HAYATI
Journal of Biosciences

Vol. 28 No. 1, January 2021 48-53
DOI:10.4308/hjb.28.1.48
ISSN: 1978-3019
EISSN: 2086-4094

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

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ARTICLE INFO

Article history:
Received November 20, 2019
Received in revised form October 5, 2020
Accepted October 9, 2020

KEYWORDS:
Apple Cucumber,
Cucurbitaceae,
ITS region,
phylogeny

ABSTRACT

Cucurbitaceae is one of the largest family in Angiosperm in which the most member of this family is important fruit crops in Indonesia such as Cucumber, Melon, Watermelon, and Apple Cucumber. In particular, Apple Cucumber, currently attracts attention to many researchers due to its phylogeneticand taxonomic problem. In term of its appearance, the fruit looks like an apple but the taste is melon. The purpose of this study was to elucidatephylogenetic 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 Begoniaas 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.

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, leading to phylogenetic (the origin and relationships) and taxonomic (the naming) problems of this plant in the family Cucurbitaceae. However, so far no studies have been conducted to address these problems.

This present study was aimed at elucidating phylogenetic relationships between Apple Cucumber and other species of Cucurbitaceae using variation of DNA sequences of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA, and eventually giving taxonomic identity for Apple Cucumber. The ITS regions used here is in part due to its small size (less than 700 bp) and tandem repeat array (Baldwin *et al.* 1995), so that easy to handle. The ITS regions have been used successfully for addressing phylogenetic problem in many family of plants (e.g. Suetsugu *et al.* 2018).

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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.

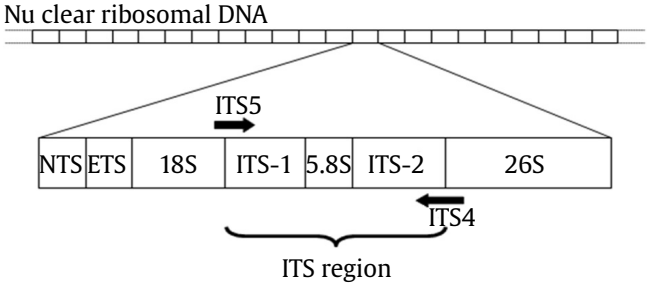

Figure 1. Position of primers applied in this study

Table 1. Plant materials examined in this study

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

*outgroup

3. Results

The alignment process resulted in 650 characters after adjusment 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.

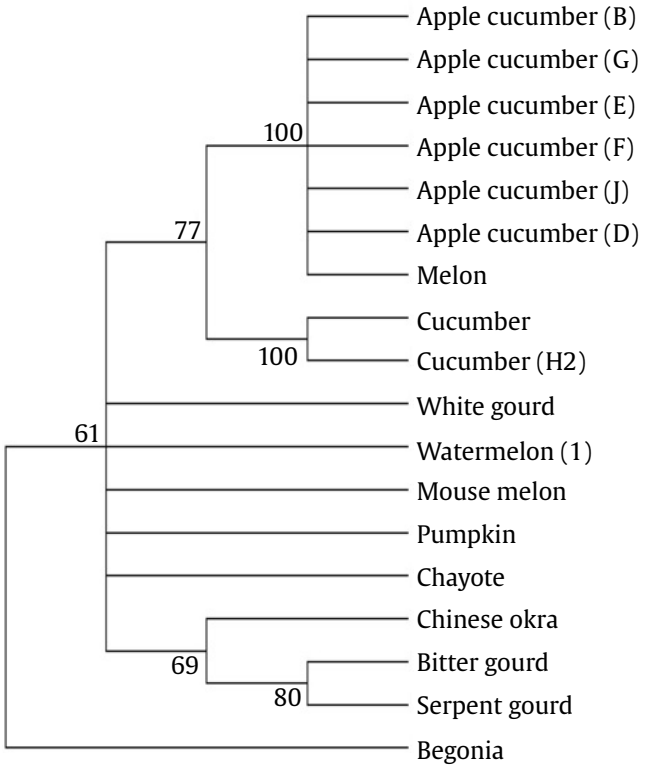

Figure 2. Phylogenetic tree of apple cucumber based on the ITS region. Only bootstrap values of more than 50 is displayed on the branch

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 throughout the ITS sequences (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

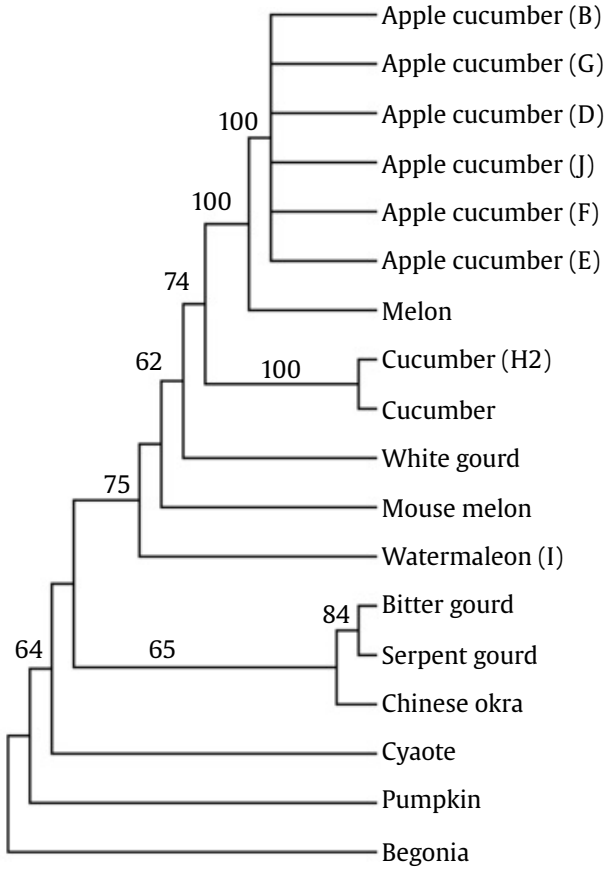

Figure 3. A single phylogenetic tree constructed by combining molecular and morphological data (748 steps of length, 0.722 of CI, and 0.709 of RI)

(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

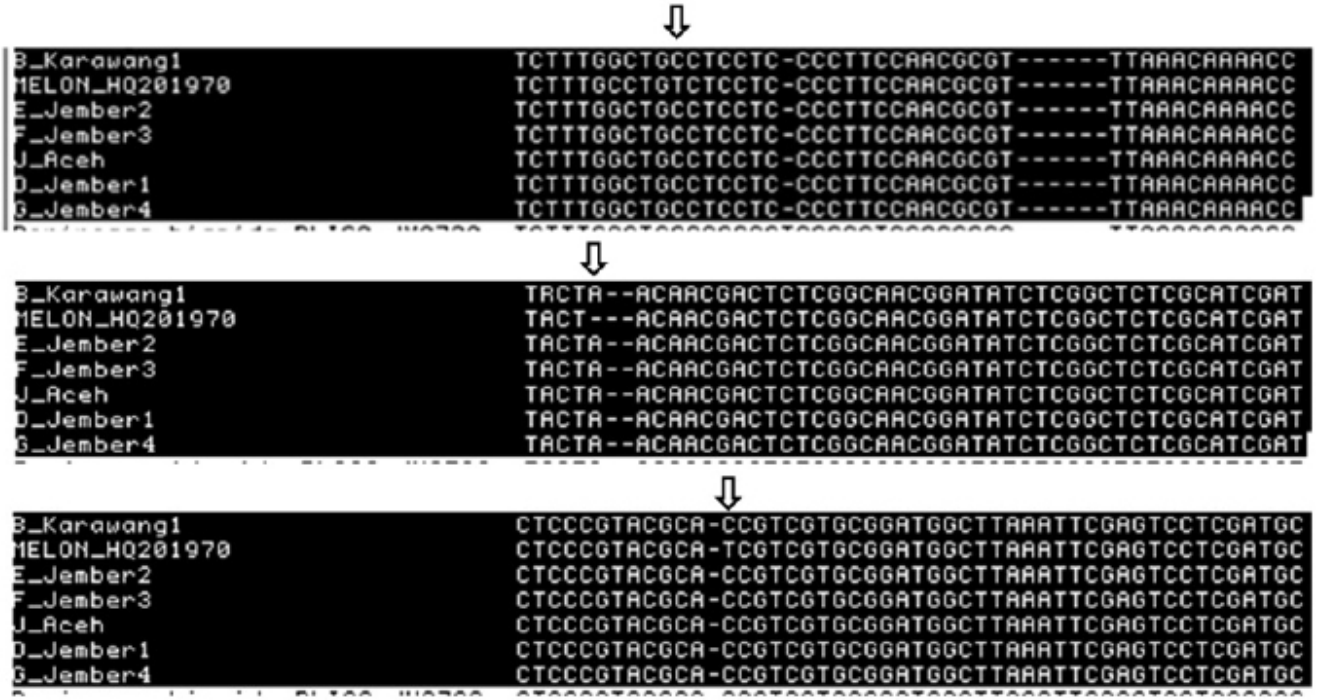

Figure 4. Nucleotide differences between Apple cucumber and melon in three locations (arrow) throughout the ITS region (650 nucleotides)

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)						Medium (approx. 10 cm)
Fruit shape	Flattened						Globular
Fruit size	Small (approx. 450 g)						Small to intermediate (approx. 800 g)
Days to first mature fruit	53 days	55 days	54 days	52 days	55 days	55 days	73 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						Finely wrinkled
Fruit corking/ netting distribution	Absence						Presence (partially covers fruit)
Fruit corking/ netting intensity	Absence						Presence (pronounced
Fruit corking/ netting pattern	Absence						Presence (netted)
Fruit skin hairiness	Presence (very short)						Absence
Diameter of peduncle	Small						Intermediate
Main colour of flesh	White						Pale green
Flesh flavour	Intermediate						Sweet
Flesh thickness	50 mm						500 mm
Placenta colour	White						Orange
Placenta diameter	16 mm	27 mm	23 mm	26 mm	22 mm	23 mm	50 mm
Cavity diameter	23 mm	24 mm	26 mm	21 mm	24 mm	25 mm	41 mm

*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.

Acknowledgements

The authors acknowledge a research funding by Indonesia Ministry of Research Technology and Higher Education (No. 092/SP2H/DRPM/2018). We also thank Anggi Meinawan Insanie, S.Si. for her skillful in laboratory work.

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