AGRIVITA Journal of Agricultural Science. 2016. 38(2): 155-162

UTILIZATION OF cpDNA SEQUENCES TO IDENTIFY 15 MANGO ACCESSIONS

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Received: December 30, 2015/ Accepted: April 22, 2016

ABSTRACT

Information about genetic relationship and genetic diversity are an inevitable need in mango breeding program. Base substitution value, genetic distance and grouping of 15 mango accessions based on accessions in chloroplast DNA (cpDNA) among 15 mango accessions were assessed. The samples were originated from Mango Germplasm, Cukur Gondang Research Station of Indonesian Tropical Fruits Research Institute, Pasuruan, East Java. Sequencing of Chloroplast DNA was used to obtain nucleotide sequence data. Paired specific primer rpl20 F - rps12 R and atp F - rbcL R were used for amplification of non-coding area of mango DNA chloroplast and sequencing processes as well. All data were analyzed using Software MEGA 6. The result showed that total numbers and nucleotide base sequences varied among all accessions. All accessions were grouped in five different clusters that might be used as source of parental breeding.

Keywords: cpDNA; genetic relationships; mango; sequencing; variability

INTRODUCTION

Mango is a popular and prominent fruit in Asia and South America countries. India, China, Thailand, Pakistan, Mexico, and Indonesia are the major producers of mango, mangosteen and guava (FAOSTAT, 2012). As the fourth mango producer in the world, Indonesia has not involved yet to the major mango exporters (Saave, 2011) because of the low quality of the fruit to meet the international market requirements (Mukherjee and Litz, 2009). However, the quality of Indonesian mango could still be improved through breeding programs. Recently, breeding program of mango is focused on the production of better quality of mango accessions, which have improved in performance and resistance to both biotic and abiotic stresses (Lavi *et al*., 2004).

High diversity of mango and the relatively uncomplicated vegetative multiplication of the plant make the extensive cultivation and the generation of new superior mango accessions are possible (Lavi *et al*., 2004). The attempt to create new mango accessions is needed to add genetic diversity and to fulfill the market preference. One of the characteristics to meet the international preferences is mango cultivars with red blush (Sauco, 2004).

The selection of parental crossing in mango breeding program is still based on phenotype characteristics (Lavi *et al*., 1998). Naturally, mango is a heterozigous tree. Hence, phenotype based crossing will lead to the unpredictable hybrids (Usman *et al*., 2001). Lavi *et al*., (2006) stated that molecular markers can be used to identify and distinguish different type of cultivars with more precision result. The differences among individual plant could be determined by the genetic code (base sequences) contained in DNA (Semagn *et al*., 2006). The development of the genetic markers has further reduced the uncertainty in breeding mango and maintaining the hybrid populations in a better way. Molecular markers reflect inheritance Mendel, which make them possible to trace the fingerprints of each individual including history of its evolution (Usman *et al*., 2001). Those efforts were done through phylogenetic tree and study of genetic relationship (Hoshino *et al*., 2012). Intergenic spacer region rpl20-rps12 and atp-rbcL of chloroplast DNA of 19 mango accessions were used to differentiate intra- and inter-cultivars (Khan and Azim, 2011).The research aimed to know base substitution value, genetic distance and grouping accessions (genetic relationships) of 15 mango accessions using intergenic spacer

Cite this as: Sukartini, Taryono, P. Basunanda and R.H. Murti. 2016. Utilization of cpDNA sequences to identify 15 mango accessions. AGRIVITA Journal of Agricultural Science. 38(2): 155-162. Doi: 10.17503/agrivita.v38i2.767
Accredited : **SK No. 81/DIKTI/Kep/2011**
Permalink/DOI: **http://dx.doi.org/10.17503/agrivita.v38i2.767**

region rpl20-rps12 and atp-rbcL of chloroplast DNA sequences (cpDNA).

MATERIALS AND METHODS

The research was conducted in the laboratory of genetic and breeding, Gadjah Mada University, Yogyakarta. The research was started from May to November 2013. Leaf samples of 15 mango accessions, such as: Apel Merah, Arumanis 143, Khirsapati Maldah, Irwin, Haden, Gedong Gincu, Saigon, Delima, Liar, Keitt, Agung, Madu Segoro, *Mangifera gedebi*, Bangalora, and Paw-paw were taken from mango germplasm, Cukur Gondang Research Station of ITFRI in Pasuruan, and East Java.

Doyle and Doyle (1990) method modified by (Lodhi *et al*., 1994) was used for DNA isolation. Mango shoot leaves about 7-10 days after emerging were collected for DNA extraction. Leaf samples of each mango accession of about 0.1 g were pulverized in a mortar with 800µl buffer extraction (2% CTAB; 1.4 M NaCl; 20 mM EDTA pH 8; 100 mM Tris–HCl pH 8; 1% PVP-40; 1% Mercaptoethanol). The sample was then transferred into 1.5 ml micro tube, and mixed well using vortex machine. Sample was incubated into water bath at 65°C for 60 minutes and transferred to the room temperature (25°C) for 2 minutes. A mixture of 500 µl of chloroform and isoamil alcohol (CIAA) with a ratio of 24:1 was then added to each sample.The solution was mixed through inverted-reverse and followed by a vortex for 5 minutes and centrifuged at 12,000 rpm for 15 minutes.

The new solution (supernatant) was transferred into new micro tube and added with sodium acetate 3 M as much as 1/10 the volume of supernatant. The solution was inverted reverse for 5 minutes and then added with cold isopropanol as much as 2/3 from total volume (supernatant + sodium acetate) and incubated in a freezer for 24 hours. The sample was centrifuged at 12,000 rpm for 10 minutes. The upper solution was discarded and DNA sediment was air dried. DNA sediment was dissolved in 50 µl of aqua-bidest. The quantification of DNA was done in the absorbance of 260 nm. DNA concentration for PCR was assigned in 5 ng μl^{-1}. Paired specific primers were used for PCR amplification of non-coding area of mango DNA chloroplast (Table 1).

PCR reactions were done in 10 µl of 5 µl Go Taq© Green Master Mix, 2.25 µl nuclease F-W, 0.25 µl primer (0.125 µl each for F and R) and 2.5 µl DNA. Touchdown method was used with 35 cycles with an initial denaturation cycle of 3 minutes at 94ºC, followed by denaturation for 40 seconds at 94ºC, and 40 seconds annealing at 50ºC. Temperature was decreased 2ºC every 5 cycles until the end of the process. The end of the process must reach 38ºC, one minutes of extension at 72 ºC, with a final extension of 10 minutes at 72 ºC.

The result of DNA amplification was observed by electrophoresis at 1.5% gel metaphor agarose, with 10 µl DNA resulted from amplification of 3 µl KAPA dye, 5 µl marker at 80 volt, 400 Am for 70 minutes. The result of electrophoresis was observed under ultraviolet light. Sanger method was used for sequencing of amplification result that was observed by the same primer.

RESULT AND DISCUSSION

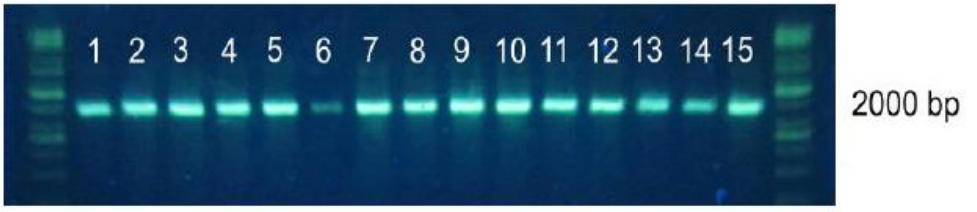
Amplification Result

DNA chloroplast intergenic area of the 15 mango accessions could be recognized by both paired primers. It is characterized by the appearance of all of the same band at the same position or at 2000 bp (Figure 1 and 2).

Base on Figure 1 and 2, the primers were fitted for 15 accessions. Non-coding area such as intergenic and intron are areas, in which mutation possibly happened. Mutation is source of diversity (intra and intra-specific) from base sequences in DNA or genotype (Intrieri *et al*., 2007).

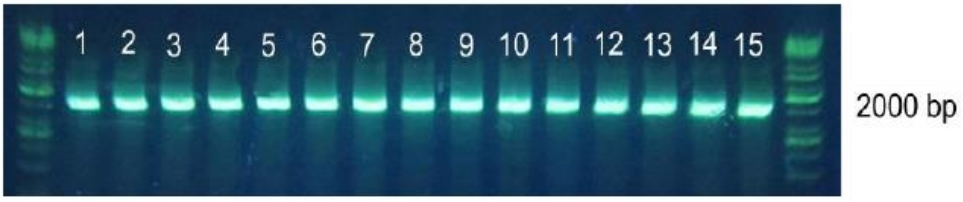
Table 1. Primers were used for amplification

No.	Primer name	Sequence 5'-3'	Literature
1.	rpl20 F	TTTGTTCTACGTCTCCGAGC	Hamilton (1999)
	rps 12 R	GTCGAGGAACATGTACTAGG	
2.	atp F	GAAGTAGTAGGATTGATTCTC	Samuel *et al*. (1997)
	rbcL R	CCCTACAACTCATGAATTAAG	



Remarks: 1. Arumanis 143, 2. Saigon, 3. *Mangifera gedebe*, 4. Irwin, 5. Haden, 6. Li'ar, 7. Khirsapati Maldah, 8. Paw-paw, 9. Bangalora, 10. Madu Segoro, 11. Gedong Gincu, 12. Keitt, 13. Agung, 14. Delima, 15. Apel Merah

Figure 1. The result of amplification with utilization of primer rpl20 F - rps 12 R



Remarks: 1. Arumanis 143, 2. Saigon, 3. *Mangifera gedebe*, 4. Irwin, 5. Haden, 6. Li'ar, 7. Khirsapati Maldah, 8. Paw-paw, 9. Bangalora, 10. Madu Segoro, 11. Gedong Gincu, 12. Keitt, 13. Agung, 14. Delima, 15. Apel Merah

Figure 2. The result of amplification with utilization of primer atp F - rbcL R

Table 2. Number of nucleotides of 15 mango accessions

No.	Accessions	Number of Nucleotides Base			
		rpl20 F	rps12 R	atp F	rbcL R
1.	Agung	810	815	791	769
2.	Apel Merah	810	810	762	713
3.	Arumanis 143	810	815	791	733
4.	Bangalora	809	814	788	768
5.	Delima	813	808	766	712
6.	*Mangifera gedebi*	811	808	773	748
7.	Gedong Gincu	810	811	787	713
8.	Haden	808	809	764	767
9.	Irwin	809	807	783	758
10.	Keitt	809	811	765	717
11.	Khirsapati Maldah	808	811	790	768
12.	Li'ar	812	808	775	714
13.	Madu Segoro	812	814	772	767
14.	Paw-paw	366	812	764	762
15.	Saigon	809	810	771	731

Sequencing

The sequencing was done by using the same primers and resulted diverse number of nucleotide bases (Table 2). Total number of base resulted from DNA sorting was more than 1000 bases. The number was considered sufficient to create a true phylogeny as suggested by Tateno *et al*. (1994). Number of nucleotides was between 700 and 800. The least number of nucleotides was observed in Paw paw that had only 366 bases.

Homogeneity Test

The Disparity Index Test analysis of all molecular data showed that the data were not homogeneous. In homogeneous data were caused by the outlier of nucleotide number found in paw-paw accessions (Tabel 2). The biased data were

marked by yellow color in a result matrix analysis (Table 3). Tamura *et al.* (2013) revealed that inhomogeneous data could not be utilized to establish topology and to count distance because it will lead to a bias result. To avoid uncertainty analyses, molecular data from primer rps 12 R, atp F and rbcL R were the only data used for further analysis.

Bases Substitutions

The level of difference bases among accessions in detail was reflected in the differences of the base composition inter-accessions. Two accessions which have the same number of bases were not necessarily having the same base composition in codon (Table 4).

Table 3. *Disparity Index Test* data as result of DNA sorting

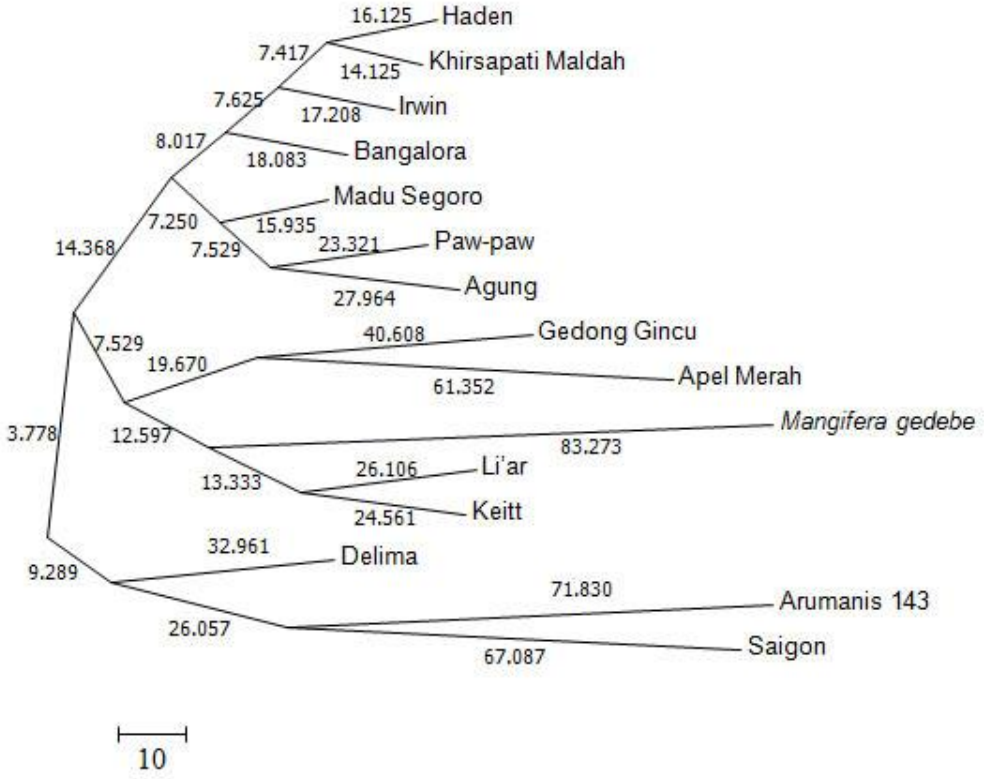
	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]
[1]		0.00	0.00	0.00	0.00	0.00	0.00	2.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[2]	1.00		0.00	0.00	0.00	0.00	0.00	2.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[3]	1.00	1.00		0.00	0.00	0.00	0.00	2.87	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[4]	1.00	1.00	1.00		0.00	0.00	0.00	2.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[5]	1.00	1.00	1.00	1.00		0.00	0.00	1.62	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[6]	1.00	1.00	1.00	1.00	1.00		0.00	2.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[7]	1.00	1.00	1.00	1.00	1.00	1.00		1.89	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[8]	0.01	0.01	0.00	0.01	0.02	0.00	0.01	1.90	2.40	1.97	1.83	2.18	3.20	1.61	
[9]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	
[10]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00		0.00	0.00	0.00	0.00	0.00
[11]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00		0.00	0.00	0.00	0.00
[12]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.01	1.00	1.00	1.00		0.00	0.00	0.00
[13]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00		0.00	0.00
[14]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00		0.00
[15]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.03	1.00	1.00	1.00	1.00	1.00	1.00	

Remarks: [1] = Arumanis 143; [2] = Saigon; [3] = *Mangifera gedebe*; [4] = Irwin; [5] = Haden; [6] = Li'ar; [7] Khirsapati Maldah; [8] = Paw-paw; [9] = Bangalora; [10] = Madu Segoro; [11] = Gedong Gincu; [12] = Keitt; [13] = Agung; [14] = Delima; [15] = Apel Merah; numbers above the diagonal = standarderror; Numbers under the diagonal = inter-accessions distance; yellow mark = biases data

Table 4. Bases composition inter-accessions

	T	**C**	**A**	**G**	**Total**	**T-1**	**C-1**	**A-1**	**G-1**	**Pos #1**	**T-2**	**C-2**	**A-2**	**G-2**	**Pos #2**	**T-3**	**C-3**	**A-3**	**G-3**	**Pos #3**
[1]	30.7	16.1	36.1	17.1	2339	29.8	18.1	35.0	17.1	778	32.0	16.0	36.2	15.7	781	30.3	14.1	37.1	18.6	780
[2]	30.5	16.4	35.7	17.4	2312	29.5	18.3	34.7	17.6	767	31.9	16.9	36.2	15.0	771	30.2	14.1	36.2	19.5	774
[3]	32.8	15.3	35.4	16.5	2328	32.4	16.6	33.9	17.1	771	33.3	15.3	37.0	14.4	778	32.6	14.1	35.3	18.0	779
[4]	31.0	15.8	37.6	15.5	2348	30.3	17.0	36.5	16.1	781	32.2	16.1	38.1	13.7	783	30.6	14.4	38.1	16.8	784
[5]	30.5	16.0	37.8	15.7	2340	29.8	17.6	36.6	15.9	778	31.2	16.1	38.7	14.0	781	30.3	14.3	38.2	17.2	781
[6]	30.9	15.5	37.5	16.2	2297	30.2	17.1	36.1	16.5	764	31.8	15.6	38.2	14.5	765	30.7	13.7	38.2	17.4	768
[7]	30.0	16.5	38.2	15.3	2368	29.1	18.4	36.9	15.6	788	31.1	16.1	39.2	13.6	788	29.8	14.9	38.5	16.8	792
[8]	30.8	15.5	38.2	15.4	2337	30.2	16.8	37.2	15.8	779	31.1	15.9	38.9	14.0	778	31.2	13.8	38.5	16.5	780
[9]	30.6	16.0	38.4	15.0	2370	29.9	17.4	37.1	15.6	788	31.2	15.9	39.1	13.8	788	30.6	14.9	38.9	15.6	794
[10]	30.9	15.9	37.8	15.4	2353	30.2	17.9	36.7	15.2	782	31.8	15.8	38.5	13.9	785	30.5	14.1	38.3	17.0	786
[11]	31.0	16.1	36.6	16.3	2313	30.8	17.8	35.1	16.3	769	31.8	16.0	37.0	15.2	770	30.4	14.5	37.7	17.4	774
[12]	30.9	15.6	36.7	16.8	2293	30.2	16.9	35.8	17.1	762	31.5	15.9	37.4	15.3	763	31.1	13.9	36.8	18.1	768
[13]	30.9	15.9	38.1	15.2	2375	30.5	17.6	36.3	15.7	791	31.4	15.9	39.0	13.7	790	30.7	14.1	38.9	16.2	794
[14]	30.4	15.8	37.3	16.5	2284	29.1	17.7	36.2	16.9	756	31.9	15.5	38.5	14.2	762	30.2	14.2	37.3	18.3	766
[15]	31.0	15.8	36.4	16.8	2284	29.5	17.9	36.6	15.9	759	32.7	15.4	36.1	15.9	762	30.8	14.3	36.4	18.5	763

Remarks: All base frekuensi in %; [1]=Arumanis 143, [2]=Saigon, [3]=*Mangifera gedebe*, [4]=Irwin, [5]=Haden, [6]=Liar, [7]=Khirsapati Maldah, [8]=Paw-paw, [9]=Bangalora, [10]=Madu Segoro, [11]=Gedong Gincu, [12]=Keitt, [13]=Agung, [14]=Delima, [15]=Apel Merah; Pos#1=first base in codon, Pos#2=second base in codon, Pos#3=third base in codon.



Remarks: Branch length numbers were exhibited number of base substitution

Figure 3. Base substitutionss of 15 studied mango accessions

Differences in base composition reflected the base substitution at each accession. Base substitution value was determined by the value of branch length in phylogenic tree (Figure 3). These results were in accordance with the opinion of Yang (1996), who stated that the difference value of base sequence was caused by base substitution. Tamura *et al.* (2013) asserted that the degree of difference on DNA base sequences could lead to the degree similarity or difference characters.

The greater branch length value showed a difference in the characters of accession. *Mangifera gedebe* species is an accession that has the largest branch length value. These indicate the high difference in base composition between *Mangifera gedebe* and others. The anatomy character of *Mangifera gedebe* fruits is one of the striking differences that may differentiate the species with the other *Mangifera indica* accessions. The mesocarp of *Mangifera gedebe* fruits was not developed (the exocarp was directly attached to endocarp).

Genetic Distance and Grouping Accessions

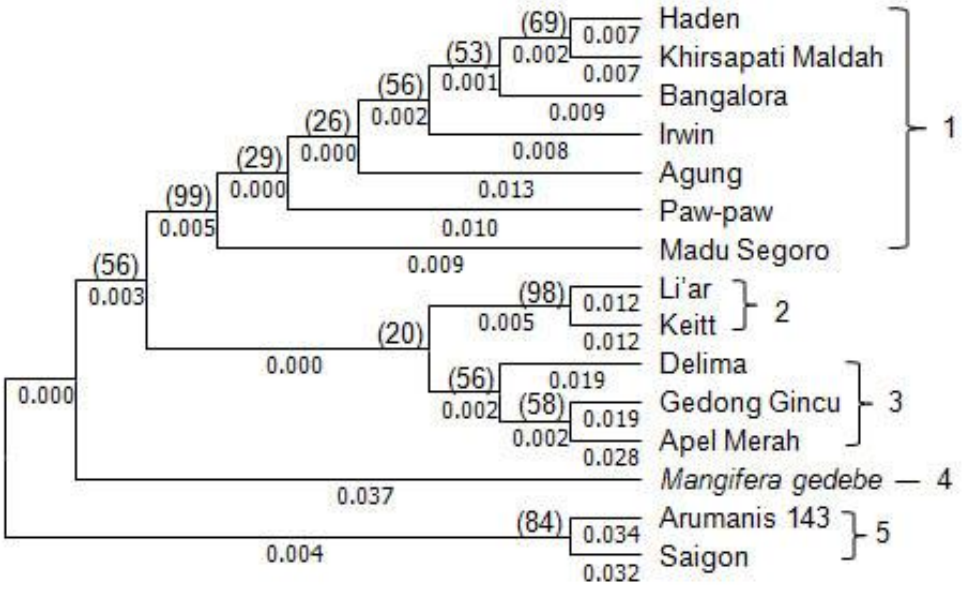
Maximum Composite Likelihood method was used to count the value of genetic distance (Table 5). The tree branch pattern or geometries relationship of inter-accessions (topology) and grouping accessions in phylogenic tree or genetic relationship were presented in Figure 4.

Overall, genetic distance inter-accessions were relatively diverse, though some accessions showed equal values. This was caused by similarity and difference of genetic distance value inter-accessions and determined by similarity and difference composition of each base that was used to form genetic distance matrix. The lowest genetic distance was between Haden and Khir-sapati Maldah i.e. 0.014, while the highest was observed between species *Mangifera gedebe* and Apel Merah accessions viz. 0.075.

Table 5. Genetic distance matrix inter-accessions

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]
[1]															
[2]	0.066														
[3]	0.074	0.074													
[4]	0.056	0.054	0.054												
[5]	0.057	0.056	0.058	0.017											
[6]	0.057	0.056	0.055	0.036	0.034										
[7]	0.062	0.057	0.061	0.017	0.014	0.033									
[8]	0.054	0.054	0.055	0.019	0.025	0.033	0.025								
[9]	0.062	0.056	0.054	0.020	0.018	0.036	0.017	0.023							
[10]	0.056	0.054	0.054	0.020	0.020	0.030	0.022	0.020	0.022						
[11]	0.059	0.061	0.062	0.039	0.038	0.042	0.039	0.040	0.043	0.040					
[12]	0.058	0.055	0.056	0.033	0.033	0.023	0.033	0.033	0.035	0.029	0.042				
[13]	0.062	0.059	0.055	0.024	0.023	0.037	0.026	0.024	0.022	0.022	0.039	0.038			
[14]	0.058	0.056	0.067	0.038	0.036	0.037	0.036	0.037	0.038	0.034	0.042	0.036	0.043		
[15]	0.069	0.068	0.075	0.051	0.046	0.044	0.047	0.049	0.048	0.049	0.047	0.049	0.052	0.046	

Remarks: [1]=Arumanis 143, [2]=Saigon, [3]=*Mangifera gedebe*, [4]=Irwin, [5]=Haden, [6]=Li'ar, [7]=Khirsapati Maldah, [8]=Paw-paw, [9]=Bangalora, [10]=Madu Segoro, [11]=Gedong Gincu, [12]=Keitt, [13]=Agung, [14]=Delima, [15]=Apel Merah



Note: Numbers in parentheses indicate the frequency bootstrap

Figure 4. Grouping accessions of 15 studied mango accessions in a phylogeny tree

Grouping accessions were based on the availability of branch and value of bootstrap. Two or more accessions were classfied into the same group when the values of bootstrap were equal or more than 95% (Felsenstein, 1985; Brown, 1994). Based on this determination, the 15 mango accessions were classified into five difference clades. A close genetic relationship was shown among accessions within one clade. Genetic relationship and genetic distances are very useful in breeding programs. Prospective parental crossing can be selected to obtain hybrids with specific properties. Gvozdenovic *et al*. (2009) declared that hybrids appearance can

be estimated based on the appearance of parental crossing. Selection of parental crossing can be determined based on the information of genetic relationship and genetic distance. One of them is to estimate the heterosis phenomenon.

CONCLUSION

Based on chloroplast DNA (cpDNA), the accessions of fifteen mango were grouped into five different clusters based on similarity and difference composition of bases with very close relationship among accessions in each group.

ACKNOWLEDGEMENT

We deeply thank Tantri Swandari, a laboratory technician at genetic and breeding laboratory, Gadjah Mada University for her help during laboratorial work. This study was financed by Graduate Faculty of Agricultural Research Grant, Gadjah Mada University, year of 2013.

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