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Cytological Analysis of *Aerides odorata* Lour. from Sleman, Special Region of Yogyakarta

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ABSTRACT
Aerides odorata is a species that belongs to the Orchidaceae family and lives as epiphytic orchids. This orchid is distributed across Southeast Asia, India, and China. This orchid occupies one type of habitat. Morphological characters of flowers in this species vary, depending on the habitat occupied. The cytological analysis includes mitotic phase, chromosome number, chromosome morphology, chromosome shape, and karyotyping. Those can be used as cytological data to predict speciation mechanism, phylogenetic analysis, and evolution among species. This research aims to acquire cytological information about flower morphology, stage of mitosis, and karyotype of *A. odorata* from Sleman, Yogyakarta. In addition, the cytological analysis of this orchid is to compare the number of chromosomes possessed with research that has been done previously. The method in this study consisted of observing the morphological flower characters, chromosome preparation, visualization of mitotic phases, chromosome shape, and karyotyping. The steps in squash consist of fixation with 45% glacial acetic acid, maceration with 1N HCl, staining with Feulgen solution, and squashing. The data were analyzed using Corel Draw 2018 (64 bit), Image Raster 3.0, and Microsoft Office Excel 2019. The orchid flowers found in Sleman, Yogyakarta have various morphology in flower, mainly in color. The phases of mitosis include prophase, prometaphase, metaphase, anaphase, and telophase. This orchid has 38 chromosomes that consisting of 14 metacentric chromosomes and 24 telocentric chromosomes with the karyotype formula obtained is 2n=2x=38=14m+24t.

Keywords: *Aerides odorata, Chromosome, Cytology, Morphology, Sleman.*

1. INTRODUCTION

Aerides odorata which known as "Anggrek Kuku Macan" in Indonesia, has a wide distribution in India, China, Southeast Asia, and Papua New Guinea. This orchid grows in forest areas especially in lowland with an altitude of 0-400 meter above sea level (masl), the average daytime temperature is around 34-38 ˚C and the temperature at night is 22-24 ˚C [1-2]. The mean elevation of the lowlands in Sleman is 150 masl [3]. This orchid lives epiphytically on Dao trees (*Dracontomelon dao*), Teak trees (*Tectona grandis*), and Putat trees (*Barringtonia acutangula*), or trees that can withstand rainwater, has a normal and shady canopy, smooth skin, large diameter stems, and flat branches and is not easily peeled off and overgrown with moss [4-5]. *A. odorata* is often found near the coast [1]. This orchid plant has one type of habitat, namely lowland forest which has high light intensity and low humidity [6], but it has excellent adaptability. This species can flower in dry areas during the dry season [2]. Flowers are white with purple spots at the corners of the crown, have a long column with lateral sepals and unified labellum, a racemose inflorescence type, located at the axils of the leaves, star-shaped, the petals are 2-3 cm wide, white sepals, and the pollinia are two in number. The labellum consists of 3 prominent lobes [2,5,7]. This species has a myriad of potentials that can

be exploited. It is often used as an ornamental plant and a medicinal plant because it contains bioactive compounds ethyl acetate and methanol which function as anti-cancer drugs [2,8]. This orchid plant is widely spread in several parts of Indonesia, such as Java, Yogyakarta, and Sulawesi with relatively large quantities depending on the appropriate environmental conditions. It can be found in Mount Langgar Forest, Lampung; Tropical Rain Forest on Rubiah Island, Aceh; Curug Setawing Forest; Wanagama Forest; South Slope of Mount Merapi Yogyakarta; Kalisegoro Village, Central Java; Mount Semahung; Mount Penanggungan, East Java; Butu Tahuana Hill, Mambutapua Village, Kalimbua Village, Sulawesi; Central Java Bantarbolang Nature Reserve; Bantimurung Bulusaurung National Park; and Tanjung Peropa National Park Southeast Sulawesi [1-3;5-6;9-13]. Currently, this orchid plant is not included in the species protected by the Indonesian government. However, the trade status of this orchid is regulated by CITES and is included in the Appendix II category [13].

Chromosomes in plants can be observed in actively dividing cells such as root tips [14]. Chromosome analysis or characterization in plants is generally carried out using cytogenetic techniques [15]. It is useful to see the complex phenomena of chromosomes at the molecular level based on chromosomes and the evolution that occurs at the level of certain taxa [16]. Chromosome analysis using cytogenetic techniques includes the number, morphology, karyotype, and shape of chromosomes [17-18]. The results of the cytogenetic analysis will show the mechanism of species diversity and phylogenetic relationships taking into account the evolution between these species [19].

Species from the Orchidaceae family have a relatively high diversity of chromosome numbers [20]. This causes large variations in the number of chromosomes between species or even within one species [21]. In addition, there are variations in the size, morphology, karyotype, and shape of the chromosomes in the Orchidaceae family [22]. Variations in the number of chromosomes reported in the Epidendroideae subfamily were 2n = 28, 30, 32, 34, 36, 38, 40, 42, 46, 48, and 50 [23].

Meanwhile, the Orchidoideae subfamily has a chromosome number of 2n = 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50 [24]. Most species of the genus *Aerides* have 38 chromosomes. *Aerides odorata, A. crassifolia, A. crispa, A. falcata, A. houllettiana, A. maculosa, A. multiflora, A. ringens*, and *A. rosea* have basic chromosomes with n=19 and 2n=38. However, there are variations in the number of chromosomes found in other species of the genus *Aerides*, namely *A. lawrenceae* with a chromosome formula of 2n=40. Meanwhile, *A. odorata* var. *immaculata* which is the synonym of *A. odorata* has formula of chromosome 2n=76 [25].

The karyotype is an arrangement of the chromosomes of an individual based on the number and size of chromosomes in a somatic cell [26]. A karyotype can be used to analyze changes in chromosomal structure, see taxonomic relationships among species, evolutionary processes, and phylogenetics [27-28]. Karyotype analysis includes the number and composition of chromosomes [29]. It is an important basis in the characterization of chromosomes using cytogenetic techniques [28]. Chromosomal morphology is one of the stable characters during cytological analysis. Meanwhile, the shape and structure of chromosomes are diverse characters. Each level of family, subfamily, genus, and species can have a different karyotype [30].

Chromosomes play an important role in cytotaxonomic studies to see the taxonomic status of an individual [31]. Indirect cytotaxonomy studies also play an important role in the development of plant breeding [32]. Cytological analysis of *A. odorata* from Sleman is important to carry out taxonomic verification of this species based on its chromosomal character with the relevant literature.

2. METHOD

2.1. Morphological observation

The photograph of *A. odorata* on the trees from around Gadjah Mada University's alumnae office has been taken. Observations were made on the habitus characters, roots, stems, leaves, and flowers. The observed morphological characters were then determined with the *Orchids of Java* literature [33] and analyzed descriptively.

2.2. Chromosome preparation

The *A. odorata* chromosomes were prepared using squash method [34]. The light green root tips of *A. odorata* were washed in running water and cut ± 1 cm at 09.00. The root tips of *A. odorata* were then dried and fixed by adding 45% glacial acetic acid into a 1.5 ml microtube until the root tips were completely submerged. After that, the samples were incubated at 4 ˚C for 15 minutes. The sample was then washed with distilled water three times. Maceration was done by adding 1N HCl to the microtube until the root tips were completely submerged and incubated at 62 ˚C for 30 minutes. Next, the root sample was washed again with distilled water three times. The staining step was carried out by incubating the samples in Feulgen solution at room temperature for 1 hour. The tip of the root was cut to ±2-3 mm size and taken with a brush then it was placed on a glass object. The glycerin was added to the

tip then covered with a cover glass. Squashing is done by carefully pressing the surface of the cover glass with the flat base of a pen. The root slide then observed using a light microscope (Olympus BX41, Japan) with 400X magnification to see the phases of mitosis and to determine a clear prometaphase image for the chromosomal reconstructions [14,35].

2.3. Visualization of the phases of mitosis, chromosome shape, and karyotype

The cytological analysis includes variables of mitotic phases, chromosome number, chromosome shape, chromosome size, ideogram reconstruction, and karyotype. The phases of mitosis include prophase, prometaphase, metaphase, anaphase, and telophase. The number of chromosomes is based on the number of chromosomes visualized at prometaphase. The shape observations were observed under a light microscope and visualized using Image Raster 3.0 and Corel Draw 2018 (64 bit) software. The first step is to reconstruct the chromosome shape with the help of Corel Draw 2018 software. Reconstruction is done using the shape tool menu and the freehand tool. Then, the chromosomes are numbered with a pointer line using the freehand tool and text tool features. Then, the short arm length (p) and long arm (q) were measured using Image Raster 3.0. Then, the sequence of chromosomes is carried out based on the size obtained from previous measurements to produce a data karyotype. Next, the ratio of the arm length of the chromosome and the centromeric index was calculated to determine the shape of the chromosome. Finally, the shape of the identity chromosomes and ideograms made with Microsoft Excel 2019 formulas.

2.4. Data analysis

The number of chromosomes was calculated during observations in the prometaphase phase. Karyotypes and ideograms are made by including analysis of the measurement of the short arm (p) and long arm (q) of the chromosome, measuring the Centromeric Index (CI), forming the actual shape of the chromosome, and making an ideogram. The creation of karyotypes and ideograms is carried out with the help of Microsoft Excel 2019 software, Image Raster 3.0, and Corel Draw 2018. The Equation (1) used to calculate the Centromeric Index (CI) [36] value is as follows:

$$CI = \frac{Length\ of\ the\ short\ arm\ of\ the\ chromosome\ (p)}{Absolute\ length\ of\ the\ chromosome\ (p+q)} x100 \quad (1)$$

The results of CI calculations are used to determine the shape of the chromosomes. Chromosomal classification can be determined based on the CI value to be telocentric (CI: 0-12.5); acrocentric/sub-telocentric (CI: 12.5-25); submetacentric (CI: 25-37.5), and metacentric (CI: 37.5-50) [37].

3. RESULTS AND DISCUSSION

Figure 1 Morphological character of *A. odorata*. (A) Habitus, (B) Flower. A bar = 30 cm, B bar = 1 cm.

The tiger nail orchid (*A. odorata*) is a member of the family Orchidaceae, subfamily Epidendroideae, subtribe Aeridinae which spreads in various areas [38-39]. The results of this study showed that the morphology of *A. odorata* found in Sleman, Yogyakarta is herbaceous and epiphytic with a height of ±85 cm. The roots of *A. odorata* are dorsiventral. The stem is brownish green, irregularly cylindrical in shape, has a monopodial growth direction, and has nodes and internodes. Leaves are green, ± 12 in number, arranged alternately. The young leaves arrangement is duplicative or double, oblong with split ends of leaves. The flower has a length of ±3.44 cm and a width of 4.06 cm, modified into a labellum consisting of the hypochillium (base), mesochillium (middle), and epichillium (tip). The labellum is equipped with a spur that resembles the shape of a protruding pouch. The inflorescences are compound with a pleurant type of implantation (between the two axils of the leaves). The type of flowering is raceme. Flowers are white with purple spots at the tips of sepals and petals, the base and the median of labellum. The results obtained show variations in flower color from research conducted by Comber [33]. Differences in morphological characters such as flower color occur due to its adaptation for the habitat so that it expresses new genes [40].

The chromosome shapes can be observed and the chromosome sizes can be measured at the time of mitotic division [41]. Mitosis occurs in several phases, namely prophase, metaphase, anaphase, and telophase [42]. At the time of prophase, chromosomes consisting of chromatin threads will thicken, shorten, and appear. In addition, the nuclear wall of the cell begins to disappear. The transitional phase from prophase to

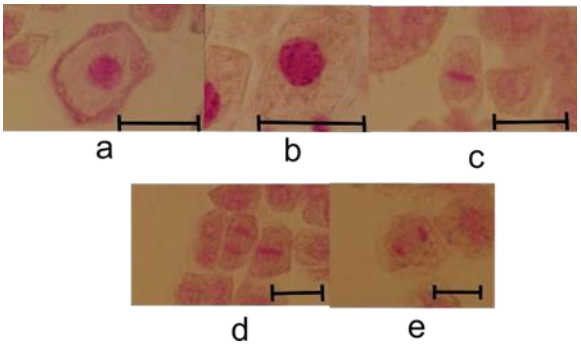

Figure 2. The mitosis phases of *A. odorata*. (a) prophase, (b) prometaphase, (c) metaphase, (d) anaphase, (e) telophase. a bar = 14µm, b bar = 26µm, c bar = 9µm, d bar = 8µm, e bar =7 µm.

metaphase is called prometaphase. In prometaphase, the chromosomes begin to disperse and their structure is easy to observe. In metaphase, the chromosomes appear to be lined up in the equatorial plate. In anaphase, the chromosomes separate and the chromatids move to the opposite poles. In telophase, the stem cell splits into two parts and a cell wall is formed [14]. Chromosome characterization by making karyotype will be easier to observe during prometaphase. This is because at the time of prometaphase, the chromosomes appear intact, flat, and spread out so that the calculation of the number and observation of the shape is easier to do [43]. In addition, prometaphase is a mitotic phase that is often used in the cytological analysis [44].

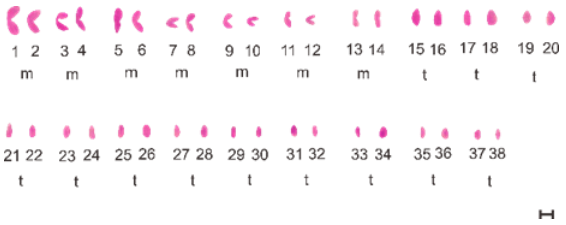

Figure 3. Karyotype of *A. odorata* chromosome. Bar = 1µm.

Based on the observation of the chromosomes of *A. odorata*, it can be found that the number of chromosomes in *A. odorata* are 38 (Figure 3). Species from the Orchidaceae family have a relatively high diversity of chromosomes [20]. This causes large variations in the number of chromosomes between species or even within one species [21]. In addition, there are variations in the size, morphology, karyotype, and shape of the chromosomes in the Orchidaceae family [22]. Variations in the number of chromosomes reported in the Epidendroideae subfamily were 2n = 28, 30, 32, 34, 36, 38, 40, 42, 46, 48, and 50 [23]. Meanwhile, the Orchidoideae subfamily has a chromosome number of 2n = 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, and 50 [24]. Most species of the genus *Aerides* have 38 chromosomes with the formula n=19 or 2n=38 [25].

Meanwhile, based on data obtained from The Chromosome Counts Database (CCDB), the number of chromosomes found in *A. odorata* varies, namely 2n = 36, 38, 40, and 76 [45]. These results are based on research that has been conducted in 1963 - 1987. Chromosome pairs arranged according to cytogenetic rules can be used to construct karyograms and compare karyotypes between one taxa group and another as an evolutionary process [46]. Karyotype was made by taking into account the cytometric and morphometric image data [17]. The shape, number, size of the long and short arms of chromosomes can indicate an evolutionary process [47]. This is because those three characters are unstable so that variations or diversity can be found in one taxon [48].

Variations in the number of chromosomes can be found in closely related species [49]. *A. odorata* has 38 chromosomes [25] and the same number of chromosomes was also found in *A. crassifolia*, *A. crispa*, *A. falcata*, *A. houllettiana*, *A. maculosa*, *A. multiflora*, *A. ringens*, and *A. rosea* (n=19 and 2n=38). However, there were differences in the number of chromosomes found in other species of the genus *Aerides*, namely *A. lawrenceae* (2n=40). Meanwhile, *A. odorata* var. *immaculata* which is the synonym of *A. odorata* has formula of chromosome 2n=76 [25]. The difference in the number of chromosomes shows the existence of genetic diversity and phylogenetic relationships in orchid species from the genus *Aerides* [50]. The phenomenon of variation in the number of chromosomes in one genus or one species can occur due to several factors such as demographic differences, natural habitat, domestication, and hybridization [49, 51]. These factors can lead to speciation and thereby causing evolution [52].

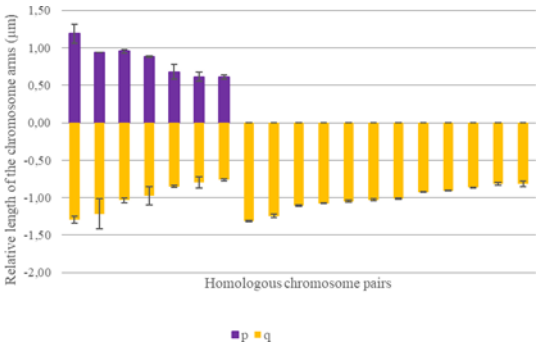

Figure 4. Chromosome ideogram of *A. odorata*.

The arrangement of karyotypes and ideograms is based on the shape, size, and number of chromosomes [53]. The shape of the chromosome is determined based on the position of the centromere [54]. Based on the karyotyping results, it can be seen that *A. odorata* has a karyotype formula of 2n=2x=14m+24t (Figure 3). The

arm length of the chromosomes ranged from 0,765-2,485 µm. The chromosome ideogram also showed that the chromosome pairs number 1-7 (chromosome numbers 1-14) are metacentric and the chromosome pairs number 8-19 (chromosome number 15-38) are telocentric (Figure 4). Thus, there are two variations of chromosome shape found in *A. odorata*, namely metacentric and telocentric with short arms ranging from 0- 1,19µm and long arms between 0,765-1,295 µm.

It can be concluded that *A. odorata* found in Sleman, Yogyakarta Special Region has morphological characters in flower. The specific characteristic of this orchid flower is the presence of white and purple spots at the tips of sepals and petals, as well as at the base and the median of labellum. The number of chromosomes in *A. odorata* found in Sleman is 38 chromosomes and in accordance with research conducted by Felix and Guerra [25]. This orchid has karyotype formula 2n=14m+24t, which consist of metacentric and telocentric chromosomes. The forms of chromosomes that can be found in *A. odorata* include metacentric and telocentric. Molecular data such as RAPD, RFLP, ISSR, SSR, sequencing, and FISH are needed to enrich the information and strengthen the results obtained.

AUTHORS' CONTRIBUTIONS

KAN did morphological observation, karyotyping, data analysis, full paper writing, review and editing, FYK did chromosome preparation, full paper writing, review and editing, ADRR did visualization of phases of mitosis, chromosome shape, review and editing, AK did full paper writing, review and editing, HPLI did full paper writing, review and editing, ES did review and editing.

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