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Genetic variation and genomic constitution in orchid *Dendrobium* hybrid section *Spatulata* derived from interspecific hybridization based on sequence related amplified polymorphism marker

Aziz Purwantoro[1,*], Agus Budi Setiawan[1], Rikcy Setiaji Nugraha[2,3], Sairoh Bisirotil Mujtaba[4], and Aditya Hanung Setyadi[4]

[1]Laboratory of Plant Breeding, Departement of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Jalan Flora Bulaksumur, Yogyakarta 55281, Indonesia
[2]Graduate School of Plant Breeding, Faculty of Agriculture, Gadjah Mada University, Universitas Gadjah Mada, Jalan Flora Bulaksumur, Yogyakarta 55281, Indonesia
[3]Agri Orchids Nursery, Gandekan, Guwosari, Pajangan, Bantul, D.I. Yogyakarta 55751, Indonesia
[4]Reseacher Assistant of Plant Breeding Laboratory, Departement of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Jalan Flora Bulaksumur, Yogyakarta 55281, Indonesia
*Corresponding author: azizp@ugm.ac.id

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ABSTRACT *Dendrobium* hybrid section *Spatulata* is widely cultivated in Indonesia due to its ease of cultivation, high economic value and adaptability, also extended flower shelf life. Various attempts to meet the rising market demand for *Dendrobium* hybrid section *Spatulata*, including the development of new varieties with unique flower traits such as flower color, a longer and bigger horn, and disease resistance. In this study, we conducted a breeding program aimed at developing a new cultivar of *Dendrobium* hybrid section *Spatulata* (antelope orchids) through interspecific hybridization. The study aimed to investigate the genetic variation and genomic constitution of the eight hybrids and their corresponding parental lines that resulted from interspecific hybridization using sequence-related amplified polymorphism (SRAP) marker. Six species of *Dendrobium* section *Spatulata i.e.*, *Dendrobium* Sri Mulyani, *D. Cochliodes*, *D. strepsiceros*, *D. stratiotes*, *D.* Alice Noda, *D. helix*, and several hybrids of antelope orchids derived from three hybridizations including *D.* Sri Mulyani × *D. cochliodes*, *D. stratiotes* × *D. strepsiceros*, and *D.* Alice Noda × *D. helix*, respectively, were subjected into SRAP markers for genotyping analysis. *Dendrobium* hybrid section *Spatulata* hybrids produced by interspecific hybridization were genuine hybrids with substantial genetic variability based on flower morphology, including labellum shapes and color intensities, as well as curly horn shapes and color intensities. The SRAP marker, which was used to genotype the hybrid and parental lines, exhibited a significant degree of polymorphism, and might be used to distinguish each accession. It produced a unique DNA amplicon that ranged from 180 to 530 bp and inherited a certain progeny line. The unweighted pair group mean average (UPGMA) dendrogram and Principal Coordinate Analysis (PCoA) biplot showed that all the hybrids were grouped into three major clusters according to their corresponding parental lines based on their genetic background and genomic constitution. These findings are critical for the genetic improvement of the Antelope orchid to develop novel varieties.

KEYWORDS *Dendrobium Spatulata*, Genetic variability, Genomic constitution, SRAP marker

1. Introduction

Dendrobium is a member of the Orchidaceae subfamily, which includes over 888 generas and over 33,000 species found worldwide (Chase et al. 2015; Christenhusz and Byng 2016; Han et al. 2020; Ketsa and Warrington 2023). The genus *Dendrobium* is found in a variety of environments over most of south, east, and southeast Asia, including India, China, Japan, Philippines, Vietnam, Indonesia, New Guinea, Australia, and other Pacific islands (Ketsa and Warrington 2023). This orchid is the one of the most extensively grown species in Indonesia due to its ease of cultivation and adaptability in lowland altitude. *Dendrobium* can be cultivated as flowering potted plants, cut flower production, medicinal and cosmetic production since its varied bioactive compounds (Hinsley et al. 2018; Ketsa and Warrington 2023). Section *Spatulata* of the *Dendrobium* genus is the most widely grown and commercially valued orchid in Indonesia. *Spatulata* orchids, also known as Antelope Dendrobiums, have long and spiral horns, colorful, beautiful, and long-lasting flowering, and make it more intriguing and distinctive than other *Dendrobium* sections (Yam and Lee 2013). *Dendrobium* section *Spatulata* has a large geographical range, extending

from the northern Philippines southward to the Australian state of Queensland, and from the western portion of Java eastward to the Pacific islands and Samoa (Arobaya et al. 2022).

Orchid breeders are being pushed to meet the rising market demand and trade in *Dendrobium* section *Spatulata* to develop new varieties with unique flower traits such as flower color, the longer and bigger horn, and the disease resistance. This genetic improvement can be reached using traditional and molecular breeding (Li et al. 2021). Genetic variation is important for orchid breeding to develop new variety with desired traits. Evaluating and utilizing Antelope orchid germplasm is critical for selecting appropriate parental lines for the development of novel hybrid cultivars of Dendrobium section Spatulata. Section Spatulata species are essential parents in the development of potted plant and cut-flower cultivars (Kamemoto et al. 1999). Yam and Lee (2013) reported that the section Spatulata orchid offers a significant potential for developing new cultivars through interspecific hybridization with the high genetic recombination rate and fertility of the hybrids. Another study reported that genetic variation of Spatulata orchid can be induced through mutation using gamma irradiation (Handini and Aprilianti 2020). In this study, we have conducted a breeding program for developing new cultivar of *Dendrobium* section *Spatulata* (antelope orchids) through interspecific hybridization. The section *Spatulata* hybrids exhibit great variations in flower morphologies including flower colors, labellum shapes and colors, horn shapes and colors, and flower bloom shelf life. Consequently, evaluating the genetic variation of these hybrids is critical for providing fundamental genetic information and genetic improvement of section *Spatulata* orchid.

Genetic variation can be evaluated using morphological traits. Several studies have been conducted to identify genetic diversity of *Dendrobium* section *Spatulata* based on morphological traits in Indonesia (Arobaya et al. 2022; Hidayati et al. 2016). However, there are several disadvantages of morphological evaluation, such as time consuming, laborious, and frequently influenced by environmental factors (Sormin et al. 2021). The genetic variation also can be investigated by using DNA markers approach. The orchid genomes have been sequenced (Cai et al. 2015; Yan et al. 2015), to utilize and develop the molecular genetic marker. Plant genotyping using DNA markers is reliable and has been extensively applied in orchid (Hartati and Muliawati 2020; Hsu et al. 2019, 2020; Lal et al. 2023) and other plant species, such as melon, cucumber and mungbean citepFatmawati2021, Setiawan2019, Setiawan2020, Sormin2021. The sequence-related amplified polymorphism (SRAP) is dominant marker, and this marker employs a set of customized primers to amplify the region-specific open reading frames (ORFs) in a certain gene (Li and Quiros 2001). SRAP is more reproducible than RAPD and ISSR (Budak et al. 2004; Hartati and Muliawati 2020; Lal et al. 2023), because the specific DNA fragment amplified by SRAP might correspond to specific regulatory gene instead of a repetitive DNA sequence that might amplified by RFLP, RAPD, and ISSR markers. In addition, it has been also used to assess genetic diversity and population structure in orchids (Cai et al. 2011).

In this study, the genetic variation and genomic constitution of the eight hybrids and their corresponding parental lines resulted from interspecific hybridization was investigated using SRAP markers. The genetic variation and genomic constitution of the hybrids can be clearly discriminated, and they were clustered based on their respective genetic background.

2. Materials and Methods

2.1. Plant Materials

Six species of *Dendrobium* section *Spatulata i.e.*, *Dendrobium* Sri Mulyani (P1S), *D. cochliodes* (P1C), *D. strepsiceros* (P2St), *D. stratiotes* (P2Sp), *D.* Alice Noda (P3A), *D. helix* (P3H), were used as parental lines for the interspecific hybridization. The antelope orchid hybrid derived from three hybridizations including *D.* Sri Mulyani × *D. cochliodes*, *D. stratiotes* × *D. strepsiceros*, and *D.* Alice Noda × *D. helix*, respectively, together with their parental lines were also used in this study. The parental lines and their hybrids were grown in the low altitude (65 m above sea level) and maintained at the Agri Orchid Nursery, Gandekan, Guwosari, Pajangan, Bantul, D.I. Yogyakarta, Indonesia.

2.2. DNA Isolation

Total DNA was extracted from all parental and progenies lines of orchid leaves using CTAB (hexadecyltrimethylammonium bromide) method Doyle (1990). In brief, the DNA from 0.5 g of leaves were extracted using CTAB isolation buffer, followed by purification using 200 µL of 24:1 chloroform:isoamyl alcohol (CIAA) and DNA precipitation using cold isopropanol. Then, the DNA samples were air-dried and resuspended in 100 µL using TE Buffer. Genomic DNA quantification was determined by NanoDrop (2000c Spectrometer, Thermo Scientific, USA) and the DNA was diluted using TE Buffer into the final concentration of 100 ng/µL.

2.3. PCR Amplification

The DNA was amplified using a T100™ thermal cycler (Bio-Rad, USA). The PCR used 50 ng of gDNA, 0.2 mM dNTPs, 0.2 µM primer, 1X GoTaq® Green Master Mix (Promega, USA), 1.25 U/µL GoTaq® polymerase, and Nuclease Free Water into final volume 10 µL. The PCR condition was conducted in accordance with (Li and Quiros 2001) protocol. In brief, pre-denaturation at 94 °C for 5 min; the first five cycles comprised of denaturation at 94 °C for 1 min, annealing at 35 °C for 1 min, and extension at 72 °C for 2 min. The annealing temperature was elevated to 50 °C for 1 min in an additional 30 cycles, followed by an 8 min extension at 72 °C. The ten primer combinations were used and listed in Table 1. The PCR amplified products were separated using 1.5% agarose gel

(w/v) electrophoresis and stained using FloroSafe DNA (1st BASE, Singapore).

2.4. Data Analysis

The amplified DNA were scored into binary data. The data were analyzed using online Marker Efficiency Calculator (iMEC) (Amiryousefi et al. 2018). The value of polymorphic information content (PIC), total amplified band, polymorphic band, and degree of polymorphism were measured to determine the primer efficiency. Principal coordinate analysis (PCoA) and Jaccard similarity coefficient was used to estimate genetic similarity among the parental and progeny lines using the SIMQUAL program embedded in GenAIEx 6.50. Clustering analysis was conducted with an unweighted pair group mean average (UPGMA) method with arithmetic averaging using the SAHN program in NTSYS PC 2.2 (Rohlf 2009).

3. Results and Discussion

3.1. Flower Morphology of Parental and Progeny Lines of Antelope Orchids

Six parental lines comprised of *Dendrobium* Sri Mulyani (P1S), *D. Cochliodes* (P1C), *D. strepsiceros* (P2St), *D. stratiotes* (P2Sp), *D.* Alice Noda (P3A), *D. helix* (P3H) were used to develop new hybrids of *Spatulata* orchids. The hybridization between *D.* Sri Mulyani × *D. cochliodes* produced four surviving plants and successfully developed with distinct flower colors including labellum shapes and color intensities, and the curly horn shapes and color intensities (purple to dark purple) (Figure 1). The progenies from this parent hybridization were registered as *Dendrobium* Mataram at Royal Horticultural Society (RHS) on September 22, 2020. The data were deposited at Bluenanta database (https://orchidroots.com/detail/information/?pid=101038975&role=). Furthermore, two hybrids were produced from the hybridization between two species of antelope orchids [*D. stratiotes* × *D. strepsiceros*]. The hybrids have upright curled long horns that are partially pink and little pink labellum (Figure 1). The hybrids were registered as *Dendrobium* Puntadewa at Royal Horticultural Society (RHS) on September 30, 2020 and the data were deposited at Bluenanta database (https://orchidroots.com/detail/information/?pid=101039108&role=pub). The hybridization between *D.* Alice Noda × *D. helix* produced two hybrids with distinct brown intensities of sepal, petal, and labellum colors (yellow and pink) (Figure 1). These hybrids were registered as *Dendrobium* Fapertagama at Royal Horticultural Society (RHS) on March 03, 2021 and the data were deposited at Bluenanta database (https://orchidroots.com/detail/information/?pid=101041538&role=pub). These re-

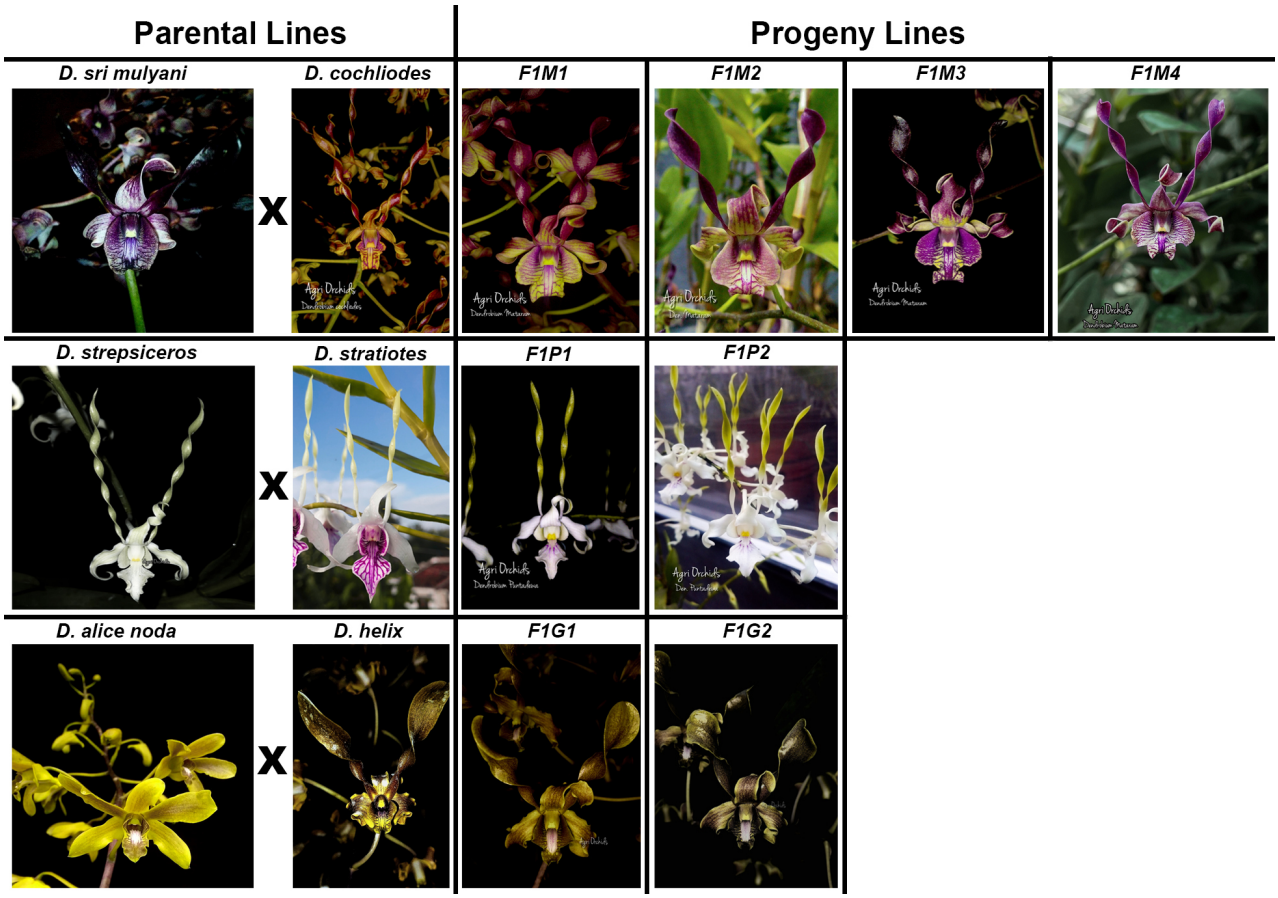

FIGURE 1 The flower morphologies of the parents and hybrids of *Dendrobium* section *Spatulata* derived from interspecific hybridization.

TABLE 1 The data of polymorphic information of *Spatulata* orchids amplified using SRAP markers.

Primer Combinations	Primer sequence (5'-3')	Size Range (bp)	Total Amplified Bands	Total Polymorphic Bands	Degree of Polymorphism (%)	PIC Values
ME5 - EM5	ME5 (TGA GTC CAA ACC GGAAG) EM5 (GAC TGC GTA CGA ATT AAC)	110 - 1200	14	14	100.00%	0.1795
ME5 - EM6	ME5 (TGA GTC CAA ACC GGAAG) EM6 (GAC TGC GTA CGA ATT GCA)	100 - 800	4	2	50.00%	0.3478
ME5 -EM7	ME5 (TGA GTC CAA ACC GGAAG) EM7 (GAC TGC GTA CGA ATT CAA)	100 - 950	13	13	100.00%	0.3191
ME6 - EM6	ME6 (TGA GTC CAA ACC GGACA) EM6 (GAC TGC GTA CGA ATT GCA)	150 - 1400	16	16	100.00%	0.2990
ME6 - EM7	ME6 (TGA GTC CAA ACC GGACA) EM7 (GAC TGC GTA CGA ATT CAA)	150 - 950	10	10	100.00%	0.3381
ME6 - EM8	ME6 (TGA GTC CAA ACC GGACA) EM8 (GAC TGC GTA CGA ATT CAC)	100 - 1100	14	13	92.86%	0.3469
ME7 - EM6	ME7 (TGA GTC CAA ACC GGACG) EM6 (GAC TGC GTA CGA ATT GCA)	200 - 950	12	10	83.33%	0.3669
ME8 - EM5	ME8 (TGA GTC CAA ACC GGACT) EM5 (GAC TGC GTA CGA ATT AAC)	100 - 2800	24	24	100.00%	0.2950
ME8 - EM6	ME8 (TGA GTC CAA ACC GGACT) EM6 (GAC TGC GTA CGA ATT GCA)	250 - 1600	10	10	100.00%	0.3212
ME8 - EM7	ME8 (TGA GTC CAA ACC GGACT) EM7 (GAC TGC GTA CGA ATT CAA)	120 - 1300	16	16	100.00%	0.3271
Total			133	128		
Mean			13.3	12.8	96.24%	0.3141

sults suggested that interspecific hybridization can contribute to increase the genetic variation of *Dendrobium* section *Spatulata*. Another study reported that hybridization between two different species produced high variation in all progenies of orchid (Dwiati et al. 2020).

The parental lines and the hybrids have different flower bloom shelf lives. The flowers of *Dendrobium* Sri Mulyani (P1S) have a shelf life for 3 months, *D. Cochliodes* (P1C) for 2.5 months, *D. strepsiceros* (P2St) for 2 months, *D. stratiotes* (P2Sp) for 2.5 months, *D.* Alice Noda (P3A) for 3 months, *D. helix* (P3H) for 1.5 months. The hybrids of *D.* Sri Mulyani × *D. cochliodes* have the flower shelf life ranging from 2.5 to 3 months. The floral shelf life for the hybrids of *D. stratiotes* × *D. strepsiceros* and *D.* Alice Noda × *D. helix* is 2.5 months. This coincides with Kamemoto et al. (1999) and Yam and Lee (2013), who found that *Dendrobium* section *Spatulata* has a long flower shelf life. Another strategy can be applied to improve the shelf life of *Dendrobium* section *Spatulata* using modification of postharvest handling (Poonsri 2020).

3.2. DNA Amplification of Antelope Orchids using SRAP Marker

The interspecific hybridization among *Dendrobium* section *Spatulata* exhibited phenotypic variation in flower morphologies including labellum shapes and color intensities, and the curly horn shapes and color intensities (Figure 1). This phenotypic variation was supported by the genetic constitution and confirmed by the genotyping of the hybrids using SRAP markers (Figure 2). The result clearly showed that the hybrids inherited the DNA fragment from their parents.

The DNA was successfully amplified from six parents and eight hybrids of antelope orchids using ten primer combinations of SRAP. The primer produced 133 of total amplified bands with the degree of polymorphism ranged from 50% to 100% (Table 1). The average of polymorphism degree is 96.24% with the amplicon size ranged from 100 to 2800 bp. In this study, the degree of polymorphism produced by SRAP marker is higher than RAPD marker used in the analysis of Dendrobium genetic variation that reported by Choopeng et al. (2019). This result suggested that SRAP marker is efficient and good marker to evaluate the differentiate and identify genetic background of the orchid hybrids. In addition, the PIC

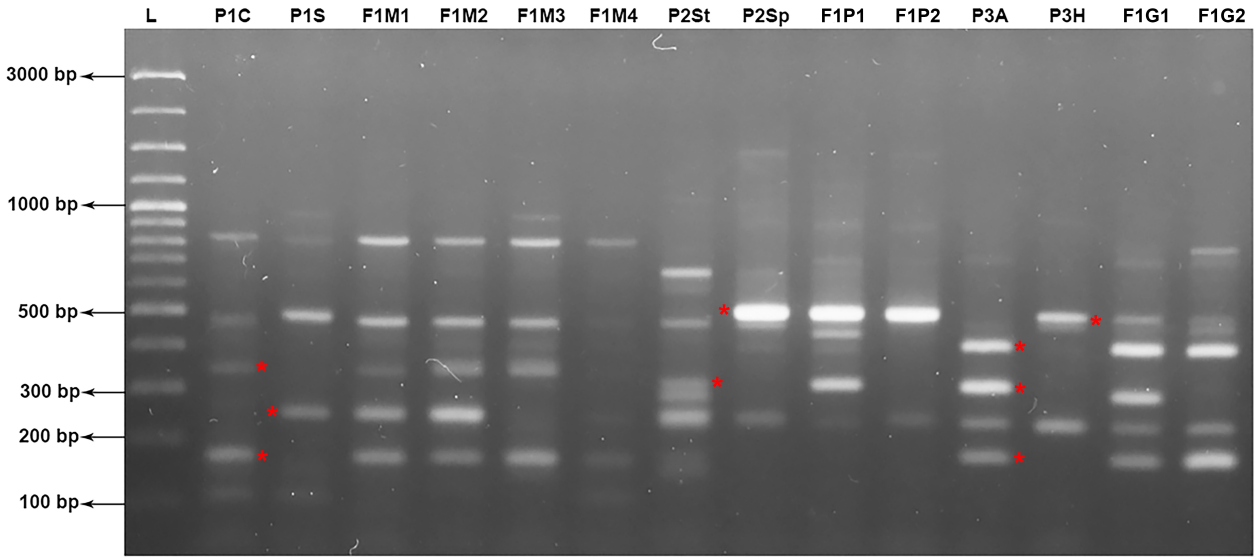

FIGURE 2 The amplified DNA produced by combination of ME 5 and EM 7 primers in all parental and hybrid lines. Red asterisks depict the specific band amplified from each parent and inherited into the hybrids of *Spatulata* orchids. F1M1 to F1M4 are the hybrids derived from *D.* Sri Mulyani × *D. cochliodes*, F1P1 and F1P2 are the the hybrids derived from *D. stratiotes* × *D. strepsiceros*, and F1G1 and F1G2 are the the hybrids derived from *D.* Alice Noda × *D. helix*.

TABLE 2 Pairwise Jaccard genetic similarity index among 14 genotypes of *Spatulata* orchids.

	P1C	P1S	F1M1	F1M2	F1M3	F1M4	P2St	P2Sp	F1P1	F1P2	P3A	P3H	F1G1	F1G2
P1C	1													
P1S	0.44	1												
F1M1	0.38	0.32	1											
F1M2	0.28	0.34	0.20	1										
F1M3	0.38	0.34	0.22	0.20	1									
F1M4	0.40	0.36	0.20	0.34	0.28	1								
P2St	0.52	0.48	0.54	0.56	0.58	0.60	1							
P2Sp	0.59	0.47	0.59	0.61	0.63	0.63	0.31	1						
F1P1	0.53	0.41	0.53	0.51	0.53	0.57	0.29	0.22	1					
F1P2	0.58	0.46	0.54	0.58	0.58	0.62	0.34	0.35	0.31	1				
P3A	0.54	0.38	0.48	0.52	0.50	0.54	0.48	0.51	0.43	0.36	1			
P3H	0.56	0.30	0.36	0.44	0.38	0.38	0.54	0.47	0.39	0.40	0.38	1		
F1G1	0.55	0.31	0.37	0.49	0.45	0.43	0.55	0.58	0.48	0.45	0.27	0.23	1	
F1G2	0.52	0.30	0.30	0.42	0.38	0.38	0.58	0.55	0.49	0.46	0.34	0.18	0.09	1

Remarks: F1M1-4, F1P1-2, F1G1-2 represent the progeny line numbers derive from crossing of P1C x P1S, P2St x P2Sp, P3A x P3H, respectively.

value varied from 0.1795 to 0.3669, with the average being 0.3141 (Table 1). This result suggested that the SRAP is dominant marker. The marker is classified into dominant marker when the PIC valued is ≤ 0.5 (Alikhani et al. 2014). High PIC value indicates that this marker is adequate for identifying the genetic variation in the tested orchid population.

The DNA amplification of the hybrids resulted from three hybridizations of parental lines confirmed that the progenies are genuine hybrids. The DNA of parental lines, when amplified with the primer combination of ME 5 and EM 7 generated the specific bands that only present in each female or male parents and these unique band were inherited into specific progenies (Figure 2). These specific amplified bands ranged from 180 to 530 bp. Fatmawati et al. (2021) reported that the genuine hybrids produced by interspecific hybridization must have inherited the DNA fragment from their respective parental lines.

Our results confirmed that the hybrids were phenotypically and genetically different. However, further study is required to be conducted to characterize and confirm the specific amplified DNA present in the hybrids is related to a certain phenotypic variation. The SRAP is dominant marker and could not distinguish the heterozygote genotype of the hybrids compare to co-dominant marker such as simple sequence repeat (SSR) marker. However, numerous SSR primer must be designed from the specific microsatellite DNA sequence present in the antelope orchids genome and must be tested through PCR. Currently, there is no draft genome of antelope orchids in the genome database to design this marker (Chen et al. 2022).

3.3. *Genetic Similarity and Clustering Analysis Among The Hybrids and Parents of Antelope Orchid*

Jaccard's similarity coefficient was used to estimate genetic relationship among the hybrids and parental lines of Antelope orchid. The coefficient similarity was ranged from 0.09 to 0.63 (Table 2). *Dendrobium strepsiceros* had the highest similarity index with the F1M3 and F1M4 hybrids, whereas the lowest similarity index was between F1G1 and F1G2. This result suggested a high genetic variation between the hybrids and the parental lines. In addition, the UPGMA dendrogram separated the accessions of antelope orchid into three major cluster (Figure 3a). The first cluster (cluster I) was comprised of *D.* Alice Noda, *D. helix*, and their two hybrids. The second cluster (cluster II) was consisted of *D.* Sri Mulyani, *D. cochliodes*, and their four hybrids. The third cluster (cluster III) consisted of *D. stratiotes*, *D. strepsiceros*, and their two hybrids. The PCoA biplot also confirmed and supported the dendrogram result (Figure 3b) in which all the hybrids were grouped with their corresponding parental lines. Our results suggested that the hybrids derived from interspecific hybridization of *Spatulata* parental lines exhibited a great variation in labellum shapes and color intensities, and the curly horn shapes and color intensities. Li et al. (2021) reported that the F1 orchid progenies generated from two parents with distinguishing desired features typically show significant phenotypic variations. The hybrid progenies derived from *D.* Sri Mulyani × *D. cochliodes* showed dominant purple color in their long-horned curly and slightly purple in their labellum (Figure 1). The hybrids of *D. stratiotes* × *D. strepsiceros* also exhibited a little purple color in their labellum inherited from *D. stratiotes*. Purple color corresponds to anthocyanin pigmen, and widely distributed in orchids (Hsiao et al. 2011). Orchid flowers with

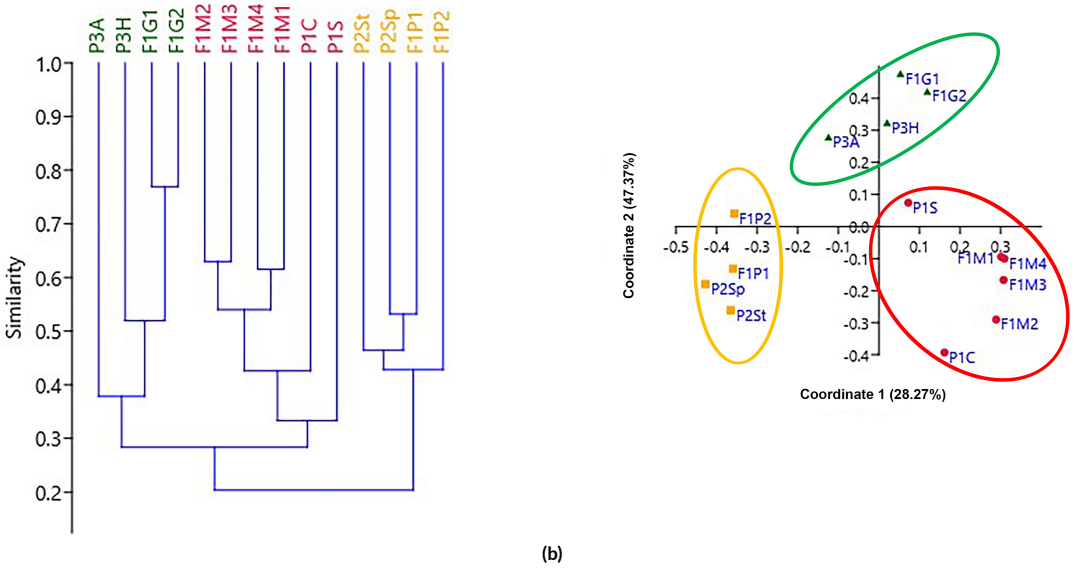

FIGURE 3 Cluster analysis of parents and 10 interspecific hybrids of *Spatulata* orchids. Dendrogram (a) and PCoA biplot (b) generated using SRAP marker.

purple coloration are presenting due to a sufficient expression of MYB11, the key regulatory R2R3-MYB transcription factor for controlling the production of purple color (Hsu et al. 2019). The hybrids of *D.* Alice Noda × *D. helix* showed a dark yellow flower might be due to high accumulation of carotenoid. Yellow color in orchid was regulated by RcPCP1 gene in which promoted α-carotene and lutein accumulation (Li et al. 2020).

4. Conclusions

The hybrids of *Dendrobium* hybrid section *Spatulata* resulting from interspecific hybridization are genuine hybrids and these had high genetic variation based on their flower morphology including labellum shapes and color intensities, and the curly horn shapes and color intensities. The SRAP marker was used for genotyping the hybrid and parental lines and it showed high degree of polymorphism, which can be used to differentiate each accession. The SRAP marker produced a unique DNA amplicon ranged from 180 to 530 bp and inherited into a certain progeny line that may corresponding to specific regulatory gene in the antelope orchids. The UPGMA dendrogram and PCoA biplot showed that all the hybrids were grouped with their corresponding parental lines based on their genetic background and genomic constitution. These results are important for genetic improvement of antelope orchid in developing new varieties.

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Authors' contributions

AP designed and supervised the research, interpreted the data, writing the manuscript. ABS supervised the experiment, analyzed data, and wrote the manuscript. RSN performed the experiment, grown and maintained the plant materials, analyzed the data. SBM and AHS supporting the laboratory experiment and analyzed the data.

Competing interests

All authors declare that there are not any conflicts of interest.

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