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Genetic Diversity Analysis Using Resistance Gene Analog-Based Markers to Support Morphological Characterization of Shallots

(Analisis Keragaman Genetik Menggunakan Marka Berbasis Analogi Gen Resisten untuk Mendukung Karakterisasi Morfologis Bawang Merah)

Lina Herlina[1], Reflinur[1]*, Kristianto Nugroho[1], Rerenstradika T. Terryana[1], Sobir[2], Awang Maharijaya[2], and Suryo Wiyono[3]

[1]Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Jl. Tentara Pelajar 3A, Bogor 16111 Indonesia
Telp. (0251) 8622833; Faks. (0251) 8622833; *E-mail: reflinur@yahoo.com
[2]Department of Agriculture and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Jl. Meranti, BAU Dramaga Campus, Bogor 16680 Indonesia
[3]Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University, Jl. Kamper, BAU Dramaga Campus, Bogor 16680 Indonesia

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ABSTRAK

Bawang merah (*Allium cepa* var. *aggregatum*) merupakan salah satu tanaman sayuran penting di Indonesia. Keterbatasan pengetahuan mengenai keragaman genetik dan adanya ancaman penyakit merupakan masalah utama dalam mempertahankan produksi bawang merah yang tinggi di Indonesia. Pengembangan marka molekuler terkait karakter ketahanan penyakit dibutuhkan dalam kegiatan pemuliaan molekuler bawang merah. Penelitian ini bertujuan mengevaluasi keragaman genetik 36 genotipe bawang merah asal Indonesia berdasarkan penanda yang berasal dari daerah konservatif analogi gen ketahanan (*resistance gene analog*/RGA) untuk melengkapi karakterisasi morfologis. Sebanyak dua belas karakter morfologis dan lima belas penanda molekuler diinvestigasi dalam karakterisasi dan diskriminasi genotipe bawang merah Indonesia. Karakterisasi pada tingkat morfologis menunjukkan bahwa variasi fenotipik terbesar terdapat pada total bobot umbi (TWB, cv = 99,39%) dan terkecil pada karakter tinggi tanaman (PH, cv = 28,16%). Analisis korelasi antar karakter morfologis bawang merah menunjukkan korelasi positif antara karakter TWB dan jumlah umbi (NB), TWB dan berat umbi per tanaman (WB), serta antara WB dan PH. Total 1.512 alel dengan rata-rata 1.946 alel per lokus diperoleh pada analisis variasi molekuler. Nilai Polymorphism Information Content (PIC) yang dihasilkan dari primer RGA berkisar antara 0,253 dan 0,676 dan 6 dari 15 marka bersifat sangat informatif dengan nilai PIC ≥0,50. Berdasarkan analisis klaster, ke-36 genotipe bawang merah Indonesia terbagi atas enam kelompok besar. Hasil penelitian ini menegaskan bahwa marka berbasis RGA dapat mendukung karakterisasi morfologis dalam mengevaluasi keragaman genetik bawang merah.

Kata kunci: *Allium cepa*, *Fusarium*, keragaman genetik, gen ketahanan.

ABSTRACT

Shallot (*Allium cepa* var. *aggregatum*) is one of the most important vegetable crops grown in Indonesia. The limited knowledge available on the genetic diversity and the threat of plant disease have been major problems to maintain high shallot production in Indonesia. Development of molecular markers linked to disease resistance is required for molecular breeding activity in this crop. This study aimed to assess the genetic diversity at conserved domain of resistance gene analog (RGA) in a set of 36 Indonesian shallot genotypes to complement morphological characterization. Twelve morphological and fifteen molecular markers traits were investigated in an attempt to characterize and to discriminate the Indonesian shallots genotypes. Characterization at morphological level indicated that phenotypic variance was highest for total bulb weight (TWB, cv = 99.39%) and the least for the plant height (PH, cv = 28.16%). The correlation analysis between traits showed that TWB and number of bulb (NB), TWB and bulb weight per plant (WB), NB and WB, and WB and PH were positively correlated. Molecular analysis revealed a total of 1,512 alleles with an average of 1.946 alleles per locus. The Polymorphism Information Content (PIC) values ranged from 0.253 to 0.676 and six out of 15 RGA markers were highly informative with PIC values ≥0.50. Based on cluster analysis, the 36 Indonesian shallot genotypes were clearly discriminated into six major groups. These results revealed that the RGA-based markers could support the morphological characterization in evaluating the genetic diversity of shallots.

Keywords: *Allium cepa*, *Fusarium*, genetic diversity, resistance gene.

INTRODUCTION

Bulb/basal rot disease caused by *Fusarium oxysporum* (Isniah and Widodo 2015) is one of the most devastating diseases in shallot (*Allium cepa* var. *aggregatum*) causing severe yield losses of up to 100% (Dean et al. 2012). The development of shallot cultivars resistant to the bulb/basal rot disease is highly desirable as an effective strategy to reduce yield losses. Diversity in plant genetic resources provides opportunity for plant breeders in developing new and improved cultivars with desirable characteristics, which include both farmer-preferred traits, such as yield potential and large seed, and breeder-preferred traits, such as pest and disease resistance and photosensitivity (Govindaraj et al. 2015). In this sense, a better understanding of the genetic diversity in the shallot germplasm is needed in order to explore new sources of the resistance genes to certain diseases to be incorporated into new cultivars.

Resistance gene analogs (RGAs) are large class of potential *R*-genes that have conserved domains and structural features. These *R*-genes mostly comprised of a conserved nucleotide binding site-leucine-rich repeat (NBS-LRR) domain structure (Saha et al. 2013). The RGAs are important resources for the development of molecular markers, hence enhancing numerous disease resistance breeding efforts (Ameline-Torregrosa et al. 2008; Perazzolli et al. 2014). The conserved domain in genes such as the sequences of NBS-LRR genes represent opportunities for designing PCR-based strategies with degenerate primers for amplification and isolation of numerous related sequences in other plant species (Sharma et al. 2009). Several DNA markers developed from the isolated RGA sequences have been utilized for several purposes, such as genetic diversity analysis, tagging disease resistance related traits, and discovering candidate genes in several crops (Nordberg et al. 2014). In regards to Alliaceae crops, such as onion (*A. cepa* sp.) and wild-onion (*A. fistulosum*), a few RGA sequences have also been identified, published,

Table 1. List of 36 Indonesian shallot genotypes with their collection sites used in this study.

Genotype code	Genotype name	Collection site/province	Additional information (source of collection)
A-01	BM Bandung	West Java	Local farmers/breeders
A-02	BM Yuwono	Central Java	Local traditional market
A-03	BM Maja	West Java	Government breeding station
A-04	BM Kramat-1	Central Java	Government breeding station
A-05	BM Boyolali	East Java	Local farmers/breeders
A-06	BM Tajuk	East Java	Local farmers/breeders
A-07	BM Kramat-2	West Java	Government breeding station
A-08	BM AG-1	West Java	Government breeding station
A-09	BM Trisula	West Java	Government breeding station
A-10	BM Sumenep-I	East Java	Local farmers/breeders
A-11	BM Bali Karet	East Java	Local farmers/breeders
A-12	BM AG-2	West Java	Government breeding station
A-13	BM Sembrani	East Java	Government breeding station
A-14	BM Manjung	West Java	Government breeding station
A-15	BM Pikatan	West Java	Government breeding station
A-16	BM Mentes	Central Java	Government breeding station
A-17	BM Katumi	East Java	Local farmers/breeders
A-18	BM-14	West Java	Local traditional market
A-19	BM-15	West Java	Local traditional market
A-20	BM Demak	Central Java	Local farmers/breeders
A-21	BM Sumenep	East Java	Local farmers/breeders
A-22	BM-17	West Java	Local traditional market
A-23	BM-18	West Java	Local traditional market
A-24	BM Brebes	Central Java	Local farmers/breeders
A-25	BM-20	West Java	Local traditional market
A-26	BM-21	West Java	Local traditional market
A-27	BM-22	West Java	Local traditional market
A-28	BM-23	West Java	Local traditional market
A-29	BM-29	West Java	Local traditional market
A-30	BM-24	West Java	Local traditional market
A-31	BM BT Ijo	East Java	Local farmers/breeders
A-32	BM Biru Lancor	East Java	Local farmers/breeders
A-33	BM Bauji	East Java	Local farmers/breeders
A-34	BM SuperPhilips	East Java	Local farmers/breeders
A-35	BM Bima Curut	West Java	Local farmers/breeders
A-36	BM Bombay	West Java	Local supermarket

and registered in NCBI database (Rout et al. 2014). In Indonesia, the shallot genetic analyses using molecular markers have been reported (Pinilih et al. 2015; Sari 2017). However, no molecular markers derived from the RGAs has been used. Therefore, the application of DNA marker to differentiate Indonesian shallot genotypes on the basis of RGA would be very helpful to develop shallot cultivars resistant to the bulb/basal rot disease. The objectives of this study were to assess the genetic diversity at conserved domain of RGA in a set of 36 Indonesian shallot genotypes to complement morphological characterization. This study would provide beneficial information to support breeding activities on this crop particularly to develop resistant genotype of shallot against Fusarium disease.

MATERIALS AND METHODS

A total of 36 shallot genotypes were used as plant genetic materials in this study (Table 1). Of these, Sumenep was known as a superior shallot cultivar in terms of resistance to several kinds of diseases and it is also expected to have high resistance to *F. oxysporum* (Baswarsiati et al. 2010).

Phenotypic Evaluation

Phenotypic evaluation was conducted in the green house and the experiment was carried out in a Completely Randomized Design with ten replications. Each shallot genotype was planted with ten unit polybags with two bulbs per polybag. Twelve importance morpho-agronomic traits consisted of four quantitative and eight qualititative traits were observed from the 36 shallot genotypes. The four quantitative traits consisted of plant height (PH), total weight of bulb (TWB), number of bulb (NB), and weight of bulb per plant (WB). The eight qualitative traits consisted of leaf color (LC), shape of bulb (SB), skin color (SC), intensity of skin color (ISC), terminal basal bulb (TB), division rate of bulb (DB) which is the tendency of bulbs to split into bublets, curvature of plant canopy (CP), and erectness of plant canopy (EP). As a guidance for characterization of the morpho-agronomic traits with any adjustment, the Calibration Book Onion and Shallot was used (Naktuinbouw 2010).

DNA Extraction and PCR Analysis

Total genomic DNA was extracted from freshly-harvested shallot bulbs according to the *cetyl trimethyl ammonium bromide* (CTAB) method as described by Doyle and Doyle (1990). A total of 36 Indonesian shallot genotypes were amplified using 15 RGA markers which were developed from the RGA of *Allium* sp. (AsRGAs) sequences accessible in the public database (https://www.ncbi.nlm.nih.gov/). List of the primers is presented in Table 2.

The PCR reaction was performed in a total volume of 20 μl which contained 10 ng DNA template, 0.15 μM of each primer, and 1× Kapa2G Fast ReadyMix (Kapa Biosystems). PCR reaction was run on a 96-well GeneAmp® PCR 9700 (Applied Biosystems) using the following program, initial denaturation at 94ºC for 4 min, 40 cycles of 94ºC for 30 sec, 46–56ºC (depending upon primers annealing temperature) for 45 sec, and 72ºC for 2 min. The final 7 min was then done at 72ºC. The amplified products were separated by electrophoresis using 1.5% (w/v) agarose gel in 0.5× TBE buffer. The PCR products were then stained using ethidium bromide (10 mg/ml) and documented using ChemiDoc™ XRS (Bio-Rad) transilluminator under UV light.

Table 2. List of RGA primers used in this study.

Primer name	Sequence (5' → 3')	Product size (bp)
AsRGA-3	gaaaccgatacctgggaaca//agctgcccagttcttcaaaa	226
AsRGA-12	caccctgtatcaggcgattt//ctgatcccacacatcatcca	241
AsRGA-20	caaactgattgcgtttgtgg//acagatcttccacgccaatc	173
AsRGA-21	tatctggtggtgctggatga//tccagcatcataatgcgttc	152
AsRGA-28	caccctgtatcaggcgattt//ctgatcccacacatcatcca	241
AsRGA-31	aagtgctgtttcgccagttt//tatcatccagcaccagcaga	210
AsRGA-34	tatctggtggtgctggatga//acgctttttcttcgcacagt	178
AsRGA-37	cgcgtgaaatatatgctgga//acagatcttccacgccaatc	217
AsRGA-40	ccctgtatcaggcggtgtat//ctgatcccacacatcatcca	239
AsRGA-44	agccagaactttagcgtgga//caaaagcgttcccagctatc	179
AsRGA-45	tatctggtggtgctggatga//tccagcatcataatgcgttc	152
AsRGA-51	agccagaactttagcgtgga//caaaagcgttcccagctatc	179
AsRGA-53	agccagaactttagcgtgga//caaaagcgttcccagctatc	179
AsRGA-6	ccctgtatcaggcggtgtat//tgcgataatcggtgttgcta	177
AsRGA-29	gcgtgagccagaaatttagc//atcggtttcccacacatcat	170

Data Analysis

The quantitative traits data were subjected to analysis of variance (ANOVA) using Minitab software package (Ryan and Joiner 2001) and the differences between pairs of means were considered at significant level value of p <0.05. The estimated genetic similarity matrix from the morphological and agronomic traits data was used to construct a dendrogram with the neighbour-joining (NJ) method using DARwin software (Perrier and Flori 2003) in 10,000 bootstrapped replicates.

The PCR product from each marker was scored in a binary form with '1' indicating the presence of an allele, while '0' indicating its absence. Statistical analysis for various genetic parameters, such as number of amplified loci (n), the maximum (Max) and minimum (Min) band size, the percentage of polymorphism (Ppl), and number of effective alleles (Neff), loci and their standard deviations (SD) were computed for each AsRGA marker using POPGENE software version 1.31 (Yeh et al. 1999). Ppl refers to the number of loci divided by the number of total loci. Other diversity indices, such as allele frequency (pi) and Polymorphic Information Content (PIC) were also estimated. The pi for primers was calculated for each single sample and among all samples as given by pi = 1–([1–Fb]^{¼}), where Fb is referred to the band frequency. PIC value were calculated using the online PICcalc program (Nagy et al. 2012).

RESULTS

Phenotypic Evaluation

All measured phenotypic traits were shown in Table 3. In general, phenotypic evaluation of the 36 shallot genotypes across twelve morpho-agronomic traits resulted in a wide range of phenotypic variation with the range of coefficient values of greater than 10%. The greatest coefficient of phenotypic variation was observed in TWB (89.57%), whereas the smallest ones was in PH (25.16%). The significant correlation was observed in the following variables: NB, WB, and PH, of which p-value ranged from 0 to 0.298 (Table 4).

Clustering Based on Morpho-Agronomic Traits

Cluster analysis of all genotypes on the basis of morpho-agronomic traits revealed five distinct clusters (Figure 1). Bali Karet was separated apart from other shallot genotypes with genetic similarity coefficient of 16.24 (called as node 1). On the other

Table 3. Descriptive statistics of four quantitative and eight qualitative traits of 36 Indonesian shallot genotypes.

Variable	Mean	Standard deviation	Coefficient of variation	Squares	Range	Skewness	Kurtosis
PH	26.76	7.18	26.85	27581.64	25.15	0.72	-0.40
TWB	2.143	1.920	89.57	294423836	6973	1.34	0.80
NB	6.067	1.974	32.54	1461.547	8.1	-0.05	-0.64
WB	9.46	6.16	65.17	4551.59	26.36	1.40	1.77
LC	2.139	1.018	47.62	201	3	-0.12	-1.79
SB	4.583	1.933	42.17	887	7	-0.10	-1.01
SC	5.500	1.384	25.16	1156	6	-0.86	1.42
ISC	4.278	1.523	35.61	740	4	0.73	-0.86
TB	3.222	1.476	45.80	450	4	-0.41	-1.23
DB	3.500	1.935	55.28	572	4	-0.54	-1.78
CP	1.2571	0.5606	44.59	66	2	2.15	3.86
EP	1.3529	0.544	40.21	72	2	1.23	0.64

PH = plant height, TWB = total weight of bulb, NB = number of bulb per plant, WB = weight of bulb per plant, LC = leaf color, SB = shape of bulb, SC = skin color, ISC = intensity of skin color, TB = terminal of basal bulb, DB = divison rate of bulb, CP = curvature of canopy plant, EP = erectness of canopy plant.

Table 4. Matrix correlation among morphological traits*.

	PH	TWB	NB
TWB	0.254		
	0.008		
NB	0.101	0.349**	
	0.298	0.000	
WB	0.488*	0.711**	0.308**
	0.000	0.000	0.001

PH = plant height, TWB = total weight of bulb, NB = number of bulb per plant, WB = weight of bulb per plant.

*First line: R-value, second line: p-value.

**Significant at p-value <0.001.

hand, the remaining four nodes consisted of five shallot genotypes in node 2, six genotypes in node 3, four genotypes in node 4, and 23 genotypes in node 5. In comparison to the five clusters, the 23 shallots clustered in the node 5 showed a closely relationship among genotypes with shared the genetic similarity of 40.93% at least.

AsRGA Marker Analysis

A total of 547 alleles (g) derived from 36 shallot cultivars genotyped by 15 AsRGA markers were resolved in this study (Table 5). The amplified fragments ranged from 50 bp to 3.5 kb in size. The number of bands obtained for each primer varied from 3 (primer AsRGA6) to 15 (primer AsRGA12 and AsRGA34) bands, with an average of 8.93 ± 3.83 bands per primer. Of these, a total of 82 (61.19%) bands were polymorphism across shallot cultivars. All the AsRGA primers screened in the thist study revealed marked polymorphism in the shallot genotypes, with a maximum of polymorphic loci of 14% (AsRGA34) and 11% (AsRGA12) and a minimum value of 2.4% (AsRGA3 and AsRGA51).

The PIC values ranged from 0.253 (AsRGA51) to 0.676 (AsRGA28) with an average of 0.444 (Table 6). The PIC provides an estimate of the discriminatory

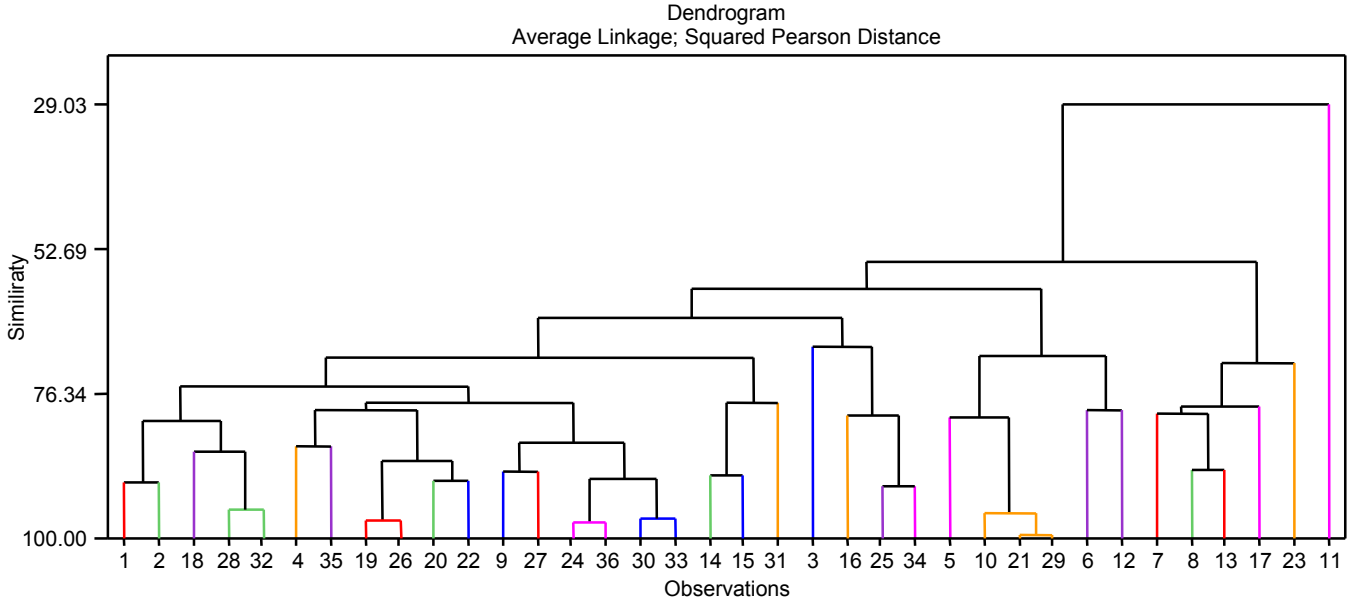

Figure 1. Dendrogram of the 36 Indonesian shallot genotypes based on morpho-agronomic traits using Average Linkage-Squared Pearson Distance analysis.

Table 5. Diversity indices of the AsRGA loci observed in this study.

Primer	Number of effective alleles (Neff)	Number of polymorphic locus (pl)	Number of amplified loci (n)	Percentage of polymorphism (Ppl)	Maximal band size (Max-b)	Minimal band size (Min-b)	Number ofobserved allelle	Frequency of band (Fb)	Allele frequency (pi) ± SD	PIC value
	A	B	c	d	E	F	g	h	i	j
AsRGA3	1	2	11	18.18	110	50	9	0.011	9.00 ± 0.00	0.375
AsRGA12	2	11	15	73.33	1,500	90	11	0.014	5.50 ± 0.50	0.373
AsRGA20	2	5	8	62.50	1,500	50	52	0.066	26.00 ± 10.00	0.335
AsRGA21	1	3	4	75.00	3,000	50	21	0.027	23.00 ± 0.00	0.375
AsRGA28	4	6	8	75.00	1,500	50	62	0.078	15.50 ± 4.72	0.676
AsRGA31	3	8	10	80.00	1,500	50	40	0.051	13.33 ± 8.73	0.455
AsRGA34	3	14	15	93.33	1,400	50	42	0.053	14.00 ± 5.10	0.551
AsRGA37	3	7	11	63.64	1,000	50	37	0.047	12.33 ± 1.70	0.586
AsRGA40	3	4	10	40.00	2,500	50	25	0.032	8.33 ± 3.09	0.541
AsRGA44	2	4	8	50.00	3,000	70	28	0.035	14.00 ± 8.0	0.280
AsRGA45	3	7	14	50.00	3,500	50	59	0.075	19.67 ± 8.81	0.532
AsRGA51	2	2	6	33.33	3,500	50	33	0.042	16.50 ± 10.50	0.253
AsRGA53	3	3	6	50.00	800	75	31	0.039	10.33 ± 6.60	0.420
AsRGA6	3	3	3	100.00	300	100	63	0.080	21.00 ± 2.94	0.585
AsRGA29	2	3	5	60.00	600	50	34	0.043	17.00 ± 7.00	0.329
Sum	37	82	134				547			
Average	2.47	5.47	8.93	61.62	1,714.00	59.00	36.47	0.046	15.03	0.444
SD	0.83	3.46	3.83	22.17	1,125.84	16.71	16.99	0.020	5.69	0.130

SD = standard deviation, PIC = Polymorphism Information Content.

power of a locus by taking into account the number and the relative frequencies of the alleles. The higher PIC value, the more effective the marker in discriminating individual within a population.

Genetic diversity of shallot genotypes on the basis of AsRGA markers (as loci) was resumed in Table 6. These data were also an indication of the presence of high diversity among the genotypes. The. The polymorphisms identified were used to generate the genetic dissimilarity. These loci produced a total of 1,512 alleles with an average of 1.946 alleles per marker.

Clustering Based on RGA Markers

Cluster analysis of the 36 Indonesian genotypes on the basis of RGA markers showed six main branching nodes with all genotypes were well separated (Figure 2). At node 1, No. 25 (BM-20) appeared to be separated apart from others (GS = 0.74). Similarly, genotype No. 24 (Bima Brebes) was also isolated from the others with genetic similarity of 0.70. This result indicated that the two genotypes, BM-20 and Bima Brebes, had high genetic variation on the resistance gene loci in comparison to those in the others. On the other hand, the three shallot genotypes, No. 36, 34, and 35 which are referred as Super Philips, Bima Curut, dan Bawang Bombay, respectively, could be clearly assigned in a different small group with genetic similarity of 0.69.

DISCUSSION

The correlation among morpho-agronomics traits in shallot is taken into consideration as an important parameter to be carried out. In the present study, three agronomic characters (TWB, WB, and PH) were significantly and positively correlated in good agreement with the regression analysis which showed that the main traits contributed to TWB are

Table 6. Genetic diversity indices of shallot genotypes based on AsRGA marker used in this study.

Genotype	Total loci	Neff	pi	Average allele per loci ± SD	Average allele per marker ± SD
BM-1	50	27	54.00	1.00 ± 0.720	2.381 ± 0.881
BM-2	36	19	52.78	0.72 ± 0.712	1.714 ± 0.868
BM-4	47	25	53.19	0.94 ± 0.712	2.238 ± 0.906
BM-5	61	32	52.46	1.22 ± 0.750	2.905 ± 0.906
BM-56	33	18	54.55	0.66 ± 0.580	1.571 ± 0.710
BM-55	33	18	54.55	0.66 ± 0.653	1.571 ± 0.888
BM-44	46	24	52.17	0.92 ± 0.756	2.190 ± 1.037
BM-45	55	29	52.73	1.10 ± 0.772	2.619 ± 0.999
BM-46	39	20	51.28	0.78 ± 0.671	1.857 ± 0.844
BM-47	44	23	52.27	0.88 ± 0.702	2.095 ± 0.868
BM-48	33	17	51.52	0.66 ± 0.675	1.571 ± 0.906
BM-49	46	24	52.17	0.92 ± 0.775	2.190 ± 1.082
BM-51	58	30	51.72	1.16 ± 0.845	2.762 ± 1.178
BM-53	37	19	51.35	0.74 ± 0.695	1.762 ± 0.921
BM-54	43	22	51.16	0.86 ± 0.705	2.048 ± 0.898
BM-8	38	20	52.63	0.76 ± 0.694	1.81 ± 0.898
BM-21	31	16	51.61	0.62 ± 0.580	1.476 ± 0.683
BM-27	51	27	52.94	1.02 ± 0.715	2.429 ± 0.881
BM-34	43	22	51.16	0.86 ± 0.726	2.048 ± 0.950
BM-35	29	15	51.72	0.58 ± 0.602	1.381 ± 0.765
BM-38	28	14	50.00	0.56 ± 0.575	1.333 ± 0.713
BM-39	57	30	52.63	1.14 ± 0.740	2.714 ± 0.904
BM-40	58	30	51.72	1.16 ± 0.753	2.762 ± 0.955
BM-42	44	23	52.27	0.88 ± 0.659	2.095 ± 0.811
BM-43	38	20	52.63	0.76 ± 0.673	1.810 ± 0.898
BM-3	36	19	52.78	0.72 ± 0.606	1.714 ± 0.750
BM-25	41	21	51.22	0.82 ± 0.730	1.952 ± 0.976
BM-24	35	18	51.43	0.70 ± 0.630	1.667 ± 0.774
BM-52	52	27	51.92	1.04 ± 0.769	2.476 ± 0.983
BM-41	52	28	53.85	1.04 ± 0.689	2.476 ± 0.777
BM-50	40	22	55.00	0.80 ± 0.647	1.905 ± 0.785
BM-9	48	26	54.17	0.96 ± 0.666	2.286 ± 0.750
BM-10	37	20	54.05	0.74 ± 0.652	1.762 ± 0.844
BM-7	35	18	51.43	0.70 ± 0.653	1.667 ± 0.833
BM-29	14	7	50.00	0.28 ± 0.439	0.667 ± 0.563
BM-28	28	15	53.57	0.56 ± 0.575	1.333 ± 0.700
BM-30	16	8	50.00	0.32 ± 0.458	0.760 ± 0.575
Average	40.865	21.432	27.03	0.817 ± 0.673	1.946 ± 0.856

Neff = number of effective alleles, pi = allele frequency, SD = standard deviation..

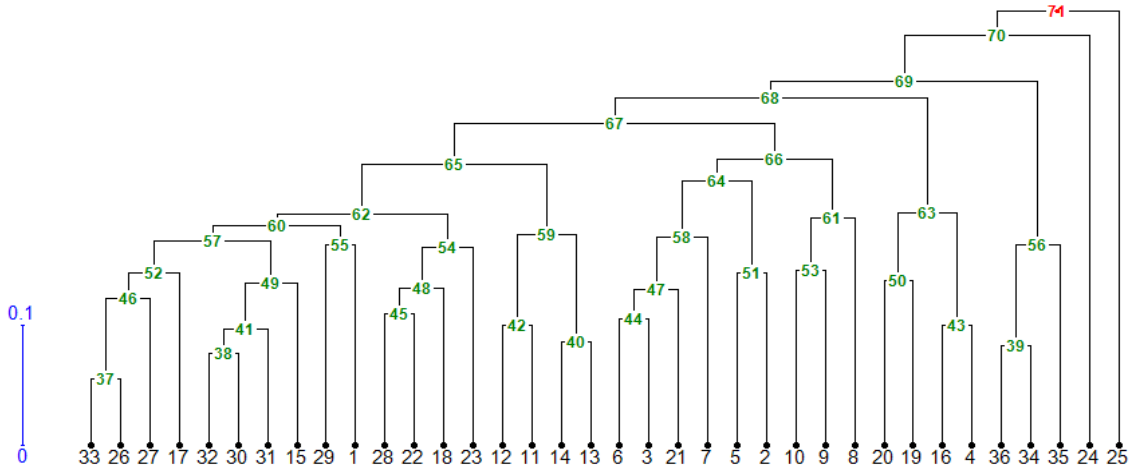

Figure 3. Clustering analysis of 36 shallot genotype based on Unweighted Pair Group Method using Arithmetic Averages (UPGMA).

PH, WB, and NB (data not shown). These results indicated that the increase of PH, WB, and NB contributed to TWB. This reflected the increase of yield potential resulting from the contribution of significant association of growth attributes, such as PH, bulb weight, and other traits. It is a fact that both correlation and regression analyses would provide benefit to plant breeders for development of new shallot breeding strategy in the future.

On the basis of the twelve morpho-agronomic traits, the 36 shallot genotypes were clustered into five groups. However, the clustering analysis which only derived from the phenotypic traits data might not provide enough information on determination of shallot cultivars relationship, in particular to select appropriate genetic materials to perform a new breeding program. Their genetic relationship may change in heterosis across diverse environmental conditions. Hence, the genetic diversity of shallots at molecular level using RGA would be more powerful in assisting breeders in the development of new breeding materials resistant to Fusarium disease.

The application of RGA as molecular markers in plant breeding has already spread since the last two decades. Since many plant genomes have been sequenced (Monaco et al. 2014; Nordberg et al. 2014), abundance of RGAs were discovered and more molecular markers developed from the RGA sequences become available for routine marker assisted selection in the future. Recently, many RGAs have been mapped through the gene(s) association mapping approach to be linked to resistance loci to a number of diseases (Ren et al. 2013). Chen et al. (2015) conducted the gene family analysis of RGAs in relation to Verticillium disease resistance genes in cotton. Similarly, various QTL mapping studies related to disease resistance on the basis of RGAs in several crops have also been reported. These include studies in chili pepper (Pflieger et al. 1999), soybeans (Kanazin et al. 1996), sunflower (Bouzidi et al. 2018), common bean (Mutlu et al. 2008), and rice (Selvaraj et al. 2011). These progressive RGA marker studies are a great sign of its usefulness in plant breeding.

Wang et al. (2000) and Ren et al. (2013) have used the RGA markers to analyse the genetic structure and geographic distribution in a diverse collection of rice germplasm. RGAs may also provide clues about the complex mechanisms of resistance, the interactions involved in pathogen recognition, and the evolution of the *R*-genes (Aarts et al. 1998). Li et al. (2016) reported that the RGA-based marker development strategies have been successfully applied for the development of diagnostic markers in several crops. The strategy involved four iterative steps, i.e. identification of genome-wide RGAs, identification of potential RGA candidates in the vicinity of the target resistance gene using comparative genomics analysis, designing of SNP markers for candidate RGAs, and marker evaluation using biparental genetic populations and/or association panels.

To our best knowledge, the implementation of RGA markers in shallot breeding program has never been done in Indonesia and our study is the first genetic diversity study on Indonesian shallot genotypes by using RGA markers. Less application of RGA-based markers in supporting the conventional shallot breeding program in Indonesia might be caused by various factors, such as limited numbers in the shallot genetic resources as well as the lack of their genetic diversity knowledge, lack of the desired traits information needed by shallot breeders,

insufficient shallot breeders, and lack of the shallot collection being evaluated. To initiate the application of RGA markers on the improvement of shallot crop resistant to *Fusarium*, therefore, a total of the 15 polymorphic RGA markers developed in the present study were evaluated for assaying the genetic diversity of shallot genotypes which generated six shallot genotype groups.

The genetic diversity using both the RGA markers and morpho-agronomic traits showed high genetic variation in the 36 shallot genotypes. In comparison to the two clustering methods, both clusterings classified the shallot genotypes into different large groups, within different similarity level ranges. In the morpho-agronomic traits-based cluster, the difference in percentage of similarity between the first and last group reached 34.69%, whereas that in the RGA-based cluster reached 15%. These results implied that RGA markers distinguished shallot genotypes more thoroughly compared to morphological characters.

Previous study resumed that RGAs were irregularly distributed in plant genomes and that many reside in clusters (Sekhwal et al. 2015). This might be other reasons for the differences found in the two clustering methods. In general, the shallot genotypes could not be discriminated based on their collection sites (production centers) either with the morphological or molecular clustering approaches. Similarly, Laila et al. (2013) also reported that genetic diversity analysis using morphological characters on 16 red onions cultivars collected from three different topographical regions (Central Java, West Java, and East Java) did not cluster together according their regions of origin. Hartl et al. (1988) explained that morphological traits was influenced by environmental condition due to unknown mechanism of genetic control and great environmental effects in the process of trait expression. Thus, genetic variation should be the main factor for discrimination of shallot genotypes rather than morphological traits because the morphological traits were not always reliable in the estimation of their genetic relationship.

Clustering based on both approaches revealed that four shallot genotypes (Tajuk, Sumenep, Sumenep-I, and Lokal Boyolali) were always in the same group, implying their high genetic similarities. Emphasing on the probability abundance of the RGAs within each genotype, the results also implied the potential use of Tajuk and Boyolali as alternative resources of resistance genes for the future breeding program in developing resistant genotypes against Fusarium disease in shallot. Nevertheless, the type and number of RGAs within both shallot genotypes might be different from those of Sumenep. Liu (2003) reported some RGAs represented highly divergent defense system in plants containing large and diverse family of genes with conserved motifs and domains. Thus, the clustered distribution of RGAs provides a reservoir of genetic variation to drive the evolution of new *R*-gene specificities (Michelmore 1998; Young 2000; Zhou et al. 2007). Tajuk has been known as one of superior genotype which is commonly grown by farmers in East Java province, a production center of shallot in Indonesia (Baswarsiati et al. 2010). These potential parental genotypes resistant to Fusarium disease would be helpful for breeding activities in the future in Indonesia.

CONCLUSION

Both the RGA markers and morpho-agronomic traits informed the genetic diversity of the 36 shallot genotypes. RGA markers could provide a better shallot differentiation into different groups in comparison to morpho-agronomic traits. The genetic relationship of shallot genotypes obtained in this study would potentially be useful for selection of ideal combination of parental lines to be used in the development of new shallot cultivar resistant to bulb/basal rot of shallot in Indonesia.

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