Analysis of Stomata, Chloroplast, and Chromosome of Local Mandarin Citrus (*Citrus reticulata*) Plants Grown from Endosperm Culture

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Abstract. This research aimed to identify accessions of jeruk keprok Garut plants (*Citrus reticulata*) grown from endosperm culture with stomata analysis, chloroplasts analysis, and chromosome analysis. This research was conducted at the Plant Breeding Laboratory of Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI), Malang. This research used seven accessions of jeruk keprok Garut (local mandarin citrus) from endosperm culture and mother plant of Garut mandarin. Data analysis was carried out with the Statistical Package for the Social Sciences (SPSS) application Application 22.0. The alignment analysis showed that all one accession (GT 224) out of seven accessions of Garut mandarin grown from the endosperm cultures is consistent with the mother plant according to chromosome number observation, stomata number observation, and chloroplast number observation.

Keywords: local mandarin, endosperm culture, stomata, chloroplast, and chromosomes

1. Introduction

Citrus plants are one of the most important horticultural crops. Citrus plants are generally cultivated to produce fruit for fresh consumption (Purwito *et al.*, 2015). Local commercial citrus commodities in Indonesia (citrus, mandarin, and pamelo) in production and quality have not been able to meet the need for domestic consumption (Martasari, 2008). The need for fresh consumption of citrus fruits in Indonesia is 3.26 kg/capita/year (assuming that the consumption of oranges is 10 percent of the fruit consumption, Food and Agricultural Organization/FAO standard). The consumption of citrus in Indonesia in the coming years is estimated to reach 11.85 million tonnes per year or equivalent to 580,000 ha with a production of 20 ton/ha/year. The prospect and potential of the citrus market are very large, so it is necessary to increase the quantity, quality, and continuity of the citrus administration in Indonesia (Martasari, 2008).
Central Bureau for Statistics and Directorate General of Horticultural (2015) stated that the growth of Indonesian citrus production in 2014 to 2015 decreased by 3.6 percent. The decline in domestic citrus production made Indonesia the second-largest citrus importing country in ASEAN after Malaysia. The growth of orange imports by 11% annually in five years has made Indonesia a promising market share for other countries in marketing its products. The production of Indonesian citrus that has not enough consumption needs of domestic oranges to provide challenges and opportunities for both farmers, citrus entrepreneurs, and governments in the effort to increase the production of citrus (Soelarso, 1996).

Central Bureau for Statistics (2015) reported that North Sumatera province, East Java, and West Kalimantan are three of the largest mandarin citrus provinces to national production. The production of mandarin citrus in North Sumatra province amounted to 483,006 tonnes (27.68 percent), East Java amounted to 480,396 tons (27.54 percent), and last West Kalimantan by 147,371 tonnes (8.44 percent).

Citrus fruits that are in the interest of society generally have a sweet, easily peeled, attractive and smooth fruit skin color, and no seeds (seedless) (Purwito et al., 2015). One of plant breeding systems that is applied to create citrus plants without seeds is an endosperm culture (Purnama et al., 2017). The endosperm culture is an alternative technique to produce a triploid plant directly by taking one stage. The endosperm tissue is triploid because it is a fusion result between two polar cores and one sperm (Sukamto, 2010). The condition can be utilized to form a new crop of triploid by conforming and generalizing endosperm cells. Plants that have ploidi triploid will usually be a crop of no-seed or seed but sterile (Purnama et al., 2017).The method used to identify mandarin citrus as a result of endosperm culture by performing cytological analysis. The cytological analyses carried out include stomata analysis, chloroplast analysis, and chromosome analysis. The results of each cytological analysis will be obtained by the number of stomata, the number of chloroplasts, and the number of chromosomes.

This research aimed to 1) identify mandarin citrus (Citrus reticulata) plants grown from endosperm culture with stomata analysis, chloroplasts analysis, and h chromosome analysis.

2. Materials and methods
This research was conducted at the Plant Breeding Laboratory of Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI), Malang.

2.1. Plant Materials and tools
Plant materials used in this study included seven accessions of Garut mandarin (C. reticulata) from endosperm culture and one mother plant of Garut mandarin (Table 1). Materials were glacial acetic
acid (CH$_3$COOH) 45 percent solution, 1 N hydrochloric acid solution (HCl), Orsein, emersion oil, distilled water, clear nail polish, 70 percent alcohol, and 1 percent silver nitrate (AgNO$_3$) solution. The tools used in this study were dropper pipettes, cover glass, glass preparations, razor blades, scalpels, tweezers, 2 µl tubes, Olympus BX 51 microscopes, memert ovens, fume hoods, refrigerators, stationery, markers, tissue, scissors, sticky tape, masks, gloves, and cameras.

| Accession Code | Accession Code |
|----------------|----------------|
| Mother plant of Garut mandarin | GT 113 |
| GT 12 | GT 224 |
| GT 110 | GT 109 |
| GT 2 | GT 6 |

2.2. Data analysis

Data analysis was performed based on the results of photographs obtained from the samples. Then the data is tabulated into an observation table and averaged. After obtaining the mean value, then it is calculated using standard deviations and an analysis of the kinship by using the Statistical Package for the Social Sciences (SPSS) application Application 22.0.

3. Results and discussion

3.1. Stomata analysis

![Figure 1. Triploid accession GT 224 stomata with a magnification of 400x.](image-url)
Table 2. Number of stomata of seven accessions of Garut mandarin grown from the endosperm culture

| Accession Code | Number stomata/fields of view | Accession Code | Number stomata/fields of view |
|----------------|-------------------------------|----------------|-------------------------------|
| Mother plant of Garut mandarin | 26.92 ± 3.37 | GT 113 | 30.52 ± 5.46 |
| GT 12 | 39.20 ± 7.43 | GT 224 | 37.60 ± 6.19 |
| GT 110 | 39.88 ± 6.30 | GT 109 | 39.88 ± 6.35 |
| GT 2 | 20.36 ± 3.43 | GT 6 | 19.56 ± 3.47 |

Note: Average ± Standard Deviation

The average number of stomata results from the seven accessions of mandarin citrus from endosperm culture and mother plant of Garut mandarin can be obtained in terms of the similarity of the values of each accession as presented in Figure 2.

Figure 2 shows that the accessions of Garut mandarin were consisted of three groups that have similar values, namely group 1 consisting of accessions GT 110, GT 109, GT 12, and GT 224 with an average number of stomata 37-39; group 2 consisted of GT 2 and GT 6 accessions with an average number of stomata of 20-26, and group 3 consisted of accessions of Garut mandarin and GT 113 with an average number of stomata of 26-30. Accessions in group 2 and group 3 have similar values because group 2 has an average stomata number of 20-26 and group 3 has an average number of stomata of 26-30. Group 1 is the accession that has the highest number of stomata compared to accessions in other groups.

The number of stomata in the arrowroot mother plant of Garut mandarin has the least amount when compared to the accession of mandarin citrus from endosperm culture, this is thought to be influenced by the number of chromosomes because mother plant of Garut mandarin has a number of chromosomes $2n$ and the accession of mandarin citrus resulting from endosperm culture has a
Increasing the number of chromosomes will be followed by an increase in the size of the stomata which results in an effect on the density of the stomata. Also, the amount of stomata will increase in line with the increase in the number of chromosomes.

Haryanti (2010) stated that the number of stomata per unit area varies not only between types but also within one type, due to their relationship with environmental factors during growth. The number and size of stomata are influenced by genotype and environment. Closing cells that surround the stomata control the opening and closing of the stomata. Closure of stomata is important to prevent water loss when the water supply is limited while limiting CO$_2$ uptake for photosynthesis. Stomata open during the day and close at night. The process of opening and closing the stomata is affected by turgor pressure on the closing cells. Increasing and decreasing the size of guard cell openings is a result of changes in turgor pressure on guard cells (Meriko and Abizar, 2017).

According to Yulianti et al., (2015), the length of the stomata is related to the amount of chloroplast in the guard cell. Increasing the amount of chloroplasts in guard cells causes the size of the stomata to become larger. Increasing stomata size results in lower stomata density. Diploid plants have smaller stomata size compared to tetraploid derivatives.

### 3.2. Chloroplast analysis

![Image of chloroplast and stomata of mandarin citrus from endosperm accession GT 224 with 1000x magnification.](image_url)

**Table 3. Number of chloroplast 7 accessions of Garut mandarin from endosperm culture**

| Accession Code     | The number of chloroplasts/guard cells | Accession Code | The number of chloroplasts/guard cells |
|--------------------|----------------------------------------|----------------|----------------------------------------|
| Mother plant of Garut mandarin | 16 ± 0                                  | GT 113         | 18 ± 0                                  |
| GT 12              | 13,66 ± 2,08                           | GT 224         | 16,66 ± 1,15                           |
| GT 110             | 14,66 ± 1,53                           | GT 109         | 16,66 ± 1,15                           |
| GT 2               | 14,66 ± 2,31                           | GT 6           | 15 ± 0                                  |

Note: Average ± Standard Deviation
The average result of the number of chloroplasts from the seven accessions of mandarin citrus from endosperm culture with the parent plant of Garut mandarin can be obtained from the similarity dendogram in Figure 4.

Figure 4 shows that the accessions of Garut mandarin have 2 groups of accessions that have similar values, namely group 1 consisting of accessions GT 224 and GT 109 with an average number of chloroplasts 16 and group 2 consisting of accessions GT 110, GT 2, and GT 6 with an average number chloroplast 13-15. Accessions in group 1 and accessions of mother plant of Garut mandarin have similar values with the average number of chloroplasts 16. Accessions in group 2 with accessions GT 12 have similar values because group 2 has an average number of chloroplast 13-15 and GT 12 accessions have the average number of chloroplasts 13. Accessions in group 1 are accessions that have the highest mean number of chloroplasts when compared to accessions in other groups. The Accession of the mother plant of Garut mandarin was in group 1 with a mean value of 16 chloroplasts.

The amount of chloroplasts in mother plant of Garut mandarin has the least amount compared to accessions of mandarin citrus from endosperm culture. This shows that the number of chloroplast mandarin citrus resulting from endosperm culture will increase a lot followed by increasing the size of the stomata. This is due to the increasing size of the stomata resulting in the number of chloroplasts becoming more. The size of the stomata increases in harmony with the increase in the number of chromosomes. According to Kartiman et al., (2018), stomatal size is related to the number of chloroplasts in stomatal guard cells. Increasing the amount of chloroplasts causes the size of the stomata (length and width) to be larger. The greater the size of the stomata, the better it absorbs CO₂ for photosynthesis. Changes that occur in the number of chloroplasts in stoma guard cells and stomata density can be accurate, fast, and easy indicators for the identification of various levels of plant ploidy. The number of chloroplasts guarding the stoma of the tetraploid plant is approximately two times
more than that of the original diploid plant. Changes in stomata density negatively correlated with the level of ploidy, the higher the level of ploidy, the lower the stomata density (Yulianti et al., 2015).

3.3. Chromosome analysis

![Figure 5](image)

Figure 5. A mandarin citrus chromosome from the endosperm of GT 224 accession with 1000x magnification.

| Accession Code | Number of Chromosomes | Accession Code | Number of Chromosomes |
|----------------|-----------------------|----------------|-----------------------|
| GT 224         | 25.10 ± 2.64          | GT 224         | 25.10 ± 2.64          |
| GT 109         | 22.50 ± 3.89          | GT 109         | 22.50 ± 3.89          |
| GT 6           | 22.80 ± 2.10          | GT 6           | 22.80 ± 2.10          |
| GT 113         | 26.60 ± 1.35          | GT 113         | 26.60 ± 1.35          |
| GT 110         | 23.70 ± 2             | GT 110         | 23.70 ± 2             |
| GT 12          | 24 ± 1.73             | GT 12          | 24 ± 1.73             |

Table 4. Number of chromosomes the seven accessions of Garut mandarins from endosperm culture

Note: Average ± Standard Deviation

The results of the average number of chromosomes from 7 accessions of mandarin citrus from endosperm culture with mother plant of Garut mandarin can be obtained by the similarity of dendogram values in Figure 6.

![Figure 6](image)

Figure 6. Dendogram of chromosome accessions of Garut mandarin.

Figure 6 shows that the accessions of Garut mandarin have 3 groups of accessions that have similar values, namely group 1 consisting of accessions of Garut and GT 2 with an average number of
chromosomes 18; group 2 consisted of accessions GT 109, GT 6, GT 12, and GT 110 with a mean number of chromosomes 22-24, and group 3 consisted of accessions GT 113 and GT 224 with a mean number of chromosomes 25-26. Accessions in group 2 and group 3 have similar values because group 2 has an average number of chromosomes 22-24 and group 3 has an average number of chromosomes 25-26. Accessions in group 3 are accessions that have the highest number of chromosomes compared to accessions in other groups. Mother plant of Garut mandarin are in one group with accession of GT 2 with an average number of chromosomes 18, this is because the accession of GT 2 should have an average number of chromosomes $3n = 2x = 27$ but only have an average number of chromosomes $2n = 2x = 18$ so that accessions of GT 2 has the same rate as the mother plant of Garut mandarin.

The results of the calculation of the number of chromosomes can describe the genetics of the plants used where endosperm culture plants have several chromosomes $3n$ and the parent plant has several chromosomes $2n$. The number of these chromosomes will also be related to the number of stomata and the number of chloroplasts, because with the addition of the number of chromosomes will be in harmony with the increasing number of chloroplasts and increasing the size of the stomata. This is by following the opinion of Nurwanti (2010) which states that cells that have increased the number of chromosomes will experience an increase in the number of chloroplasts in the Anthurium plowmanii plant. Calculation of the number of chloroplasts in stomatal guard cells is reported to be more stable in describing genetic background. The chromosome ploidy level of a plant is in harmony with the size of its leaf stomata. The higher the level of ploidy chromosomes, the greater the leaf stomata (Sukamto et al., 2010).

3.4. Alignment between Number of Chromosomes, Number of Stomata, and Number of Chloroplasts

Alignment between the number of stomata, the number of chloroplasts, and the number of chromosomes is done to find out the consistency of the accessions used in this study. This alignment refers to the number of chromosomes obtained because observations of the number of chromosomes can determine the level of ploidy from the accession.
Table 5. Alignment between the number of chromosomes, the number of stomata, and the number of chloroplast accessions of Garut mandarin.

| Group (Number of chromosomes) | Accession Code | Group (Number of stomata) | Accession Code | Group (Number of chloroplasts) | Accession Code |
|-------------------------------|---------------|----------------------------|---------------|-------------------------------|---------------|
| 1 (18) GT 224                 | 1 (37-39) GT 110 | 1 (16) GT 110             | 1 (18) GT 224 | 1 (16) GT 110                 | 1 (16) GT 224 |
| 2 (22-24) GT 109 GT 224 GT 6  | GT 109 GT 12 GT 224 | GT 12 GT 6               | 2 (13-15) GT 109 | 2 (13-15) GT 109             | 2 (13-15) GT 109 |
| 3 (25-26) GT 224 GT 113       | 3 (26-30) Garut GT 113 | 3 (26-30) Garut           | 3 (26-30) Garut GT 113 | 3 (26-30) Garut GT 113       | 3 (26-30) Garut GT 113 |

Note: **Group** = has the most number
**GT 224** = accession that has consistency

The results of alignment between the number of chromosomes, the number of stomata, and the number of chloroplast accessions of Garut mandarin are presented in Table 5 shows that the accessions that have the most number of chromosomes are GT 113 accessions with chromosome number 26, 60 ± 1.35 and GT 224 with the number of chromosome 25.10 ± 2.64. Accessions that have the most number of stomata are GT 110 accessions with 39.88 ± 6.30 stomata; GT 109 with stomata of 39.88 ± 6.35;

GT 12 with the stomata number 39.20 ± 7.43; GT 224 with the number of stomata 37.60 ± 6.19. The accession which has the most number of chloroplasts is GT 224 with chloroplast 16.66 ± 1.15 and GT 109 with chloroplast 16.66 ± 1.15.

4. Conclusions
Alignment results from 7 accessions of Garut mandarin from endosperm culture and one mother plant of Garut mandarin indicate that the accession GT 224 is an accession that has consistency on the number of chromosomes, the number of stomata, and the number of chloroplasts.

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