Pak. J. Bot., 54(2): 483-489, 2022. DOI: http://dx.doi.org/10.30848/PJB2022-2(40)

DE NOVO TRANSCRIPTOME ASSEMBLY AND DEVELOPMENT OF EST-SSR MARKERS OF ENDANGERED *DENDREBIUM NOBILE* (ORCHIDACEAE)

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Abstract

Dendrobium nobile of the family Orchidaceae has been widely used as an important traditional Chinese herbal medicine and a popular garden flower worldwide. It is mainly distributed in southern Tsinling Mountains of China. However, the wild *D. nobile* is getting endangered due to its habitat destruction and overexploitation. Deciphering genomic information of *D. nobile* and developing genetic markers are essential for the formulation of scientific protection measures. Here, the comprehensive transcriptome data of a wild *D. nobile* ecotype, "chishuijinchai", was obtained by *de novo* assembly based on Illumina RNA-seq data. A total of 138,628,272 clean reads were generated after removing the adapters and low-quality reads. All the reads were assembled into 114,191 unigenes with an average length of 918 base pairs (bp). Out of 114,191 unigenes, 39,373 unigenes (34.5%) were annotated based on any of the COG, KEGG, KOG, Pfam, Swiss-Prot and Nr databases. Additionally, 27,614 (24.18%) unigenes were annotated into 3 main GO categories. Moreover, a total of 29,380 SSR (simple sequence repeats) loci located on 22,572 unigenes were identified by MISA. Around 100 primers were randomly selected for polymorphism validation across 8 *Dendrobium* species. Out of these selected primers, 62 and 29 primers showed inter- and intra-specific polymorphisms, respectively. These findings provide fundamental basis for evolution and population genetic diversity studies of *D. nobile*, which should be beneficial for the protection of its germplasm resources

Key words: Transcriptome; *De novo*; *Dendrobium nobile*; EST-SSR markers

Abbreviations: AFLP, amplified fragment length polymorphism; EST-SSR, expressed sequence tag-derived simple sequence repeat; SRAP, Sequence-related amplified polymorphism; RAPD, Random Amplified Polymorphic; ISSR, inter-simple sequence repeat.

Introduction

Dendrobium nobile Lindl. (*Orchidaceae*), is mainly distributed in southern Tsinling mountains in China (Chen & Tsi, 2000). Wild *D. nobile* populations are epiphytics or lithophytics, which can grow on tree trunks or bare rocks within natural forests (Liu *et al*., 2014). The stem of *D. nobile* has been widely used as traditional Chinese medicine to treat various diseases (Ng *et al*., 2012; Yang *et al*., 2014; Kim *et al*., 2015). Moreover, *D. nobile* became a kind of popular garden plant due to its attractive violet flower and unique flowering pattern (Martin & Madassery, 2006). Recent studies have reported many active components from *D. nobile*, such as polysaccharides (Wang *et al*., 2010; Luo *et al*., 2010), alkaloids (Yang *et al*., 2014; Li *et al*., 2017), and phenolics (Zhou *et al*., 2017; Bhattacharyya *et al*., 2019). These active components are believed to be involved in health related functions such as antitumor, antiviral, antihyperglycemic, and immunomodulatory activities.

However, its over exploitation due to the increasing market demand and also its habitat destruction, the natural populations of *D. nobile* are being destroyed at an alarming rate (Bhattacharyya & Kumaria, 2015). Thus, special attentions are required and immediate conservation procedure should be followed. There is an urgent need to study the hereditary diversity and structure of wild *D. nobile* populations, which is believed to be the prerequisite for wild resource conservation. Several studies have employed some molecular markers such as AFLP, TRAP, RAPD, DAMD, ISSR and SCoT, to identify the genetic diversity, population structure, and phylogenetic relationship among *Dendrobium* species (Bhattacharyya & Kumaria, 2015; Feng *et al*., 2015; Bhattacharyya *et al*., 2015, 2017). However, these markers are likely not able to overcome their own deficiencies, such as low rate of genome coverage, difficult in distinguishing polymorphism, and poor repeatability (Zheng *et al*., 2018), which eventually provide unreliable results. Simple sequence repeats (SSRs), which are ubiquitous in eukaryotic genomes, have been widely approached in plant science for construction of genetic linkage map (Liu *et al*., 1996), and decipherment of phylogenetic relationship (Agrama & Tuinstra, 2003; Guo *et al*., 2014; Ma *et al*., 2019). Moreover, attributing to advances in the technology of DNA sequencing, the large number of SSR markers can be easily developed from the high throughput sequencing data, particularly these cost-effective Illumina reads (Xu *et al*., 2018; Chen *et al*., 2019; Liu *et al*., 2019). Recently, SSR has been employed in the genetic studies of *Dendrobium* species (Xu *et al*., 2017; Zheng *et al*., 2018), guaranteed its potential values in the identification of *Dendrobium* species. Several studies have developed SSRs using expressed sequence tags (ESTs) of *D. nobile* and utilized for its phylogenetic analysis (Lu *et al*., 2013; Lu *et al*., 2014), however, the number of SSR makers is only about one hundred and is quite limited for genetic research.

The ecotype of *D. nobile*, "chishuijinchai", mainly distributed in Chishui City of Guizhou Province, China, is known for its excellent quality and valuable medicinal

benefits owning to the unique geographical advantage. *In vitro* culture of *D. nobile* has been successfully developed and used for large-scale propagation (Lee *et al*., 1995; Fan *et al*., 2020). The tissues isolated for culture were derived from only several individuals, which resulted in narrower genetic diversities of *D. nobile*. Therefore, it was essential to develop stable markers to analyze the genetic diversity of *D. nobile* populations. Here, *de novo* assembles of the transcriptome data of the ecotype of *D. nobile* "chishuijinchai" using Illumina reads were performed. A total of 114,191 unigenes were detected with an average length of 918 bp. After screening the unigenes, 29,380 SSRs located on 22,572 unigenes were identified. The PCR amplification of 100 randomly selected primers confirmed the stability and application of these predicted SSR markers. Our results provide fundamental basis for evolution and population genetic diversity studies, as well as the germplasm conservation of *D. nobile*.

Material and Methods

Plant materials and RNA extraction: Wild plants *of D. nobile* were gathered from Yanziyan National Forest Park, Chishui City, Guizhou Province, China (28°24′43″N, 105°44′38″E), and grown in a greenhouse with a control condition of 16-h day/8-h night light photoperiod in Guizhou Normal University. Total RNA was extracted from a mixed tissues pool (1:1:1, root: stem: leaf) following the instruction of the commercial RNA kit (Invitrogen, USA) protocol. The Qubit (Invitrogen, USA) was used to check the quality as well as the quantity of total RNA. The Agilent Technologies 2100 Bioanalyzer (Agilent) was also employed to assess the RNA integrity number (RIN). And the total RNA with RIN < 8 was excluded for further analysis. Three biological replicates were prepared in present study. The genomic DNA was isolated from young leaves of *D. nobile* for EST-SSRs validation.

cDNA library construction and Illumina sequencing: Total RNA per biological replicate (1.5 μg) was prepared to construct the cDNA library according to the TruSeq RNA Sample Prep Kit (Illumina, USA). To isolate mRNA from total RNA, the poly-T oligo-attached magnetic beads (Illumina, USA) were applied following the company's analysis process. Then fragmentation buffer was used to segments the mRNA into ~200 bp length. After that, the first-strand cDNA was synthesized based on short fragmented mRNA. Subsequently, adapter was added onto the end-paired, A-tailed cDNA fragments. Eventually, the cDNA was amplified by PCR for 15 cycles.

The cDNA library of *D. nobile* were sequenced by the Illumina HiSeq^{TM} 4000 platform with the PE150 approach, which gave rise to 150-bp end-paired reads. The raw sequence data were checked for data quality control by FastQC version 0.11.9 based on per base sequence quality scores, per sequence GC content, per sequence quality scores, sequence length distribution and overrepresented sequences (Brown et al., 2017).

De novo assembly of *D. nobile* transcriptome: To generate clean data, Trimmomatic version 0.33 (Bolger *et al*., 2014) was used to remove the adapters and low-quality reads with the following parameters: leading: 3, trailing: 3, slidingwindow: 4:15, minlen: 36, leading: 3, trailing: 3, slidingwindow: 4:15, minlen: 36, and tophred: 33. Then the clean reads were assembled by Trinity -2.1.0 with default parameters (Grabherr *et al*., 2011). After that, the R project (v. 3.4.3) with TransRate and CD-HIT package was applied to optimize the assembly and obtain the non-redundant result using default parameters (Geniza & Jaiswal, 2017). All the RNA-Seq data, including raw data and process documentation used in this study are available at the BIG Sub (http://bigd.big.ac.cn.), under the accession number: CRA002590.

Unigenes annotation: The NCBI BLAST 2.2.28+ was used to annotate the assembled unigenes of *D. nobile*. The following 6 public databases are referenced, including the nonredundant protein sequences (NR), nucleotide sequences (NT), Kyoto Encyclopedia of Genes, Genomes (KEGG), Protein family (Pfam), Swiss-Prot protein and the eu-Karyotic Ortholog Groups (KOG) database) (Camacho *et al*., 2009). The Blast2GO was then applied to predict the gene function using the NR and Pfam databases with the E value of 0.001 as threshold (Conesa *et al*., 2005).

EST-SSR prediction and primer design: To detect the expressed sequence tag derived-simple sequence repeats (EST-SSRs) of these unigenes, the Misa web service (https://webblast.ipk-gatersleben.de/misa/) was employed (Beier *et al*., 2017) with minimal repeat numbers set at 10 repeats for mononucleotides, 6 for dinucleotides, 5 repeats for trinucleotides, tetranucleotides, pentanucleotides, and hexanucleotides. And the maximum interruption between 2 SSR loci within a compound microsatellite was 100 bp. Then the Primer 5.0 was employed to design the EST-SSR primers based on these standards: (i) annealing temperature (Tm) from 57˚C to 63˚C, (ii) primer size from 18 bp to 27 bp, (iii) Tm max difference between forward and reverse primer was less than 2˚C, (iv) and the production size of primer ranged 100–300 bp.

EST-SSR markers validation: To validate these EST-SSR loci of *D. nobile*, totally 100 EST-SSR primers were randomly synthesized (Sangon, China). Altogether, 2 different ecotypes of *D. nobile* and 6 other *Dendrobium* species were further analyzed. Total DNA of these samples were extracted from young and fresh leaves using the CTAB method. The NanoDrop 2000 (Thermo Fisher Scientific, USA) was used to assess the DNA quality and quantity. After that, PCR amplification was performed to test the polymorphism of these primers using a reaction of 10 μl, containing 5 μl 2×Master MIX (CWBIO, China), 1 μl DNA template (50 ng), 1.0 μl of primer mixture (100 μM), and 3 μl ddH_2O. DNA fragments were amplified using an initial 5-min denaturation at 94˚C followed by 30 cycles (94˚C for 45 sec, 53˚C-57˚C for 30 sec, and 72˚C for 45 sec), and a final 10-min elongation step at 72˚C. Finally, PCR products were checked by 1% high resolution agarose gels (Beijing XMJ Scientific Co., Ltd, China), which can identify 20-bp differences of PCR production.

Results

De novo assembly and unigenes annotation: Illumina sequencing of the *D. nobile* samples generated a total of 142,279,996 raw reads (~20.8 Gb data), and 138,628,272 clean reads were obtained after trimming process. Subsequently, these clean reads were assembled into a reference of 158,421,349 bp with an overall 39.63% GC content. This reference comprised of 114,191 unigenes with an average length of 918 bp which gave rise to a total of 172,509 transcripts (Table 1), indicating a widespread alternative splicing of the unigenes in *D. nobile*.

Table 1. Summary of the de novo assembly of the transcriptome of *Dendrobium noble*.

Items	Number
De novo assembled reference (bp)	158,421,349
Total unigenes	114,191
Total transcripts	172,509
Largest unigene (bp)	13,857
Smallest unigene (bp)	210
Average length (bp)	918.34
N50	1,385
GC content (%)	39.63

Among the 114,191 assembled unigenes, a total of 39,373 (34.48%) unigenes were annotated based on any of the 5 public databases, resulting in 37,794 (33.10%) unigenes in NR database, 25,497 (22.33%) unigenes in Swiss-Prot, 13,881 (12.16%) in KOG, 7,123 (6.24%) in KEGG, and 3,477 (3.04%) in COG databases, and 1,649 unigenes (4.19%) (Table 2; Fig. 1A). Out of 172,509 transcripts, 77,969 (45.20%) were annotated at least once, 76,076 (44.10%) in NR database, 54,914 (31.83%) unigenes in Swiss-Prot, 30,204 (17.51%) in KOG, 15,487 (8.98%) in KEGG, and 8,366 (4.85%) in COG databases, furthermore, 3,804 uniquely annotated transcripts were identified (Table 2; Fig. 1B).

GO classification: The Blast2GO was employed to obtain the GO functional annotation of unigenes. A total of 27,614 (24.18%) unigenes of *D. nobile* were annotated into 3 main categories of GO, including Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) (Fig. 2). Majority of annotated unigenes (24,617 unigenes, 89.15%) were involved in MF categories, and these unigenes constructed 14 terms at second level, which were dominantly represented by "binding" (17,869 unigenes, 72.59%) and "catalytic activity" (12,692 unigenes, 51.56%). In the BP category, 15,397 unigenes (55.76%) were clustered into 20 terms at second level, and the terms of "cellular process" (13,140 unigenes, 85.34%), "metabolic process" (12,976 unigenes, 84.28%), and "single-organism process" (4,307 unigenes, 27.97%) were the top 3 subcategories. In the CC category, 12,781 unigenes (51.92%) were enriched into 14 subgroups, of which most were represented by the term of "cell" (7,556 unigenes, 59.12%), "cell part" (7,447 unigenes, 58.27%), "membrane" (6,970 unigenes, 54.53%), "membrane part" (6,548 unigenes, 51.23%), and "organelle" (5,825 unigenes, 45.58%).

Table 2. Summary of unigenes/transcripts function blasted with NR, Swiss-Prot, Pfam, COG, and KEGG public databases.

Items	Transcripts	Percentage (%)	Unigenes	Percentage (%)
COG	8366	4.85	3477	3.04
KOG	30204	17.51	13881	12.16
KEGG	15487	8.98	7123	6.24
NR	76076	44.10	37794	33.10
Swiss-Prot	54914	31.83	25497	22.33
Annotated in all	3804	4.88	1649	4.19
Total annotated	77969	45.20	39373	34.48

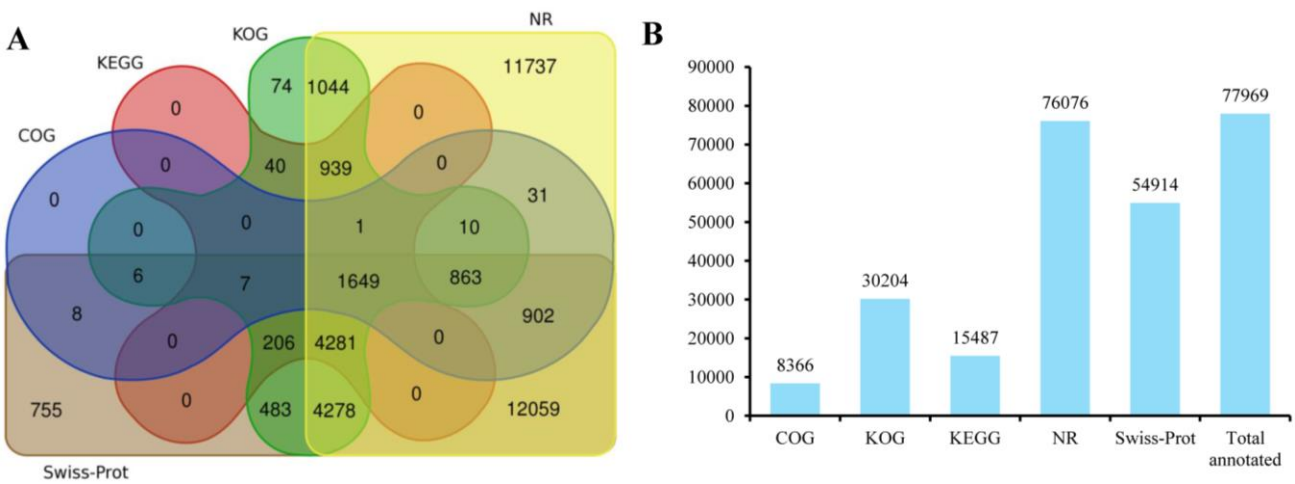

Fig. 1. The annotation of unigenes and transcripts of *D. nobile* transcriptome in public databases. (A) Venn diagram of unigenes annotated in public databases; (B) Number of transcripts annotated in public databases.

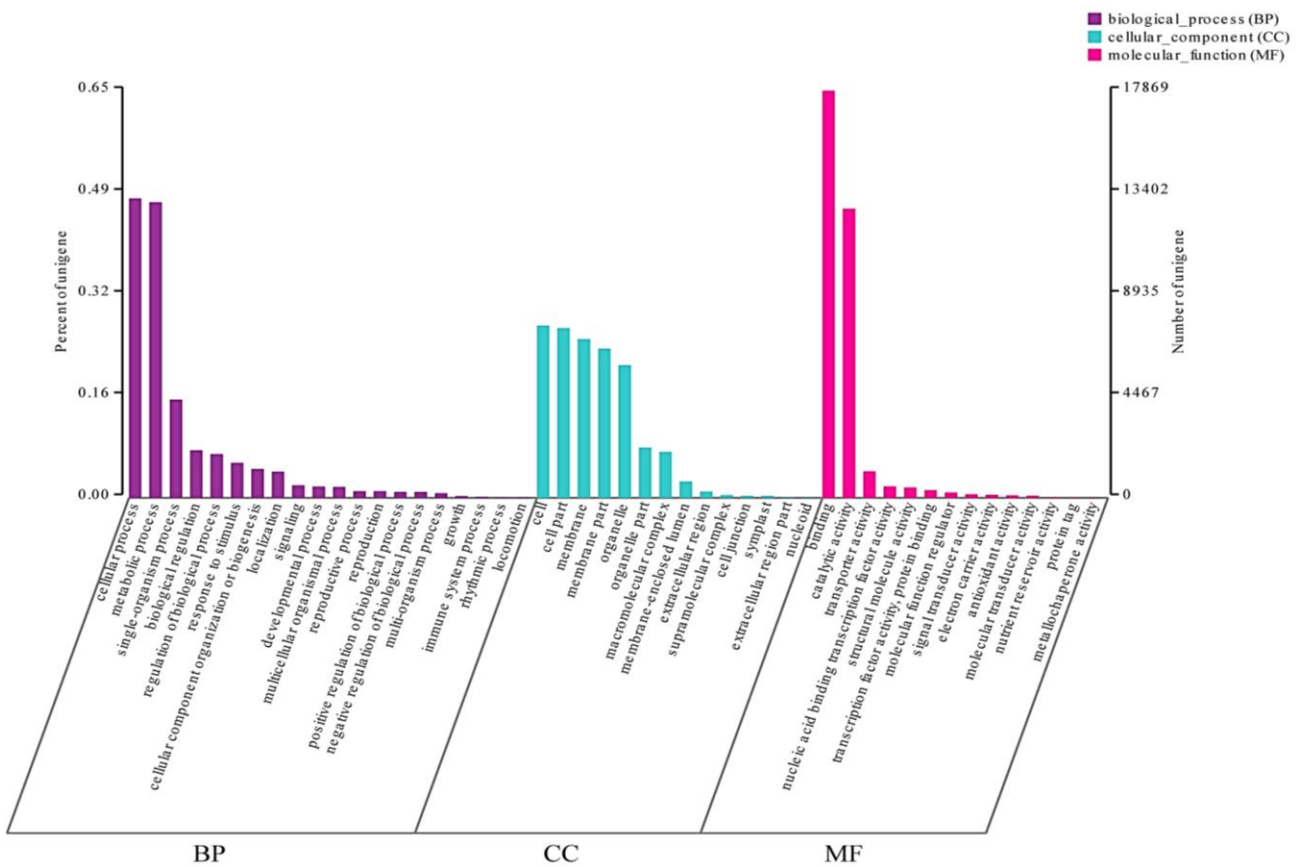

Fig. 2. GO assignment of annotated unigenes of *D. nobile* transcriptome. Only the GO terms at second level were showed in this

Table 3. Information of SSR types of the assembled unigenes.

Iterms	Description
Number of unigenes containing SSRs (%)	22,572 (19.77)
Number of unigenes containing more than one SSRs (%)	3,905 (17.30)
Mono-nucleotide repeats (%)	20,255 (68.94)
Di-nucleotide repeats (%)	5,626 (19.15)
Tri-nucleotide repeats (%)	3,303 (11.24)
Tetra-nucleotide repeats (%)	142 (0.48)
Penta-nucleotide repeats (%)	23 (0.08)
Hexa-nucleotide repeats (%)	31 (0.10)
Number of compound SSRs (%)	1,798 (6.12)
Total SSRs	29,380

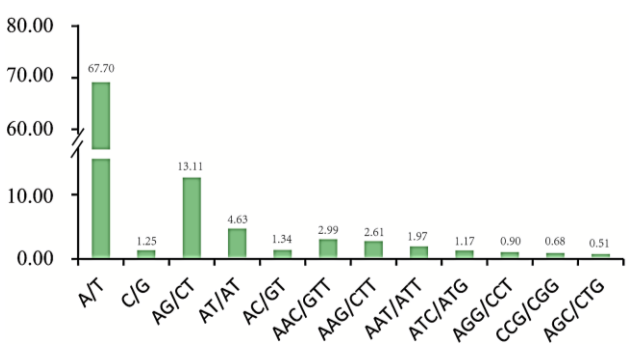

Fig. 3. The dominantly represented SSR motifs in assembled unigenes of *D. nobile.*

The distribution of SSR loci: The web-service of MISA was employed to detect the SSR loci of the assembled unigenes. We identified 29,380 SSR loci in 22,572 unigenes, which only represented 19.77% of the total annotated unigenes. And 3,905 unigenes (17.30% of identified unigenes containing SSR) harbored more than one SSR locus (Table 3). Among these SSR loci, mononucleotide motifs (20,255 loci) were the most abundant comprising of 68.94% of the total SSR loci, followed by di- (5,626, 19.15%), tri- (3,303, 11.24%), tetra- (142, 0.48%), hexa- (31, 0.10%) and penta-nucleotide (23 0.08%) motif repeats. Additionally, 1,798 (6.12%) compounding SSR loci, in which the interval within the SSRs was no more than 100 bp, were identified.

Among these SSR loci, a total of 72 different types were detected. For the mononucleotide repeats, the abundant type was A/T (19,889 loci), occupying 67.70% of the total SSR loci, whereas the type of C/G (366 loci) was only 1.25%. Among the dinucleotide repeats, the type of AG/CT (3,851, 13.11%) was the most frequent, followed by AT/AT (1,361, 4.63%), AC/GT (394, 1.34%) and CG/CG (20, 0.07%). The types of AAC/GTT (877, 2.99%), AAG/CTT (767, 2.61%) and AAT/ATT (579, 1.97%) were the top 3 motifs for the trinucleotide repeats. Out of 19 types in tetranucleotide repeats, the AAAT/ATTT (69, 0.23%) motif was the most abundant type (Fig. 3).

Development and validation of EST-SSR Markers: The Primer 5.0 was used to design the primers for the EST-SSR loci based on the criterions detail described in the Method section. According to these criterions, up to 3 primers per SSR locus (21,990, 74.85%) were eventually designed. To validate these designed primers, 100 primers were randomly selected to amplify in 8 *Dendrobium* species. All of the primers selected successfully generated the amplified

production with the number of bands ranging from 1 to 6. A total of 91 primers (91%) gave rise to polymorphic amplification across 8 selected *Dendrobium* species. Out of 91 polymorphic primers, 62 primers (68.13% of polymorphic primers) had the polymorphic productions between *D. nobile* and other species of *Dendrobium* genus (inter-specific polymorphisms), and 29 primers (31.87%) showed intra-specific polymorphisms, of which the amplified productions polymorphisms could be detected between the 2 ecotypes of *D.* nobile.

Based on the genetic similarity coefficients of the validated EST-SSR, the genetic clustering dendrogram of *Dendrobium* species was constructed by the UPGMA method (Drummond & Rodrigo 2000). The result of UPGMA dendrogram showed that the 8 *Dendrobium* species could be clearly divided into two clusters at the coefficient value of 0.52 (r=0.99) (Fig. 4). The cluster I comprised of *D. trotile*, *D. officinale*, *D. findlayanum*, *D. hybrid*, *D. thyrsiflorum*, and 2 ecotypes of *D. nobile* and *D. aphyllum* was the only one clustered in the group II.

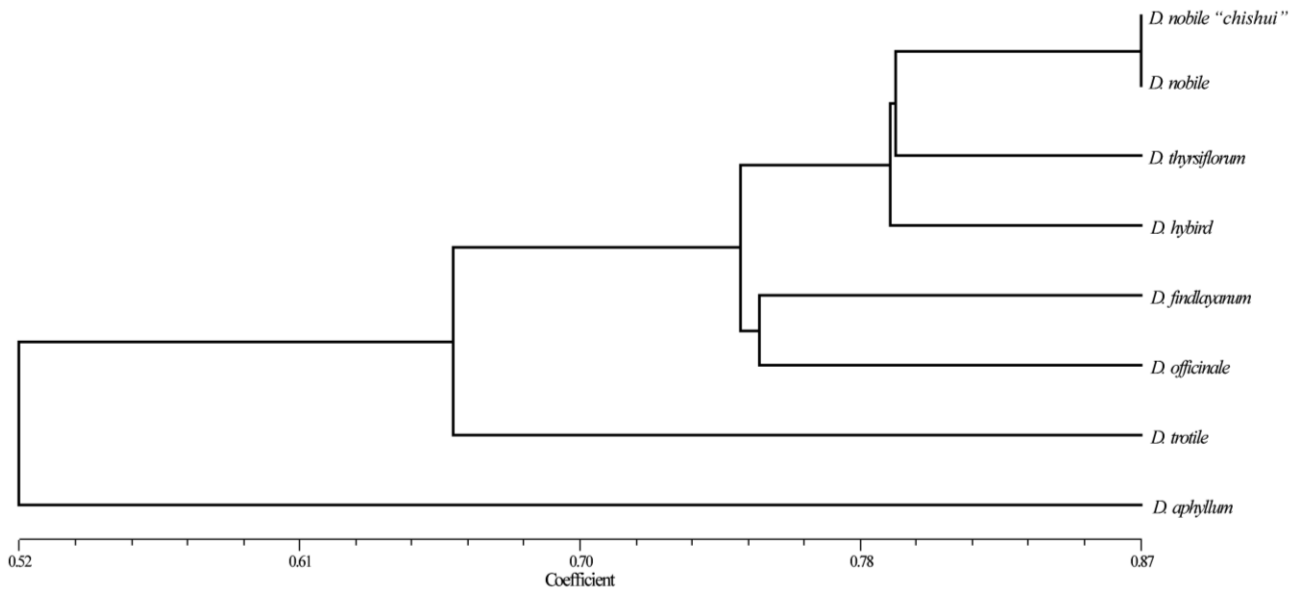

Fig. 4. The phylogenetic relationship of the selected *Dendrobium* species constructed by EST-SSR markers. The NTSYS 2.1 software under the UPGMA method was employed to construct the phylogenetic trees.

Discussion

D. nobile is an important traditional Chinese herbal medicine and a popular ornamental plant, its active components and the functions have been widely studied (Wang *et al*., 2010; Luo *et al*., 2010; Li *et al*., 2017; Xu *et al*., 2017; Ma *et al*., 2019), however, due to the lack of available DNA makers, studies referring to the genome information, population structure, genetic diversity, and genetic linkage have not been comprehensively reported. Here, de novo assembly was performed on the transcriptome data of a wild ecotype of *D. nobile* "chishuijinchai" and developed a mass of EST-SSR makers, which would facilitate the studies of evolution and population genetic diversity of *D. nobile*. A total of 114,191 unigenes were obtained, comparable to that of *D. officinale* (Zhang *et al*., 2016) and another *D. nobile* transcriptome analysis (Wen *et al*., 2017), but far lower than that of *D. huoshanense* transcriptome analysis (Yuan *et al*., 2018), in which 478,361 unigenes were obtained. For the annotation of unigenes, a relatively low proportion, 45.20% (77,969) of all unigenes, were totally annotated in these 5 common databases (NR, Swiss-Prot, KEGG, COG and KOG). Similar situations were observed in *D. officinale* (Zhang *et al*., 2016), and *D. huoshanense* (Yuan *et al*., 2018), likely attributed to the deficiency of complete genome sequences of *Dendrobium* species.

Molecular markers have been widely used in plants for the study of genetic diversity, population structure, and genetic improvement analysis (Feng *et al*., 2015; Bhattacharyya *et al*., 2015; Zheng *et al*., 2018; Ma *et al*., 2019). For decades, these makers, such as SRAP, AFLP, RAPD, DAMD, ISSR and SCoT, have been employed for germplasm conservation and identification of *Dendrobium* species (Feng *et al*., 2014; Feng *et al*., 2015; Bhattacharyya *et al*., 2015, 2017). The co-dominant SSR markers have also been specifically developed for genetic study of *D. nobile* (Lu *et al*., 2013; Lu *et al*., 2014). However, the number of SSRs developed in these studies is limited, indicating a limited availability for genetic research. In this study, based on the transcriptome data of *D. nobile*, we identified 29,380 putative SSRs motifs from 22,572 (19.77%) unigenes, which was comparable to the observation in *D. officinale* (Xu *et al*., 2017). Among these SSR motifs, the most abundant type was A/T (67.70%), likely attributed to poly-A tail of the unigenes. For the dinucleotide repeats, the type of AG/CT (13.11%) was over represented, akin to the results in SSR studies on *D.* officinale (Xu *et al*., 2017) and other species. Gao *et al*., (2014) and Chen *et al*., (2017) suggested that these sequences might be preferentially reserved during plants evolution. For the trinucleotide repeats, the popular motif was AAC/GTT (2.99%), whereas the dominant trinucleotide repeat was AAG/CTT in *D. officinale* (Xu *et al*., 2017), twice more than the type of AAC/GTT. These

results indicated that the trinucleotide repeats might be prone to variable against selective pressure, and could be used as powerful markers to detect the genetic diversity among *Dendrobium* species.

Given that the *Dendrobium* genus harbors a large reserve of over 1,000 species (Schuiteman, 2011), with similar morphological and cytological characteristics (Feng *et al*., 2014; Zheng *et al*., 2018), thus, developing a rapid and accurate species identification method is a prerequisite for germplasm conservation, sustainable utilization, and genetic improvement for the *Dendrobium* species. SSRs markers are considered to have multiple uses among related species due to their high level of transferability and the detection ability of conserved orthologous loci (Kang *et al*., 2015). In present study, 100 SSR markers were randomly selected to amplify across 8 selected *Dendrobium* species. A total of 91 primers generated polymorphic products, including 62 inter-specific polymorphic primers (68.13%) and 29 intra-specific polymorphic primers (31.87%), confirmed the stability and application of these predicted SSR markers. Our results will provide fundamental basis for evolution and population genetic diversity studies, as well as the germplasm conservation of *D. nobile*.

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(Received for publication 3 September 2020)