WL, WT and XM) were the most divergent between them respectively owing to all pairwise F_{st} values above 0.25, while FM population and FX population were considered identical because the pairwise F_{st} value was 0.0 between the two populations. Correlation analysis showed that highly significant correlation was observed between genetic distance and actual geographical distance ($r = 0.854$, $P < 0.0001$), indicating that the genetic differentiation of the 10 *D. nobile* populations conformed to the geographical isolation model. Analysis of molecular variance (AMOVA) revealed that the genetic variation was greater within populations (87.8%) than among populations (12.2%). This confirmed that intra-population variation was the main source of genetic variation in 10 *D. nobile* populations. The results also showed that $N_m = 1.799 > 1$, indicating that there was gene exchange between different populations. Analysis of unweighted pair-group method with arithmetic mean (UPGMA) suggested that the 10 populations were classified into three groups (Group I, Group II and Group III), Group III could be further divided into two subgroups (Group IIIa and Group IIIb). The results will not only provide valuable information for the level of genetic diversity of *D. nobile* growing in southwestern of China but also help for formulation of strategies for resource protection and utilization. Moreover, GBS appears as an efficient tool to detect intra-population variation.

Introduction

Dendrobium nobile Lindl., an epiphytic orchid, is found to be distributed in tropical and subtropical regions of Asia, including Thailand, Laos, Vietnam, Sikkim, Bhutan, Burma, and China. In China, it is found to be present in southern regions of the Tsinling Mountain, such as Yunnan, Guangxi, Guizhou, Sichuan, and Hubei provinces (Tsi et al. 1999). It is one of the most important and representative species in the genus *Dendrobium* with approximately 1500 species in the worldwide, and is considered as a favorite ornamental plants, known for unique type, bright color and long-lasting flowers. In addition, *D. nobile* plants has been used as precious and traditional Chinese medicine, which has the special efficacy of enhancing immunity, treating neurological diseases, digestive system diseases, ophthalmic diseases and anti-tumor owing to containing a variety of bioactive substances such as dendrobine, polysaccharide, flavonoids, and etc (Yang et al. 2014; Bhattacharyya et al. 2015). It is listed in Pharmacopoeia of the People's Republic of China (Chinese pharmacopoeia Committee 2020). Therefore, *D. nobile* has high economic value, ecological value and scientific research value.

However, owing to the pressure of human activities, over-exploitation, natural habitat destruction and fragmentation, like other *Dendrobium* plants, *D. nobile* is facing the serious threat of endangered and is contained in Appendices I and II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Wang et al. 2018). In order to protect wild resources of *D. nobile*, the plants have been cultivated in China, particularly in southwestern regions of China for the market demand. Despite a long time of cultivation, few reports are yet available on genetic variation and population structure of *D. nobile*. Diversity in cultivated population is crucial for genetic improvement and for the related ornamental industry (Satya et al. 2015; Kang et al. 2015). It is necessary to study population structure and access the level of genetic diversity of the species. An analysis of genetic diversity using molecular marker is a trusted and reliable approach which could provide useful baseline information of various plants or population (Bhattacharyya et al. 2013).

Molecular markers have been developed and a variety of molecular markers have been used to detect genetic diversity and genetic relationships among species in the genus *Dendrobium* to date, including random-amplified polymorphic DNA (RAPD) (Zhu et al. 2011), inter simple sequence repeat (ISSR) (Yang et al. 2011), simple sequence repeat (SSR) (Lu et al. 2013; Kang et al. 2015), sequence

related amplified polymorphism (SRAP) (Feng et al. 2014), inter primer binding repeats site (iPBs) (Cui et al. 2020), target region amplification polymorphism (trap) (Feng et al. 2015), sequence-characterized amplified regions (scar) (Zheng et al. 2021), these markers are based on electrophoretic map. Other markers are based on sequence differences, including nuclear genome such as *ITS*, chloroplast genome, including *matK*, *rbcl*, *psbA-trnH* and mitochondrial genome (Ye et al. 2017; Wang et al. 2018; Nguyen et al. 2020). These studies showed that highly genetic differentiation and rich genetic diversity existed among different *Dendrobium* species. The revealed variabilities have allowed for better understanding of the extent of diversity present in the genus.

Efforts have also been made to assess the genetic diversity within species in the genus *Dendrobium* using different molecular markers. These studies revealed that genetic diversity and the variation within populations was greater than among populations in *D. officinale* (Jiang et al. 2016; Zhang et al. 2019; Xie et al. 2020) and in *D. moniliforme* (Ye et al. 2015). While most of the genetic variation found to be existed among populations in *D. fimbriatum* (Ma et al. 2009). Some studies aimed at understanding the genetic diversity of *D. nobile* using molecular markers for the analysis of population structure, and genetic variation had been reported in natural populations. The results showed that the genetic variation among natural populations from Northeast India was higher than within populations by Bhattacharyya et al. using SCoT (2013), RAPD (2015), ISSR and DAMD (2015) markers respectively. Other results also showed higher genetic differentiation among populations from Qinba mountain area in China using ISSR marker (Jiang et al. 2016). Whereas the genetic variation of *D. nobile* within populations from Yunnan Province, China was higher than among populations, using DALP marker (Zhang et al. 2013). In these studies, the natural population of *D. nobile* was mainly taken as the research object, and the sampling range were respectively located in Northeast India, Yunnan province or Qinba mountain area of China. However, the research on genetic variation of cultivated population was rarely reported.

In recent years, a helpful tool to enhance genetic analysis has emerged called genotyping-by-sequencing (GBS) (Chen et al. 2015), which is a new method based on next-generation-sequence (NGS) (Zhu et al. 2019) and massive sequencing which is able to discover a large number of single nucleotide polymorphisms (SNPs) (Wang et al. 2017). The main advantage of the method over previous approaches is that they can still work in the absence of a reference genome (Perea et al. 2016). It has dramatically reduced costs and time and provides a rapid, high-throughput and cost-effective tool for exploring plant genetic diversity on a genome-wide scale (Taranto et al. 2016). Therefore, GBS technology has been widely used for population structure and genetic diversity studies in various plant species (Taranto et al. 2016; Pavan et al. 2017; Wang et al. 2017; Cai et al. 2018; Guo et al. 2019; Morigengova et al. 2019; Niu et al. 2019; Wang et al. 2019; Zhou et al. 2019; Jia et al. 2020; Zhang et al. 2021).

To our knowledge, no population structure, and genetic diversity studies were available of *D. nobile* in previous study using GBS. In this study, we gathered 129 *D. nobile* accessions from 10 cultivated populations of Yunnan, Guizhou, and Sichuan provinces in southwestern China. The objectives of our study were to apply GBS technology (1) to discover SNPs for *D. nobile* species, (2) to assess the level of genetic diversity in a set of 129 accessions, (3) to evaluate the utility of the GBS in the genetic diversity analysis of *D. nobile*, an endangered medicinal plant.

Materials And Methods

Plant Materials

A total of 129 accessions of *D. nobile* were analyzed in this study. All samples were collected originally between 2019 and 2020 from 10 imitation-wild cultivated populations growing in Sichuan, Guizhou and Yunnan of Southwestern China. The species collected was identified by authors. The GPS coordinates of the 10 populations were recorded. Young leaves which were fresh, healthy and disease-free were cut off from randomly selected plants and immediately put into self-sealing bags containing dry Silica Gel, then they were taken back to the laboratory and stored in the refrigerator at -20°C prior to use. Provenances of the 10 populations used in this study were listed in Table 1.

Table 1
Provenances of the 10 populations used in this study

Population code	Geographic location	Longitude	Latitude	Altitude (m)	Sample Number
WL	Wanglong Town in Chishui City of Guizhou Province	E105°52′	N28°30′	536	15
FX	Fuxing Town in Chishui City of Guizhou Province	E105°45′	N28°27′	388	15
WT	Wutong Town in Hejiang County of Sichuan Province	E105°33′	N28°30′	377	15
FM	Fengming Town in Hejiang County of Sichuan Province	E105°52′	N28°39′	430	14
FB	Fubao Town in Hejiang County of Sichuan Province	E106°06′	N28°44′	579	15
SP	Sipo Town in Yibin City of Sichuan Province	E104°26′	N28°48′	407	11
SJ	Songjia Town in Yibin City of Sichuan Province	E104°51′	N28°44′	368	9
LJ	Liujiang Town in Hongya County of Sichuan Province	E103°14′	N29°43′	615	12
XM	Xiema Town in Jiajiang County of Sichuan Province	E103°22′	N29°46′	465	14
ML	Mengla Town in Mengla County of Yunnan Province	E101°34′	N21°29′	640	9

Genomic DNA Extraction

Genomic DNA was extracted from young leaf tissues of *D. nobile* using the cetyl-trimethyl-ammonium-bromide (CTAB) method or plant DNA Kit (Tiangen Biotech Co., Ltd., Beijing). The detection of DNA mainly included the 3 following kinds: (1) The integrity of DNA was verified by electrophoresis on 1% agarose gel. (2) The purity of DNA was measured by Nanodrop Spectrophotometer (nanodrop, np-1000 USA), the ratio of OD260/OD280 was between 1.8 and 2.0. (3) The concentration of DNA was quantified by the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA).

Genotyping by Sequencing

Qualified DNA was first digested using the restriction enzymes (*Pst*I, *Msp*I), and barcoded adapters were ligated to the digested fragments, then PCR amplification was carried out, the PCR products were pooled, fragments between 350 and 600 bp were isolated from agarose gel and purified, the library was constructed. After the library was tested to be qualified, paired-end sequencing was performed on the Illumina novaseq6000 platform at Huazhi Biotech Co., Ltd. in Changsha, China.

All the raw pair-end sequencing data in FASTQ format were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with BioProject accession PRJNA747120. The original data were split, low-quality reads were removed, and clean reads were finally generated, which was a total of 135 G. The quality of all sequencing was evaluated using fastqc version 0.11.8 software. The results showed that all samples had no quality problems and the sequencing quality was good. 10000 reads were randomly selected from the FASTQ file of sequencing data and aligned to sequencing in NCBI data using blast version 2.2.26 software (Altschul et al. 1990) to detect the pollution of sequencing.

Using BWA version 0.7.13 software (Li and Durbin 2009), the clean reads were aligned to the reference genome sequence of *Dendrobium catenatum* Lindl. (GCF_001605985.2_ASM160598v2_genomic.fna) (Zhang et al. 2016).

Variation was detected using FreeBayes version 0.9.21 software (Ebbert et al. 2016) and filtered using bcftools software (parameters: view-m2-M2-v snps). SNP locus with allele frequency (MAF) less than 0.05 and missing data rate higher than 20% were filtered out using vcftools version 4.2 software with parameter: -minGQ 5 -maf 0.05 -max-missing 0.8 (Garrison and Marth 2012). SNPs information was statisticed using vcf-stats software (Danecek et al. 2011). After the filtering, 836,786 SNPs of 129 accessions were retained and used for further analysis.

Data Analysis

Based on the filtered SNPs, the population structure of 129 accessions was conducted using Admixture software, the number of subgroup varied from 1 to 12, the output of STRUCTURE was evaluated to determine the most appropriate k value, which will exhibit

the low cross-validation error compared to other k values. Samples were considered to belong to one of the subgroup when the value of the corresponding membership coefficient (Q) was higher than 0.6, if not, the sample was considered as admixed at assigned k.

The genetic distance matrix was calculated using plink software and phylogenetic tree was constructed using Neighbor Joining algorithm method. The entire accessions were divided into subgroups based on the major nodes of the phylogenetic tree.

Principal component analysis (PCA) was performed to assess genetic distinctness and redundancy, and to assess the sample association plots of the first two resulting principal components using GCTA version 1.26.0 software (Yang et al. 2011).

Genetic diversity parameters including observed heterozygosity (*Ho*), expected heterozygosity (*He*), effective number of alleles (*Ne*), polymorphism information content (*PI*C), Shannon's information index (*I*), gene flow (*Nm*), were calculated using Arlequin version3.2 software (Excoffier et al. 2005). These parameters were used for the analysis and the comparison of the diversity of the 10 populations.

In addition, using Arlequin version3.2 software, genetic differentiation index (Fst) value was calculated, and the pairwise genetic distances among the 10 populations were evaluated, and the average values of Fst provided a measure of genetic distance between populations. In the meantime, unweighted pair-group method with arithmetic mean (UPGMA) dendrogram based on the pairwise genetic distances among the 10 populations obtained was used to assess inter-population differentiation and distinctness. An analysis of molecular variance (AMOVA) was performed to quantify the genetic variation present within and among the 10 populations.

The correlation coefficient between genetic distance and actual geographical distance was conducted using R version 3.6.3 software (Martin et al. 2014) to assess their correlation respectively.

Results

A total of 129 *D. nobile* accessions were sequenced using GBS technology in order to understand the genetic diversity and population structure of the 10 populations. After filtering and quality controlling, raw reads were split and finally generated a total of 135G clean reads. A total of 836,786 SNPs of high quality data was yielded and were used for final analysis. More than 60% of sequencing aligned to the reference genome, the depth of sequencing was 1×- 3×, and the coverage was basically more than 11%. Statistics on sequence data (Table 2) further showed that the number of total reads ranged from 6,497,132 (XM population) to 17,566,739 (WT population); The number of total bases ranged from 887,060,448 (XM population) to 2,373,845,107 (WT population); the quality value 20(Q20) ≥ 92.61%, with an average of 95.26%; the quality value 30(Q30) ≥ 82.38%, with an average of 87.60%; the GC contents distributed in a range of 37.58% (SJ population) to 38.82% (XM population). It was suggested that GBS was a valuable molecular method qualified for population genetic characterization of *D. nobile*.

Table 2
Summary statistics of sequencing reads, bases, GC content and Q20(Q30) of the 129 accessions of *Dendrobium nobile* Lindl. from the 10 populations

Populations	Total Reads	Total Bases	GC content (%)	Q20 (%)	Q30 (%)
FB	14,663,845	2,000,153,894	38.33	92.61	82.38
FM	14,258,947	1,928,206,553	37.84	93.58	84.05
FX	15,163,263	2,056,901,952	38.06	96.26	89.76
LJ	14,913,211	2,024,594,136	38.27	94.29	85.47
ML	12,749,467	1,723,895,648	38.59	94.37	85.62
SJ	15,522,683	2,102,755,840	37.58	95.73	88.54
SP	10,440,432	1,413,377,961	38.23	96.51	90.26
WL	16,553,946	2,249,397,485	38.21	96.65	90.53
WT	17,566,739	2,373,845,107	38.00	96.34	89.78
XM	6,497,132	887,060,448	38.82	96.24	89.63

It was shown in Table 3, from the genomic level, the total number of SNP sites in the 10 populations of *D. nobile* varied from 170,167 (XM population) to 255,053 (LJ population); The range of homozygous loci was 63,748 (SJ population)-141,624 (ML population), and

the percentage of that was between 28.54% (FB population) -75.03% (ML population); the range of heterozygous loci was 46,452 (ML population) -168,225 (FB population), and the percentage of that was 24.97% (ML population)-71.46% (FB population). It was also found that more transitions than transversions, and the ratio of transition/transversion varied from 1.804 to 1.911.

Table 3
Summary statistics of SNPs of *Dendrobium nobile* Lindl. of the 10 populations

Populations	No. of SNP sites	No. of homozygous loci	No. of heterozygous loci	Homozygous Rate (%)	Heterozygous Rate (%)	No. of Transition	No. of Trans version	Ratio of Transition/Transversion
FB	234,918	66,692	168,225	28.54	71.46	71,462	39,463	1.811
FM	234,451	80,601	153,850	34.20	65.80	71,231	39,486	1.804
FX	233,722	76,868	156,853	32.70	67.30	71,194	39,179	1.817
LJ	255,053	120,028	135,025	47.12	52.88	77,672	42,733	1.818
ML	188,076	141,624	46,452	75.03	24.97	57,950	30,329	1.911
SJ	211,806	63,748	148,058	30.26	69.74	64,252	35,685	1.801
SP	188,446	66,465	121,981	36.40	63.60	57,298	31,534	1.817
WL	230,915	76,260	154,655	32.73	67.27	70,205	38,739	1.812
WT	241,389	100,930	140,459	41.63	58.37	73,330	40,537	1.809
XM	170,167	90,086	80,081	52.61	47.39	52,102	28,183	1.849

STRUCTURE analysis was used to study the population structure of *D. nobile* based on 836,786 high quality SNPs from the 129 accessions. STRUCTURE software was run and the cross validation error (CV error) was calculated with k ranging from 1 to 12 to determine the optimal k value and the number of groups (k) was plotted. The results showed that (Fig. 1): when the k value is from 1 to 2, the CV error value decreased rapidly, at k = 3, a distinct inflection point was observed, the CV error value decreased to the minimum value, and then gradually increased to 8, after a rapid, gentle and linear rising respectively, finally the CV error value reached the maximum value at k = 12. So the most appropriate number was found to be k=3, which produced the lowest CV error value compared to other k values.

As a consequence, a total of 129 *D. nobile* accessions were classified into three groups. To classify groups, accessions unequivocally were assigned to a group when the threshold of the membership coefficient (Q) was higher than 0.6, while those with the Q lower than 0.6 were assigned to the admixed group. It was shown in Fig. 2, the first group, highlighted in red, consisted of 11 accessions, accounting for 8.53% of all accessions, with 8 accessions from LJ population, 1 accession from FM-WT and XM population, respectively. The second group which was the largest group, highlighted in blue, accounting for 58.14% of all accessions, consisted of 75 accessions, including 15 accessions from FB population, 11 accessions from SP and WL population, respectively, 10 accessions from FX population, 9 accessions from FM and SJ population, respectively, 6 accessions from XM population and 4 from WT population. The third group, highlighted in green, consisted of 29 accessions, accounting for 22.48% of all accessions, including 9 accessions from ML population, 7 accessions from XM and WT population respectively, 3 accessions from WL population, and 1 accession from FM, FX and LJ population respectively. In addition, the 14 accessions, with Q value of each group was less than 0.6, accounting for 10.85% of all accessions, were in the admixed group, that could not be unequivocally assigned to any of the above three groups.

The genetic distance matrix of all *D. nobile* accessions from 10 populations was calculated by p-link software and cluster analysis was carried out to construct the phylogenetic tree, as shown in Fig. 3. The phylogenetic tree was further generated that classified 129 accessions into three major clusters, except for the remains of 14 accessions (gray in color) in admixed cluster also. By comparing STRUCTURE groups versus NJ-tree clusters, it was possible to observe that all the accessions in group I, II, III and admixed were distributed in the cluster I, II, III and admixed, respectively. Cluster I (red in color) was the smallest cluster consisting 11 accessions, most of them from LJ population, and others from FM-WT and XM population, respectively. Cluster II (blue in color) was the largest cluster consisting of 75 accessions from 8 populations respectively, except for ML and LJ populations. Cluster III (green in color) was

comparatively smaller cluster, consisting of 29 accessions from 7 populations, including ML, XM, WT, WL, FM, FX and LJ population respectively.

The clustering in phylogenetic tree revealed genetic relationship of 129 accessions from the 10 populations. The green cluster and the red cluster were close together, indicating that accessions in the two clusters had a close genetic relationship. The blue cluster was far away from the red and green cluster, indicating that the genetic relationship between them is also far away. Different color clusters had different degrees of accessions mixing of different populations, indicating that there was a certain phenomenon of gene exchange between different populations.

Further principal component analysis (PCA) was performed to identify patterns of genetic variation among individual accessions and the 10 populations. All accessions were divided into four groups in the PCA plot (Fig. 4a). "0(orange in color)", "1(dark green in color)", "2(cyan in color)" and "3(purple in color)" represented admixed group, group I, II, and III in the STRUCTURE group respectively. The first principal component explained 15.02% of variation and the second explained 8.23%, both explained 23.25% of the total variation. All accessions in group II and a small amount of accessions in group III were placed in the positive (upper) portion of the plot of PC2 axis, while all accessions in group I, in admixed group and most accessions in group III were placed in the negative (lower) portion of the plot of PC2 axis. Only group I in the positive (right) portion along the plot of PC1 axis, the other three groups were placed in the negative (left) portion along the plot of PC1 axis. Moreover, it had been found that accessions in the group III showed a loose distribution, while in the group I and II showed centralized in the PCA plot indicating the larger genetic diversity present in group III.

In Fig. 4b, an initial analysis of population structure was performed using PCA. The 10 populations were represented by 10 different colors in the plot. It had been found that accessions from LJ and WT population respectively showed high dispersal, indicating high and higher genetic diversity in the two populations; while accessions from ML population clustered tightly together, indicating lower genetic diversity and it could be distinguished from other populations at the PCA plot. Moreover, different populations, except for ML population, appeared to overlap and became undistinguishable clearly with PCA, indicating that their genetic background was similar.

From the above, we concluded that the results of STRUCTURE analysis, PCA, and the phylogenetic tree showed a high degree of similarity. All accessions could be classified into three groups and one admixed group and there was a consistent one-to-one match among the three methods.

In order to understand the genetic diversity of the ten populations, the diversity parameters were calculated in Table 4. It indicated that the mean values of *Ho*, *He*, *Ne*, *PIC*, and *I* ranged from 0.184 - 0.345, 0.315 - 0.351, 1.287 - 1.512, 0.141 - 0.256, 0.205 - 0.360, with an average of 0.276, 0.328, 1.441, 0.222, 0.284 respectively. The results suggested that the ML population showed the lower genetic diversity compared to other populations by the *Ne* = 1.287, *PIC* = 0.141, and *I* = 0.205, while the WT population showed the slightly higher genetic diversity by the *Ne* = 1.512, *PIC* = 0.256, and *I* = 0.360.

Table 4
Genetic diversity parameters for the 10 populations. Observed heterozygosity (H_o), expected heterozygosity (H_e), number of effective alleles (N_e), Polymorphic information content (PIC), Shannon diversity index (I)

Populations	H_o	H_e	N_e	PIC	I
FB	0.344	0.339	1.459	0.232	0.250
FM	0.273	0.319	1.494	0.247	0.332
FX	0.277	0.315	1.486	0.242	0.316
LJ	0.263	0.327	1.483	0.238	0.320
ML	0.184	0.337	1.287	0.141	0.205
SJ	0.345	0.351	1.395	0.216	0.210
SP	0.324	0.342	1.393	0.198	0.231
WL	0.2749	0.320	1.481	0.243	0.301
WT	0.2466	0.320	1.512	0.256	0.360
XM	0.2311	0.315	1.424	0.209	0.315

The genetic difference among the 10 populations was estimated by comparative analysis of genetic differentiation coefficient (F_{st}) (Table 5). The pairwise F_{st} value and genetic distances among the 10 populations were different. The results revealed that the highest values of F_{st} were between ML population and other nine populations, with F_{st} values ranged from 0.271 to 0.446, respectively. Meantime, the F_{st} values between LJ population and FB, SJ, SP populations were 0.277, 0.283, 0.288 respectively, all above 0.25 indicating that the largest difference between them. A slightly higher pairwise F_{st} values (0.164, 0.161, 0.184, 0.198), suggested a high differentiation between LJ population and XM, FM, FX, WL populations. The pairwise F_{st} values between WT population and FM, FX, LJ, SJ, SP, WL, XM populations; XM population and FM, FX, SJ, SP, WL populations; FB population and WT, XM populations ranged from 0.061 to 0.147 respectively, suggested that there was a moderate amount of differentiation among these populations, indicating that these populations were relatively genetically distinct. The pairwise F_{st} values between other populations showed slight genetic differentiation, indicating these populations had lower variability. Particularly, FM and FX population were considered identical because the F_{st} value was 0.0 between the two populations.

Table 5
Pairwise values of genetic differentiation index (F_{st}) among the 10 populations of *Dendrobium nobile* Lindl.

populations	FB	XM	FM	FX	LJ	SJ	SP	WL	WT	ML
FB	0.000									
XM	0.141*	0.000								
FM	0.024*	0.080*	0.000							
FX	0.016*	0.091*	0.000	0.000						
LJ	0.277*	0.164*	0.161*	0.184*	0.000					
SJ	0.005*	0.138*	0.025*	0.013*	0.283*	0.000				
SP	0.012*	0.134*	0.031*	0.019*	0.288*	0.003	0.000			
WL	0.023*	0.091*	0.009	0.001	0.198*	0.019	0.023	0.000		
WT	0.144*	0.085*	0.068*	0.080*	0.100*	0.143*	0.147*	0.061*	0.000	
ML	0.413*	0.334*	0.336*	0.356*	0.309*	0.446*	0.446*	0.356*	0.271*	0.000
Note: Indicates significant pairwise F_{st} after sequential Bonferroni corrections ($\alpha = 0.05$)										

Furthermore, correlation between genetic distance and actual geographical distance of the 10 populations were also analyzed. Highly significant correlation was observed between genetic distance and actual geographical distance ($r = 0.854$, $P < 0.0001$), indicating that

the genetic differentiation of the 10 *D.nobile* populations conformed to the geographical isolation model.

In order to define the patterns of genetic variation and to estimate the variation components among the populations, an analysis of molecular variance (AMOVA) was performed based on the pairwise genetic distances between the 10 *D.nobile* populations. The AMOVA revealed that much greater variation within populations (28.96%+58.84%=87.8%) than among populations (12.2%, $p<0.001$) (Table 6). In other words, 28.96% and 58.84% of the variation was attributed to the differences among individuals within populations and within individuals respectively, whereas among the populations, account for 12.2% of the total variation. This confirmed that intra-population variation was the main source of genetic variation in the 10 *D. nobile* populations. The results also showed that $Nm = 1.799 > 1$, indicating that there was gene exchange between different populations.

Table 6
Analysis of molecular variance (AMOVA) of *Dendrobium nobile* Lindl. for the 10 populations

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation	P value	Nm
Among populations	9	4266042.36	14918.48	12.20	<0.001	1.799
Among individuals within populations	119	14577322.96	35409.85	28.96	<0.001	
Within individuals	129	8513143.50	71952.95	58.84	<0.001	
Total	257	27356508.82	122281.28			

Analysis of unweighted pair-group method with arithmetic mean (UPGMA) dendrogram based on the AMOVA results revealed the 10 populations were classified into three distinct groups at the Phi statistic of 0.075 or more. Group I and Group II consisted of only one population of ML and LJ, respectively; Group III was the largest group, with 8 populations consisting of SJ, SP, FB, WL, FM, FX, XM, and WT populations. At the statistic of 0.05, Group III could be further divided into two subgroups named Group IIIa and Group IIIb. In Group IIIa, there were two populations, including XM and WT population; In Group IIIb, there were 6 populations, including SJ, SP, FB, WL, FM, and FX populations.

Discussion

Phenotypic traits and molecular markers are two different tools for assessing genetic diversity. In previous study, the diversity of 18 phenotypic traits of the same 10 populations was analyzed, and the results showed that inter-population variation was the main source of phenotypic traits variation of *D.nobile* (He et al. 2021). In the present study, the AMOVA analysis of the same 10 populations based on GBS molecular markers revealed that the intra-population variation was the major genetic variation of *D. nobile*, indicating morphological traits were often influenced by the environment.

At the same time, in previous study, the cluster analysis of the 10 populations of *D. nobile* based on 18 phenotypic traits showed that at the Euclidean distance of 6.5, the 10 populations could be divided into 3 groups, which generally showed the rule that populations with similar geographical location and latitude gathered together, and phenotypic traits showed the pattern of geographical variation. In this study, the same 10 populations of *D. nobile* were clustered by UPGMA method according to the genetic distance. At the genetic distance of 0.075, the 10 populations could also be divided into three groups. There were similarities and differences between clustering based on molecular marker and phenotypic traits. The similarities were as follows: in the two kinds of clusters, ML population, at the southernmost end of Yunnan Province was clustered alone. Differences were as follows: in phenotypic clustering, XM population and LJ population gathered together; while in molecular clustering, LJ population gathered solely, the XM population gathered with WT population firstly to form subgroup, and then with the other 6 populations clustering in another subgroup gathered to form a group, indicating that the genetic background of *D. nobile* resources from XM and WT population was similar, and accessions of XM population might be introduced from Wutong town in Hejiang county of Sichuan province. In addition, in phenotypic clustering, FX population was closer to WT population, but in molecular clustering, FX population was closer to FM population, indicating that the genetic background between the two populations was similar. The SP and SJ population clustered together in molecular clustering, which was grouped independently in phenotypic clustering. Similar reports had been reported that there were inconsistencies between molecular clustering and phenotypic clustering in *D. officinale* and other plants species (Zhang et al. 2019; Yang et al. 2016).

The results of cluster analysis were well supported by the analysis of genetic differentiation among the 10 populations based on the genetic differentiation coefficient (F_{st}). The pairwise values of F_{st} between ML population and the other 9 populations were the largest,

varied from 0.271 to 0.446, all above 0.25, indicating obvious genetic differences between them. It could explain why ML population is not clustered with the other 9 populations. The F_{st} value between FX population and FM population was 0, showing a high degree of genetic homogeneity, no genetic differentiation between the two populations.

In addition, the results were inconsistent with the report by Bhattacharyya et al. (2013, 2015), that a greater degree of variation existed among the populations compared to that within the populations of *D. nobile* in Northeast India using SCoT, ISSR, and DAMD markers. The other studies had reported the similar results of *D. nobile* (Zhang et al. 2013; Jiang et al. 2016). Similar reports in *D. moniliforme*, another species of the *Dendrobium* genus (Ye et al. 2017). The reasons were considered that there had been high genetic differentiation in the long-term evolution process owing to geographic isolation, fragmented distribution and lack of exchange between natural populations. In our study, the accessions of *D. nobile* were from cultivated populations, the N_m value for the 10 populations was 1.799, implicating that genetic exchange occurred among the *D. nobile* populations in the neighboring distributing regions.

A different level of genetic diversity of the 10 cultivated populations from southwestern China was observed by analysis of genetic parameters. For example, it was shown the higher genetic diversity in WT population, while the lower genetic diversity in ML population. Firstly, it could be related to its own breeding system which was asexual reproduction. In a separate population far away from other populations and within its own small population, the distribution area might have genetic drift due to geographical isolation and lack of gene exchange with other populations, thus reducing the level of genetic diversity of ML population. This result was consistent with the conclusion of previous report (Yan et al. 2015). Their study found that *D. nobile* from Hainan Island in China showed a low level of genetic diversity, and there was a high degree of genetic differentiation between *D. nobile* from mainland China. Due to geographical isolation, there were genetic differences and significant regional specificity. There was a relatively higher level of genetic diversity in WT population, the reason was that Wutong town located in Hejiang county of Sichuan province and at the junction with Chishui city of Guizhou province. It is convenient to communicate with Sichuan and Guizhou of two provinces so the genetic diversity in the population was high. Geographical factors also had an impact on genetic diversity. The 10 cultivated populations distributed in different regions, and the longitude and latitude span a large range. Different geographical differences also lead to different levels of genetic diversity to adapt to environment. The results were consistent with the reported that the latitude and altitude had a positive effect and average annual temperature had negative influence on the population genetic diversity of *Gymnocarpus przewalskii* (Xu et al. 2018). As Mousadik et al. (1996) reported that the genetic variations had been affected by its own breeding system, distribution area, habitat change, individual cell mutation, founder effect, species legacy areas produced in the Pleistocene, etc.

Molecular clustering still showed obvious geographical characteristics, indicating that genetic differentiation conformed to geographical isolation mode. Through correlation analysis, there was a significant correlation between genetic distance and geographical distance. The reason might be that the accessions collected in this study distributed widely, spanning 3 Provinces, including Sichuan, Guizhou and Yunnan province which distributed in southwestern China. It showed different climatic and ecological conditions in different geographical regions. There existed a different degree of genetic differentiation between these populations could be related to their adaptability to different environments. Similar reports had been reported in *D. officinale* (Yuan et al. 2013), as well as other families such as naked fruit wood (Xu et al. 2018) and cassava in Brazil (Carvalho and Schaal 2001).

In the current study, a combination of different three analysis methods was employed in studying the genetic diversity and population structure of *D. nobile* from the 10 populations in southwestern China. Interestingly, the three methods, including STRUCTURE analysis, phylogenetic tree analysis, and principal component analysis (PCA) in general showed the same results and supported each other. The all accessions of *D. nobile* could be divided into three groups using the three methods respectively, except for the 14 accessions which were in admixed group. Moreover, the accessions classified by the three methods correspond to each other one by one. In other words, the three methods play a mutually reinforcing role.

In this study, comprehensive analysis showed that GBS technique could be suitable for the genetic diversity and population structure studies of *D. nobile*, an endangered medicinal plant. In the future, with the continuous improvement of the method, it would be optimized and used in the *D. nobile* for various purposes, such as construction of high density genetic maps, genetic mapping of complex traits through Genome-Wide Association Studies (GWAS), and estimation of breeding values in genetic selection as reported by Taranto et al. (2016).

Declarations

Author contributions

Tao He contributed to experimental design, data analysis, and writing of this article. Changrong Ye and Qin Zeng contributed to GBS sequencing and bioinformatics. Xiaoli Fan and Tianfang Huang contributed to participating in experiments, and data analysis.

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Conflict of interest

The authors declare that they have no conflicts of interest or competing interests.

Consent for publication

All authors consent to publish.

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Figures

Figure 1
 A curve graph of cross-validation (CV) error values at different k value

Figure 2
 A structure plot of the 129 accessions of *Dendrobium nobile* Lindl. by k=3 using 836,786 SNPs

Figure 3

Phylogenetic dendrogram of 129 accessions of *Dendrobium nobile* Lindl. from the ten populations based on 836,786 SNPs

Figure 4

Principal component analysis (PCA) plots of the 129 accessions *Dendrobium nobile* Lindl. based on 836,786 SNPs a Numbers (0-3) represent subgroup derived from structure analysis, colors represent different subgroup b Colors represent the 10 populations used in the study

Figure 5

UPGMA dendrogram of the 10 populations based on pairwise F_{st} value