


Article

Molecular Diversity of Tidal Swamp Rice (*Oryza sativa* L.) in South Kalimantan, Indonesia

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Abstract: Tidal swamp rice has long been cultivated by the local people of the South Kalimantan, Indonesia. This germplasm possess some important traits for adapted to a wide range of abiotic and biotic stresses. In this study, a total of 16 cultivars of the tidal swamp rice, consisting of 15 from the South Kalimantan Provinces and 1 from South Sumatera, Indonesia (an outgroup) were analyzed phylogenetically based on the chloroplast *trnL-F* and nuclear intergenic spacer region (IGS). The results showed that this germplasm has a relatively more extraordinary genetic diversity than other local rice. On a nucleotide level, the tidal swamp rice showed a genetic diversity of 0.61 for nuclear IGS and 0.58 for *trnL-F*. The phylogenetic reconstruction also exhibited that the tidal swamp rice has the unique phylogenetic trees, particularly for the combined sequence datasets. This information would be useful for the rice conservation and breeding programs in the future.

Keywords: *Oryza sativa*; genetic diversity; tidal swamp; breeding and conservation programs

1. Introduction

Tidal swamps are a potential area to be developed for future agricultural activity in many Asian countries with a total of 20.1 million hectares still available [1]. While such regions are considered the marginal areas, primarily due to natural problems, they support an excessive amount of indigenous rice cultivars [1]. Hundreds of the tidal swamp rice cultivars have been found in the region, and they show some beneficial agronomic traits. As a result of the domestication process and through long-term natural selection, this germplasm shows better adaptation to the local conditions and cultures [2]. Tidal swamp rice also exhibited both a tremendous genetic diversity and substantial genes for promoting the development of new rice cultivars in the future [3–5].

In South Kalimantan for example, one of the biggest rice producing regions in Indonesia, there are about 70 tidal swamp rice cultivars which are still found and preserved by the local farmers [6]. Some of them show important traits for the future breeding, such as Siam Pandak which has a high level of Fe content [6]. Nevertheless, the genetics of these germplasms have not yet been adequately studied and incorporated optimally into the rice breeding programs [7,8]. In general, characterization of rice germplasm has been undertaken based on morphological markers, such as grain shape and color, as well as the plant height and culm diameter [9,10]. However, these markers were strongly influenced by the environmental factors [11].

The molecular marker application may contribute in-depth information on the potential and genetic diversity of the rice germplasm [12]. These markers are more effective, efficient, accurate, and stable to environmental changes than morphological markers [11]. DNA sequencing is a part of molecular applications that constitutes a substantial contribution to the study of phylogenetics,

in plants with both distant and close relationships [13,14]. In this case, the chloroplast *trnL-F* genes and nuclear intergenic spacer (IGS) region sequences could be used for that purpose [15,16]. It is because both have an elevated mutation rate, as well as the genetic diversity [17–20]. Some plant germplasms have been revealed by these markers, such as *Brassica* [21], *Oryza* [14,22], tomato [23], and *Annonaceae* [24].

This study was directed to investigate and determine the genetic diversity and relationship of the tidal swamp rice of the South Kalimantan Province, Indonesia using the chloroplast *trnL-F* and nuclear IGS sequences. In a local scale, such studies could reveal the complex interaction between the germplasm and farmers cultivation practices in good view [25]. Hence, the results of this study are expected to be used as a reference nationally, even internationally, in the genetic improvement and conservation programs of rice in the future.

2. Materials and Methods

2.1. Plant Materials

A total of 16 cultivars of tidal swamp rice, consisting of 15 from South Kalimantan and 1 from South Sumatera, Indonesia (an outgroup) were sampled for this study (Table 1, Figure 1). An outgroup cultivar was obtained from the Indonesian Agriculture of the Tidal Swamp Research Institute, South Kalimantan, Indonesia. Seeds of different cultivars were germinated after being soaked in a fungicide solution and rinsed thoroughly in warm water (30–35 °C). Seeds then were transplanted into pots, 30 cm in diameter and 20 cm in height, with soil collected from a paddy field and placed in a greenhouse. Leaf samples of, at least, three individual seedlings of each rice cultivar were collected for DNA assay.

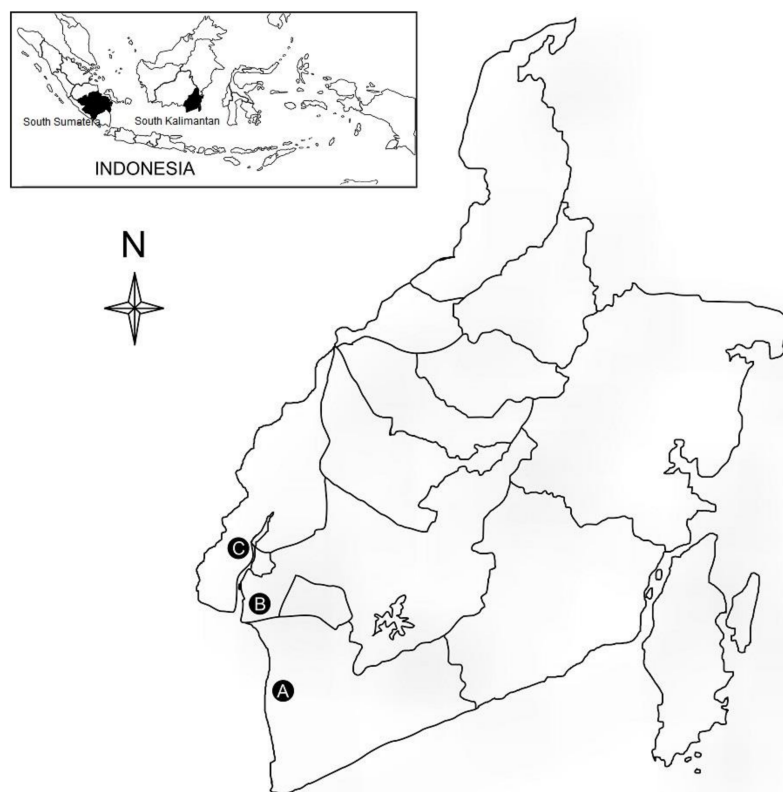


Figure 1. A map of Indonesia showing three collecting sites of the tidal swamp rice in the South Kalimantan Province, Indonesia: Tanah Laut (A), Banjar (B), and Barito Kuala (C).

Table 1. List of the tidal swamp rice employed in this study.

Name of Cultivars	Genetic Status	Grain Type	Sub-Species	Origin
Adil Ganai	Landrace	Non-glutinous	Javanica	Banjar, South Kalimantan
Banih Putih	Landrace	Non-glutinous	Javanica	Banjar, South Kalimantan
Bayar Papuyu	Landrace	Non-glutinous	Close to Japonica	Banjar, South Kalimantan
Bayar Putih	Landrace	Non-glutinous	Javanica	Banjar, South Kalimantan
Ciherang	Improved	Non-glutinous	Javanica	Tanah Laut, South Kalimantan
Ganai Perak	Landrace	Non-glutinous	Javanica	Banjar, South Kalimantan
Lakatan Siam	Landrace	Glutinous	Javanica	Banjar, South Kalimantan
Lakatan Pacar	Landrace	Glutinous	Javanica	Barito Kuala, South Kalimantan
Lakatan Wangi	Landrace	Glutinous	Javanica	Banjar, South Kalimantan
Pandak Laut	Landrace	Non-glutinous	Intermediate	Banjar, South Kalimantan
Pandak Putih	Landrace	Non-glutinous	Javanica	Banjar, South Kalimantan
Siam Babirik	Landrace	Non-glutinous	Intermediate	Tanah Laut, South Kalimantan
Siam Kuning	Landrace	Non-glutinous	Javanica	Tanah Laut, South Kalimantan
Siam Mutiara	Landrace	Non-glutinous	Indica	Barito Kuala, South Kalimantan
Siam Oon	Landrace	Non-glutinous	Indica	Banjar, South Kalimantan
Sardani ¹	Landrace	Non-glutinous	Close to Indica	South Sumatera

¹ An outgroup.

2.2. DNA Isolation, Amplification, and Sequencing

Genomic DNAs were isolated using three-weeks-old of each rice leaf sample, following the Mini Kit of DNA Extraction protocol (Geneaid, New Taipei City, Taiwan). The isolated DNAs were measured using UV-vis spectrophotometer (NanoVue, GE Healthcare, Little Chalfont, UK), at wavelengths of 260 nm and 280 nm [26]. The isolated DNA sequences were then amplified using the chloroplast *trnL-F* and nuclear IGS primers (Table 2). The PCR was carried out at a total volume of 25 µL, consisted of 1.25 µL of 10 ng DNA template, 4.75 µL of the PCR grade water, 1.25 µL each primer (0.5 µM), and 16.50 µL master mix PCR (Kapa Biosystems). This master mix PCR contains 50 mmol KCl, 10 mmol Tris-HCl pH 8.8, 0.1% Triton-X, 1.5 mmol MgCl₂, 200 µmol dNTP, and 0.5 U Taq DNA polymerase (Kapa Biosystems, Wilmington, MA, USA). All amplification reactions were performed in a PCR thermocycler (Biorad T100, Hercules, CA, USA) for 35 cycles, and programmed with the following conditions [14]: (1) An initial denaturation for 3 min at 95 °C; (2) denaturation for 30 s at 95 °C, (3) annealing for 30 s at 55 °C for *trnL-F* and 53 °C for IGS; (4) extension for 1 min at 72 °C; and (5) final extension for 5 min at 72 °C. PCR products were separated on 1.5% agarose gels. The amplified DNA fragments which corresponding to the expected size (Table 2) were then collected from the gels, purified by the QIAquick gel extraction kit (Qiagen, Germantown, MD, USA) and sequenced using Sanger dideoxy sequencing methods (Integrated DNA Technologies, Singapore). All sequences can be accessed in GenBank database with the accession numbers of MH150976 to MH151008.

Table 2. Primers used for PCR amplification and for cycle sequencing.

Marker	Primer	Sequences (5' to 3')	Expected Size (bp)	Ta/Tm (°C)	Reference
<i>trnL-F</i>	Forward	CGAAATCGGTAGACGCTACG	950	55	Taberlet et al. [20]
	Reverse	ATTGAACTGGTGACACGAG			
IGS	Forward	TAGGCCTCTCTAAGTCCGCT	1612	53	Cordes et al. [27]
	Reverse	GTCCAAAGCAGCTCATACGC			

2.3. Data Analysis

The DNA sequences from the two datasets were separately aligned using the Clustal X version 2.0 [28] and refined manually. Indels (insertions or deletions) were introduced into the alignment coded in the following ways. Shared indels were treated as single characters. Indels of uniform length were coded as absence (1) or presence (0) characters independent of the indel length. The gapped

regions in the alignment were excluded from subsequent analysis unless some positions included nucleotide diversity. Gaps were treated as missing entries [29]. Maximum likelihood (ML) analysis was performed by heuristic search with nearest neighbor interchange (NNI) options, strong branch swap filter, and initial tree automatically (maximum parsimony). The sequence data were also analyzed with a neighbor-joining method using the Kimura two-parameter distance estimates [30]. Topological robustness was assessed by bootstrap analysis with 1000 replicates using simple taxon addition [31]. The characters were weighted equally in all phylogenetic analyses. The phylogenetic analyses were conducted by a software of MEGA7 [32].

3. Results

3.1. Sequence Characteristics and Genetic Diversity

Approximately 0.89 kilobase pairs (kb) of two sets of DNA fragments from the chloroplast *trnL-F* and nuclear IGS genomes were sequenced separately from 16 tidal swamp rice cultivars. The phylogenetic information was generated by both sequences given in Table 3. In general, the aligned sequences of nuclear IGS indicate a more extraordinary value of the number of segregating sites, Bayesian information criterion (BIC), and Akaike information criterion (AICc) than chloroplast *trnL-F*. Similarly, GC content and nucleotide diversity were higher in IGS sequences than the chloroplast *trnL-F*. However, both sequences have a similar value of Ti/Tv ratio (1.13) (see Table 3). Moreover, the Tajima's test of neutrality shows that these sequences have a progressive value of D greater than zero ($D > 0$).

Table 3. Comparison of phylogenetic information for the tidal swamp rice genome from two datasets ¹.

Parameter	Chloroplast <i>trnL-F</i>	Nuclear IGS	Combined
Range of sequence length (bp)	936–953	849–1405	1796–2352
Total number of sites (n)	936	849	1780
Number of polymorphic sites (S)	929	845	266
Parameters	30	30	30
Bayesian information criterion (BIC)	20,221.72	29,081.93	60,694.01
Akaike information criterion (AICc)	19,993.42	28,856.57	60,446.29
Maximum likelihood value ($\ln L$)	−9966.65	−14,398.22	−30,193.11
Transition/transversion bias value (R)	0.58	0.56	0.68
Ti/Tv ratio	1.13	1.13	0.89
GC content (%)	35.71	69.10	51.60
Nucleotide diversity (π)	0.58	0.61	0.04
Tajima's test of neutrality (D)	4.12	4.57	−0.54

¹ Based on Kimura two-parameter model.

3.2. Phylogenetic Relationship Based on the Chloroplast *trnL-F* Spacer

The phylogenetic tree was obtained for the chloroplast *trnL-F* sequences from the tidal swamp rice (Figure 2). In general, four separate groups with relatively high values of bootstrap support (bs) formed on this tree. Based on this tree, the *Lakatan Pacar* was closely related to *Sardani*, an outgroup, with a bootstrap value of 100%. Similarly, the *Pandak Laut* was closely related to *Ciherang*, an improved cultivar, at bs = 85%. However, the *Sardani* has far related to *Pandak Putih* (bs = 99%).

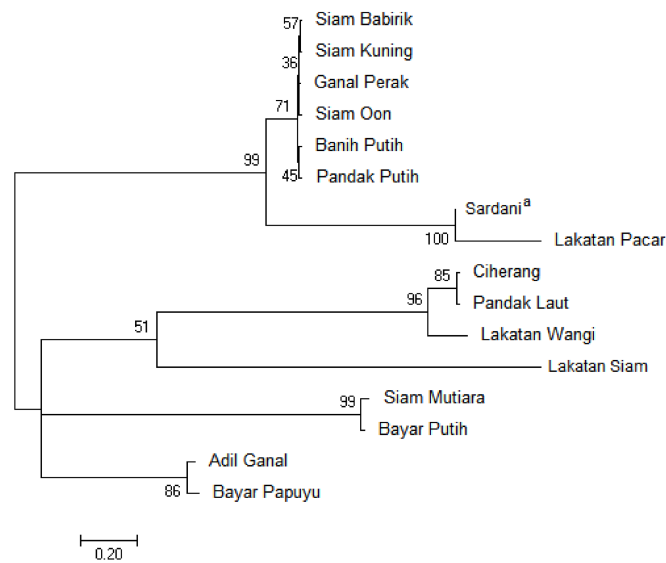


Figure 2. Phylogenetic tree of the tidal swamp rice based on the chloroplast *trnL-F* sequences. The numbers above branches indicate bootstrap values inferred from 1000 replicates (^a an outgroup).

3.3. Phylogenetic Relationship Based on the Nuclear IGS Region

The phylogenetic relationship was obtained for the IGS sequences from the tidal swamp rice. The IGS tree was shown in Figure 3. In general, two independent groups of these germplasms were resolved by the IGS sequences. Differ from the *trnL-F*, the IGS was able to combine the *Sardani* with three tidal swamp rice of the South Kalimantan, such as *Lakatan Pacar*, *Banih Putih*, and *Ganal Perak*, at a bootstrap value of 96%. Whereas the *Ciherang* clustered with *Pandak Putih*, not with *Pandak Laut*, in a similar group (bs = 100%) as well. In this tree, the distant relationship is shown by *Lakatan Pacar* and *Sardani* (Figure 3).

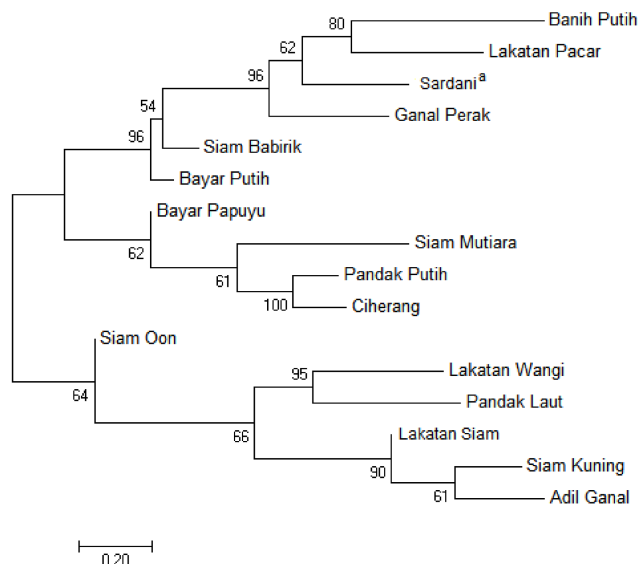


Figure 3. Phylogenetic tree of the tidal swamp rice based on the nuclear IGS sequences. The numbers above branches indicate bootstrap values inferred from 1000 replicates (^a an outgroup).

3.4. Phylogenetic Relationship of Combined Chloroplast and Nuclear Datasets

One phylogenetic tree was reconstructed based on the combination of two individual sequencing datasets (Figure 4). This combined dataset produced many changes in a phylogenetic tree of the tidal swamp rice, including their bootstrap values. In general, two independent groups formed on this tree, and relatively similar to the topology of the IGS phylogenetic tree. In this case, *Lakatan Pacar* has closely related to *Sardani* at 100% bootstrap support, whereas *Ciherang* to *Lakatan Wangi* and *Pandak Laut* (bs = 100%). Based on this tree, *Sardani* and *Ganal Perak* were two cultivars with the most distant relationship.

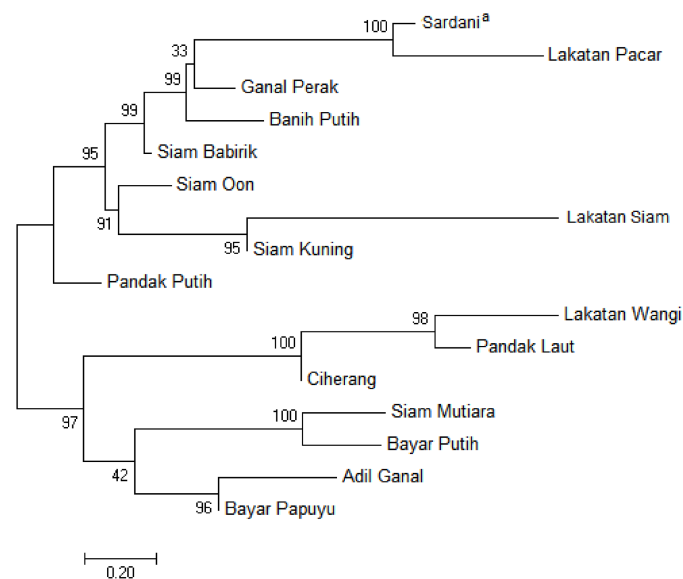


Figure 4. Phylogenetic tree of the tidal swamp rice inferred from the combined of chloroplast *trnL-F* and nuclear IGS sequences. The numbers above branches indicate bootstrap values inferred from 1000 replicates (^a an outgroup).

4. Discussion

Genetic diversity was manifested by differences in many characters, such as morphology, anatomy, and in the proteins; enzymes; and DNA sequences of almost all organisms [33]. It was required for populations to adapt the environmental changes [33]. In our results, the aligned sequences of nuclear IGS show a relatively higher genetic diversity than chloroplast *trnL-F*, indicated both by nucleotide diversity and the number of segregating sites (Table 1). Moreover, this region strongly influenced by some mutations, like deletions and substitutions, as reported by Cordesse et al. [27]. In the *Oryza* genus, the IGS shows a high genetic diversity as well [27]. According to Chung et al. [34], nuclear IGS have been changing quickly, both in the number of copies and its chromosome segments. Furthermore, Chung et al. [34] reported that this region on some chromosomes of 10 genera of *Oryza* has an excessive number of repeated elements, located at the end of the short arm of chromosome 9 [34].

Based on the result, while the chloroplast *trnL-F* has relatively lower genetic diversity than IGS, it would be interesting for future studies [20]. Drábková et al. [35] reported that *trnL-F* has a higher substitution rate than an insertion–deletion (indel), making it a valuable tool for studying short-term evolutionary processes. Furthermore, Bayer and Starr [36] confirmed that this region is a primary tool for phylogenetic studies because of mutation rates of insertion–deletion. Thus, both sequences (*trnL-F* and IGS) have a good chance to apply in the future rice breeding program or developing new rice cultivars.

Overall, the tidal swamp rice of the South Kalimantan, Indonesia demonstrated a relatively more extraordinary genetic diversity than other local rice germplasm. For instance, in this study, our rice

germplasm showed a genetic diversity by 0.61 for nuclear IGS and 0.58 for *trnL-F*, whereas the South Korean local rice, using SSR markers, only had 0.035 [22]. Thus, this indicates that the tidal swamp rice germplasm experienced a long-term period of domestication process [37]. In fact, this process may generate the changes of an individual genome [38]. Furthermore, the Tajima test of neutrality indicated that selection balance (there are no rare alleles) has occurred in this rice population [39]. Thus, the more accurate application of other molecular markers, such as single nucleotide polymorphism (SNP), to study the local rice population is profoundly important to do.

Furthermore, studies on phylogenetic relationships are indispensable to support rice breeding programs in the future [40], particularly in choosing the parents for developing new rice cultivars. These studies are extensively conducted by many researchers—such as Kim et al. [22], Choudhury et al. [41], Liu et al. [11], Zhang et al. [42], etc.—using different molecular markers, both in a local and a global scale. Nevertheless, such studies are still limited to the tidal swamp rice germplasm.

Our results revealed the phylogenetic relationships of the tidal swamp rice germplasm of the South Kalimantan, Indonesia based on the chloroplast *trnL-F* and nuclear IGS sequences. Both reflected the differences in clustering when analyzed separately, four groups for *trnL-F* (Figure 2) and two for IGS (Figure 3). While these trees were generated by two aligned sequences, both have a low resolution, indicated by the average of bootstrap values for each tree. Such a condition can be caused by the weakness of sequence variation used [14]. For this reason, Anderberg et al. [43] suggested the combined sequence data use in increasing the phylogenetic tree resolution.

In this study, the combined sequences of both regions provided a better resolution of a phylogenetic tree than sequence data used separately (Figure 4) but generated a similar pattern of a tree by IGS. This tree, however, could not show the germplasm groupings based on the geographical origin as well. Using the combined sequences data of chloroplast *trnL-F*, nuclear ITS, and mitochondrial *nad1* of the *Oryza* genus, Duan et al. [14] reported a different result of this study, where the phylogenetic tree has a high-resolution with a geographical origin associated.

Regardless of there being no association of geographical origin, the phylogenetic trees provided a unique illustration, where the tidal swamp rice has a monophyletic divergence (derived from a common ancestor). Using the chloroplast *trnL-F* sequences, Hall et al. [44] succeeded to show and reconstruct the genetic relationships of *Capparaceae* and *Brassicaceae* and concluded that their genetic divergences are monophyletic. Such cases are also reported on *Diseae* (*Orchidoideae: Orchidaceae*) [45]. However, in *Annonaceae*, Pirie et al. [24] revealed a different phylogenetic signal to this marker. According to Pirie et al. [24] this was probably due to the gene paralogy, caused by duplicating events, in the past, where the second copy of *trnL-F* diverged from the first *trnL-F* directly from *Annonaceae* ancestor.

Using the chloroplasts and nuclear genome on clustering, Kim et al. [22] succeeded in classifying and reconstructing the phylogenetic relationships of 9 species of *Oryza* into 3 different groups. The same grouping is also shown by Duan et al. [14] when analyzing the *Oryza* genus using a combined sequence data of chloroplast *trnL-F* and nuclear ITS, as well as mitochondrial *nad1*. Thus, the markers can be used in other phylogenetic studies [46]. Whereas, this result may provide a particular opportunity to explore phylogenetic relationships of the tidal swamp rice from different angles in the future.

5. Conclusions

The tidal swamp rice cultivars of South Kalimantan, Indonesia demonstrated a relatively more extraordinary genetic diversity than other local rice cultivars. In this study, these germplasms showed a genetic polymorphism of 0.61 for nuclear IGS and 0.58 for *trnL-F*. The phylogenetic reconstruction also exhibited that the tidal swamp rice has the unique phylogenetic trees, particularly for the combined sequence datasets. Thus, this information would be useful for the rice conservation and breeding programs in the future.

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