

Full Length Article

The pretreatment condition for chromosome count and karyotype analysis of *Dimocarpus longan* from Thailand

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Abstract

Chromosome number and karyotype analysis reveal an important information for plant evolutionary study and plant breeding program. However, very few studies were conducted on cytogenetics of *Dimocarpus longan* (Longan), an economic important subtropical fruit in Thailand. This research aims to develop the practical procedure for longan chromosome preparation to investigate the chromosome number and karyotype analysis in 8 longan cultivars from Thailand. The chromosome preparation was optimized using longan shoot tip and root tip cells. Two pretreatment chemicals which are *p*-dichlorobenzene and 8-hydroxyquinoline were selected and treated at 4°C in different time points consisted of 1, 3, 6 and 24 hours. All treated samples were then hydrolysed in 1N HCl at 60°C for 7 minutes and stained with carbol fuchsin at room temperature for 15 minutes. The result showed that root sample pretreated with *p*-dichlorobenzene at 4°C for 1 hour was the best condition for longan chromosome analysis. This condition was further used for chromosome investigation in 8 Thai's longan cultivars which are Baiyoke, Plueakkhao, Phetsakorn, Krob-Ka-Ti, Haewkrae, E-daw, BiewKhiew Chiangmai and Pingpong. The chromosome number of all studied cultivars was determined as $2n = 30$. The variation of the total chromosome length from 0.499 to 1.293 μm while the relative length (RL) and size of chromosomes group L, M and S were observed among 8 longan cultivars. From this study, the pretreatment method can be used to investigate chromosomes of all longan cultivars. The data obtained from this study will be important information for plant breeders to develop longan varieties in Thailand. However, further chromosome investigation using approaches such as Fluorescence in situ hybridization (FISH) would be more informative for study evolution of longan in the future.

Keywords: chromosome preparation, chromosome number, karyotype, *Dimocarpus longan*

Introduction

Chromosome count and karyotyping provide an important information for evolutionary study in plants. Chromosome number analysis attracts many cytotaxonomists for investigation because it is the quickest, cheapest, and easiest way to obtain the substantial data about the genome of a given species (Guerra, 2008). Squashing method is one of the most common method for the preparation of plant chromosome. The method includes pretreatment, fixation, hydrolysis and staining. The first step for chromosome analysis of any plant sample is to obtain meristematic tissue. Currently, young root tip meristem is the most used tissue for analysis because of its high rate of cell proliferation (de Paula & Pinto-Maglio, 2015). Pretreatment is another important step in the process of preparations of plant tissue to investigate the chromosome number and to establish the karyotype of a species (Rachma & Salamah, 2018). Several pretreatment agents can be used for plant chromosomes study. The most commonly used agents are colchicine, 8-hydroxyquinoline, α -bromonaphthalene, *p*-dichlorobenzene (PDB) and cold water, all of which inhibits the mitotic spindle (Ekong et al., 2014). Lavania (1988) used either *p*-dichlorobenzene, 8-hydroxyquinoline, or their mixtures with a variation of 3-5 hours of immersion at 12-14°C from the root tips of 11 species and 7 chemotypes in *Cymbopogon*. The results showed that 3-7 well scattered and properly contracted metaphase plates were observed in different species by their agents. In addition, Rachma et al. (2018) used cold water, *p*-dichlorobenzene (P), 8-hydroxyquinoline (O) and PO (their mixture) for pretreatment of the shoot tips. Each pretreatment was carried out for 3, 6, 12 and 14 hours and the results showed that different pretreatments have their own optimum condition for accumulate cells in late prophase and metaphase.

Dimocarpus longan is an important subtropical evergreen fruit tree that is grown commercially in many countries. It originated from South China or Southeast Asia and is commonly called longan (dragon eye) in Asia (Lin et al., 2017). Longan is a major source of national income for Thailand since it is the biggest exporter of longan worldwide. Major longan planted area is in the upper northern provinces of Thailand, include Lamphun, Chiang Mai, Chiang Rai, Nan, Phra Yao, Lampang, Phrae and Chanthaburi (Choo, 2003). Longan is one of the “Product Champion” of Thailand regarded by Ministry of Agriculture and Cooperative and Ministry of Commerce (Jealviriyapan et al., 2000; Ramingwong et al., 2005). However, there are more than 30 longan cultivars found in Thailand and some cultivars have similar morphological characteristics. Perhaps theirs are the same cultivar but different in name because of planted location. It is difficult to distinguish the cultivar through only morphological data. Therefore, chromosome number and karyotype analysis are important genetic information for identifying cultivars more clearly.

In a previous study, chromosome investigation and karyotype analysis of longan were carried out in China. The karyotype formulas of longan are $2n = 30 = 16m(2sat) + 8sm + 6sat$ and satellite chromosome of longan was located in No.12 chromosome (Liuxin, 1994). In Thailand, Ramingwong et al. 2005 collected the root tips at 1 hour intervals from 7:00 a.m. – 12:00 p.m. to determine the appropriate time for investigating longan chromosome number. The results showed that the suitable time for chromosome counting was between 9:00 a.m. – 10:00 a.m. However, the duration of immersion and details of cultivar analyzed were not mentioned in their study. This study attempted to establish the appropriate time and tissues processing for investigation of longan chromosome number and karyotypes of 8 longan cultivars in Thailand. The data obtained from this research provided a well reproducible method for longan’s karyotype analysis which will be useful in breeding programs, evolution, systematic and conservation of longan in the future.

The limitation of pretreatment condition was pretreatment solution and immersion time. Accumulating evidence has suggested that different tissues may require different pretreatments and none seems to be of general applicability (Battaglia, 1957). Pretreatment solutions used in this research are *p*-dichlorobenzene and 8-hydroxyquinoline. Root tips and shoot tips are immerse in a pretreatment solution for 1, 3, 6 and 24 hours. The immersion period influences the size of the chromosome. The longer the immersion in the pretreatment solution the shorter the size of the chromosome (Rachma & Salamah, 2018). Therefore, the suitable condition is important to properly observe the number of chromosomes.

Materials and Methods

Plant materials

Root and shoot tips samples of 8 longan cultivars were collected from mature trees (10-15 years) planted at Maejo University, Chiang Mai, Thailand. The air layering branches from individual cultivars were used for root tips sample collection. Actively growing root and shoot tips were cut into 0.2 - 0.5 cm long and used for chromosome preparation.

Chromosome preparation

For chromosome investigation, a Feulgen squash method of Dyer (1979) was used with a slight modification. Two pretreatment chemicals which are *p*-dichlorobenzene and 8-hydroxyquinoline were selected to compare the efficiency of metaphases accumulation. The effect of pretreatment was observed in different time points consisted of 1, 3, 6 and 24 hours by keeping the tissue sample at 4°C. Each pretreatment group was fixed in newly prepared Carnoy's fixative (3: 1 absolute ethanol: glacial acetic acid) for at least 24 hours and then stored in 70% ethanol at 4°C until used. To prepare a microscope slide sample, the tissues were rinsed in distilled water to remove the fixative solution and treated with 1N HCl for 7 minutes at 60°C. The tissues were removed from 1N HCl by washing in distilled water then transferred to carbol fuchsin solution for 15 minutes. The tissues were squashed by dissecting needle on a slide glass, then covered with a cover glass and observed under a microscope (Olympus CX23). The best pretreatment condition was selected for chromosome preparation for all 8 longan cultivars by observing 10 well-spread metaphases and used for karyotype analysis.

Karyotype analysis

Chromosome number was counted and the parameters of the chromosomes including total chromosome length (LT), the relative length (RL) and standard deviation of RL were analyzed from 10 metaphase cells of each individual longan cultivar. The parameters of the chromosomes derived from measurements of the metaphase chromosomes in photomicrographs according to Chaiyasut's protocol (1989). The idiograms were generated by total chromosome length (LT). The size of chromosomes of all 8 longan cultivars were classified into 3 groups consisting of Group L (Chromosome with large size), Group M (Chromosome with medium size) and Group S (Chromosome with small size).

Results and Discussion

A simple, rapid, and reliable chromosome staining procedure for determining chromosome number is needed for plant-breeding program (Owen & Miller, 1993). A main purpose of breeding program is to transfer the genetic variability from related species into crops.

So far, 'E-Daw' is the only commercially grown longan variety, about 98 percent of the total longan production (Jaroenkit et al., 2014). For this reason, bringing concern if there are some effects on 'E-Daw' may impact longan production in the future. Plant breeders have used interspecific genetic crosses and alien introgression lines to overcome this problem such as transfer resistance genes against pathogens (Prieto, 2020). Thus, chromosome number and karyotype of longan are one of the genetic information for plant breeder to select and combine the greatest parental plants to obtain the next generation with the best characteristics. However, investigating chromosome number of longans is hindered due to their small chromosome size. Furthermore, it is difficult to prepare the metaphase cell of longan where well-spread chromosomes are all visible in a single focal plane. At present, there is no universal agent for pretreatment and perhaps individual organisms exhibit a differential sensitivity (Battaglia, 1957). But as the prefixing agent, *p*-dichlorobenzene has been commonly recommended for chromosome analysis due to its highly effectiveness (Sarbhoy, 1980). In this research, the pretreatment of tissues with 8-hydroxyquinoline failed to accumulate mitotic cells in metaphase, and prophase were relatively more abundant in any duration of immersion. In addition, pretreatment of shoot tips with *p*-dichlorobenzene also gave poor results in every immersion period. This may be due to some fatty suspension in the shoot tip cells (Ramingwong et al., 2005) which are difficult to squash. Nevertheless, the pretreatment of root tips with *p*-dichlorobenzene produced preparations superior to pretreatment with 8-hydroxyquinoline (Sharma & Mookerjea, 1955). However, the variation of immersion period with *p*-dichlorobenzene showed significant difference on chromosome size and arrangement. Root tip treated with *p*-dichlorobenzene for 3, 6 and 24 hours revealed that the chromosomes were stacked and crossed with each other, causing chromosome count to be biased. Moreover, the *p*-dichlorobenzene treated root tip at 24 hours resulted in the shortest chromosome size compared with other immersion periods. Based on observations made in this study, the pretreatment with *p*-dichlorobenzene for 1 hour is the best condition for counting longan chromosome preparation. While pretreatment conditions are important for chromosome preparation, it is imperative that hydrolysis time is also taken into consideration as this affects the chromosome as well. Inappropriate time of hydrolysis can induce differential chromosome staining with carbol fuchsin solution (Chiamamonrat, 1990). Fortunately, this study's chromosome preparation by hydrolysis using 1N HCl for 7 minutes at 60°C resulted in the most optimal condition for longan chromosome investigation. The example of metaphase cells from 8 longan cultivars pretreated with *p*-dichlorobenzene for 1 hour at 4°C and hydrolysed in 1N HCl for 7 minutes at 60°C was shown in Figure 1. Although, this is the best condition for counting the chromosomes, it failed to provide centromeric constrictions. The chromosomes clumped excessively that even other cell cycle stages could be obtained, thus the primary constriction was observed only in some chromosome arms as shown from the arrow in Figure 2. Therefore, to figure out the evolution of longan in Thailand further studies are required to gain more information on the karyotype using Fluorescence in situ hybridization (FISH) or other chromosome banding chromosome preparations.

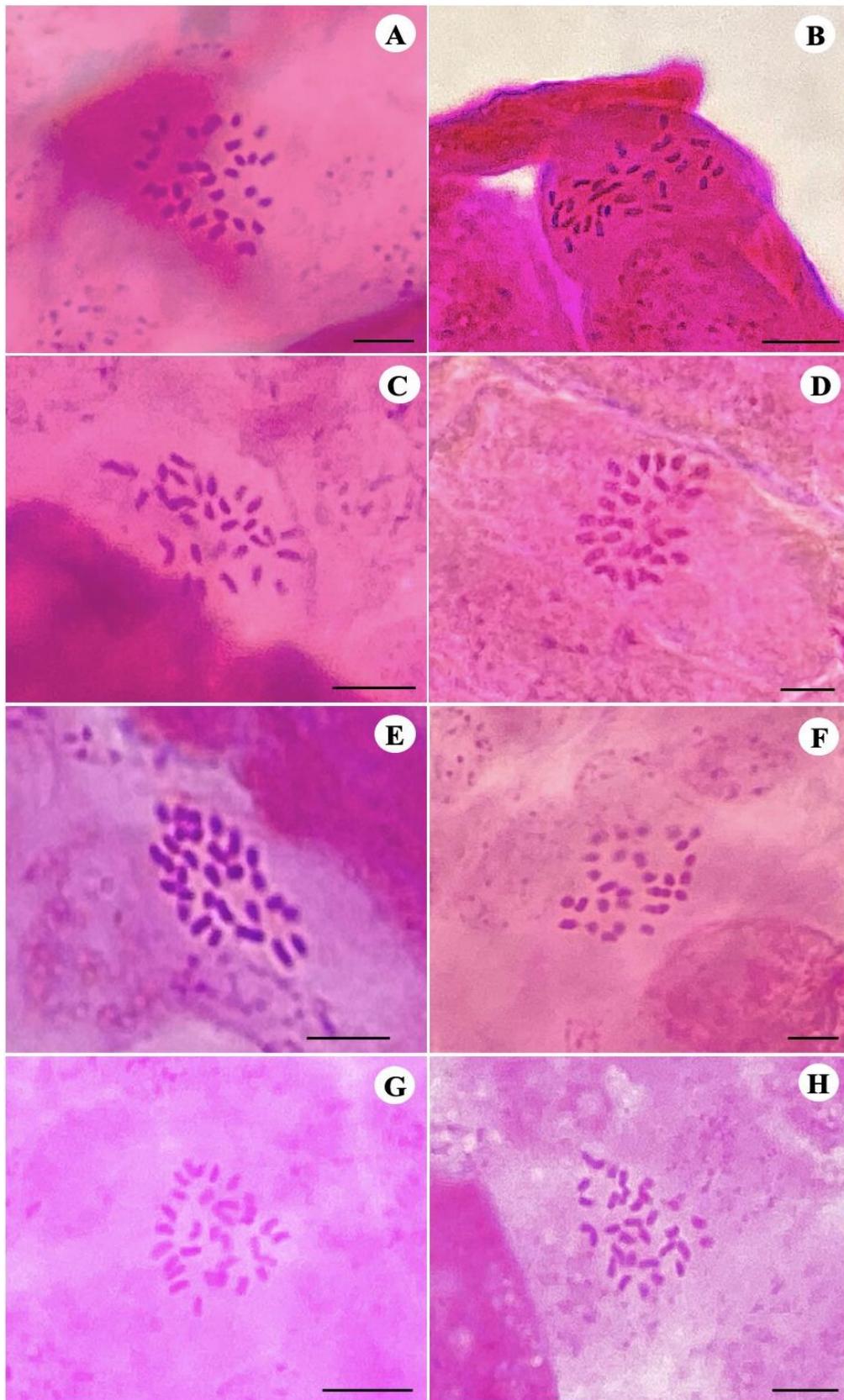


Figure 1. Metaphase chromosomes of 8 longan cultivars. A: Baiyoke, B: Plueakkhao, C: Phetsakorn, D: Krob-Ka-Ti, E: Haewkrae, F: E-daw, G: BiewKhiew Chiangmai, H: Pingpong. Scale bars = 3 μ m.

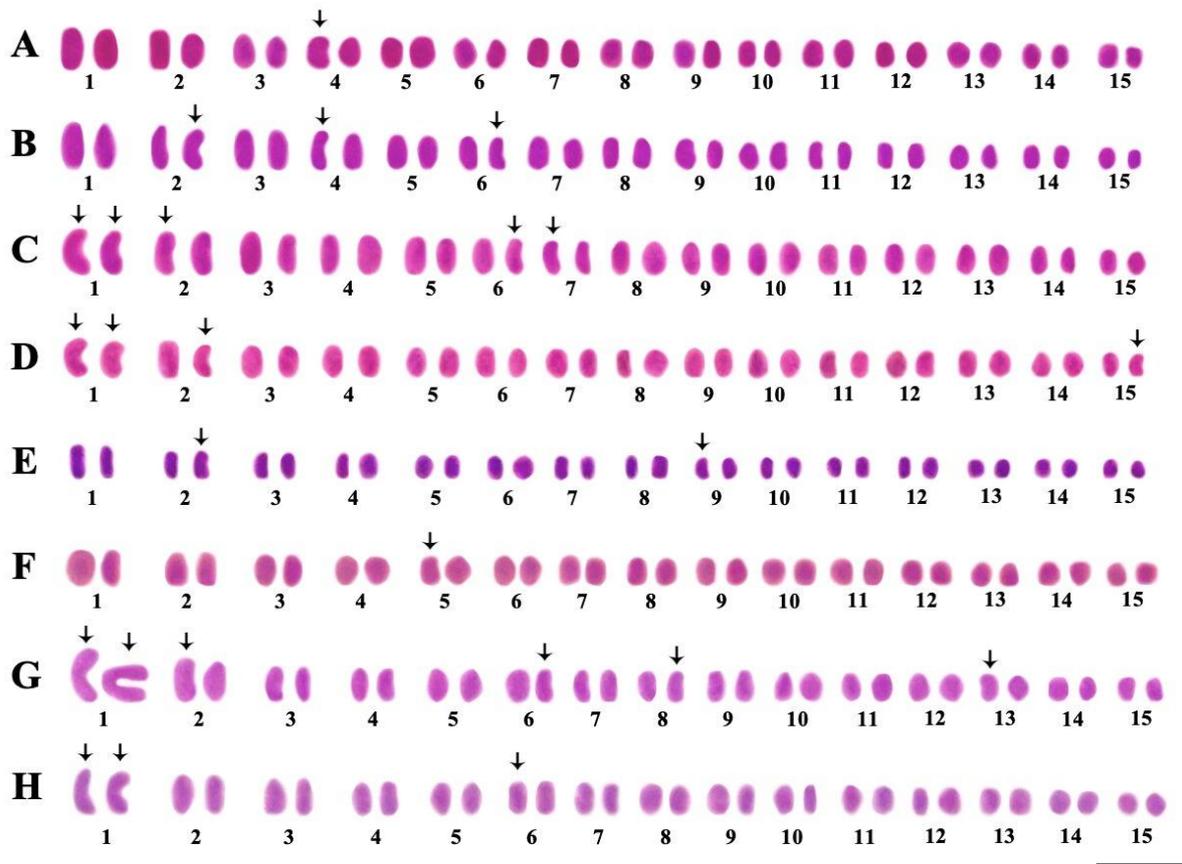


Figure 2. Karyotypes of 8 longan cultivars. A: Baiyoke, B: Plueakkhao, C: Phetsakorn, D: Krob-Ka-Ti, E: Haewkrae, F: E-daw, G: BiewKhiew Chiangmai, H: Pingpong. Scale bars = 3 μ m.

All karyotypic parameters of 8 longan cultivars were measured as presented in Table 1. The somatic chromosome number of all cultivars was found to be $2n = 30$. The total chromosome length (LT) was a determined minimum of 0.499 μ m in Phetsakorn and maximum 1.293 μ m in Baiyoke, whereas the lowest relative length of 4.6% was found in Baiyoke and Plueakkhao, and the highest relative length of 9.7% was observed in BiewKhiew Chiangmai. Based on the chromosome size, the idiograms were made and 5 groups of longans can be classified. Finally, the idiograms of all 8 longan cultivars showed the gradually decreasing length of the chromosome without the centromere position and different size of the chromosome pair as shown in Figure 3. The chromosome morphology of D. longan is an interesting point and should be explored further in the future.

Table 1. Mean of total chromosomes length (LT), relative length (RL) and standard deviation (SD) of RL from metaphase chromosomes in 10 cells of *Dimocarpus longan* ssp. *longan* var. *longan*, 2n (diploid) = 30.

| Group | Cultivar name | 2n | LT (μm) | | RL \pm SD | | Chromosome pair | | |
|-------|---------------------|----|----------------------|-------|-----------------|-----------------|-----------------|--------|-------|
| | | | Min | Max | Min | Max | Large | Medium | Small |
| 1 | Baiyoke | 30 | 0.640 | 1.293 | 4.6 \pm 0.005 | 9.3 \pm 0.005 | 6 | 8 | 1 |
| | Plueakkhao | 30 | 0.528 | 1.068 | 4.6 \pm 0.004 | 9.2 \pm 0.005 | 6 | 8 | 1 |
| 2 | Phetsakorn | 30 | 0.499 | 0.956 | 4.8 \pm 0.003 | 9.2 \pm 0.007 | 5 | 10 | 0 |
| | Krob-Ka-Ti | 30 | 0.664 | 1.280 | 4.8 \pm 0.003 | 9.3 \pm 0.006 | 5 | 10 | 0 |
| | Haewkrae | 30 | 0.634 | 1.212 | 4.9 \pm 0.003 | 9.4 \pm 0.006 | 5 | 10 | 0 |
| 3 | E-daw | 30 | 0.636 | 1.289 | 4.7 \pm 0.006 | 9.6 \pm 0.006 | 5 | 9 | 1 |
| 4 | BiewKhiew Chiangmai | 30 | 0.605 | 1.234 | 4.8 \pm 0.002 | 9.7 \pm 0.012 | 4 | 10 | 1 |
| 5 | Pingpong | 30 | 0.675 | 1.282 | 5.1 \pm 0.004 | 9.6 \pm 0.008 | 3 | 12 | 0 |

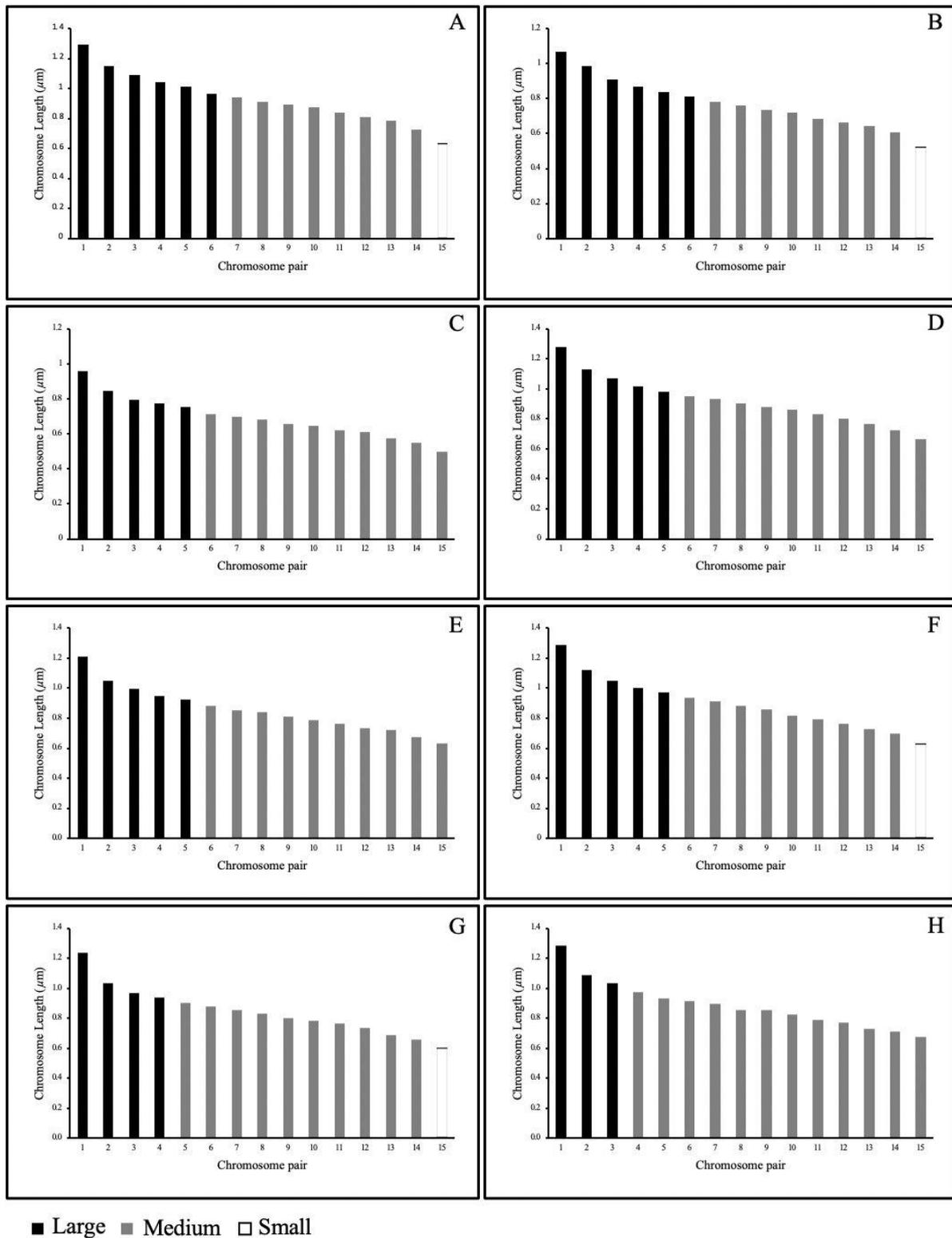


Figure 3. Idiogram showing lengths and sizes of chromosomes of 8 longan cultivars. A: Baiyoke, B: Plueakkhao, C: Phetsakorn, D: Krob-Ka-Ti, E: Haewkrae, F: E-daw, G: BiewKhiew Chiangmai, H: Pingpong.

Conclusion

It can be concluded that the use of the root tip for chromosome counting is better than the shoot tip. The pretreatment of the longan root tip with *p*-dichlorobenzene for 1 hour at 4°C is effective in arresting the chromosome at metaphase and hydrolysed in 1N HCl at 60°C for 7 minutes gave the best staining results while 8-hydroxyquinoline gave unsatisfied result. This established protocol can be used with all longan cultivars in Thailand but centromeric constrictions were not readily observable.

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Author contribution

PP and SKL performed research. SKL and PL designed the research study. PP and SKL collected and provided samples. PP, SKL, PL and IP analyzed the data. PP, SKL and PL wrote the manuscript. All authors approved the final version of this manuscript.

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