Article

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Diversity of Unusual Ribosomal Genes and Ecological Origin of Rice

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Abstract: Tandemly organized *rRNA* genes are the typical example of a multigene family, where individual members evolve coordinately within but independently between species due to gene conversion and unequal crossing over. More frequently, in eukaryotic species with an inter-species hybrid origin, expression of unhomogenized *rRNA* genes from one progenitor is epigenetically silenced because of nucleolus dominance, and distinct *rRNA* genes may lose functionality and evolve faster. Interestingly, we obtained unusual ribosomal gene sequences from *Oryza* species that showed great diversity and did not appear in the present rice genomic sequence. The diversity of rDNA sequences indicated that the homogenization in rice is incomplete and explains the introgression of distinct *rRNA* gene families into ancestral rice genome before speciation and continent separation. The divergent large subunit (LSU) ribosomal genes are expressed, some of them differentially, depending on the N-fertilization of plants. Detection of differential transcripts of the *rRNA* genes suggested that *rRNA* gene families are not functionally equivalent. Phylogenetic analysis assigned *Oryza* species branching order to monocots, and monocot lineages probably have the same ecological origin by molecular clock calculation. Therefore, our results suggested that the geographical distances of continents separation cause barriers to the gene flow and homogenization among *Oryza* species which requires further explanation,

Keywords: ecological origin; *Oryza* spices; *rRNA* genes; diversity; differential expression

1. Introduction

Genera of the *Gramineae*, *Oryza*, does not only provide food for half of the world's population but is also used as a model plant by the scientific community. The genus *Oryza* contains about 27 species but is remarkable in the diverse ecological adaptations of its species, in total 11 genome types; the diploid six (AA, BB, CC, EE, FF, and GG), and tetraploid five (BBCC, CCDD, HHJJ, HHKK, and KKLL)[1,2]. For thousands of years, Rice has grown uniquely in sustainable high-output agroecosystems. In relation to genera containing other cereals, *Oryza* occupies a distinct phylogenetic position in a separate subfamily, the *Ehrhartoideae* [3]. Species in the genus *Oryza* and other genera closely related to *Oryza* have been extensively studied either because of their agronomically useful traits in rice genetic improvement (wild species in *Oryza* and *Porteresia*) or because of their economic value as part of cuisine (Zizania) and forage (Leersia)[4]. The origin and diversification of this tribe, in particular the origin of the genus *Oryza* and its divergence, remains largely unclear [5].

Protein production occurs on Ribosomes in all living cells. Ribosomes exhibit substantial differences across species, although universally conserved[6,7]. Some variations are observed in ribosomal RNAs (*rRNAs*) which play the role of central interface for hundreds of proteins. These proteins are ribosome assembly factors (AFs) and ribosomal proteins (RPs) which are highly variable in terms of length and sequence in various species [8–10]. Interestingly, eukaryotic RNAs have species-specific expansion segments (ESs) in comparison to prokaryotic rRNAs which are the hot spot of variations among different eukaryotic species[11,12]. Furthermore, in prokaryotes, ES/ES-like segments were also reported which are not common in eukaryotic species even heterogeneous *rRNAs* with differential expression of sequences have also been observed in various eukaryotic species, e.g., Plasmodium, zebrafish, mice, and Homo sapiens[10,13].

The duplication of single genes or of whole genomes followed by functional gene diversification are important processes that lead to evolutionary innovation[14]. The large proportion of genes with no recognizable homolog in the genome of the diploid *Oryza sativa spp. indica* suggests that the rice genome had been duplicated one or several times during evolution [15] and that *Oryza sativa* as vertebrates [16] and most other angiosperms [17] may be degenerate, ancient polyploids. The contribution of hybridization, autopolyploidy, or allopolyploidy to the origin of the rice genome could be not estimated yet, because rice genes show unique gradients in GC content, codon, and amino acid usage which confound genome analyses based on comparisons of protein-encoding regions[18]. However, in some cases, the contribution of interspecies hybridization to the origin of a particular species could be also traced back by inference of the evolutionary history of its conserved *rRNA* genes. The highly conserved genes of 16S-18S and 23S-28S RNA genes are usually contained head to tail in repeated tandem units and evolve in a concerted manner. Within most species, they have nearly identical sequences among individual copies due to homogenization processes, which allowed them to treat them as single-copy genes in a phylogenetic context. Following interbreeding between two different species, *xenologous rRNA* genes from one parent are usually epigenetically silenced because of nucleolar dominance and probably deleted later. Alternatively, *xenologous rRNA* genes may be also converted to one sequence type [19], or as in *Quercus robur* and *Quercus petraea*, parental rDNA families may be even maintained in the nucleus[20].

Genetic distance is the measurement of evolutionary changes between sequences from different organisms and is calculated for a pair of sequences by simply counting the number of nucleotides or amino acids that differ between them (Morgenstern et al. 2015). A small genetic distance between two sequences may suggest a recent common ancestor but is also consistent with a slower rate of sequence change and a more ancient common ancestor. Evolutionary rates depend on a combination of factors: generation time, population size, metabolic rate, the efficacy of DNA repair, and the degree to which mutations are beneficial or deleterious, all of which may vary among species. Often paleontology or biogeography can provide a date for one or more points in a phylogeny, which are then used to "calibrate" the timescale for the rest of the phylogeny[21]. Remarkably, sample sequencing at different times is the estimation of evolution which requires a faster evolutionary rate and/or negligible sampling times [22]. Therefore, evolution requires the usual phylogeny assuming that all its branches evolve at the same rate following a static molecular clock of sequence change [23] calculating the origin time and fossils existence of the organisms.

We are interested in understanding the evolution of *rRNA* genes due to the uneven distribution of crossing over which evolves coordinately within but independently between species [24]. Here, we have undertaken the functional characterization of unusual ribosomal gene sequences from *Oryza* species that showed great diversity but are absent in the present genomic sequence. We also estimated the divergence between the *rRNA* families in rice. Moreover, we observed that rice rDNA homogenization is not complete and provides an explanation to the introgression of distinct *rRNA* gene families into ancestral rice before speciation. Furthermore, our study showed that the large subunit (LSU) of ribosomal genes of all families are expressed depending on N fertilization of plants.

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be carefully reviewed and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the principal conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets—e.g., [1] or [2,3], or [4–6]. See the end of the document for further details on references.

2. Materials and Methods

DNA and RNA extraction

Oryza officinalis and 17 *Oryza species*, *O. alta* (ACC105143, with IRGC accession number), *O. australiensis* (ACC100882), *O. glaberrima* (TOG5674), *O. grandiglumis* (ACC105669), *O. granulata* (ACC102119), *O. latifolia* (ACC100190), *O. longiglumis* (ACC105148), *O. longistaminata*, *O. malampuzhaensis* (ACC105329), *O. minuta* (ACC101141), *O. nivara* (ACC105763), *O. officinalis* (ACC101399), *O. punctata* (ACC105690), *O. rhizomatis* (ACC105432), *O. ridleyi* (ACC100821), *O. rufipogon* (ACC106423), *O. sativa subsp. indica* (IR36), were selected for further analysis. Plants including three-way hybrid *Zea mays* were grown in unfertilized rice field soil. For some experiments, plants were fertilized with 2 g NH_4NO_3 per kg soil. DNA and *rRNA* preparations from ribosomes were extracted from fresh leaves or roots kept in liquid nitrogen according to standard methods [25].

Gene sequencing

28S *rRNA* genes corresponding to the positions 184 to 1101 in the Saccharomyces cerevisiae 28S rRNA gene were amplified by PCR with forward primer 28f1 (5′-GAC CCC AGG TCA GGC GGG ACT ACC-3′) and reverse primer 28r1 (5′-GCT ATC CTG AGG GAA ACT TCG GAG G-3′) and sequenced with the ALF Express (Pharmacia, Uppsala, Sweden (SI-1). For specific amplification of a partial RDF2 rRNA tandem unit we used primers NS7 and 98rev. PCR conditions were as follows: initial denaturation at 95°C for 2 min, 30 cycles with denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final denaturation at 72°C for 10 minutes. PCR products were cloned into the TOPO vector (Cat. No. K4500-40, Invitrogen Co.) according to the manufacturer's instructions and sequenced with standard primers. Reverse transcription PCR (RT-PCR) on total RNA or ribosomal RNA were done with RT-PCR beads (Amersham Pharmacia Biotech, Freiburg, Germany) following the manufacturer's protocol. Reverse transcription was carried out with primer 25r1. Direct sequencing of cDNA PCR products was carried out with forward primer 283fCy5 (5′-GCA (AG) CC CAA AT (CT) (AG) GG (ACT) G (AG) TAA AC-3′) and reverse primer 406rCy5 (5′-CA (AC) GCA CT (GCT) TTT GAC TCT CTT TTC-3′). For specific amplification of cDNA products of RC1, RC2 and RC3 rDNA families, the following primer pairs for nested PCR were used 98f2 (5′-CGC ACC GTT CGA ACT GTA GTC-3′) and 98r2 (5′-GTT ACA GCG TGG CAC CCC AAG G-3′), 914f (5′-GCC CAA CGT GAA AAT CGG GCA G-3′) and 914r (5′-GTA TCA CTT TGA GCC TCC ACC-3′), 931f (5′-CCC TAA TAA CCG AAT TGT AGT CTG G-3′) and 931r (5′-CTA GAT GGT TCG ATT GGT CTC ATC C-3′), respectively (SI-1). PCR conditions were as given above. Additionally, for *O. officinalis* and for *O. longistaminata* genomic libraries were constructed in the lambda ZAP express vector (Stratagene, San Diego, CA) and screened with 5′-digoxigenin labeled primer 28f1. The obtained 94 sequences were deposited in GenBank under accession numbers AF363835 to AF363923, AF375550, AF375551, AY097331, and AY097327 to AY097329.

Probes (RC and RDF) labeled and northern hybridization

Three specific probes RCp1, RCp2, and RCp3 digested from 28S *rDNA* clones (AF363841, AF363902, and AF363882 representing RC1, RC2, and RC3, respectively) with *Ahd I* or *Bsm I* and resulting 225, 261 and 276 bp fragments, respectively. Furthermore, RDF1, RDF2, and RDF3 were digested each with *Ahd I* or *Bsm I* and resulting 225 to 276 bp fragments corresponding to nucleotides 7-232, 7-268, or 7-283 of equivalent PCR products. These all were labeled with digoxigenin by random primed labeling. Labeled fragments correspond to variable regions B10-B18 of the secondary structure model of the Saccharomyces cerevisiae 28S *rRNA* gene. The RDF2 18S probe is a 3′ and 5′ with digoxigenin end labeled primer and corresponds in length to the sequence (SI-2B). Probes were hybridized at high stringency and visualized by CDP-Star (Roche, Mannheim, Germany) on an X-ray film according to the manual's guide.

Terminated restriction fragment length polymorphism (T-RFLP)

PCR products were obtained from the cDNA templates with primer 98f2 with 5'-labeled Cy5. PCR products purified after agarose gel electrophoresis were digested with restriction enzymes and analyzed on an ALF Express automatic sequencer (Pharmacia, Uppsala, Sweden).

Sequences data analysis

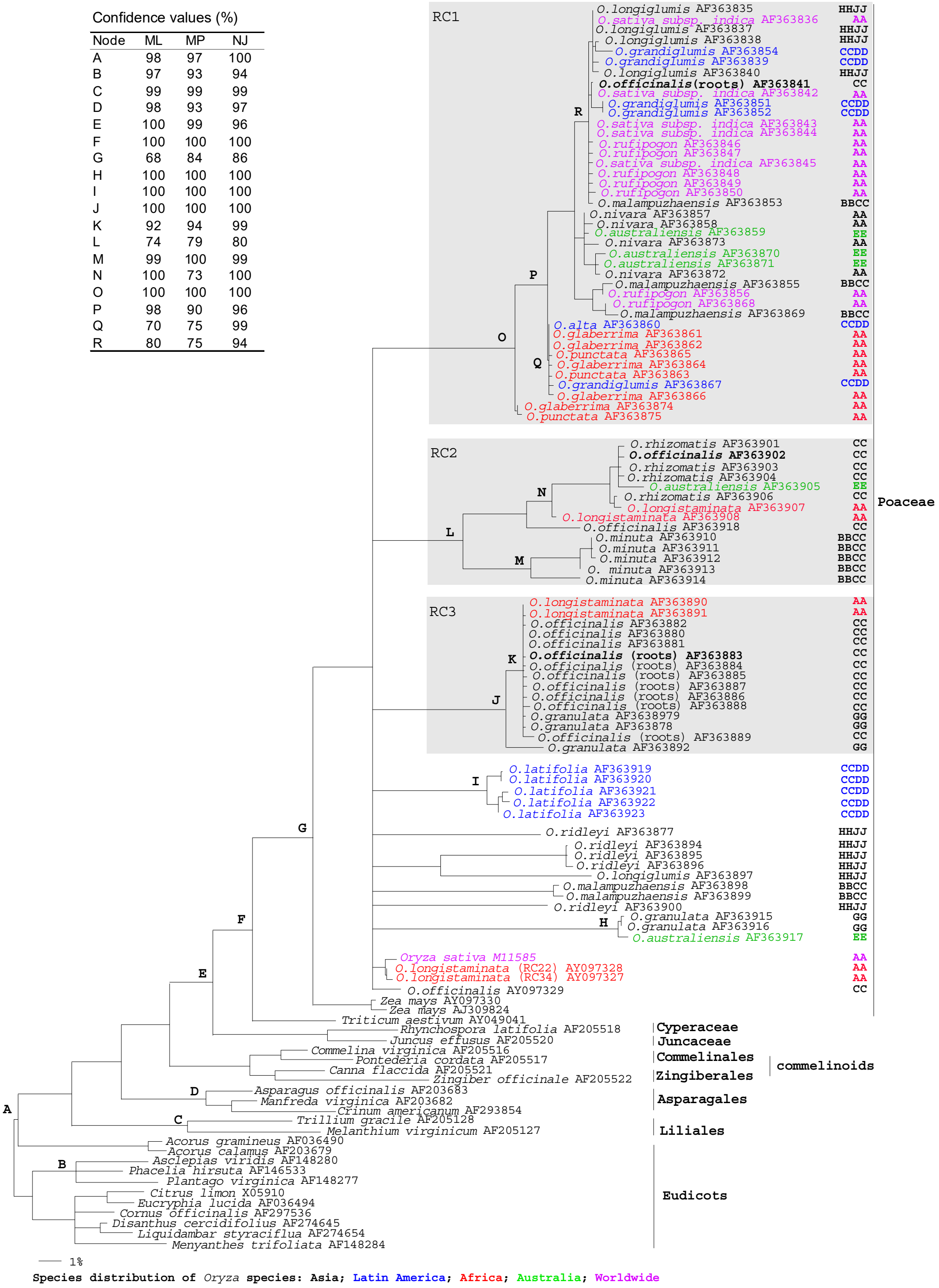
DNA sequences were aligned by the Ribosomal Database II 28S (http://rdp.cme.msu.edu/cgis/seq_align.cgi?su=LSU) and the MultAlin alignment tools (http://prodes.toulouse.inra.fr/multalin/multalin.html). The alignment producing the shortest tree was used for (equal weighting) maximum parsimony, neighbor-joining, and maximum likelihood analyses with PAUP version 4.0b2a [5]. Parsimony jackknifing (1000 replicates) with TBR branch swapping, 10 random sequence additions, and gaps treated as missing data were used to provide estimates for internal support of clades. Neighbor-joining trees were inferred from maximum likelihood distances and 1000 bootstrap re-samplings. Maximum likelihood trees (1000 replicates) were estimated by a heuristic search procedure using the GTR+G+I and a four-category discrete gamma model of among-site rate variation. *A*verage distances were estimated with the software package PHYLTEST, version 2.0 [26]. The gamma distribution shape parameter alpha was estimated from the data set using PAUP version 4.0 [27].

3. Results

3.1. Phylogeny inferred from concatenated sequences of the genus Oryza

Recombinant DNA technology has become an extremely important tool in biological sciences for manipulating DNA fragments to obtain desired characteristics. The rDNA families reported here were obtained in geographically isolated and widely distributed wild and cultivated species of the genus *Oryza* (Figure 1). We sequenced 94 clones obtained from PCR amplifications, containing partial sequences of 28S *rRNA* genes of 17 *Oryza* species. Sequence alignment, restriction enzyme sites, and used primers are listed in supplementary information 1 (SI-1). Phylogenetic analyses depict the descendent evolutionary lines of different genes, organisms, or species from a common ancestor. Therefore, our phylogenetic analyses are based on 864 alignment positions with 723-845 nucleotides and are consistent with previous results on the phylogeny of monocots based on 18S rDNA, *rbcL*, and *atpB* genes [28]. Further, we analyzed the genealogical relationships within rice cluster I (RCI) by networking approach allowing the assessment of a putative evolutionary origin of the ribosomal sequences through estimation of root probabilities. This analysis identified two networks (A and B) in RCI. Network A comprises two ribosomal sequences of *O. glaberrima* and *O. punctata* from Africa (AF363874, AF363875) which are located on a single branch in the phylogenetic tree (Figure 1). Network B is represented by all other sequences which are located on the well supported sister branch. In this network the highest root probability (0.114) was estimated for sequence AF363842 from *O. sativa*. For sequences AF363848, AF363856, AF363857, AF363864, and AF363868 root probabilities of 0.057 and for the rest of 0.028 were calculated. This result is consistent with an important role of *O. sativa* in the transcontinental distribution of the rRNA families by interspecific hybridization and gene flow even to species of wild rice like *O. longiglumis* and *O. australiensis*, which are not known to exchange genes with *O. sativa*. The possibility that *O. sativa* plays an important role in gene flow between rice species does not contradict our conclusion that the *rRNA* families in rice have been originally brought together by an ancient hybridization event predating speciation. Because in the genome of *O. sativa* RC2 is missing but is present in the genome of all other rice species subjected by us to genomic Southern hybridization, this LSU *rRNA* family like the other ones was probably present in wild species of rice previous to interspecific hybridization with *O. sativa*.

Topologically congruent trees were obtained from all three phylogeny reconstruction methods. Three major rDNA families were assigned as RC1, RC2, and RC3 (Figure 1). Gene fragments obtained were highly divergent from the published *O. sativa* sequence (M11585). Interestingly, most of the obtained 28S rRNA gene sequences were low homology to that of the *O. sativa* sequence (M11585), even if the genome sequences of the indica and japonica subspecies have been assembled by whole-genome shotgun sequencing. The assembled sequence covers 93% of the 420-megabase genome. Homologs of 98% of the sequenced maize, wheat, and barley proteins are found in rice [29] providing the base for the development of cereals.



Confidence values (%)

Node	ML	MP	NJ
A	98	97	100
B	97	93	94
C	99	99	99
D	98	93	97
E	100	99	96
F	100	100	100
G	68	84	86
H	100	100	100
I	100	100	100
J	100	100	100
K	92	94	99
L	74	79	80
M	99	100	99
N	100	73	100
O	100	100	100
P	98	90	96
Q	70	75	99
R	80	75	94

Figure 1. Maximum likelihood (ML) and maximum parsimony (MP) consensus tree (for groups receiving >50% support) inferred from partial 28S *rRNA* gene sequences showing phylogenetic relationships of *Oryza* species and other angiosperms. Nodes are labeled with jackknife support values from MP, ML, or bootstrap values from neighbor-joining analyses (NJ). The lowest support value >55% in all three analyses is shown A to R. Three well-supported 28S *rRNA* gene clusters are assigned as RC1-3. Genome types are given for each *Oryza* species. Dicot sequences were used as outgroup. This is a figure. Schemes follow the same formatting.

3.2. Validation of the PCR sequence artifacts through southern hybridization

To exclude the possibility of PCR artifacts for the diverged rDNA families, genomic southern hybridization with specific probes for 3 different rDNA families (RC1, RC2, and RC3) revealed that the different rDNA families were not PCR artifacts caused by sequence contamination or PCR recombination [30] (Figure 2). In a blot of genomic DNA, *BseRI*- and *XcmI*-digested total DNAs from five rice species were hybridized with the three specific probes. *BseRI* does not digest RC2 but RC1 and RC3 at nucleotides 60-63 (corresponding to the positions of Saccharomyces cerevisiae 28S rRNA gene). *XcmI* digests three rDNA families RC1, RC2, and RC3 at nucleotides 861-864, 929-931, and 914-918 (corresponding to the positions of Saccharomyces cerevisiae 28S rRNA gene) respectively. Accordingly, genomic DNA of RC1 and RC3 digested with *BseRI* and *XcmI* generated 800 bp and 860 bp fragments, respectively, were exhibited by hybridization (Figure 2). In contrast, in the specific RCp2 probed blot only 4.5 kb and 10 kb fragments hybridized due to absence of the *BseRI* restriction sites. The other hybridizing fragments (RC2, lanes 5 and 6; RC3, lane 6,) indicated that the rDNA families in rice are polymorphic.

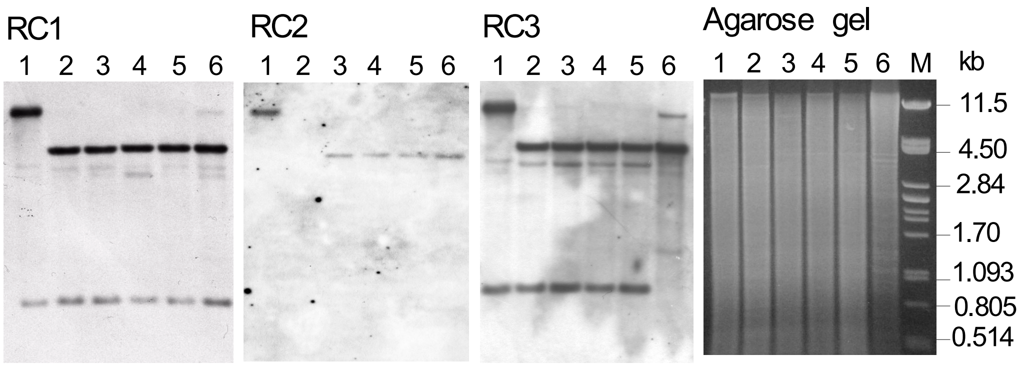


Figure 2. Southern hybridization showed the occurrence of the rDNA families RC1, RC2 and RC3 in rice genome with specific probes RCp1, RCp2 and RCp3, respectively; 1, 2, 3, 4, 5, 6 indicated genomic DNA from *O. officinalis*, *O. sativa subsp. indica* IR36, *O. longistaminata*, *O. rufipogon* from Nepal, *O. rufipogon* from IRRI and *O. glaberrima* digested with *Bse RI* and *Xcm I*, separately; agarose gel showed the electrophoresis of digested genomic DNA; M, lambda DNA/Pst I Marker, 29 fragments (size in bp): 11501, 5077, 4749, 4507, 2838, 2556, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 72, 15.

3.3. Fragments hybridization of RDF1-4

In control blots, RDF1-3 18S probes only hybridized with 18S gene fragments from the same but not from a different rDNA family (SI-2B-D). In a blot of genomic DNA, *Bse RI*-and *Xcm I*-digested total DNAs from five rice species were hybridized with the three RDF 28S probes. Bse RI does not restrict RDF2- but cuts RDF1- and RDF3 28S PCR fragments at nucleotides 68-71. *Xcm I* cuts all retrieved RDF1-, RDF2-, and RDF3 PCR fragments. Accordingly, in RDF1 and RDF3 probed lanes 800 bp and 860 bp fragments, respectively, were hybridizing (SI-2C).

3.4. Analyses of 28S genes in ribosome of O. officinalis

After conforming the polymorphic behavior of these rDNA fragments, we further analyzed 28S rDNA which are conserved universal DNA barcodes for living organisms including plants (Thompson and Christensen, 2011). Northern analyses from ribosome preparations showed that 28S genes belonging to rDNA families RC1, RC2, and RC3 are co-dominantly expressed and present in the ribosome of *O. officinalis* (Figure 3). A further indication that 28S rDNA family genes in rice might be functional came from expression analyses. This is surprising because their A + T content is increased (0.26% O. sativa; 0.24% *O. longistaminata*; 0.24% *O. officinalis*) in comparison to the much less diverged rice LSU rRNAs (0.21%; M11585, AY097330, AY097328, AY097327, respectively) within the same genome. An increased A + T content in alleles usually indicates a relaxation in selection which can follow duplication of genes [31]. This kind of *rRNA* gene transcription would indicate that rDNA family genes were not silenced and may have remained functional because of positive selection. Apart from the common 5′-cap-structure-dependent initiation of translation, also complementarity of *rRNA* within internal ribosome entry sides (IRES) of particular *mRNAs* [32], or binary interactions of ribosomal subunits with *mRNA* in general may be important for controlling the levels of particular proteins in eukaryotes [33]. In the ancestor(s) of cereal plants *rRNA* genes may have been selected for, which maintained this type of translational control for certain genes. This model predicts that *mRNAs* of particular genes are preferentially translated by *rDNA* family, or rDNA family hybrid ribosomes in rice. The high heterogeneity of rice ribosomes will provide a unique opportunity to test the ribosome filter hypothesis [33] which predicts that ribosomes will translate individual *mRNAs* with different relative efficiencies.

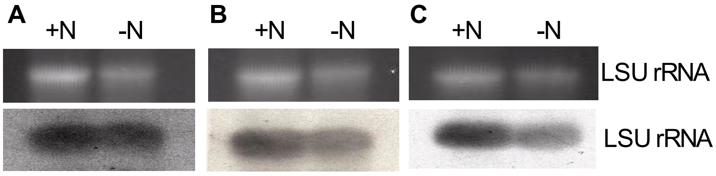


Figure 3. Northern hybridization showed the co-dominant expression of rDNA families RC1, RC2 and RC3 in rice plants. A, B, C, total RNA extracted from *O. officinalis* with specific probe RCp1, RCp2 and RCp3 hybridization, respectively; $^{+}$N, $^{-}$N indicated *O. officinalis* grown in soil fertilized and not fertilized. Agarose gels (upper panel) and Northern blots (lower panel).

3.5. Conservation of RNA secondary structure

RNA secondary structure is similar to an alignment of protein or nucleic acid sequences, except that the RNA sequence folds back on itself by a “complementary bases" pair, and it is also much more conserved than the sequence. The implications of conserved secondary structure changed 1 base of a pair, its partner is also changed so as to conserve that base pair. Ribosomal functions such as peptidyl transferase activity and interaction sites for translation factors and *tRNAs* have been mapped to this phylogenetically conserved structure. Parts of the LSU rRNA secondary structure of three copies of *O. officinalis* (AF363841, AF363902, and AF363882) are shown in Figure 4. The proposed secondary structure of one copy (AF363841) lost the B13_1 region (Figure 4a). The other changes also happened in B10, B11, B12, C1_1, and C1_3 regions. These changes may influence the interaction between *rRNA* and *mRNA* during the protein translation.

The consequences of changes in the region of the LSU *rRNAs* sequenced so far are unknown. However, a comparison of representative secondary structures with the variability map for eukaryotic LSU [34] gave no indication that LSU *rRNA* family genes are nonfunctional. Most conserved stem and loop structures in helixes B, C, and D are preserved with most of the differences in the variable parts. A further indication that LSU *rRNA* family genes in rice might be functional came from expression analyses.

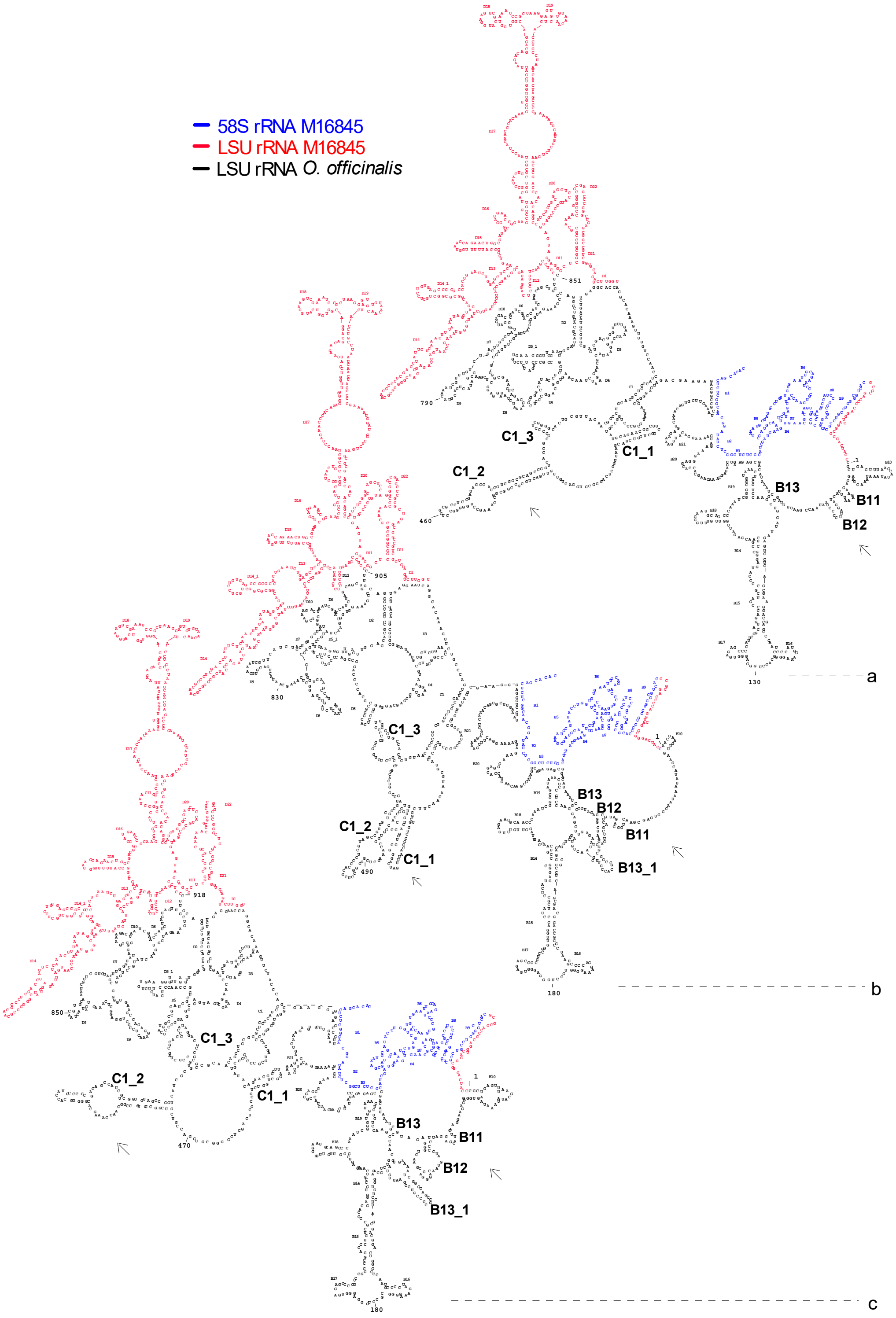


Figure 4. Proposed secondary structure of three copies of *rRNA* genes in *O. officinalis*. Arrows indicated the differences among the related loops or stems of secondary structure (B10, B11, B12, B13_1, C1_1 to C1_3). a, *O. officinalis* AF363841 represented cluster RC1; b, *O. officinalis* AF363902 represented cluster RC2; c, *O. officinalis* AF363882 represented cluster RC3.

3.6. Differential expression of 28S ribosomal genes with and without fertilization

Applying a different fertilization regime with and without combined nitrogen on pot-grown *O. officinalis* plants revealed no differences in expression of RC1, RC2, and RC3 of 28S ribosomal genes (Figure 3). But further inspection of ribosomal gene expression after RC2-specific reverse transcription PCR followed by a terminated restriction fragments length polymorphism (T-RFLP)

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analyses of the products showed that dependent on N fertilization treatment, some RC2 28S *rRNA* gene were differentially expressed (Figure 5A,B). Differential expression was also confirmed by northern hybridization with specific 5′-digoxigenin labeled primers targeting sequences AF363902 and AF363911. 400 bp of readable sequence obtained from unfertilized plants are identical to AF363902 in RC2. Readable sequences from fertilized plants correspond to AF363902 and AF363911. This result suggests that switching of *rRNA* transcription units does not occur between *rDNA* families but within rDNA families in rice. In eukaryotes, switching of ribosome types has been first observed in Plasmodium [35], where several different *rRNA* genes are differentially transcribed during parasite development. However differential regulation of *rRNA* gene transcription within a repeat type has been not reported yet. It is unlikely that this mode of transcriptional regulation of *rRNA* genes happens by chance. Because nitrogen is the nutrient that is most limiting to plant growth in many ecosystems, a nitrogen-mediated regulation of *rRNA* expression may reflect an adaptation at the translational level to balance the supply of biologically available nitrogen under adverse conditions.

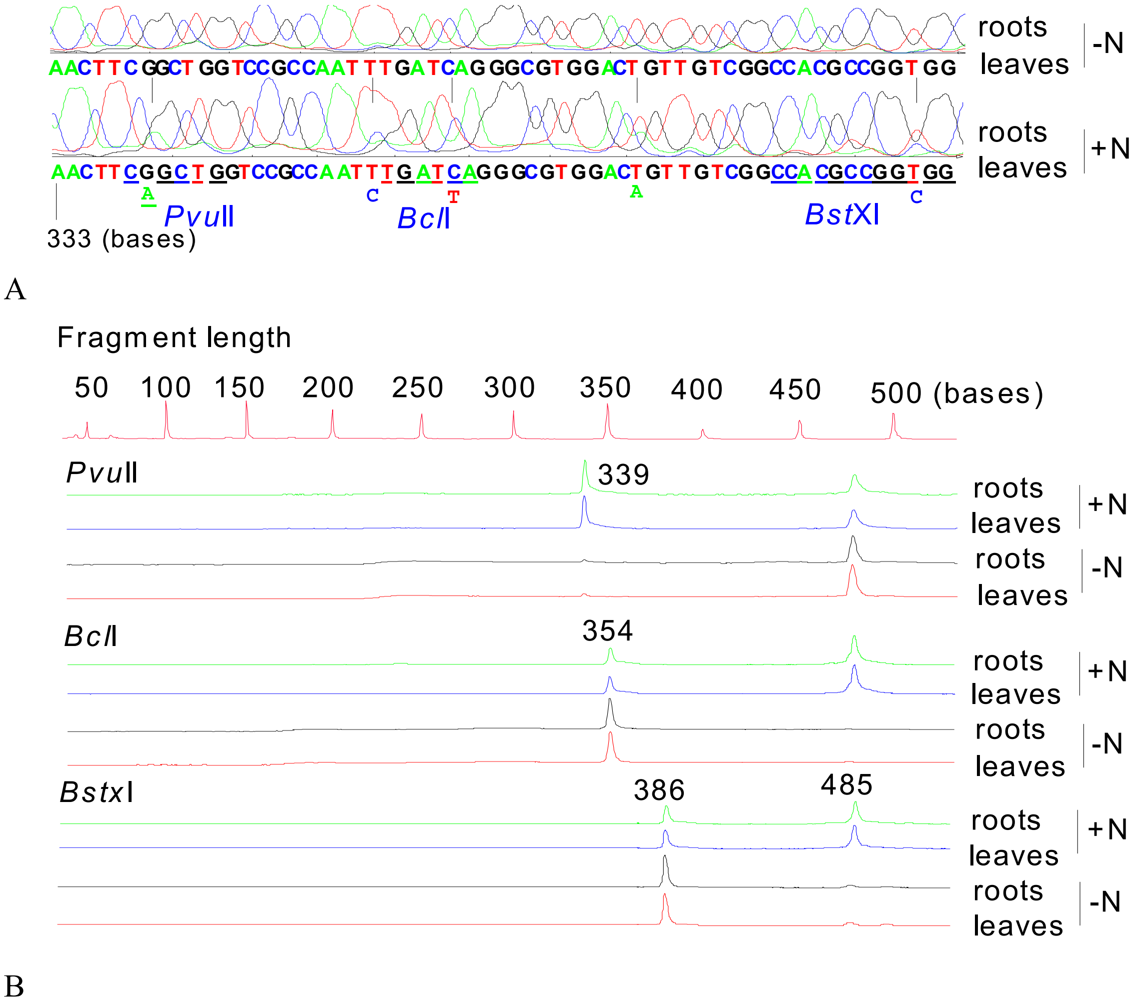

Figure 5. 28S rDNA-based cDNA sequence printouts (A) and corresponding terminal restriction fragment length polymorphism (T-RFLP) fingerprint patterns (B) showed the differential expression of 28S *rRNA* genes in rDNA family RC2. 28S *rRNA* genes extracted from roots and leaves of *O. officinalis* grown in soil fertilized (+N) and unfertilized (-N) with combined nitrogen. For T-RFLP analyses restriction sites of *Pvu II*, *Bcl I* and *Bst XI* were underlined in the sequence printout.

4. Discussion

The relationships of species within the genus *Oryza* which consists of 16 diploid and 7 allotetraploid species could not be resolved in 28S *rRNA* genes constructed phylogenetic tree. In the diploid species, *O. longistaminata*, *O. officinalis*, or *O. australiensis* from Africa, Asia, and Australia, respectively, three rDNA families RC1, RC2, and RC3 were detected within the same species. As in *Quercus*, the intra- and interspecific sequence differences within the clades were usually very low, which was explained for the two oak species by ongoing homogenization within but not between rDNA families [19]. The same observation is exhibited in rice. Some of the sequences on these branches namely from *O. officinalis*, *O. sativa*, and *O. alta* were identical to sequences from other species (*O. longistaminata*, *O. longiglumis*, and *O. glaberrima* respectively), which may be explained by species interbreeding, or very ancient speciation. In contrast to the *Quercus* species, which often hybridized with each other [36], natural crosses between wild rice species are unknown [37]. However, natural hybridization is common between wild rice and the worldwide cultivated *O. sativa* [38]. Species interbreeding as the reason for very similar or even identical 28S *rRNA* gene sequences in different species of *Oryza* can be excluded because comparative genomic mapping with AFLP markers had shown previously that all rice species have diverged independently from each other [39]. Recent speciation can be excluded because the phylogenetic analyses of the AFLP markers clearly indicated that the evolution of *Oryza* followed a polyphyletic pattern with eight monophyletic groups of species including those with identical sequences being separated from each other already early in evolution [40]. Furthermore, the finding of RC1 homologs in the draft genome sequences of O. sativa ssp. Indica [41] and japonica [42] confirmed the presence of RC1 in this species. Comparing 28S sequence AF363845 of RC1 rDNA family from O. sativa L. spp. indica IR36 to the draft genome sequence of the same subspecies by means of BLASTN gave a hit with 99.6% identity to the blasted 851 nucleotides in contig 21607 (http://btn.genomics.org.cn /blast/blast.php?name=rice) and a hit with 98% identity in contig HTC1232751301.1.1 when this sequence was blasted against the genome sequence of *O. sativa L. ssp. japonica* (http://www.tmri.org /index.html). No other high hits (>90% identity) were found, suggesting that RC1 genes are either not high copy genes or have not been sequenced or assembled yet. However, due to the high signal strength observed in Southern hybridization (Figure 2) at short exposure times (<5 min), the possibility that RC1-3 genes represent single copy genes can be excluded.

Sequence analyses of a partial PCR-amplified RDF2 tandem unit from *O. officinalis* (SI-2B) identified a unique truncated 18S *rRNA* gene in standard head to tail orientation upstream of the RDF2 18S *rRNA* gene. We found two distinct INDELs (insertion/deletion) in the P-site required for tRNA binding, one 48-base-pair (bp) deletion and one 14 bp insertion (SI-2A). Southern (SI-2C) and Northern (SI-2D) hybridization with a specific probe, revealed that this unique truncated 18S rDNA is widespread in rice and occurs even in maize. This result confirms that in the last common ancestor of maize, rice and all other cereals 50 Mya, this unique truncated 18S *rRNA* gene was already present, supporting the ancient origin of the rDNA families.

These results showed unambiguously, that in the diploid genomes of *O. officinalis*, *O. longistaminata*, *O. glaberrima*, *O. sativa*, and *O. rufipogon* all three rDNA families are present. Genomic southern hybridization also indicated that the rDNA families are more widespread in the genus *Oryza* than the initial PCR results suggested. The high diversity of 28S *rRNA* genes is consistent with a high variability in the positions of rDNA loci on the chromosomes in the genus *Oryza* [43]. This variability may be even higher than expected from fluorescence in situ hybridization, because for example in *O. officinalis* only three 28S rDNA clusters on chromosomes 4, 9 and 11 have been located [43] as opposed to the 4 highly diverged 28S rDNA sequences detected in our study.

Our results also showed that the rDNA families are ancient and predate the species split in the genus *Oryza*, because 28S gene sequences of several rice species were distributed among several rDNA families. The very little intraspecific sequence variability (Figure 1) within RC1-3 in combination with the high signal strength in southern hybridization (Figure 2) suggests high rates of concerted evolution within each rDNA family in spite of a large sequence divergence among families. In the *rRNA* multigene family of eukaryotes arrays of paralogous gene copies with low sequence divergence are typically homogenized by concerted evolution to nearly identical sequences within a species. Therefore, the presence of highly diverged copies within a multigene family would indicate that recombination between arrays is somehow suppressed, which may be attributed to a high sensitivity of the mismatch repair system to high sequence divergence [44] or to a non-telomere location on the chromosome [45]. This would suggest that diverged members of *rRNA* multigene families within a species are always *xenologous*, because a paralogous origin would be difficult to explain otherwise. Accordingly, the most plausible explanation for the evolutionary origin of the divergent rDNA families in rice as in *Quercus* [19] is that they were brought together by an ancient hybridization event.

In order to get a clue about the timing of this evolutionary event we tried to detect the *xenologous* rDNA families in another grass. The well-established phylogeny on the grass family and molecular-clock-based estimates indicate that maize and rice share their last common ancestor with the vast majority of the grass family and emerged 50 My ago, respectively [46]. These evolutionary relationships make maize an excellent candidate to evaluate the distribution of the rDNA families of rice within grasses. Rice species with several identical rDNA families in their genome, like *O. officinalis*, *O. longistaminata*, and *O. australiensis*, and possibly also *O. grandiglumis* are restricted to Asia, Africa, Australia, and South America ecological region, respectively [47]. Chang suggested that because of this allopatric distribution, speciation in the genus Oryza might have been driven by the successive breakup of Gondwanaland, which would place the origin of grasses into the Jurassic period (~140-210 My ago) (Table 1), much earlier than current estimates based on the first appearance of fossil grass pollen grains at the end of the Cretaceous (55-70 Mya) [48]. Monocots are probably not well preserved in the fossil record in the early Creteous (125-115 million years) [49], which raises the possibility that similar to vertebrate evolution [50], divergence times may significantly precede the appearance of relevant groups in the fossil record. The high statistical support for the branching nodes G within the rDNA families of maize-rice (Figure 1) allows us to infer maize and *Oryza* spices existed before the successive break-ups of Gondwanaland (SI-3). Therefore it was a possible Gondwanaland ancestry of rice based on our data.

Table 1. Dates of divergence of *Oryza* 28S rDNA lineages RC1, RC2 and RC3.

Taxon pair[a]	Average distance	Divergence time (My ago) [b]
Monocots (106) vs. dicots(9)	0.2214 ± 0.0163	170-235
RC1 (41) vs. RC3 (15)	0.1862 ± 0.0205	143-198
RC1 (41 vs. RC2 (14)	0.1990 ± 0.0165	153-211
RC2 (14) vs. RC3 (15)	0.1785 ± 0.0174	137-190

[a] Numbers in brackets give the number of sequences used for the analysis as represented in Figure 1. [b]A divergence time of 170-235 My ago was assumed for the monocot-dicot split (Yang et al., 1999).

5. Conclusions

Natural crosses between wild species of *Oryza* with different genomic types are unknown. However natural hybridization is common between wild species of rice and the worldwide cultivated *O. sativa*. Several evidence indicate that the distribution of the *rRNA* families in rice species was not a consequence of interspecific hybridization after speciation of the genus *Oryza*. (i) Comparative genomic fingerprints strongly support an independent evolution of *Oryza* species [51]. (ii) Before the onset of the worldwide distribution of *O. sativa* about 300 years ago, the geographic isolation of many rice species and short-lived pollen at least in *Oryza sativa* represented constraints making cross-continental interspecific hybridization within the genus *Oryza* by long-range transport of pollen. (iii) Chromosomal incompatibilities between rice species like *O. sativa* and *O. officinalis* or *O. granulata* either produce completely male sterile hybrids in sexual crosses with *O. sativa* or totally prevent species interbreeding. Nonetheless, the identity of partial LSU rDNA sequences affiliated with different *rRNA* families from different species is striking. If we consider the long span of genetic isolation after continent separation, the presence of identical *rRNA* gene sequences in different species appears to be unlikely but could be explained by the exchange of *rRNA* families through natural species interbreeding.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary 1 (Table S1: Used primers in this study); Supplementary 2; Figure SI-1. Codominant expression of RDF1-3 in rice. Presence of truncated RDF2 18S in ribosome preparations. (A) The portion of the aligned matrix from a unique truncated O. officinalis 18S ribosomal gene linked to RDF2 28S rRNA (AY097331) shows two INDELs in the decoding region that distinguishes it from other 18S rRNA genes. Dots indicate nucleotides identical to the top sequence, and dashes indicate gaps. The deleted and a putative alternative P-site are labeled. (B-D) Occurrence of the truncated 18S rRNA gene in rice. (B) Control Southern blot of EcoR I digested from rDNA clones with normal and truncated 18S rRNA genes (AY097331) hybridized with the RDF2 18S probe. Vector labeled with a star. (C). Presence of the truncated 18S rRNA in ribosome preparations from leaves of Zea mays and O. longistaminata (Oryza l.) grown in soil fertilized (+N) or not fertilized (-N) with combined nitrogen (D). Agarose gels (upper panel) and Northern blots (lower panel).; Figure S1: title

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