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Research Article

Genetic diversity among *Vanda celebica*, *Vanda dearei*, and their hybrids based on ISSR markers

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ABSTRACT

A cross between two different orchid species is a way to improve the horticultural performance of the hybrids. Inter-simple sequence repeat (ISSR) is a method of molecular markers that could be used to analyze genetic diversity among parents and their hybrid progenies. The purpose of this study was to examine the genetic diversity of the parent species of Vanda dearei and Vanda celebica, the hybrid progenies of (♀ Vanda dearei x ♂ Vanda celebica)-cross and the hybrid progenies of their reciprocal cross. DNA amplification using 5 ISSR primers, namely UBC 810, UBC 815, UBC 834, UBC 835, and UBC 880, produced 60 DNA bands with a size of 200-2800 bp, with the percentage of polymorphism ranging from 81.8%-100% and the percentage of monomorphism as much as 0%-18.2%. Analysis of genetic diversity showed that the genetic diversity among hybrid progenies of (♀V. dearei x ♂V. celebica) was 8%. In comparison, those among hybrid progenies of its reciprocal cross (♀V. celebica x ♂V. dearei) were 13%. In conclusion, deploying V. celebica as a female parent and V. dearei as a male parent will most likely produce higher diversity among its progenies, compared to the reciprocal cross.

Keywords: DNA bands; diversity; marker, orchid; reciprocal cross

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INTRODUCTION

One of the orchid genera, Vandaceous, is widely cultivated as cut flowers and ornamental plants. Numerous Vandaceous are crossed to form new hybrids because they have various flower colors. A cross between two different orchid species is a way to improve the horticultural performance of the hybrids. Vanda is difficult to find in nature, so crossing efforts are being made to add new genetic diversity as an orchid conservation effort (Kasutjianingati & Firgiyanto, 2018).

Vanda celebica was discovered in 1899 by Rolfe on the islands of Sulawesi and East Kalimantan. *Vanda celebica* has pale greenish-yellow sepals, and slightly reddish-brown speckled petals with a red lip (Gardiner et al., 2013). *Vanda dearei* is a species of orchid endemic to Kalimantan which is spread around Kinabatangan and Tenom in Sabah, Kuching in Sarawak, Sekayan River in West Kalimantan, and Kutao in East Kalimantan. *Vanda dearei* is listed as an endangered species in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). This species has large beautiful flowers and a strong aroma that blooms all year round (Jualang et al., 2014).

Selection of parents and hybridization techniques is the first step taken in crossbreeding plants which affects the genetic diversity of the hybrids. The selection of parents with high morphological compatibility determines the success of the

hybridization program (Hartati et al., 2019). One of the hybridization techniques is reciprocal cross or the opposite cross by changing male parents to female parents and *vice-versa*. Molecular markers were used to analyze genetic diversity in parents and hybridization results. Among the PCR-based molecular markers, inter-simple sequence repeat (ISSR) is one of the methods that generate distinct DNA bands.

ISSR is a kind of DNA marker that is constructed from repeating primers between simple sequence repeats (SSR) regions. ISSR can analyze many loci because it has a long DNA sequence. The polymorphism produced by ISSR is higher than that of RAPD (Ma et al., 2019; Rajaram et al., 2019; Tang et al., 2021). ISSR has been used to identify genetic diversity in the *Dendrobium nobile* orchid population and resulted in 90% polymorphism (Bhattacharyya et al., 2015) and *Coelogyne* spp. with polymorphism reaching 98.9% (Hartati, 2017). Crosses of *Vanda celebica* with *Vanda dearei*, with *Vanda celebica* as the female parent, have a success of 33%-100% crossing, whereas if *Vanda celebica* is used as the male parent the success achieved is 0% - 67% (Hartati et al., 2017). The purpose of this study was to examine the genetic diversity of the parent species, namely the *Vanda celebica*, *Vanda dearei*, and their hybrid progenies using ISSR markers.

MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Breeding, Faculty of Agriculture, The Sebelas Maret University, Surakarta. The material resources used in this study were *Vanda dearei* (P1), and *Vanda celebica* (P2) as parents, *Vanda dearei* x *Vanda celebica* (C1, C2) as F_1 hybrids, and a reciprocal cross between *Vanda celebica* x *Vanda dearei* (R01, R02) which is described in (Table 1). The young leaves of five-year-old Vanda spp. orchids and three-year-old hybrids from the Spisy Klaten orchid garden were used for DNA extraction. The ISSR primers used were UBC-810, UBC-815, UBC-834, UBC-835, UBC-880 (Table 2).

Table 1. Genetic resources of orchids evaluated.

Code	Species name	Origin
P1	*Vanda dearei*	Kalimantan
P2	*Vanda celebica*	Sulawesi
C1, C2	F_1 hybrids	♀ *Vanda dearei* x ♂ *Vanda celebica*
R01, R02	F_1 hybrids of reciprocal cross	♀ *Vanda celebica* x ♂ *Vanda dearei*

Table 2. ISSR Primer sequences.

Primer	Sequence of nucleotides (5'-3')
UBC 810	AGA GAG GAG GAG AGA AT
UBC 815	CTC TCT CTC TCT CTC TG
UBC 834	AGA GAG AGA GAG AGA GYT
UBC 835	AGA GAG AGA GAG A GA GTC
UBC 880	GGA GAG GAG AGG AGA

The DNA isolation method from orchid plants is based on the isolation protocol from Doyle and Doyle (1990) with modifications that include the addition of a 3% Polyvinyl Pyrrolidone (PVP) buffer. The leaves sample was weighed at 0.1 g. Subsequently, each sample was placed into a 2 mL microtube, followed by the addition of 1,400 µL CTAB and 2 buckshot pellets. The samples were incubated in a water bath at 60 °C for 1 hour and turned upside down for 10 minutes; after 2 minutes rest, 500 µL of CIAA (Chloroform: Isoamyl alcohol 24:1) was added to the 6 samples. After that, the sample was vortexed until homogeneous and centrifuged at 12,000 rpm for 15 minutes to separate the DNA solution from debris. About 1000 µL of supernatant was collected, and 500 µL of CIAA was added, vortexed, and centrifuged at 12,000 rpm for 15 minutes. Then, 800 µL of supernatant was collected, then 80 µL sodium acetate and 586.7 µL cold isopropanol were added.

Before amplifying DNA, it was dissolved with 50 µL ddH_2O per sample, while 10 µl of ISSR primer was dissolved with 90 µL ddH_2O. The PCR machine program was initially

heated at a temperature of 94 °C for 2 min, followed by 45 cycles consisting of denaturation at a temperature of 94 °C for 1 min and annealing at a temperature of 36 °C for 1 min. The PCR cycle for the ISSR marker ended with one final extension cycle at 72 °C for 5 min and cooling at 25 °C.

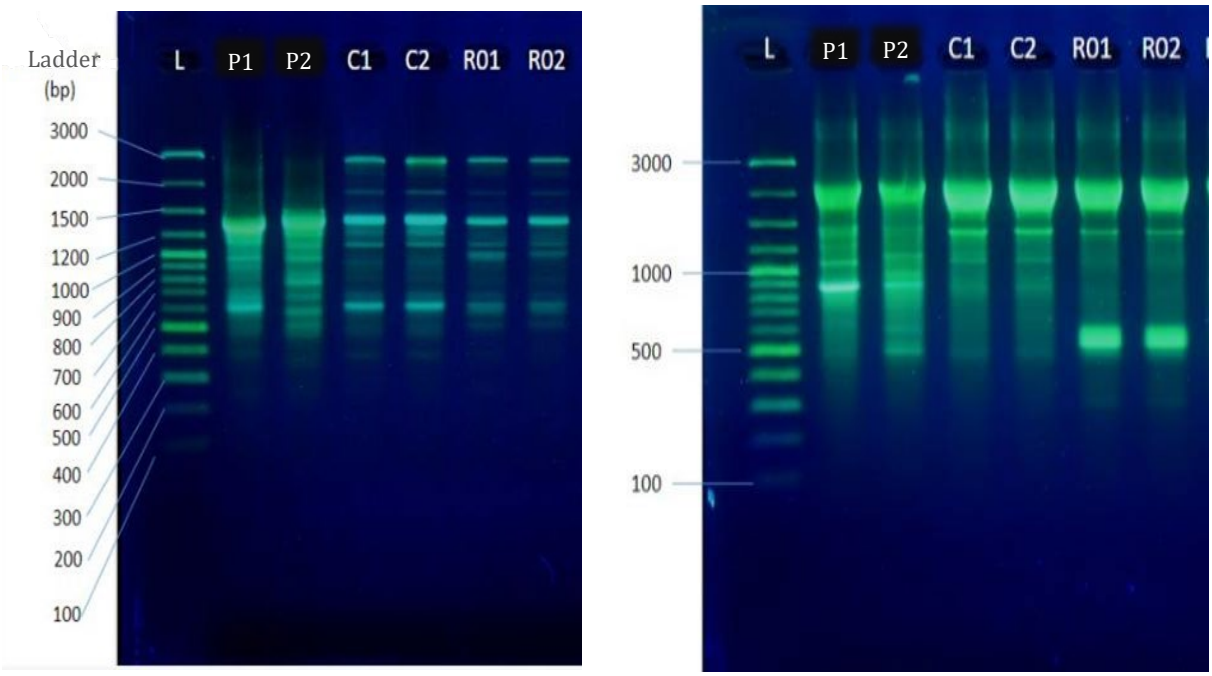
PCR amplification products were analyzed using electrophoresis with a 1.5% agarose concentration. Agarose (GeneDirex) 0.45 g was added to Buffer 1X TBE 30 mL in an Erlenmeyer flask. The Erlenmeyer-containing agarose was heated in a microwave for 1 min, and then after it was homogeneous, 5 µL of fluorosafe DNA staining was added. The agarose solution was poured into the mold; then, the comb was placed in position and left for about 45 min until the agarose solidified. TBE 1X buffer was poured into the electrophoresis tank, and then the agarose gel was placed into the tank with the negative pole. The PCR product in the form of DNA was inserted into the gel well, and the DNA marker was inserted into the right and left edges of the gel. The electrophoresis chamber was set to a voltage of 100V, 400 mA, for 70 min. This process was carried out until the dye loading moved to close to 1/3 of the bottom of the gel. The agarose gel resulting from electrophoresis was visualized with UV light with DNA fragments appearing blue and documented using a camera.

Data analysis was carried out by assigning scores to the bands that appeared. A score of 1 was given for bands that were present, while absent bands were scored as 0. Dendogram analysis was conducted using NTSYSpc (Numeral Taxonomic System) software version 2.02 with the SIMQUAL (Similarity Qualitative) UPGMA (Unweight Pair Group Method Arithmetic) method. The similarity index is calculated using the DICE formula.

RESULTS AND DISCUSSION

Genetic variation describes the dynamics and viability of a population (Gholami et al., 2021). Low genetic variation in a population is the impact of genetic drift resulting from a decrease in population size that can limit gene flow in the population. Polymorphisms produced by each primer have a different percentage, this is due to the difference in sequences between DNA. Giri et al. (2017) suggested that plant breeding programs could utilize ISSR markers and morphological, especially if the linkage between plants to be crossed was unavailable. Figure 1 shows that the bands produced by primers UBC 834 and UBC 880 have unequal positions. This might be due to differences in DNA sequences between samples, resulting in polymorphism in these species. According to Ellegren & Galtier (2016), Each species has a certain DNA sequence that is different from other species, these differences form genetic diversity known as polymorphism of a species.

Primer UBC 834 amplified 9 DNA bands with a size of 500-2800 bp, and resulted in 88.9% polymorphism with 1 monomorphic band at a size of 2800 bp, while the primer UBC 880 amplified 11 DNA bands measuring 800-2800 bp, and yielded 81.8% polymorphic band with 2 monomorphic bands measuring 1600 bp and 2100 bp. The DNA bands of samples C1 and C2 (♀ *Vanda dearei* x ♂ *Vanda celebica*) had the same pattern as the UBC 834 primer and were slightly different on the UBC 880, namely the difference in the size of 800 bp, and 2600 bp whereas at that size there was a C2 DNA band but no DNA band at C1. Samples R01 and R02 (♀ *Vanda celebica* x ♂ *Vanda dearei*) had the same banding pattern in the UBC 834 primer and 1 different DNA band in the UBC 880 primer at size 2600 (Table 3, Figure 1).



UBC 834 UBC 880

Figure 1. The ISSR banding pattern of five Vanda species was obtained with UBC 834 and UBC 880. L: ladder (1 kb); Lane (P_1) *Vanda dearei*, (P_2) *Vanda celebica*, (C1, C2) ♀ *Vanda dearei* x ♂ *Vanda celebica*), (R01, R02) ♀ *Vanda celebica* x ♂*Vanda dearei*

According to Ramekar et al. (2015), DNA bands that appear in hybrids but are not found in parents may be caused by recombination or mutation. Recombination, mutation, and rearrangement of chromosomes are some of the main mechanisms that cause genetic diversity. Recombination is an event during meiosis that involves the physical exchange of material between chromatids of homologous chromosomes. According to Wolter et al. (2019), the plant breeding program has succeeded in providing new plant varieties with new traits that are better than their parental traits. The efficiency of plant breeding can be achieved by selection based on molecular markers so that parents can be selected and produce superior hybrids.

All primers used amplified 60 bands sized 200-2800 bp, with the percentage of polymorphism ranging from 81.8-100% and the percentage of monomorphism as much as 0-18.2%. UBC 835 primer produced the most bands (15 DNA bands) with a size of 200-2800 bp, with 100% polymorphic. High polymorphism indicates high genetic diversity (Mehmood et al. (2013). ISSR markers have analyzed the genetic diversity of orchids, for example in the terrestrial orchid *Cypripedium japonicum* with a genetic diversity reaching 80% (Tian et al., 2018), *Cattleya granulosa* with a polymorphism percentage of more than 70%, and a genetic diversity of 30% (Fajardo et al., 2017). Qian et al. (2013), found that the rare orchid *Calathea tsoongiana* has a high polymorphism of 98.8%.

Table 3. Description of the ISSR product obtained from *Vanda dearei* (P1), *Vanda celebica* (P2), ♀ *Vanda dearei* x ♂ *Vanda celebica* (C1, C2), ♀ *Vanda celebica* x ♂ *Vanda dearei* (R01, R02).

No	Primary	Size (bp)	Number of bands	Polymorphic bands	Monomorphic bands	% Polymorphic	% Monomorphic
1	UBC 810	600-2400	13	13	0	100.00	0.00
2	UBC 815	500-2800	12	11	1	91.70	8.30
3	UBC 834	500-2800	9	8	1	88.90	11.10
4	UBC 835	200-2800	15	15	0	100.00	0.00
6	UBC 880	800-2800	11	9	2	81.80	18.20

Table 4. Similarity index of *Vanda dearei* (P1), *Vanda celebica* (P2), ♀ *Vanda dearei* x ♂ *Vanda celebica* (C1, C2), ♀ *Vanda celebica* x ♂ *Vanda dearei* (R01, R02).

	P1	P2	C1	C2	R01	R02
P1	1.000					
P2	0.582	1.000				
C1	0.552	0.316	1.000			
C2	0.603	0.387	0.923	1.000		
R01	0.453	0.423	0.618	0.600	1.000	
R02	0.500	0.473	0.552	0.603	0.868	1.000

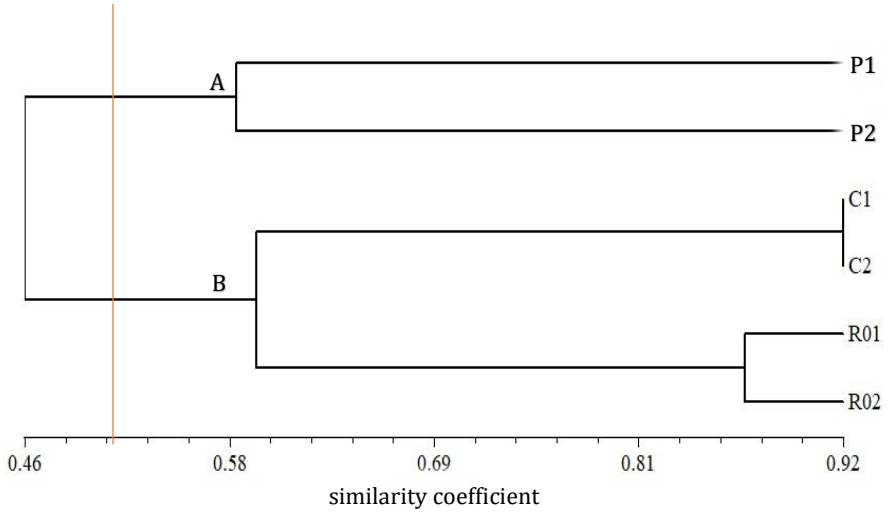


Figure 2. UPGMA dendrogram of ISSR markers showing the genetic relationship between *Vanda dearei* (P1), *Vanda celebica* (P2), ♀ *Vanda dearei* x ♂ *Vanda celebica* (C1, C2), ♀ *Vanda celebica* x ♂ *Vanda dearei* (R01, R02).

At a similarity coefficient of 0.50, the genotypes were divided into two clusters (Figure 2). Cluster A contained the parents of *Vanda dearei* (P1), and *Vanda celebica* (P2) which have a similarity coefficient of 58% or genetic diversity of 42%. Cluster B segregated into two sub-clusters, namely sub-cluster I contained hybrids from the cross ♀ *Vanda dearei* x ♂ *Vanda celebica* (C1, C2), places *Vanda dearei* as a female parent, with high genetic similarity of 92%, or with a very low genetic diversity of only 8%. The sub-cluster II contained crosses showing the hybrids of ♀ *Vanda celebica* x ♂ *Vanda dearei* (R01, R02), with *Vanda dearei* as the male parent, resulting in a genetic similarity of 87% or genetic diversity of 13% (Table 4, Figure 2). Thus, the hybrids from a reciprocal cross with *Vanda celebica* as the female parent and *Vanda dearei* as the male parent generated a genetic diversity of 5% higher than those of the reciprocal cross.

Table 4 shows that the hybrid accessions had more genetic similarities to the parent *Vanda dearei* (P1). The similarity coefficient of the hybrids (C1, C2, R01, R02) with *Vanda dearei* (P1) was greater than with *Vanda celebica* (P2). As reported by Fu (2015), different plant breeding methods show different impacts on plant genetic diversity. Parental selection could increase genetic diversity in hybridization results, while intraspecific hybridization can reduce genetic diversity. Parents were selected based on the magnitude of genetic closeness due to their high compatibility. *V. celebica* and *V. dearei* are used as female or male hybridization parents to obtain new commercial hybrid orchids.

CONCLUSIONS

Genetic diversity of *Vanda dearie*, *Vanda celebica*, and hybrids resulting from cross ♀*Vanda dearei* x ♂*Vanda celebica* and the reciprocal cross of ♀*Vanda celebica* x ♂*Vanda dearei* was detected using the ISSR markers. All ISSR markers produced 60 DNA bands with a size of 200-2800 bp, with the percentage of polymorphism ranging from 81.8-100%. Analysis of genetic diversity showed that the hybrids from *Vanda dearei* as female parents had a genetic diversity of 8% while the reciprocal cross had a genetic diversity of 13%. The genetic diversity of hybrids resulting from the reciprocal cross with *Vanda celebica* as the female parent and *Vanda dearei* as the male parent produced a genetic diversity that was 5% higher than those of the reciprocal cross.

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REFERENCES

Bhattacharyya, P., Kumaria, S., & Tandon, P. (2015). Applicability of ISSR and DAMD markers for phyto-molecular characterization and association with some important biochemical traits of *Dendrobium nobile*, an endangered medicinal orchid. *Phytochemistry, 117(1),* 306–316. https://doi.org/10.1016/j.phytochem.2015.06.022

Doyle, J. J., & Doyle, J. L. (1990). Isolation of Plant DNA from Fresh Tissue. *Focus*, *12*(1), 13-15.

Ellegren, H., & Galtier, N. (2016). Determinants of genetic diversity. *Nature Reviews Genetics, 17(7)*, 422–433. https://doi.org/10.1038/nrg.2016.58

Fajardo, C. G., de Almeida Vieira, F., Felix, L. P., & Molina, W. F. (2017). Negligence in the Atlantic forest, northern Brazil: a case study of an endangered orchid. *Biodiversity and Conservation, 26*(5), 1047–1063. https://doi.org/10.1007/s10531-016-1285-5

Fu, Y. B. (2015). Understanding crop genetic diversity under modern plant breeding. *Theoretical and Applied Genetics, 128*(11), 2131–2142. https://doi.org/10.1007/s00122-015-2585-y

Gardiner, L. M., Kocyan, A., Motes, M., Roberts, D. L, & Emerson, B. C. (2013). Molecular phylogenetics of Vanda and related genera (Orchidaceae). *Botanical Journal of the Linnean Society, 173*(4), 549–572. https://doi.org/10.1111/boj.12102

Gholami, S., Vafaee, Y., Nazari, F., & Ghorbani, A. (2021). Molecular characterization of endangered Iranian terrestrial orchids using ISSR markers and association with floral and tuber-related phenotypic traits. *Physiology and Molecular Biology of Plants, 27*(1), 53–68. https://doi.org/10.1007/s12298-0200-0920-0

Giri, L., Jugran, A. K, Bahukhandi, A., Dhyani, P., Bhatt, I. D., Rawal, R. S., Nandi, S. K., & Dhar , U. (2017). Population genetic structure and marker-trait associations using morphological, phytochemical, and molecular parameters in *Habendaia edgeworthii*—a threatened medicinal orchid of West Himalaya, India. *Applied Biochemistry and Biotechnology, 181*(1), 267–282. https://doi.org/10.1007/s12010-016-2211-8

Hartati, S. (2017). Study of genetic diversity on six species of Indonesian *Coelogyne* spp. based on ISSR markers. *Pakistan Journal of Biological Sciences, 20*(11), 577–583. https://doi.org/10.3923/pjbs.2017.577.583

Hartati, S., Nandariyah, Yunus, A., & Djoar, D. W. (2019). Hybridization technique of black orchid (*Coelogyne pandurata* Lindley) to enrich the genetic diversity and to rescue the genetic extinction. *Bulgarian Journal of Agricultural Science, 25*(4), 751–755.

Hartati, S., Sumijati, S., Pardono, P., & Cahyono, O. (2017). Genetic improvement of natural orchids of *Vanda* spp through interspecific crosses to support the development of orchids in Indonesia. *Caraka Tani: Journal of Sustainable Agriculture, 29*(1), 31. https://doi.org/10.20961/carakatani.v29i1.13304

Jualang, A. G., Devina, D., Hartinie, M., Sharon, J. S., & Roslina, J. (2014). Asymbiotic seed germination and seedling development of *Vanda dearei*. *Malaysian Applied Biology, 43*(2), 25–34.

Kasutjianingati, K., & Firgiyanto, R. (2018). Characterization of morphology from orchid *Vanda* sp. as a genetic information source for preservation and agribusiness of orchids in Indonesia. *IOP Conference Series: Earth and Environmental Science, 207*(1). https://doi.org/10.1088/1755-1315/207/1/012006

Ma, M., Dong, X., Liao, K., Hao, F., Qin, R., & Liu, H. (2019). Outcrossing rates and gene flow in the natural population of the endangered endemic aquatic lycophyte *Isoetes yunguiensis* as revealed by ISSR markers. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 47*(2), 339–346. https://doi.org/10.15835/nbha47111264

Mehmood, A., Jaskani, M. J., Ahmad, S., & Ahmad, R. (2013). Evaluation of genetic diversity in open-pollinated guava by iPBS primers. *Pakistan Journal of Agricultural Sciences, 50*(4), 591–597.

Qian, X., Wang, C. xia, & Tian, M. (2013). Genetic diversity and population differentiation of *Calanthe tsoongiana*, a rare and endemic orchid in China. *International Journal of Molecular Sciences, 14*(10), 20399–20413. https://doi.org/10.3390/ijms141020399

Rajaram, M. C., Yong, C. S., Gansau, J. A., Go, R. (2019). DNA barcoding of endangered Paphiopedilum species (Orchidaceae) of Peninsular Malaysia. *Phytotaxa, 387*(2), 94-104. https://doi.org/10.11646/phytotaxa.387.2.2

Ramekar, R. V., Sa, K. J., Woo, S. Y., & Lee, J. K. (2015). Non-parental banding patterns in recombinant inbred line population of maize with SSR markers. *Genetics and Molecular Research, 14*(3), 8420–8430. https://doi.org/10.4238/2015.July.28.9

Tang, H., Tang, L., Shao, S., Peng, Y., Li, L., Luo, Y. (2021). Chloroplast genomic diversity in Bulbophyllumsection Macrocaulia (Orchidaceae, Epidendroideae, Malaxideae): Insights into species divergence and adaptive evolution. *Plant Diversity, 43*(5), 350-361. https://doi.org/10.1016/j.pld.2021.01.003.

Tian, H. Z., Han, L. X., Zhang, J. L., Li, X. L., Kawahara, T., Yukawa, T., López-Pujol, J., Kumar, P., Chung, M. G., & Chung, M. Y. (2018). Genetic diversity in the endangered terrestrial orchid *Cypripedium japonicum* in East Asia: Insights into population history and implications for conservation. *Scientific Reports, 8*(1), 1–13. https://doi.org/10.1038/s41598-018-24912-z

Wolter, F., Schindele, P., & Puchta, H. (2019). Plant breeding at the speed of light: The power of CRISPR/Cas to generate directed genetic diversity at multiple sites. *BMC Plant Biology, 19*(1), 1–8. https://doi.org/10.1186/s12870-019-1775-1