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Original Research Article

Genetic Variation of Rice Populations Estimated Using nrDNA ITS Region Sequence

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Abstract - The rice belonging to *Oryza sativa* is not only has significant economic importance, for it is the major source of nutrition for about 3 billion all around the world. But also plays a vital role as a model organism, because it has a number of advantages to be a model plant, such as efficient transformation system and small genome size. Many methods and techniques have been conducted to attempt to distinguish different *Oryza sativa* species, such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and so on. However, studies using sequence analysis of internal transcribed spacer (ITS), a region of ribosomal RNA has not been reported until now. This study was undertaken with an aim to understand the phylogenetic relationships among sixteen isolates of *Oryza sativa* collected from abroad and fifteen isolates collected from Korea, using ribosomal RNA (rRNA) internal transcribed spacer (ITS) sequences to compare the phylogeny relationships among different *Oryza sativa* species. The size variation obtained among sequenced nuclear ribosomal DNA (nrDNA) ITS region ranged from 515bp to 1000bp. The highest interspecific genetic distance (GD) was found between Sfejare 45 (FR12) and Anapuruna (FR15). Taebong isolate showed the least dissimilarity of the ITS region sequence with other thirty isolates. This consequence will help us further understanding molecular diversification in intra-species population and their phylogenetic analysis.

Key words - Genetic divergence, Homology analysis, Molecular identification, *Oryza sativa*, Phylogeny

Introduction

Rice (*Oryza sativa*) is an , and it can grow to more than one meter tall, depending on the species and soil fertility. It is not only the major source of nutrition for about 3 billion people (DUNCAN *et al*., 1992), but also a model organism (Yoshiaki *et al*., 1998). It has a couple of advantages to be a model plant. At the beginning, it has relatively small genome size (Arumuganathan *et al*., 1991). What's more, the transformation system in rice is efficient (Shimamoto *et al*., 1989; Hiei *et al*. 1994; Song *et al*., 1995). Then, molecular genetic maps in rice are dense (Causse *et al*., 1994; Nagamura *et al*., 1997). In addition, rice has large-insert libraries (Umehara *et al*., 1997) and plenty of genetic resources.

As far as we concerned, taxon classification using simple conventional morphological methodology has not satisfied the increasing demands of more refined identification. Molecular identification based on DNA cloning and sequencing has been considered to be a more efficient, faster way compared to the conventional identification methods, such as SSR (simple sequence repeat: Zietkiewicz *et al*., 1994) method for mono-locus analysis of microsatellites, RAPD (random amplified polymorphic DNA: Hadrys *et al*., 1992), ISSR (inter simple sequence repeat: Nagaoka *et al*., 1997) and AFLP (amplified fragment length polymorphism: Vekemans *et al*., 2002), through which larger portion of the genome can be studied. Among various molecular identification means, the sequence analysis of internal transcribed spacer (ITS: Sang *et al*., 1995), a region of ribosomal RNA is considered to be an effective method. ITS reference to a piece of non-functional situated between structural (rRNA), which were excised during rRNA maturation. Because, ITS is not only easy to amplify even from small amount of DNA but also has a high degree of variation even between closely related species. As a result, sequence comparison of the ITS region is widely used in taxon classification.

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Many investigations have been conducted to do taxon classification, including the method based on microsatellite analysis (Zhou *et al*., 2003), RFLP analysis (Zhang *et al*., 1992), PCR-based markers (Xiao *et al*., 1996) and quantitative trait loci (QTLs) analysis (Xiao *et al*., 1995). As for the infra-generic relationship between the genus *Oryza sativa* L., many botanists have their own opinion.

Zhou *et al*. (2003) investigated the genus and provided an infra-generic classification, concluded that the habitat destruction and degradation throughout the geographic range may be the main factor attributed to high genetic differentiation among populations. Xiao *et al*. (1996) indicated that genetic distance measures using RAPDs and SSRs may be useful for predicting yield potential and heterosis of intra-subspecific hybrids other than inter-subspecies hybrids. Tan *et al*. (2001) further recognized that because of natural and human selection, with the evolution from wild rice to cultivated rice, many alleles were lost. It leaded to the lower genetic diversity of the cultivated rice. Besides above, much attention was paid to genetic research in recent years using various approaches. While there were no research reported conducted ITS method.

In this work, we developed a new identification method by constructing the 18S-26S nuclear ribosomal DNA (nrDNA) ITS1 and ITS2 sequence variations for different *Oryza sativa* populations to distinguish similar species in molecular levels. This study would provide more phylogenetic information on *Oryza sativa* species and it will help to further understand the nucleotide variations among different populations of *Oryza sativa* species based on ITS method.

Material and Methods

Plant materials

Thirty one samples of the plant used in this study were harvested from laboratory of Plant Development Engineering, department of Bio-health Technology, college of Biomedical Science, Kangwon National University. The plants were grown from seeds collected from various regions in South Korea and abroad.

70% ethanol were used to surface-sterilize the mature seeds of the species for 30 seconds and then rinsed five times with sterile water. The sterilized seeds were then transferred to a pot containing sterilized fertilized soil in the greenhouse condition. The fresh leaves were used for DNA extraction. Fresh leaf tissues were harvested and sampled in liquid nitrogen.

Sixteen isolates of foreign rice were named as FR1 to FR16 and fifteen isolates of domestic rice were named as DR1 to DR15 for the convenience of sample management (Table 1).

The ITS sequence information of the rice species have published by GenBank, NCBI, the data of all populations, abbreviations and GenBank accession numbers were listed in Table 2.

Table 1. Abbreviation of sixteen isolates of foreign rice and fifteen isolates of domestic rice for the convenience of sample management

Species	Isolate	Abbreviation
Foreign	Vesem	FR1
	Kimaje	FR2
	Tadukau	FR3
	Silewah	FR4
	Drew	FR5
	Raminad 3	FR6
	Teqmg	FR7
	Tepep	FR8
	Dular	FR9
	Kanfo 51	FR10
	Toride	FR11
	Sfejare 45	FR12
	Nipponbare	FR13
	Gumei No. 4	FR14
	Anapuruna	FR15
	Morobereloau	FR16
Domestic	Taebong	DR1
	Central	DR2
	Resides	DR3
	Flowering	DR4
	Bongwang	DR5
	Samchan	DR6
	1 Ru	DR7
	Jinbong	DR8
	Naebong	DR9
	Huadong	DR10
	Pyongyang No. 33	DR11
	Shangzucha	DR12
	Hapcheon aengmi	DR13
	Huashancha	DR14
	Milyang No. 160	DR15

DNA extraction

DNA was extracted from fresh leaves of rice using the modified cetyl-trimethyl-lammonium bromide (CTAB) method described by Arumuganathan *et al*. (1991).

PCR amplification of the nuclear ribosomal ITS sequences

ITS primer pairs ITS5, 5'-GAA AGT AAA AGT CGT AAC AAG G-3' and ITS4, 5'- TCC TCC GCT TAT TGA TAT GC-3' were used to amplify the nrDNA ITS region including ITS1, 5.8S rRNA, ITS2 sequences. PCR amplification was conducted using the primer pairs with the following program: 35 cycles of denaturation at 95℃ for 1 min, annealing at 52℃ for 1 min and a final extension step at 72℃ for 1.5 min. Finally, a 7 min extension at 72℃ followed the 35 cycles to ensure the completion of novel strands. All PCR products were purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at MACRO GEN Advancing through Genomics (Korea).

Table 2. ITS region sequence information about accession number, the length (bp) of sixteen foreign isolates of rice and fifteen domestic isolates of rice

Species	Isolate	GeneBank Accession No.	ITS Length (bp)
Foreign	FR1	KF761364	572
	FR2	KF761365	611
	FR3	KF761366	523
	FR4	KF761367	610
	FR5	KF761368	571
	FR6	KF761369	569
	FR7	KF761370	574
	FR8	KF761371	644
	FR9	KF761372	655
	FR10	KF761373	515
	FR11	KF761374	1000
	FR12	KF761375	572
	FR13	KF761376	1023
	FR14	KF761377	965
	FR15	KF761378	962
	FR16	KF761379	1000
Domestic	DR1	KF761380	1000
	DR2	KF761381	597
	DR3	KF761382	601
	DR4	KF761383	571
	DR5	KF761384	612
	DR6	KF761385	533
	DR7	KF761386	657
	DR8	KF761387	637
	DR9	KF761388	666
	DR10	KF761389	660
	DR11	KF761390	574
	DR12	KF761391	566
	DR13	KF761392	1000
	DR14	KF761393	569
	DR15	KF761394	573

Sequence analysis

Homology analysis of the ITS region sequences from thirty one isolates were performed by DNAMAN 6.0 software according to the Observed Divergency Distance Method. The phylogenetic tree of thirty one rice species were also constructed based on neighbor joining method using DNAMAN 6.0.

Results and Discussion

Total ITS sequence analysis of sixteen foreign *Oryza sativa* species

Primer set, ITS5 and ITS4 were used to amplify the total rRNA ITS sequences from sixteen isolates of foreign *Oryza sativa* species collecting form abroad, containing complete ITS1 region, 5.8S rRNA gene and ITS2 region sequences. These sequences have been submitted to GenBank, NCBI database, with accession numbers in Table 2.

ITS sequences of the sixteen isolates showed size variation, ranging from 515bp to 1000bp. Symmetric matrix of Jaccard coefficients of total ITS region sequences showed some identity ranging from 38.7 to 97.2% (Table 3). FR11 and FR7 showed the least Jaccard coefficient, while the largest Jaccard coefficients appeared between FR15 and FR12. Some samples were collected from the same geographical region, however, there were still genetic variation existing in the ITS region sequences.

Total ITS sequence analysis of fifteen domestic *Oryza sativa* species

The total rRNA ITS sequences of fifteen isolates of

Table 3. Homology matrix (%) of sixteen foreign isolates of rice total ITS region sequences

Isolate	FR1	FR2	FR3	FR4	FR5	FR6	FR7	FR8	FR9	FR10	FR11	FR12	FR13	FR14	FR15	FR16
FR1	100															
FR2	89.9	100														
FR3	47.1	46.6	100													
FR4	45.7	45.2	95.2	100												
FR5	80.4	79.1	46.0	44.0	100											
FR6	75.4	75.4	45.2	44.0	85.1	100										
FR7	45.9	45.7	90.2	84.5	43.9	44.1	100									
FR8	90.5	88.9	46.1	45.1	81.4	80.1	45.2	100								
FR9	46.2	44.8	95.6	88.0	44.1	44.1	84.7	46.6	100							
FR10	47.1	47.9	95.7	94.5	45.7	45.7	89.3	47.4	94.0	100						
FR11	63.5	63.8	39.7	39.6	57.0	59.6	38.7	66.5	40.8	40.5	100					
FR12	46.3	46.9	95.9	91.0	45.4	46.0	84.5	46.7	91.6	94.9	42.8	100				
FR13	46.5	46.3	94.2	85.4	45.1	44.8	84.6	46.0	83.2	92.6	40.8	91.4	100			
FR14	41.5	42.7	40.6	43.1	39.8	41.1	41.8	45.3	42.0	40.8	41.8	47.9	43.7	100		
FR15	47.1	47.0	95.8	89.6	45.5	45.0	85.3	47.3	86.8	95.1	41.1	97.2	69.1	50.4	100	
FR16	46.1	44.8	87.6	80.2	44.9	44.6	80.3	46.4	78.2	88.1	42.2	84.7	63.3	38.9	66.4	100

Table 4. Homology matrix (%) of fifteen domestic isolates of rice total ITS region sequences

Isolate	DR1	DR2	DR3	DR4	DR5	DR6	DR7	DR8	DR9	DR10	DR11	DR12	DR13	DR14	DR15
DR1	100														
DR2	34.0	100													
DR3	32.4	38.1	100												
DR4	34.0	54.6	44.3	100											
DR5	30.7	33.0	33.3	33.9	100										
DR6	35.2	38.3	31.3	40.7	29.6	100									
DR7	33.9	47.9	44.3	60.8	32.6	37.8	100								
DR8	36.0	39.9	47.1	50.7	32.4	36.3	52.9	100							
DR9	31.8	43.5	43.9	64.2	33.6	32.6	58.6	47.4	100						
DR10	33.2	39.0	91.6	45.4	34.4	33.5	44.6	47.4	45.6	100					
DR11	34.9	47.8	45.1	67.8	34.7	37.4	83.4	54.2	68.7	46.4	100				
DR12	31.4	31.1	34.4	32.3	49.2	30.8	33.6	34.6	32.2	34.7	33.8	100			
DR13	34.0	32.1	32.6	30.9	46.9	32.2	32.8	32.4	32.1	34.3	33.3	75.4	100		
DR14	36.9	41.0	48.1	50.8	34.7	35.2	55.8	89.1	50.8	48.5	54.2	34.7	34.0	100	
DR15	32.0	53.3	39.2	78.4	32.1	37.1	55.6	44.7	55.4	39.0	57.6	31.0	30.5	44.7	100

domestic *Oryza sativa* species were amplified using the primer set, ITS5 and ITS4, containing complete ITS1 region, 5.8S rRNA gene and ITS2 region sequences. These sequences have been submitted to GenBank, NCBI database, with accession numbers in Table 2.

ITS sequences size variation were observed among the fifteen isolates, ranging from 533bp to 1000bp. Symmetric matrix of Jaccard coefficients of total ITS region sequences showed some identity ranging from 29.6 to 91.6% (Table 4), The least Jaccard coefficient appeared between DR6 and DR5, while the largest Jaccard coefficients appeared between DR10 and DR3. Genetic variation still exist in the ITS region sequences of samples that were collected from the same geographical region.

Homology analysis of sixteen foreign *Oryza sativa* species

Among sixteen foreign *Oryza sativa* species, FR11 had the lowest homology of 38.7% with FR7, according to the total

ITS region sequence analysis (Table 3). There were numerous length and nucleotides variations in ITS region (Table 3). The dissimilarity was mainly owing to the sequence variation among the sixteen species in ITS region, with a low homology of 38.7% (Table 3). FR14 species showed relatively low homology with all the other species (Table 3). FR14 species not only showed relatively low homology with other species, but also appeared to be more information sites when aligned with other ITS region sequences.

From above results we can conclude that the ITS region sequences of all the isolates used in this study were not intra-specifically conversed. In our study, the nucleotide variations appeared in the total ITS regions of different foreign *Oryza sativa* isolates. Some species showed more similar ITS regions with others, for example FR15 had higher homology with FR12 compared to other isolates.

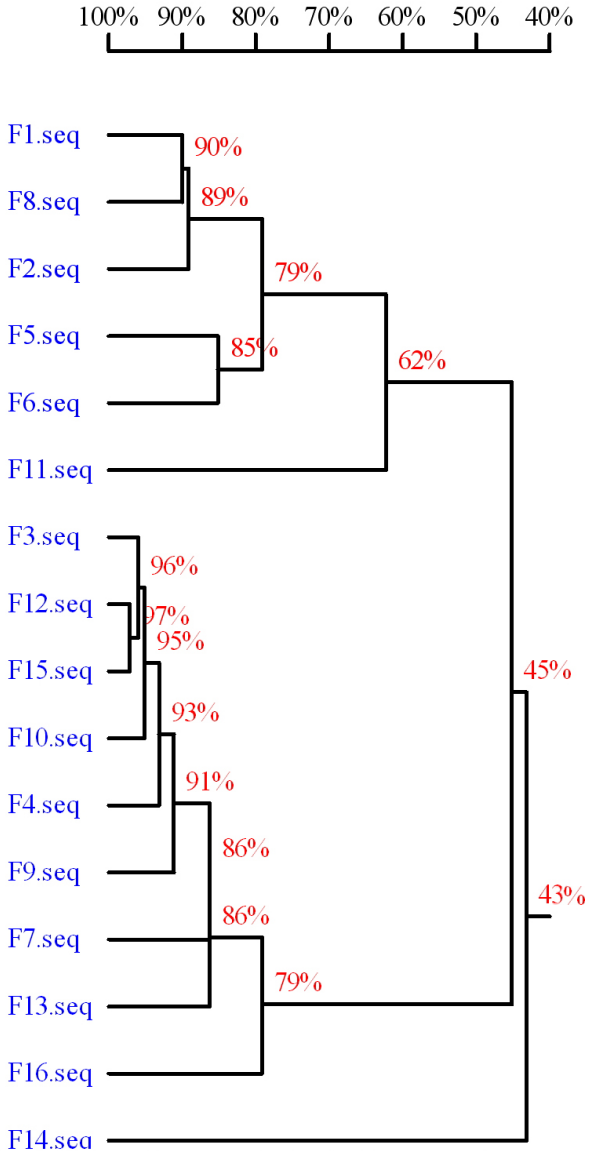

Fig. 1. Phylogenetic tree constructed by total ITS region sequences of sixteen foreign isolates of rice.

Homology analysis of fifteen domestic *Oryza sativa* species

DR6 had the lowest homology of 29.6% with DR5 among fifteen domestic *Oryza sativa* species, according to the total ITS region sequence analysis (Table 4). Numerous length and nucleotides variations happened in ITS region (Table 4). DR1 species showed relatively low homology with all the other species (Table 4). The dissimilarity was mainly owing to the sequence variation among the fifteen species in ITS region, with a low homology of 29.6% (Table 4). DR1 species not only showed relatively low homology with other species, but also appeared to be more information sites when aligned with other ITS region sequences.

In our study, the nucleotide variations appeared in the total ITS regions of different domestic *Oryza sativa* isolates. Some species showed more similar ITS regions with others, for example DR10 had higher homology with DR3 compared to other isolates. We can conclude from above results that the ITS region sequences of all the isolates used in this study were not intra-specifically conversed.

Phylogeny of sixteen foreign *Oryza sativa* species

The result of phylogenetic tree showed that all sequences amplified in this experiment were divided into three groups (Fig. 1). Among them, FR14 formed one clade, FR1, FR8, FR2, FR5, FR6 and FR11 formed one clade, the other isolates formed another clade. Each isolates in the same group showed more than 62% similarity to each other. The FR14 population showed the highest dissimilarity with all populations, sharing about 43% similarity according to clustering analysis (Fig. 1). The group formed by FR1, FR8, FR2, FR5, FR6 and FR11 populations shared the lowest similarity of 62%, while the FR12 and FR15 populations shared 97% similarity (Fig. 1).

The clustering analysis on the sixteen foreign species showed that all species were not form distantly separate clusters. To further understand the phylogeny of them, a broader concept

of samples should be investigated with the same molecular phylogenetic analysis

Phylogeny of fifteen domestic *Oryza sativa* species

All sequences amplified in this experiment were divided into four groups according to the result of phylogenetic tree (Fig. 2). Among them, DR5, DR12 and DR13 formed one clade, DR1 formed one clade, DR6 formed one clade, the other isolates formed another clade, composed by DR4 and DR15 group, DR7, D11 and DR9 group, DR8 and DR14 group, DR3 and DR10 group. The DR1 population showed the highest dissimilarity with all populations, sharing about 34% similarity according to clustering analysis (Fig. 2). The group formed by DR5, DR12 and DR13 populations shared the lowest similarity of 48%, while the DR3 and DR10 populations shared 92% similarity (Fig. 2).

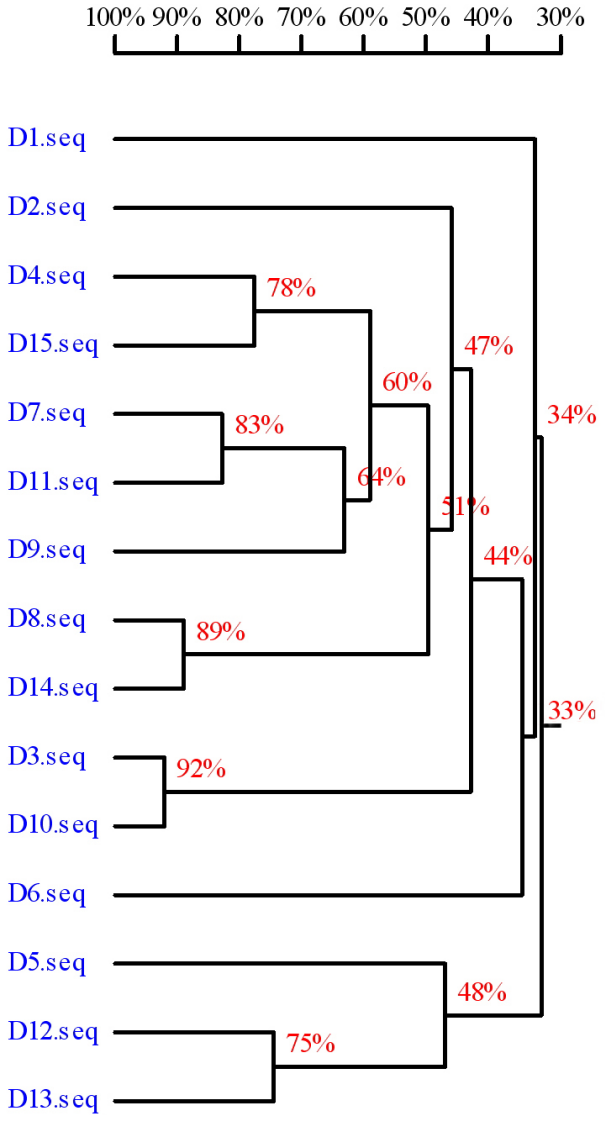

Fig. 2. Phylogenetic tree constructed by total ITS region sequences of fifteen domestic isolates of rice.

As a conclusion, it is the first time that reported the new method by taking advantage of constructing the 18S-26S nuclear ribosomal DNA (nrDNA) ITS1 and ITS2 sequence variations for different *Oryza sativa* populations to distinguish similar species in molecular levels. Through using common primer set, ITS4/ITS5, the total ITS sequences of *Oryza sativa* populations collected from abroad and Korea were successfully amplified. However, they could not yet be authenticated with the method of ITS region sequences analysis in the present study because of limited sampling of species. To make correct identification of *Oryza sativa* populations, additional markers and identification methods should be applied.

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