First Phylogenetic Treatment of Apple Cucumber (Family Cucurbitaceae) from Indonesia Utilizing DNA Variation of Internal Transcribed Spacer Region

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1. Introduction

Cucurbitaceae is huge and diverse family in angiosperm, comprising about 96 genera with approximately 1,000 species (Renner and Schaefer 2017). The most member of this family plays important role as the main fruit crop commodities in Indonesia. Cucumis is among the most popular genus in the family since many species of Cucumis are favored by the community due to their rich in sources of vitamins and minerals, such as C. sativus (Cucumber) and C. melo (Melon). Cucumber and Melon are two common fruit crops that has been known worldwide.

For a long time peoples in Indonesia have been surprised by the present of “new comer” tropical fruit, namely Apple Cucumber, especially in Aceh, and recently this plant has spread in Karawang, Jember and other regions. Apple cucumber is assumed to be a natural hybrid between Cucumber and Melon (no previous report about this), and is likely to have originated in China (Sebastian et al. 2010; Zhang et al. 2012). Its appearance looks like an apple but the taste is melon. The purpose of this study was to elucidate phylogenetic relationship between Apple Cucumber and other species of Cucurbitaceae based on variation of DNA sequences derived from internal transcribed spacer (ITS) region. As many as six individuals of Apple Cucumber collected from Karawang, Jember, and Aceh were examined. The ITS sequences of some species of family Cucurbitaceae were retrieved from GenBank, and put them in the analysis. Phylogenetic analysis based on parsimony method with using Begonia as outgroup reveals that Apple Cucumber are nested in the same clade as Melon (Cucumis melo) with high bootstrap value (100%), suggesting that Apple Cucumber is under the same species as Melon. However, on the basis of morphological characters of fruit, apple cucumber is different with that of Melon. This considerably first phylogenetics treatment provides fundamental knowledge for establishing a subspecies of Melon.
2. Materials and Methods

2.1. Plant Materials

In total, six individuals of apple cucumber distributed in Karawang, Jember, and Aceh were analyzed with a single genus Begonia were used as outgroup. The ITS sequences of the outgroup along with some species of family Cucurbitaceae were retrieved from GenBank (www.ncbi.nlm.nih.gov). Table 1 describes detail information about plant materials and ITS sequences used in this study.

2.2. Amplification and Sequencing

Total DNA was extracted from fresh materials using a GeneJET Plant Genomic Purification Mini Kit (Thermo Scientific, USA) following manufacturer’s instructions. Primer pairs ITS-5 (5’-TAGAGGAAGGAGAAGTCGTAACAA-3’) as forward and ITS-4 (5’-CCCGCCTGACCTGGGGTCGC-3’) as reverse primer following Hidayat et al. (2008) were used (see Figure 1). The PCR profile consisted of an initial 2 min premelt at 95°C and 35 cycles of 30 s at 95°C (denaturation), 2 min at 57°C (annealing), and 2 min at 72°C (extension), followed by a final 10 min extension at 72°C. The PCR products were sent to Macrogen, South Korea for DNA sequencing.

2.3. Phylogenetic Analysis

DNA sequences of the ITS region obtained were aligned with Clustal X (Thompson et al. 1997) and were adjusted manually. Phylogenetic tree reconstruction based on parsimony method was performed using PAUP* version 4.0b10 (Swofford 1998). Insertion and deletion were treated as missing data. All characters were equally weighted and unordered (Fitch 1971). The evaluation the internal support of clades was conducted by bootstrap analysis (Felsenstein 1985) utilizing 1,000 replicates. The number of steps, consistency indices (CI), and retention indices (RI) were calculated using the TREE SCORE command in PAUP*.

2.4. Morphological Observation

Diagnostic morphological characters were analysed according to The International Plant Genetic Resources Institute (IPGRI 2003) in order to provide more evidences.

Table 1. Plant materials examined in this study

| Plant (local name) | Species             | Code | Location | Accession number |
|--------------------|---------------------|------|----------|------------------|
| Apple cucumber     | Cucumis melo        | B    | Karawang | HQ201970         |
| Apple cucumber     | Cucumis sativus     | D    | Jember   | AY833602         |
| Apple cucumber     | Cucumis sativus     | E    | Jember   | JX073074         |
| Apple cucumber     | Benincasa hispida   | F    | Jember   | GU799500         |
| Apple cucumber     | Citrulus lanatus    | G    | Jember   | FJ915110         |
| Apple cucumber     | Melothria scabra    | H2   |          | AM981178         |
| Melon               | Cucumis moschata    | I    |          | KC329521         |
| Cucumber            | Sechium edule       |      |          | HQ201988         |
| White gourd         | Luffa acutangula    |      |          | GQ240882         |
| Watermelon          | Trichosanthes cucumerina |    |          | HQ729030         |
| Mouse melon         | Begonia sp.         |      |          |                  |

*outgroup
3. Results

The alignment process resulted in 650 characters after adjustment by eye, of which 283 (42%) were constant, 181 (28%) were uninformative, and 186 (30%) were parsimony informative. Parsimony analysis produced a single tree (Figure 2) with 727 steps, CI and RI value are 0.714 and 0.660, respectively.

The tree (Figure 2) confirms that Apple Cucumber is the hybridization result between Melon and Cucumber. Moreover, the tree places Apple Cucumber examined in the same clade with Melon (C. melo) with strong bootstrap value (100%), suggesting they are belong to the same species of Melon, C. melo. This result has addressed the puzzle of what species Apple Cucumber belongs to.

![Phylogenetic tree of apple cucumber based on the ITS region. Only bootstrap values of more than 50 is displayed on the branch](image)

4. Discussion

In many phylogenetic studies, using only a single data set, even molecular data, might not elucidate phylogenetic relationships and taxonomic identity of the organisms examined (e.g. Doyle 2013; Bagheri et al. 2016). This is because every single data represents different evolutionary history (Lang et al. 1999) and may lead to the wrong conclusions about the relationships (Qi et al. 2013). Therefore, the use of multiple data set and their combination could provide more reliable results. On the other hand, despite its superiority in molecular phylogenetic studies, the ITS region has some disadvantages especially related with problem in failure of concerted evolution. Every ITS unit (Figure 2) along a hundred or thousand copies that arrange nrDNA (nuclear ribosomal DNA) evolves independently, and this is subjected to paralogous (Baldwin et al. 1995).

Regarding this situation, DNA sequences of the ITS region utilised in this present study has been accompanied by morphological characters to provide the most informative tree. Thus, 141 morpho-characters were added to molecular data, and were resulted in more robust phylogenetic tree (Figure 3) than previous one (Figure 2). All samples of Apple Cucumber form their own clade and become a sister group of Melon. It is meaning that Apple Cucumber and Melon are different, although on the basis of DNA variation, they differ only in three locations (Figure 4). From this combined analysis and more detailed morphological observation, we identified that as many as 21 characters are informative (Table 2) to distinct Apple Cucumber from Melon.

Another things should be pointed out here is lack support of morphological data to the ITS data. We identified nucleotides difference of ITS sequences between Apple Cucumber and Melon only in three locations (Figure 4), whereas morphologically (mostly fruit) they are very different (Table 2). In eukaryotes, this situation is not rare, without exception in plant...
Figure 3. A single phylogenetic tree constructed by combining molecular and morphological data (748 steps of length, 0.722 of CI, and 0.709 of RI)

Figure 4. Nucleotide differences between Apple cucumber and melon in three locations (arrow) throughout the ITS region (650 nucleotides)

(e.g. Stepanovic et al. 2016). Epigenetics phenomenon in plant is remarkable (Pikaard and Scheid 2014). Less variation of DNA sequences do not always bring to less variation of morphology, but this often causes a wide phenotypic diversity (Carvalho et al. 2017).

The taste of Apple Cucumber is very much like Melon, although the shape of fruit looks like an apple. This is in accordance with position of Apple Cucumber and Melon in the tree (Figure 3). Apple Cucumber is considered to be a subspecies of Melon in Indonesia as suggested by this study. Not only this study, a new subspecies through phylogenetic analysis has been proposed by many researchers in angiosperm group (Zeng et al. 2014) such as in Dasyphyllum (Ferreira et al. 2019). On the basis of detailed quantitative and qualitative morpho-agronomic characters (141 characters), these two plants are different (Saputro et al. 2020).

In the end, this study clearly shows that Apple Cucumber is more closely related with Melon (C. melo) rather than Cucumber (C. sativus). This suggests that scientific name of Apple Cucumber would be C. melo. In addition, we identified 21 key characters (mostly character of fruit) that can be used to distinguish Apple Cucumber from that of Melon, providing a fundamental knowledge for establishing a subspecies of Melon. Further phylogenetic studies, however, with extensive sampling and utilizing more
Table 2. Diagnostic characters between Apple Cucumber and Melon

| Characters                      | Plant materials* |
|---------------------------------|------------------|
|                                 | AC (B) | AC (D) | AC (E) | AC (F) | AC (G) | AC (J) | M               |
| Length of leaf petiole          | Short (approx. 3 cm) | Flattened | Small (approx. 450 g) | Medium (approx. 10 cm) | Globular | Small to intermediate (approx. 800 g) | 73 days |
| Fruit shape                     |        |        |        |        |        |        |                 |
| Fruit size                      | 53 days | 55 days | 54 days | 52 days | 55 days | 55 days |                  |
| Total fruit weight per plant    | 5.5 Kg  | 6 Kg    | 5 Kg   | 5 Kg   | 5.3 Kg  | 5 Kg   | 15 Kg           |
| Predominant fruit skin colour   | White   |        |        |        |        |        | Green           |
| Secondary fruit skin colour     | Green   |        |        |        |        |        | Grey            |
| Secondary colour of immature fruit | Absence | |        |        |        |        | Presence (dark green) |
| Secondary skin colour pattern   | Absence | |        |        |        |        | Presence (speckled; spots <0.5 cm) |
| Fruit surface                   | Smooth  | Absence |         |        |        |        |                  |
| Fruit corking/netting distribution | Absence | |        |        |        |        | Finely wrinkled Presence (partially covers fruit) |
| Fruit corking/netting intensity | Absence | |        |        |        |        | Presence (pronounced) |
| Fruit corking/netting pattern  | Presence (very short) | |        |        |        |        | Absence |
| Fruit skin hairiness            | Small   | |        |        |        |        | Intermediate |
| Diameter of peduncle            | White   | |        |        |        |        | Pale green |
| Main colour of flesh            | Intermediate 50 mm White | |        |        |        |        | Sweet 500 mm Orange |
| Flesh flavour                   | |        |        |        |        |        |                 |
| Flesh thickness                 | 16 mm   | 27 mm  | 23 mm  | 26 mm  | 22 mm  | 23 mm  | 50 mm           |
| Placenta colour                 | |        |        |        |        |        |                 |
| Placenta diameter               | 23 mm   | 24 mm  | 26 mm  | 21 mm  | 24 mm  | 25 mm  | 41 mm           |
| Cavity diameter                 |        |        |        |        |        |        |                 |

*AC = Apple Cucumber, M = Melon, B, D, E, F, G, J = the code of plant materials that correspond to Table 1
genetic markers are desirable to carry out in the future in order to provide more plausible evidence for Apple Cucumber in phylogenetic and taxonomic context.

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