Annual Research & Review in Biology
10(5): 1-11, 2016, Article no.ARRB.27375
ISSN: 2347-565X, NLM ID: 101632869

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Species-specific Loci of Three Indonesian Durio Inferred from ISSR Fingerprinting

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Authors' contributions

This work was carried out in collaboration between all authors. Author PAR performed the experiments and drafted the manuscript. Authors Miftahudin and TC arranged the molecular genetics and statistical analyses of the study. Author Suharsono finalized the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2016/27375
Editor(s):
(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Amusa D. Oluwafemi, University of Lagos, Nigeria.
(2) Aparajita Mohanty, Gargi College, University of Delhi, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/15501

Original Research Article

Received 30th May 2016
Accepted 24th June 2016
Published 24th July 2016

ABSTRACT

Aims: To assess molecular diversity and to determine species-specific loci of Indonesian durio, based on Inter-Simple Sequence Repeat (ISSR) markers.
Study Design: Genomic DNA isolation from fresh durio leaves and then DNA amplification. Electrophoresis PCR product and bands scoring. Calculating the diversity genetic parameter and identification specific loci.
Place and Duration of Study: The research was done in Plant Biology Laboratory, Biology Departments, Bogor Agricultural University, start from April 2014 until January 2016.
Methodology: Ten ISSR primers were used to reveal the genetic diversity of 58 varieties of three species (*D. zibethinus*, *D. kutejensis*, and *D. tanjungpurensis*) sampled from Mekarsari and Cipaku

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Garden, West Java, and from Sekadau Regency, West Kalimantan, Indonesia. Genetic diversity parameters were estimated based on binary data of PCR bands appearance which calculation using GenAlEx. Construction phenogram using Mega software and validating of loci specific with Chi-Square method invoked SAS® software.

Results: The diversity genetic parameter showed that the mean number of observed alleles, the mean number of effective alleles, Shannon's Information Index, the genetic diversity, and the percentage of polymorphic loci were 1.25, 1.34, 0.30, 0.20, and 58.3%, respectively. UPGMA based cluster analysis and principal coordinate analysis classified them into three groups according to the species. We found 13 loci from 7 ISSR primers linked to species-specific loci.

Conclusion: The genetic diversity of durio can be assessed using the ISSR markers, which can also be used for species identification of Indonesian durio.

Keywords: Durio; genetic diversity; ISSR marker; fingerprinting; species-specific loci.

1. INTRODUCTION

Durio spp is a tree genus belonging to the family Malvaceae, order Malvales, class Magnoliopsida, and division Magnoliophyta [1,2]. This genus is easily distinguished from other genera by having a dense indumentum that covers the lower side of leaves, and producing big spiny fruit with large seeds that are covered with a fleshy aril [3].

In this study, we focus on three durio species, specifically *Durio zibethinus* Murr., *Durio kutejensis* Becc., and *Durio tanjungpurensis* Navia. The durian (*D. zibethinus* Murr.) is a thorny-fruited plant known as the "king fruit" with various cultivars spread throughout Southeast Asia countries, such as Indonesia, Burma, Malaysia, Thailand, and the Philippines. Durian cultivars have specific designation names in Indonesia, such as Kane, Matahari, Ajimah, Sitokong, Petruk, Sukun, Layung, and Manalagi. Durian has considerable diversity in Indonesia, which is very valuable for durian development [4]. It grows widely in natural forest, contributing to a steady food chain, and is also cultivated in gardens. The durian fruit is highly preferred by local residents, although it has a strong smell. Besides durian, Lai (*D. kutejensis* Becc.) is a rare species that originate from Kalimantan. Morphologically, Lai has large leaves (20-33 cm long, 6-12 cm wide), a milder smell than durian, red or yellow flowers, and all stamens free [3,5]. Another species, *D. tanjungpurensis* Navia, is an endemic species known as Durian Tengkurak, which is closely related to *D. testudinarum* Becc. with fruits at either the base of the tree's trunk or buttress root, and stamens fused for more than half of stamens' length [6].

Durian is the superior perenial plant which flesh of the seed contains good nutritional compounds and antibacterial activity. It has high levels of fiber, carbohydrate, sugar, fatty acids, carotenoids, β-carotene, and low levels of antioxidant [7]. Durian is also reported as containing flavonoids with antidiabetic effect in rats [8]. Other chemical properties of durian extracts, which obtained from different sources of fruit, has also been previously reported by several authors. Antimicrobial activity against bacteria and yeast has been found in crude polysaccharide gel extracted from the fruit-hull of durian using in-vitro agar medium test [9], while a compound extracted using chloroform from the fruit pulp of *D. zibethinus*, 7,8-dimethoxy-13,13-dimethyl-2,13dihydro-3H-Pyrano[5,6-c]quinolin2-one, showed significant antibacterial and antifungal activity against a range of microorganisms tested [10].

In recent years, molecular genetic studies of durian have been carried out by several authors. Due to the limitations of morphological based studies for example the environment effect on the size of vegetative organs, DNA marker studies of *Durio* are needed to support the species' sustainability and to save the abundant resources of durian. Many efforts are being made by researchers to identify the diversity within the durian germplasm using inter-simple sequence repeat (ISSR) markers. The markers were first developed by Ziętkiewicz et al. [11] based on the amplification of a single primer which are related to the amplification of the DNA region located between two inverted microsatellites loci [12]. ISSR-marker analysis has been utilized on many plant species, with various objectives, such as the identification of genetic diversity of cereal crops [13], the characterization of date palm germplasm [14], the conservation of japonica tea in China and Japan [15], the sex determination of India green potato [16], the characterization of the Brazilian sweet potato germplasm [17], the determination of the homogeneity of in-vitro clones grapes [18], the collection and conservation of the African citrus [19], and the

evolution and speciation study of the Asteraceae [20]. Considering the potentials of the DNA marker based genetic diversity analysis, the aims of the present study is to use ISSR markers to investigate the genetic diversity of two cultivated *Durio* species, *D. zibethinus* Murr. and *D. kutejensis* Becc. in Indonesia, and to evaluate cross species amplification of ISSR markers among three durio by adding the third of durio species, *D. tanjungpurensis* Navia. The genetic profile may be used not only to help preserve the plant but also provide genetic authentication and fidelity for plant collection of conservation programs and for plant commercialization.

2. MATERIALS AND METHODS

2.1 Sample Source

A total of 48 accessions of durian fresh leave samples were taken from both Cipaku and Mekarsari Garden and examined in this research (Table 1). Cipaku Garden is an ex-situ conservation field owned by the Agricultural Research and Development Agency, the Indonesian Ministry of Agriculture, and is located at ±125 m asl and covering on 6°38'12.5"S, 106°48'36.2"E to 6°38'12.2"S, 106°48'42.4"E, in Cipaku, Bogor Regency, West Java, Indonesia. The other field site, Mekarsari Garden, is an ecotourism and a business fruit garden belongs to the Mekar Unggul Sari private company established in 1990, and is located at ±78 m asl and covering on 6°24'57.7"S, 106°58'18.1"E to 6°25'6.2"S, 106°59'46.4"E, in Cileungsi, Bogor Regency, West Java, Indonesia. The *D. zibethinus* were sampled from both gardens, and *D. kutejensis* were taken only from Cipaku Garden. The ISSR data of ten *D. tanjungpurensis* accessions were generously provided from the previous study [21], and used as comparison for the other two species ISSR data (Table 2). In that previous study, *D. tanjungpurensis* were collected from Tembaga, Sekadau Regency, West Kalimantan, Indonesia.

2.2 DNA Isolation

DNA was isolated using the CTAB method [22], with some modifications. Fresh leaves were weighed as much as 0.2 g and crushed into a powder with liquid nitrogen using a mortar, then transferred into 2 ml microtube. The tube was then added with 1 ml of CTAB lyses buffer, followed by 2 µl of 2-mercaptoethanol (Sigma Chemical Co., USA). The sample tubes were incubated in a water bath (65°C) for 60 min, and inverted every 15 min. The homogenates were then centrifuged (Microcentrifuge 16D Galaxy VWRTM, IL) at 9,900 ×g for 10 minutes. The supernatant was transferred into a new microtube, then added with chloroform:isoamyl alcohol (24:1) at 1x of the supernatant volume, and centrifuged again. The supernatant was then transferred into a new microtube, to which cold isopropanol (0.8x of the supernatant volume) and sodium acetate (0.1 of the supernatant volume) were added. The samples were incubated in the -20^{o}C freezer overnight. The suspension was then centrifuged at 16,000 ×g to obtain a pellet of DNA. The DNA pellet was washed with 500 µL of cold 70% ethanol, centrifuged and dried. The DNA pellets were diluted using 200 µL of sterile water.

2.3 DNA Amplification

The composition of the PCR reaction for a total volume of 25 µl was 12.5 µl GoTaq® Green Master Mix (Promega, USA, catalog number M7122), 0.15 µl BSA [20 mg/ml], 0.15 µl $MgCl_2$ 20 mM, 1 µl primer [10 pmol/µl], 3 µl DNA template [5 ng/µl], and 8.2 µl ddH_2O nuclease-free. A total of 10 ISSR primers, originating from references, were used for the amplification of the DNA (Table 3). DNA amplification was undertaken in a T-Gradient thermocycler PCR machine (Biometra, Göttingen, Germany) with a cycle time and temperature program as follows: initial denaturation at 95°C for 5min, followed by 35 cycles of 94°C for 1 min, annealing temperature ranged at 42.5 to 54°C, depending on the primer (Table 3), for 1 min, elongation at 72°C for 1 min, and final extension cycle of 72°C for 10 min. Three microliters of each PCR product were electrophoresed on 0.85% (w/v) on agarose gel (Top Vision^{TM} Agarose, Fermentas, USA) in 1x TBE buffer at 75 volts for 110 min. The number and size of the band were distinguished by electrophoresis technique. The PCR fragments were visualized using an UV transilluminator and documented using a digital CCD camera (Daihan WiseDoc® Portable WGD-20 Gel Documentation System, Korea).

2.4 Data Analysis

The DNA bands were scored manually for presence (1) or absence (0) of bands which guided by marker ladders. The scoring was repeated three times of the same gel for each primer to improve the reliability of the results. The data matrix of the ISSR profiles were compiled in Microsoft® Office Excel, which was

used to create a genetic distance matrix and five genetic diversity parameters, i.e. the number of observed alleles, the number of effective alleles, Shannon's Information Index, the genetic diversity, and the percentage of polymorphic loci, which was measured using GenAlEx software [25]. Based on a distance matrix, a Principal Coordinate Analysis (PCoA) was

Table 1. Sample code, accession names, species name, collection sites of forty-eight durio accessions investigated in the study

No	Sample code	Accession names	Species name	Place of collection
1	ZC1	Ajimah	*D. zibethinus*	Cipaku Garden
2	ZC3	D-04	*D. zibethinus*	Cipaku Garden
3	ZC4	D-06	*D. zibethinus*	Cipaku Garden
4	ZC5	D-24	*D. zibethinus*	Cipaku Garden
5	ZC6	Gapu	*D. zibethinus*	Cipaku Garden
6	ZC7	Tanpa Sekat	*D. zibethinus*	Cipaku Garden
7	ZC10	Kani/Kane	*D. zibethinus*	Cipaku Garden
8	ZC14	Kirik	*D. zibethinus*	Cipaku Garden
9	ZC20	Matahari	*D. zibethinus*	Cipaku Garden
10	ZC21	Menoreh Kuning	*D. zibethinus*	Cipaku Garden
11	ZC23	Namlung	*D. zibethinus*	Cipaku Garden
12	ZC24	Nyamat	*D. zibethinus*	Cipaku Garden
13	ZC25	Otong short leaf	*D. zibethinus*	Cipaku Garden
14	ZC26	Otong long leaf	*D. zibethinus*	Cipaku Garden
15	ZC29	Pelangi	*D. zibethinus*	Cipaku Garden
16	ZC31	Petruk	*D. zibethinus*	Cipaku Garden
17	ZC33	Ripto	*D. zibethinus*	Cipaku Garden
18	ZC36	Sitokong	*D. zibethinus*	Cipaku Garden
19	ZC37	Sukun	*D. zibethinus*	Cipaku Garden
20	ZY1	Local Soya1	*D. zibethinus*	Cipaku Garden
21	ZY2	Local Soya2	*D. zibethinus*	Cipaku Garden
22	ZS1	Matahari	*D. zibethinus*	Mekarsari Garden
23	ZS2	Ajimah	*D. zibethinus*	Mekarsari Garden
24	ZS3	Aden	*D. zibethinus*	Mekarsari Garden
25	ZS4	Sihejo/Hejo	*D. zibethinus*	Mekarsari Garden
26	ZS5	Monthong	*D. zibethinus*	Mekarsari Garden
27	ZS6	Sililin	*D. zibethinus*	Mekarsari Garden
28	ZS7	Kukusan	*D. zibethinus*	Mekarsari Garden
29	ZS8	Wisma Lerem	*D. zibethinus*	Mekarsari Garden
30	ZS9	Surya	*D. zibethinus*	Mekarsari Garden
31	ZS10	Sibakul	*D. zibethinus*	Mekarsari Garden
32	ZS11	Sikapal	*D. zibethinus*	Mekarsari Garden
33	ZS12	Soekarno	*D. zibethinus*	Mekarsari Garden
34	ZS13	Sihepe/Hepi	*D. zibethinus*	Mekarsari Garden
35	ZS14	Kamarung	*D. zibethinus*	Mekarsari Garden
36	ZS15	Jarian	*D. zibethinus*	Mekarsari Garden
37	ZS16	Lai-Mas	*D. zibethinus*	Mekarsari Garden
38	ZS17	Simas Cipaku	*D. zibethinus*	Mekarsari Garden
39	ZS18	Simas Cikalong	*D. zibethinus*	Mekarsari Garden
40	ZS19	Musangking	*D. zibethinus*	Mekarsari Garden
41	ZS20	Perkasa	*D. zibethinus*	Mekarsari Garden
42	LC41	Lai Kalimantan	*D. kutejensis*	Cipaku Garden
43	LC42	Lai Kutai	*D. kutejensis*	Cipaku Garden
44	LC43	Lai Parung	*D. kutejensis*	Cipaku Garden
45	LC44	Lai-2	*D. kutejensis*	Cipaku Garden
46	LC45	Lai-3	*D. kutejensis*	Cipaku Garden
47	LC46	Lai-4	*D. kutejensis*	Cipaku Garden
48	LC47	Lai-4b	*D. kutejensis*	Cipaku Garden

Table 2. Ten accessions of *D. tanjungpurensis*, in which the ISSR data were used in this study

No	Sample code	Accessions name	Species name	Source
1	DT1	Tembaga1	*D. tanjungpurensis*	[21]
2	DT2	Tembaga2	*D. tanjungpurensis*	"
3	DT3	Tembaga3	*D. tanjungpurensis*	"
4	DT4	Tembaga4	*D. tanjungpurensis*	"
5	DT5	Tembaga5	*D. tanjungpurensis*	"
6	DT6	Tembaga6	*D. tanjungpurensis*	"
7	DT7	Tembaga7	*D. tanjungpurensis*	"
8	DT8	Tembaga8	*D. tanjungpurensis*	"
9	DT9	Tembaga9	*D. tanjungpurensis*	"
10	DT10	Tembaga10	*D. tanjungpurensis*	"

Table 3. List of ISSR primer names, sequences, primer lengths, and annealing temperature used in the study

ISSR primer	Sequence (5'–3')	Primer length	Annealing temperature (^{o}C)	References
ISSR1	$(AGG)_5$	15	49.5	[23]
ISSR3	$(AGA)_4AGT$	15	42.5	"
ISSR4	$(GAG)_5AC$	17	50.0	"
ISSR5	$(GAG)_5AT$	17	51.6	"
ISSR9	$(GGGGT)_3$	15	53.0	"
PKBT2	$(AC)_8TT$	18	52.0	[24]
PKBT3	$(AG)_8T$	17	47.5	"
PKBT7	$(GA)_9A$	19	50.7	"
PKBT8	$(GA)_9C$	19	52.8	"
PKBT12	$(GT)_9T$	19	54.0	"

conducted to construct a three-dimensional array of the durio accessions that was performed using GenAlEx [25] and was visualized using SAS® Graph v9 [26] software. Binary data was calculated to genetic distance data using GenAlEx, and then used to construct a phenogram using the statistical method of unweighted pair-group method with arithmetic means (UPGMA), and to conduct bootstraps methods for testing the inference phenogram tree with 1000 replications using Mega 5.2 software [27]. After determining the candidate species-specific loci which most often appear in one species but was not in other species, three repetitions of independent gel electrophoresis were done for reliable evaluating the specific fragments. Statistically, we validated the chance of the species-specific loci by employing the Fisher's Exact Test of the Chi-Square analysis with SAS® v9 software.

3. RESULTS AND DISCUSSION

3.1 Cross Species ISSR Amplification and Marker Profiles

Ten ISSR primers successfully amplified DNA genome of three durio species and produces a total of 3021 bands across all examined accessions. Among those 3021 bands, a total of 164 scorable polymorphic PCR fragments of 58 durio accessions were identified (Table 4). The number of PCR fragments among durio accessions for particular primers varied from 14 to 19 bands, and their size ranged from 200 to 2000 bp.

Cross-species amplification showed that the content of specific genetic material owned by accessions based species. This result will make it easier to do comparisons screening and selection at the locus where the ISSR primer is specific to a species of durio. Thus, the application of ISSR markers can be utilized to distinguish each durio species at the DNA molecular level. The dominant ISSR markers were highly polymorphic percentage for durio accessions (Table 4).

3.2 Genetic Diversity Analysis

Among all accessions, the number of alleles varied from 0.84 (*D. kutejensis* from Cipaku) to 1.95 (*D. zibethinus* from Cipaku-Mekarsari) with an average of 1.25, and the number of effective

alleles ranged from 1.21 to 1.54 with an average of 1.34 (Table 5). The Shannon's index score ranged from 0.18 to 0.48, and the genetic diversity score varied from 0.12 to 0.32. The average of the polymorphic percentage loci was 58.3%, with results ranging from 31.1% to 97.6%, where the lowest and highest polymorphic percentages were found in *D. kutejensis* and *D. zibethinus*, respectively. A high level of genetic differentiation among the accessions was detected by the high indices of genetic diversity, Shannon's diversity, and polymorphic percentage.

3.3 Genetic Relationship Analysis

Cluster analysis was conducted based on 164 informative loci revealing three clusters at 40% phenogram' scale (Fig. 1), *D. zibethinus* from Cipaku-Mekarsari, *D. kutejensis* from Cipaku, and *D. tanjungpurensis* from Sekadau Regency. The cluster analysis was also confirmed by PCoA output (Fig. 2), which clustered the accessions into three groups separated according to the species of durio. Comparing all three groups genetically, *D. zibethinus* is the most diverse group; however, *D. zibethinus* is more similar in fenotipe to the *D. tanjungpurensis*, than to the *D. kutejensis*. Therefore, sampling size of accessions of *D. zibethinus* is much more compared to the other two species may not influence.

3.4 Species-specific Loci

Species-specific loci were determined by checking all 164 loci. A candidate locus specific to a certain species was chosen when the locus (band) is very unique, or only present in one species and was not found in the other species. The bands were then validated to assure their validity and their specificity to a species using Chi Square analysis. There are 13 loci yielded by 7 ISSR primers, which can be used partially as a species-specific locus, while 3 other primers (ISSR3, PKBT2, and PKBT3) did not produce any species loci (Table 6). The species-specific loci amplified with ISSR1 primer and PKBT7 primer were shown with arrows in Fig. 3.

Table 4. ISSR name, PCR product size, number of bands scored, and the number of polymorphic bands amplified from 58 durio accessions using ten ISSR primers

ISSR primer	Size range (bp)	Number of bands scored	Number of polymorphic bands	Percent of polymorphic bands
ISSR1	200-1800	17	17	100
ISSR3	300-1500	15	13	87
ISSR4	300-1500	14	14	100
ISSR5	200-1800	19	19	100
ISSR9	300-2000	16	16	100
PKBT2	350-1400	15	14	93
PKBT3	250-1400	15	15	100
PKBT7	250-1500	19	19	100
PKBT8	200-1400	18	18	100
PKBT12	400-1900	16	16	100
Total		164	161	980
Averages		16.4	16.1	98.0

Table 5. Summary of diversity parameters among durio species

Durio species	No. sample	Na	Ne	I	h	%P
D. zibethinus	41	1.95	1.54	0.48	0.32	97.6%
D. kutejensis	7	0.84	1.21	0.18	0.12	31.1%
D. tanjungpurensis	10	0.96	1.27	0.24	0.16	46.3%
Mean		1.25	1.34	0.30	0.20	58.3%
SE(±)		0.04	0.02	0.01	0.01	20.1%

Na=No. Alleles; Ne=No. Effective Alleles; I=Shannon's Information Index; h=Genetic Diversity; %P= Percentage of Polymorphic Loci.

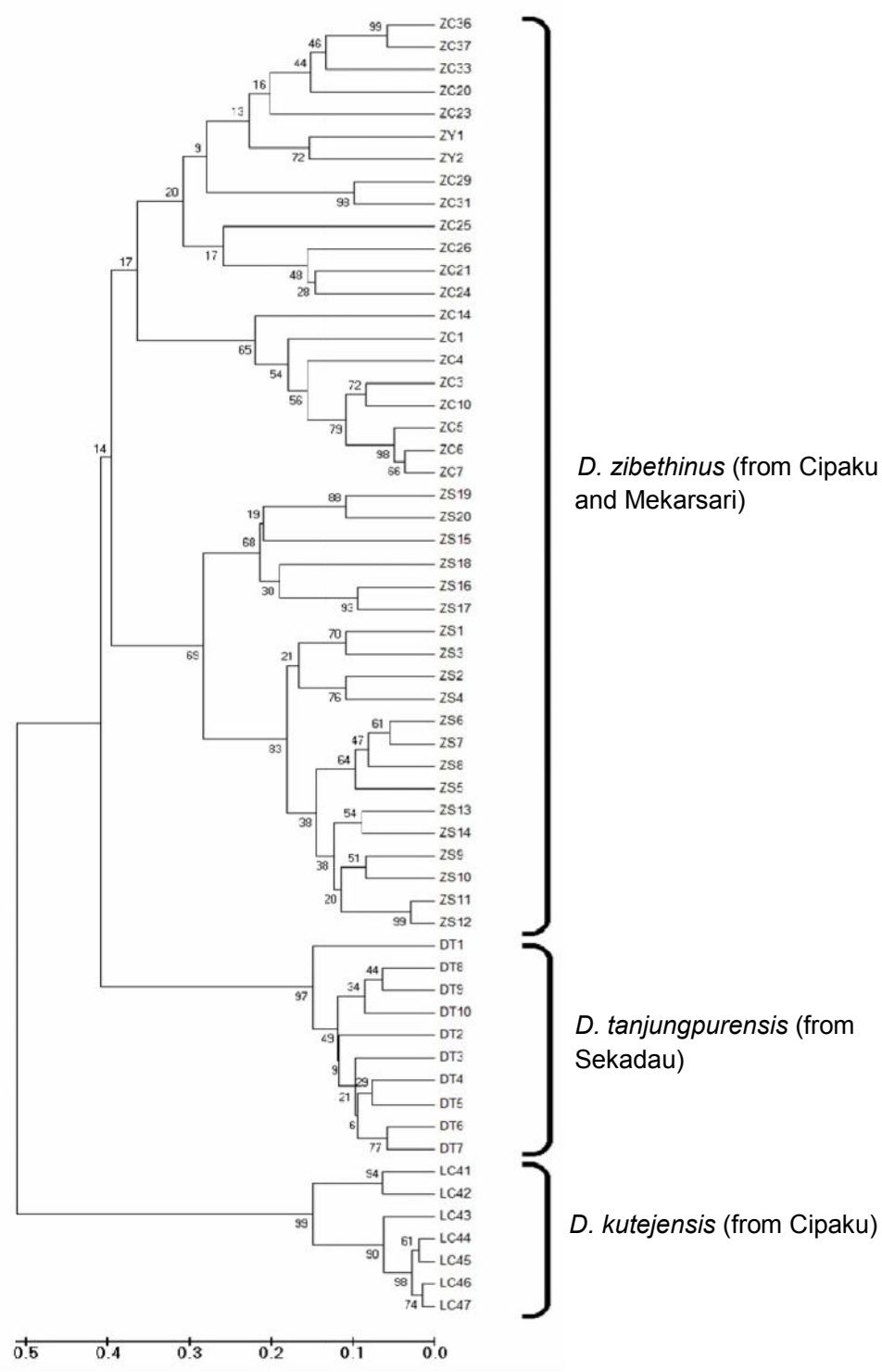

Fig. 1. Phenogram of durio accessions based on ISSR markers constructed using the UPGMA method. The bootstraps values are shown under the branch line

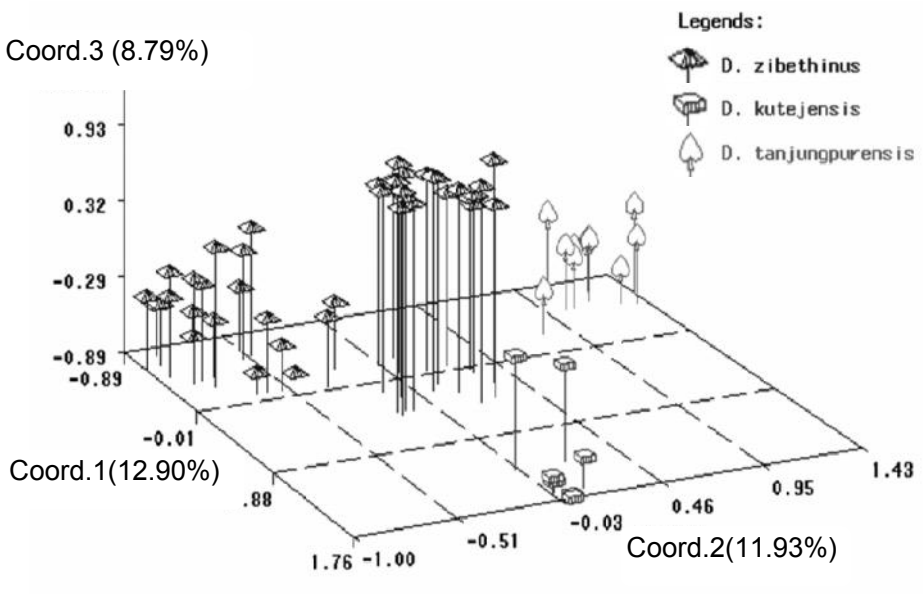

Fig. 2. Matrix plot for PCoA based on ISSR markers showing separation of the three main groups. Distribution of 58 accessions of durio species was presented in a three-dimensional PCoA, with a value of percentage cumulative variation of 33.54%. It appears that the genetic profile between the three species differs from each other

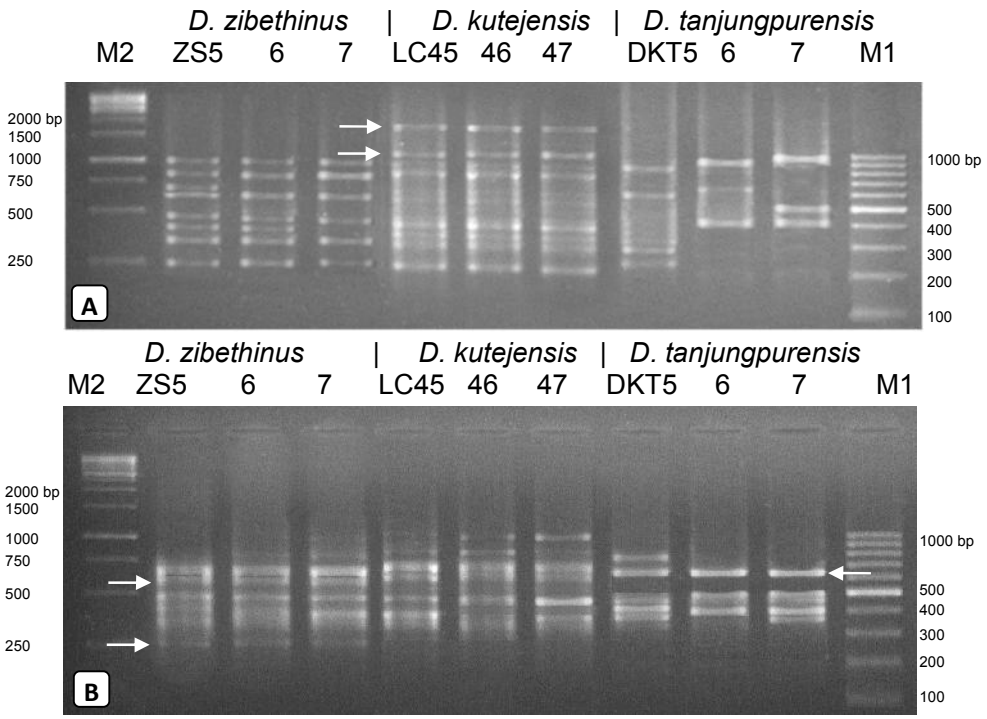

Fig. 3. Species-specific loci revealed from (A) ISSR1 primer and (B) PKBT7 primer for three Indonesian durio of three selected sample. Arrows indicate unique bands from each primer. Sample codes correspond to those accessions listed in Table 1 and Table 2 Marker Ladder: 100 bp (M1) (Thermo Scientific, cat. #SM0241) and 1 kb (M2) (Thermo Scientific, cat. #SM0311)

Table 6. Species-specific loci based on ISSR primer for three Indonesian durio

Primer	Specific band of the species (bp)			Fisher's Test value	Validity
	D. zibethinus	*D. kutejensis*	*D. tanjungpurensis*		
ISSR1	–	1800	–	2.66E-08	Valid
	–	1100	–	3.18E-05	Valid
ISSR4	900	–	–	1.08E-07	Valid
	–	1000	–	2.53E-04	Valid
	–	300	–	3.33E-09	Valid
ISSR5	–	200	–	4.58E-06	Valid
ISSR9	–	–	650	6.35E-07	Valid
PKBT7	550	–	–	3.74E-06	Valid
	250	–	–	3.43E-06	Valid
	–	–	650	1.94E-09	Valid
PKBT8	500	–	–	1.33E-10	Valid
PKBT12	–	750	–	2.66E-08	Valid
	–	–	1500	4.75E-07	Valid

Notes: Statistics validation used Chi-Square with Fisher's Exact Test (P = .001). ISSR3, PKBT2, and PKBT3 primer were not available for species-specific loci, and were not displayed

4. CONCLUSION

A high level of genetic diversity in the three *Durio* species, *D. zibethinus*, *D. kutejensis*, and *D. tanjungpurensis* were assessed utilizing ISSR markers, which are useful for genetic diversity analysis. We identified three main groups among the accessions, where *D. zibethinus* is the most diverse group. Thirteen species-specific loci, which can be used to authenticate three Indonesian durio germplasms, were identified. This will help preserve the durio conservation programs at *ex-situ* Cipaku Garden or at ecotourism Mekarsari Garden, beside the *in-situ* conservation at Sekadau Regency, West Kalimantan, Indonesia.

ACKNOWLEDGEMENTS

This study was supported by grants from The Indonesian Ministry of Education and Culture and the General Directorate of Higher Education Postgraduate Scholarship from 2010 to 2014. Additional fund were supplied by Dr Ir Tatik Chikmawati via DIPA IPB 2013 for research project no. 2913.089.521219. The authors are thankful to Syasti Hastriani, the undergraduate student at Bogor Agricultural University, for helping in laboratory works. We would also acknowledge the contributions of Dr. Daniela Guicking, Systematik und Morphologie der Pflanzen, Universität Kassel Germany for the technical discussions, and to Gregori Granadi Hambali, MS, Director of Research and Development Agency, PT Sasaran Ehsan Mekarsari, Mekarsari Ecotourism Garden, West Java, for the admission to the durian field. We are also thankful for peer reviewers and their questioning on the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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