Development of cDNA-derived SSR markers and their efficiency in diversity assessment of Cymbidium accessions

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Abstract Cymbidium spp. are popular flowering plants. Assessment of the genetic diversity in cultivated Cymbidium facilitates conservation of germplasm and subsequent cultivar improvement. Thus, it is important to develop more efficient polymorphic DNA markers. Although more motifs (403) were identified and more primers (206) were designed in the genomic library compared to the cDNA library, a larger number of successful primers were obtained from the cDNA library (59.9%) than from genomic DNA library (51.1%). However, higher PIC and gene diversity were identified in genomic SSRs. The average allele number per locus was also higher in genomic SSRs (7.3) than EST-SSRs (5.2), among the 24 evaluated Cymbidium accessions. AT/TA was comparatively high in EST-SSRs, while this motif was not as common in genomic SSRs. The GTT/AAC/TGT/ACA/TTG/CAA was the most frequent in genomic SSRs. The number of repeats ranged from 3 to 12 in EST-SSRs. Currently, 52 novel polymorphic SSR markers have been evaluated, which will be useful for germplasm assessments, core set construction, evaluation of genetic diversity, and marker assisted selection (MAS) based Cymbidium breeding.

Keywords: cDNA library, Cymbidium, enriched library, molecular diversity, SSR

INTRODUCTION

The genus Cymbidium, a member of the Orchidaceae, is terrestrial, epiphytic, and lithophytic, composed of 44 species and distributed in Northwest India, China, Japan, Korea, the Malay Archipelago, and North and East Australia (Du Puy and Cribb, 1988; Obara-Okeyo and Kako, 1998). The oriental Cymbidium is a popular ornamental orchid, and is important in the Korean floricultural industry. Cymbidium spp. have highly variable floral morphologies, pollinator relationships, and diversities in ecological habitats (Arditti, 1992; Judd et al. 1999). However, the genetic relationship between many of the Cymbidium spp. lineages remains unclear (Choi et al. 2006). It is important to properly characterize and evaluate the genetic diversity of Cymbidium to effectively conserve and use this species (Park et al. 2009). Thus, it is essential to identify novel polymorphic markers.

Parchman et al. (2010) discussed the advantages of 454 sequencing as a cost and time effective way to discover novel SSRs. Next-generation sequencing facilitates developing such markers, not only because enormous amounts of sequencing data are generated, but also because the novel markers are gene-based (Parchman et al. 2010). A 454 sequencer is a large-scale parallel pyrosequencing system, using a genome sequence (GS) FLX titanium instrument, with the ability to sequence 400-600 million base pairs per run with 400-500 base-pair read lengths. The GS FLX system includes a simplified sample preparation work-flow and emulsion (em) PCR automation. Pyrosequencing is a powerful tool that has been used in genome and functional expression analyses. It is also an attractive
approach to discover novel transcripts, unknown gene functions, sequences of high quality, base discrepancies, and alternative splice variants (Bainbridge et al. 2006). The combination of long, accurate reads and high throughput makes 454 pyrosequencing analysis on the FLX genome sequence well suited for detailed investigations (Jarvie and Harkins, 2008). Thus, 454 pyrosequencing is increasingly being used in many investigations.

Previous studies have focused on intra and inter-specific genetic diversity and/or evolutionary relationships in *Cymbidium* using isozymes, random amplified polymorphic DNA, amplified fragment length polymorphisms, inter-simple sequence repeats, and internal transcribed spacer sequence. Currently, simple sequence repeats (SSRs) or microsatellite markers have been used to study genetic diversity, phylogenetic relationships, classification, evolutionary processes and quantitative trait loci in many crops. They have some advantages, such as technical simplicity, relatively low cost, high genetic resolution power, and being highly polymorphic. Moreover, they are reliable and easy to score (Gupta and Varshney, 2000). They are clusters of short tandem repeat nucleotide bases distributed throughout the genome and are co-dominant, multi-allelic, and require a small amount of DNA for scoring. Therefore, they have been recognized as useful molecular tools for marker-assisted selection in various species (Agrama et al. 2007). Thus, Xia et al. (2008) designed and applied nine novel SSR markers in *Cymbidium sinensis*. However, *Cymbidium* genomic research has lagged behind other crop species due to a lack of polymorphic DNA markers. Thus, it is important to develop and identify polymorphisms of SSR markers in *Cymbidium*. In the past, SSRs were developed by screening several thousand clones through colony hybridization with repeat-containing probes. Presently, they are developed by constructing genomic and/or complementary DNA (cDNA) libraries and followed by sequencing. This study investigated and developed SSR markers, and discussed strategies to develop these markers from genomic DNA and cDNA libraries.

**MATERIALS AND METHODS**

**Plant materials**

DNA and RNA were extracted from the Korean *Cymbidium* species *goeringii*. RNA was extracted from fresh green leaves to synthesize the cDNA library. To confirm that the novel SSR markers were polymorphic, we evaluated 24 *Cymbidium* accessions, including 20 *C. goeringii* collected from different geographical origins and four *C. sinensis* from the National Institute of Horticultural and Herbal Science of the Rural Development Administration, Republic of Korea (Table 1).

**cDNA synthesis and library preparation**

Total RNA isolation, mRNA purification, cDNA synthesis, fragmentation by nebulization, and adaptor ligation were performed prior to 454 sequencing. Total RNA was isolated using Trizol RNA isolation protocol (modified by D. Francis from Edgar Hultema) and the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA), following the manufacturer’s protocol. Fresh green leaves (100 mg) of *C goeringii* were frozen in liquid nitrogen, ground into a powder, and total RNA was extracted. RNA concentration was determined using the NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA) and agarose gel electrophoresis. mRNAs were purified with the PolyATract mRNA Isolation System (Promega, Madison, WI, USA). The purified products were used as template to synthesize full-length cDNA using the ZAP-cDNA Synthesis kit (Stratagene, Santa Clara, CA, USA). The cDNA was fragmented by nebulization for library construction.

From the cDNA, a single-stranded template DNA library was generated. The cDNA was fragmented by nebulization using an Agilent 2100 bioanalyzer (Waldbronn, Germany) with a mean fragment size of approximately 600 bp. Approximately 1 µg of cDNA was used to generate a genome sequencing library using a FLX Titanium analyzer (Roche, Mannheim, Germany). The cDNA fragment ends were polished (blunted), and two short adapters were ligated to the ends according to standard procedures (Margulies et al. 2005). The adapters provided priming sequences for amplification and sequencing of the sample library fragments, as well as a “sequencing key,” which is a short sequence of four nucleotides used by the system software for base calling. The sequencing key also releases the unbound strand of each fragment (with a 5’ adaptor, A) following DNA repairs in the double-stranded library. The quality of the single-stranded DNA fragment library was assessed using the 2100
bioanalyzer. The library was also quantified to determine the optimal concentration of library to use for emulsion-based clonal amplification.

454 pyrosequencing of the cDNA library

Single copies of template species from the DNA library were hybridized to DNA capture beads. The immobilized library was re-suspended in amplification solution, and the mixture was emulsified, followed by PCR amplification. After amplification, the DNA-conjugated beads were recovered from the emulsion and enriched. The sequencing primer was annealed to the immobilized amplified DNA templates. After amplification, single DNA-carrying bead was placed into each well of a PicoTiterPlate (PTP) device. The PTP was inserted into the FLX genome titanium sequencer for pyrosequencing (Ronaghi, 2001; Elahi and Ronaghi, 2004), and sequencing reagents were sequentially flowed over the plate. Information from the PTP wells was captured simultaneously by a camera, and the images were processed in real time by an onboard computer. After sequencing, sequence assembly was performed using the GS De Novo Assembler software to get contigs and singletons. All sequence data was confirmed with references using GS Reference Mapper software. The resulting sequences were trimmed using SeqClean and the Lucy program.

Investigation of SSR motifs and designing DNA markers

All sequences generated by 454 pyrosequencing were investigated for SSR motifs using the ARGOS program 1.46 (SSRManager) at the default setting (Kim et al. 2007). Of the identified SSR motifs, only motifs having sufficiently large flanking sequences were used to design primer pairs. The SSRs detected were categorized as perfect di-, tri-, tetra-, penta-, or hexa-nucleotide motifs.

SSR Genotyping using the ABI (Applied BioSystems) genetic analyzer

PCR amplification was first confirmed using 4 different samples for each SSR primer. M13-tail PCR method was used to measure the size of the PCR products (Schuelke, 2000). Only primers yielding amplified product were used for the M13-tail PCR. PCR amplification conditions were as follows: 94°C for 3 min, 30 cycles each at 94°C for 30 sec, 55~60°C for 45 sec, 72°C for 1 min, followed by 10 cycles of 94°C for 30 sec, 53°C for 45 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. SSR alleles were resolved on an ABI 3500 Genetic Analyzer (Applied Biosystems) using the GeneMapper 4.1 software and sized precisely using GeneScan installation Kit DS-33 and GeneScan 600 LIZ size standard v2.0. The GeneScan installation standard DS-33 consists of pooled PCR products labeled with 6-FAM, VIC, NED, and PET dyes.

Data analysis

Locus variability was measured in terms of the number of alleles, heterozygosity (H), major allele frequency (MAF), gene diversity (GD), and polymorphic information content (PIC) using PowerMarker 3.25 (Liu and Muse, 2005). The UPGMA algorithm was used to construct a phylogram for the entire population using shared allele distances with the MEGA4 software (Tamura et al. 2007) embedded in PowerMarker.

RESULTS

Sequencing and evaluation of SSRs

A summary of the genomic and cDNA sequencing from C. goeringii, and SSR identification, is shown in Table 2. SSR-enriched genomic library results were taken from our previous published paper (Moe et al. 2010) and were also shown in Table 2. From a genomic library of 525 sequenced clones, we identified 56 clones (10.67%) that were redundant and 322 clones (61.33%) that had microsatellite repeating motifs. Four hundreds and three SSR motifs were indentified from 322 clones using the SSRManager, ARGOS program 1.46. In total, 206 (51.11% of total repeat motifs) primer pairs were designed from the flanking sequences of SSR-containing clones, and screened for polymorphisms in a
panel of 10 C. goeringii accessions, using the procedure described previously (Dixit et al. 2005). Only 14 primer pairs produced reproducible polymorphic bands (Moe et al. 2010), and these were further evaluated against 24 Cymbidium accessions.

In total, 4238 contigs and singletons were assembled by De Novo Assembly after 454 pyrosequencing from a cDNA library. Among them, 166 (3.9%) were found to be redundant and 4072 (96.1%) were found as unit express sequence tags (ESTs). Although 312 SSR motifs were investigated from unit ESTs, only 187 (59.93%) had sufficiently large flanking sequences to allow for primer design by ARGOS program 1.46 (Table 2 and Table 3). Currently, we have screened 70 (39.33% of the total designed primers) primer pairs for polymorphisms against the 24 Cymbidium accessions. We selected 70 primers proportionally to its designed primers each of di-, tri-, tetra- and others nucleotide motifs. Of the 70 primers tested, only 52 (74.3% of total tested primers) showed reproducible polymorphic bands against 24 tested accessions.

Comparisons of SSR repeat motifs

SSR development and its characteristic features are summarized in Table 4. Sequence analysis of all SSR-containing clones revealed a high number of di-nucleotide SSRs (82.88%), compared with tri-nucleotide SSRs (16.63%), from the genomic library. The CT/AG/TC/GA class of repeat motifs was most frequently identified (69.46% of the total di-nucleotide motif type) among the di-nucleotides, followed by the TG/CA/GT/AC class (27.54%). Among the tri-nucleotide SSRs, the GTT/AAC/TGT/ACA/TGT/CAA class of repeat motifs was predominant (56.72%), followed by CTT/AAG/TGC/GCT/GAG/GAA (19.41%) and TGC/GCA/GCT/AGC/CTG/CAG (14.93% of total trinucleotide motif type).

Different SSR motif distributions were found in EST-SSRs identified from the cDNA library. Similar proportions of di-nucleotide (47.44%) and tri-nucleotide (47.12%) SSRs were found. A small number of tetra-nucleotide (3.85%) and other motifs (1.6%) were also identified. Among the di-nucleotides, CT/AG/TC/GA (61.49%) was the most common SSR class, followed by TA/AT (33.78%) and TG/CA/GT/AC (4.73% of total di-nucleotide type). There was no GC/CG class of di-nucleotides found in either case. The CTT/AAG/TGC/GCT/GAG/GAA class was the most abundant (27.89%) among trinucleotide nucleotide motif type, followed by TGC/GCA/GCT/AGC/CTG/CAG (14.96% of total trinucleotide motif type).

Figure 1 presents the distribution of different repeat motifs (di-, tri-, others) and their respective number of repeats, regardless of the motif class. The highest value was observed for tri-nucleotides with 4 repeat motifs, followed by 5 and 3 repeat motifs. This trend was not consistent in the di-nucleotides. In this case, the highest number was still observed for 4 repeat motifs, but followed by 3, 6, 7, and finally 5 repeat motifs. The lowest numbers were seen at 9 repeat tri-nucleotide motifs, 12 repeat di-nucleotide motifs, and at 6 repeats of other motif types.

Designing SSR primers and screening for polymorphism

Primer pairs were designed for all available SSR motifs detected. However, not all SSR motifs can be designed as primer pairs, it can be designed only if the SSR motifs had sufficiently large flanking sequences. Among the 402 and 312 SSR motifs, only 51.11% (206) and 59.93% (187) of the motifs of genomic SSRs and EST-SSRs could be used for primer design, respectively. Respective SSR motifs and successfully designed primer pairs (EST-SSR primers) were presented in Table 3.

The 70 novel markers were classified according to the nucleotide type, di-, tri-, and others, in their polymorphism. In the screening analysis, 25 di-nucleotide markers, 40 tri-nucleotide markers, and 5 of other nucleotide types were included. Although 84% (21) of the di-nucleotides, 67.5% (27) of the tri-nucleotides, and 80% (4) of the other nucleotide types were polymorphic, it was difficult to deduce whether the di-nucleotides were more efficient in the polymorphism test (Table 5). There was no correlation between the number of motif repeats and the polymorphic efficiency in Cymbidium (data not shown).

The SSR markers were analyzed to confirm that they were polymorphic against 24 Cymbidium accessions. Variability at each SSR locus was measured in terms of the numbers of alleles,
heterozygosity, gene diversity, and PIC using the PowerMarker 3.23 software (Liu and Muse, 2005). In total, 102 alleles were detected, with an average of 7.3 alleles per locus, by 14 genomic SSRs (Table 6). The KNU-CC-32 locus had the highest number (15) of alleles, followed by KNU-CC-35 (9), while only two alleles were observed at KNU-CC-42. Allele size ranged from 105 to 380 bp. Heterozygosity values ranged from 0.000 to 1.000 (mean, 0.416). The average gene diversity and PIC values were 0.621 and 0.589, with ranges from 0.363 (KNU-CC-52) to 0.896 (KNU-CC-32), and from 0.323 (KNU-CC-42) to 0.887 (KNU-CC-32), respectively.

In total, 271 alleles, with an average of 5.2 alleles per locus, were detected by 52 EST-SSRs across 24 Cymbidium accessions (Table 7). The highest number of alleles was found in CG-cSSR-67 locus (13), followed by CG-cSSR-9 (11), while only two alleles per locus were observed at seven other loci. The alleles ranged from 72 to 449 bp. The heterozygosity value ranged from 0.000 to 1.000, with an average of 0.601. The average gene diversity and PIC values (0.545 and 0.497) identified from 52 EST-SSRs was slightly lower than the 14 genomic SSRs against 24 Cymbidium accessions. The gene diversity ranged from 0.117 (CG-cSSR-20) to 0.842 (CG-cSSR-27), while PIC ranged from 0.110 (CG-cSSR-20) to 0.887 (CG-cSSR-20) in EST-SSRs.

Genetic diversity

As described above, of the 70 markers evaluated, a total of 56 (80%) microsatellites amplified well in the survey panel, and these were used for the polymorphism survey in the germplasm panel and diversity analysis. In this final group of 56 amplified markers, 4 (5.71%) were monomorphic and 52 (74.29%) were polymorphic (Table 3).

A genetic distance-based analysis was performed to determine how useful these novel SSRs could be in studying genetic variations and phylogenetic relationships among 24 germplasm collections. A UPGMA dendrogram was constructed using the Mega 4.0 program (Tamura et al. 2007) embedded in the PowerMarker program (Liu and Muse, 2005). The greater value of genetic diversity (0.629) and PIC (0.589) across the 24 different Cymbidium accessions was revealed by genomic SSRs markers than by EST-SSRs. It reflected a high level of polymorphism was in genomic DNA. All Cymbidium accessions were clustered into two main groups (G1 and G2) in the UPGMA phylogram by 14 sets of genomic SSRs. When Cymbidium accessions were evaluated using 52 EST-SSRs, they were classified into 3 groups and one outstanding accession (G1, G2, G3 and KNU-085; Figure 2a and 2b). Group 1 (G1) included all C. goeringii accessions (20) and group 2 (G2) consisted of all (4) C. sinensis accessions, based on the genomic SSRs. Two C. goeringii accessions (KNU-017 and KNU-032) were separated from G1, and one C. sinensis accession (KNU-085) was separated from G2, when analyzed using the EST-SSRs.

DISCUSSION

Cymbidium spp. are popular flowering plants. Assessment of the genetic diversity in cultivated Cymbidium facilitates conservation of germplasm and subsequent cultivar improvement. However, few molecular studies focusing on the genetic diversity and conservation of these species have been performed. Only a limited number of genetic markers, such as 224 ISSR (Wang et al. 2009) and 38 SSR (Xia et al. 2008; Moe et al. 2010; Huang et al. 2011) have been developed. Thus, it is important to characterize novel, efficient polymorphic DNA markers. Previously, to develop novel markers, several thousand clones were screened through colony hybridization with repeat-containing probes. This method is extremely tedious and inefficient for plant species with low SSR frequencies (Zane et al. 2002). Some studies have reported construction of simple sequence repeat (SSR)-enriched genomic libraries to facilitate the development of SSR markers for crop plants (Gwag et al. 2006; Cho et al. 2010). Previously, we used a SSR-enriched Cymbidium goeringii genomic library to search for novel SSRs. In this study, we prepared a cDNA library from mRNA to develop Cymbidium genetic markers. Aside from marker development, cDNA libraries play an important role in gene separation and cloning. Target genes can be isolated from cDNA and used directly for expression. Thus, this library is a basic tool to evaluate and characterize novel genes (Jun, 2007). With advances in molecular biological technologies, the construction and use of cDNA libraries have improved. The use of next-generation cDNA sequencing has become popular to develop markers, not only because enormous amounts of sequence data are available from which markers can be identified, but also because the novel markers are gene-based (Parchman et al. 2010).
Previous studies have shown that microsatellite enrichment levels ranged from 11% to 99% (Zane et al. 2002; Ueno et al. 2003; Pandey et al. 2004; Zhao et al. 2005; Cho et al. 2010). The efficacy of SSR development in *C. goeringii* was higher (61.3%) than in other plants, for which enrichment efficiencies ranged between 10% and 22% (Ferguson et al. 2004; Moretzsohn et al. 2004). Primer pairs cannot always be designed from the SSR repeat motifs. Although more motifs (403) were identified and more primers (206) were designed in the genomic library compared to the cDNA library, a larger number of successful primers were obtained from the cDNA library (59.9%) than from genomic DNA library (51.1%). These values were slightly lower than the percentage of successful primers available in wheat (69.6%) (Gadaleta et al. 2010) and mungbean (65.2%) (Blaire et al. 2011), but much higher than ginseng (8.59%) (Chengjun et al. 2008). It was surprising that in *Cymbidium* a high percentage of the EST-SSR markers (74.3%) were polymorphic, as opposed to genomic SSRs (6.8%). In other crops, such as mungbean 32.5% and 39.8%, and ginseng 60.5% of the EST-SSR markers are polymorphic, respectively (Chengjun et al. 2008; Blaire et al. 2011). Although we used the same accession (*Cymbidium goeringii*) and program to design the SSR markers, a clear difference in polymorphic efficiency was observed. There are three possible reasons for these differences: (1) the use of different number of *Cymbidium* accessions during polymorphic screening, (2) only 39.3% of designed primers (not all primers) can be screened in the EST-SSRs, and (3) polymorphic primer pairs may be selected by chance while primer selection, although they were selected proportionally according to their repeat motifs types. In comparison, amplification with non-gene based microsatellites is prone to some pitfalls for AT-rich hybridization-derived genomic microsatellites (Blair et al. 2011). Differences between genic and other types of genomic microsatellites have been observed in marker sets of other crops (De Campos et al. 2007; Hanai et al. 2007).

Of the EST-SSRs, similar ratios of 47.4% and 47.1% di- and tri-nucleotide repeat motifs were identified, respectively. This differs from others crops, such as mungbeans, peanuts, and wheat, in which tri-nucleotides are the most common motif, followed by di- and other nucleotide types (Wang et al. 2009; Gadaleta et al. 2010; Teh et al. 2010; Blair et al. 2011). However, for genomic SSRs, di-nucleotides are the most abundant (82.9%), followed by tri-nucleotides (16.6%). This is not consistent with other crops, such as proso millet and Italian millet, in which tri-nucleotides are more common than di-nucleotides (Cho et al. 2010; Zhao et al. 2012). Although the AT/TA class was comparatively high in the EST-SSR di-nucleotides, it was less so in genomic SSR. It was previously reported that the most common di-nucleotide repeat in plants is TA (Tóth et al. 2000). However, this repeat is not suitable for hybridization because of its ability to auto-complement (Jia et al. 2009). The CTT/AAG/TCT/AGA/GAA and TGC/GCA/GCT/AGC/CTG/CAG motifs were the most frequent tri-nucleotide classes in EST-SSRs, while GTT/AAC/TGT/ACA/TTG/CAA motif class was the most frequent genomic SSR tri-nucleotide. The number of repeats ranged from 3 to 12 in EST-SSRs.

The average levels of polymorphism in the selected genomic SSRs were 0.589, while EST-SSRs were 0.497. The average gene diversities were 0.621 and 0.545 for genomic and genomic SSRs, respectively. A high PIC and gene diversity was identified in genomic SSRs. It is believed that polymorphism should be primarily present at the genomic level, and less so at the EST level. The average number of alleles per locus was also higher in genomic SSRs (7.3) compared with EST-SSRs (5.2), among the 24 evaluated *Cymbidium* accessions. Generally, as the number of genetic markers increases, higher genotyping resolution can be expected. Thus, we completed accurate grouping of the 52 EST-SSRs, identifying KNU085 as a unique class. The 52 SSR markers, rich in polymorphisms, will be useful for germplasm assessments, core set construction, assessment of genetic diversity, MAS-based crop breeding, and other *Cymbidium* improvements programs.

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REFERENCES


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How to reference this article:

## TABLES

Table 1. Cymbidium accessions used in this study showing the UPGMA results analyzed by 14 genomic SSR markers and 52 EST SSR markers.

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<th>No.</th>
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Table 2. A summary sequencing information and SSR marker designed by SSR Manager (ARGOS program).

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<td>4 Redundant clones</td>
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<td>5 Clones that had SSR motifs</td>
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<td>Total Primer designed</td>
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<td>59.93</td>
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<td>Tested for Polymorphism with 24 accessions</td>
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<td>Polymorphic markers</td>
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Table 3. Primer sequences and simple sequence repeat motif for new set of cDNA-derived (micorsatellite derived from cDNA sequence) series markers.

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<th>Sr No.</th>
<th>Primer Name</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>Motif</th>
<th>Expected Product Size</th>
<th>Amplification</th>
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<td>CGCCACCATAACCCAGTGT</td>
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<td>ATGGCCCAAGTTGACAG</td>
<td>(TA)9</td>
<td>251</td>
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<td>AATCTTGTCAACAAATGACAA</td>
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<td>CAGGCACTAGGAGCATGAGA</td>
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<td>TCCACTCACCAAAATAGTTTC</td>
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<td>GCAGTTTTGTCTGCGTGTA</td>
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<td>CCAAGGCTCACCACCA</td>
<td>(GA)5</td>
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Development of cDNA-derived SSR markers and their efficiency in diversity assessment of Cymbidium accessions

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<tr>
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<tr>
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<tr>
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DOI: 10.2225/vol15-issue2-fulltext-4
Table 4. Characteristics of the SSR sequences identified from genomic DNA library and cDNA library of *C. goeringii*.

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<th>Genomic DNA library</th>
<th>cDNA library</th>
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<td></td>
<td></td>
<td>Repeated motifs</td>
<td>(%)</td>
</tr>
<tr>
<td>Di</td>
<td>CT/AG/TC/GA</td>
<td>232</td>
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<tr>
<td></td>
<td>TG/CA/GT/AC</td>
<td>92</td>
<td>27.54</td>
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<tr>
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<td>TA/AT</td>
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<td>Tri</td>
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<tr>
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<td>TCGA/CGAT/GATC/ATCG</td>
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<td>AGCC/GCCA/CCAG/CAGC</td>
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<td></td>
<td>TTTT/TTCT/TCTT/CTTT</td>
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<td>8.33</td>
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<tr>
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<tr>
<td></td>
<td>Total</td>
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<tr>
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<td>Total</td>
<td>403</td>
<td>206 (51.11%)</td>
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Table 5. Amplification and polymorphism of 70 tested simple sequence repeat markers according to its repeated types using 24 *Cymbidium* accessions.

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<th>Others</th>
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<td>2</td>
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<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>21(84%)</td>
<td>21(84%)</td>
</tr>
<tr>
<td>Grand total</td>
<td>70</td>
<td>56(80%)</td>
<td>52(74.3%)</td>
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</table>
Table 6. Characterization of 14 simple sequence repeat markers identified as polymorphic markers using 24 cymbidium accessions.

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<th>Marker</th>
<th>Size Range</th>
<th>Major Allele Frequency</th>
<th>Allele No</th>
<th>Gene Diversity</th>
<th>Heterozygosity</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNU-CC-01</td>
<td>157-181</td>
<td>0.46</td>
<td>6</td>
<td>0.688</td>
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<td>0.641</td>
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<tr>
<td>KNU-CC-25</td>
<td>166-266</td>
<td>0.55</td>
<td>6</td>
<td>0.620</td>
<td>0.000</td>
<td>0.569</td>
</tr>
<tr>
<td>KNU-CC-30</td>
<td>116-284</td>
<td>0.79</td>
<td>4</td>
<td>0.354</td>
<td>0.000</td>
<td>0.330</td>
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<tr>
<td>KNU-CC-32</td>
<td>202-238</td>
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<td>15</td>
<td>0.896</td>
<td>0.913</td>
<td>0.887</td>
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<td>KNU-CC-34</td>
<td>169-243</td>
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<td>6</td>
<td>0.447</td>
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<td>KNU-CC-35</td>
<td>173-225</td>
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<td>9</td>
<td>0.808</td>
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<td>KNU-CC-40</td>
<td>218-250</td>
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<td>7</td>
<td>0.728</td>
<td>1.000</td>
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<td>KNU-CC-42</td>
<td>207-209</td>
<td>0.72</td>
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<td>0.405</td>
<td>0.478</td>
<td>0.323</td>
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<tr>
<td>KNU-CC-43</td>
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<td>0.709</td>
<td>0.636</td>
<td>0.687</td>
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<tr>
<td>KNU-CC-52</td>
<td>236-260</td>
<td>0.79</td>
<td>7</td>
<td>0.363</td>
<td>0.375</td>
<td>0.350</td>
</tr>
<tr>
<td>KNU-CC-55</td>
<td>105-243</td>
<td>0.23</td>
<td>8</td>
<td>0.833</td>
<td>0.792</td>
<td>0.812</td>
</tr>
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<td>105-243</td>
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<td>8</td>
<td>0.597</td>
<td>0.500</td>
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<tr>
<td>KNU-CC-76</td>
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<td>0.125</td>
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<td>0.621</td>
<td>0.416</td>
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Table 7. Characterization of 52 simple sequence repeat markers identified as polymorphic markers using 24 *Cymbidium* accessions.

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<th>Marker</th>
<th>Size Range</th>
<th>Major Allele Frequency</th>
<th>Allele No</th>
<th>Gene Diversity</th>
<th>Heterozygosity</th>
<th>PIC</th>
</tr>
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<td>CG-cSSR-1</td>
<td>122-379</td>
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<td>CG-cSSR-4</td>
<td>83-216</td>
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<td>0.167</td>
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<td>0.492</td>
<td>0.875</td>
<td>0.371</td>
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<td>CG-cSSR-8</td>
<td>171-336</td>
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<td>5</td>
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<td>0.708</td>
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<td>11</td>
<td>0.694</td>
<td>0.958</td>
<td>0.665</td>
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<td>0.250</td>
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**Total** | **271** |
| **Mean** | 0.58 | 5.2 | 0.545 | 0.601 | 0.497 |
Fig 1. Distribution of EST-SSR marker sizes. Coloured bars show the number of markers from di-nucleotide, tri-nucleotide, and other (tetra-, penta- and hexa-nucleotide) categories with different numbers of repeats.
Fig 2. UPGMA dendrograms showing phylogenetic relationships among the 24 Cymbidium accessions analyzed by (a) 14 genomic SSRs markers and (b) 52 EST-SSRs markers.