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GENETIC VARIABILITY AND RELATIONSHIP OF TEMU GLENYEH *(Curcuma soloensis* Val.) FROM JAVA, INDONESIA

M. JALIL[1,2,*], A. PURWANTORO[3], B.S. DARYONO[4] and PURNOMO[5*]

[1]Postgraduate Student, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2]Faculty of Tarbiyah, Institute Agama Islam Negeri Kudus, Indonesia
[3]Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia
[4]Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[5]Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
*Corresponding author email: emjie.jack@gmail.com, purnomods@ugm.ac.id
Email addresses of coauthors: ronsasm@hotmail.com, bs_daryono@mail.ugm.ac.id

SUMMARY

Temu Glenyeh (*Curcuma soloensis* Val.) belongs to the family Zingiberaceae. Although it is synonymous with *Curcuma longa* L., the two are different species. Given that the morphological characters of this herb overlap with those of other *Curcuma* species, distinguishing these species on the basis of morphological criteria is difficult. The aim of this study was to determine the genetic variation and relationship of *C. soloensis* Val. on the basis of molecular characters by using intersimple sequence repeats (ISSRs). The samples consisted of 32 accessions and were obtained from 12 districts in Java Island. Ten ISSR primers were used in amplification. Molecular data were analyzed numerically in the form of binary data. The similarity indexes were obtained on the basis of the Jaccard coefficient by using the UPGMA method. Cluster and principal component analyses (PCA) were performed with the MVSP 3.1 program and UPGMA method. This investigation revealed that the 10 ISSR primers provided 37 ISSR bands comprising 28 polymorphic and nine monomorphic bands. Cluster analysis yielded two large groups, namely A (*Curcuma zanthorrizha* Roxb.) and B (*C. soloensis* Val. and *Cucurma longa* L.) with a coefficient of 0.566. *C. zanthorrizha* Roxb. (Temulawak) and *C. longa* L. (turmeric) belonged to the same genus as *C. soloensis* Val. The 0.70 phenon line showed that two accessions of *C. longa* L. (CL-1 and CL-3) clumped with all other accessions of C*. soloensis* Val. from Java. The PCA findings indicated that the characters that played the most prominent role in managing the grouping of *C. soloensis* Val. accessions were the primers SPS3 (515 and 425 bp), HB12 (207 bp), HB14 (351 bp), P3 (1083 and 651 bp), 811 (345), 817 (502 and 402 bp), and 824 (371 bp).

Keywords: Genetic variability, relationship, morphological traits, principal component analysis, ISSR markers, *Curcuma soloensis* Val., *Curcuma longa* L., *Curcuma zanthorrizha* Roxb

Key findings: By using ISSR markers, this investigation detected sufficient genetic variability among the accessions of *C. soloensis* Val. from Java, Indonesia, for classification and grouping purposes.

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INTRODUCTION

Curcuma soloensis Val. (Temu Glenyeh) is a medicinal plant that belongs to family Zingiberaceae. Valeton (1918) stated that this species was received from Surakarta under the name of "*Gelenje*" or "*Belenje*". This species is widely cultivated in Southeast Asia (Zhang *et al*. 2011). *C. soloensis* Val. is synonymous with *Curcuma longa* L. (Kew Science, 2020). *C. soloensis* Val. and *Curcuma longa* L. are considered as separate species due to their rhizome colors (Valeton, 1918). However, collectively, both plants belong to the species *Curcuma viridiflora* Roxb (Backer and van-den-Brink, 1968).

Although morphological markers, such as leaves and rhizomes, are often used for identification, these parameters remain confusing (Kress *et al*., 2002; Islam *et al*., 2007). Morphological characters have several weaknesses given their high level of plasticity but can be used quickly (Singh, 2010). *C. soloensis* Val. can be distinguished from *C. longa* L. on the basis of their leaves, pseudo stems, rhizomes, and several other characters (Zaveska *et al*., 2012; Sungkawati *et al*., 2019). Flowering traits are considered effective in identifying the genus *Curcuma*; however, the inflorescence of this genus only blooms once in a year for few days (Sirirugsa *et al*., 2007). Field observations have shown that the flower of *C. soloensis* Val. is terminal and appears once in a year during January to May (Figure 1). Characterization on the basis of morphological characters reported that *C. soloensis* Val. and *C. longa* L. from Java Island, Indonesia, has the similarity index (SI) of 0.665 (Jalil *et al*., 2020). Given that Singh (2010) established the similarity value of 0.65 as the threshold for genera, *C. soloensis* Val. and *C. longa* L. should stand alone as separate species (Singh, 2010). This interpretation can be confirmed through molecular approaches (Das *et al*., 2011).

C. soloensis Val. is an annual herb that has an important role as a medicinal and ornamental plant and is used in cosmetics and kitchen spices (Valeton, 1918; Zhang *et al*., 2011). The plant structure of *C. soloensis* Val. is composed of a rhizome, pseudostem, and compound flowers (Sirirugsa *et al*., 2007; Subositi and Wahyono, 2019). The rhizome of *C. soloensis* Val. also contains compounds obtained in the form of a yellow liquid, namely, sesquiterpenes (Marliyana *et al*., 2018). The extract of *C. soloensis* Val. plants can be used as biological fungicide to control fungal pathogens with a minimum inhibitory concentration of 50–200 mg/µL (Diastuti *et al*., 2019). Heyne (1988) reported that *C. soloensis* Val. is believed by the Javanese to heal itchy wounds, ulcers, and scabies. From a molecular point of view, no systematic research has been previously carried out on *C. soloensis* Val. in Java. Therefore, molecular research is expected to be capable of revealing the genetic variation in *C. soloensis* Val. in Java. Contemporary developments in molecular biology have greatly assisted in the exploitation of the genetic diversity in *Curcuma* (Jatoi *et al*., 2007; Sirirugsa *et al*., 2007).

Molecular techniques, i.e., intersimple sequence repeats (ISSRs), can easily describe the genetic diversity and relationship of *C. soloensis* Val. from Java. ISSR markers are repetitive DNA with sizes of 100–3000 base pairs and are widely distributed throughout the genome; they have higher reproducibility than RAPD markers (Zietkiewicz *et al*., 1994; Karp *et al*., 1997; Souframanien and Gopalakrishna, 2004; Ng and Tan, 2015). ISSR markers are ideal for various studies, especially for studies on genetic variation and diversity (Sikdar *et al*.,

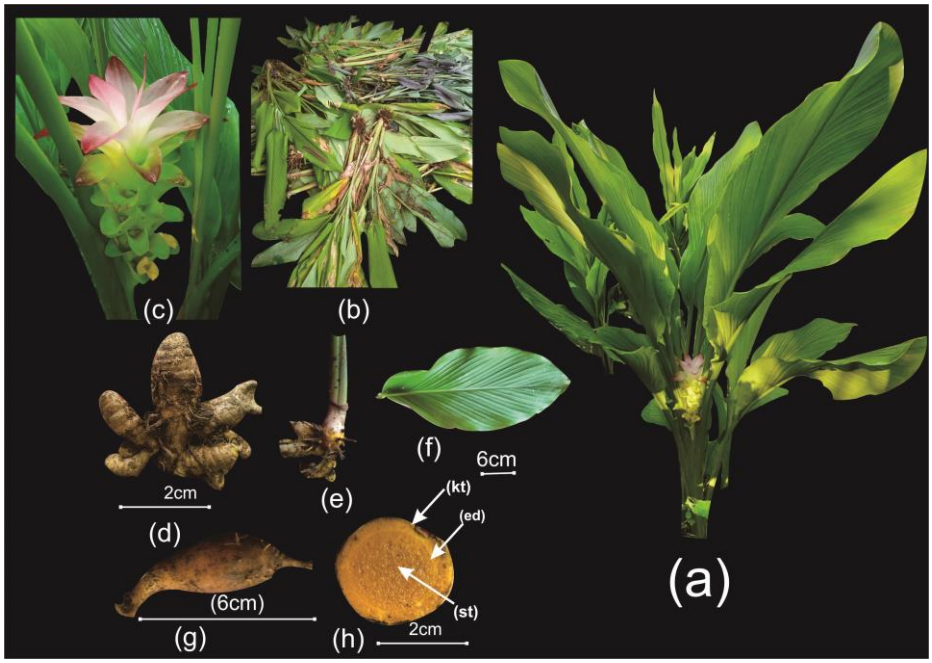

Figure 1. *C. soloensis* Val.: (A) Habit; (b) *C. soloensis* Val. is considered as a a weed; (c) Inflorescence: terminal; (d) Rhizome; (e) stem; (f) leaf: oblong-lanceolate; (g) stipitate tubers; (h) rhizome meat color.

2010; Mohanty *et al*., 2014), DNA fingerprinting (Chaveerach *et al*., 2008), and phylogenetics (Ismail *et al*., 2008). ISSR markers can resolve the various confusing issues in various similar species of *Curcuma* (Syamkumar and Sasikumar, 2007; Das *et al*., 2011). ISSR markers have successfully revealed the percentage of polymorphisms in *Curcuma* species, i.e., 95.60% (Chaveerach *et al*., 2007). The use of ISSR markers in studying genetic variability in the family Zingiberaceae (*Curcuma*) ranks second with a value of 16% (Ismail *et al*., 2016).

Considering the above background, this research is expected to be capable of determining the genetic variation and relationship of *C. soloensis* in Java by using ISSR molecular markers. Genetic variation is necessary for avoiding the possibility of using genotypically similar genotypes, for determining the parenthood of *C. soloensis* Val. varieties, and for support the *C. soloensis* Val. conservation program in Java Island, Indonesia (Jan *et al*., 2010). Therefore, this study aimed to determine the genetic variation and relationship of *C. soloensis* Val. in Java Island on the basis of ISSR molecular characters.

MATERIALS AND METHODS

Genetic material

The sampling of all the accessions was carried out in 12 different districts of Java, Indonesia (Table 1). This molecular study used 32 accessions that consisted of 26 accessions of *C. soloensis* Val. and three each accessions of *C. longa* L. and *Curcuma zanthorrizha* Roxb. The samples were collected on the basis of the information and knowledge of the Javanese people about the plant morphology of *C. soloensis* Val., *C. longa* L., and *C. zanthorrizha* Roxb.

Table 1. Vernacular name, research location, rhizome color, and inflorescence of *C. soloensis* Val. from Java Island, Indonesia.

Accessions	Species Name	Vernacular Name	SubDistrict	Altitude (m asl)	Latitude	Longitude	Rhizome Colour	Presence of Inflorescence
CS-01	C. *soloensis* Val.	Temu Blenyeh	Imogiri	427	7°55’44”S	110°25’58”E	Gray-Orange Group 163-strong orange yellow B	Absesnt
CS-02	C. *soloensis* Val.	-do-	Tirtomoyo	382	7°55’58”S	111°07’31”E	Gray-Orange Group 163-strong orange yellow B	Absent
C2-03	C. *soloensis* Val.	-do-	Tirtomoyo	393	7°55’58”S	111°07’30”E	Gray-Orange Group 163-deep orange yellow A	Absent
CS-04	C. *soloensis* Val.	-do-	Tawangmangu	826	7°38’32”S	111°06’15”E	Gray-Orange Group 163-strong orange yellow B	Absent
CS-05	C. *soloensis* Val.	-do-	Tegalrejo	114	7°47’22”S	110°21’01”E	Gray-Orange Group 163-strong orange yellow B	Present
CS-06	C. *soloensis* Val.	-do-	Karangmojo	226	7°53’19”S	110°41’10”E	Gray-Orange Group 163-strong orange yellow B	Absent
CS-07	C. *soloensis* Val.	-do-	Patuk	153	7°52’47”S	110°31’33”E	Gray-Orange Group 163-deep orange yellow A	Absent
CS-08	C. *soloensis* Val.	-do-	Pabelan	575	7°18’50”S	110°31’39”E	Gray-Orange Group 163-strong orange yellow B	Absent
CS-09	C. *soloensis* Val.	-do-	Pakis	706	7°27’30”S	110°19’05”E	Gray-Orange Group 163-deep orange yellow A	Absent
CS-10	C. *soloensis* Val.	-do-	Dlingo	365	7°55’42”S	110°25’19”E	Gray-Orange Group 163-strong orange yellow B	Absent
CS-11	C. *soloensis* Val.	-do-	Ngadirojo	195	7°51’26”S	110°59’00”E	Gray-Orange Group 163-deep orange yellow A	Absent
CS-12	C. *soloensis* Val.	-do-	Tirtomoyo	168	7°56’45”S	111°02’21”E	Gray-Orange Group 163-strong orange yellow B	Absent
CS-13	C. *soloensis* Val.	-do-	Nawangan	809	7°58’13”S	111°07’48”E	Gray-Orange Group N167-Brownish Orange A	Absent
CS-14	C. *soloensis* Val.	-do-	Nawangan	815	7°58’13”S	111°07’48”E	Gray-Orange Group 163-deep orange yellow A	Absent
CS-15	C. *soloensis* Val.	-do-	Nawangan	816	7°58’13”S	111°07’48”E	Gray-orange group 164-Brownish orange A	Absent
CS-16	C. *soloensis* Val.	Kunir Kuning	Sawoo	239	7°59’35”S	111°34’39”E	Gray-Orange Group 163-deep orange yellow A	Absent
CS-17	C. *soloensis* Val.	-do-	Bendungan	319	8°00’17”S	111°41’54”E	Gray-Orange Group N167-Brownish Orange A	Absent
CS-18	C. *soloensis* Val.	-do-	Tugu	151	8°01’38”S	111°36’30”E	Gray-Orange Group N167-Brownish Orange A	Absent
CS-19	C. *soloensis* Val.	Temu Blenyeh	Tugu	221	8°02’35”S	111°35’50”E	Gray-Orange Group N167-Brownish Orange A	Absent
CS-20	C. *soloensis* Val.	Temu Blenyeh	Sawoo	394	8°01’44”S	111°34’33”E	Gray-Orange Group N167-Brownish Orange A	Absent

Table 1 (cont'd).

Accessions	Species Name	Vernacular Name	SubDistrict	Altitude (m asl)	Latitude	Longitude	Rhizome Colour	Presence of Inflorescence
CS-21	*C. soloensis* Val.	Temu Blenyeh	Pajangan	147	7°51'19"S	110°17'51"E	Gray-Orange Group 163-strong orange yellow B	Present
CS-22	*C. soloensis* Val.	Tembu Blenyeh	Pajangan	147	7°51'17"S	110°17'51"E	Gray-Orange Group 163-strong orange yellow B	Present
CS-23	*C. soloensis* Val.	Temu Blenyeh	Pajangan	148	7°51'18"S	110°17'51"E	Gray-Orange Group 163-strong orange yellow B	Present
CS-24	*C. soloensis* Val.	Temu Blenyeh	Pajangan	145	7°51'19"S	110°17'51"E	Gray-Orange Group 163-strong orange yellow B	Present
CS-25	*C. soloensis* Val.	Temu Blenyeh	Pajangan	145	7°51'20"S	110°17'51"E	Gray-Orange Group 163-strong orange yellow B	Present
CS-26	*C. soloensis* Val.	Temu Blenyeh	Imogiri	427	7°55'44"S	110°25'58"E	Gray-Orange Group 163-strong orange yellow B	Absent
CL-01	*C. longa* L.	Kunir	Tegalrejo	114	7°47'23"S	110°21'02"E	Yellow group 9-vivid yellow A	Present
CX-01	*C. zanthorrizha* Roxb.	Temulawak	Tegalrejo	115	7°47'20"S	110°21'09"E	Gray-Orange Group 163-deep orange yellow A	Absent
CX-02	*C. zanthorrizha* Roxb.	Temulawak	Patuk	148	7°52'45"S	110°31'35"E	Gray-Orange Group 163-deep orange yellow A	Present
CL-03	*C. longa* L.	Kunyit	Sindangkasih	517	7°15'57"	108°13'04"E	Yellow group 9-vivid yellow A	Absent
CL-04	*C. longa* L.	Kunyit	Indihiang	407	7°17'37"	108°11'51"E	Yellow group 9-vivid yellow A	Absent
CX-03	*C. zanthorrizha* Roxb.	Temulawak	Bendungan	529	7°57'33"S	111°42'06"E	Gray-Orange Group 163-deep orange yellow A	Present

Table 2. ISSR primers and base sequences used in PCR amplification.

No.	Primers	Primer sequence	Concentration (μM)	Annealing temperature (°C)
1	SPS 3	(GACA)4 = GACAGACAGACAGACA	20	47.5
2	HB 12	(CAC)3GC = CACCACCACGC	20	44.9
3	HB 13	(GAG)3GC = GAGGAGGAGGC	20	41.2
4	HB 14	(CTC)3GC = CTC CTCCTCGC	20	41.8
5	P3	(AG)8TG = AGAGAGAGAGAGAGAGTG	20	49.5
6	P8	(CAC)5 = CACCACCACCACCAC	20	53.1
7	811	(GA)8C = GA GA GA GA GAC	20	46.7
8	817	(CA)8A = CACACACACACACACAA	20	50.5
9	824	(TC)8G = TCTCTCTCTCTCTCTCG	20	47.5
10	17898B	(CA)6GT = CACACACACACAGT	20	44.6

Table 3. ISSR molecular characters in a binary form used for analysis using UPGMA.

No.	Primers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	SPS3-907 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
2	SPS3-692 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
3	SPS3-515 bp	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	0	0
4	SPS3-425 bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
5	SPS3-302 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0
6	HB12-1092 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	HB12-946 bp	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1
8	HB12-810 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
9	HB12-630 bp	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1
10	HB12-450 bp	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	HB12-400 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	HB12-330 bp	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0
13	HB12-207 bp	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	0	0	0	1
14	HB13-300 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	HB14-885bp	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	HB14-720 bp	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
17	HB14-525bp	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	HB14-351 bp	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1
19	P3-1083 bp	0	1	1	1	1	1	1	1	0	1	1	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1
20	P3-651 bp	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	1	0
21	P3-478 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	P8-1013bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	P8-793 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24	P8-607 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1
25	P8-414 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
26	811-1136 bp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
27	811-1105 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
28	811-578 bp	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
29	811-466 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30	811-345 bp	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0
31	811-220 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
32	817-502 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1
33	817-402 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
34	824-1191 bp	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
35	824-803 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
36	824-371 bp	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0
37	17898B-384 bp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1

Sample order 1. CS-1; 2. CS-2; 3. CS-3; 4. CS-4;5. CS-5; 6. CS-6; 7. CS-7; 8. CS-8; 9. CS-9; 10. CS-10; 11. CS-11; 12. CS-12; 13. CS-13; 14. CS-14; 15. CS-15; 16. CS-16; 17. CS-17; 18. CS-18; 19. CS-19; 20. CS-20; 21. CS-21; 22. CS-22; 23. CS-23; 24. CS-24; 25. CS-25; 26. CS-26; 27. CL-1; 28. CL-3; 29. CX-1; 30. CX-2; 31. CX-3; 32. CL-4.

Isolation of DNA

Isolation was performed with a kit in accordance with the genomic DNA mini kit (plant) procedure from Geneaid. The sample was diluted by using TE buffer (pH 0.8). Isolation was performed with the fresh leaves of *C. soloensis* Val., *C. longa* L., and *C. zanthorrizha* Roxb. on the basis of the protocol of Geneaid's Genomic Kit. Amplification was performed with a PCR mixture with a total volume of 25 µL in a 200 µL tube. The PCR reaction volume was composed of ddH_2O (8.5 µL), MyTaq HS Red Mix (Bioline) (2×) (12.5 µL), ISSR primers (2 µL) at the concentration of 20 µM, and DNA template (2 µL). The initial denaturation reaction was performed at 95 °C for 3 min. Denaturation was performed at 95 °C for 45 s. annealing was performed at 41.2 °C–53.1 °C for 30 s. Extension was performed at 72 °C for 45 s. The final extension was performed at 72 °C (Table 2). The DNA amplification process (PCR) from denaturation to extension comprised 35 cycles. The amplified DNA was then electrophoresed on 2% agarose gel containing 2.5 µL of fluoresce at 50 V for 40 min. The results of the electrophoresis were observed with a UV transilluminator, and the DNA banding pattern (profile) was then photographed.

Data analysis

The data obtained were in the form of binary data, specifically, 0 for the absence of a band and 1 for the presence of a band, through the visualization of the results via electrophoresis (Table 3). Molecular data were numerically analyzed. The SI was calculated on the basis of the Jaccard formula (Sneath and Sokal, 1973). Cluster analysis was performed with the unweighted-pair group method with arithmetic means (UPGMA), and a dendrogram was generated by using MVSP 3.1 software to determine relationships.

RESULTS

The visualization of DNA electrophoresis by using a UV transilluminator with 10 ISSR primers revealed 37 bands comprising 28 polymorphic and nine monomorphic bands (Figure 2). The types of primers, nucleotide arrangement, number, and size of the ISSR bands are presented in Table 4. The ISSR primer that provided the highest number of bands was HB12 (eight DNA bands) with a size of 207–1092 bp. The primers that displayed the most polymorphic bands were SPS3 and 811 with five DNA bands. In previous studies, the primer SPS3 was used to determine the genetic variation in *Curcuma* genotypes; this primer produced 22 DNA bands (Mohanty *et al*., 2014). Polymorphic bands were found with the primers SPS3 (five bands), HB12 (four DNA bands), HB14 (four DNA bands), P3 (two DNA bands), P8 (two DNA bands), 811 (five DNA bands), 817 (two DNA bands), 824 (three DNA bands), and 17898B (one DNA band). The most monomorphic bands were observed with primer HB12 (four DNA bands), then with four other primers i.e., HB13 (one DNA band), P3 (one DNA band), P8 (two DNA bands), and 811 (one DNA band). The primer HB13 showed a single monomorphic band.

On the basis of the appearance of the ISSR bands, cluster analysis was carried out by using the MVSP 3.1 program. Two large groups, namely, groups A and B with a coefficient of 0.566, were generated (Figure 3). Group A consisted of three accessions, whereas group B comprised 29 accessions. In accordance with cluster analysis, group A was further divided into two groups: group A-1 with one accession, namely, CX-2 (*C. zanthorrizha* Roxb. Patuk), and group A-2 with two accessions, specifically, accessions CX-1 (*C. zanthorrizha* Roxb. Tegalrejo) and CX-3 (*C. zanthorrizha* Roxb. Bendungan). Group A separated with a similarity coefficient of 0.745.

Table 4. Primers, nucleotide arrangement, and number of ISSR bands (Chaveerach et al., 2008; Das et al., 2011).

No.	Primers	Primer Sequence	Total bands	Polymorphic bands	Monomorphic bands	Band sizes (bp)
1	SPS3	5'-GACAGACAGACAGACA-3'	5	5	0	302–907 bp
2	HB12	5'-CACCACCACGC-3'	8	4	4	207–1092 bp
3	HB13	5'-GAGGAGGAGGC-3'	1	0	1	300 bp
4	HB14	5'-CTCCTCCTCGC-3'	4	4	0	351–885 bp
5	P3	5'-AGAGAGAGAGAGAGAGTG-3'	3	2	1	478–1083 bp
6	P8	5'-CACCACCACCACCAC-3'	4	2	2	414–1013 bp
7	811	5'-GAGAGAGAGAGAGAGAC-3'	6	5	1	220–1136 bp
8	817	5'-CACACACACACACACAA-3'	2	2	0	402–502 bp
9	824	5'-TCTCTCTCTCTCTCTCG-3'	3	3	0	371–1191 bp
10	17898B	5'-CACACACACACAGT-3'	1	1	0	384 bp
Total			37	28	9	

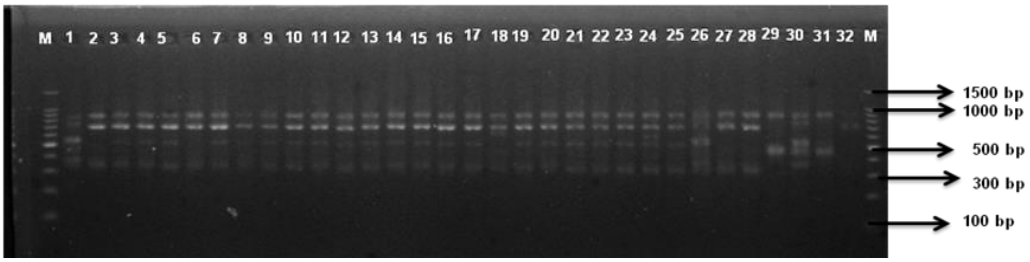

Figure 2. Primary SPS3: 1. CS-1; 2. CS-2; 3. CS-3; 4. CS-4; 5. CS-5; 6. CS-6; 7. CS-7; 8. CS-8; 9. CS-9; 10. CS-10; 11. CS-11; 12. CS-12; 13. CS-13; 14. CS-14; 15. CS-15; 16. CS-16; 17. CS-17; 18. CS-18; 19. CS-19; 20. CS-20; 21. CS-21; 22. CS-22; 23. CS-23; 24. CS-24; 25. CS-25; 26. CS-26; 27. CL-1; 28. CL-3; 29. CX-1; 30. CX-2; 31. CX-3; 32. CL-4.

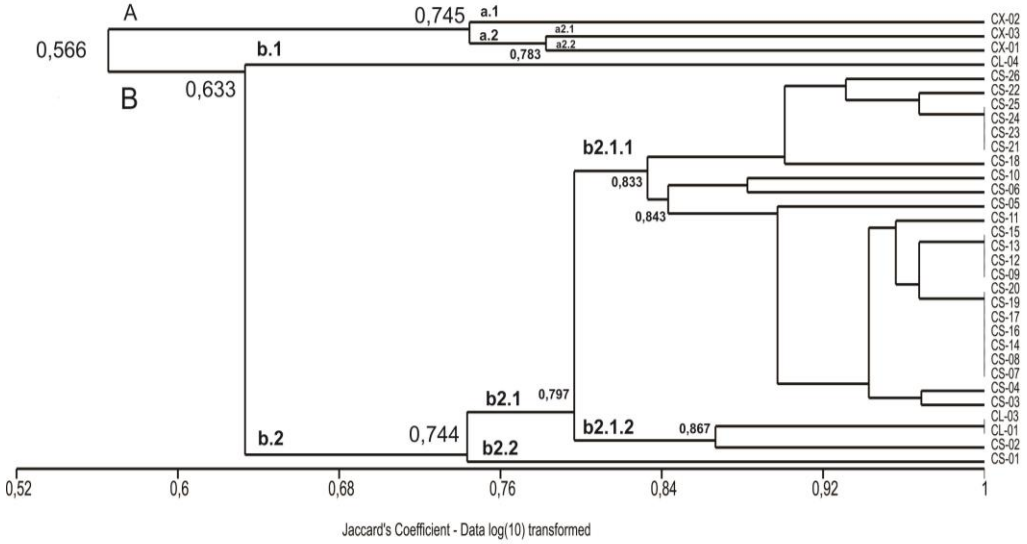

Figure 3. Dendrogram of *C. soloensis* Val. from Java Island, Indonesia, based on ISSR molecular markers.

Subgroup A-2 consisted of subgroup A-2.1 with accession CX-03 (*C. zanthorrizha* Roxb. Bendungan), and subgroup A-2.2 consisted of accession CX-01. The group A-2 subgroups had the similarity coefficient of 0.783. *C. zanthorrizha* Roxb. clustered because of the primers SPS3 (692, 515, and 302 bp) and HB14 (720 bp). Observations in Pacitan and Trenggalek, East Java, revealed that empon-empon farmers admit to selling *C. longa* L. (Turmeric), *C. zanthorrizha* Roxb. (Temulawak), and *C. aeruginosa* Roxb (*Temu Ireng*) to Surakarta and Yogyakarta. The embryos that arrived in Yogyakarta were also likely to be planted by the surrounding community. Thus, *C. zanthorrizha* Roxb. Trenggalek and Yogyakarta were closely related on the ISSR molecular basis and and clustered in clade A (Figure 3). Group A was grouped on the basis of the primary equation SPS3 (907 bp, 425 bp), HB12 (946, 630, 330, and 207 bp), HB14 (885 and 525 bp), P3 (1083 and 651 bp), P8 (607 bp), 811 (345 and 220 bp), 817 (502 and 402 bp), 824 (1191, 803, and 371 bp), and 17898B (384 bp).

Group B consisted of 26 accessions of *C. soloensis* and three accessions of *C. longa* L. (Figure 3). However, three accessions belonging to *C. longa* L. were identified with the accession codes CL-01, CL-03, and CL-04. The determination of the three accessions of *C. longa* L. based on information from the local knowledge of the Javanese community was confirmed in accordance with the morphological criteria of *C. longa* L. Group B was divided into subgroups B-1 and B-2 with a similarity coefficient of 0.633. This grouping was based on primers SPS3 (692 bp), P8 (414 bp), and 811 (1136 bp). Subgroup B-1 only had one accession, i.e., CL-04 (*C. longa* L. Tasikmalaya). These accessions were grouped on the basis of the bands shown by the following primers: HB12 (1092, 810, 450, and 400 bp), HB13 (300 bp), P3 (478 bp), P8 (1013 and 793 bp), and 811 (466 bp).

Subgroup B-2 consisted of 26 *C. soloensis* accessions and two *C. longa* L. accessions, i.e., CL-01, and CL-03. This grouping was based on the similarity coefficient of 0.744 (Figure 3). The ISSR primers that categorized the B-2 subgroup accessions were SPS3 (302 bp) and 811 (1105 bp). Subgroup B-2 consisted of subgroups B-2.1 and B-2.2. Subgroup B-2.2 had only one accession, namely, CS-01 (*C. soloensis* Val. Imogiri).

Subgroup B-2.1 consisted of two subgroups, i.e., B-2.1.1 and B-2.1.2. Primary character 811 (578 bp) separated subgroup B-2.1 with a similarity of 0.797. Subgroup B-2.1.1 consisted of 24 accessions of *C. soloensis* Val. with the accession codes CS-03, CS-04, CS-05, CS-06, CS-07, CS-08, CS-09, CS-10, CS-11, CS-12, CS-13, CS-14, CS-15, CS-16, CS-17, CS-18, CS-20, CS-21, CS-22, CS-23, CS-24, CS-25, and CS-26. Subgroup B-2.1.2 consisted of three accessions, namely, CL-01, CL-01, and CS-02.

Based on the dendrogram and the information obtained for the *C. longa* L. and *C. soloensis* Val. phenon line 70, *C. longa* L. and *C. soloensis* Val., except for CL-04, grouped into the same clade. However, all were grouped into the phenone 63 line. Considering that the similarity values ranged from 0.68 to 0.94, all the accessions were assumed to belong to one species. The dendrogram showed that the similarity value was 1 (100%) for CL-01 with CL-01 (*C. longa* L. Tegalrejo) and CL-03 (*C. longa* L. Ciamis). *C. soloensis* Val. accessions with code CS-07 (Patuk), CS-08 (Pabelan), CS-14 (Nawangan 2), CS-16 (Sawoo), CS-17 (Bendungan), CS-19 (Kucur-Kucur Tugu), and CS-20 (Blumbang Sawoo) had a similarity value of 100%. Likewise, *C. soloensis* Val. CS-09 (Pakis), CS-12 (Tirtomoyo), CS-13 (Nawangan 1), and CS-15 (Nawangan 3) had 100% similarity. Four samples of *C. soloensis* Val. obtained from Pajangan District, namely, CS-21, CS-23, CS-24, and CS-25, also had a similarity value of 100%.

Principal component analysis (PCA) was carried out by using the MVSP 3.1 program. The variables were 32 accessions and 37 cases of ISSR primary characters. PCA was performed to determine the pattern of accession

grouping and to show the role of each character in the grouping process. The results of the PCA of the ISSR molecular characters could be seen in Figure 4. The results further revealed that the characters that played the most important role in the grouping were primers SPS3 (515 and 425 bp), HB12 (207 bp), HB14 (351 bp), P3 (1083 and 651 bp).), 811 (345), 817 (502 and 402 bp), and 824 (371 bp).

DISCUSSION

The results showed that polymorphism in *C. soloensis* Val. reached 75.68%. which confirmed the considerable genetic diversity of this species in Java Island, Indonesia (Table 4). This percentage indicated that the genetic diversity of *C. soloensis* Val. could be ascribed to natural mutation, recombination, and gene migration due to ex-situ domestication (Damayanti, 2012). Sexual crossover is limited in *C. soloensis* Val. accessions. Therefore, the diversity among the genotypes might be due to spontaneous mutation. *Curcuma* flowers generally have a sterile part of the bractea (Delin and Larsen, 2000; Sasikumar, 2005), and this species is propagated through rhizomes (Apavatjrut *et al*., 1999). Genetic diversity caused by mutation may also involve different processes of crossing over, i.e., substitution, inversion, translocation, and deletion. Alterations at the DNA base level that boost diversity can be detected molecularly (Tanksley *et al*., 1995). The detected high variation might be due to essence of the ISSRs, which can detect even small changes in the genome. ISSR primers can detect more DNA bands than RAPD primers, thus showing better potential in evaluating germplasm and identifying the accuracy of identification and exploration of polymorphism in *Curcuma* accessions (Das *et al*., 2011; Ismail *et al*., 2016).

Cluster analysis revealed two major groups, namely, groups A and B, and each group was further divided into subgroups. This grouping was also similar to clustering with morphological characters, i.e., *C. zanthorrizha* Roxb. Bendungan clustered with *C. zanthorrizha* Roxb. Tegalrejo (Jalil *et al*., 2020). Morphologically, *C. zanthorrizha* Roxb. was separated from *C. soloensis* Val. and *C. longa* L. into different collective species (Backer and van-den-Brink., 1968). In past studies, the three species were separated into the Mesantha and Exantha sections (Valeton, 2018). The ISSR molecular data also separated the *C. zedoaria* (Berg.) Roscoe group from the *C. viridiflora* Roxb group (Islam *et al*., 2007). Other past studies also revealed that ISSR markers have the capability to separate the accessions of *C. longa* L. from those of *C. zedoaria* (Berg.) Roscoe. (Das *et al*., 2011). Imogiri traders deliberately mix *C. soloensis* Val., *C. longa* L., and *C. zanthorrizha* Roxb. to prepare herbal concoctions because they have morphological similarities. The result of another study showed that *C. zanthorrizha* Roxb. from East Java (Nganjuk, Kediri) groups with that from the Yogyakarta region (Daryono *et al*., 2011). On average, the said genotype has a plant height of approximately 140 cm and an average biomass content of 263.92 g; however, the *C. soloensis* Val. exhibited diverse quantitative characters (Table 5).

The genus *Curcuma* is difficult to distinguish through the morphological approach because of its large variation due to hybridization (Zaveska *et al*., 2012). Therefore, this genus can be characterized through molecular approaches, such as DNA barcoding (Vinitha *et al*., 2014), and ISSR (Kuras *et al*., 2004; Chaveerach *et al*., 2007; Das *et al*., 2011). *C. soloensis* Val. Tirtomoyo was closer to *C. longa* L. Tegalrejo and Ciamis (Figure 3). *C. soloensis* Val. Tirtomoyo lives wild in the edges of bamboo groves and roadsides, whereas *C. longa* L. is deliberately planted under teak trees. Jalil *et al*. (2020) reported that *C. soloensis* Val. is considered as a weed, and its rhizome is deliberately removed at the edges (Figure 1). ISSR data can be

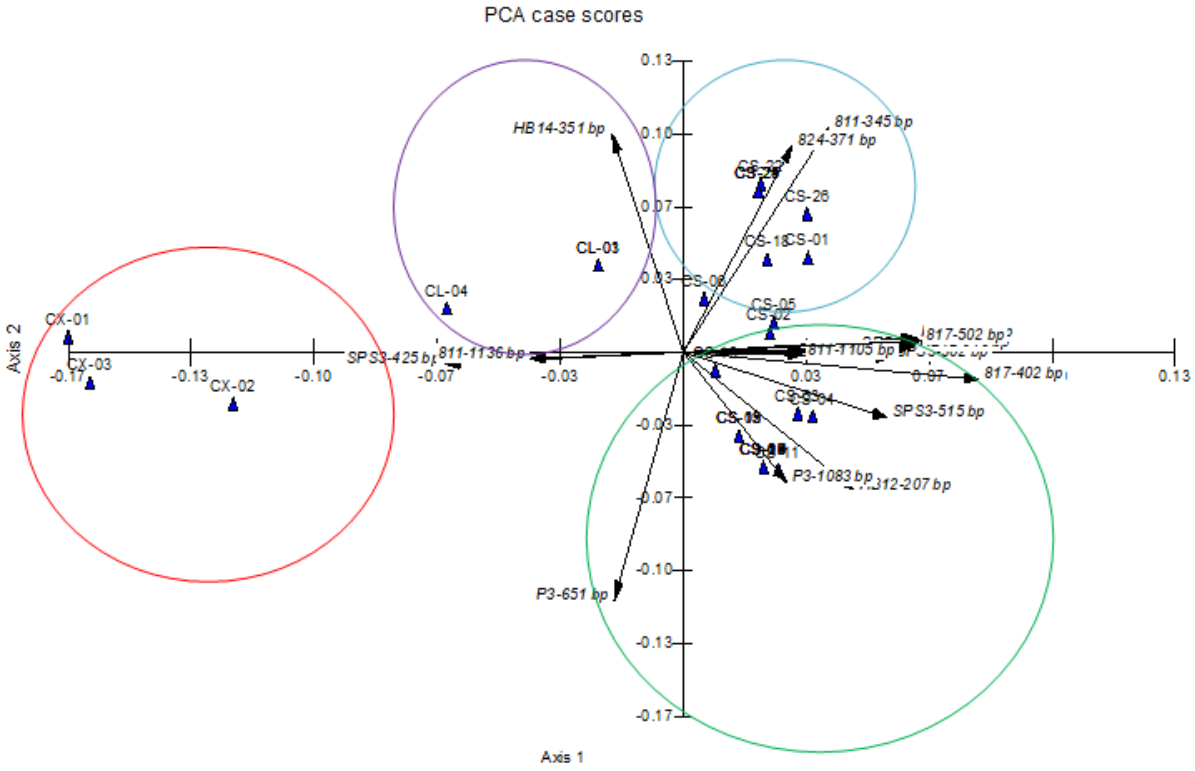

Figure 4. Analysis of the main components of the ISSR molecular characters showing the grouping of *C. soloensis* accessions. The characters that played a vital role in the clustering are shown on the longest arrow and marked with a colored circle. Red color: primary SPS3-425 bp (*C. zanthorrizha* Roxb group. Coded CX). Purple color: HB-14-351 bp primer containing the *C. longa* L. group coded CL. Blue color: primary 811–345 bp, 824–371 bp containing *C. soloensis* Val group with the code CS). Green color: primary 817–502 bp, 817–402 bp, SPS3-515 bp, HB12-207, P3-1083 bp, and P3-651 bp containing a *C. soloensis* Val group with the code CS.

Table 5. An overview showing the characteristics of the plant *C. soloensis* Val. from Java Island, Indonesia.

No.	Characters	Mean	Std. Deviation	Variance	Coeficient of Variation
1	Plant biomass (g)	263.92	163.74	26811.08	0.62
2	Plant height (cm)	140.12	39.14	1531.94	0.28
3	Number of pseudo-stems	12.00	9.714	94.36	0.80
4	Vagina length (cm)	72.72	24.78	614.04	0.34
5	Lamina length (cm)	62.00	17.91	320.58	0.29
6	Leaf width (cm)	17.64	3.93	15.41	0.22
7	Number of leaves in a pseudo-stem	6.00	1.66	2.75	0.27
8	Root length (cm)	13.68	6.01	36.14	0.44
9	Number of branching rhizomes	4.00	1.44	2.08	0.35
10	Number of rhizome segments	9.00	1.88	3.53	0.21
11	Diameter of primary rhizomes (cm)	4.36	0.86	0.74	0.20
12	Perimeter of primary rhizomes (cm)	13.00	2.16	4.67	0.17
13	Length of secondary rhizomes (cm)	8.20	2.48	6.17	0.30
14	Diameter of secondary rhizomes (cm)	2.208	0.50	0.25	0.23
15	Perimeter of secondary rhizomes (cm)	7.36	1.04	1.07	0.14

used to evaluate genetic diversity at the species level and below the species level, such as cultivars (Isshiki *et al*., 2008; Lal *et al*., 2012).

PCA confirmed the role of each character in the grouping of *Curcuma* accessions. Usually, influential characters have an eigen value ≥ 2.00 (Steven and Tello, 2014) as shown by arrows of different lengths. Long arrows are indicative of the character's imported role in the grouping. PCA revealed that seven ISSR primers, i.e., SPS3, HB12, HB14, P3, 811, 817, and 824, played a vital role in the grouping. On the basis of previous findings, the seven primers were polymorphic and were used to distinguish the infrageneric genus *Curcuma* (Chaveerach *et al*., 2008; Das *et al*., 2011). The ISSRs showed sufficient polymorphism to distinguish among various genotypes of *Curcuma* spp. (Reddy *et al*. 2002), Zingiberaceae (Mohanty *et al*., 2014; Ismail *et al*., 2016), and other families (Pratami *et al*., 2020).

CONCLUSIONS

The populations of *C. soloensis* Val. in Java, Indonesia were concluded to reveal the highest genetic variability on the basis of the data obtained with ISSR markers. Ten ISSR primers yielded 37 ISSR bands that consisted of 28 polymorphic and nine monomorphic bands. The cluster analysis revealed two larger groups, namely, A (*C. zanthorrizha* Roxb.) and B (*C. soloensis* Val. and *C. longa* L.) with a coefficient of 0.566. The phenon line (0.70) showed that two accessions (CL-1 and CL-3) of *C. longa* L. clumped with all other accessions of *C. soloensis* Val. The PCA results revealed that the characters that played the most prominent role in the grouping were primers SPS3 (515 and 425 bp), HB12 (207 bp), HB14 (351 bp), P3 (1083 and 651 bp), 811 (345), 817 (502 and 402 bp), and 824 (371 bp).

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