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Trimanto ✉; R. T. Probojati; D. Wahyudi; L. Hapsari ✉

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RAPD Analysis to Reveal the Genetic Diversity among Closely Related *Etlingera* Species of *Achasma* Group, Zingiberaceae

Trimanto[1, a)], R T Probojati[2, b)], D Wahyudi[3, c)] and L Hapsari[1, d)]

[1]*Research Center for Plant Conservation, Botanic Gardens, and Forestry, National Research and Innovation Agency, Bogor, Indonesia*
[2]*Department of Agrotechnology, Faculty of Agriculture, University of Kadiri, Kediri, Indonesia*
[3]*Department of Biology, Faculty of Science and Technology, University of State Islamic Maulana Malik Ibrahim, Malang, Indonesia*

[a)] Corresponding author: triman.bios08@gmail.com
[b)] rasyadan@genbinesia.or.id
[c)] didik_wahyudi@bio.uin-malang.ac.id
[d)] Corresponding author: hapsari.lia@gmail.com

Abstract. The morphological characteristics among closely related *Etlingera* species of *Achasma* group were found overlapping which needs to be confirmed molecularly. Hence, the purposes of this study were to reveal the genetic diversity and relationship of four species, namely *E. megalocheilos*, *E. littoralis*, *E. coccinea* and *E. metriocheilos* using RAPD markers, also to differentiate the species identity of *E. cf. megalocheilos* specimens from Sulawesi and Java. The results showed that 13 out of 20 OPA primers were successfully amplifying the DNAs target. Five highly polymorphic primers were selected as recommended markers *i.e.* OPA09, OPA10, OPA11, OPA18, and OPA20. Diversity analysis showed high genetic richness with Shannon index of 0.417 and the number of polymorphic loci of 98.90%. Clustering and PcoA analyses resulted in three clusters following their species identity. Cluster 1 comprised of three specimens of *E. littoralis* (similarity value of 44.44% to 61.79%). Cluster 2 comprised of *E. coccinea* and *E. metriocheilos* (similarity value of 40.32%). Cluster 3 comprised of *E. cf. megalocheilos* Sulawesi and *E. megalocheilos* from Java (similarity value of 34.78%). This study has confirmed that the four *Etlingera* spp. were genetically distinct, and *E. cf. megalocheilos* from Sulawesi and Java were possibly the same species but still unclear. Further research using DNA barcodes for a more precise identification is required.

Keywords: *Etlingera*, Molecular, RAPD, Polymorphism, Relationship, Zingiberaceae

INTRODUCTION

Etlingera Giseke is a large and morphologically diverse genus in the Zingiberaceae family with approximately 150-200 species available in nature [1,2]. Their native range is tropical and subtropical Asia to the South Pacific [3]. Many species of *Etlingera* have been cultivated and utilized by the community, such as for herbs, food ingredients, traditional medicine, as well as for ornamental flowers [4,5]. *Etlingera* is known to have high essential oil content and potential as modern medicine [6]. The typical character of *Etlingera* is a terrestrial perennial herb with inflorescences separated from the leafy shoots with a plant height of 1-8 meters. Several species are clump-forming with stilt roots [7]. The inflorescence shoots are often so short with some floral parts grow below ground. All that can be seen is a circle of flowers with prominent bright red-yellow petal-like structures (labellum) radiating out from the ground [8]. Flowers are pollinated by stingless bees. Fruits ripen below ground, and the seeds are thought to be dispersed by animals such as wild pigs [8,9].

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There are at least six species of *Etlingera* which formerly identified as a species of *Achasma*, with some possible synonymies. Thus, they are considered as *Etlingera* of *Achasma* group [10,11]. In particular, about four closely related *Etlingera* species of *Achasma* group were recognized for their overlapping characteristics, specifically the inflorescences. First, *Etlingera megalocheilos* (Griff.) A.D. Poulsen which is widely distributed in Sundaland [12]. The labellum characteristics are red with pale red or yellowish lateral margins. The labellum margins are not inrolled and upper half anther dehiscent [1]. The second is *Etlingera littoralis* (J.Koenig) Giseke which is widely distributed in the Malay Peninsula. It has labellum median red and yellow at lateral part [13]. Then, *Etlingera coccinea* (Blume) S.Sakai & Nagam. which is native to Thailand, Malay Peninsula, Sumatra, Java, and throughout Borneo, recently recorded in the Philippines [14]. This species is more similar to *E. megalocheilos*, but it has a long-elongated labellum, broad yellow median and red margin. Fourth, *Etlingera metriocheilos* (Griff.) R.M.Sm. which is distributed in Peninsula and Borneo [12]. This species has a purple-red lip with a white margin and a purple-red (almost black) stigma [11].

Therefore, a molecular approach is required to confirm the species identity and delimitation of these four species of *Etlingera* of *Achasma* group. Furthermore, molecular confirmation was also required to verify the identity of *E. cf. megalocheilos* specimens from Sulawesi in comparison to *E. megalocheilos* from Java. The species was previously claimed as a new record for the Sulawesi region or expanded from Sundaland to Wallacea by Trimanto and Hapsari [15] but rejected by Ardiyani and Poulsen [16]. The species was considered as *E. penicillata* (K.Schum.) A.D. Poulsen, the only *Achasma* group represented in Sulawesi [16]. Through molecular confirmation, data support will be obtained regarding the genetic diversity and relationship of the *Etlingera* species of *Achasma* group for a better decision.

Random Amplification of Polymorphic DNA (RAPD) is one molecular method that is often used to analyze the genetic diversity of Zingiberaceae [17]. It is a PCR-based method using arbitrary primers which bind to the nonspecific sites on the DNA and amplify them. The amplified fragments then migrated on the agarose gel and a different banding pattern was observed [18]. This method has some advantages because of cost-effectiveness and technical ease. Furthermore, it provides less accurate estimation to distantly related populations but more accurate to closely related populations [19], making it suitable for this study. Nevertheless, there are several disadvantages of RAPD, including not being locus-specific (dominant marker) so that it unable to differentiate whether the amplified DNA segment comes from a homozygous or heterozygous locus, and low reproducibility so that it can produce false bands [20].

Hence, the purposes of this study were to reveal the genetic diversity and relationship of four closely related *Etlingera* species of *Achasma* group using RAPD markers, namely *E. megalocheilos*, *E. littoralis*, *E. coccinea* and *E. metriocheilos*, also to confirm the species identity of *E. cf. megalocheilos* from Sulawesi and *E. megalocheilos* from Java. This study results are expected to be the supporting data for the taxonomy and delimitation of the *Etlingera* species.

MATERIALS AND METHODS

Plant Materials

The plant materials examined in this study were seven specimens of *Etlingera* species of *Achasma* group, which comprised of four closely related species (Table 1). The leaf samples were collected from Purwodadi Botanic Garden (East Java, Indonesia) and Suriana Botanic Conservation Garden (Pulau Pinang, Malaysia).

TABLE 1. List of *Etlingera* species of *achasma* group examined in this study

Sample Code	Species Name	No. Registration	Sample Collection Locality
E1	*Etlingera cf. megalocheilos* (Griff.) A.D. Poulsen	P19930724	Pani Binangga Nature Reserve, Parigi Moutong, Central Sulawesi, Indonesia
E2	*Etlingera megalocheilos* (Griff.) A.D. Poulsen	P199109107	Alas Purwo National Park, Banyuwangi, East Java, Indonesia
E3	*Etlingera littoralis* (J.Koenig) Giseke, Pedu	L14008	Balik Pulau, Pulau Pinang, Malaysia
E4	*Etlingera littoralis* (J.Koenig) Giseke, Moongate	L14018	Balik Pulau, Pulau Pinang, Malaysia
E5	*Etlingera littoralis* (J.Koenig) Giseke, Summit Red	L14019	Balik Pulau, Pulau Pinang, Malaysia

E6	*Etlingera coccinea* (Blume) S.Sakai & Nagam.	L14016	Balik Pulau, Pulau Pinang, Malaysia
E7	*Etlingera metriocheilos* (Griff.) R.M.Sm.	L14020	Balik Pulau, Pulau Pinang, Malaysia

Methods

The whole-genome DNAs were isolated from leaf samples using DNA extraction kit (Promega), referring the guidelines for plants. The DNA yields were then confirmed qualitatively using 1% agarose gel electrophoresis. The PCR-RAPD assays were performed using a thermal cycler (BioRAD) with 20 OPA primers comprised of OPA01 to OPA20 (Operon Technologies Inc.). The PCR cocktail was conducted in a total volume of 10 µL comprised of 1 µL DNA sample, 1 µL primer (10 pmol), 5 µL DreamTaq DNA polymerase, and 3 µL nuclease-free water. The PCR cycle protocol started with 4 minutes pre-denaturation at 94 °C, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at a different temperature according to each primer requirements, following previous study by Probojati *et al*. [21] for 45 seconds and extension at 72°C for 2 minutes. The cycle was ended by post-extension at 72 °C for 5 minutes. The PCR amplicons were confirmed using electrophoresis on agarose gel 2% with DNA ladder 100 bp, then visualized under UV-transilluminator.

Data Analysis

The visualized RAPD fragments on gel agarose were first evaluated for the reproducibility. Only the reproducible and consistent bands were selected for further data analysis. The RAPD band patterns of each primer from individual specimens were read using GelAnalyzer 19.1. The band size read results were converted into binary matrix of absent (0) or present (1). The final data were then analyzed to determine the most polymorphic primer for amplification, using four parameters *i.e.* polymorphic information content, effective multiplex ratio, markers index, and resolving power [22,23].

Analysis of genetic diversity were conducted using GenAlEx 6.5 [24]. The parameters observed including number of effective alleles, number of observed alleles, Shannon information index, expected heterozygosity, unbiased expected heterozigosity and percentage of polymorphic loci. Furthermore, analysis of clustering and principal coordinate (PcoA) were conducted to binary data matrix using Palaeontological Statistics (PAST) 3.0 software based on unweighted pair group method with arithmetic mean, Dice similarity index, and 1000 bootstraps [25].

RESULTS AND DISCUSSION

RAPD Polymorphisms and Marker Informativeness

The RAPD amplification of *Etlingera* species of *Achasma* group found to be very challenging, only 13 out of 20 OPA primers were successful and reproducible (Table 2). The whole-genome DNA of *Etlingera* spp. are presumably high in GC, so that difficult to amplify. Annealing temperature and times become important factors in the amplification success of GC-rich DNA templates [26]. Some samples were found not to produced bands or absent on certain primers. It may due to the absence of homolog primer sequences information in the genome [21]. In total, 182 amplified bands were observed with sizes ranging from 200-1,500 bp (Fig. 1). The results showed that among seven specimens of closely related *Etlingera* spp. has high polymorphic bands in average 98.90%. RAPD marker using a single primer can detect polymorphisms, although there is no specific nucleotide sequence information. RAPD polymorphisms result from changes in nucleotide bases that alter the primary binding site or insertion or deletion within the amplified region. Differences in polymorphisms may be due to genetic variations that exist among/within species. Polymorphisms can be used to construct individual genetic maps and relationships [19,20,27].

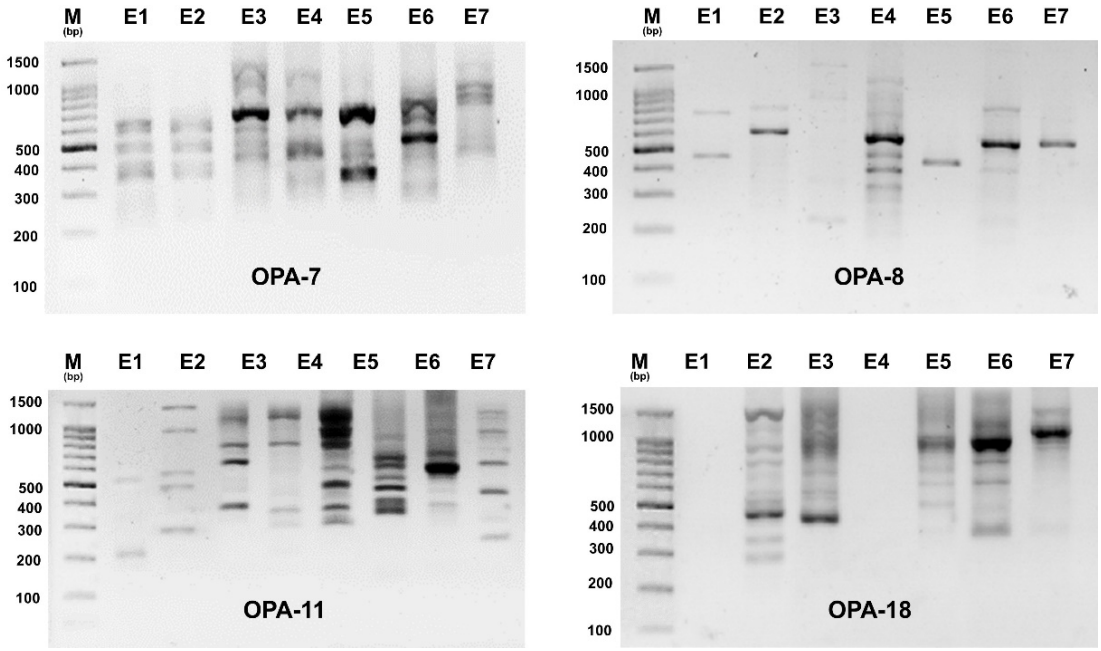

FIGURE 1. Visualization gel electrophoresis of some RAPD amplification of *Etlingera* species of *Achasma* group. Notes: E1= *E. cf. megalocheilos*_Sulawesi, E2= *E. megalocheilos*_Java, E3= *E. littoralis*_Pedu, E4= *E. littoralis*_Moongate, E5= *E. littoralis*_Summit Red, E6= *E. coccinea*, E7= *E. metriocheilos*

TABLE 2. RAPD polymorphisms and marker informativeness of *Etlingera* species of *Achasma* group

No.	Primer	TNB	NPB	PB (%)	PIC	EMR	MI	RP
1	OPA04	15	15	100	0.33	225	74.69	6.86
2	OPA05	13	12	92.31	0.34	169	57.31	13.43
3	OPA06	7	7	100	0.31	49	15.43	2.86
4	OPA07	13	12	92.31	0.36	169	61.55	11.14
5	OPA08	14	14	100	0.37	196	72.00	7.71
6	**OPA09**	**17**	**17**	**100**	**0.39**	**289**	**112.41**	**13.14**
7	**OPA10**	**19**	**19**	**100**	**0.40**	**361**	**142.69**	**12.86**
8	**OPA11**	**21**	**21**	**100**	**0.40**	**441**	**176.57**	**13.71**
9	OPA15	11	11	100	0.39	121	46.69	6.86
10	OPA17	10	10	100	0.26	100	26.12	3.14
11	**OPA18**	**15**	**15**	**100**	**0.41**	**225**	**91.84**	**11.14**
12	OPA19	11	11	100	0.27	121	33.22	3.71
13	**OPA20**	**16**	**16**	**100**	**0.35**	**256**	**90.12**	**8.00**
Total		**182**	**180**	**1284.62**	**4.58**	**2722**	**1000.64**	**114.56**
Average		**14.00**	**13.85**	**98.90**	**0.35**	**209.38**	**76.97**	**8.81**

Notes: TNB= total number of bands, NPB= number of polymorphic bands, PB (%)= polymorphic bands percentage, PIC= polymorphic information content, EMR= effective multiplex ratio, MI= marker index, RP= resolving power

RAPD primer of OPA-11 was generated the highest number of bands (21), followed by OPA10 (19), OPA9 (17), OPA20 (16), and OPA18 (15). The values of PIC were ranging from 0.26 (OPA18) to 0.41 (OPA17), in average of 0.35. If the PIC value is closer to the maximum value (0.5), thus the primers will be more informative for use in genetic diversity analysis [23]. The EMR calculated were ranged between 441 (OPA11) and 49 (OPA06), in average of 209.38. Primers with high EMR value will be more effective in producing polymorphic bands [22]. The highest value of MI and RP were shown by OPA11 *i.e.* 176.57 and 13.71, respectively. The high MI value is important to determine the most efficient primer in analyzing a number of bands simultaneously [22], while RP is to measure the primary strength in producing polymorphic bands [23]. Hence, based on the marker informativeness parameter analysis, the five highly polymorphic primers were selected, including OPA09, OPA10, OPA11, OPA18, and OPA20 (Table 2).

Those five selected RAPD primers from this study may become a reference as recommended markers for further studies to reveal the diversity of another *Etlingera* spp. in particular, and Zingiberaceae in general.

Genetic Diversity Parameters

The genetic diversity parameters describes the genetic richness of the examined taxa. As evidently by RAPD markers, genetic diversity analysis among closely related species of *Etlingera* species of *Achasma* group in this study showed high genetic richness in contrast to morphological observation (Table 3). The RAPD primers showed 182 number of loci with polymorphic loci reaching 98.90%. The average number of dominant alleles observed 1.989 with 69.93% as effective alleles. The Shannon index value and heterozygosity were also considered high. The value of heterozygosity more than 0.20 for common plant families particularly rare species is considered high [28]. The genetic diversity level of *Etlingera* spp. in this study was considered higher than the previous study on *Zingiber* spp. with I = 0.327±0.261 [29], but lower than *Curcuma* spp. with I = 0.53 ± 0.19 [30].

TABLE 3. RAPD genetic diversity parameters of *Etlingera* species of *Achasma* group

Parameters	Mean value ± Standard Error
Number of samples (N)	7
Number of loci (Nl)	182
Number of observed alleles (Na)	1.989 ± 0.008
Number of effective alleles (Ne)	1.391 ± 0.019
Shannon's information index (I)	0.417 ± 0.011
Expected heterozygosity (He)	0.259 ± 0.009
Unbiased expected heterozygosity (uHe)	0.279 ± 0.010
Polymorphic loci percentage (P)	98.90%

Clustering and PcoA Scatter Plot Pattern

The result of clustering analysis of seven specimens of *Etlingera* species of *Achasma* group based on RAPD markers were divided into three clusters following their species identity, supported by low to strong bootstraps (58-100) and a high confidence level (cophenetic correlation) of 0.8773 (Fig. 2 & 3). Furthermore, the scatter plot PcoA was confirmed the clustering analysis result. The PcoA represents the data variability in a two-dimension set of axes [29]. The minimum spanning tree separates the specimens into three clusters. The PCoA of first two principal coordinates generated eigenvalues of 0.54 and 0.29, respectively, and contributed to a cumulative 59.58% of the total variance. The considerable polymorphisms and minimum spanning tree may also illustrate the possibility of finding genetic divergence among taxa examined [29].

Cluster 1 comprised three specimens of *E. littoralis* with genetic similarity value of 44.44% to 61.79%, supported by strong bootstraps (87-93). Species *E. coccinea* and *E. metriocheilos* were clustered with a genetic similarity value of 40.32% in Cluster 2. Furthermore, Cluster 1 was found as a sister group to Cluster 1, separated at a genetic similarity value of 33.59%. Meanwhile, species *E. cf. megalocheilos* from Sulawesi and *E. megalocheilos* from Java were clustered with a genetic similarity value of 34.78% in Cluster 3 and served as an outgroup with strong bootstrap support (100). Cluster 3 was separated from Cluster 1 and Cluster 2 at a genetic similarity value of 26.17% (Fig. 2 & 3, Table 4).

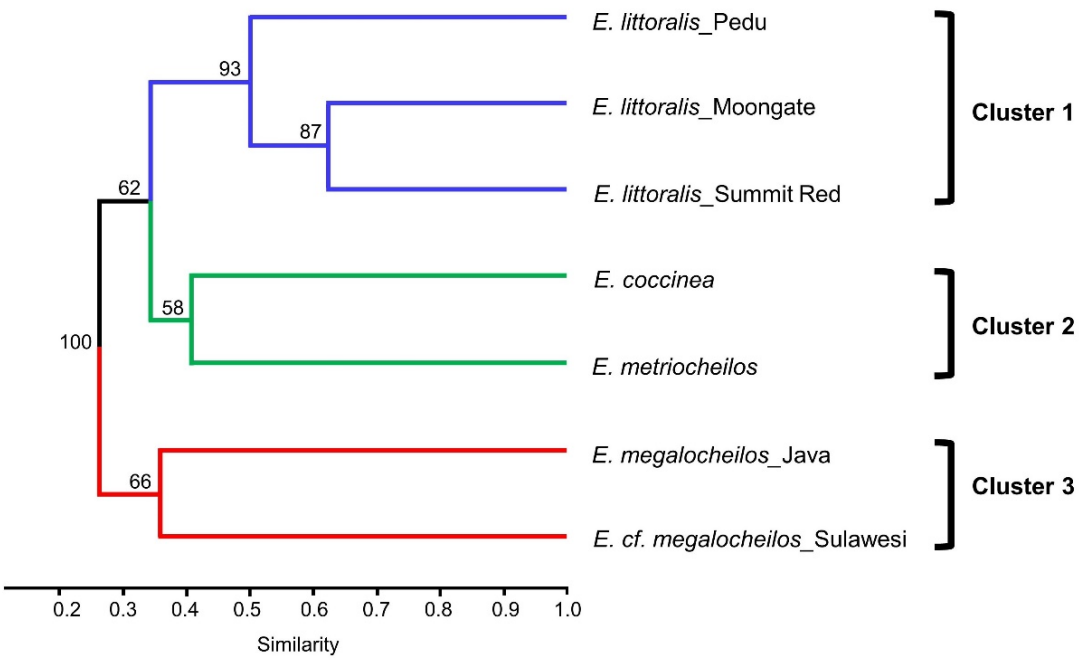

FIGURE 2. RAPD dendogram clustering of *Etlingera* species of *Achasma* group

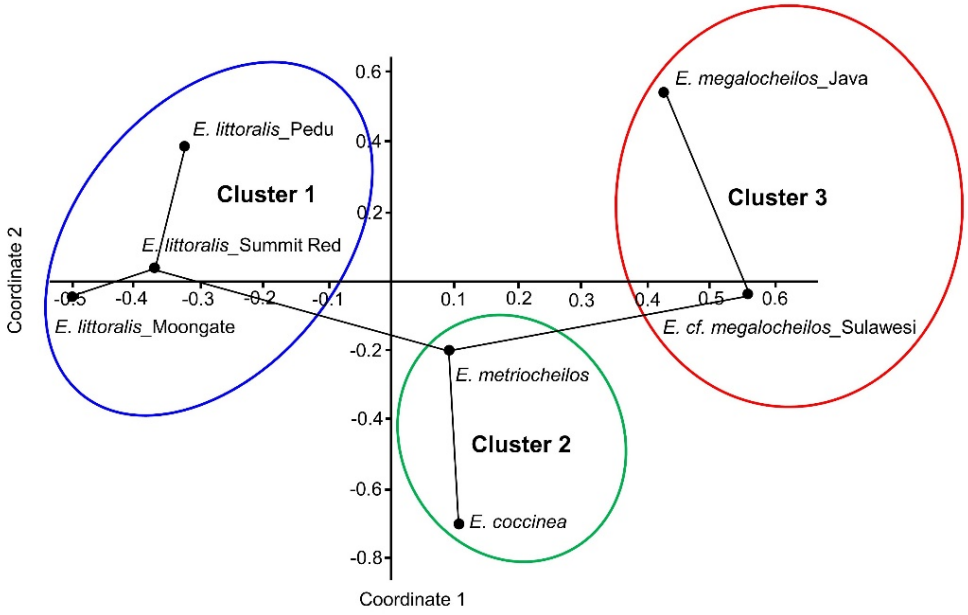

FIGURE 3. RAPD scatter plot PcoA of *Etlingera* species of *Achasma* group

TABLE 4. RAPD dice similarity index (%) of *Etlingera* species of *Achasma* group

No.	Species name	E1	E2	E3	E4	E5	E6	E7
E1	*E. cf. megalocheilos*_Sulawesi	100						
E2	*E. megalocheilos*_Java	34.78	100					
E3	*E. littoralis*_Pedu	17.65	31.58	100				
E4	*E. littoralis*_Moongate	20.20	14.41	54.55	100			
E5	*E. littoralis*_Summit Red	17.31	29.31	44.44	61.79	100		
E6	*E. coccinea*	26.17	26.89	26.36	30.16	33.59	100	
E7	*E. metriocheilos*	32.99	33.03	38.66	32.76	39.67	40.32	100

General Discussion

The morphological characteristics of *Etlingera* species of *Achasma* group, *i.e. E. megalocheilos*, are considerably confusing with *E. littoralis*, also with *E. coccinea* and *E. metriocheilos*. Most of them has similar inflorescence characteristics embedded in the ground with labellum color median red and yellow margin [13,15] (Fig. 4 A-E). Meanwhile, *E. coccinea* specimen in the Philippines has a yellow long lip with an inrolled and red margin [12] (Fig. 4F), and *E. metriocheilos* differentiated by labellum with red and white margin [11] (Fig. 4G). Morphological characteristic can be influenced by the environment. In the same species, variations occur because environmental factors are more dominant than genetic factors. For example, temperature and sunlight have an effect on stem-leaf ratio and morphological modifications in herbaceous plants [31]. Phenotypic plasticity is considered as one of the main ways that plants can overcome the variability of environmental factors [32]. A single genotype can produce varying phenotypes under different environmental conditions [33]. The high species diversity can also be caused by natural hybridization between species [34]. The RAPD analysis from this study has confirmed that those four species of closely related *Etlingera* are distinct species.

Previous study of *E. littoralis* specimens collected from 13 provinces in Thailand exhibits high morphological variation [13] (Fig. 4E). The three morphologically variables of *E. littoralis* in this study were clustered together in Cluster 1 (Fig. 2 & 3). This result indicates that indeed the tree specimens were closely related and considered as the same species. Furthermore, *E. coccinea* which is clearly morphologically different from *E. littoralis* and *E. megalocheilos* confirmed genetically distinct, as separated in different cluster. Species *E. coccinea* was found separated in Cluster 2 and closer related to *E. metriocheilos* (Fig. 2 & 3). Meanwhile, research on morphology and genetic diversity of *E. metriocheilos* is still very limited. This species was cited as different name into *E. sphaerocephala* [35]. However, a more in-depth research is needed on this species to clarify its taxonomic status.

In particular of *E. megalocheilos*, the inclusion of this species as a new record in Sulawesi is still being debated. It was argued that the description of the *E. cf. megalocheilos* specimen from Sulawesi has intermediate characters with *E. penicillata* in the flower, labellum, and lamina length but differs in flowers number (5-7 vs. 10-12), length of staminal tubes, corolla lobes and color of labellum margin (yellow vs plain red). Furthermore, *E. penicillata* was speculated to have those variations [16]. However, according to Trimanto and Hapsari [15] the specimen *E. megalocheilos* from Java and *E. cf. megalocheilos* from Sulawesi considered highly similar to have the same labellum character, the red-orange labelum with yellow margins (Fig. 4 A-D). They differ only slightly in the color intensity of the bracts where specimens from Sulawesi have a darker red color. In addition, our observations in the garden showed that both species are flowering in the same month between November-December. Plant specimens from Sulawesi is found produce more inflorescences than specimens from Java. Specimens from Java only found 1-2 inflorescences per clump. Both specimens never found fruiting in the garden; they may require a special pollinator such as stingless bees [9]. This RAPD study showed that *E. cf. megalocheilos* from Sulawesi and *E. megalocheilos* from Java were clustered together in Cluster 3 with strong bootstrap, indicating that both specimens were possibly the same species but still unclear due to the low level of genetic similarity value (34.78%). Further study to make a decision is needed using DNA sequences/barcodes for a more precise genetic markers such as *ITS*, *RbcL*, *matK*, etc.

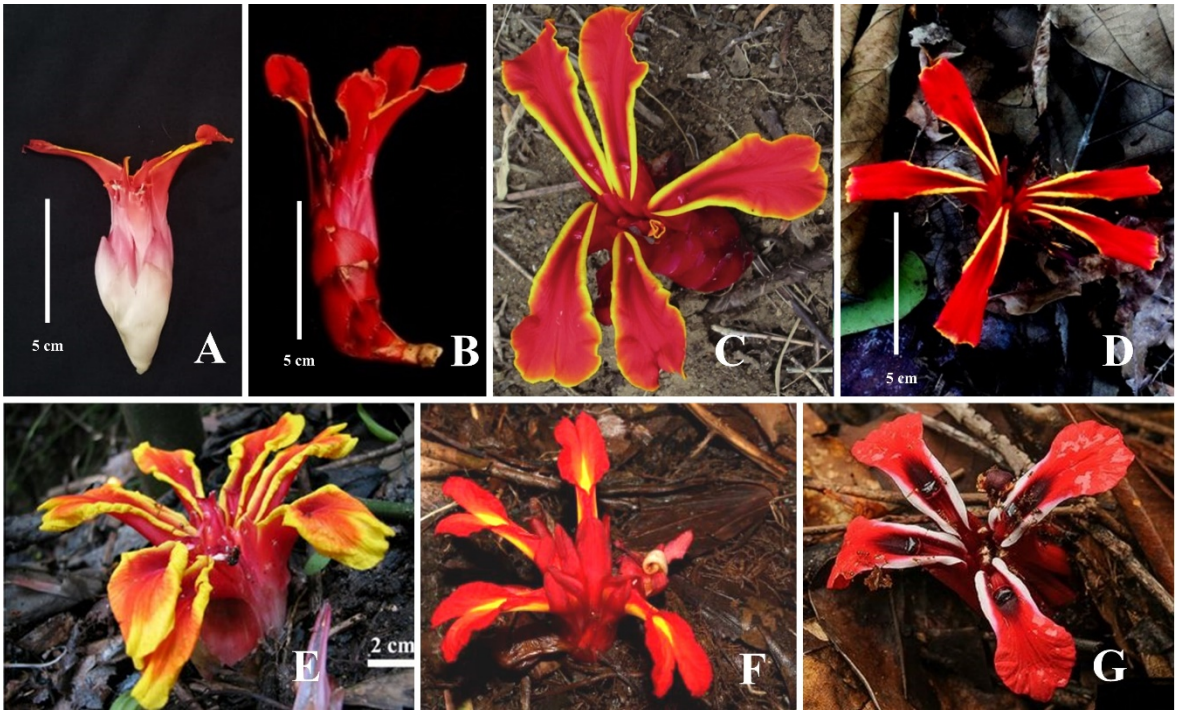

FIGURE 4. Inflorescence characteristics of *Etlingera* species of *Achasma* group: A. *E. megalocheilos* from Java (side view), B. *E. cf. megalocheilos* from Sulawesi (side view), C. *E. megalocheilos* from Java (aerial view) [1], D. *E. cf. megalocheilos* from Sulawesi (aerial view), E. *E. littoralis* from Thailand [9], F. *E. coccinea* from the Philippines [14], and G. *E. metriocheilos* from Malaysia [36]

CONCLUSIONS

In conclusion, RAPD markers showed high genetic diversity among closely related *Etlingera* species of *Achasma* group. The clustering analysis and PcoA resulted in three clusters following their species identity. It confirms that the four closely related species were distinct, *i.e. E. megalocheilos*, *E. littoralis*, *E. coccinea* and *E. metriocheilos*. In particular, *E. cf. megalocheilos* from Sulawesi and *E. megalocheilos* from Java were possibly the same species but still unclear. Further advanced molecular study is required for a more precise identification conclusion.

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