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Karyological studies of four species of Lady's Slipper Orchids (*Paphiopedilum*) collected in the Bogor Botanical Garden, Indonesia

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Abstract. *Paphiopedilum* is one of the most widely grown and hybridized of all orchid genera due to its distinctive flower morphology. This genus consists of 139 accepted species and is native to southern China to tropical Asia. Karyological studies on the genus *Paphiopedilum* have been reported by many cytologists in different countries. However, many Indonesian members of *Paphiopedilum* have remained comparatively limited in investigated cytologically. This study aimed to analyze karyological characters of four species of *Paphiopedilum* collected in the Bogor Botanical Garden, Indonesia, namely *P. armeniacum*, *P. hirsutissimum, P. primulinum,* and *P. superbiens*. Karyological studies were conducted by root tips squash method. The results showed that four species of *Paphiopedilum* have a basic chromosome number of x = 15. They are diploid with 2n=30, instead of the common diploid chromosome number of *Paphiopedilum* (2n = 26). *Paphiopedilum hirsutissimum* and *P. primulinum* possess 16 m + 14 sm chromosome formulae. Whereas *Paphiopedilum armeniacum* and *P. superbiens* revealed 16 m + 14 sm and 18 m + 12 sm chromosome formulae, respectively. A Robertsonian change in chromosome number generated by the fission of chromosomes would best explain the origin of the new diploid chromosome number and karyotypes of these species.

Keywords: Bogor Botanical Garden, chromosome, diploid, karyotype, *Paphiopedilum.*

INTRODUCTION

Paphiopedilum Pfitzer (Orchidaceae) is known as a lady's slipper orchid and belongs to the subfamily Cypripedioideae Lindley (1840) with other four genera, namely *Cypripedium, Mexipedium, Phragmipedium*, and *Selenipedium*. The subfamily was separated from other subfamilies based on its special characteristic of having two separated fertile anthers (Cribb 1998). *Paphiopedilum* has unique characters that distinguished from genera *Cypripedium* and *Selenipedium*, viz. conduplicate coriaceous leaves, as opposed to the pli-

cate persistent leaves of the latter two genera. *Paphiopedilum* differs from *Phragmipedium* and *Mexipedium*, as they have imbricate sepal vernation, different chromosome base numbers, and a unilocular ovary (Albert and Chase 1992; Albert 1994).

Paphiopedilum is the most popular orchid genus for ornamental plants, because of its distinctive flower morphology (Lee et al. 2011). Therefore, members of this genus are widely grown and hybridized of all orchids (Cribb 1998; Lan and Albert 2011). Ecologically, species of *Paphiopedilum* are important to narrow endemics in various mainland and islands habitat and range from seaside cliffs to montane rainforests (Cribb 1998). Based on the morphological characteristics of inflorescence, leaf type, floral morphology, and molecular data on ITS sequences, Cribb (1997) divided this genus into three subgenera, namely *Parvisepalum*, *Brachypetalum,* and *Paphiopedilum*. After Cribb (1997), several new species and treatments have been described for this genus, therefore this genus consisted of approximately 98 species worldwide by the year 2000 (Koopowitz 2000). The subgenus *Paphiopedilum* is classified into five sections, viz. *Coryopedilum*, *Pardalopetalum*, *Cochlopetalum*, *Paphiopedillum*, and *Barbata* (Lee et al. 2017; Tsai et al. 2020) based on morphological, cytological and molecular data (Cox et al. 1997; Cribb 1998; Chochai et al. 2012).

Paphiopedilum is distributed in four of 25 biodiversity hotspots (Myers et al. 2000), the Indo-Burma, Sundaland, Wallacea, and Philippines, which are also the "major evolutionary hotspots" (de Bruyn et al. 2014). According to Tsai et al. (2020), phylogeny and historical biogeography of *Paphiopedilum* reveals the relevance of the differentiation of *Paphiopedilum* in Southeast Asia and geological history. The mountain forests of Indonesia are home to great diversity of the endemic *Paphiopedilum* species. It is predicted that at least 50% of the Lady's Slipper Orchids of the world can be found naturally in the mountains forest of Indonesia, most of them are endemic to the mainland of Indonesia. For example, *P. agusii* Cavestro & N Bougourd, *P. anitanum* Cavestro, *P. braemii* H.Mohr ., *P. dodyanum* Cavestro, *P. glaucophyllum* J.J. Smith, *P. intaniae* Cavestro, and *P. javanicum* (Reinw. ex Lindl.) Pfitzer are endemic to Java (Govaerts et al. 2018).

Chromosome number and karyomorphological analysis is very significant for understanding the genome structure, its organization, and evolution within a genus of certain plant taxon at inter- and intra-specific levels (Ehrendorfer 1980). Analyses of chromosome numbers are very important, because they represent a fundamental step in the study of any taxa of organisms. Chromosome counts provide indispensable information on genetic discontinuities within and among species, and they contribute to the understanding of phylogenetic relationships at all taxonomic levels (Windham and Yatskievych 2003).

Extensive chromosome account and karyotype analysis of the Lady's Slipper Orchids have been published. Chromosome counts are published for almost all species of *Paphiopedilum* and the detailed karyotypes are also available for most species of the genus (Kamemoto et al. 1963; Karasawa 1979; Karasawa and Aoyama 1980; Karasawa 1982, 1986; Cox et al. 1998). However, chromosome account and karyotype analysis for Indonesian *Paphiopedilum* are scanty although most of species of this genus are distributed in Indonesia. This study aimed to observe chromosome numbers and conduct the karyotipe analysis of some species of Indonesian *Paphiopedilum*. Accumulation of cytological data would be very important in providing reference to breeding programs.

MATERIALS AND METHODS

Plant materials

Root tips of four species of *Paphiopedilum* in various subgenera were taken from the orchids in vitro culture collection of the Plant Tissue Culture Laboratory and the orchid plants collection of the Bogor Botanical Garden (BBG). *Paphiopedilum primulinum, P. superbiens* were collected from the wild population, whereas *P. armeniacum* and *P. hirsutissimum* were introduced plants (Table 1).

Somatic chromosome observations

Procedures for somatic chromosome observation followed by Manton (1950) and modified by Praptosuwiryo and Darnaedi (2008). The actively growing roots were used for chromosome preparation. Root tips pretreated with 0.001 M 8-hydroxyquinolin at 4°C for 24-26 hours. The root tips then were fixed in 45% acetic acid for 10 minutes at room temperature after being rinsed with distilled water. Root tips were macerated with 45% acetic acid (CH_3COOH): 1N HCl (1:3) at 60°C for 4 minutes, and then stained in 1% aceto-orcein. The meristematic cells were squashed in a drop of 1% acetic acid orcein under a coverslip of 22 x 22 mm on a microscope slide. Chromosome observation was performed under the microscope using a 100x magnification objective with the addition of immersion oil. An Olympus micro-

Table 1. Living material for karyological studies of the genus *Paphiopedilum* collected in the Bogor Botanical Garden, Indonesia.

No.	Species	Subgenus/Section	Distribution	Status
1.	*P. armeniacum* S.C.Chen & F.Y.Liu	*Parvisepalum/ Parvisepalum*	China	introduced
2.	*P. hirsutissimum (*Lindl. ex Hook.) Stein	*Paphiopedilum /Paphiopedilum*	China, India, Lao PDR, Myanmar, Thailand, Vietnam	introduced
3.	*P. primulinum* M.W.Wood & P.Taylor	*Paphiopedilum/Cochlopetalum*	Sumatra	wild, collected from Aceh
4.	*P. superbiens* (Rchb.f.) Stein	*Paphiopedilum /Barbata*	Sumatra	wild, collected from North Sumatra

Table 2. Chromosome shape classification based on long and short arms ratio.

Arm Ratio (AR)	Sentromer position	Chromosome shape
1.0 ≤ AR < 1.7	median	metasentric (m)
1.7 ≤ AR < 3.0	submedian	submetasentric (sm)
3.0 ≤ AR < 7.0	subterminal	subtelosentric (st)
7.0 ≤ AR < ∞	near terminal	acrosentric (a)
∞	terminal	telosentric (t)

scope U-TV0 with the objective 100x connected to a digital camera (5XC-3 5H12344) with a computer monitor was used to capture the images of well-spread chromosome complements.

Data analysis

The long-arm and the short-arm length of each chromosome were recorded using 5 μm as the unit. Each chromosome picture was cut out and arranged in descending order of length. Karyotype analysis was based on mitotic metaphase cells from each species. Chromosome shape at metaphase was classified based on arm ratio (AR) (Levan et al. 1964) (Table 2). Chromosome characteristics were measured using Ideokar 1.2 software (Mirzaghaderia and Marzangib 2015).

RESULTS

In the present investigation, the numbers of somatic chromosomes of four species of *Paphiopedilum* were counted and presented in Table 3 and Figures 1.

The four species of *Paphiopedilum* disclosed the same chromosome number of 2n=30. Somatic chromosomes at metaphase and the idiogram of four species of *Paphiopedilum* are shown in Figure 1. The chromosome shapes of *P. armeniacum*, *P. hirsutissimum*, *P. primulinum*, and *P. superbiens* are 16 m + 14 sm, 24 m + 6 sm, 24 m + 6 sm, and 18 m + 12 sm, respectively (Table 3.).

DISCUSSION

Paphiopedilum possesses unusually large chromosomes for orchids (Kamemoto et al. 1963). The basic cytology of *Paphiopedilum* is reasonably well studied and chromosome numbers have been published for many species (Karasawa 1979, 1986; Karasawa and Aoyama 1980, 1988; Karasawa and Tanaka 1980, 1981; Karasawa and Saito 1982; Cox et al. 1998). However, almost all species reported were outsite of Indonesia.

Four species of *Paphiopedilum* cultivated in the Bogor Botanical Garden have chromosome number of 2n = 30 (Figure 1). Chromosome numbers of the four species were reported for the first time. Karyological studies of *Paphiopedilum* have shown considerable chromosomal variation, which ranges from 2n = 26 to 2n =

Table 3. Karyotypic characters of four species of *Paphiopedilum* collected in the Bogor Botanical Garden.

No.	Species	Chromosome					
		Chromosome number (2n)	Long arm (μm)	Short arm (μm)	Total arm Length (μm)	Arm ratio (μm)	Chromosome shape
1.	*P. armeniacum* S.C.Chen & F.Y.Liu	30	3.23–5.24	1.12–4.60	4.32–9.80	1.13–1.25	16 m + 14 sm
2.	*P. hirsutissimum (*Lindl. ex Hook.) Stein	30	2.36–5.73	1.21–4.33	3.58–10.05	1.14–2.03	24 m + 6 sm
3.	*P. primulinum* M.W.Wood & P.Taylor	30	1.58–7.07	1.21–5.31	3.00–12.38	1.11–1.76	24 m + 6 sm
4.	*P. superbiens* (Rchb.f.) Stein	30	2.36–5.73	1.21–4.33	3.58–10.05	1.27–1.95	18 m + 12 sm

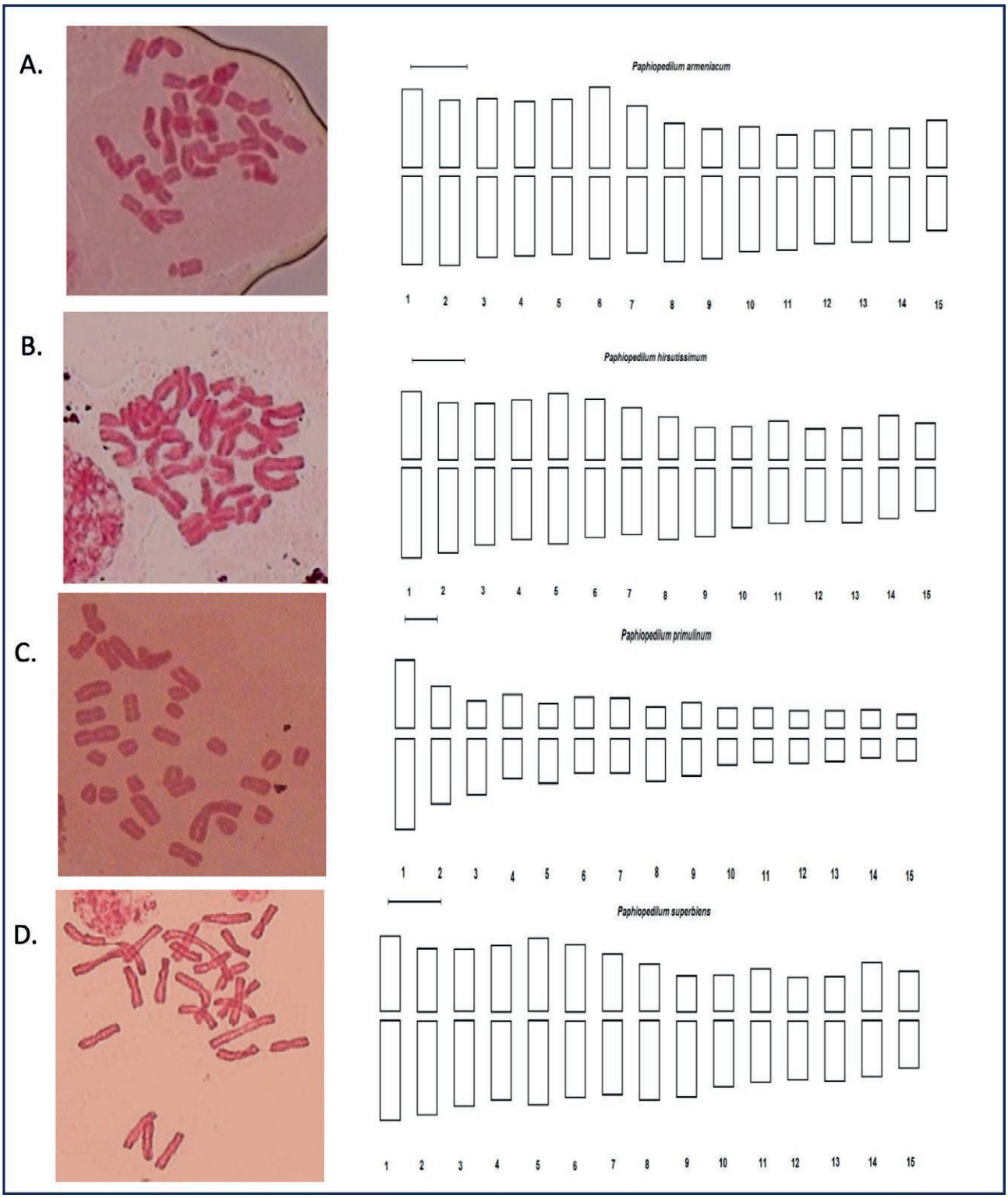

Figure 1. Somatic and ideogram metaphase chromosomes of *Paphiopedilum*, Ideogram of each species is on the right side of the image: (A) *P. armeniacum* (2n = 30), (B) *P. hirsutissimum* (2n = 30), (C) *P. primulinum* (2n = 30), (D) *P. superbiens* (2n = 30). Scale bars: 5 μm.

42 (Karasawa 1979). However, species of *Paphiopedilum* have the multiple chromosomes of the basic chromosome number x = 13 as reported by Lee et al. (2017). Species in the sections *Barbata* and *Cochlopetalum* of subgenus *Paphiopedilum* have a variety of chromosome numbers ranging from 2n = 28 to 42 and 2n = 30 to 37, respectively (Cox et al. 1998).

Paphiopedilum armeniacum S.C.Chen & F.Y.Liu

Paphiopedilum armeniacum belongs to subgenus *Parvisepalu*m, section *Parvisepalu*m (Lan and Albert 2011). This species is currently listed as endangered in The IUCN Red List of Threatened Species, version 2014.3 and grows in the restricted area to a river valley in Yunnan, China on limestone substrates in rocky and brushy habitat (Rankou and Averyanov 2015).

The chromosome account of *P. armeniacum* cultivated in the Bogor Botanical Garden with 2n = 30 is a new record for chromosome number for this species. Lee et al. (2018) reported a cytotype of *P. armeniacum* with 2n = 26 from Taiwan. This species is usually reported to have chromosome 2n = 26 (Lan and Albert 2011).

The new karyotype of *P. armeniacum*, with 2n = 30, with the centromeric formula of chromosomes 16 m + 14 sm reported here (Figure 1A), expands the chromosome number range for subgenus *Parvisepalum*, which was previously considered to be conserved at 2n = 26 (Chochai et al. 2012). The members of subgenus *Parvisepalum* usually show 2n = 26m (Lee et al. 2011).

Paphiopedilum hirsutissimum (Lindl. ex Hook.) Stein.

Paphiopedilum hirsutissimum is a member of subgenus *Paphiopedilum,* section *Paphiopedilum* (Lan and Albert 2011). This species is distributed in northern and western Guangxi, southern and western Guizhou, southern and eastern Guizhou, northern and eastern India, Laos, Thailand, and northern Vietnam (Liu et al. 2009; Li et al. 2015; Chen et al. 2018). This species usually grows on the crevices on shaded cliffs or rocky and well-drained places in forests or thickets in limestone areas at the elevation of 700–1500 m asl.

Chromosome number of *P. hirsutissimum* cultivated in the Bogor Botanical Garden reported here is 2n = 30. Formerly cytological records showed that most species of the subgenus *Paphiopedilum*, section *Paphiopedilum*, have diploid chromosome number 2n = 26 (*P. fairrieanum, P. henryanum, P. hirsutissimum, P. tigrinum*), except *P. druyi* (2n = 30) (Lan and Albert 2011).

The chromosome formula of *P. hirsutissimum* reported here is 2n = 24 m + 6 sm (Figure 1B). Chromosome formula in the subgenus *Paphiopedilum* are vary, for example, 2n = 26 m of *P. rothschildianum* (section *Coryopedilum*), 2n = 20m + 12t of *P. callosum* (section *Barbata*), 2n = 14m + 22t of *P. glaucophyllum* (section *Cochlopetalum*) (Lee et al. 2011).

Paphiopedilum primulinum M.W.Wood & P.Taylor.

Paphiopedilum primulinum is endemic to Sumatra (southern Aceh). It is a member of section *Cochlopetalum* and closely related to *P. liemianum* based the molecular data, such as nuclear ribosomal ITS, plastid *trnL* intron, *trnL-F* spacer, and *atpB-rbcL* spacer (Tsai et al. 2020).

The chromosome account of *P. prmulinum* with 2n = 30 is reported here for the first time. Formerly cytological study *P. primulinum* of Sumatra has been reported to have 2n = 32 (Lan and Albert 2011). The chromosome formula for *P. primulinum* reported here is 2n = 24 m + 6 sm (Figure 1C).

Paphiopedilum superbiens (Rchb.f.) Stein.

Paphiopedilum superbiens belongs to subgenus *Sigmatopetalum,* section *Barbata* (Karasawa and Saito 1982). According to Braem and Chiron (2003), this species is included in the *Ciliolare* complex of subsection *Loripetalum*. Based on the phylogenetic relationships resulting from analysis of the combined molecular data matrix, such as nuclear ribosomal ITS, plastid *trnL* intron, *trnL-F* spacer, and *atpB-rbcL* spacer, *P. superbiens* is closely related to *P. curtisii* and come into section *Barbata* (Tsai et al. 2020).

The chromosome account of *P. superbiens* with 2n = 30 is reported for the first time. A formerly cytological study of the members of the section *Barbata* revealed 2n = 36 for *P. curtisii* and *P. dayanum*, 2n = 38 for *P. acmodontum* and *P. sangii,* 2n = 40 for *P. sukhakulii* and *P. venustum* (Lan and Albert 2011). The chromosome formula for *P. superbiens* reported here is 2n = 18 m + 12 sm (Figure 1D).

Paphiopedilum (Orchidaceae: Cypripedioideae) is characterized by considerable chromosome number variation (2n = 26–42). Chromosome numbers vary from 2n = 28–42 and 2n = 30–37 in the sections *Barbata* and *Cochlopetalum* of subgenus *Paphiopedilum*, respectively (Lee et al. 2018). According to Cox et al. (1998), the most common diploid chromosome number in *Paphiopedilum* is 26 metacentric chromosomes, as displayed in subgenus *Parvisepalum, Brachypetalum* and *Paphiopedilum*, mainly sections *Pardalopetalum*, *Coryopedilum*, and *Paphiopedilum*. However, the chromosome number 2n = 30 was also reported. This diploid chromosome number occured in *P. druryi* and *P. spicerianum* (Cox et al. 1998). The chromosome account of *Paphiopedilum* obtained from this current study supported the existence of 2n = 30 for the genus. Prior to this study, 2n = 30 was

reported for the hybrid species of *P. rothschildianum* × *P. moquetteanum* (Lee et al. 2011).

The changes in chromosome number and karyotype symmetry were considered as a consequence of Robertsonian centric fission (Jones 1998). A Robertsonian relationship between the different karyotypes whereby changes in chromosome number are generated by the fission or fusion of chromosomes at or near the centromere to generate either telocentric or metacentric chromosomes, respectively (Robertson 1916). Duncan and MacLeod (1949, 1950) proposed Robertsonian change to explain the maintenance of arm number in the two genera of Orchidaceae, namely *Paphiopedilum* and *Phragmipedium*. Cox et al. (1997) postulated centric fission of metacentric chromosomes into telocentrics as the predominant mechanism of karyotype evolution in *Phragmipedium* and *Paphiopedilum* and that karyotype orthoselection is operating in some species groups. *Paphiopedilum* species display many chromosomal rearrangements like duplications, translocations, and inversions. Therefore the genus is a good model system for the study of complex chromosomal evolution in plants (Lan and Albert 2011).

The phylogeny of *Paphiopedilum* has been reported by Chochai et al. (2012) and Tsai et al. (2020) using molecular data (nuclear and plastid DNA) which each species studied here are separated in different clades both subgenus and section levels. However, their chromosome shape does not correlate with clade separation, mainly in subgenus *Paphiopedilum*. *P. hirsutissimum* (sect. *Paphiopedilum*) and *P. primulinum* (sect. *Cochlopetalum*) have similar chromosome shape of 24 m + 6 sm, but based on ITS nrDNA and plastid DNA sequence data, they were separated in different clade. It tends that clade separation among subgenus and section within subgenus *Paphiopedilum* are strongly correlated with morphological and molecular data. However, extensive chromosome account and karyotype analysis of *Paphiopedilum* are necessary to construct their relationships and evolution in the future.

CONCLUSION

Chromosome account and the karyotypes of four species of *Paphiopedilum* cultivated in the Bogor Botanical Garden, Indonesia were investigated. New diploid chromosome number was reported. The chromosome number was uniformly 2n = 30. Chromosome account of *Paphiopedilum* used in the study supports the finding that this genus has variations in the number of chromosomes. *P. hirsutissimum* and *P. primulinum* exhibited remarkably similar karyotypes, with centromeric formulae 24 m + 6 sm. Whereas *Paphiopedilum armeniacum* and *P. superbiens* possessed 16 m + 14 sm and 18 m + 12 sm chromosome formulae, respectively. These findings supported the statement that *Paphiopedilum* is a good model system for the study of complex chromosomal evolution in plants.

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AUTHORS' CONTRIBUTIONS

TNgP, RVG, and EH conceived the study. TNgP designed the study; TNgP, JRW, and IAF wrote the manuscript; TNgP, RVG, and EH conducted chromosome observations and studied morphological comparison among species studied; JRW and IAF conducted the karyotypic analysis. All authors read and approved the final manuscript.

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