INTERNATIONAL JOURNAL OF CONSERVATION SCIENCE
ISSN: 2067-533X Volume 12, Issue 2, April-June 2021: 585-598 www.ijcs.ro

GENETIC DIVERSITY AND NETWORK WITHIN DESSERT BANANAS (*MUSA ACUMINATA* CV. AA AND AAA) INFERRED BY NEWLY DESIGNED *MAT*K MARKER

Sucipto HARIYANTO[1], Rojaunnajah Kartika AINIYAH[1], Edy Setiti Wida UTAMI[1], Lia HAPSARI[2,*]

[1] Department of Biology, Faculty of Science and Technology, Airlangga University, St. Dr. Ir. H. Soekarno, Mulyorejo, Surabaya, 60115, East Java, Indonesia
[2] Purwodadi Botanic Garden – Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences, St. Surabaya – Malang Km 65, Purwodadi, Pasuruan, 67163, East Java, Indonesia

Abstract

This study aims to evaluate the genetic diversity and network within ten local cultivars of dessert bananas (Musa acuminata cv. AA and AAA) from Indonesia using newly designed matK marker. Result showed that PCR amplifications were successfully carried out. Total aligned and selected sequences were 729 bp, with nucleotide composition high in AT (67.60%) than GC (32.40%). Sequences variation shows high conservation level, comprising 97.94% monomorphics, 0.82% polymorphics, 0.96% InDels, and 0.27% missing data; with high genetic similarity 99.44%-100%. Haplotype diversity was high (Hd=0.844±0.103), forming six haplotypes but low in nucleotide diversity (π=0.231±0.053). Both haplotype network and phylogenetic tree were separated into two clades, following its genomic subgrouping. Clade I comprised of Inarnibal (AA), Cavendish (AAA) and Gros Michel (AAA). Clade II comprised of Sucrier (AA) and Red (AAA). The genetic diversity within genomic group AAA is higher than AA. Both genomic groups cannot be clearly separated due to continuous genetic variation and mutations. Six point mutations were identified; mostly due to substitution of transversion than transition (Ti/Tv=0.5). The finding of this study suggests that the newly designed matK marker is effective for PCR and sufficient to discriminate at intraspecific level of desert bananas.

Keywords*: Genetic conservation; Haplotype; Molecular barcode; Phylogenetic; Polymorhism*

Introduction

The genus *Musa* L. belongs to the family Musaceae, a member of the order Zingiberales, comprises of economically important bananas [1]. The Indo-Malesian region (incl. Indonesia) is considered the homeland of bananas both wild species and cultivars [2-4]. Banana is a fruit plant of worldwide popularity with a global production reached up to 114 million tonnes in 2017 [5]. Edible banana cultivars contain high nutrient values to support food security include high carbohydrates, total sugar, vitamin C, and potassium; moderate protein and low fat [6]. Further, it also has importance values in social and cultural, particularly in rural communities and in developing countries [7, 8].

The vast majority banana cultivars were derived from natural hybridization between wild diploid *Musa* species i.e., *Musa acuminata* (A genome, x = 11) and *Musa balbisiana* (B genome, x = 11). Both inter and intraspecific crosses involving those two wild diploids resulted

* Corresponding author: lia.hapsari@lipi.go.id, hapsari.lia@gmail.com

in wide ploidy levels and genomics groups of current cultivated bananas such as diploids (AA, BB, AB), triploids (AAA, AAB, ABB), and tetraploids (AAAB, AABB, ABBB) [9, 10]. Nonetheless, the evolution process of those edible bananas are very complex involving multiple stages of hybridization, mutation, elaborated by human migration, domestication and adaptation over thousands of years and separated by time and places [2-4].

The most economically important edible bananas consumed and widely grown as dessert or freshly eaten fruit are diploid (AA) and triploid (AAA). The diploid AA cultivars have been largely displaced by the generally more productive triploid cultivars (AAA) but are still popular cultivated in Malaysia, Indonesia, India, Papua New Guinea and the Solomon Islands [4]. Both diploid and triploid cultivars (AA and AAA) are seedless, with bright peel colour, white-yellow-orange flesh, soft and fine flesh texture, sweet taste, and aromatic flavours; thus, they have become favourites [11, 12]. Some subgroups of various banana diploid AA cultivars recognized worldwide such as Inarnibal, Pisang Lilin, Sucrier, Samba, Tjau Lagada, etc. Meanwhile triploid AAA cultivars include Cavendish, Gros Michel, Red, Lakatan, Ibota, Mutika, Lujugira etc. [7, 13].

Elucidating the genetic diversity of bananas is important to provide valuable information for further breeding and conservation prioritization. Indonesia possessed rich diversity of potential local cultivar of dessert bananas (AA and AAA) which needs to be evaluated its genetic diversity. Genetic diversity is the basic material for adaptation, flexibility and responsiveness of evolution in facing various pressures including environmental stresses due to climate change, pests or diseases etc. [14]. In addition, varietal diversity can help farmers to ensure food and nutrition security, by providing them with more options to manage climatic risks and strengthen the resilience of their farms and surrounding landscapes [15]. Most of dessert bananas of AA and AAA groups are considered susceptible to some diseases such as bunchy top [16] and Panama diseases [17]. Thus, advocating and promoting utilization of more local cultivars of dessert bananas are necessary.

Molecular DNA barcode is widely used of biological tool to address basic questions of natural world in systematics, ecology, evolutionary biology and conservation, species interaction networks, taxonomic discovery, assessing priority areas for environmental protection etc. [18]. Some of DNA barcode markers for plants have been developed and used, from plastid and nuclear genomic sources, both coding and non-coding region [19]. One of recommended barcode for plant is *mat*K gene. The *mat*K gene is encoding the maturase enzyme subunit K. The full sequence length is approximately 1,570bp, and is located in plastid genome, inside the intron of *trn*K gene [20, 21]. Some previous studies have reported the use of *mat*K marker to elucidate genetic variations in many plants such as Dipterocarpaceae [21], Solanaceae, Euphorbiaceae [22], ferns [23], Acanthaceae [24]; Liliopsida [25], Orchidaceae [26], Poaceae [27], Zingiberaceae [28, 29], Musaceae [30] etc.

Hitherto, there was still limited information available of the usage of *mat*K barcode to study the genetic diversity within banana cultivars, particularly from Indonesia. Furthermore, PCR amplification of the *mat*K barcoding region is often difficult when dealing with multiple Angiosperm families [20, 31]. Our preliminary study using universal *mat*K primers recommended by CBOL [32] was proven not successful to amplify the DNA sample of banana cultivars. Hence, the aims of this study were to reveal the genetic diversity and networks within dessert bananas (*M. acuminata* cv. AA and AAA) from Indonesia using newly designed *mat*K barcode based on *Musa* sequences reference available in GenBank. The newly designed *mat*K primers from this study is proposed as effective marker to study the genetic diversity for bananas (Musaceae) in particular and Angiosperms in general. A better knowledge and understanding of genetic diversity and networks of dessert bananas from Indonesia will be useful as genetic basis for further dessert banana breeding and genetic conservation strategy.

Experimental

Plant materials

In total, ten cultivars of dessert bananas (*M. acuminata* cv.) were examined in this study. It comprised of four diploids (AA) and six triploids (AAA) cultivars from the living collections of Purwodadi Botanic Garden located in Pasuruan, East Java. They were locally collected from several areas in Indonesia including East Java, Yogyakarta, Central Java, and West Nusa Tenggara (Table 1).

Table 1. Plant materials of *M. acuminata* cv. AA and AAA examined in this study

Sample code	Local name	Synonim	Subgroup	Genomic group	Collection locality	
					Region	Island
S1	Pisang Berlin	Señorita, Pisang Empat Puluh Hari	Inarnibal	AA	Pasuruan, East Java	Java
S2	Pisang Orlin	-	Inarnibal	AA	Probolinggo, Central Java	Java
S3	Pisang Rayap	-	Sucrier	AA	Yogyakarta	Java
S4	Pisang Mas	Lady Finger, Amas, Kluai Khai	Sucrier	AA	Bangkalan, East Java	Madura
S5	Pisang Billa	-	Red	AAA	Sumbawa, West Nusa Tenggara	Sumbawa
S6	Pisang Moseng	Red Dacca, Morado, Pisang Raja Udang	Red	AAA	Sampang, East Java	Madura
S7	Pisang Ambon Hong	Pisang Embun, Kluai Hom Thong	Gros Michel	AAA	Pasuruan, East Java	Java
S8	Pisang Kongkong	-	Gros Michel	AAA	Malang, East Java	Java
S9	Pisang Kreas	-	Cavendish	AAA	Tegal, Central Java	Java
S10	Pisang Cebol	Dwarf cavendish	Cavendish	AAA	Lumajang, East Java	Java

Methods

Morphological observation

Morphological observation was conducted by minimal descriptors in order to differentiate among genomic groups and subgroups following descriptors for bananas from IPGRI-INIBAP/CIRAD [33], and supported by previous studies and references.

Primer design

The construction of new *mat*K primers (Table 2) was based on *mat*K gene sequences available on GenBank with accession number MG041493, *M. acuminata* isolate MN10, cultivar None laphu (AA) from India as reference [34]. The *mat*K gene sequences were evaluated with BioEdit 7.0.5.3. The primers were designed using Primer3 0.4.0 [35]. Later, the primers produced were confirmed in silico using the Primer-BLAST menu on GenBank to determine the accurate position of the primers attachment to the *mat*K gene.

Table 2. Newly designed primers to amplify the *mat*K gene of *M. acuminata cv. AA and AAA* examined in this study

Primer name	Primer direction	Primer sequences (5'-3')	Size (bp)	T_m (°C)	GC (%)	Thermal cycling conditions*
*mat*K F	Forward	CTA CAT ATC CGG CCA AAT CG	20	58.4	50.0	[94/30, 54/45, 72/60] x 40
*mat*K R	Reverse	CGA AAT CCT GGT CCA AAT CC	20	58.4	50.0	

*Temperature (°C)/time (s) for denaturation, annealing, and extension steps; out of thermal cycling conditions included an initial denaturation of 94 °C for 3 minutes and a final extension of 72 °C for 7 minutes

Molecular procedure

Molecular analysis was conducted at the Molecular Genetics Laboratory of Biology Department, Faculty of Science and Technology, Airlangga University, Surabaya, Indonesia. A

fresh young leaf sample was taken for molecular analysis (one individual per cultivar). Total genomic DNAs were extracted using Promega Wizard® Genomic DNA Purification Kit (Madison, WI, USA) following the manufacturer's protocols for plants. Amplifications were conducted in a 35µL volume PCR reaction, which contained of 17.5µL of Go Taq® Green Master Mix (2x) from Promega (DNA polymerase, dNTPs, and $MgCl_2$), 2µL each of forward and reverse newly designed *mat*K primers, 6.5µL of nuclease-free water, and 7µL of DNA template. PCR thermal cycling condition was following Table 2. Confirmation of successful amplification was conducted by electrophoresis separation on 1.5% agarose gel, stained with 1 µg/ml of Ethidium bromide in TBE buffer, then visualised under UV light. Generuller 10 Kb DNA ladder (Thermo Scientific, CA, USA) was used to estimate the size of amplicons. Further, the amplicons were then purified and sequenced at 1st BASE Laboratory, Singapore.

Data analysis

DNA sequences of *mat*K gene were evaluated using BioEdit 7.0.5.3. Homology of *mat*K gene sequences was detected using Basic Local Alignment Tool (BLAST) in GenBank. Multiple sequence alignment was performed using ClustalW program followed by visual adjustment in MEGA 7.0.26. Genetic diversity and neutrality tests were conducted with DnaSP 6.12.03. Median joining analysis was employed using Haplotype Network ver. 5.0.1.1 to reconstruct haplotype networks map [36]. Genetic relationship and pairwise distance analyses were employed in MEGA7.0.26 using Kimura 2 parameter evolution model, Maximum Parsimony, 1000 bootstrap replicates [37].

Results and discussion

Morphological features of M. acuminata cv. AA and AAA

All ten cultivars examined are valuable genetic resources which exhibited varying morphological characteristics following its genomic group and subgroup (Fig. 1), with several important and potential traits. Banana cultivars with an AA genome are generally characterized by horizontally bunches, fruit small size, straight with rounded transverse section and bottle-necked to pointed apex with attractive bright yellow peel and pulp color, soft textured, and sweet-sugary tastes when ripen [11]. Inarnibal subgroup was differentiated with Sucrier by its very thin skin, when ripe the skin easily cracks and the fruits tend to falls from hand by themselves (deciduous). This fragility makes it difficult to store or transport them over long distances [6, 38]. Meanwhile Sucrier subgroup also has thin skin, but it is not easily cracking, and the fruit is persistent [39, 40]. Pisang Orlin has slender fruit than Pisang Berlin (Fig. 1: S1-S2). Likewise, Pisang Rayap has slender and pointed fruit than Pisang Mas (Fig. 1: S3-S4). Both Pisang Berlin and Pisang Mas are relatively popular commercial dessert bananas in the local market in East Java.

Fig. 1. Morphological features of *M. acuminata* cv. AA and AAA examined in this study

The triploid AAA group are more vigorous than the diploids. It bears heavy symmetrical and hanging bunches, with large fruit, markedly curved, creamy white flesh, soft and fine textures, sweet taste and aromatic flavour when ripen so they have become popular [12]. Red subgroup is distinctly characterized by pigmented plant with deep red pseudo stem, petioles, and midribs; it also produces moderately sized bunches of fruit with reddish green to deep maroon skin depends on direct sunlight exposure [38]. The ripen pulp is light orange flesh which indicated high carotenoid level, potential to alleviate vitamin A deficiency [39]. Both Pisang Billa and Pisang Moseng showed similar characteristics of Red Subgroup (Fig. 1: S5-S6). They are not popular commercial cultivars but still available in the local market and people mostly used as ornamental plants in home garden.

Meanwhile Gros Michel and Cavendish subgroup are popular commercial cultivars; both are very close related subgroup with similar characteristics. Cultivars in Gros Michel subgroup can be distinguished from those in Cavendish subgroup by their green/pale pink versus bright red undersheath pseudostem, bottle-necked to pointed fruit, short pedicel with green to full yellow color. The fruit of Cavendish subgroup at cooler temperature or when artificially ripened turn to attractive bright yellow color. Both has a long transport life if harvested at the correct maturity [12, 38]. The various cultivars are similar except for their height and characteristics of the bunch and fruit. Both Pisang Ambon Hong and Kongkong showed similar characteristics of Gros Michel. Pisang Kongkong has spiral bunch shape (all fruits are attached to a unique crown coiled around the stalk) whilst Pisang Ambong Hong is cylindrical (Fig. 1: S7-S8). Pisang Cebol has short pseudo stem and compact fruits in a bunch, whilst Pisang Kreas has normal height (Fig. 1: S9-S10). Pisang Cebol is included as dwarf Cavendish (Cebol = dwarf). It is well suited for home garden, commercial, and agroforestry cultivation.

New matK primers evaluation

The *mat*K primers in this study have designed following the terms and conditions of ideal parameters for efficient and effective PCR [41, 42]. Nucleotide length of the newly designed *mat*K primers is 20 bp each, with melting temperature (Tm) 58.4°C, GC contents 50%, and annealing temperature (Ta) 54°C (Table 2). Primer length is associated with efficiency of PCR process, the longer oligonucleotides primer will highly be possible to form a secondary structure which disrupt the PCR process. Whilst GC contents and Tm levels are important to avoid miss-priming the target sequences. In addition, the presence of G/C clamp at the 3' end of each primer greatly helps to stabilize the bond between the primer and the DNA template needed for initiation during PCR process. Further, in silico simulation using Primer-BLAST on GenBank showed that the newly *mat*K primers were conform with *mat*K gene target of *M. acuminata* with approximately length of 756 bp. It was also conforming with other *Musa* species such as *M. itinerans*, *M. ornata*, *M. balbisiana*, *M. paradisiaca*, *M. schizocarpa*, etc.

Amplifications of matK marker on M. acuminata cv. AA and AAA

Amplification of the *mat*K gene using newly designed primer was successfully carried out to all banana specimens examined. Visualization on 0.8% agarose gel electrophoresis was shown by the presence of a specific DNA band in the sample lane at the length between 700 and 750bp (Fig. 2). The *mat*K gene sequences within *M. acuminata* cultivars both forward and reverse produced were high in trace score, with sequences length resulted were 724 to 731bp. This length range of *mat*K gene sequences was in accordance with some previous studies in some species of the Zingiberales order, such as in *Zingiber* spp., *Curcuma* spp., *Amomum* spp., and *Kaempferia* spp. 300 to 925bp [29], wild *Musa* spp. 720-950bp [30], *Alpinia* spp. 789 to 854bp [43], *Costus* spp. 740-1542bp [43] etc. Further, BLASTn to Genbank database showed high homology with *mat*K gene sequences of species from Musaceae family (such as *M. acuminata* ssp., *M. acuminata* cultivars, *Musa* hybrid cultivars, etc.) with high similarity query cover values and per identity 98 to 99.50%.

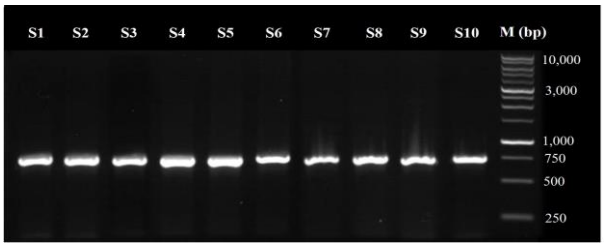

Fig. 2. Electrophoregram amplicons of *mat*K gene within *M. acuminata* cv. AA and AAA

The problematic for PCR amplification and sequencing in the *mat*K region is leading to the design of new taxon-specific primers or modifications of existing primers [20, 31]. The findings of this current study suggest that the newly designed *mat*K primer for DNA barcoding is effective for amplification of bananas on *M. acuminata* cv. AA and AAA groups with 100% succession rate. It is also effective for amplification to other *Musa* species, Musaceae family, Zingiberales order and Monocots as suggested by in silico simulation. Nonetheless, further testing for its universality is required by involving taxonomically diverse samples across Angiosperms.

Sequence polymorphisms within M. acuminata cv. AA and AAA

Total aligned and selected *mat*K gene sequences within *M. acuminata* cv. AA and AAA examined were 729 bp. Nucleotide composition in average were high in AT (67.60%) than GC (32.40%) (Table 3). The *mat*K gene as a coding region or exon was known to have high AT bases content because it was associated with their functions in transcription and protein translation. It is proposed to aid splicing of seven different chloroplast group IIA introns that reside within precursor RNAs for essential elements of chloroplast function [20, 26]. Low GC content means low spots of mutation and recombination rates. Mutation or loss of *mat*K gene, the principle targets for *mat*K protein activity were also lost, suggesting co-evolutionary reduction [26]. Evidently, *M. acuminata* cv. AAA group have higher GC content than AA group, so it is more likely that mutations will occur in triploid bananas. The highest GC content was found in Pisang Cebol, followed by Pisang Billa and Pisang Kreas (Table 3).

Table 3. Nucleotide composition of *mat*K gene within *M. acuminata* cv. AA and AAA

Sample code	OTU	Nucleotide composition (%)					
		T(U)	C	A	G	A+T	G+C
S1	Pisang Berlin	29.22	15.24	38.50	17.04	67.73	32.27
S2	Pisang Orlin	29.14	15.19	38.54	17.13	67.68	32.32
S3	Pisang Rayap	29.40	15.26	38.28	17.06	67.68	32.32
S4	Pisang Mas	29.28	15.19	38.40	17.13	67.68	32.32
S5	Pisang Billa	29.42	15.33	38.12	17.13	67.54	32.46
S6	Pisang Moseng	29.20	15.15	38.29	17.36	67.49	32.51
S7	Pisang Ambon Hong	29.09	15.24	38.50	17.17	67.59	32.41
S8	Pisang Kongkong	29.09	15.24	38.50	17.17	67.59	32.41
S9	Pisang Kreas	29.13	15.26	38.42	17.20	67.55	32.45
S10	Pisang Cebol	29.10	15.17	38.34	17.38	67.45	32.55
Average		29.21	15.23	38.39	17.18	67.60	32.40

Remarks: OTU = Operational Taxonomic Unit, G = Guanin, A = Adenin, C = Cytosin, and T = Thymin

The *mat*K gene sequences variation within *M. acuminata* cv. AA and AAA shows high conservation level, comprising 714 (97.94%) monomorphic sites, 6 (0.82%) polymorphic sites, 7 (0.96%) insertion and deletions (InDels), and 2 (0.27%) sites with missing data. Pairwise distance analysis resulted high genetic similarity of 99.44% to 100% (low genetic divergence 0-0.006). The *mat*K gene sequences at low level intraspecific taxa within *M. acuminata* cv. AA and AAA in this study was considered lack of polymorphisms. However, *mat*K gene sequences

polymorphisms of higher taxa at interspecies (same genus) on Zingiberaceae as close related family of Musaceae also showed low polymorphic sites, such as within *Aframomum* 0.96%, *Curcuma* 0.77%, *Elettariopsis* 0.59%, and *Hedychium* 0.38% [28].

Further polymorphism analysis, the polymorphic sites were comprised of 5 (0.68%) singleton variable sites and 1 (0.14%) parsimony informative sites. It was identified six point mutations which considered as single nucleotide polymorphism/SNP in six banana cultivars i.e. Pisang Billa, Pisang Moseng, Pisang Kreas, Pisang Berlin, Pisang Mas and Pisang Rayap. A SNP is a variation at a single position in a DNA sequence among individuals. Bananas of Red subgroup (AAA) i.e. Pisang Billa and Pisang Moseng were experienced higher mutations compared to others (Table 4).

The point mutations occurred were mostly due to substitution of transversion than transition (Table 4). A transversion is considered to be a more drastic change than a transition, because substitution of one-ring to two-ring chemical structure or vice versa (transversions) requires more energy than substitution without change in the ring structure (transitions). It can be caused by high-energy sources such as radiation, chemicals, and high environmental pressure, or appear spontaneously during DNA replication. The transition and transversion ratio (Ti/Tv) of this study was 0.5, which means the substitution mutations occur randomly, because basically there are two possible transitions and four possible transversions. The Ti/Tv ratio has been used as an important parameter in many studies such as phylogenetic tree reconstruction and estimation of divergence [45].

Table 4. Nucleotide variations of *mat*K gene within *M. acuminata* cv. AA and AAA

Polymorphic site	Site position	SNP	Mutation type	OTU member
Singleton variable	4	A-T	Transition	Billa
	5	A-C	Transversion	Billa
	704	A-G	Transversion	Moseng
	721	G-A	Transversion	Berlin
	727	T-A	Transition	Kreas
Parsimony informative	416	G-T	Transversion	Rayap, Mas, Billa, Moseng

Remarks: OTU = Operational Taxonomic Unit, SNP = single nucleotide polymorphism, G = Guanin, A = Adenin, C = Cytosin, and T = Thymin

Genetic diversity within M. acuminata cv. AA and AAA

Genetic diversity within a population can be assessed based on haplotype diversity (Hd) and nucleotide diversity (π). Haplotype diversity represents probability of two randomly selected alleles to be different from each other. Whilst nucleotide diversity reflects nucleotide divergence of two individuals with respect to one locus [46]. The haplotype diversity (Hd) based on *mat*K gene sequences of ten *M. acuminata* cv. AA and AAA was high (Hd>0.5), forming six haplotypes but low in nucleotide diversity (π < 0.5%) among haplotypes (Table 5). Further, neutrality tests of Tajima's D and Fu's FS showed negative values, indicating deviations from neutrality and both suggested recent population expansion of *M. acuminata* cultivars. Exception for *M. acuminata* cv. AA was showed positive Tajima's D which signifies low levels of polymorphisms frequency, indicating a decrease in population size and/or balancing selection [46, 47]. Genetic diversity within *M. acuminata* cv. AAA was revealed higher than AA as evidenced by its nucleotide diversity and polymorphisms (Table 5).

Table 5. Genetic diversity indices and neutrality tests of *mat*K gene sequences within *M. acuminata* cv. AA and AAA

OTU	OTU number	Haplotype number	Hd±SD	π±SD (%)	Eta	Fu's Fs	Tajima's D
Overall *M. acuminata* cv.	10	6	0.84±0.10	0.23±0.05	6	-2.56	-1.15
M. acuminata cv. AA	4	3	0.83±0.22	0.16±0.05	2	-0.66	0.59
M. acuminata cv. AAA	6	3	0.80±0.03	0.26±0.08	5	-0.63	-0.83

Remarks: Hd = haplotype diversity, π = nucleotide diversity, SD = standard deviation, Eta = total number of mutation/polymorphic site

Haplotype network and phylogenetic tree within M. acuminata cv. AA and AAA

Haplotype networks are connected graphs with cycles represents evolutionary relationships (genealogies) at the intraspecific level. It is considered more informative than conventional phylogenetic trees to display intraspecific DNA sequence variation by the additional feature of putative recombination events in which not shown in the phylogenetic tree [36, 49]. The genetic network resulted both from haplotype and phylogenetic analyses based on *mat*K gene sequences were separated into two polyphyletic clades, the genomic grouping AA and AAA were nested on both clades. Interestingly, the separation of each clade was following the genomic subgrouping. Inarnibal subgroup (AA) was close related with Gros Michel subgroup (AAA), with similarity 99.72%-100%. Whilst Sucrier Subgroup (AA) was close related with Red subgroup (AAA), with similarity 99.58%-100% (Figs. 3 and 4).

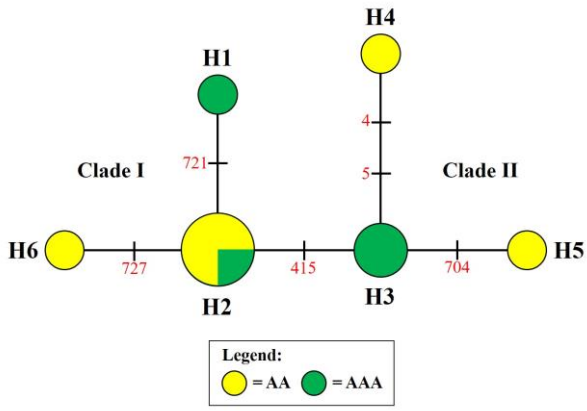

Fig. 3. Haplotype network within *M. acuminata* cv. AA and AAA based on *mat*K gene sequences. Note: H1=Pisang Berlin; H2=Pisang Orlin, Pisang Ambon Hong, Pisang Kongkong, Pisang Cebol; H3=Pisang Mas, Pisang Rayap; H4=Pisang Billa; H5=Pisang Moseng; H6=Pisang Kreas.

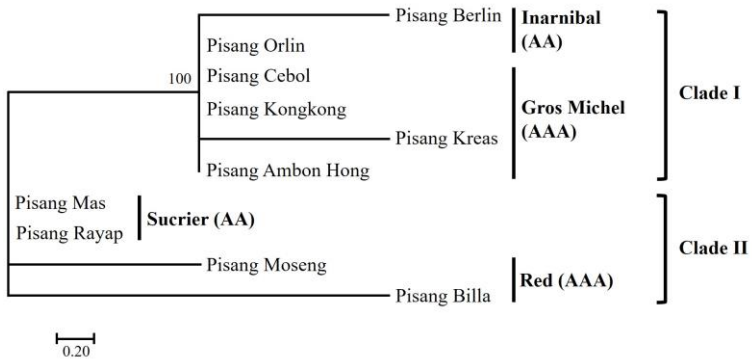

Fig. 4. Phylogenetic tree within *M. acuminata* cv. AA and AAA based on *mat*K gene sequences (Maximum Parsimony)

Previous study using sequences of nuclear ribosomal Internal Transcribed Spacer/ITS sequences also showed that banana cultivars AA group were nested together with AAA group [49]. The *mat*K gene located in chloroplast genome is uniparental inherited exclusively from the female parent, since pollen (from male parent) having no plastids [50]. Whilst ITS located in nuclear genome is biparental inherited from their both male and female parents. This result showed that both chloroplast and nuclear genomes marker resulted similar pattern genealogies of complex edible *M. acuminata* cv. AA and AAA. The genomic groups of AA and AAA

possibly cannot be clearly separated since both are very closely related and derived from the same of a particular species and subspecies of ancestors i.e. *M. acuminata* with continuous variation and mutations. Further, the addition of one set of chromosomes through autopolyploidy also did not change the cultivar genetic constitutions [51].

During evolution, banana cultivars may first exist from clone selection of wild cultivated *M. acuminata* population (AAw) and then in regions where the diffusion of plants occurred (exchange or via human migration) hybridization with other *M. acuminata* subspecies wild cultivated populations and partly fertile clones (AA) from different origins led to the generation of more sterile AA diploids, and the more vigorous and nearly sterile triploids (AAA) [3]. Banana cultivars are reproducing asexually with rhizomes, thus maintaining those constituents of genetic variation from natural hybridization and mutations.

In details, Clade I comprise of Inarnibal Subgroup (Pisang Berlin, Pisang Orlin), Cavendish subgroup (Pisang Cebol, Pisang Kreas) and Gros Michel subgroup (Pisang Kongkong and Pisang Ambon Hong), which separated into three haplotypes (Figs. 3 and 4). Subgroups Cavendish and Gros Michel were identical (similarity 100%) and clustered in Haplotype 2, except Pisang Kreas which separated by a single point mutation (site position 727, transition) in Haplotype 6 (similarity 99.86%). Within Inarnibal Subgroup, Pisang Orlin was found identical with Cavendish and Gros Michel (H2), whilst Pisang Berlin was separated from Pisang Orlin by a single point mutation (site position 721, transversion) in Haplotype 1 (similarity 99.86%).

Clade II comprises of Sucrier subgroup (Pisang Mas, Pisang Rayap) and Red Subgroup (Pisang Moseng, Pisang Billa), which separated into three haplotypes (Figs. 3 and 4). Within Sucrier subgroup, Pisang Mas and Pisang Rayap were identical clustered in Haplotype 3 (similarity 100%). Meanwhile within Red subgroup, Pisang Billa and Pisang Moseng were separated into their own haplotypes (H4 and H5) connected to Haplotype 3 with similarity 99.58%. The haplotype separation of both cultivars possibly due to adaptation to their specific habitat i.e. Sumbawa Island and Madura Island, respectively. The occurrence of several polymorphic nucleotides between haplotype pairs indicates that the target region has a high substitution rate [52]. Pisang Billa has the highest substitution rate separated with Haplotype 3 with two polymorphic nucleotides (site position 4 & 5). Mutations are very important for the evolutionary process, enable organisms to reproduce more effectively and adapt well to changing environments [53]. The finding of this study suggests that genetic diversity of *mat*K gene sequences within *M. acuminata* cv. AA and AAA are highly conserved, lack of polymorphisms, low nucleotide diversity, and high genetic similarity; but considered sufficient to discriminate at intraspecific level of banana cultivars through haplotype network and phylogenetic analysis.

Implications for conservation and banana breeding

Assessment of genetic diversity within *M. acuminata* cv. AA and AAA provides important information genetic basis which imply to conservation prioritization strategy and for further dessert banana breeding and development purposes. For ex-situ conservation strategy, banana cultivars which genetically revealed identical (placed at the same haplotype) are ineffective and inefficient. Banana cultivars clustered in Haplotype 2 (Pisang Orlin, Pisang Ambon Hong, Pisang Kongkong, and Pisang Cebol) and Haplotype 3 (Pisang Mas and Pisang Rayap) were revealed identical, thus if resources are limited, it suggested being chosen one of them (each haplotype) as a representative collection. However, for in-situ/ on-farm conservation is encouraged to plant diverse banana local cultivars to manage climatic risks, strengthen the resilience of pest and diseases outbreaks, and to secure food sources. Further, there are evidence of local banana genetic erosion through genetic uniformity of common cultivated commercial cultivars which replaced and reduced the cultivation of potential local cultivars [7].

All bananas *M. acuminata* cv. AA and AAA examined in this study are valuable genetic resources with several important and potential traits although considered less genetically

diverse based on *mat*K marker. Some local/traditional cultivars are having breeding potentials such as partial resistance to pests and diseases, high nutrition values, post-harvest quality, etc. [54]. Particularly for *M. acuminata* cv. AA can be used as the male parent in crosses hybridization with tetraploids to produce triploids in banana breeding [55]. Later, banana breeding through genomic tools enable to exploit similarities between close-related bananas to find, identify and evaluate the genes responsible for particular traits and how to bring them together in a new cultivar. Thus, further research using functional DNA-based markers is suggested to evaluate the diversity of banana germplasm and its precise properties, such as to characterize disease resistances, stress-related and other genes, and in the future can be expected to enable the measurement and mapping of quantitative trait loci [56]. As well as exploration and conservation of banana genetic resources also utilization of banana genetic diversity using novel approaches for understanding are required urgently.

Conclusions

The newly designed *mat*K marker has proven to be effective for PCR within desert bananas of *M. acuminata* cv. AA and AAA. Further testing for its universality is required by involving taxonomically diverse samples across Angiosperms. Genetic diversity of *mat*K gene sequences within *M. acuminata* cv. AA and AAA are highly conserved, lack of polymorphisms, low nucleotide diversity, and high genetic similarity. However, it was sufficient to discriminate at intraspecific level of desert bananas. Both haplotype network and phylogenetic tree were separated into two clades, following its genomic subgrouping. The genetic diversity within genomic group AAA is higher than AA. Both genomic groups cannot be clearly separated due to continuous genetic variation and mutations. All bananas *M. acuminata* cv. AA and AAA examined in this study are valuable genetic resources with several important and potential traits although considered less genetically diverse based on *mat*K marker. Further research using functional DNA-based markers is suggested to evaluate the diversity of banana germplasm and its precise properties.

Acknowledgments

The authors gratefully acknowledge Purwodadi Botanic Garden, Indonesian Institute of Science for providing the living plant materials and the Ministry of Research, Technology and Higher Education, Republic of Indonesia for funding the molecular part of this study with Master's Research Fellowship grant number 1520/UN3/2019. Sincere thanks also addressed to the Molecular Genetic Laboratory of the Biology Department, Airlangga University for the molecular and genetic facilities.

Author's contribution: Conceptualization, S.H.; Methodology, R.K.A., L.H.; Performed the lab. experiment, R.K.A.; Data analysis, R.K.A., L.H.; Writing–original draft manuscript, S.H., R.K.A., L.H.; Supervised the research activities and results, S.H., E.S.W.U, L.H. All authors (S.H., R.K.A., E.S.W.U., L.H.) commented and contributed to the review and editing of the manuscript.

References

[1] A. Lamere, A.M. Otaghvari, S.R. Rao, *Phylogenetic implications of the Internal Transcribed Spacers of nrDNA and chloroplast DNA fragments of Musa in deciphering the ambiguities related to the sectional classification of the genus*, **Genetic Resources of Crop and Evolution**, **64**(6), 2017, pp. 1241-1251.

[2] J. Kennedy, *Bananas and people in the homeland of Genus Musa: Not just pretty fruit*, **Ethnobotany Research and Application**, **7**, 2009, pp. 179-197.
[3] E. De Langhe, L. Vrydaghs, P. De Maret, X. Perrier, T. Denham, *Why bananas matter: An introduction to the history of banana domestication*, **Ethnobotany Research and Application, 7**, 2009, pp. 165-177.
[4] X. Perrier, E. De Langhe, M. Donohue, C. Lentfer, et al., *Multidisciplinary perspectives on banana (Musa spp.) domestication*. **Proceedings of the National Academy of Sciences of the United States of America**, **108**(28), 2011, pp. 11311-11318.
[5] * * *, **Banana Facts and Figures: Banana Production in 2017**, FAO (Food and Agriculture of United Nations). Available online at http://www.fao.org/economic/est/est-commodities/bananas/bananafacts/en/#.XruVv2gzY2w, [Downloaded on 1st April 2020].
[6] L. Hapsari, D.A. Lestari, *Fruit characteristic and nutrient values of four Indonesian banana cultivars (Musa spp.) at different genomic groups*, **Agrivita**, **38**(3), 2016, pp. 303-311.
[7] L. Hapsari, J. Kennedy, D.A. Lestari, A. Masrum, W. Lestarini, *Ethnobotanical survey of bananas (Musaceae) in six districts of East Java, Indonesia*, **Biodiversitas, 18**(1), 2017, pp. 160-174.
[8] K. Ashokkumar, S. Elayabalan, V.G. Shobana, P. Sivakumar, M. Pandiyan, *Nutritional value of cultivars of banana (Musa spp.) and its future prospects*, **Journal of Pharmacognosy and Phytochemistry**, **7**(3), 2018, pp. 2972-2977.
[9] G. Martin, F. Carreel, O. Coriton, C. Hervouet, C. Cardi, et al., *Evolution of the banana genome (Musa acuminata) is impacted by large chromosomal translocations*, **Molecular Biology and Evolution**, **34**(9), 2017, pp. 2140-2152.
[10] Z. Wang, H. Miao, J. Liu, B. Xu, X. Yao, et al., *Musa balbisiana genome reveals subgenome evolution and functional divergence*. **Nature Plants**, **5**, 2019, pp. 810-821.
[11] L. Hapsari, A. Masrum, *Diversity and characteristic of banana Musa acuminata cultivar diploid AA collection of Purwodadi Botanic Garden*, **Proceeding of National Seminar 159th Anniversary of Cibodas Botanic Garden** (Editors: D. Widyatmoko, Puspitaningtyas, R. Hendrian, Irawati, I.A. Fijridiyanto, et al.), Cibodas Botanic Garden-Indonesian Institute of Sciences, 2011, pp. 225-229.
[12] L. Hapsari, *Performance of seven accessions banana cultivars triploid Musa acuminata group (AAA) collection of Purwodadi Botanic Garden*, **Proceeding of Humboldt Kolleg: Synergy, Networking and The Role of Fundamental Development in Asean in conjunction with: The International Conference on Natural Sciences 2011 (**Editors: H. Scheer, B. Pradhan, T.H.P. Brotosudarmo, E. Sadtono, B.K. Ane), Shaker Verlag Publisher, Germany, 2013, pp. 283-287.
[13] F. Bakry, F. Carreel, C. Jenny, J-P. Horry, *Genetic improvement of banana*, **Breeding Plantation tree crops: tropical species** (Editors: S.M. Jain, P.M. Priyadarshan), Springer, New York, 2009, pp. 3-51.
[14] C. Bellard, C. Bertelsmeier, P. Leadley, W. Thuiller, F. Courchamp, *Impacts of climate change on the future of biodiversity*, **Ecology Letters**, **15**(4), 2012, pp. 365–377.
[15] D. Mijatovic´, F. Van Oudenhoven, P. Eyzaguirre, T. Hodgkin, *The role of agricultural biodiversity in strengthening resilience to climate change: towards an analytical framework*, **International Journal of Agriculture Sustainability**, **11**(2), 2013, pp. 95-107.
[16] L. Hapsari, A. Masrum, *Preliminary screening resistance of Musa germplasm for banana bunchy top disease in Purwodadi Botanic Garden, Pasuruan, East Java*, **Buletin Kebun Raya**, **15**(2), 2012, pp. 57-70.

[17] M. Dita, M. Barquero, D. Heck, E.S.G. Mizubuti, C.P. Staver, *Fusarium wilt of banana: Current knowledge on epidemiology and research needs toward sustainable disease management*, **Frontiers of Plant Science**, **9**, 2018, pp. 1468.
[18] J.W. Kress, *Plant DNA barcodes: Applications today and in the future*, **Journal of Systematics and Evolution**, **55**(4), 2017, pp. 291-307.
[19] P.M. Hollingsworth, S.W. Graham, D.P. Little, *Choosing and using a plant DNA barcode*, **PLoS ONE**, **6**(5), 2011, Article Number: e19254.
[20] J. Yu, J-H. Xue, S-L. Zhou, *New universal matK primers for DNA barcoding Angiosperms*, **Journal of Systematics and Evolution**, **49**(3), 2011, pp. 176–181.
[21] E. Harnelly, Z. Thomy, N. Fathiya, *Phylogenetic analysis of Dipterocarpaceae in Ketambe Research Station, Gunung Leuser National Park (Sumatra, Indonesia) based on rbcL and matK genes*, **Biodiversitas**, **19**(3), 2018, pp. 1074-1080.
[22] J.I. Wattoo, M.Z. Saleem, M.S. Shahzad, A. Arif, A. Hameed, M.A. Saleem, *DNA barcoding: amplification and sequences of rbcL and matK genome regions in three divergent plant species*, **Advance on Life Science**, **4**(1), 2016, pp. 03-07.
[23] F-W. Li, L-Y. Kuo, C.J. Rothfels, A. Ebihara, W-L. Chiou, M.D. Windham, K.M. Pryer, *rbcL and matK earn two thumbs up as the core DNA barcode for ferns*, **PLos ONE**, **6**(10), 2011, Article Number: 26597.
[24] M.F. Arif, G.R. Aristya, D. Subositi, A.N. Sari, R. Kasiamdari, *Short Communication: rbcL and matK chloroplast DNA composition of green chireta (Andrographis paniculata) from Indonesia*, **Biodiversitas**, **20**(12), 2019, pp. 3575-3583.
[25] B.J. Kolondam, *Applying matK gene for identification of Liliopsida plant species from North Sulawesi through BOLD Systems*, **International Journal of Applied Biology and Pharmaceutical Technology**, **6**(2), 2015, pp. 242-245.
[26] M. Barthet, K. Moukarzel, K.N. Smith, J. Patel, K.W. Hilu, *Alternative translation initiation codons for the plastid maturase matK: unraveling the pseudogene misconception in the Orchidaceae*, **BMC Evolutionary Biology**, **15**(210), 2015, pp. 1-15.
[27] S.A. Osman, W.A. Ramadan, *DNA barcoding of different Triticum species*, **Bulletin of the National Research Centre**, **43**(174), 2019, pp. 1-13.
[28] D. Selvaraj, R.K. Sarma, R. Sathishkumar, *Phylogenetic analysis of chloroplast matK gene from Zingiberaceae for plant DNA barcoding*, **Bioinformation**, **3**(1), 2008, pp. 24-27.
[29] M.R. Vinitha, U.S. Kumar, K. Aishwarya, M. Sabu, G. Thomas, *Prospects for discriminating Zingiberaceae species in India using DNA barcodes*, **Journal of Integrative Plant Biology**, **56**(8), 2014, pp. 760-773.
[30] L-F. Li, H-Y. Wang, C. Zhang, X-F. Wang, F-X. Shi, et al., *Origins and domestication of cultivated banana inferred from chloroplast and nuclear genes*, **PLoS ONE**, **8**(11), 2013, Article Number: e80502.
[31] J. Heckenhauer, M.H.J. Barfuss, R. Samuel, *Universal multiplexable matK primers for DNA barcoding of Angiosperms*, **Applied Plant Science**, **4**(6), 2016, Article Number: 1500137.
[32] CBoL (Consortium for the Barcoding of Life) Plant Working Group, *A DNA barcode for land plants*, **Proceedings of the National Academy of Sciences of the United States of America**, **106**, 2009, pp. 12794-12797.
[33] * * *, **Descriptors for Banana (*Musa* spp.)**, International Plant Genetic Resources Institute-International Network for the Improvement of Banana and Plantain/Centre de Cooperation International en Recherche Agronomique pour le Developpement (IPGRI-INIBAP/CIRAD), Montpellier, France, 1996, pp. 1-57.
[34] P. Thangjam, R. Thangjam, **Phylogenetic study of wild banana found in North East India**, Mizoram University, Tanhril, Aizawl, Mizoram, India. Unpublished, 2017.

[35] A. Untergasser, I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm, S.G. Rozen, *Primer3 - new capabilities and interfaces*, **Nucleic Acids Research**, **40**(15), 2012, Article Number: e115.

[36] E. Paradis, *Analysis of haplotype networks: The randomized minimum spanning tree method*, **Methods in Ecology and Evolution**, **9**, 2018, pp. 1308-1317.

[37] S. Kumar, G. Stecher, K. Tamura, *MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets*, **Molecular Biology and Evolution**, **33**, 2016, pp. 1870-1874.

[38] R.C. Ploetz, A.K. Kepler, J. Daniells, S.C. Nelson, *Banana and plantain-an overview with emphasis on Pacific island cultivars ver. 1*. **Species Profiles for Pacific Island Agroforestry** (Editor: C.R. Elevitch), Permanent Agriculture Resources, Hōlualoa, Hawai'i, 2007, pp. 1-27.

[39] A. Sunandar, D. Kurniasih, *Fruit morphological characteristics and β-carotene content of three Indonesian dessert and cooking banana cultivars*, **Biosaintifika**, **11**(1), 2019, pp. 171-177.

[40] R. Ningsih, R. Megia, *Folic acid content and fruit characteristics of five Indonesian dessert banana cultivars*, **Biodiversitas**, **20**(1), 2019, pp. 144-151.

[41] S.A. Bustin, R. Mueller, T, Nolan, *Parameters for successful PCR primer design*, **Quantitative Real-Time PCR: Methods and Protocols, Methods in Molecular Biology vol. 2065** (Editors: R. Biassoni, A. Raso), Springer Science and Business Media, LLC, part of Springer Nature, 2020, pp. 5-22.

[42] X. Yang, B.E. Scheffler, L.A. Weston, *Recent developments in primer design for DNA polymorphism and mRNA profiling in higher plants*, **Plant Methods**, **2**(4), 2006, pp. 1-10.

[43] W.J. Kress, A-Z. Liu, M.F. Newman, Q-J. Li, *The molecular phylogeny of Alpinia (Zingiberaceae): a complex and polyphyletic genus of gingers*, **American Journal of Botany**, **92**(1), 2005, pp. 167-178.

[44] W.J. Kress, L.M. Prince, K.J. Williams, *The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data*, **American Journal of Botany**, **89**, 2002, pp. 1682-1696.

[45] S. Duchêne, S.Y.W. Ho, E.C. Holmes, *Declining transition/transversion ratios through time reveal limitations to the accuracy of nucleotide substitution models*, **BMC Evolutionary Biology**, **15**(36), 2015, pp. 1-10.

[46] M. Sharma, B.A. Fomda, S. Mazta, R. Sehgal, B.B. Singh, N. Malla, *Genetic diversity and population genetic structure analysis of Echinococcus granulosus sensu stricto complex based on mitochondrial DNA signature*, **PLoS ONE**, **8**(12), 2013, Article Number: e82904.

[47] M. Ashfaq, P.D.N. Hebert, M.S. Mirza, A.M. Khan, S. Mansoor, et al., *DNA barcoding of Bemisia tabaci complex (Hemiptera: Aleyrodidae) reveals southerly expansion of the dominant whitefly species on cotton in Pakistan*, **PLoS ONE**, **9**(8), 2014, Article Number: e104485.

[48] P. Mardulyn, *Trees and/or networks to display intraspecific DNA sequence variation?*, **Molecular Ecology**, **21**, 2012, pp. 3385-3390.

[49] L. Hapsari, R. Azrianingsih, E.L. Arumingtyas, *Genetic variability and relationship of banana cultivars (Musa L.) from East Java, Indonesia based on the Internal Transcribed Spacer region nrDNA sequences*, **Journal of Tropical Biology and Conservation**, **15**, 2018, pp. 101-120.

[50] C.W. Birky, *Uniparental inheritance of organelle genes*, **Current Biology**, **18**(16), 2008, pp. R692-R695.

[51] A. Retnoningsih, R. Megia, A. Hartana, *Characterization and evaluation of Musa acuminata cultivars in Indonesia based on microsatellite markers*, **Floribunda**, **4**(1), 2010, pp. 21–32.
[52] N. Nanayakkara, V. Herath, D.V. Jayatilake, *Haplotype diversity analysis of bacterial leaf blight resistance gene Xa21 in rice*, **Tropical Agriculture Research**, **30**(1), 2018, pp. 56-68.
[53] J.L. Carlin, *Mutations are the raw materials of evolution*, **Nature Education Knowledge, 3**(10), 2011, Article Number: 10.
[54] U. Subbaraya, **Farmer's knowledge of wild *Musa* in India**, Food and Agriculture Organization of the United Nations, Rome, Italy, 2006.
[54] Y.S. Poerba, F. Achmad, *Genetic variation of diploid banana cultivars (AA) from collection of Cibinong Science Center based on RAPD and ISSR markers*, **Biota**, **15**(3), 2010, pp. 308−315.
[56] J.S. Heslop-Harrison, *Genomics, banana breeding and superdomestication*, **Acta Horticulturae**, **897**, 2011, pp. 55-62.

Received: May 18, 2020
Accepted: February 23, 2021