Point mutation of ITS-nrDNA sequences as specific markers of three durian species: *Durio zibethinus*, *D. kutejensis* and *D. lowianus*

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Abstract. Molecular markers are considered more efficient tools than morphological markers for species identification. This research aimed to get specific molecular markers among three durian species *Durio zibhetinus*, *D. kutejensis* and *D. lowianus* using ITS-nrDNA sequences. A number of 139, 7 and 1 ITS sequences of *D. zibethinus*, *D. kutejensis* and *D. lowianus*, respectively, were used in this experiment. Each group of sequences was then aligned to make one consensus sequence. In order to identify the point mutations, the aligned sequences were cut with restriction enzyme *in silico* using Genious ver. 7 software. The simulations found that each consensus sequences has different point mutation forming different restriction site. Consensus sequences of *D. zibethinus* has *Eco*R1 site on base number 280, *D. kutejensis* has *Ama*871 site on base number 394 and *D. lowianus* has *Aco*1 site on base number 135. These signals could be used as specific markers for the three durian species.

Keywords: *Durio*, ITS-nrDNA, point mutation, species markers.

1. Introduction

Indonesia is the centre of origin and distribution of the genus *Durio*, and is found to have high diversity of durian genetic resources [1,2]. Every natural durian production area consists of very high genotype variation; each single tree is a different genotype [3]. Complex germplasm variation, therefore, could create conflict in determining and naming a durian type among community groups. Different naming occurred in the same genotypes. Although, high genetic variation is an essntial resource to variety improvement, either through selection [4] or breeding programs [5].

Phylogenetic analysis is one of the important steps whether to select parent trees or determine breeding strategies. This analysis could determine accessions which genetically contain wild traits that we need, or which accessions have already been developed. Phylogenetic studies are also useful in helping rationalize the number of accessions in conservation activities [6–8]. In this way, it could facilitate an efficient collection management through maintain fewer accession but still contain high genetic diversity.

Phylogeny among organisms is now generally studied based on DNA sequencing polymorphisms from sustainable genes that function as bar codes such as Internal Transcribed Spacers (ITS) [9]. The

rate of mutation in this gene is suitable for phylogenetic study in angiosperms and eukaryotes at low taxonomic levels [9]. ITS-nrDNA has also been used for studies of various plants at the intra-species level [10–12]. The massive use of this gene for phylogenetic studies is due to its advantages among others, including a high number of repetitions in the core genome, fairly rapid evolution through gene crossing and conversion, short size (<700 nt), and conserved sequence areas on both sides which leads to easy amplification [9].

Besides being used for phylogenetic analysis, in this article ITS gene demonstrated to be suitable markers for determination of three durian species, *D. zibethinus*, *D. kutejensis* and *D. lowianus*. It could specifically determine the species based on the differences in Single Nucleotide Polymorphism (SNP) in the ITS sequence which is related to areas that contain point mutation forming restriction enzymes sites.

2. Materials and methods

Materials used in this study were leaf samples from 7 accessions of *D. ketejensis* and 139 accessions of *D. zibethinus*. Isolation and amplification of durian genomic DNA were performed using genomic DNA isolation kits for plants (Geneaid™). Other materials include liquid nitrogen, PVP-40, β-mercaptoethanol, absolute ethanol, isopropanol, TAE buffer, agarose gel, PCR mix reagent (KAPA 2G), deionized water, 1 Kb DNA ladder, loading dye, DMSO, ITS5 primer (5'-TAG AGA AAG GAG AAG TCA TAA CAA-3') and ITS4 (5'-CCC GCC TGA CCT GGG GTC GC 3').

Isolation of durian genomic DNA was carried out following the protocol of the Geneaid™ isolation kit. Modifications were made during three isolation steps: first, by adding PVP-40 as much as 10% of the sample weight; second, 1% β-mercaptoethanol of the supernatant volume was added before incubating in a water bath; third, the incubation period at 65°C for 10 minutes was extended to 180 minutes.

Amplification of the target area of ITS-nrDNA using 50 ul PCR mix was prepared in a 0.2-ml PCR tube consisting of 1× Master Mix KAPA, 20 ng DNA template, 4% DMSO, and 20 µM primers. PCR mix was then amplified in PCR machine type Thermal Cycler 2720 (Applied Biosystem), with pre-denaturation reaction at 94°C for 3 minutes, followed by 25 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 60 seconds. Then, PCR was ended with final elongation at 72°C for 5 minutes. The amplification results were confirmed using electrophoresis method. Samples resulting in positive PCR were then directly sequenced using services of a third party (Macrogen Inc., South Korea).

The total of 7 ITS sequences from *D. ketejensis* and 139 from *D. zibethinus* were each aligned to produce one consensus sequence each. The two consensus sequences were then compared with the D. *lowianus* sequence downloaded from NCBI Genebank. The alignment results were then digested *in silico* using restriction enzymes listed in Genious ver. 7 software to determine the position of the mutation points that can be used as PCR-RFLP specific markers.

3. Results and discussion

A product of about 700 nucleotides was obtained from PCR amplification of durian genomic DNA samples using the ITS primers. This product is in accordance with what was conveyed by [9]. The alignment and sequence analysis of the 3 closely related species *D. kutejensis*, *D. zibethinus* and *D. lowianus* indicate that there were different bases in the consensus sequences of the three species which are point mutations. Thirteen point mutations were obtained from *D. kutejensis* sequences. Their positions are bases number 23, 41, 51, 57, 105, 106, 114, 127, 162, 197, 394, 395 and 509. One point mutation was obtained from *D. zibethinus* sequences, which is base number 283. Meanwhile, three point mutations were obtained from *D. lowianus* sequences, which are bases number 52, 135 and 160 (Figure 1).

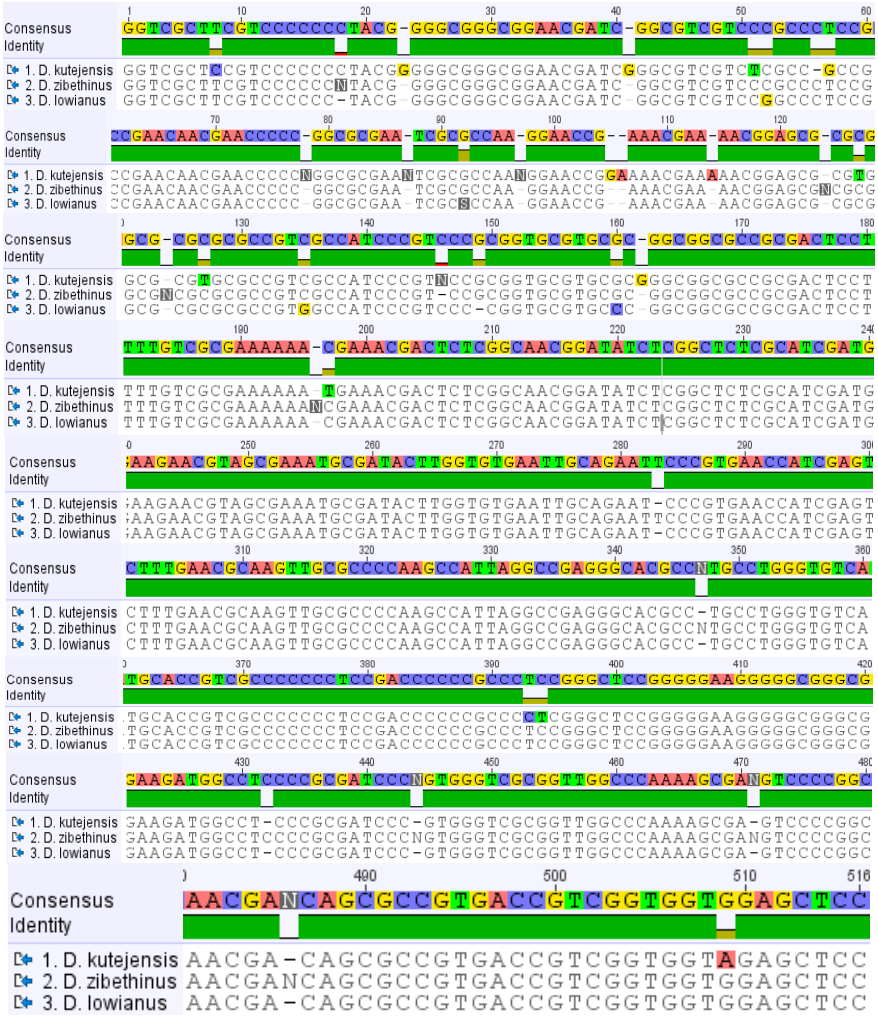

Figure 1. Alignment of ITS-nrDNA sequences of three durian spesies, *D. kutejensis*, *D. zibethinus* and *D. lowianus*, to show the point mutation sites.

In silico analysis amongst three consensus sequences showed the presence of specific restriction sites on each ITS sequences. These restriction sites were the same as one of the point mutations found in the consensus sequence of the three species. Consensus sequences of *D. kutejensis* has *Ama*871 restriction site at base number 394, consensus sequences of *D. zibethinus* has *Eco*R1 restriction site at base number 280, and consensus sequences of *D. lowianus* has *Aco*1 restriction site at base number 135 (Figure 2). The different restriction site found in each consensus sequences showed that ITS-nrDNA sequences have different species signal which could be used as specific markers to differentiate three durian species, *D. zibethinus*, *D. kutejensis* and *D. lowianus*. This finding also showed the evidence that *D. lowianus* stands as different species, whom some researcher consider it to be only as a variation of *D. zibethinus* [1].

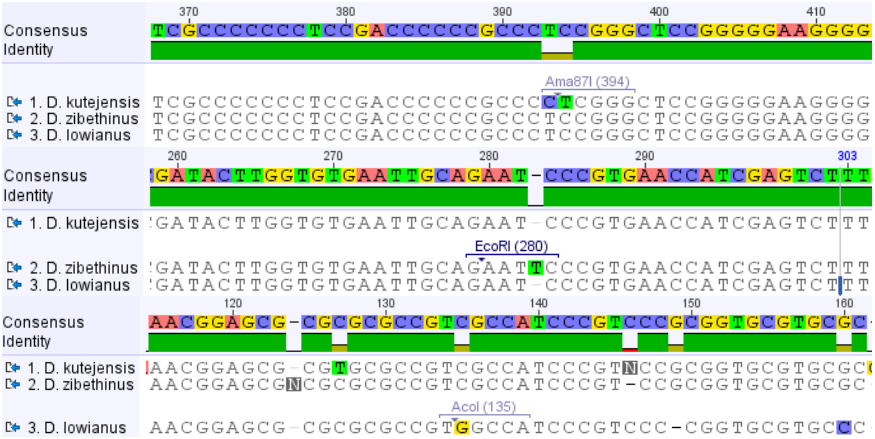

Figure 2. *In silico* analysis of point mutations on ITS-nrDNA sequences among three durian spesies, *D. kutejensis*, *D. zibethinus* and *D. lowianus*, to show enzyme restriction sites as species signals.

The difference of each restriction sites of these sequences can be used as species markers which the detection can be done simply by using PCR-RFLP technique with their respective enzymes without going through sequencing. In this way, the ITS-nrDNA sequences of *D. kutejensis* will be cut into two fragments using the *Ama*871 restriction enzyme, the ITS-nrDNA of *D. zibethinus* will be cut into two fragments using the *Eco*R1 restriction enzyme and the ITS-nrDNA of *D. lowianus* will be cut into two fragments using the *Aco*1 restriction enzyme.

4. Conclusions

Among the three related species *D. kutejensis*, *D. zibethinus* and *D. lowianus*, each of their ITS-nrDNA sequence has a specific restriction site that forms specific signal and could be used as species markers. Further determination for the three species could be simply done by using PCR-RFLP technique.

5. Acknowledgement

This research was funded by Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, Republic of Indonesia through Cooperation Scheme of KKP3T year 2013.

6. References

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