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Berlian, SOJ A3 and Jawa Local Rice Varieties Phylogenetically Distinct From *Oryza sativa* Indica and *Oryza sativa* Japonica

Yudrik Lathif[1], Riri Wiyanti Retnaningtyas[1], Dwi Listyorini[1,3*], Suharti[2,3]

[1]*Department of Biology, FMIPA Universitas Negeri Malang*
[2]*Department of Chemistry, FMIPA Universitas Negeri Malang*
[3]*Biotechnology Division Central Laboratory of Mineral and Advanced Material FMIPA Universitas Negeri Malang*

[*]*Corresponding author*
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Abstract

The genetic resources identification of Indonesian local rice varieties is a crucial work should be done to conserve our native germplasm. This research aimed to know the taxonomical position of East Java local rice varieties including Jawa (JW), Berlian (BR), and SOJ A3 (SJ) using DNA barcode based on rbcL gene. Total DNA of each sample was isolated from leaves. A pair of forward 5'-ATG TCA CCA CAA ACA SJA AC-3' and reverse 5'-TCG GTA CCT GCA GTA GC-3' primers were used to amplify fragments of rbcL gene resulting in 751bp, 755bp, and 754bp fragments from BR, SJ, and JW varieties, respectively. Phylogenetic tree reconstruction revealed that our three local varieties were forming a cluster separated from the widely cultivated subspecies Oryza sativa Indica and Oryza sativa Japonica. However, further studies are necessary to reveal a more precise position of the local varieties in a phylogenetic tree on the species level.

1. INTRODUCTION

Since the dawn of agricultural revolution in the history of humankind, rice (*Oryza sativa*) has been one of the most cultivated crops on Earth (Harari, 2014), and becomes the world's most important cereal grain (Khush, 1997; Gross & Zhao, 2014) that sustain over half the human population as it mainly cultivated in Afro-Asia region (Torre, et al., 2015; Khush, 1997; Gonzalez, et al., 2009). With the record on rice domestication as early as 10000-8000 BC (Sweeney & McCouch, 2007; Gross & Zhao, 2014). Asian culture revolves around rice cultivation (Fuller, 2011), implying the considerable dependency on rice by Asian in general including Indonesia (Fuller & Weisskopf, 2011).

As one of the Southeast Asian countries with agriculture deeply rooted in its culture and economy, Indonesia's dependency on rice is undeniable (Iskandar, et al., 2018). Indonesia is recorded as a country with high rice consumption; up to 150 kilograms per capita in 2017 (Cox, et al., 2017). This dependency on rice has encouraged cultivations of rice, including hybridization to create the most desirable traits of rice and to increase the crop yield (Gross & Zhao, 2014; Fuller & Weisskopf, 2011). A program called Revolusi Hijau (Green Revolution) in Indonesia in circa 1960 was one of the examples of an endeavor in establishing food security for Indonesian by increasing the crop yield as well as the quality to suffice the high consumption of rice (Permana, 2015). This program introduced hybrid rice varieties possessing higher crop yield and other attributes to local farmers. However, the introduction of hybrid rice varieties somehow affects the survival of local rice varieties (Iskandar, et al., 2018).

Berlian, SOJ A3, and Jawa are several amongst others of East Javan local varieties which face the same threat from Green Revolution. Berlian and SOJ A3 are mostly cultivated in Banyuwangi, and Jawa is primarily cultivated in Malang. Despite their potentials, these local varieties are threatened as less and fewer farmers plant them (Iskandar et al., 2018).

So far, these local varieties are less known for their origin and taxonomy since taxonomical identification on these local varieties are mostly carried out based on phenotypic characteristics (Wahab, et al., 2014). However, when it comes to the exact taxonomical status or their exact evolutionary origin, phenotypic identification will generate even more grey areas due to the phenotypic plasticity in plants, especially in cultivated crops (Pregitzer, et al., 2013). Meanwhile, to promote their importance in agriculture, further studies are necessary especially about their phylogenetic relationship to the widely cultivated rice *Oryza sativa* Indica and *Oryza sativa* Japonica.

A study on a molecular level such as DNA barcoding provides an alternative method of taxonomical identification with more accurate and unbiased (Virgilio, et al., n.d.). The molecular marker that is used in this study is rbcL gene, that can serve as DNA barcode in plants (Savolainen et al., 2000; Hollingsworth et al., 2016). The chloroplast gene rbcL (cpDNA) (Suzuki & Makino, 2013) is one of the molecular markers commonly used for a plant genetic identification (Kumar, et al., 2015) as it is used for most crop and other plants (Hollingsworth, et al., 2011). Moreover, the phylogenetic tree based on this gene will reveal the genetic diversity as well as genetic relationship of local varieties of rice in East Java and shed a light on their origin and taxonomic status as a stepping stone in further studies about the genetic diversities and evolutionary origin of local rice in East Java (Savolainen, et al., 2000). In light of that, this study aims to reveal the genetic relationship of East Javan local rice varieties and to reconstruct a phylogenetic tree based on the rbcL gene.

2. MATERIALS AND METHODS

DNA Isolation

The samples in this study were the leaves of Berlian (BR), SOJ A3 (SJ) and Jawa (JW) taken from their respective original plantations in Banyuwangi for BR and SJ; and Malang for JW. As much as 100 grams of leaf samples from each local variety were ground with liquid nitrogen to produce fine powder before the lysis process in DNA isolation. The DNA isolation of the respective samples were carried out according to the DNA isolation protocol provided by *Nucleospin*™ (Macherey Nagel™, Germany). The DNA quantification of each sample was carried out using *NanoDrop* Spectrophotometer.

Gene Amplification

*rbc*L gene was amplified using forward and 5 '-ATG TCA CCA CAA ACA SJA AC-3 ' and reverse 5 '-TCG GTA CCT GCA GTA GC-3 ' primers (Bafeel, et al., 2011). The amplification process was performed on Techno® thermal cycler with 94 °C pre-denaturation for 4 minutes, and 35 cycles of denaturation in 94 °C for 30 seconds, annealing in 55 °C for 30 seconds, and extension in 72 °C for 1 minute, followed by a final-extension in 72 °C for 7 minutes (Singh, et al., 2017 with modifications). Electrophoresis of the PCR product was carried out using 1 % of aSJrose gel in 100 v for 30 minutes.

DNA Sequencing

The sequencing of the rbcL gene was performed using the Sanger method in FirstBase Laboratories, Malaysia.

Sequence Analysis

The chromatogram of each DNA sequence of BR, SJ, and JW was read using FinchTV and analyzed BioEdit software. Consensus sequences from forward and reverse sequences were developed using DNABaser software. BLAST analysis was then done to confirm whether the amplified fragments are the fragment of a targeted gene or others. The alignment of the sample was carried out using ClustalX 2.1 software, with reference sequences mined from GenBank based on BLAST, such as *Oryza sativa* Indica and *Oryza sativa* Japonica with *Zea mays* as outgroup. Following that was phylogenetic tree reconstruction using Neighbor-Joining Method based on Kimura 2-parameter with the bootstrap value of 1000 and pairwise genetic distance to compute the genetic distance of BR, SJ, and JW sequences compared to that of the reference sequences. NJ method can deal with a large amount of sequence information on a personal computer, and a bootstrap test can be easily performed (Kang et al., 2017). The optimal topology was easily generated when an evolutionary tree was built by using the NJ method (Lahaye et al., 2008).

3. Results

The total DNA of 92.01 ng/µl from BR, 136.65 ng/µl from SJ and 186.68 ng/µl from JW were obtained with A260/280 values within the range of 1.8 – 2.0 (Table 1); indicating that all the DNA samples were sufficient and pure to serve as DNA template for the gene amplification process.

Table 1. Spectrophotometry results of local rice East Java.

Sample	A260/A280	Consentration (ng/µl)
BR	1,86	92,01
SJ	1,79	136,65
JW	1,67	186,68

The *rbc*L gene amplification and electrophoresis results indicated that the targeted gene of approximately 700 bp was obtained (Figure 1). The sequencing of BR, SJ, and JW resulted in 751bp, 755bp, and 754bp gene fragments from BR, SJ, and JW rice varieties, respectively; with some with some mutations including substitution, deletion, or insertion (Fig. 4). BLAST analysis confirmed that the amplified fragments are *rbc*L gene as all the samples showed an average 99 % of similarity with the reference sequences on *GeneBank* database with the query coverage of 99-100 % (Figure 2, Figure 3).

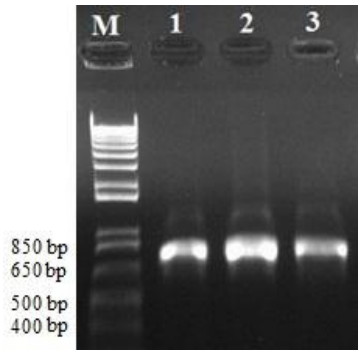
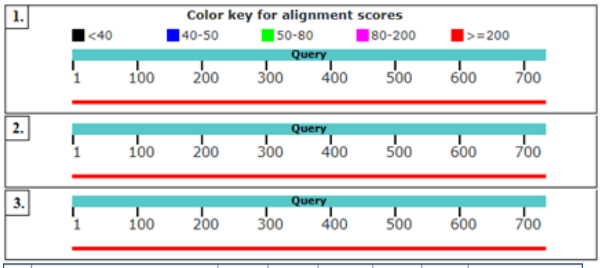
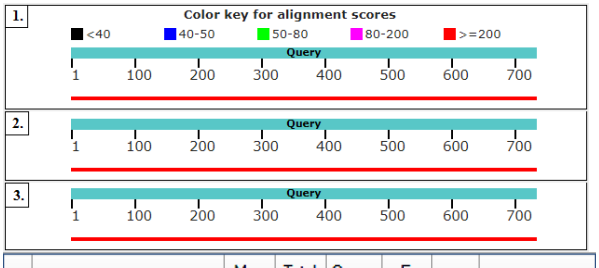


Figure 1. Electrogram of amplified *rbc*L gene results from East Java local rice varieties. M = Marker 1kb plus DNA ladder; 1 = BR; 2 = SJ; 3 = JW varieties



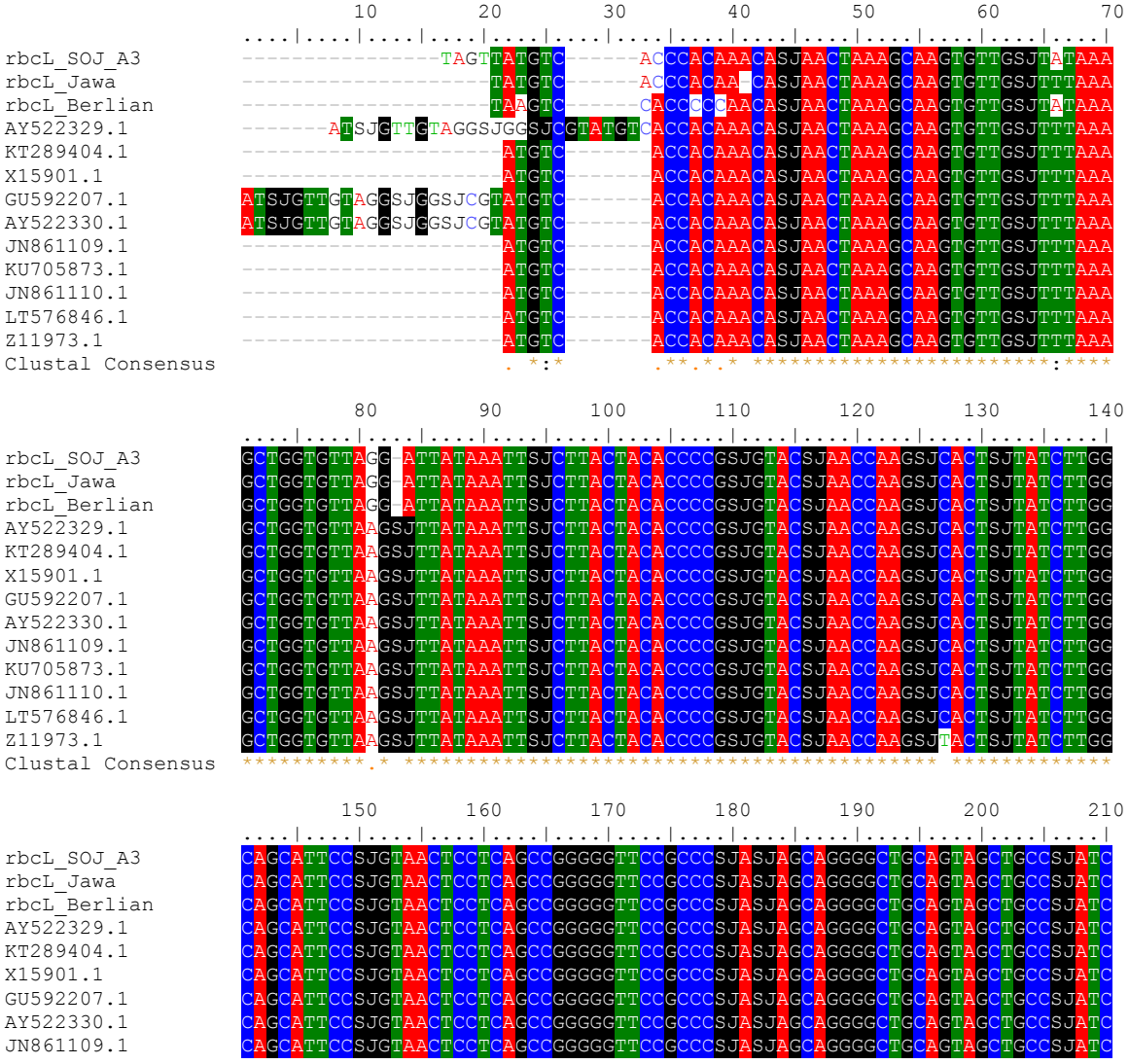
Description	Max score	Total score	Query cover	E value	Ident	Accession
rbcL Berlian	1279	1279	100%	0.0	98%	Query_148673
rbcL SOJ A3	1293	1293	99%	0.0	99%	Query_48853
rbcL Jawa	1304	1304	99%	0.0	99%	Query_52047

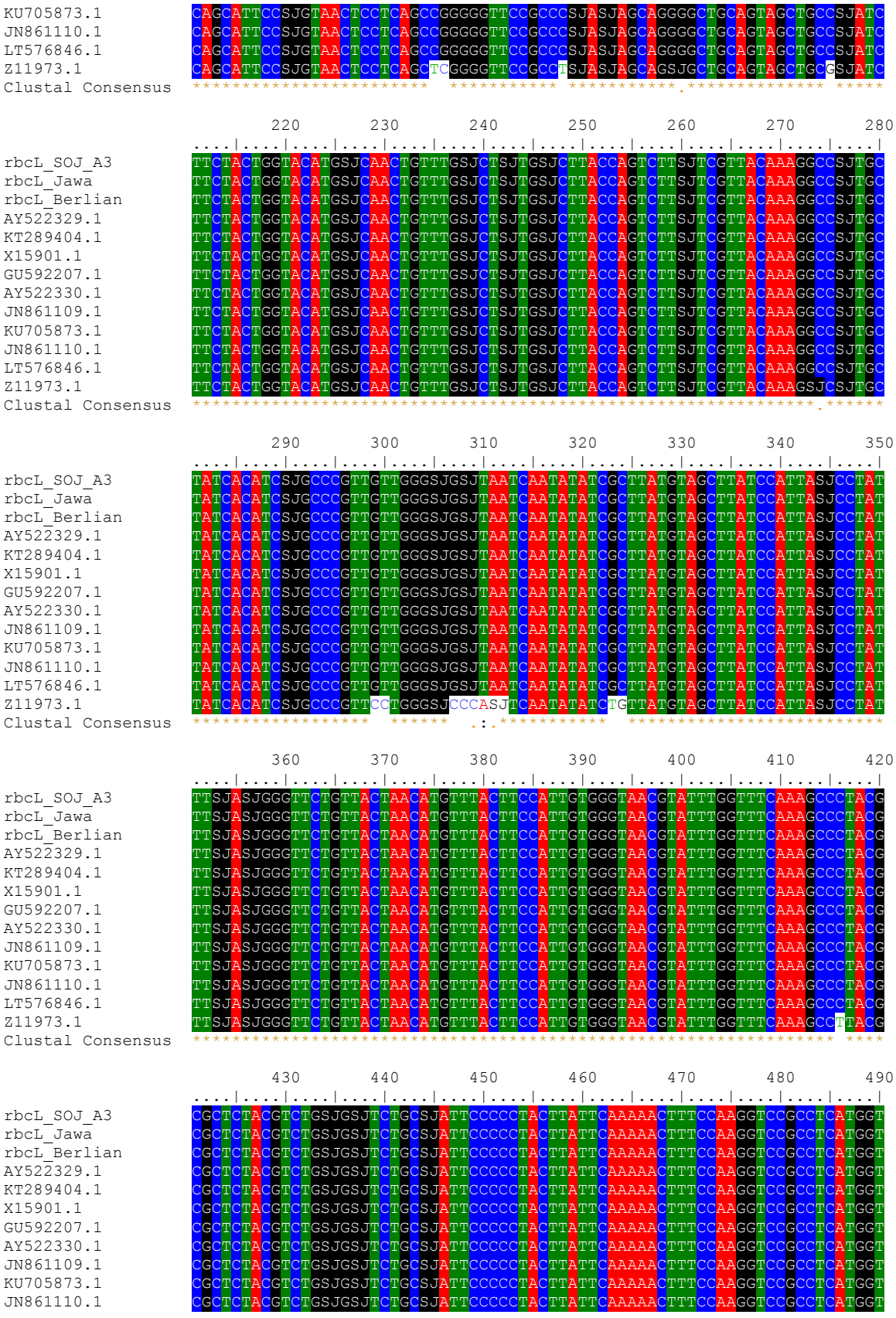
Figure 2. BLAST analysis result of *rbc*L gene from East Java local rice varieties compared to *Oryza sativa* Indica. 1) BR; 2) SJ; 3) JW varieties



Description	Max score	Total score	Query cover	E value	Ident	Accession
rbcL Berlian	1279	1279	100%	0.0	98%	Query_166661
rbcL SOJ A3	1293	1293	99%	0.0	99%	Query_165347
rbcL Jawa	1304	1304	99%	0.0	99%	Query_44251

Figure 3. BLAST analysis result of *rbc*L gene from East Java local rice varieties compared to *Oryza sativa* Japonica. 1) BR; 2) SJ; 3) JW varieties.




KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
220 230 240 250 260 270 280
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
290 300 310 320 330 340 350
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
360 370 380 390 400 410 420
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
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AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
430 440 450 460 470 480 490
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
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GU592207.1
AY522330.1
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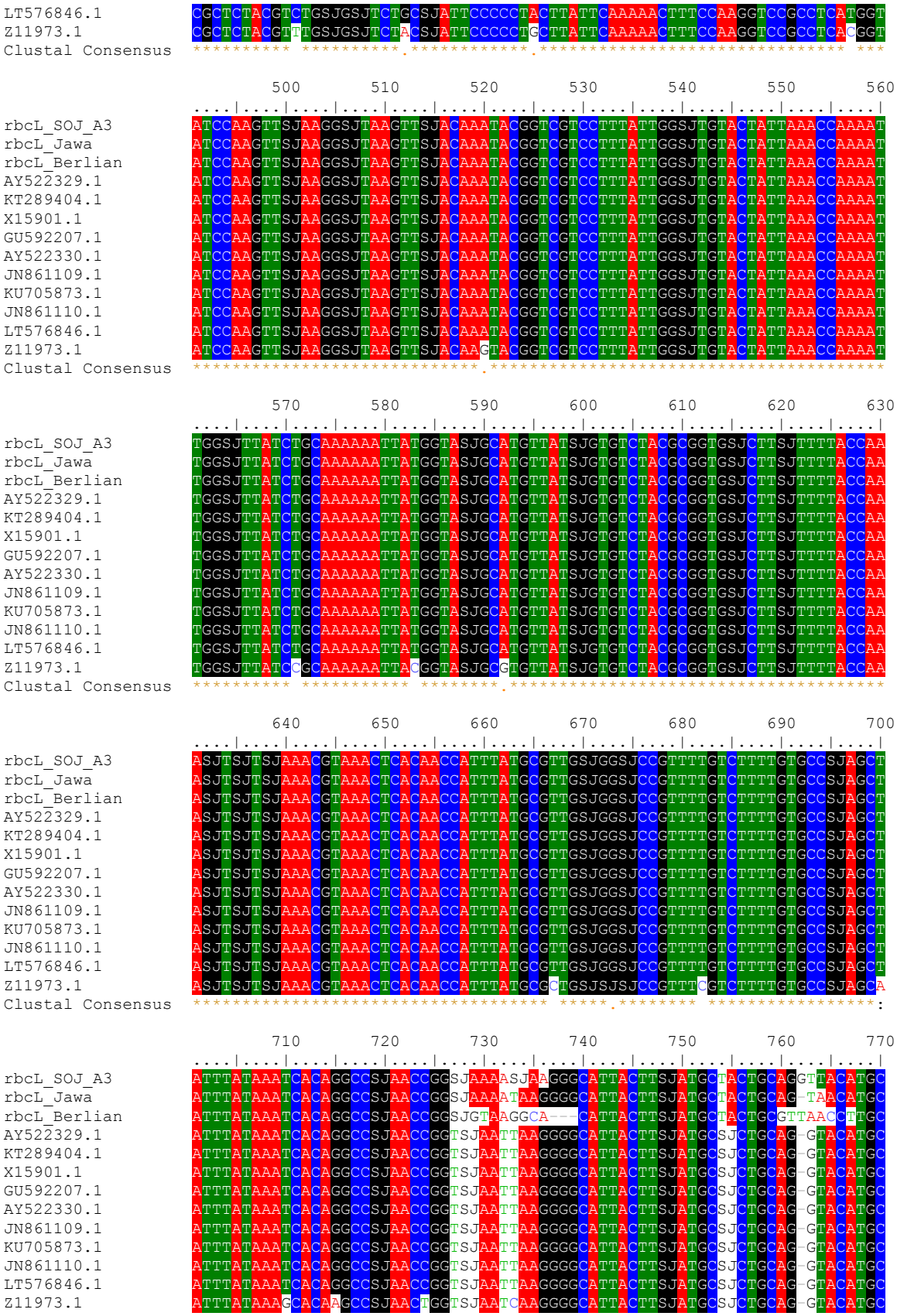
KU705873.1
JN861110.1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Z11973.1
Clustal Consensus
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rbcL_Jawa
rbcL_Berlian
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KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
570 580 590 600 610 620 630
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
640 650 660 670 680 690 700
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
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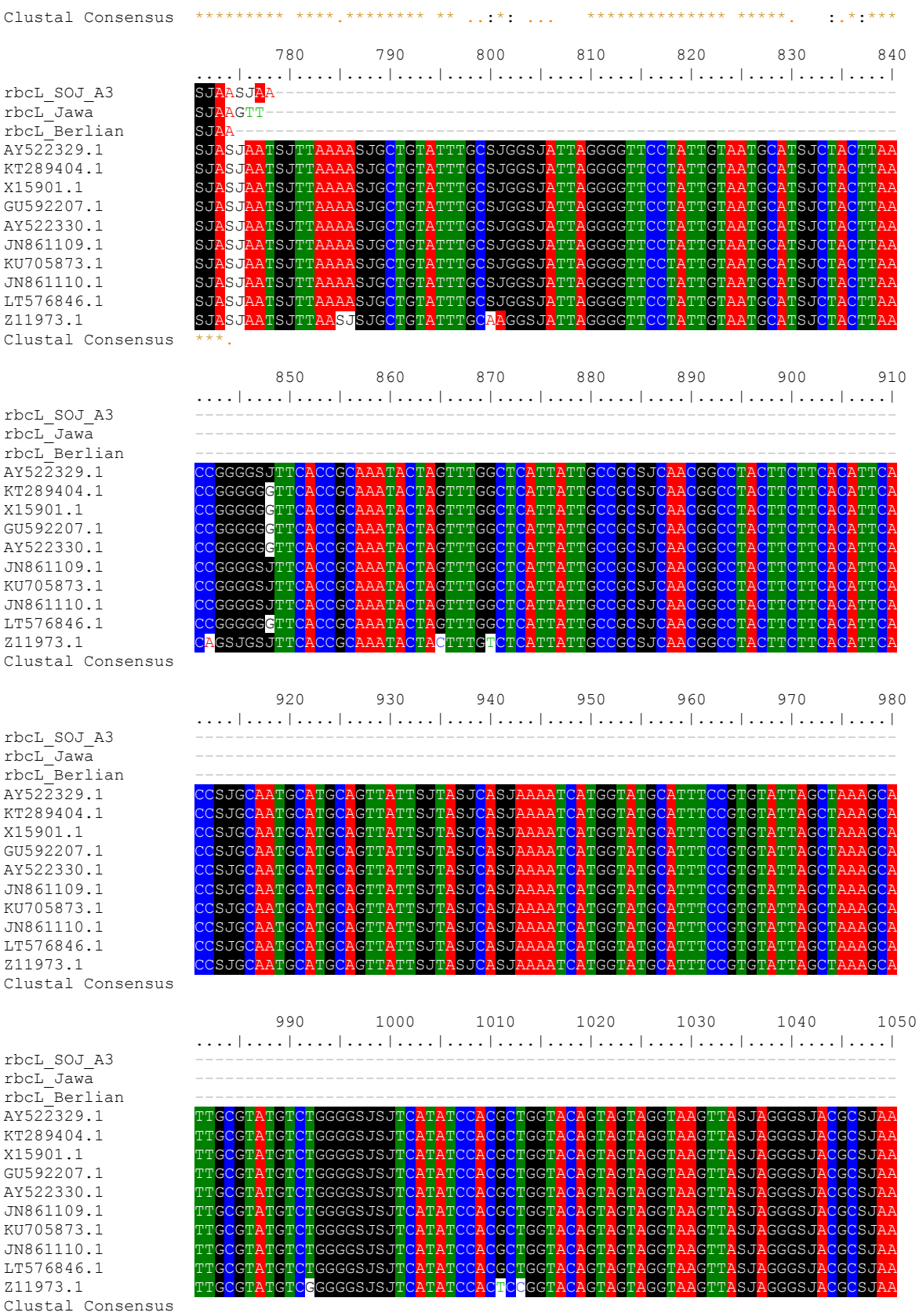
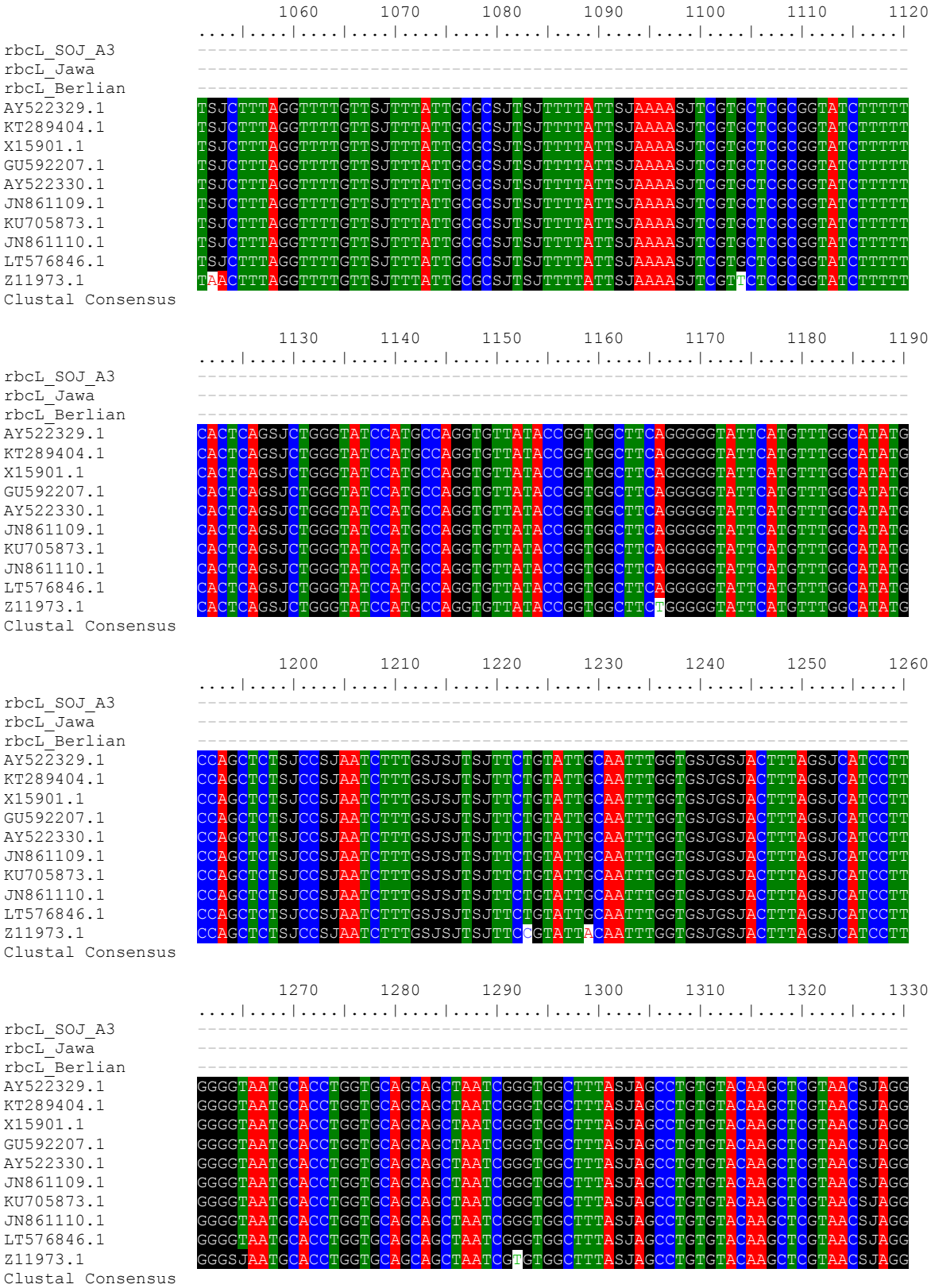
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
710 720 730 740 750 760 770
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1


Clustal Consensus
780 790 800 810 820 830 840
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
850 860 870 880 890 900 910
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
920 930 940 950 960 970 980
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
990 1000 1010 1020 1030 1040 1050
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus


1060 1070 1080 1090 1100 1110 1120
rbcL_SOJ_A3
rbcL_Jawa
rbcL_Berlian
AY522329.1
KT289404.1
X15901.1
GU592207.1
AY522330.1
JN861109.1
KU705873.1
JN861110.1
LT576846.1
Z11973.1
Clustal Consensus
1130 1140 1150 1160 1170 1180 1190
1200 1210 1220 1230 1240 1250 1260
1270 1280 1290 1300 1310 1320 1330

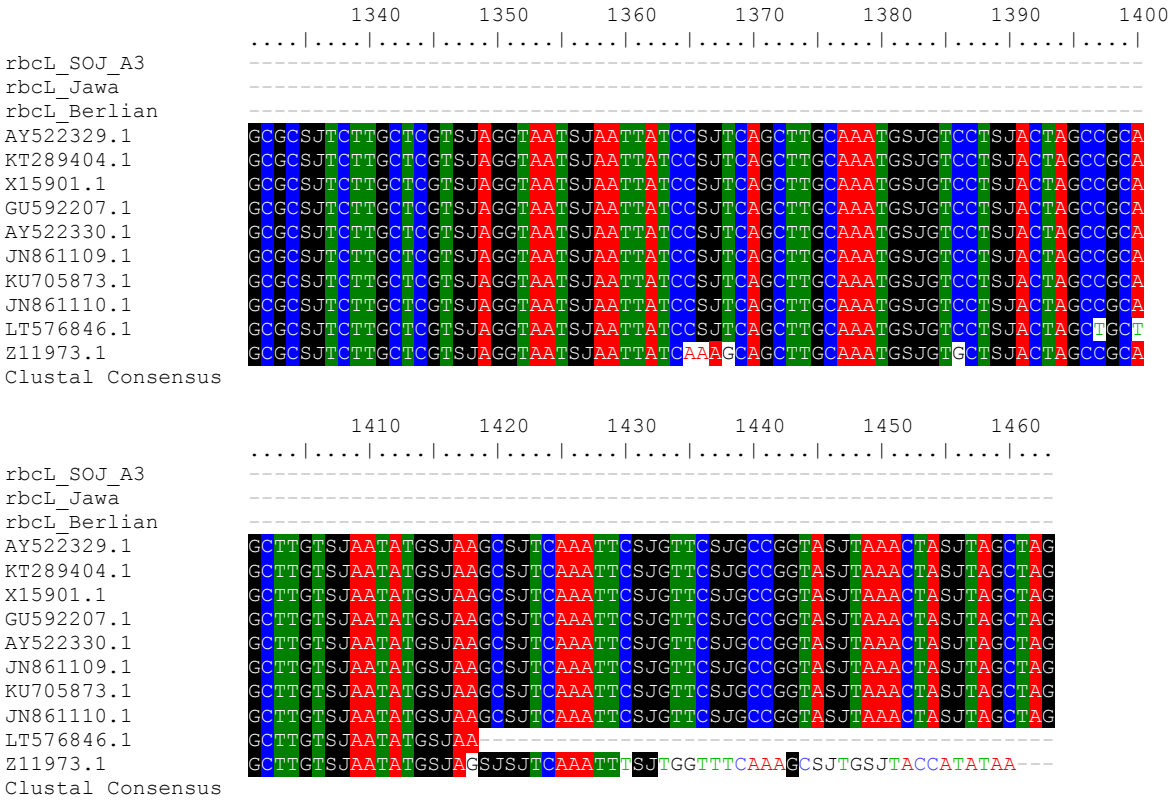


Figure 4. Multiple alignment of *rbc*L gene fragments from local rice varieties of East Java with *Oryza sativa* Indica and *Oryza sativa* Japonica as references.

The BLAST analysis result served as the basis in mining reference sequences from GenBank. In this research BR, SJ and JW were aligned with *Oryza sativa* Indica and *Oryza sativa* Japonica from GeneBank. This alignment was used as a basis to calculate the genetic distance of each sequence. The data from genetic distance was then used as a ground for phylogenetic reconstruction using the Neighbor-Joining method. According to neighbor-joining (NJ) tree reconstruction, BR, SJ, and JW formed a distinct cluster apart from *Oryza sativa* Indica and *Oryza sativa* Japonica. Within the local varieties cluster (BR, SJ, and JW), BR and SJ resided on the same clade despite some genetic divergences on BR side; both of them diverged from JW. All the three local varieties in this research split from the reference *Oryza sativa* Indica and *Oryza sativa* Japonica to form a different cluster, implying the genetic divergence of East Javan varieties (Fig. 5).

Based on the pairwise genetic distance values, all samples belonged to the same sub-species as all samples showed a low level of genetic divergence. However, as the genetic distance numbers on each sample compared to the reference sequences were all more than 0.000, these values indicated genetic variations or genetic divergences from that of Oryza sativa Indica and Oryza sativa Japonica (Table 2)Figure 4. B. cereus antagonist treatment against C. capsici; A is the appearance of the surface of the cup and the appearance of the cup. The red arrow indicates C. capsici mycelia and green arrows show B. cereus colonies.

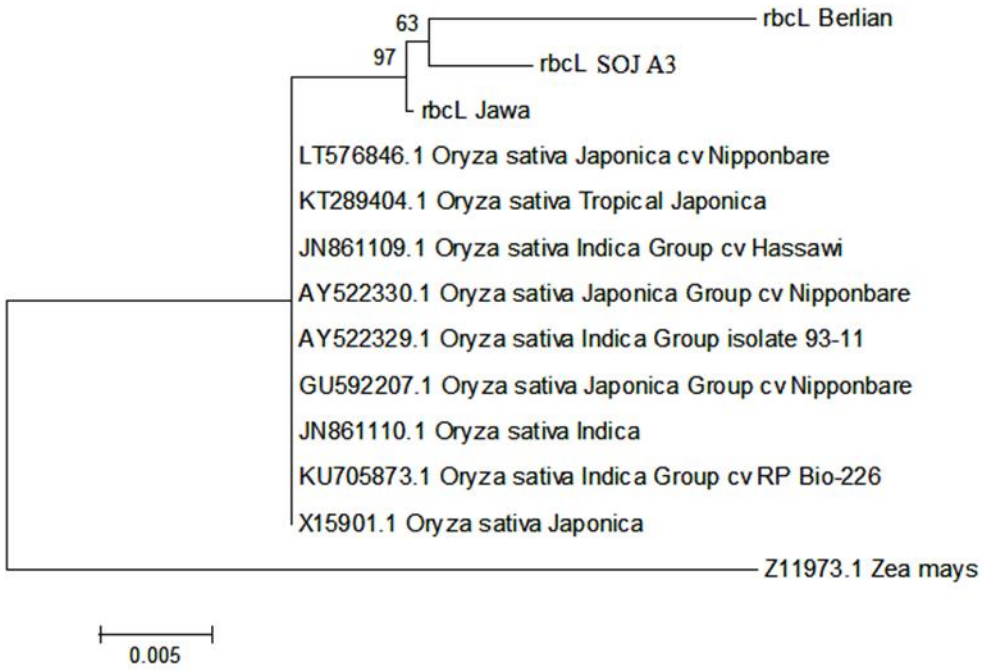


Figure 5. Phylogenetic tree of local rice varieties East Java based on the *rbc*L gene using Neighbour-Joining method showed that the local varieties *rbc*L Berlian and *rbc*L SOJ A3 belong to the same clade although the genetic variation inside their clade and both of them diverged from the local variety *rbc*L Jawa. All the three local varieties in this research split from their comparison varieties *Oryza sativa* Indica and *Oryza sativa* Japonica to form a different cluster, implying the genetic divergence of the local varieties.

Table 2. Genetic distance coefficient between germplasm of local rice varieties East Java.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. AY522329.1 Oryza sativa Indica Group isolate 93-11		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.003	0.000	0.008
2. AY522330.1 Oryza sativa Japonica Group cv Nipponbare	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.003	0.000	0.008
3. GU592207.1 Oryza sativa Japonica Group cv Nipponbare	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.003	0.000	0.008
4. JN861109.1 Oryza sativa Indica Group cv Hassawi	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.005	0.004	0.003	0.000	0.008
5. JN861110.1 Oryza sativa Indica	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.005	0.004	0.003	0.000	0.008
6. KT289404.1 Oryza sativa Tropical Japonica	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.005	0.004	0.003	0.000	0.008
7. KU705873.1 Oryza sativa Indica Group cv RP Bio-226	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.005	0.004	0.003	0.000	0.008
8. LT576846.1 Oryza sativa Japonica cv Nipponbare	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.005	0.004	0.003	0.000	0.008
9. rbcL Berlian	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021		0.005	0.005	0.005	0.010
10. SOJ A3	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.019		0.003	0.004	0.009
11. rbcL Jawa	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.016	0.005		0.003	0.009
12. X15901.1 Oryza sativa Japonica	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.011	0.005		0.008
13. Z11973.1 Zea mays	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.068	0.056	0.052	0.046	

4. DISCUSSION

BLAST analysis result with the reference sequence of the widely cultivated *Oryza sativa* Indica and *Oryza sativa* Japonica confirms the obtained gene sequence as *rbc*L gene. The multiple alignment of BR, SJ and JW in comparison with *Oryza sativa* Indica and *Oryza sativa* Japonica also show mutations such as substitutions (Porceddu & Camiolo, 2017), insertion and deletion (Capella-Gutiérrez & SJbaldón, 2013) (Fig. 4); from which it can roughly be inferred that SJ has a closer relationship to BR compared to JW.

Pairwise Genetic distance analysis also indicated that the genetic variation occurred in BR and SJ varieties were higher than that of

JW varieties, which resulted in the cluster separation of JW variety from BR and SJ. This conclusion was supported by the phylogenetic tree reconstruction using the Neighbor-Joining method. The NJ tree showed that the local varieties Berlian (BR) and SOJ A3 (SJ) belonged to the same clade although the genetic variation inside their clade and both of them diverged from the local variety Jawa (JW). The separation of BR and SJ from JW are due to the differences the area of origin of the sample, which explain the existence of gene flow which denotes the contribution of the environment to influence genetic differences between samples (Khoiriyah, 2014; Sexton, et al., 2014).

However, all the three local varieties in this research split from their comparison varieties Oryza sativa Indica and Oryza sativa Japonica to form a different cluster, implying the genetic divergence of the local varieties. However, the genetic variations occurred in the local varieties were too low to be detected as a significant divergence; thus the pairwise genetic distance analysis result suggested that BR, SJ, and JW belong to the same subspecies as Indica and Japonica. Meanwhile, the previous studies based on both molecular and phenotypic characters clearly separated Indica from Japonica due to their distinct morphological features including leaf colors, seed size and apiculus hair length, as well as consistent genetic distinctiveness which indicated the different origin of the two subspecies (Gross & Zhao, 2014) some research even mention differences in physiological traits and their substantial yet incomplete sterility barrier (Oka, 1953). However, the characters are not apparent enough to classify these two subspecies as distinct species since some domestication traits are controlled by the same alleles in Japonica and Indica, and these alleles are originated in Japonica; yet the two subspecies are divergent at neutral loci, indicating the possibility of introgression between Indica and Japonica during the domestication process throughout the millennia despite their separate origins (Gross & Zhao, 2014). All the genetics analyses also confirmed the existence of subgroups within these groups (SJrris, et al., 2005), a third group which was earlier identified based on morphology and was referred to as Javanica (Matsuo, 1952).

This identification of the third group Javanica somehow explains the divergent of BR, SJ, and JW from Indica and Japonica in this study, even though further studies are needed to confirm their taxonomic status as some ambiguities in the pairwise genetic distance values and its incongruence with the NJ tree reconstruction. The grouping of Indica and Japonica as the same subspecies and the separation of the local varieties despite their low level of genetic divergence are the proofs that there are some areas that *rbc*L gene cannot shed light into. This is because the *rbc*L gene has some limitations in identifying plants up to the species level proven by the incongruence between the phylogenetic tree and the pairwise genetic distance results. The boundaries of the *rbc*L gene marker are due to the low level of sequence differences (APG IV, 2016). The variation in *rbc*L sequence mainly exists at the above-species level, and variety is seldom found at the species level 7-10, resulting in poor abilities in species discrimination (CBOL Plant Working Group, et al., 2009; Gonzalez, et al., 2009) suggesting that *rbc*L is DNA barcode limited to identifying genus and family-level evolutionary relationships for plants (Kang, et al., 2017). Consequently, further studies using different and more specific molecular markers are needed to reveal the exact taxonomic status and the origin of East Javan local varieties Berlian, SOJ A3, and Jawa.

5. Conclusion

From our study we conclude that the local East Javan varieties Jawa (JW), Berlian (BR), and SOJ A3 (SJ) separated from the widely cultivated subspecies *Oryza sativa* Indica and *Oryza sativa* Japonica; with the separation of

JW variety from BR and SJ varieties within their respective cluster. However, further studies are necessary to reveal a more precise position of the local variations in a phylogenetic tree on the species level.

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