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Genetic Diversity of Black and Aromatic Rice Cultivar (*Oryza sativa* L.*)* from Various Regions in Indonesia using Random Amplified Polymorphic DNA Markers (RAPD)

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***Abstract*— Indonesia is one of many countries with a high potential for genetic diversity resource of rice plants, both black rice, and aromatic rice. Therefore, the kinship relationship in each cultivar of which results are not influenced by the environment can be determined by molecular methods, one of which is by using RAPD analysis. This research employed 20 RAPD primers and DNA genome materials used in isolation from ten black rice cultivars, and 15 aromatic rice cultivars. The research stage began with a collection of black rice and aromatic rice germ plasma in several regions in Indonesia, seed nursery, DNA isolation, DNA quantity, and quality testing, primary selection, RAPD, running RAPD, and phylogenetic analysis. The objectives of this research were to obtain phylogenetic diversity of black and aromatic rice in this analysis. Twenty RAPD primers used to amplify 10 black rice cultivars and 15 aromatic rice cultivars obtained 180 polymorphic DNA Ribbons, with an average percentage of 89.2%. The genetic diversity of all cultivars analyzed was considerably high. At 84% coefficient, two major groups were obtained: black rice and aromatic rice, divided into eight small groups. The results of the phylogenetic analysis demonstrate high diversity between black rice and aromatic rice. Such a thing can be a first step for selecting rice plants which will be taken as plant parent in the crossbreeding.**

***Keywords*— RAPD; cultivar; black rice; aromatic rice.**

I. INTRODUCTION

Considerably-vast Indonesian territory with the characteristic of the agricultural country has enormous potential in terms of crops' genetic resources, especially rice plants. In the view of [1], Indonesia is one of the producers of black rice and aromatic rice in the world, where Indonesia ranks third in the distribution source of black rice accession in the world, with a distribution percentage of 7.2%. China ranks the highest in terms of access distribution at 62%, followed by Sri Lanka at 8.2%, India at 5.1%, and the Philippines at 4.3%, while Malaysia and Thailand trailing behind. With this potential, black rice and aromatic rice cultivars can be further developed as raw material for assembling superior varieties of aromatic black rice.

The need for rice, both in Indonesia and the world, is very extensive since rice is included as one of the staple food sources; and dependence on rice consumption nearly reaches 60%, while dominating the intake and consumption pattern of staple foods at the present time. The community in general starts to selectively choose rice with health benefits, one of which is black rice. Black rice has high anthocyanin content, making its antioxidant activity useful as an anti-cancer [2], hypoglycemia [3], anti-inflammatory effects [4] It is also effective in reducing cholesterol level [5]-[10], As for aromatic rice, it has the advantage of which aroma is considerably fragrant, and the rice is relatively chewier or fluffier when cooked, thus enhancing the appetite of the people. Aromatic rice is rice that gives off aroma and it comes from 1-Proline volatile compound (2AP) Compound 2 AP in aromatic rice is 10 times higher, compared to non-aromatic rice [11]-[13].

From the advantages of each black rice and aromatic rice, it is highly important to examine genetic diversity further, via molecular and plant morphology, so that for a breeder can utilizes this into usable initial selection action in selecting the parent elders plant for crossbreeding and propagating superior varieties resulted from a combination

of both, [14] one of the important standard tools commonly used by biologists is a phylogenetic analysis; in which this analysis is capable of analyzing evolutionary relationship between organism, molecules, and phenotypes, allowing the results of phylogenetic analysis to produce kinship information. Genetic distance based on the similarity of genotypes is extremely necessary for a breeder to determine the parent plants and the evolutionary process occurred [15]. At the present time, the most widely used is the phylogenetic analysis of molecular results. Such thing is caused by molecular, thus allowing more characters to observe in concluding more accurate data and is free from environmental influences. This is far different from biochemical and morphological analysis, where the environment still influences the results of the analysis [16].

Molecular use [17,18], in phylogenetic analysis can be conducted using genetic markers. The function of markers is to select the properties to examine from one individual or to trace one genotype from a plant [19], RAPD is one of the markers widely used in the molecular analysis. In its use for a molecular genetic mapping application, the characteristic of RAPD is neutral and non-biased, and certain information on genome sequences is unnecessary. Based on the description above, the author conducted a series of research entitled Random Amplified Polymorphic DNA (RAPD) Genetic Diversity of Ten Black Rice Cultivars and Fifteen Aromatic Rice Cultivators (*Oryza sativa* L.*)* from Various Regions in Indonesia using RAPD. This research aimed to explore a genetic diversity of all cultivars of black rice and aromatic rice.

II. Material and Method

A. Materials

1) Germ plasma collection from various locations in Indonesia

Germ plasma in the form of seeds was collected from various locations in Indonesia: 10 black rice cultivars and 15 aromatic rice cultivars as listed in Table 1.

2) Nursery and Planting of Rice Seeds (Oryza sativa L.)

The plant material of which leaves would be taken for extraction is prepared in advance via seed nursery and planting. The seedling was previously soaked in water for 4-5 days, and then after germination, the seeds were ready to be placed right on the ray pot, and after 12 days, it could be moved to a bucket filled with soil media.

3) Preparation for DNA Extract

DNA samples were taken from leaves of rice plants aged 2 weeks [20]. Then the measurement of DNA purity and concentration was done using spectrophotometry at 260 nm and 260/280 nm. The next step was DNA isolation by taking samples of young leaves of 0.5 grams which was then crushed using Liquid Nitrogen (N2) and inserted into the *Eppendorf* filled with 500µl of complete extraction buffer (500µl Buffer Extraction + 25µl SDS 20% + 1, 25 β-mercapto). It was subsequently unsorted until reached homogeneity and was then incubated at 65_oC for 10 minutes, swirled to taste, added with Potassium Acetate 5M 500µl, and incubated on ice for 10 minutes. Next, it was centrifuged at 12,000 rpm for 10 minutes at 4^oC. The supernatant was discarded and added with 500µl TE+15 µl RNAse and was incubated for 1 hour. 500µl of PCI was added and then centrifuged of 12,000 rpm for 10 minutes at 4^oC. The supernatant was transferred to the new Eppendorf and added with Chloroform Equal Volume, vortexed and then centrifuged at 12,000 rpm for 10 minutes at 4^oC. The supernatant was transferred to the new Eppendorf, added with 0.8 isopropanol and 0.2 NaAc/ Sodium Acetate, swirled and incubated for 1 hour at -20^oC, centrifuged of 12,000 rpm for 10 minutes at 4^oC. The supernatant was discarded and added with 600µl of Ethanol PA 70% to the pellet. Centrifuge at 12,000 rpm 10 minutes at 4^oC. The supernatant was discarded, the pellets were dried and added with 20µl of Buffer TE and 1µl of RNAse, incubation of 37^oC for 1 hour, and the results of the isolation were stored at -20^oC.

TABLE I
RICE VARIETIES AND THEIR ORIGIN.

No	Name of Cultivar	Origin
1	Banjarnegara Black Rice	Banjarnegara, Central Java
2	Purbalingga Black Rice	Purbalingga, Central Java
3	Pari Ireng	Central Java
4	Purwokerto Black Rice	Purwokerto Central Java
5	Hare Lahok	Timor Leste
6	Bantul Black Rice	Bantul, Central Java
7	Lumajang Black Rice	Lumajang, East Java
8	Toraja	South Sulawesi
9	Melik Black Rice	Central Java
10	Blitar Black Rice	Blitar, East Java
11	Mentik Wangi	Pakuncen, Jogjakarta
12	Mentik Wangi Banjarnegara	Banjarnegara, Central Java
13	Mapan 05 Banjarnegara	Banjarnegara, Central Java
14	Situpatenggang	Kartuna/TB47H-MR-10
15	Pare Pulu Mandoti	Enrekang, South Sulawesi
16	Batang Gadis	IR 64/NDR 308//IR 64
17	Radah Putih Karanganyar	Karanganyar, Central Java
18	Mentik Susu Karanganyar	Karanganyar, Central Java
19	Situbagendit (non aromatic)	Batur/S2823-7d-8-1-A//S283-7d-8-1-A
20	Celebes	Tetep/IR2415-90-4-3-2//IR19661-131-1-2
21	Rojo Lele Delanggu	Delanggu, Klaten Central Java
22	Pendok	Tuban, East Java
23	Gilirang	B6672/ Membrano
24	Kurik Kusut Karanganyar	Karanganyar, Central Java
25	Genjah Arum	Banyuwangi, East Java

4) Quality Testing and DNA Quantity

DNA quality testing used electrophoresis method [14], prepared agarose gel 1% (w/v) by dissolving 0.25 g agarose into 25 ml TAE buffer on the Erlenmeyer tube and was heated on the microwave for 1 minute until the solution seemed clear, and then added with EtBr (Stadium Bromide) 1.5 µl. furthermore, the solution was poured into a mold equipped with a comb and was left to harden. Then, the print was put into an electrophoresis device containing the TAE buffer solution and was subsequently mixed with 5 µl of genomic DNA in 1 µl loading dye using the pipetting technique. After mixing it up, then it was put into agarose wells placed in an electrophoresis device. For

electrophoresis, it was set with a voltage of 75 Volts for 60 minutes. After the electrophoresis process was completed, and then the electrophoresis results were visualized using the Gel Document tool.

DNA Quantity Testing used a spectrophotometer. Before using the tool, it must be calibrated first, which was done by putting 400 µl dd H_2O into the cuvette and pressing the blank button. Afterward, the 2 µl DNA sample was put into the cuvette and 398 µl dd H_2O was added. And the measurement of DNA concentration was at a wavelength of 260/ 280 nm. [21] At a wavelength of 260 nm, the RNA and DNA Ribbons can be absorbed optimally, and at a wavelength of 280 nm, protein can be absorbed. DNA purity will occur if the value is higher than 1.8, while the DNA indication is contaminated if the value is lower than 1.8.

5) PCR

The master mix used in the PCR reaction was Bioline with a total of 10 µl tubes, where per tube contained 1µl DNA, 3 µl ddH2O, 5 µl Master mix and 1 µl primer and then running PCR with pre-denaturation at 95^{o}C for 5 minutes, and then being denatured for 40 cycles for 30 seconds at 94^{o}C. Annealing was conducted for 30 seconds at 30^{o}C and extension was done for 30 seconds at 72^{o}C. Final extension was done for 5 minutes at 72^{o}C and soaked for 15 minutes at 16^{o}C. [22]

6) Electrophoresis of PCR Results

The PCR amplification results from the targeted genes can be seen through DNA electrophoresis, by preparing 1 gram of agarose and were dissolved in TAE buffer, heated to clear and added with 2 µl of ethidium bromide and was then poured into the mold. After solidifying, it was moved into the tank electrophoresis. Furthermore, DNA product of PCR results was inserted into the gel well, and electrophoresis ran for 40 minutes at 75V.

7) Primary and Phylogenetic Analysis

Analysis of PCR electrophoresis results began by marking DNA Ribbons using binary data, namely 1 for the DNA Ribbon that appeared and 0 for DNA Ribbon that did not appear at the same marker size position. It was then run using the NT SYS -pc, [23] resulting phylogenetic tree from PCR results. And the percentage of polymorphism allele on each primer can be calculated using the formula:

$$\% \text{ Polymorphism} = \frac{\text{Number of polymorphism alleles}}{\text{Total number of alleles}} \quad (1)$$

The information of a primary can be determined by calculating the PIC value (Polymorphic Information Content). From here on, the accuracy of genetic markers in kinship can be determined.

III. Result and Discussion

A. RAPD Analysis of Black and Aromatic Rice Samples

A total of 25 genotype samples consisting of 10 black rice genotypes and 14 aromatic rice samples and 1 non-aromatic rice sample were analyzed using 20 RAPD primers with an average primary number 9.85 Table 2. OPA 6 and OPA 11 primers produce a number DNA bands that are the least compared to other primers and the highest number of DNA bands is produced from OPA 12 and OPA 19 primers with 14 bands of DNA each. The total number of alleles produced 202 with details of 180 alleles of polymorphism and 22 alleles of monomorphism. Some primers that produce 100% polymorphisms are OPA 04, OPA 07, OPA 11, OPA 12, OPA 16, OPA 17, OPA 19, and each produces polymorphisms 7,12,6,14,13,13,14 , 10. In Figure 2, OPA 16 is shown with 100% polymorphism and Figure 1, OPA 08 is 88.9% polymorphism, while for the lowest polymorphic is generated from OPA 15 primer with 4 band bands and only 2 bands of polymorphic bands.

The analysis using 20 RAPD primers were quite effective in amplifying the DNA of 10 black rice cultivars and 15 aromatic rice cultivars. It can be seen from the three replications of running PCR with the same primer showing the same results. The role of sample DNA purity of each cultivar will greatly affect the results of fragment analysis during separation with gel electrophoresis. DNA purity and diversity result from RAPD analysis will be more apparent when DNA isolation is done in large amount, so that at the time of extraction it does not affect the breakdown of the genomic DNA chain [24] And oftentimes, overly small DNA concentrations will produce a less clear DNA ribbon and DNA prints containing phenolic compounds and polysaccharides that can also affect the DNA ribbon results.

The results of each primer show different differences in the number of DNA ribbons, as shown in Table 2. In the primer OPA 08 produces a polymorphic band 8, and in the OPA 16 primer produces 13 polymorphic ribbons.

Reading results of polymorphism DNA ribbons were then converted into binary data, where if there was a DNA band, it would be marked 1 and if there was no DNA band then it will be marked with 0, and was then analyzed using NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) program version 2.01.

The total number of polymorphic DNA ribbons produced from these 20 primers was 180 so that above average, each primer had at least 9 polymorphic ribbons with an average percentage of polymorphism of 89.2%.

Amplified DNA ribbons that are produced in PCR [25], both in terms of quantity and intensity, depending also on the primary ability to recognize the sequence of complementary DNA to the DNA mold. And for the distribution of primer attachment in the mold will bring up fragments in a large number of primers and a small number of fragments in other primers. And the selectivity of the [26] RAPD primer selection for use will also have an effect on polymorphism. This is caused by each primer having a different site attachment, which can also lead to a difference in polymorphic DNA ribbons. This difference also appears in the large number of base pairs & number of ribbons and their size as shown in Figure 1 OPA 8 primers and Figure 2 OPA 16 primers. The ribbons are scattered at different marker sizes ranging from 100 to 3000 bp.

TABLE II
TWENTY AMPLIFIED RAPD PRIMERS AND POLYMORPHISMS IN 25 RICE CULTIVARS

No	Primary	Base Order (5’ -3’)	DNA Ribbon	Monomorphic amplicons	Polymorphic amplicons	(%) of polymorphism
1	OPA-01	CAGGCCCTTC	10	1	9	90
2	OPA-02	TGCCGAGCTG	7	1	6	85.7
3	OPA-03	AGTCAGCCAC	10	6	9	90
4	OPA-04	AATCGGGCTG	7	-	7	100
5	OPA-05	AGGGGTCTTG	10	1	9	90
6	OPA-07	GAAACGGGTG	12	-	12	100
7	OPA-08	GTGACGTAGG	9	1	8	88.9
8	OPA-09	GGGTAACGCC	11	1	10	90.9
9	OPA-10	GTGATCGCAG	9	4	5	55.6
10	OPA-11	CAATCGCCGT	6	-	6	100
11	OPA-12	TCGGCGATAG	14	-	14	100
12	OPA-13	CAGCACCCAC	8	2	6	75
13	OPA-14	TCTGTGCTGG	10	1	9	90
14	OPA-15	TTCCGAACCC	4	2	2	50
15	OPA-16	AGCCAGCGAA	13	-	13	100
16	OPA-17	GACCGCTTGT	13	-	13	100
17	OPA-18	AGGTGACCGT	13	1	12	92.3
18	OPA-19	CAAACGTCGG	14	-	14	100
19	OPA-20	GTTGCGATCC	10	-	10	100
20	OPC-07	GTCCCGACGA	7	1	6	85.7
Total			197	22	180	1784.1
Average			9.85	1,1	9	89.205

OPA 08

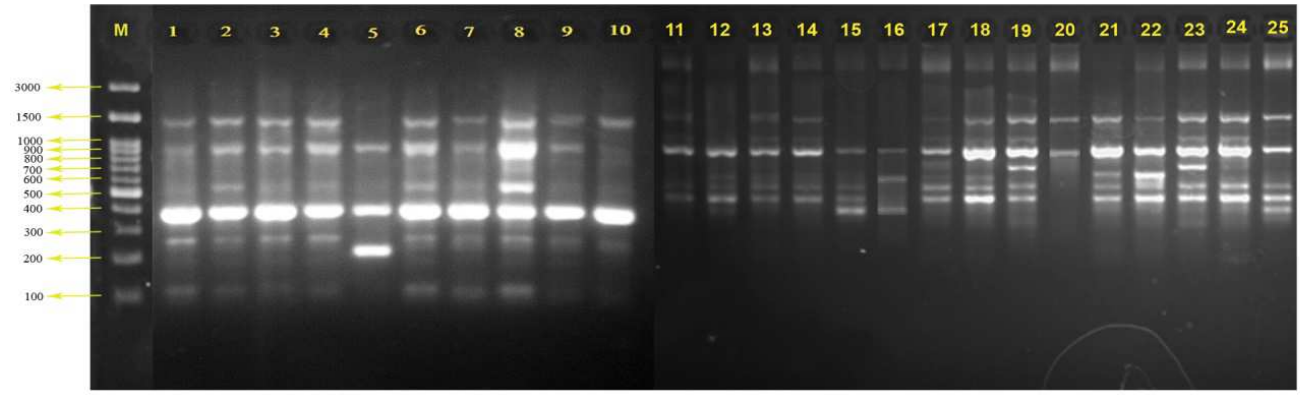

Fig 1. DNA ribbon profile of 10 black rice cultivars (1-10) and 15 aromatic rice cultivars (11-15) using RAPD OPA 08 primers with a 100-bp DNA marker size.

OPA 16

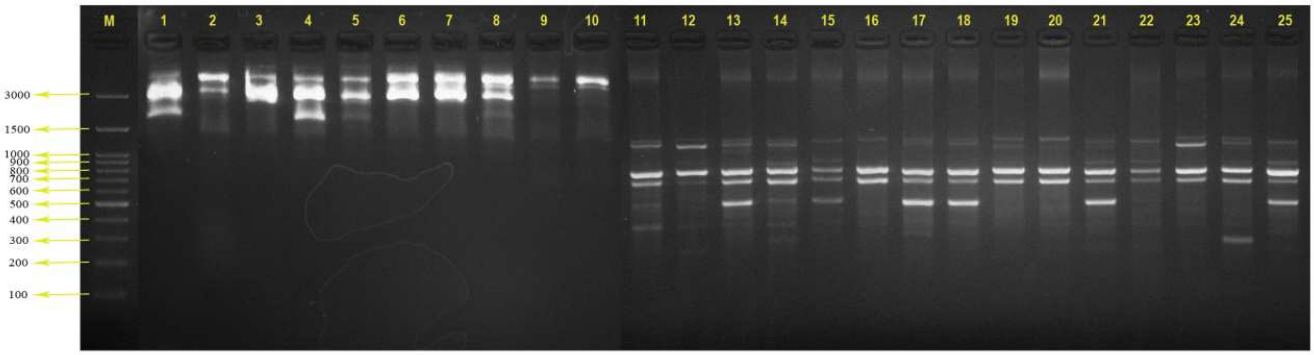

Fig 2. DNA band profiles of 10 black rice cultivars (1-10) and 15 aromatic rice cultivars (11-15) using RAPD OPA 16 primers with a 100-bp DNA marker size.

TABLE III
SIMILARITY MATRIX

	H.Banjarnegara	H.Purbalingga	Pariireng	H.Purwokerto	Harelahok	H.Bantul	H.Lumajang	H.Toraja	H.Melik	H.Blitar	Mentikwangi	MWB	M.05banjarnegara	Situpatenggang	PPM	Batanggadis	RPK	MSK	Situbagendit	Celebes	Rojoleledelangu	Pendok	Gilirang	KKK	Genjaharum
H.Banjarnegara	100																								
H.Purbalingga	81.97	100																							
Pariireng	75.43	85.56	100																						
H.Purwokerto	83.80	83.70	86.36	100																					
Harelahok	72.52	76.14	76.19	75.58	100																				
H.Bantul	80.93	83.15	83.53	87.36	74.70	100																			
H.Lumajang	87.50	81.77	79.77	88.14	76.92	90.06	100																		
H.Toraja	80.00	87.00	81.25	85.71	74.47	83.16	84.97	100																	
H.Melik	77.38	76.30	83.64	82.84	77.02	88.34	84.34	83.24	100																
H.Blitar	75.61	72.19	75.78	77.58	71.34	77.99	82.72	79.56	85.71	100															
Mentikwangi	57.75	58.33	57.61	59.57	55.56	56.04	58.38	58.82	59.89	57.80	100														
MWB	53.26	53.97	57.46	55.14	54.24	53.63	57.14	55.72	56.32	56.47	78.76	100													
M.05banjarnegara	58.95	58.46	59.89	59.69	59.02	59.46	61.70	61.84	63.33	60.23	81.41	88.78	100												
Situpatenggang	64.92	58.16	60.64	63.54	58.70	61.29	64.55	63.46	64.09	62.15	82.00	82.23	87.69	100											
PPM	50.00	47.27	44.59	47.21	50.98	46.45	46.84	45.20	46.67	46.58	66.27	65.06	67.44	68.21	100										
Batanggadis	55.32	55.96	55.14	56.09	56.35	52.46	56.99	54.63	53.93	55.17	82.23	78.35	82.00	78.61	65.88	100									
RPK	56.68	52.08	52.17	54.26	50.00	54.95	58.38	57.84	57.63	58.96	79.59	77.72	82.41	81.00	66.27	81.22	100								
MSK	57.14	53.61	54.84	57.90	54.95	55.44	57.75	56.31	58.10	54.86	83.84	80.00	84.58	84.16	64.33	82.41	84.85	100							
Situbagendit	54.14	54.84	56.18	56.04	54.02	56.82	56.98	55.56	59.65	57.49	76.84	79.14	80.83	77.32	63.80	77.49	82.11	84.38	100						
Celebes	56.00	57.78	59.30	57.96	59.52	57.65	60.12	57.29	59.39	58.39	77.17	74.03	78.08	76.60	58.60	80.00	73.91	76.34	80.90	100					
Rojoleledelangu	55.62	56.25	55.44	57.45	56.67	56.04	56.22	54.90	56.50	54.34	74.49	70.47	74.37	75.00	68.64	73.10	71.43	74.75	76.84	72.83	100				
Pendok	56.32	51.40	49.12	53.71	57.49	53.25	54.65	48.17	51.22	50.00	66.67	67.78	66.67	70.59	71.80	66.30	72.13	72.43	74.58	66.67	79.78	100			
Gilirang	56.18	52.46	52.57	55.87	54.97	54.34	57.96	55.39	57.14	58.54	78.08	78.26	81.05	79.58	60.00	75.53	77.01	81.48	85.08	75.43	74.87	72.41	100		
KKK	57.78	55.14	55.37	56.35	54.34	54.86	58.43	54.82	55.29	57.83	75.13	75.27	78.13	76.68	58.03	74.74	76.19	77.49	80.87	74.58	75.13	71.59	90.00	100	
Genjaharum	54.34	55.06	55.29	56.32	61.45	54.76	56.14	52.63	57.67	55.35	70.33	71.51	73.51	70.97	64.52	66.67	69.23	71.74	77.27	69.41	79.12	76.92	77.46	77.71	100

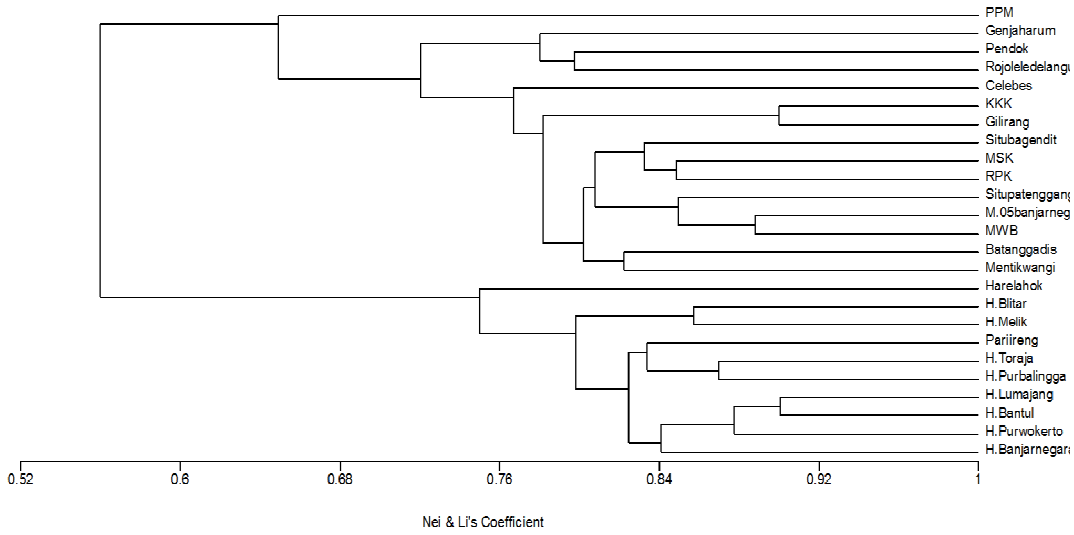

Fig 3. Phylogenetic relationship analysis of black and aromatic rice cultivars

B. Similarity and Phylogenetic Analysis

Dendograms of black and aromatic rice clusters can be obtained after knowing the genetic distance from the similarity values of 25 black and aromatic rice varieties, in Figure 3 it is clearly shown that the dendogram obtained with two large clusters. Table 3 (in appendix) describes the similarity level between one variety and another, where a high degree of similarity can be used as information to select parent plant of crossbreeding so that the success of crossbreeding has a greater chance. If viewed from the two major groups, namely black rice and aromatic rice, Lumajang black rice, and Situpatenggang aromatic rice have a similarity level of 0.649%. With a high degree of similarity, the kinship between the two cultivars is also high.

The results of the phylogenetic kinship analysis of 10 black rice cultivars and 15 aromatic rice cultivars, at a coefficient of 0.84%, show high genetic variation as shown in Figure 3.

The phylogenetic tree produced shows that there were two major groups: 10 black rice cultivars (Herelahok, Hitam Blitar, Hitam Melik, Pari Ireng, Hitam Toraja, Hitam Purbalingga, Hitam Lumajang, Hitam Bantul, Hitam Purwokerto, Hitam Banjarnegara) and 15 cultivars aromatic rice (PPM, PPM, Genjah Arum, Pondok, Rojolele Delanggu, Celebes, KKK, Gilirang, Situbagendit, Mentik Susu Karanganyar, Mentik Wangi Banjarnegara, RPK, Situpatenggang, Batang Gadis dan Mentik Wangi Karanganyar) are further divided into eight small groups, and the small group will still also be subdivided into small groups. This shows a kinship or genetic variation between the groups. The more groups being formed in phylogenetic trees, the larger diversity between cultivars, thus showing that it can be used as the first step for selecting cultivar collection to be made as parent plant in crossbreeding to obtain superior varieties.

IV. CONCLUSIONS

Twenty RAPD primers used to amplify 10 black rice cultivars and 15 aromatic rice cultivars were obtained. The total number of polymorphic DNA ribbons was 180; so that on average, in each primer, there were at least 9 polymorphic ribbons with an average polymorphism percentage of 89.2%.

The genetic diversity of all cultivars being analyzed was also seen to be quite high at 84% coefficient, where there were two major groups, i.e. black rice and aromatic rice, which was then divided into eight small groups. The results of the phylogenetic analysis demonstrate that the high diversity between black rice and aromatic rice can be made as a first step for selecting rice plants like the parent plant in crossbreeding.

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