GENETIC DIVERSITY OF THE DYE-PRODUCING JAVANESE TOM *INDIGOFERA TINCTORIA* L. FROM THREE INDONESIAN ISLANDS BASED ON SIMPLE SEQUENCE REPEATMARKERS

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Abstract

Javanese Tom (*Indigofera tinctoria* L.) is commonly used as blue dyes agent by Indonesian fabrics and batik crafters. The research was aimed to identify the genetic diversity and population structure of *I. tinctoria* L. based on the SSR markers. The research was conducted in 33 regions in three Indonesian islands, with 63 samples. The results from electrophoresis of 15 SSR primers produced 76 bands with 250–750 bp in size. From dendrogram obtained likelihood coefficient of 68–100%. Weirklei3 and Weirklei4 accessions had the highest likelihood coefficient with 100% (same). The highest diversity was recorded in Cirebonean population. The populations from Sumenep, Cirebon, and Flores were grouped as one with high like hood coefficient. Populations from Java, Madura, and Flores islands have high likelihood coefficients. Based on SSR markers, *I. tinctoria* populations from three Indonesian islands were having a high likelihood coefficient, thus unqualified to be categorized as the infra-species.

Key words: Java, Indigo, Natural dye, Eco friendly.

Introduction

Indigofera (*Indigofera tinctoria* L.) are biennial shrubs with clustered terminal flowers, compound leaves (*imparipinnate*), and cylindrical pods as the specific identifier. It has economic and ecological values because it can be used as the precursor for blue dyes and producing nontoxic waste. Blue dyes from Indigoferas were used by Indonesian peoples as the coloring agent for traditional fabrics such as *batik*, *tenun*, and *lurik*. *I. tinctoria* are distributed from lowlands to beaches, and able to grow at arid, critical, or even high salinity soils (Hassen, 2006).

Agro-morphological study is useful to characterize and identify promising genotypes (Jan *et al.*, 2017[a, b]; Shinwari *et al.*, 2011). Morphological differences had been observed in *I. tinctoria* from Java, Madura, and Flores islands. Those differences are remnants, longer petioles, more seed and dark-greenish-blue leaves only founded in Floresian accessions. Those differences also found in the color intensities from fabrics dyed with various indigo pastes. Floresian *Tenuns* fabrics were darker compared with Javanese and Maduranese *Batiks*. Those differences were allegedly linked to the status and diversity of *I. tinctoria* L.

Simple Sequence Repeat (SSR) analysis was employed to verify the status and diversity of *I. tinctoria* L. SSR analysis has been proved as the reliable method, not only to identify interspecies differences but also inter-accession differences in the species (Ercisli *et al*., 2011; Hee *et al*., 2011; Tantasawat *et al*., 2011). The biochemical and molecular markers are used to study phylogenetic relationship among different plant species (Shinwari *et al.*, 2018; Jan *et al.*, 2016; Rehman *et al.*, 2015; Pervaiz *et al.*, 2010). Therefore, the current research work was designed to study SSR based diversity of *I. tinctoria* L. and to identify the status of the Floresian species and color saturation from the fabrics dyed by Indigo pastes from Javanese, Maduranese, and Floresian *I. tinctoria* that planted in homogeneous-controlled conditions.

Materials and Methods

Plant materials: The plant materials were collected from 33 locations in Java, Madura, and Flores islands of Indonesia (Fig. 1). One to five samples were collected from each location, and the total collected samples were 63 (Table 1).

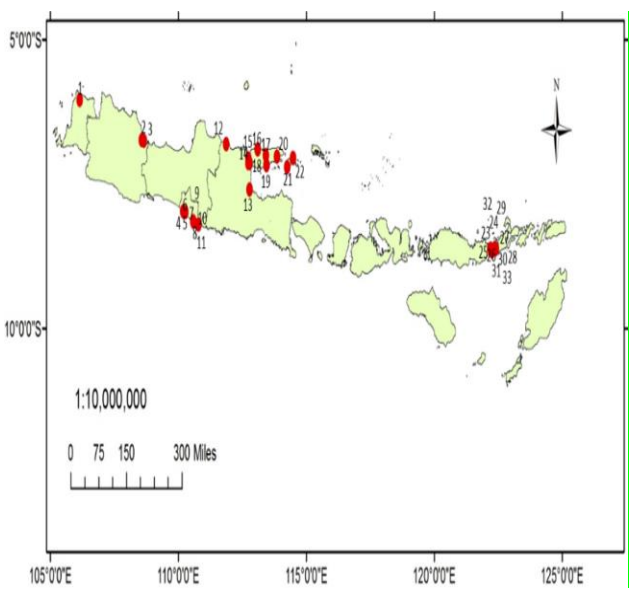

Fig. 1. The sampling locations. 1. Sukajaya (Banten); 2. Babatan, 3. Kajawenan; 4 P. Saden, 5. P. Slili; 6, Piyungan; 7. Stadion Sultan Agung Bantul; 8. Srigading, Bantul; 9. Trisik; 10. Banaran, Kulon Progo; 11. Dandeles; 12. Tuban; 13. Pasuruan; 14. Burneh; 15. Halim Perdana Kusuma; 16. Kalisoga; 17. Jukporong; 18. Bedung; 19. Jenma; 20. Andulang; 21. Gapura; 22. Pakhandangan; 23. Wairklei; 24. Kotauning; 25. Jl. Sugiyopranoto; 26. Wairpare; 27. Permatasari Hotel; 28. Wairplawu; 29. Naiora; 30. Egon; 31. Hoder; 32. Banjava; 33. Wairbleler.

Isolation and purification of DNA genomes: Genetic diversity evaluation of *I. tinctoria* L. from three Indonesian islands were done using Simple Sequence Repeats (SSR) markers. The SSR primer used in this study were the primers used by Sicard *et al.,* (2005), Al-Ashabi (2011), El Fatehi *et al.,* (2013) (Table 2).

The DNA was isolated using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle, 1987). 0.2 g of samples (dry leaves) was combined with 1000 µl washing buffers and grounded using mortar grinder. The solutions were incubated at -20°C for 20 minutes, and then centrifuged at 12000 rpm for 15 minutes. The supernatants were separated, and 1000 µl of buffer extract were added back to the tubes. The tubes were incubated at 65 °C for 20 minutes and were gently shacked after every 5 minutes. After incubation, the tubes were centrifuged at 12000 rpm for 10 minutes. The supernatants were separated to new 2 ml tubes and added with CTAB as much as 1/10 supernatant volumes, 1x volume of CI (Chloroform: Isoamyl alcohol = 24:1) and centrifuged at 12000 rpm for 10 minutes. The supernatants were moved to 1.5 ml tubes and cool isopropanol (0.6x volume) was added, the mixture was homogenized, and incubated at -20°C for 1 hour. After incubation, the tubes were centrifuged at 12,000 rpm for 10 minutes. The supernatants were separated and 1000 µl of cold-absolute ethanol was added. The mixture was centrifuged again at 12000 rpm for 4 minutes. The supernatants were removed and the pellets were dried. After drying 100 µl of TE buffer (ph8) was added to the microtubes and the DNA was stored in the freezer.

Table 1. Number and origin of *I. tinctoria* L. from Java, Madura and Flores Islands.

No.	Collection number	Location			
		Village	District	District/City	Province
1.	yin001-yin005	Piyungan	Tepus	Gunung Kidul	Yogyakarta
2.	yin006-yin008	Jl Dandeles	Purwodadi	Purworejo	Middle Java
3.	yin021-yin025	Kalisoga	Burneh	Bangkalan	Eastern Java
4.	yin 026- yin 028	Jl Halim Perdana Kusuma	Burneh	Bangkalan	Eastern Java
5.	yin 029- yin 032	Jukporong	Tanjungbumi	Bangkalan	Eastern Java
6.	yin 033- yin 035	Bedung	Tanahcileng	Pamekasan	Eastern Java
7.	yin 036- yin 038	Jelma	Tejatimur	Pamekasan	Eastern Java
8.	yin 039- yin 041	Tlanakan	Tlanakan	Sampang	Eastern Java
9.	MRH001	Pakandhangan Tengah	Bluto	Sumenep	Eastern Java
10.	MRH002-003	Gapuro Timur	Gapuro	Sumenep	Eastern Java
11.	MRH004	Andulang	Gapuro	Sumenep	Eastern Java
12.	yin045- yin057	Godonggede	Kerek	Tuban	Eastern Java
13.	yin068- yin072	Pantai Sadeng	Girisubo	Gunungkidul	Yogyakarta
14.	yin077- yin081	Pantai Slili	Tepus	Gunungkidul	Yogyakarta
15.	yin082- yin084	Trisik	Banaran	Kulonprogo	Yogyakarta
16.	yin085- yin087	Stadion Sultan Agung	Sewon	Bantul	Yogyakarta
17.	yin103- yin105	Srigading	Bambanglipuro	Bantul	Yogyakarta
18.	yin106- yin108	Babatan	Gunungjati	Cirebon	Western Java
19.	yin109- yin111	Kejawenan	Penggambiran	Cirebon.	Western Java
20.	yin115- yin117	Sukajaya	Karangantu	Serang	Banten
21.	yin132- yin134	Jl Wonosari	Piyungan.	Bantul	Yogyakarta
22.	yin148	Waiklei	Alok	Sikka	Eastern Nusa Tenggara
23.	yin151- yin153	Kotauning	Alok	Sikka	Eastern Nusa Tenggara
24.	yin160- yin162	wairpare	Alok.	Sikka	Eastern Nusa Tenggara
25.	yin163- yin165	Naiora	Kangae.	Sikka	Eastern Nusa Tenggara
26.	yin178- yin180	Wairbleler	Waigete	Sikka	Eastern Nusa Tenggara

Table 2. SSR Primer used in the DNA amplification of *I. tinctoria* L.

No.	Primer sequence	
	Forward	Reverse
1.	CCAGTACCCCATATTCTTCC	CTGTGTTTGGGTTGTGATGG
2.	AAACATACCCCTGGCAGTTCC	TTCTGACCTAAGAAAGAGCCTGG
3.	CTGTTACGGCACCTGGAAAG	GCAGAGACACACCTTAACCTTG
4.	CATCTTCCTCACCTGCATTC	TTTGGTGAAGATGACAGCCC
5.	CCAACCACATTCTTCCCTACGTC	GCGAGGCAGTTATCTTTAGGAGTG
6.	CGTTAGATCCCGCCCAATAGT	CCGTCCAGGAAGAGCGAGC
7.	CCGTTGCCTGTATTTCCCCAT	CGTGTGAAGTCATCTGGAGTGGTC
8.	GAGGGTGTTTCACTATTGTCACTGC	TTCATGGATGGTGGAGGAACAG
9.	GCCCTAAGGACTGCATTTTG	CCCCTCCTAAACCCTCAATC
10.	TTGTCTTAAATCGGATGGCT	AGCGTTACAGGGTGTTCCTG
11.	AAGAATGACGAAGAGGCGAA	TCAGAAATTCCCTCCCATTG
12.	CTGCTGATGATGTTGTGGATG	GAACACGTGTACGGAGACCA
13.	GTCAGAATCCCCATGTACACAA	CCCTCTCAAAACACCTTCCA
14.	CCCATATCACCATCACCAAA	CATTGTTGAGCATGTTGAAGG
15.	TTTGGAGGCTTTGAGCCTTA	CCCAACAGGGATACCACTTC

DNA Amplification: The DNA was amplified using PCR machine. GoTaq Green PCR mix solution was used as the reagent with 15 SSR primers that have been used for the study of *Lens culinaris* Medikus., *Phaseolus vulgaris* L., and *Vicia ervilia* (Sicard *et al.,* 2005; Al-Ashabi, 2011; El Fatehi *et al.,* 2013). For each SSR-PCR amplification the samples were amplified in 25 µl of solutions consisted of 2.5 µl of DNA, 1 µl of SSR primers, 12.5 µl of GoTaq® Reaction Buffer pH 8.5 (400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl2) and 9 µl water-free nuclease (Promega, USA). The process consisted of the initial denaturation at 94°C for 3 minutes, followed by 30 denaturation cycles at 94°C for 1 minutes, primer annealing at 45–51°C for 45–60 seconds, and elongation at 72°C for 2 minutes, and final elongation at 72°C for 10 minutes (Choudhary *et al.,* 2008; Sicard *et al.,* 2005; Al-Ashabi, 2011; El Fatehi *et al.,* 2013).

Visualization of DNA Ampliclons: The results of PCR were visualized using Agarose Gel Electrophoresis (2%). The DNA was colored using Ethidium Bromide (EtBr). Five µl of ampliclons were poured into the designed well on the gel according to the labels. The electrophoresis was conducted on the buffer solution of TBE 1x (Tris Cli 0.89M, boric acid 0.89M, and EDTA 2 mM) at 80 V for 90 minutes. The band patterns were observed under UV light and captured using UV transilluminator photography (Wise Doc, Korea).

Data analysis: The bands from ampliclons visualization were assumed as the SSR alleles. Each of the bands was scored based on whether the band was to exist or non-exist on the samples. The bands were scored as zero (0) if absent and one (1) if present on each compared samples. The scores were constructed into the binary data matrix and used to quantify the similarity scores using Similarity for Qualitative Data (SIMQUAL) and Simple Matching (SM) coefficient. The phenogram was constructed based on the similarity score using Sequential Agglomerative Hierarchical and Nested Clustering (SAHN) using Unweighted Pair-Group Method Arithmetic Average (UPGMA) method and Principal Component Analysis (PCA) with NTSys PC 2.11a (Rohlf 2004). Population structure was analyzed using Genetic Analysis in Excel (GenAlex) V. 6.501 (Peakall & Smouse, 2012).

Results and Discussions

15 SSR primer markers used to determine the biodiversity of 63 *I. tinctoria* L. samples have been successfully amplifying genomic sequences of the *I. tinctoria* L. with diverse patterns, quantities, and band sizes among samples and primers. The DNA amplification process using 15 SSR primers produced 76 bands. The number of bands amplified by each individual primer was 3–7 bands with 250–750 base pairs (bp) in length (Fig. 2). The number of polymorphic alleles was 76 with degree of polymorphism at 100% (Table 3).

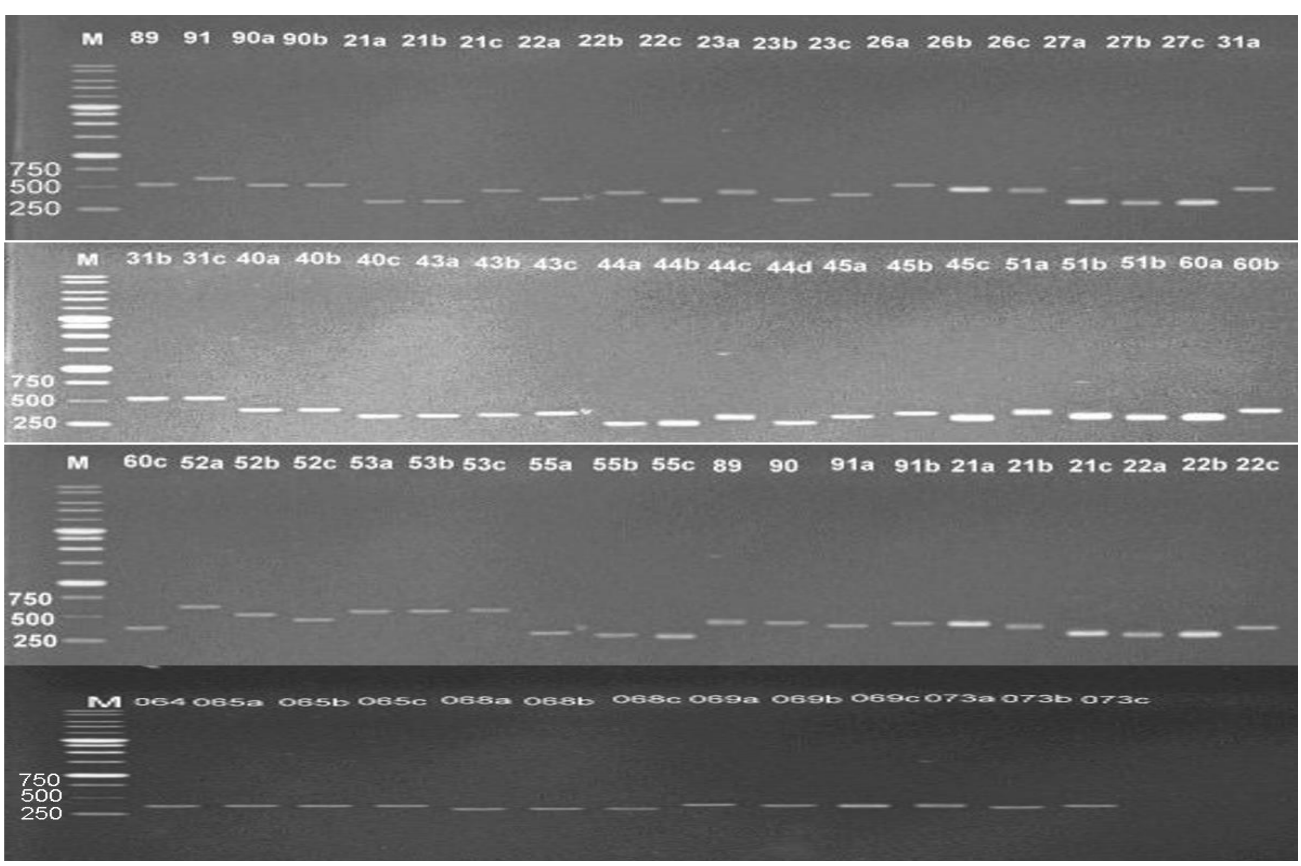

Fig. 2. DNA bands profiles of *I. tinctoria* from three islands, Primers: A1 and annealing temperature: 45–51°C. M= Marker DNA 10.000 bp; 89= Pakhandangan; 91= Andulang; 92a- b= Gapura; 21a- c= Burneh; 22a- c= HPK; 23a-c= Jukporong; 26a-c= Jenma; 27a-c= Tlanakan; 31a- c= Kerek; 40a-c= Pantai Sadeng; 44a-c= KulonProgo; 45a-c= Stadion; 51a-c= Bambanglipuro; 60a-c= Piyungan; 52a-c= Babatan; 53a-c= Kajawenan; 55a-c= Karangantu; 064= Wairklei; 065a- c= Kotauning; 068a- c= Naiora; 069a- c= Luah; 073a- c= Wairbleler

Table 3. Genomic Band Profile of *I. tinctoria* L. with 15 SSR primers.

No.	Primer codes	Fragments (bp)	Number of bands	Number of polymorphic bands
1.	A1	300–550	7	7
2.	A2	250–500	5	5
3.	A4	300–650	5	5
4.	A5	250–500	4	4
5.	B1	450–750	4	4
6.	B2	450–700	5	5
7.	B3	300–550	6	6
8.	B5	500–750	4	4
9.	C1	300–700	6	6
10.	C3	250–450	5	5
11.	C4	460–600	3	3
12.	D1	500–750	4	4
13.	D2	300–650	6	6
14.	D4	250–600	6	6
15.	D5	350–600	6	6
Total			**76**	**76**

Table 4. Genetic diversity indicator of *I. tinctoria* L. from Java, Madura, and Flores Islands.

Population	N	Na	Ne	I	Ho	He	%P
Sumenep	4	2.6	1.8	0.7	0.3	0.4	100
Bangkalan	6	2.9	2.4	0.9	0	0.5	100
Jukporong	3	1.9	1.7	0.5	0	0.4	80
Pamekasan	3	2.5	2.4	0.9	0	0.5	100
Sampang	3	2.1	1.9	0.7	0	0,4	93.3
Tuban	3	2	1.9	0.6	0	0,4	73.3
Gunung Kidul	6	2.5	2	0.7	0	0.5	100
Kulon Progo	4	2.2	2	0.7	0	0.4	86.7
Bantul	9	**3**	2	0.8	0	0.5	100
Cirebon	6	2.9	**2.5**	**1**	0	**0.6**	100
Serang	3	2.2	2.1	0.7	0	0.5	93.3
Kotauning	4	2.2	1.9	0.7	0.1	0.4	93.3
Waioti	6	1.7	1.5	0.4	0	0.2	60
Wairbleler	3	1.5	1.4	0.3	0.1	0.2	53.3
Means	**4**	**2.3**	**1.9**	**0.7**	**0**	**0.4**	**88.1**

Note: Na= Number of effective alleles, Ne= Number of effective alleles, I= Shannon's information index, Ho= Observed heterozygosis, He= Expected heterozygosis, And % P= Polymorphism

C4 primer was producing the least amount of bands (3), while A1 was producing the most (7) (Fig. 2). Visualization of the molecular data as the dendrogram was shown the DNA fragments on certain length (base pairs). DNA fragments of certain length represent an allele of a phenotype.

Results from molecular similarity analysis of 63 *I. tinctoria* samples using 76 molecular markers was shown as a dendrogram (Fig. 3) with the likelihood coefficient of 0.68-100. 100% likelihood coefficient indicates the samples was identical, as shown by the samples from Wairklei3 and Wairklei4. Thus, it can be said that the genetic similarity of *I. tinctoria* of three Indonesia islands was high because have the likelihood coefficient over 50%.

At the likelihood coefficient: 0.70, the populations were grouped into three groups. Group I consisted of 5 populations; Sumenep, a part of Cirebon, and entire Flores (Kotauning, Waioti, and Wairbleler). Group II unifying 7 populations from Bangkalan, Jukporong, Sampang, Tuban, Pamekasan, Kulon Progo, and a part of Cirebon. Group III unifying 3 populations from Gunung Kidul, Serang, and Bantul. Those groups were unifying *I. tinctoria* based on the locations but not based on the islands of origins. That grouping indicates each species within a group has similar (homogeneous) genetic structure, and each of the group was to have different genetic structures compared to others.

Likelihood coefficient of 0.68 was obtained from similarity analysis based on the molecular data, differed from the result based on the morphological data: 0.31. Those results show that the inter-specimen morphological characteristics were highly diverse compared to molecular characteristics. The lowest diversity scores were obtained by Wairklei3 and Wairklei4 genotypes. Diversity scores based on SSR markers were relatively smaller (32%) compared with the diversity scores based on the morphological characteristics (69%). Thus, it appeared that the morphological characteristic variations were due to environmental factors, so there were many specimens from different locations showed different morphological traits. The difference in grouping results based on SSR markers and morphological data were due to the morphological traits were produced from the interaction between genotypes and environmental factors. Otherwise, the SSR markers were specific sequences in the DNA which were not affected by the environmental conditions. From those results, we could say the information obtained both from morphological and molecular data were more accurate to describing the genetic diversity of *I. tinctoria* to support the efforts to develop high-quality *I. tinctoria* cultivars.

Genetic similarity can be seen from the interpopulation likelihood coefficient. High coefficients were shown by Bangkalan-Jukporong populations (0.79), and the lowest one was Kulon Progo-Sumenep population (0.15). High coefficients in Bangkalan-Jukporong populations show the specific bands amplified from the accessions have little differences, which is 300 bp bands for D2, and B3, and 450 bp band for B1. Furthermore, those high similarity scores could be caused by similar habitats and environmental conditions from the locations of those accessions.

Population structures of *I. tinctoria* L.: Genetic diversity of *I. tinctoria* based on the SSR markers varied among 14 observed populations. Numbers of Alleles (Na) were ranged from 1.5–3.0, and the effective allele numbers (Ne) were ranged from 1.4–2.5. The highest Shannon Index was from Cirebon population, and the lowest one was Wairbleler population. Genetic diversity (He) was ranged from 0.2–0.6 (Table 4). The highest genetic diversity was found in Cirebon and the lowest one was found in Waioti and Wairbleler. The high genetic diversity in Cirebon population can be seen from the dendrogram. The Cirebonese population was scattered across the different groups with low similarity. Accessions from Cirebon1 and Cirebon2 grouped with populations from Madura and Flores. Meanwhile, accession from Cirebon 3–6 grouped with other Javanese populations. Those results show that the Cirebonese specimens have great potentials as the genetic resources for Indigofera.

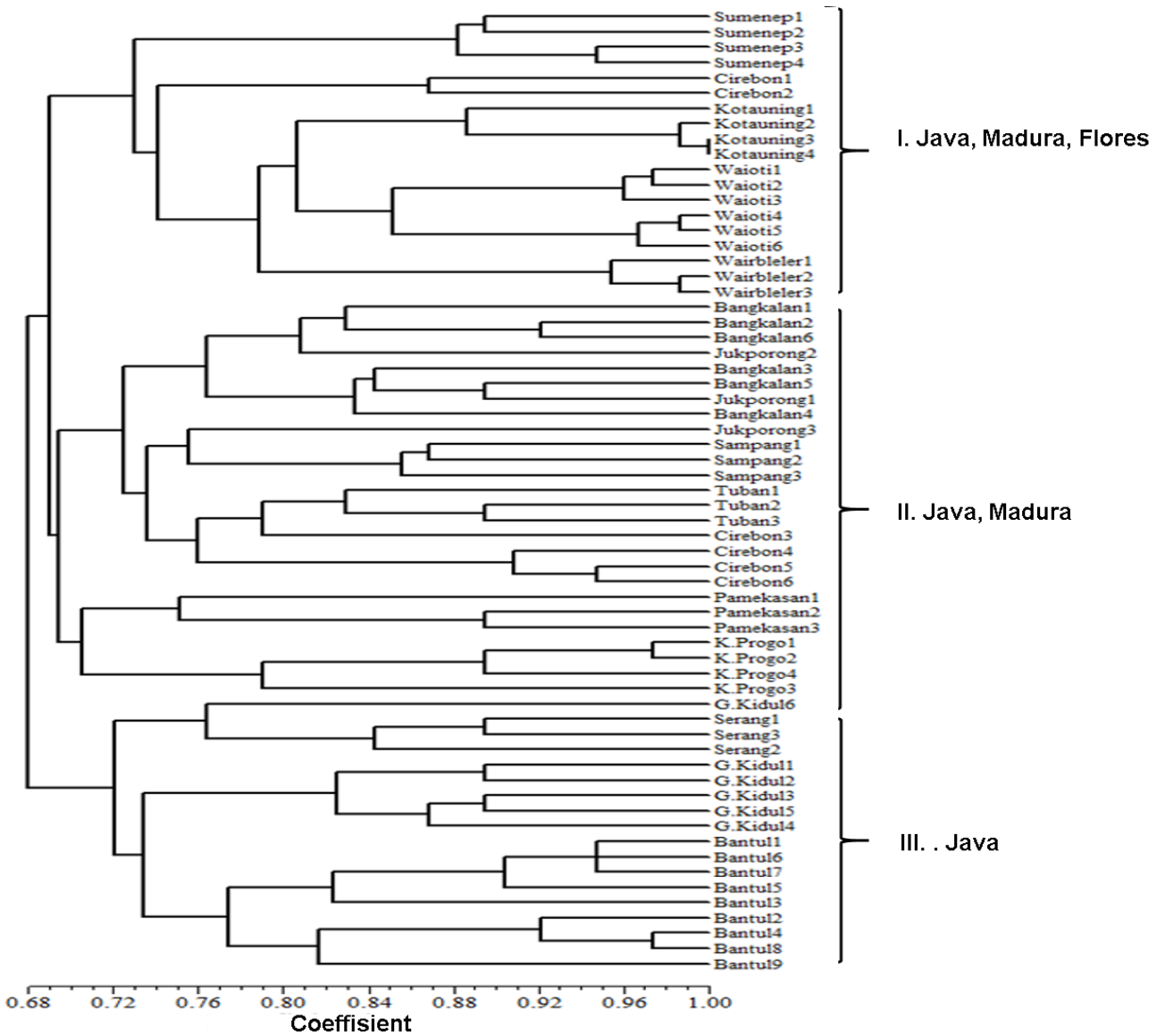

Fig. 3. Dendrogram grouping of 63 *I. tinctoria* samples based on SSR data analyzed with the likelihood coefficient and UPGMA.

High polymorphism scores (p>60) were found in 12 populations, meanwhile, two populations-Waioti and Wairbleler-had low polymorphism scores (Table 4). Polymorphism score can be used to determine the genetic diversity of a species. The higher polymorphism score, the higher genetic diversity will be. Thus, it can be said that those 12 populations have very informatics loccuses to explain the genetic diversity of studied Indigofera population. The average polymorphism score of 88.1%, and the highest of 100% found in six populations; Sumenep, Bangkalan, Pamekasan, Gunung Kidul, Bantul, and Cirebon. In the present study higher polymorphism was found in *I. tinctoria* compared to earlier studies in *Vicia faba* 55%; *V. ervilia* 65%; and *V. sativa* 62% (Suresh *et al.*, 2013; Chung *et al.*, 2013; El Fatehi *et al.*, 2013). Those variations were due to differences in the number of samples, types, used primers, and taxanomic status (Qiang *et al.*, 2015; Chung *et al.*, 2013). (*Punica granatum*) which producing the low quantity of polymorphic bands. The abundance of the amplified polymorphic bands from *I. tinctoria* genomes resulted from proper primers and markers selection.

Accessions from Sumenep and Cirebon were grouped with the Floresi with high likelihood coefficient (>50%). Accessian from Sumenep, Cirebon and Flores had high genetic similarity. Most of the morphological differences between Javanese and Floresian populations were due to differences in the habitats and environmental factors. The Floresian habitats have higher temperature and light intensity compared to Javanese and Maduranese one. From those facts, the formation of Floresian Indigofera infra-species was not yet possible.

Conclusion

SSR primers can be used to determine the genetic diversity of *I. tinctoria*. Fifteen SSR primers have been successfully amplifying DNA 63 *I. tinctoria* accessions and producing 76 polymorphic bands with 100% polymorphism score. The band patterns can be used to distinguish accession groups and locations, but not based on the island of origin. Genetic diversity of *I. tinctoria* from three Indonesian islands had the average score of 0.4, the highest one was Cirebonean populations (0.6) and

the lowest were the populations from Waioti and Wairbleler (0.2). Populations from Sumenep, Cirebon, and Flores had high genetic similarity. Breeding strategy with selections should be focused on the population with highest genetic diversity; Cirebon. The Floresian *I. tinctoria* population was a variant, and not qualified as an infra-species, although have distinct morphological traits compared with Javanese and Maduranese populations.

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