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Research Article
Study of Genetic Diversity on Six Species of Indonesian *Coelogyne* spp. Based on ISSR Markers

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Abstract

Background and Objective: Almost all of *Coelogyne* species from Indonesia are epiphytic. Some of these are facing the extincion and need to be conserved through plant breeding programs. Unfortunately, there are not many research reports on the genetic diversity of orchids which are substantial for genetic conservation and plant breeding program. The study aimed to identify the genetic diversity of some important species of genus *Coelogyne* spp., performed using inter simple sequence repeats (ISSR) molecular marker. **Materials and Methods:** The DNA of six orchid species from the genus of *Coleogyne* spp. was separated and served as samples in the PCR amplification reaction using 10 ISSR primers. **Results:** This study found that using six orchid species from the genus of *Coelogyne* spp. (*C. pandurata*, *C. massangeana*, *C. mayeriana*, *C. asperata*, *C. celebensis* and *C. rumphii*), the ISSR primers yielded as many as 106 amplified fragments which varied in size from 250-3000 bp. **Conclusion:** Moreover, this study showed that the polymorphic amplification bands reached as high as 98.9% and the similarity coefficient of the six orchid species studied revolved between 0.32-0.70, meaning that the genetic diversity of the orchid species studied was spread out between 0.30-0.68.

Key words: *Coelogyne*, genetic diversity, inter simple sequence repeats, molecular markers, orchid

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Orchidaceae, both natural and artificial hybrids, is the largest flowering plant family in the world[1]. Orchid is one of Indonesia's biological riches that have tremendous appeal. Indonesia's natural orchids were spread in various islands mostly in Sumatera, Kalimantan and Papua. The genus of *Coelogyne* Lindl. which had member more than 200 species and mainly spread out in Southeast Asia including Indonesia. Those species in general had flowers in various size which were pollinated by insects or wasps[2], they had very short (3-5 days) blooming period and many difficulties in self pollination[3]. Orchids of the genus of *Coelogyne* Lindl. were among the important orchids in Indonesia. Unfortunately, some of the species of *Coelogyne* are facing serious threat of extinction. Like what happens globally that the cause of orchid extinction is caused by the excessive exploitation without being offset by adequate rescue efforts[4], habitat destruction and difficulties of cultivation through conventional methods[3]. Fortunately, Indonesia has a collection of such threatened species which are now grown in the Bogor Botanical Gardens of Indonesia, for collections and as a source of germplasm for plant breeding programs, those were *Coelogyne pandurata* (East Kalimantan), *Coelogyne massangeana* (West Sumatra), *Coelogyne mayeriana* (Kalimantan), *Coelogyne asperata* (West Kalimantan), *Coelogyne celebensis* (South Sulawesi), *Coelogyne rumphii* (South Sulawesi).

The plant breeding program required information on the genetic diversity and classification that can indicate the level and relationship between cultivars as the basis for selection[5]. The genetic relationships or genetic distances among the cultivars are very substantial factor for the success in plant crossing program. The higher the coefficient value of similarity, the more similar phenotypic characters and the species contained in one group showed the close genetic relationship.

Inter simple sequence repeats (ISSR) have proven successful in identifying genetic diversity and kindship relationships[6-14]. The characterization of genetic diversity using the ISSR have been done in several species of orchids such as *Cymbidium*[15], *Cymbidium goeringii*[16], *Anoectochilus formosabus*[17], *Cymbidium sinense*[18], Cattleya[19], *Calanthe tsoongiana*[20], *Piperia yadonii* (*Orchidaceae*)[8], genetic diversity among populations *Rynchostylis retusa* epiphytic orchids from Goa[21], *Dendrobium officinale*[22]. *Balanophora fungosa*[23], *Cymbidium goeringii*[24]. The ISSR markers can also be used to examine the genetic stability of orchid plantlets generated by tissue culture[25]. The other benefit using the ISSR markers is that they are possible to be used for informing genetic conservation e.g., *Cymbidium ensifolium*[26], Lu *et al*.[18] Cattleya[27], Rhubarb[28]. Another benefit of the ISSR is that they may be used to select species from the same genera on herbal medicinal plants[29,30].

The study aimed to assess the genetic relationship among six important species of genus *Coelogyne* spp., which was done using molecular marker of ISSR. This information of genetic relationships is crucial factor for the success of plant breeding and for cultivar classification.

MATERIALS AND METHODS

The study was conducted at the Center for Plant Conservation Bogor Botanic Gardens, Indonesian Institute of Sciences (LIPI) from April until August, 2017.

Plant materials: Six species from genus *Coelogyne* spp., were taken from collection of Center for Plant Conservation, Kebun Raya Bogor Indonesian. Visual appearance of six species as well as their corresponding names in the collection, general geographical distribution and specific origin are presented in Table 1 and Fig. 1.

Chemicals: The chemicals required for DNA analysis with RAPD-PCR method are: CTAB, EDTA, Tris-HCL, PVPP, aquades sterile, mercaptoethanol, NaCl, quartz sands, chloroform, isoamyl, alcohol, ethanol absolute, alcohol 70%, PCR buffer reaction, dNTP mix, Taq DNA polymerase, $MgCl_2$ and primer. These all chemicals used in this study were obtained from commercial packaging products.

Method: There were 10 primers that were selected from 15 tested primers used in this study, those were UBC-835, UBC-815, UBC-880, UBC-810, UBC-834, UBC-814, UBC-826, UBC-843, UBC-844 and UBC-807.

DNA extraction: The DNA extraction was conducted as performed by Poerba and Ahmad[32] using the CTAB method of Delaporta which was modified with the addition of R Nase until the end concentration reached 250 μg mL^{-1}.

Table 1: Plant materials and the origin

Species	Locality and Habitat	Altitude (m)
Coelogyne pandurata	East Kalimantan	100
Coelogyne masangeana	West Sumatra	1150-2100
Coelogyne mayeriana	Kalimantan	100
Coelogyne asperata	West Kalimantan	320-l000
Coelogyne celebensis	South Sulawesi	826-220
Coelogyne rumphii	South Sulawesi	100-2000

Fig. 1(a-f): Species of *Coelogyne* spp., used in the study[31], (a) *C. pandurata*, (b) *C. massangeana*, (c) *C. mayeriana*, (d) *C. asperata*, (e) *C. celebensis* and (f) *C. rumphii*

DNA Amplification: The DNA amplification was performed using the method of Williams *et al.*[33], which was adopted from Poerba and Ahmad[32]. The procedures of DNA amplification we adopted from Poerba and Ahmad[32] were as follow: Ten primary ISSR (UBC-835, UBC-815, UBC-880, UBC-810, UBC-834, UBC-814, UBC-826, UBC-843, UBC-844 and UBC-807), which were previously selected and produced polymorphic bands on orchids. The PCR reaction was carried out at a total volume of 151 containing 0.2 nMdNTPs, 1X reaction buffer, 2 mM MgCl2, 25 ng DNA sample, 1 single primary prime and 1 µ of Taq DNA polymerase (Promega) using thermocycler (Takara) for 45 cycles. The first heating at 94°C for 2 min, followed by 45 cycles consisting of 1 min denaturation at 94°C, annealing 1 min at 36°C and 2 min extension at 72°C. After 45 cycles completed, followed by 5 min of extension process of DNA fragment at 72°C and cooling at 25°C. The PCR amplification results were visualized on a 2.0% agarose gel in TEA (Tris-EDTA) buffer by electrophoresis using Mini Mupid Cells for 50 min at 50 V. Then immersed in ethidium bromide solution with final concentration of 1 L/100 mL for 10 min. The DNA fragment separation results were detected using a translucent UV, then photographed using a camera. Standard size of 100 bp plus DNA ladder for setting the band size of DNA amplification yielded.

Data analysis: Genetic diversity is observed by scanning the appearance of DNA bands. The DNA tape is translated in binary data, the value of 1 means the band is exist and value of 0 means the band was absent. Analysis was done by cluster analysis using NTSYSpc (Numerical Taxonomy and Multivariative Analysis System) version 2.02[34] with Unweight Pair Group Method Arithmetic (UPGMA) method SIMQUAL (Similarity Qualitative). The grouping method used the dice coefficients of similarity for qualitative data (SIMQUAL) and Sequential Agglomerative Hierarchical and Nested (SAHN)-unweighted pair-group method arithmetic average (UPGMA)[35]. The genetic diversity among the species was then performed using the tree dendrograms.

RESULTS AND DISCUSSION

ISSR amplification: Among other PCR-based fingerprint methods, ISSR is a marker of DNA performing high polymorphism. The ten ISSR primer system used in this study

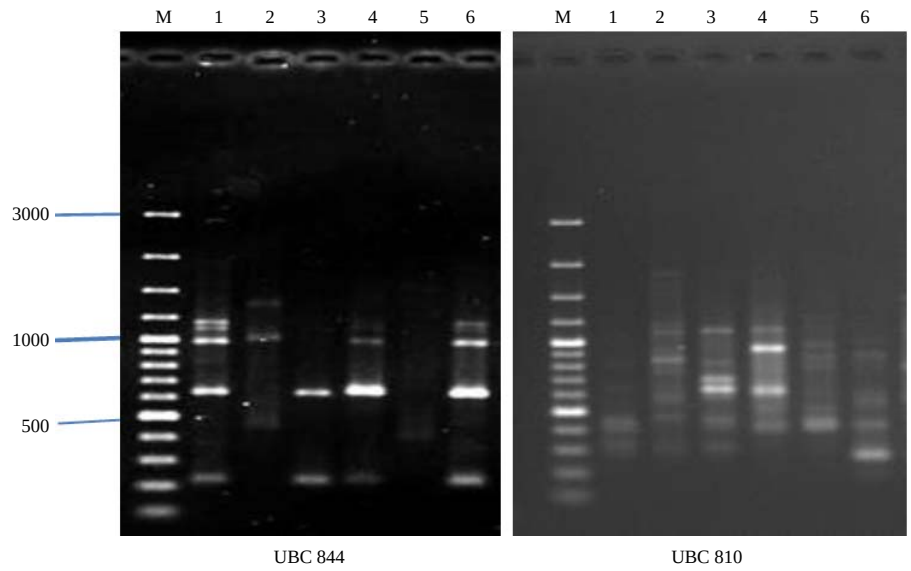

Fig. 2: DNA bands produced by ISSR UBC 844 and UBC 810: 1: *C. pandurata*, 2: *C. massangeana*, 3: *C. mayeriana*, 4: *C. asperata*, 5: *C. celebensis* and 6: *C. rumphii*

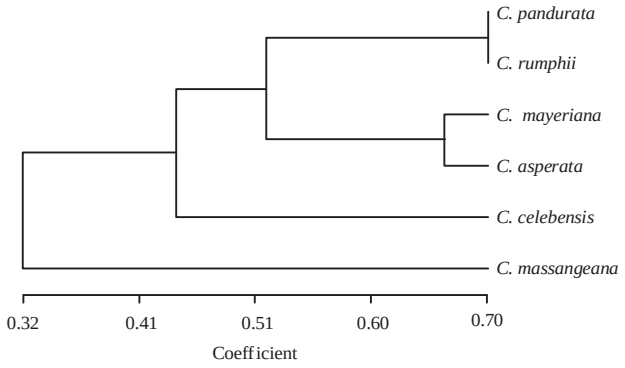

Fig. 3: Dendrogram of the six species of *Coelogyne* spp.

Table 2: ISSR primers used in DNA amplification

Primer	Sequence of Nucleotides (5'-3')
UBC-835	AGA GAG AGA GAG A GA GTC
UBC-815	CTC TCT CTC TCT CTC TG
UBC-880	GGA GAG GAG AGG AGA
UBC-810	GAG AGA GAG AGA GAG AT
UBC-834	AGA GAG AGA GAG AGA GYT
UBC-814	CTC TCT CTC TCT CTC TA
UBC-826	ACA CAC ACA CAC ACA CC
UBC-843	CTCTCTCTCTCTCTCTRA
UBC-844	CTCTCTCTCTCTCTCTRC
UBC-807	AGA GAG AGA GAG AGA GT

amplified 107 loci, meaning that one prime could amplify 10.7 loci in average (Table 2, Fig. 2). The percentage of polymorphism across all the *Coelogyne* from 89-100% with an average of 98.9% (Table 3). Parab and Krishnan[21] found that the seven ISSR primers among populations *Rynchostylis retusa* 40-80% polymorphism. The fragment number of each primer ranging from the minimum of 7 loci (found in UBC-826) to the maximum of 15 loci (observed in the UBC-843). While the size of bands was found in the range of 250-3000 bp (Table 3).

This finding corresponds to the study of Lu *et al.*[18]. They studied as many as 151 cultivar of *Cymbidium sinense* cultivars using 18 ISSR primers which produced as many as 14.478 DNA fragments having sizes in the range of 100-2500 bp. They also reported that from the 18 ISSR primers, each primer expressed fully polymorphism of the loci[18]. Research of Hartati *et al.*[31] showed that variability of six *Coelogyne* species revealed by 11 RAPD primer, the fragment number of each primer reached an average of 7.2 loci per primer. In another study, Wang *et al.*[36] identified as many as 31 *Dendrobium* species and concluded that the use of a single primer could detect an ISSR locus of 13-20.

Clustering pattern of *Coelogyne* spp.: In this study the genetic relationship between species studied was expressed by using the tree dendrogram with UPGMA link method. Results in the dendrogram showed that genetic diversity among genera *Coelogyne* had similarity coefficients between 0.32 and 0.70 (Fig. 3). It could be concluded that the genetic diversity of the studied orchid species is in the range of 0.30-0.68. These results suggest that the genetic variability of six *Coelogyne* species revealed by ISSR molecular marker in this study was higher than previous studies conducted using

Table 3: Band size and number of amplified and polymorphism loci

Primer	Band size (bp)	Number of amplified	Number of polymorphic loci	Percentage of polymorphic loci
UBC-835	250-3000	14.0	14.0	100.0
UBC-815	300-1200	10.0	10.0	100.0
UBC-880	350-2000	11.0	11.0	100.0
UBC-810	250-2000	13.0	13.0	100.0
UBC-834	400-1000	7.0	7.0	100.0
UBC-814	450-1200	10.0	10.0	100.0
UBC-826	500-1050	8.0	7.0	89.0
UBC-843	450-1900	15.0	15.0	100.0
UBC-844	250-1300	9.0	9.0	100.0
UBC-807	500-1500	10.0	10.0	100.0
	Total	107.0	106.0	989.0
	Average	10.7	10.6	98.9

RAPD markers which showed the range of 0.31-0.55 genetic similarity or the diversity was in the range of 0.45-0.69 (Hartati *et al*.)[31].

The dendrogram (Fig. 3) showed that the ten ISSR molecular marker was able to classify into tree similarity clusters at the coefficient similarity of 0.51. The first cluster consisted of *C. pandurata*, *C. rumphii*, *C. mayeriana*, *C. asperata*, the second cluster was *C. celebensis* and the third one was *C. massangeana*. The results similar to our previous studies using RAPD markers[31]. The other study by Wang *et al*.[36] stated that the genetic similarity resulted from their study using 31 species of *Dendrobium* spp., showed the similarity coefficient ranged from 0.512 (*Dendrobium chrysotoxum*/*D. moniliforme*) to 0.730 (*D. hancockii*/*D. hercoglossum*).

In the orchid *S. plicata*, from 10 primaries ISSR showed high genetic diversity up to 91.14%. Species that have a degree of genetic diversity high in the population will have a greater variety of alleles that can be selected[37]. The recent experimental results are in line with some of the experiments that have been reported in some orchids of the genus to another, as reported by Lu *et al*.[18] that the use ISSR markers was also managed to classify the genetic diversity of 151 orchid cultivars of *Cymbidium sinense* into 7 main groups. Shen *et al*.[22] also reported that the molecular markers ISSR fingerprints have also been successfully used to identify the orchids of *Dendrobium officinale* performed in 10 ISSR primers that were selected from 76 ISSR primer. The study showed that as many as 115 out of the 127 amplified DNA fragments were polymorphic (90.5%).

The closer the coefficient of similarity between one species of orchid will be greater the similarity and genetic distance, so there will be greater possibility of success in crosses[18]. Genetic distances carry implications in the plant breeding field. The higher the coefficient value of similarity then the similarity of the appearance of the plant will be higher. The results of this study provide implications for the selection of a combination of elders for crosses. The results of this study were information on which orchid species of the six species studied that can be crossed. However, to apply the results of this study for inter species crosses were that there should be available the parent plants with a flowering period simultaneously.

CONCLUSION

This study found that the genetic diversity among the six species of *Coelogyne* spp. (*C. pandurata*, *C. massangeana*, *C. mayeriana*, *C. asperata*, *C. celebensis* and *C. rumphii* spread out between 0.30-0.68. This study also concluded that the crosses having the highest probability of success were between *Coelogyne pandurata* with *Coelogyne rumphii* and between *Coelogyne mayeriana* with *Coelogyne asperata.* While the crosses between the other pairs of parents had a low success rate, because of the lower compatibility. Otherwise it would result in a new hybrid that had very different characters from the parents.

SIGNIFICANCE STATEMENT

This study discovers the genetic diversity of six species of Coleogyne orchids that can be beneficial for breeding program through cross pollination. This study will help the researcher to uncover the critical areas of crossing of orchid that many researchers were not able to explore. Thus a new theory on orchid breeding program may be arrived at.

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