International Journal of Genetics
ISSN: 0975-2862 & E-ISSN: 0975-9158, Volume 10, Issue 3, 2018, pp.-376-379.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000226

Research Article

PHYLOGENETIC RELATIONSHIP AMONG THE WILD RICE (*Oryza nivara*) AND INTRASPECIES OF RICE GENOTYPES BELONGING TO CHHATTISGARH REVEALED BY CHLOROPLAST DNA REGIONS *psbA- trnH*

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Received: March 31, 2018; Revised: April 06, 2018; Accepted: April 07, 2018; Published: April 15, 2018

Abstract- Background: DNA barcoding technology uses short DNA sequences, for identification of species, understanding evolutionary relationship and also for identification of loci capable of discriminating at intra and inter species level. **Method:** Nucleotide variation at chloroplast (Cp) region, loci *psbA-trnH* and phylogenetic diversity among 24 rice genotypes belonging to Chhattisgarh was analyzed. **Results:** The average nucleotide composition in forward, reverse and both the strand of *psbA-trnH* loci was 27.8% (A), 35.2% (T/U), 18% (C), and 18.4% (G) with transition/transversion rate ratios are k_1 = 6.758 (purines) and k_2 = 2.597 (pyrimidines). The overall transition/transversion bias was R = 2.054. The estimated maximum evolutionary divergence sequence was 0.092 minimum 0 and average 0.015 (MEGA 7). The maximum parsimony informative sites were 31 and number of variable sites was reported 103. While nucleotide diversity (per site pi) 0.01459 analyzed in (DNAsp v.5 program). The reported maximum number of haplotypes was 17 and Haplotype diversity (Hd) 0.919. **Conclusion:** On the basis of nucleotide diversity analysis the discrimination ability of *psbA-trnH* strand loci was found better as compared to *psbA-trnH-r* (reverse) or both the strand. The sequencing informative data obtained, from intra-species variation in rice using *psbA-trnH-f* loci is achievable and proved to be useful for rice genotypes discrimination at intraspecies level. Further validations of rice genotypes can be used for cataloging and characterization of diverse rice genotypes (cultivated and wild) belonging to Chhattisgarh and will advance up in strengthen barcoding of rice genotype present, in various regions of India.

Keywords- Rice, Oryza Species, *psbA-trnH*, Phylogeny, Variation. BOLD, diversity.

Citation: Singh Jyoti and Banerjee Shubha (2018) Phylogenetic Relationship Among the Wild Rice (*Oryza nivara*) And Intraspecies of Rice Genotypes Belonging to Chhattisgarh Revealed by Chloroplast DNA Regions *psbA-trnH*. International Journal of Genetics, ISSN: 0975- 2862 & E-ISSN: 0975-9158, Volume 10, Issue 3, pp.-376-379. DOI: http://dx.doi.org/10.9735/0975-2862.10.3.376-379

Introduction

Rice is grown in all states and ecologies of India. There are many intergrades reported between wild and cultivated forms, continuous array of intergrades between wild and cultivated types are found in Asia and Africa. Based on the natural classification of [25], *O. sativa* varieties were divided into two varietal group *viz., indica* and *japonica* as named by [27]. The genus *Oryza L.* is considered under the tribe *Oryzeae*, sub family *Oryzoideae* of grass family *Poaceae* (*Gramineae*) [19]. This genus includes two cultivated species and more than 22 wild species, distributed in tropical Asia, Africa, Australia and Central South America [20]. The Chhattisgarh is traditionally rich in rice diversity containing the wild progenitors of cultivated rice. The most of the area of Chhattisgarh holds vast genetic variability also reported that at the movement when domestication occurs from wild rice to cultivated rice several genes were vanished as results of natural and human selection [14]. It was the foresightedness of late Dr. R.H. Richharia, who initiated a systematic collection of rice germplasm from Madhya Pradesh including Chhattisgarh during 1972 to 1981. Chhattisgarh is known for rice diversity and considered as one of the secondary centers of diversity. Further explorations in collaboration with NBPGR, New Delhi were organized and new collections were added to the gene pool which currently has 23,500 accessions including 210 accessions of wild species. This germplasm has only partially been characterized for various biotic and abiotic stress tolerances along with morph physiological traits. A few genes for gall-midge and BPH resistance have been identified [16]. There is distinguished variation in germplasm of Chhattisgarh due to agro and eco-climatic conditions also presence of variation in cultural heritage of the inhabitants [23]. Therefore, assemblage, protection and evaluation of germplasm are essential for present as well as future crop improvement programme [24]. As Rice is a model plant with a small completely sequenced whole genome in cereal crops [3]. It needs immediate attention with high priority so that we could not loss this agriculturally important *Oryza* gene pool. Morphological features are usually used for taxonomic identification of species that requiring experienced professional taxonomist. Recently DNA barcoding have gained attention as a twofold intervention, one as a new tool in the taxonomists' toolbox supplementing their knowledge and secondly as a pioneering device for non-experts for quick identification of biological species. To have proper characterization and evaluation of landraces, the above tools are found to be useful for exploitation of the characters and the landraces in further program. But characterization of varieties based on morphological characters is not very reliable because major characters have low heritability and are genetically complex warranting more precise techniques. For this purpose, identification of different genotypes at molecular level is imperative. DNA barcoding is the one of the most important technique in which 'Barcode' gene are used to differentiate between the majority of plant species on earth has been identified. There are number of Cp barcoding loci which compromise of most variable regions. The chloroplast genome is a useful subject for evolutionary and phylogenetic study as it is mostly conserved, devoid of recombination, haploid, maternally inherited, and present in multiple copies per cell. There are different barcoding loci productively used for

phylogenetic studies at different taxonomic levels in diverse plant groups. DNA barcoding is adopted by many developing countries to solve the problems related to biodiversity conservation [4].The aim of study was to evaluate the performance highest differentiating barcoding loci combinations and its effectiveness for discrimination of the different *Oryza* species and cultivars on intra species level and phylogenetic relationship between wild rice and *Oryza* species (Those retrieve from rice germplasm collection I.G.K.V, Raipur) were analyzed based on Cp specific loci *psbA-trnH* regions sequences.

Methods

The study was conducted in the Research cum Instructional Farm, Department of Plant Molecular Biology and Biotechnology campus of College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh. The plant material includes diverse rice genotype of 24 germplasm lines, elite lines, landraces and wild rice, popular rice varieties and advanced breeding lines for drought tolerance, yield improvement and nutritional quality traits.

Genomic DNA extraction and PCR amplification

The total genomic DNA was extracted from single tagged plant, by CTAB method [26].

Specific primers for *psbA-trnH* was employed for amplification and used for PCR - trnH-f 5'- cgcgcatggtggattcacaaatc - 3' and
psbA-r 5'- tgcatggttccttggtaacttc - 3' [6, 18].

A total volume of 20 µl of PCR reaction mixture contained the following: 2 µl (50 ng /µl) DNA, 2µl 10mM dNTPs mix (Invitrogen), 2µl of 10X PCR buffer with 15mM MgCl2 (Invitrogen), 2µl of 10 pMo primer (1µl of each forward and reverse primer), 0.1µl of Taq DNA poly 5U/µl (Invitrogen) and rest was adjusted with nuclease free water (Sigma Aldrich).The PCR was done Veriti follows: 94^{0}C for 4 min, followed by 35 cycles of 94^{0}C for 30 sec, 55^{0}C for 30s, and 72^{0}C for 1 min, followed by an elongation step at 72^{0}C for 7 min.

Resolving PCR product on Agarose gel

PCR products were verified by electrophoresis in 1.5% agarose gel using stained with ethidium. One specific band was recorded at 1000bp on the gel. The PCR product was sent to the Thermo Fisher Scientific Services, New Delhi, India for sequencing based on Sanger method after purification using (Thermo Scientific Gene JET Gel Extraction Kit). The purpose of PCR cleanup is to remove salts, extra nucleotides and primers before sequencing.

Data analysis

The DNA sequences were analyzed using ClustalW [21], performed in MEGA 7.0.25 software. The analysis of DNA sequences was conducted by Neighbour-joining to assess topology with MEGA 7.0.25. The species identification and homology between the sequences was identified using BLAST method. The phylogenetic tree was developed using Neighbour-Joining (NJ) method which was tested with Kimura 2-parameter for evolutionary distances in MEGA 7.0 and node support was assessed on 1000 bootstrap replicated. Pair wise distance, transitional/transversional substitutions, and the maximum likelihood substitution matrix were estimated using MEGA 7.0.25 software [8]. Genetic variation among the rice genotypes were estimated by calculating the number of haplotypes, haplotype diversity (HD), and parsimony informative sites using the DNAsp ver. 5.10 [13]. To test population expansion [22], neutrality tests were performed in order to examine the null hypothesis that sequences are evolving according to the neutral expectations.

Results and Discussion

Rice (*Oryza sativa L.*) is regarded as one of the major cereal crops with high agronomic and nutritional importance. Rice is first crops by which complete genome was sequenced and it has been ideal model plant for study due to its relatively small genome size of 430 Mb compared to other plants [12]. Indian cultivars, landraces, wild and weedy relatives are the main source of rice diversity and are the rich source of worthy genes that plant breeders can exploit for crop improvement [5]. Collection and characterization of existing germplasm is not only important for utilizing the appropriate attribute, but also essential for protecting the intellectual property rights by correct identification of a genotypes, worldwide. Thus, scientific communities today is concerned on genetic variability and provide unique identity to organisms located at various sites of life, which has advanced greatly with the development of the molecular biology techniques. [18,16and 11]. The Consortium for the Barcode of Life (CBOL) is an international organization supporting the establishment of a DNA barcode for plants and aims to contribute to the global standard and collaborative movement in DNA barcoding. DNA barcoding is a technique, which provides quick identification of species without involving the morphological indication and physiological conditions, of the plant species chosen for study [2]. The plant DNA barcoding studies were initially restricted to the chloroplast genome to understand the variation of its gene sequences of coding *matK, rbcL* and *rpoC1* and non-coding *ITS* and *psbA-trnH* [1], team together tested the ability of *rpoB,rpoC1,matK* and *psbA-trnH* regions to discriminate among closely related species in seven genera of flowering plants with different generation times (trees, perennials, and annuals). In the study nucleotide variation at chloroplast (Cp) region, loci *psbA-trnH* and phylogenetic diversity among 24 rice genotypes belonging to Chhattisgarh was analyzed. Frequency of nucleotide substitution nucleotide frequencies 27.17% (A), 35.87% (T/U), 18.73% (C), and 18.24% (G). Maximum Composite Likelihood Estimate pattern of nucleotide substitution was obtained where each entry showed the probability of substitution (r) from one base (row) to another base (column) for simplicity, the sum of r values is made equal to 100.

Table-1 Maximum likelihood values of *transitional (bold)* and *transversional (italics)* substitution of nucleotides based on the *psbA-trnH* markers for 24 genotypes of rice calculated by MEGA 7.0.25.

psbA-trnH-f	A	T	C	G
A	0	*10.04*	*5.31*	**13.49**
T	*8.05*	0	**3.15**	*5.58*
C	*8.05*	**5.95**	0	*5.58*
G	**19.48**	*10.04*	*5.31*	0
psbA-trnH-r	**A**	**T**	**C**	**G**
A	0	*9.21*	*5.94*	**10.24**
T	*12.87*	0	**0.23**	*6.61*
C	*12.87*	**0.35**	0	*6.61*
G	**19.92**	*9.21*	*5.94*	0
psbA-trnH	**A**	**T**	**C**	**G**
A	0	*7.28*	*7.28*	**10.44**
T	*7.28*	0	**10.44**	*7.28*
C	*7.28*	**10.44**	0	*7.28*
G	**10.44**	*7.28*	*7.28*	0

Rates of different *transitional* substitutions are shown in bold and those of *transversional* substitutions are shown in italics of 24 rice genotypes were calculated by MEGA 7.0.25. The estimated of evolutionary divergence between 24 *psbA-trnH* Sequences ranged from 0.000 to 0.092 (average 0.015 (MEGA 7.0.25). Tajima's neutrality test was performed and showed the following estimation [Table-1].

Table-2 *Results from Tajima's Neutrality Test (Tajima, 1989)*

SR.NO.	MARKER	*m*	*S*	*ps*	*Θ*	*π*
1	*psbA-trnH*	21	103	0.093551	0.026003	0.014593
2	*psbA-trnH-f*	21	78	0.142596	0.039635	0.021677
3	*psbA-trnH-r*	24	37	0.067642	0.018114	0'011936

(Evolutionary analyses were conducted in MEGA 7.0.25. Abbreviations: m-number of sequence, n-total number of sites, S-numberof segregating, sites, Ps-S/n, Θ-Ps/a1, π-nucleotide diversity, and D is the tajima test statistics).

The parsimony informative sites were estimated 31 and number of variable sites reported 103. While nucleotide diversity (per site pi) was 0.01459 analyzed in DNAsp v.5 program. Number of haplotypes h-14, haplotype diversity (Hd)-0.919, Variance of haplotype diversity: 0.00245, Theta (per site) from Eta: 0.02752. Phylogeny (or a tree of life) is a theory about how organisms are related to one another through evolutionary time. Phylogenies are based on the assumption that more closely related species will be more similar to one another, and they are commonly built using genetic sequences or physical characters. *psbA-trnH* – 17 haplotypes were obtained from forward, 11 from reverse and 14 both the strand

sequences of the *psbA-trnH* loci. The loci *psbA-trnH* the forward, reverse and both the strand sequences was used for phylogenetic analysis which reveals that *O. nivara* wild rice was clustered separately in forward and both the strands of *psbA-trnH* other than the 20 cultivars of rice genotypes but in reverse strand was accompanied by Nagina-22 was found similar to *O. nivara*.

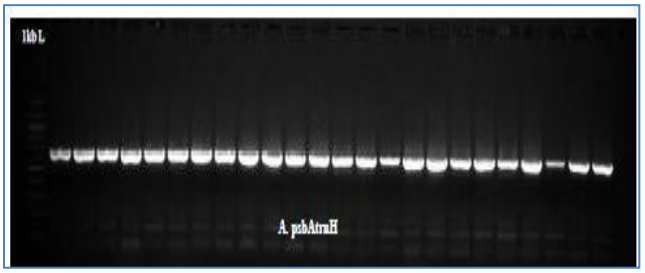
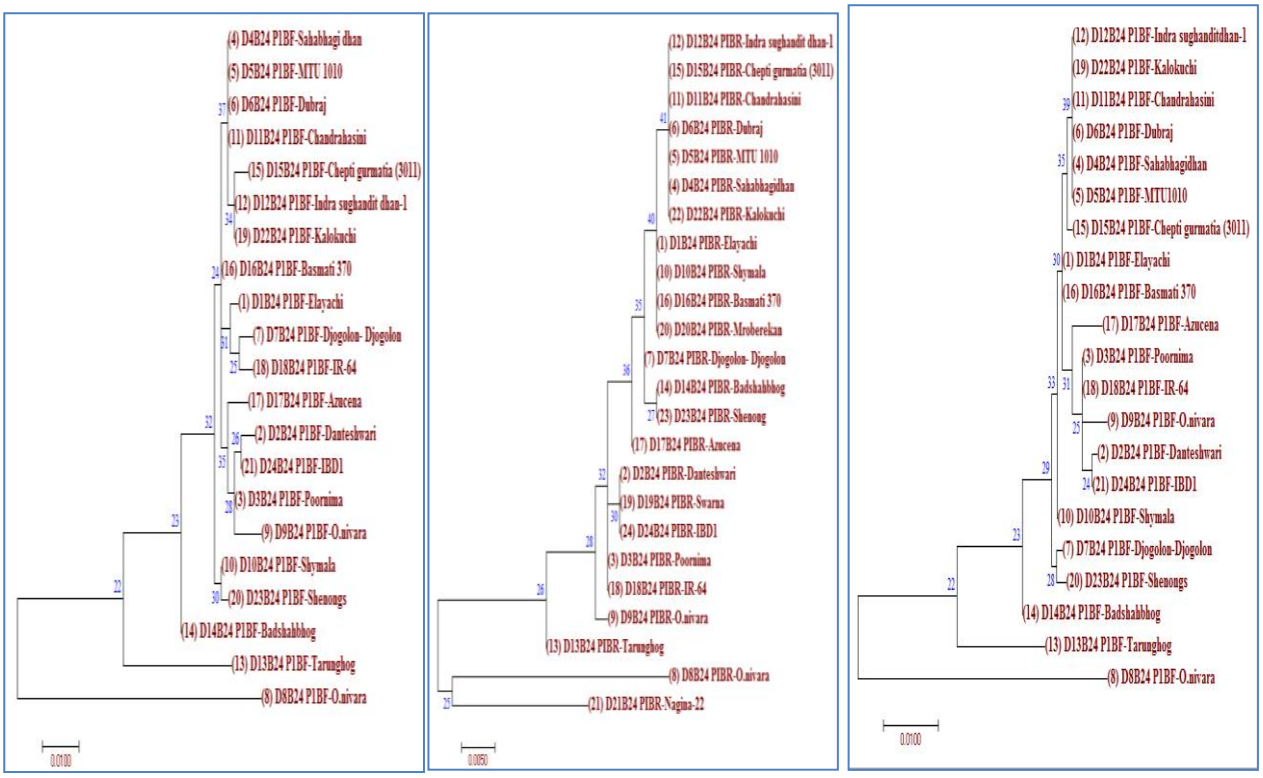


Fig-1 Amplification profiles of the chloroplast genomic loci; *psbA-trnH*

Further the C.G. specific aromatic rice line Tarunbhog and Badshahbhog were classified as most distinct among the rest of 17 cultivars of rice genotypes also Djogolon-Djogolon, IR-64, Indira sugandhit dhan-1 and Kalokuchi are more similarly grouped in one cluster as resulted in forward strand sequences of the loci *psbA-trnH*. In the same loci Chepti gurmatia (3011) shows similarity with Indiara sugandhit dhan-1 and Kalokuchi in the similar pattern Danteshwari is similar to IBD1 also Shyamala and Shenong are close but not 100% similarity is resulted. The reverse strand sequences of the *psbA-trnH* loci Badshahbhog, Shenong are most closer and poornima and IR 64, Danteshwari, Swarna and IBD1 as well, much closer shown in reverse strands of *psbA-trnH* loci. The sequences of both the strand *psbA-trnH* loci. Poornima and IR 64 in same cluster while Djogolon-Djogolon and Shenongs grouped in similar cluster showing 100% similarity. While Danteshwari and IBD1 are showing similarity but are not 100% similar. The forward and both the strand sequences of the *psbA-trnH* loci are discriminating Badshahbhog, Tarunbhog and *O.nivara* as distinct cluster as compared to other rice genotypes. On the basis of discrimination *psbA-trnH* is better as compared to *psbA-trnH-r* and both the strand *psbA-trnH* loci having maximum haplotypes 17. In accordance to the present study [15], also recommended the *psbA-trnH* an intergenic spacer as an appropriate candidate as universal barcode locus for land plants, which is in confirmation to our results in rice (*Oryza sativa L*).



psbA-trnH-f *psbA-trnH-r* *PsbA-trnH*

Fig-2 Phylogenetic tree showing Evolutionary relationship on the basis *of psbA-trnH-f,psbA-trnH-r and PsbA-trnH* lociinferred using the Maximum Likelihood method

Conclusion

O.nivara wild rice is unique of its genotypes and can be identified among other intraspecies of rice genotypes also discriminate based on *trnH-psbA* loci. Phylogenetic analysis which reveals that *O.nivara* wild rice was clustered separately in forward and both the strands of *psbA-trnH* other cultivars of rice genotypes. On the basis of nucleotide diversity analysis, the discrimination ability of *psbA-trn-f* (forward) strand loci was found better as compared to *psbA-trnH-r* (reverse) or both the strand. The sequencing informative data obtained, from intra-species variation in rice using *psbA-trnH-f* loci is achievable and proved to be useful for rice genotypes discrimination at intraspecies level. Further validations of rice genotypes can be used for cataloging and characterization of diverse rice genotypes (cultivated and wild) belonging to Chhattisgarh and will advance up in strengthen barcoding of rice genotype present, in various regions of India.

Application of research: The study provides preliminary data which is useful for wider application of DNA barcoding in rice genotype in Chhattisgarh and Variety Registration in future

Research Category: Plant Molecular Biology

List of Abbreviations

%: Percentage
^{0}C: Celcious
m-number of sequence
n-total number of sites
S-number of segregating, sites
π- nucleotide diversity

MEGA-Molecular Evolutionary Genetic Analysis
NJ-Neighbor-Joining
PCR-Polymerase Chain Reaction
Cp-Chloroplast
HD-Haplotype diversity
BPH-Brown Plant Hopper
DNA-Deoxyribonucleic Acid
BOLD-Barcode of Life Database
CBOL- Consortium for the Barcode of Life

Acknowledgement / Funding: Author thankful to Indira Gandhi Krishi Vishwavidyalaya, Raipur, 492012, Chhattisgarh, India for providing fund under RPF Project.

***Major Advisor of Research: Dr Shubha Banerjee**
University: Indira Gandhi Krishi Vishwavidyalaya, Raipur, 492012, Chhattisgarh
Research project name or number: PhD Thesis

Author Contributions: All author equally contributed

Author statement: All authors read, reviewed, agree and approved the final manuscript

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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