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RAPD Profiles of *Rhynchostylis gigantea* (Lindl.) Ridl. Collected from Puspa Nirmala Orchids Banyumas, Central Java

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ABSTRACT . *Rhynchostylis gigantea* (Lindl.) Ridl. is an orchid species spread over Southeast Asia countries. This species is very popular among ornamental plant collectors, especially due to its densely pack inflorescences. Hence, it is commercially found in many ornamental plant nursuries, such as Puspa Nirmala Orchids Banyumas, Central Java. Further development of the species should be supported by scientific data, particularly regarding the genetic variation. One of the molecular markers commonly used to study genetic variation is Random Amplified Polymorphic DNA (RAPD). This study aims to assess genetic variation of *R. gigantea* cultivars of Puspa Nirmala Orchids Banyumas collection by RAPD profiles. Genomic DNAs were extracted from leaf samples of eight *R. gigantea* individuals, while RAPD markers were amplified using five random primers (OPA-15, OPK-16, OPP-15, OPP-08 and OPO-08). Descriptive analysis was employed on the data obtained. It was revealed that all of the primers resulted in a 100% monomorphism. This indicates an extremely low genetic variation among *R. gigantea* population of Puspa Nirmala Orchids collection, which is probably due to the same origin from a selected hybrid of the same crosses.

Keyword: Genetic variation; RAPD; *Rhynchostylis gigantea*

INTRODUCTION

Rynchostylis gigantea (Lindl.) Ridl. is one of the most popular orchid species among ornamental plant collectors, particularly because of its densely pack infloresences. In addition, this species has several flower colours and patterns varying from white, white with red-purple spots to orange (Thammasiri, 2016; Adthalungrong et al., 2015). It is distributed over Southeast Asia countries (Van Minh, 2019), China and India (Pathak et al., 2013), but is currently at risk of extinction, especially due to the complexity in its reproductive processes and the effects of some environmental factors such as pathogen infestation (Dekham & Kanchanawatee, 2020; Wongwan et al., 2021). Even it is now subjected to continuously decreasing genetic variability and thus listed in the Appendix-II of CITES (Jariyajirawattana & Bunnag, 2020).

Puspa Nirmala Orchids nursery, located in Banyumas Regency Central Java, has some collections of *R. gigantea*. However, these are very limited both in number and variation, especially regarding flower colour. Only two cultivars, i.e. those with peach and white flowers, are available. Therefore, conservation and development of this orchid species by means of well-programmed breeding approaches are needed.

To further develop *R. gigantea* in Puspa Nirmala Orchids nursery, basic information on the genetic variation is required. One of the simple methods to assess genetic variation of a plant population is by the use of Random Amplified Polymorphic DNA (RAPD) method. This PCR-based technique has been widely used to evaluate genetic variation in many plant species population including orchids, mainly due to its simplicity, rapidness and relatively low cost. As well, it only needs a very small amount of DNA for analysis and is available to various random primers (Rindyastuti et al., 2015).

Here we report our study on the RAPD profiles of *R. gigantea* cultivars of Puspa Nimala Orchids collections. The RAPD profiles obtained were then used to assess genetic variation of the population. This basic molecular data is useful to support further development of the orchid species.

EXPERIMENTAL SECTION

Materials and Methods

This study has been carried out in the Laboratory of Genetics and Molecular Biology, the Faculty of Biology, Universitas Jenderal Soedirman. Eight *R. gigantea* plant samples were purchased from Puspa Nirmala Orchids, consisting of four with peach flower and another four with white flower as depicted in **Figure 1**. Genomic DNAs were extracted from the upper most leaves of the respective *R. gigantea* sample by using CTAB method (Doyle & Doyle, 1990).

Amplification of RAPD markers was performed in a PTC-100 programable thermal cycler using the

following condition: pre-denaturation at 95 °C for 3 mins, proceeded by 40 reaction cycles consisting of denaturation at 95 °C for 1 min, annealing at 35 °C for 1 min, and extension at 72 °C for 1 min. The reaction was terminated with final extension at 72 °C for 5 mins. Each PCR mixture contained 1 μL (10 ng) template DNA, 2 μL (10 pmol) RAPD primer, 5 μL (1 unit) Promega master mix, and 4 μL nuclease free water. Five random primers resulting in a considerably high polymorphism in various plant species were employed (**Table 1**).

All PCR products were visualized on a 2% agarose gel electrophoresis using 1x TBE buffer solution. Electrophoresis was run at 80 V, 500 mA for 45 mins prior to gel staining with ethidium bromide and documentation under ultraviolet transilluminator.

Data on RAPD profiles were analyzed descriptively, in which individual RAPD band amplified using each primer were scored for its occurrence regardless the intensity. A binary code was employed, where score 1 for present and score 0 for absent. The respective band size represented one locus. A locus is considered polymorphic when the frequency of common allele is less than or equals to 95%, and oppositely it is monomorphic when the frequency of common allele is greater than 95% (Hartl & Clark, 2007).

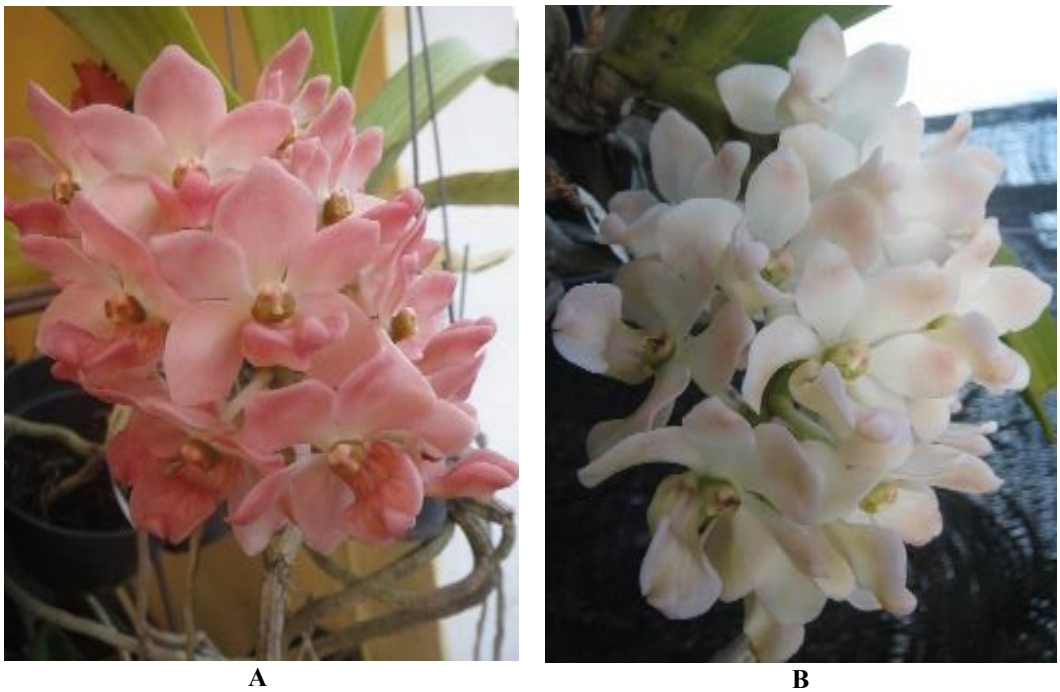
RESULTS AND DISCUSSION

All extracted genomic DNAs showed sufficiently good quality for PCR templates. Then, the amplicons produced using individual primer were run on the agarose gel electrophoresis as depicted in **Figure 2 to 6**. It can be seen from **Figure 2** that four PCR bands are produced in the respective sample, each of which are approximately 700, 1,000, 2,000 and 3,000 bp in size. Considering individual band size as a particular locus, it is simply understood that the four loci are all monomorphic. This is because all samples have bands of the same size or show similar band patterns.

Different from our finding, OPA-15 was proved to reveal 50% polymorphism in chili pepper (*Capsicum annuum*) (Bhadragoudar & Patil, 2011). Even OPA-15 was shown resulted in a 100% polymorphism in pigeon pea (*Cajanus cajan*) (Chandana et al., 2013).

Four loci or band sizes also result from OPK-16, i.e. about 500 bp, 700 bp, 900 bp and 1,100 bp respectively (**Figure 3**). Like those produced by OPA-15, they are all monomorphic. This is absolutely different from that obtained in rice (*Oryza sativa*) showing a 100% polymorphism when amplified using OPK-16 (Rajani et al., 2013). Slightly lower polymorphism (93.75%) with OPA-16 in maize (*Zea mays*) was found (Handi et al., 2013).

PCR-RAPD using OPO-08 results in only one band pattern consisting of five sizes, i.e. 350, 800, 1,000, 2,200 and 5,000 bp (**Figure 4**). All loci are monomorphic, because each band size occurs in the eight *R. gigantea* samples. On the contrary, OPO-08 resulted in a relatively high polymorphism, i.e. 85.71% in basil (*Ocimum* sp.) (Shanthy, Shalini, Santosh & Priyanka, 2014) and 62.5% in wheat (*Triticum aestivum*) (Abdellatif & AbouZeid, 2011).



A **B**

Figure 1. *Rynchostylis gigantea* (Lindl.) Ridl. from Puspa Nirmala Orchids used in this study (**A** = peach flower, **B** = white flower)

Table 1. Random primers used to amplify RAPD markers

Primer	Sequence (5′ – 3′)	T_m (°C)
OPA-15	TTCCGAACCC	34.2
OPK-16	GAGCGTCGAA	35.6
OPO-08	CCTCCAGTGT	32.0
OPP-08	ACATCGCCCA	37.6
OPP-15	GGAAGCCAAC	32.9

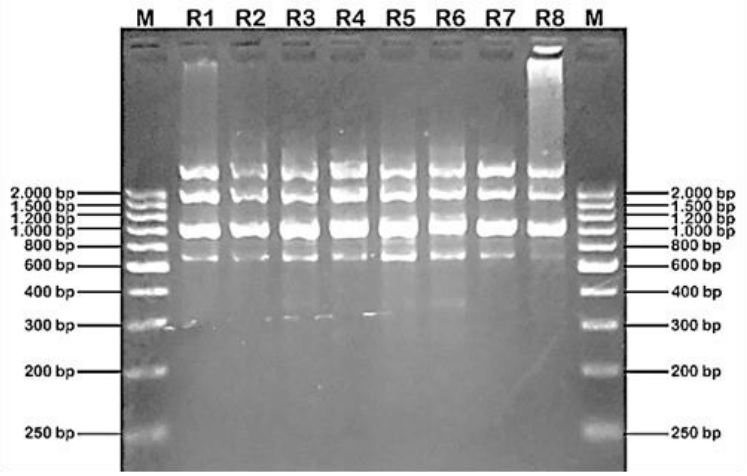


Figure 2. RAPD profile of *Rhynchostylis gigantea* (Lindl.) Ridl. from Puspa Nirmala Orchids using primer OPA-15 (M = DNA ladder, R1 – R4 = *R. gigantea* with peach flower, R5 – R8 = *R. gigantea* with white flower)

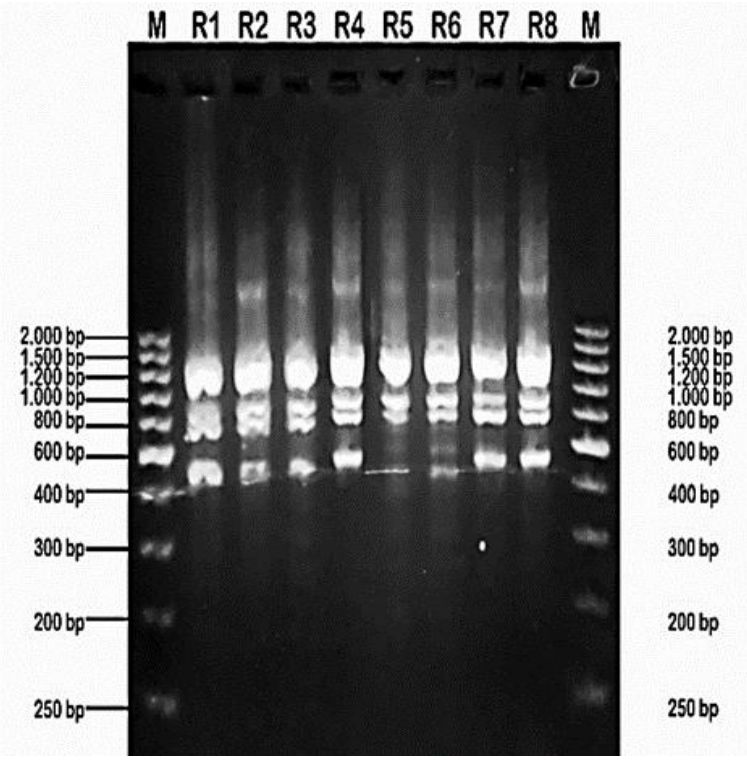


Figure 3. RAPD profile of *Rhynchostylis gigantea* (Lindl.) Ridl. from Puspa Nirmala Orchids using primer OPK-16 (M = DNA ladder, R1 – R4 = *R. gigantea* with peach flower, R5 – R8 = *R. gigantea* with white flower)

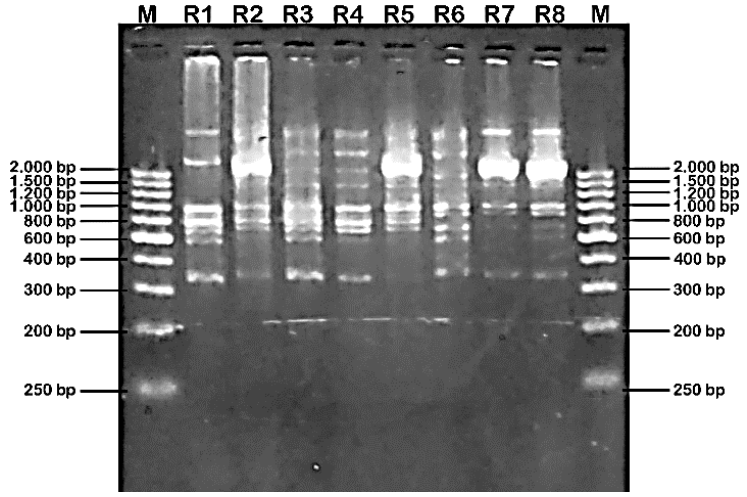


Figure 4. RAPD profile of *Rhynchostylis gigantea* (Lindl.) Ridl. from Puspa Nirmala Orchids using primer OPO-08 (M = DNA ladder, R1 – R4 = *R. gigantea* with peach flower, R5 – R8 = *R. gigantea* with white flower)

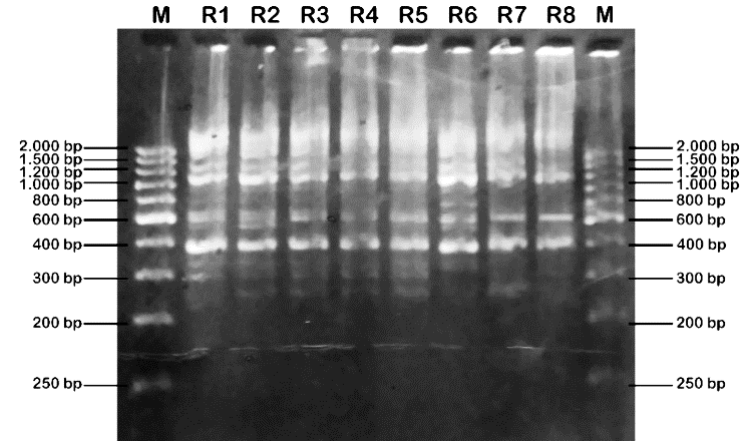


Figure 5. RAPD profile of *Rhynchostylis gigantea* (Lindl.) Ridl. from Puspa Nirmala Orchids using primer OPP-08 (M = DNA ladder, R1 – R4 = *R. gigantea* with peach flower, R5 – R8 = *R. gigantea* with white flower)

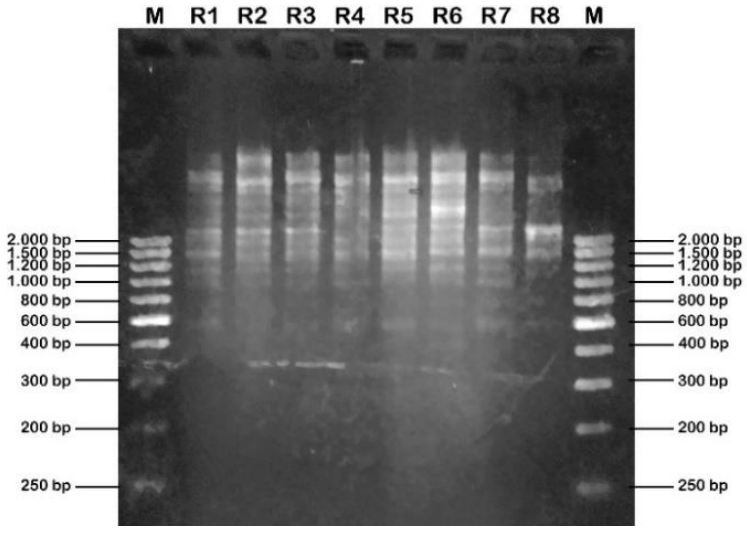


Figure 6. RAPD profile of *Rhynchostylis gigantea* (Lindl.) Ridl. from Puspa Nirmala Orchids using primer OPP-15 (M = DNA ladder, R1 – R4 = *R. gigantea* with peach flower, R5 – R8 = *R. gigantea* with white flower)

Figure 5 shows that OPP-08 also produces monomorphic bands in *R. gigantea*. Four loci of 400, 600, 1,100 and 2,200 bp in length are observed. Extremely distinct result with OPP-08 showing a 100% polymorphism in black gram (*Vigna mungo*) was reported (Vyas et al., 2016). In addition, OPP-08 resulted in 91.7% polymorphism in fenugreek (*Trigonella foenum-graecum*) (Tomar et al., 2014).

Similar to the other four primers, OPP-15 results in an complete monomorphism in *R. gigantea*. The band pattern shows four loci, i.e. 580, 1,700, 3,000 and 10,000 bp in size respectively (**Figure 6**). Conversely, much higher polymorphisms with OPP-15, i.e. 88.88% in *Terminalia* sp. (Deshmukh et al., 2009) and 61.53% in cumin (*Cuminum cyminum*) (Parashar et al., 2014) were reported.

The extremely low polymorphism of *R. gigantea* population in Puspa Nirmala Orchids indicates the absence of genetic variation in spite of the difference in flower colours. In most plant species such a fact can be due to self-fertilization occurring over many generations (Sa'diyah et al., 2013). Nevertheless, this is not commonly the case with orchids, since the stigma is adequately hidden causing very little opportunity of either insects to reach or wind blow to affect (Johnson & Steiner, 1997). Therefore, a more suitable assumption that could be suggested is that all *R. gigantea* individuals used in this study derive from a selected hybrid of the same crosses.

Transition in mating system from outcrossing to total autogamy in a widely distributed terrestrial orchid species, *Epipactis helleborine*, was observed. This led to low genetic variation and divergence, which had no relationship to either population size or spatial isolation. A strong genetic bottleneck effect after colonization of a new habitat presumably had caused the low genetic variation within population. Meanwhile, gene flow by means of easy seed dispersal might have been contributing to the low genetic differences among populations (Jacquemyn et al., 2020).

Different results with RAPD markers were obtained when genetic diversity of twenty six endemic orchid species in Papua was assessed. Ten random primers were employed resulting in a total of 54 polymorphic bands and no monomorphic one at all giving rise to the average number of 5.4 polymorphic bands. The calculated polymorphic information contents (PIC) ranged from 0.71 in OPA-12 to 0.92 in OPA-1 and OPD-11, while the number of genotypes ranged from 9 in OPA-12 to 17 in OPAW-05. Despite relatively high genetic diversity among and within Papua endemic orchid species, conservation were recommended prior to extinction (Abbas et al., 2017).

RAPD analysis has also been applied to assess genetic variability of another orchid species, *Spathoglottis plicata*. Seven random primers were selected to measure genetic variability among 17 variants of *S. plicata*, consisting of both mutants and wild types. DNA polymorphism of 53.28% was obtained, while variation in some morphological data on foliar characters and plant height were also observed. Nevertheless, no correlation between molecular and phenotypical variation was reported. Genetic variation among wild types was assumed due to somaclonal variation during *in vitro* culure. On the other hand, mutant variation was caused by random effect of induced mutation using X-ray irradiation previously applied. Much lower similarity index among mutants in comparison to that among wildtypes indicated potential formation of new cultivars (Auvira et al., 2021).

Combinations of genetic and phenotypic characteristics revealed significant phylogenetic relationship among some terrestrial orchid species in Turkey. However, it was not the case with some others. The molecular markers used were RAPD amplified with ten arbitrary primers, while the phenotypic traits involved foliar size and number, flower, stem and tuber sizes, and plant height. Overall, the results indicated the occurrence of a large quantity of gene flow at either intra- or inter-specific level in the natural population of Turkish terrestrial orchid species (Sandal Erzurumlu et al., 201s8).

Six random primers were used to amplify RAPD markers in the genetic variation analysis of intergeneric hybrids between *Coelogyne rumphii* and *C. pandurata*. Both parents were also involved in the analysis. Very high polymorphisms ranging from 87.5% to 100% resulted from all the six primers. Two large clusters were obtained in the dendogram generated by using UPGMA method. The first contained only *C. pandurata* as the male parent, while the second comprised both *C. rumphii* as the female parents and all the ten hybrids. This indicated that maternal inheritance in the intergeneric hybridization between both orchid species occurred (Hartati & Muliawati, 2020).

Characterization of some other orchid species, i.e. *Phaius* spp., by the use of RAPD markers have also been performed employing two random primers. As many as 11 PCR bands were produced with OPA-02, while 9 PCR bands resulted from OPA-16 amplification. Four *Phaius* species were used in the study, i.e. *P. tankervillae*, *P. montanus*, *P. collasus* and *P. amboinensis* (Hartati et al., 2021). Employing two other random primers, i.e. OPA-07 and OPA-09, the four *Phaius* species were shown clustered in two different groups. The first contained *P. tankervillae*, *P. montanus*, *P. collasus*, while the second consisted of only *P. amboinensis*. Even *P. amboinensis* showed higher genetic distance to the other three *Phaius* species in comparison to another orchid genus, *Dendrobium*. Four *Dendrobium* species, i.e. *D. mirbelianum*, *D. lamellatum*, *D. liniale*, *D. biggibum* belonged to one group, while *D. anosmum* was separated in another group (Yuhanna et al., 2021).

Low genetic variations were observed in the small populations of an endangered perennial orchid species, *Epipactis atrorubens*, in Northern Finland. Six micronuclear satellite loci were employed to analyze the genetic variation of the orchid populations. Among 11 populations under study, four were large populations and the other seven were small ones. Despite no significant correlation between genetic variation and fitness or population size, a positive correlation between the deterministic population growth rate and allelic richness was observed. Positive inbreeding coefficients in nearly all populations, particularly in the small ones, were found indicating that inbreeding might have been occurred. Both geitonogamy and autogamy due to pollinator deficit had presumably caused increasing probabilities of mating between genetically related individuals or even selfing rates. This in in turn might resulted in lower fitness by means of decreasing fecundity and survival rates as a consequence of deleterious recessive alleles combining in the homozygous individuals. Therefore, management should concentrate more on the conservation of large populations to avoid negative impact of stochasticity and to increase seedling recruitment rates (Hens et al., 2017).

Assessment on the genetic variations of five medicinal orchid species in Iran have been reported. They were *Anacamptis coriophora*, *Dactylorhiza umbrosa*, *Himantoglossum affine*, *Orchis mascula*, and *Ophrys schulzei*. Inter-retrotransposon amplified polymorphism (IRAP) was used as the molecular marker. A hundred percent of polymorphisms were obtained from a total of 473 IRAP bands produced. The analysis of molecular variance (AMOVA) revealed that slightly higher variation among populations was observed than those within populations. *D. umbrosa* was found as the most diverse species, while *H. affine* was the least diverse one. This findings could have a significant implication for germplasm characterization, conservation and improvement of the orchid species (Kaki et al., 2020).

RAPD markers were employed to evaluate genetic stability of micropropagated individuals of an epiphytic orchid species, *Rhyncostylis retusa*, known as both medicinal and ornamental plant species. Ten random primers were used to amplify RAPD markers in six samples comprising five *in vitro* regenerants and their mother plant. A total of 23 RAPD bands ranging from 275 bp to 1,100 bp in size were produced. This resulted in PIC values ranging from 0.28 in OPC-11 to 0.50 in OPA-3, OPA-6, OPA-07 and OPB-08. Nevertheless, absolutely high monomorphism was obtained, since similar band patterns were observed among all samples, including the mother plant. The very low polymorphism indicated considerably high genetic stability of the micropropagated *R. retusa* individuals, implying the pertinence of the *in vitro* culture technique for the orchid species conservation (Oliya et al., 2021).

High genetic stability, or in other words, low genetic variation was also found in the micropropagated *Dendrobium fimbriatum*. This ornamental and medicinal orchid species is listed in the Red Data Book of IUCN, so that *in vitro* propagation is required in supporting its conservation. RAPD markers, along with ISSR and SCoT, were used to assess the genetic stability. A hundred percent monomorphism or no polymorphism at all was found among the regenerants and also the mother plant when grown in the basal medium. Then, only 1.52%, 1.19% and 3.97% polymorphisms were observed with RAPD, ISSR and SCoT markers respectively when the plants were grown in hormone enriched medium. This considerably high genetic stability indicated a successful *in vitro* propagation of the orchid species (Tikendra et al., 2021).

RAPD and ISSR markers were used to analyze genetic variation and characterization in an ornamental and medicinal plant species, *Pseuderanthemum palatiferum* (Acanthaceae). Sixteen plant samples including the relatives, i.e. *P. bracteatum* and *Clinacanthus nutans*, collected from several sites in Vietnam were assessed for genetic variation. Both markers proved to produce a high number of polymorphic bands resulting in 100% polymorphisms. The calculated PIC from RAPD markers ranged from 0.62 in D41 to 0.90 in A39a, while those from ISSR markers varied from 0.28 in UBC811 to 0.87 in UBC810. The similarity coefficients varied from 0.53 to 0.95 with RAPD markers and from 0.32 to 0.91 with ISSR markers. This could genetically distinguish *P. palatiferum* from its two relatives. In addition, high genetic variation within *P. palatiferum* samples was also obtained from the data (Bui et al., 2021).

Two chloroplast DNA fragments, i.e. *rps* intron and *trnL-F* intergenic spacer were used to assess genetic variation and population genetic structure of a threatened orchid species, *Cypripedium tibeticum*, in China. This species, as well as many other members of genus *Cypripedium*, are found in the Red List of Chinese Species as threatened, mainly due to harvesting, breakdown in ecological connections, habitat loss and fragmentation. A total of 157 *C. tibeticum* individuals from 9 different populations in China were used as samples. Seven haplotypes were identified with a high total genetic diversity, i.e. 0.805 $\pm$ 0.078, mainly because of among-population component. Nevertheless, no substantial phylogeographic structure was observed. Two populations, i.e. Chayu and Daocheng, were recommended as the top most priority in the conservation plan due to their highest contribution to the total genetic diversity and the presence of four rare haplotypes (Guo et al., 2019).

Genetic diversity or variation can be considered the base for survival of plants in nature and for crop improvement. It is very important concerning

adaptability to various environmental conditions, especially related to climatic changes. Recombination of alleles in sexual reproduction is the primary source of genetic variation. Then, evolutionary forces such as migration, mutation, selection and genetic drift act continuously causing alteration in allelic frequency in a population and thus affect genetic variation (Bhanu et al., 2017).

The very low genetic variation in *R. gigantea* might lead to increasing risk of extinction. Anticipating this condition, rapid *in vitro* propagation of white-flower mutant of *R. gigantea* has been carried out using 4 month-old immature seed-derived protocorm-like bodies (PLBs). The calli produced were maintained by subculturing them at an interval of 2 months on half-strength MS medium containing 0.05 $mg.L^{-1}$ 6-BA and 0.2 $mg.L^{-1}$ NAA. PLBs were formed after the calli had been transferred into hormone-free MS medium, which then developed into shoots with 2 – 4 leaves within 30 days. Root development and shoot growth occurred when the shoots were transferred into half-strength MS medium containing 100 $g.L^{-1}$ banana homogenate. The established *ex vitro* plantlets showed 95% survival (Li & Xu, 2009). Another attempt to develop *in vitro* culture of *R. gigantea* has been performed by using modified VW medium supplemented with chitosan. The chitosan concentration of 10 $mg.L^{-1}$ increased leaf number, plant height and root dry weight, while that of 20 $mg.L^{-1}$ increased the longest root length. No survive seedling in VW enriched with 60 $mg.L^{-1}$ chitosan was found (Obsuwan et al., 2010). Both VW and ND medium supplemented with 2% sucrose and 15% coconut water under light or dark condition exhibited almost the same proliferation of calli in the micropropagation of *R. gigantea* (Rittirat et al., 2011).

While micropropagation approaches were continuously developed, the applications of plant growth regulators and day-length treatments have also been performed to improve the quality of *R. gigantea*. Short-day was found to induce flowering more quickly in comparison to natural condition, while GA3 had effects merely on leaf number per plant. Endogenous ABA in leaf showed highest level under natural condition that was combined with GA3 application (Phengphachanh et al., 2012).

Apart from the absolutely low genetic variation in *R. gigantea* observed in this study, phenotypic characterization on both quantitative and qualitative traits has been previously performed by another group of work. This revealed two clusters of *R. gigantea* based on geographical condition. The first lived in the low-lands, while the second was found in mountainous areas (Anuttato et al., 2017). The absence of correlation between genetic and phenotypics assessment is maybe due to the molecular markers used. Therefore, appropriate molecular markers responsible for particular phenotypic traits should be explored for better evaluation on the genetic variation of this orchid species population.

CONCLUSSIONS

The extremely low genetic variation among *R. gigantea* population of Puspa Nirmala Orchids collection is strongly asumed related to the same origin of the individuals from a selected hybrid of the same crosses. However, more appropriate molecular markers rather than RAPD should be employed for better assessment of the genetic variation.

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