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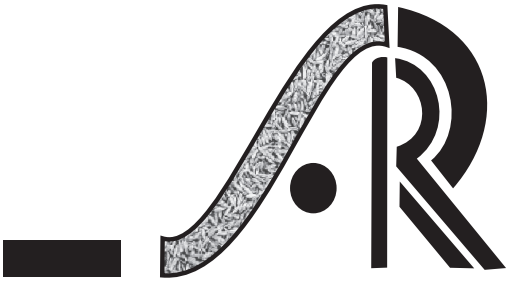

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REVIEW ARTICLE OPEN ACCESS

# Molecular assembly of starch granules: Interplay of metabolizing enzymes in rice

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## Abstract

Starch is of fundamental importance both for plant development and for energy needs upon consumption. Molecular assembly of starch granules is an important event controlling not only starch metabolism but also affect protein and lipid metabolism. Restructuring of starch granule in planta therefore affect plant metabolism. Great progress have been made by studying both crop and model systems and we approach the point of knowing the enzymatic machinery responsible for creating the massive, insoluble starch granules. Micro-structure of starch granule has been modulated by harmonising metabolic enzymes and their intermediates. Here, we summarize our current understanding of starch biosynthetic enzymes and their role in starch packing. The functional aspects of differently assembled intermediates have also been discussed. We flag-up recent observations suggesting a significant degree of flexibility during the synthesis of starch and its packing, which is contributed by enzymatic (starch synthases, branching and de-branching) as well as non-enzymatic cell matrix components (lipids, proteins, phenolics, phosphate monoesters etc). We conclude that novel experimental, both at molecular and biochemical levels along with theoretical approaches will be important to understand metabolic adjustments and redirection, tuning to various types of starch with different functional applications.

**Key words:** Resistant starch, starch packing, starch branching enzymes, debranching enzymes, retrograde starch.

## Introduction

Starch is composed of two polymers of glucose, amylose and amylopectin and is stored in the plastid (chloroplast in leaves, amyloplasts in non-photosynthetic tissues) as insoluble, semi-crystalline granules. Starch, which is the major dietary source of carbohydrates, is the most abundant storage polysaccharide in plants, and occurs as granules in the chloroplast of green leaves and the amyloplast of seeds, pulses, and tubers (Ellis *et al.,* 1998). Starch either can accumulate during the day-time photosynthesis as "transitory starch" or can accumulate in non-photosynthetic tissues and heterotrophic organs (e.g. seeds, roots and tubers) as "storage starch". Metabolic pathways regulating starch synthesis and assembly have been extensively discussed (Zeeman *et al.,* 2010). Transcriptional expression of genes involved in starch metabolism have been investigated and found to be modulated by metabolic intermediates (Tetlow *et al.,* 2004).

Starch has multiple diverse applications from industrial to health-related benefits. The structure of starch is important for effective mobilization during the grain germination process. Starch metabolism has been comprehensively characterized in rice. There are many varieties of rice grain in the world, which vary considerably in their starch accumulation, and metabolising activity. Starch polymer, amylose is a linear and relatively short polymer of glucose units linked by a (1 ® 4) bonds. Amylopectin is a branched and longer polymer where glucose units are arranged linearly through a (1®4), with branches emerging via a (1 ® 6) bonds occurring every twenty-four to thirty glucose units (Shaik *et al.,* 2014). It is well known that starch with a higher amount of amylose is more resistant to digestion (Tetlow, 2011). In addition to the amylose content, cooking (and cooling) processes can influence starch digestibility. The process and degree of gelatinisation affect retrogradation of rice starch. Gelatinisation is the irreversible collapse of molecular order (breaking of H bonds) within the starch granule, leading to starch solubilisation during hydrothermal treatment. This affects granular properties like swelling and crystallite melting (Atwell *et al.,* 1988). This leads to the higher starch availability to human digestive enzymes (Tester and Sommerville, 2003).

A type of starch got recent recognition due to its slow / incomplete digestion which doesn't contribute to blood sugar spike and act as a matrix for fermentation by helping microbiome to grow. This non-digestible starch fraction

is known as resistant starch (RS) (Englyst *et al.,* 1992). The concept regarding digestion of RS has evoked new interest in the bioavailability of starch and in its use as a source of dietary fiber with immense health benefits but its mechanism of synthesis as well as structure has not been explored yet. Resistant starch is mainly of five types – RS1, RS2, RS3, RS4 and RS5. RS1 represents starch that is resistant because it is in a physically inaccessible form such as partly milled grains and seeds and in some very dense types of processed starchy foods. RS2 represents starch that is in a certain granular form and resistant to enzyme digestion mainly due to tight packaging at granular level and relatively dehydrated. RS3 represents the most abundant fraction and is mainly retrograded amylose formed during cooling of gelatinized starch. RS4 is a type of RS where novel chemical bonds other than (1®4) or (1®6) are formed and can be obtained by various types of chemical treatments. RS5 is a complex with lipids, which resist amylolysis (Figure 1).

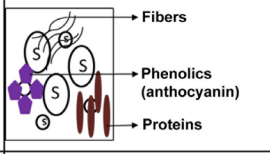
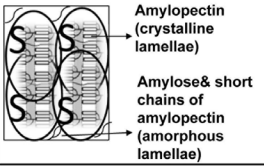
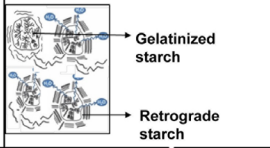
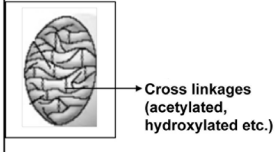
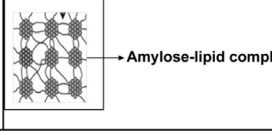
| RS type | Description | Example | Putative Structure |
|---|---|---|---|
| RS1 | Physically inaccessible starch | Coarsely ground or whole-kernel grains |   |
| RS2 | Packed crystalline structure | High-amylose starch |   |
| RS3 | Retrograded starch | Cooked and cooled starchy foods like rice |   |
| RS4 | Chemically modified starch | Cross-linked starch (acetylated, hydroxylated, octenyl succinate starch etc.) |   |
| RS5 | Amylose-lipid complex | Steric acid-complexed high-amylose starch |   |

**Figure 1. Resistant starch types and their putative structures**

In this pursuit, authors have summarized the current understanding of starch biosynthetic enzymes and their role in starch packing behind the functional aspects of starch. The existing reports suggesting the role of enzymes as well as other cellular matrix components towards imparting flexibility and resistance during digestion, has been highlighted in this review.

## Variation in RS content – possible factors

A great source of variation has been observed in RS content among the germplasm due to genetic, environmental and mutation effects. Deciphering the molecular switches, which turn normal starch to digestion resistant phenotype, is potential for future genetic engineering. Starch structure varies with their genetic origin. Additional genetic variation occurs within the genotype due to allelic variation in starch biosynthesis, branching and de-branching enzyme genes. Even though commercial rice varieties, like basmati there is little variation in RS levels (<2%), but the wild and pigmented germplasm contains substantial variation in RS content (Birt *et al.,* 2013). The field environmental conditions have an impact on starch biogenesis as well as packing, which determines the starch digestibility. It might be possibly due to the altering of the activity of various starch enzymes by temperature and moisture. Environmental variation in RS content is difficult to predict and control; therefore, it has not been used as a tool for increasing RS levels. Mutations at the loci's of starch synthases as well as branching enzymes had resulted in starch with higher apparent amylose content or long branched chain of amylopectin which resulted with more percentage of digestion RS compared to normal germplasm. These variations delivered some valuable insights on the role of various enzymes (synthase, branching, debranching and other) during granule biogenesis in coordination to environmental stimuli, which result in tight crystalline packaged structure contributed by a hierarchical intricate organization at different levels.

## Starch structure and packing

Rice has polyhedral starch granules with varying size of 3-8µm in diameter. Despite these differences, microscopic analysis of granule structure has shown some features, which appear to be constant. All granules have been shown to exhibit concentric sphere morphology, with alternating semi-crystalline and amorphous growth shells. The levels of starch organization include glucose strings, which form the basic components – amylose and amylopectin. Later it attains a double helical structure and further organize to form lamellae, lamellae combines to form super helices, which further form as blockets and growth rings, which made up to form a starch granule (Figure 2).

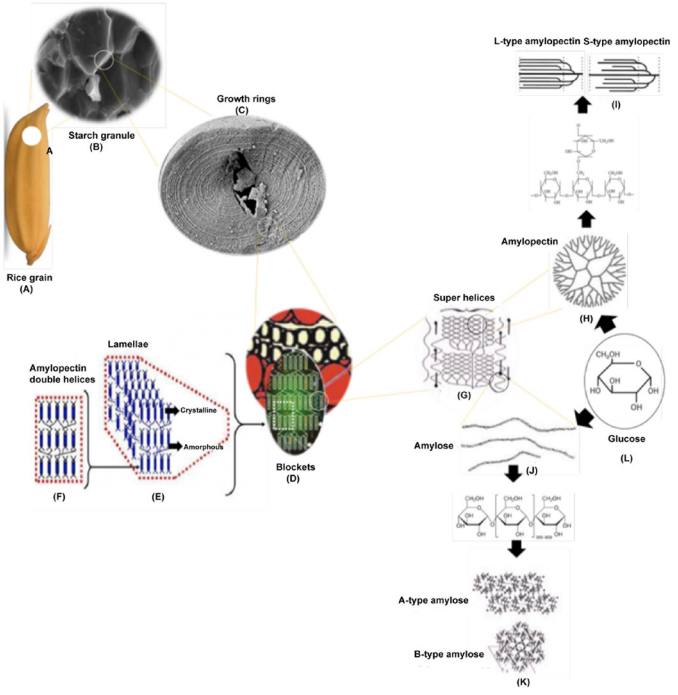



**Figure 2. Schematic representation of starch hierarchical organization**

(A) Starch accumulate in the endosperm of rice grain (B) Scanning electron micrograph of starch granule (C) Starch granule with growth rings radially organized and extending from hilum (D) Blockets, the small units of granules. Blockets consist of super helices comprising (E) Crystalline and (F) amorphous lamellae formed by double helices and branched segments of amylopectin (G) Double helices and branched fragment along with amylose form super helices (H) Glucosyl units showing α-(1,4)- and α-(1,6)-linkages known as amylopectin (I) Two types of amylopectins differing in branching pattern – S & L type (J) Glucosyl units showing α-(1,4) form linear single strand helices known as amylose (K) Amylose exist in two allomorphs – A&B type (L) Glucose, monomeric unit of starch synthesis.

Structural peculiarities are translated to physiological variations in digestibility and hence there are majorly three types of starch– rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RDS is a rapid source of energy, while SDS provides slow and sustained energy release. RS is of great interest as it belongs to special class of dietary fiber, which enriches the gut microbiome.

Despite similar organizational hierarchy, it has been observed that RS2 granules have some structural peculiarities. They contain tiny pores or channels that extend from the granule surface into the interior parts. The number and diameter of these external openings vary among the genotypes, and these act as the target place for amylolytic enzymes that enter and enlarge the pores into tunnels during digestion. The variation in RS due to environmental and mutations of various enzymes also throw light into the starch packing difference in normal vs. RS granules. Amylose-amylopectin chains are crystallized in a hexagonal lattice (B allomorph), where they pack as an array of left-handed parallel-stranded double helices fashion, which in turn form the lamellae. Short chains of amylopectin have reduced tendency to form double helical structure, less crystallite thus easily digestible, while long chains exert more molecular order and optimum packing. Such tightly packed helices contribute to the crystalline lamella and loosely packed contributed by short chains contribute to amorphous lamellar regions. Although both these amorphous and crystalline regions are arranged concentrically, crystalline regions contributed by tight packing are resistant to digestion. The presence of higher amylose also contributes to RS3 during processing, as its recrystallization on cooling occurs rapidly than amylopectin. Re-association into tight crystalline, termed as 'retrogradation' reduces starch digestibility. Higher percentage of RS1 especially in exotic, wild or pigmented germ plasm might be possibly due to the presence of various minor components, which make starch inaccessible.

Phenolics like anthocyanins, tannins, catechins have found to play role in physically trapping starch granules reducing its digestibility. Starch phosphate monoesters in native starch are essentially found along with amylopectin, which also possibly a matrix component contributing RS1 other than major components like fibers and proteins. Lipids are also found in low amounts (up to 1.5%) in much starch, especially cereal starch, in the form of free fatty acids and lysophospholipids. The lipids in cereals are associated with the amylose fraction. In these plants, the proportion of lipid-complexed amylose varies from 13 to 43%, known as RS5, an variant having resistance to amylolysis due to structural differences. Other than the components in the matrices, which make them inaccessible for enzymatic digestion, the most critical factor is the tight packing by the interplay of various key enzymes which play in coordination during starch granule biogenesis resulting in varied proportion of RS (Table 1).

**Table 1. Different types of resistant starch (RS)**

| RS type | Description | Example | Putative Structure |
|---|---|---|---|
| RS1 | Physically inaccessible starch | Coarsely ground or whole-kernel grains | Fibers; Phenolics (anthocyanin); Proteins |
| RS2 | Packed crystalline structure | High-amylose starch | Amylopectin (crystalline lamellae); Amylose& short chains of amylopectin (amorphous lamellae) |
| RS3 | Retrograded starch | Cooked and cooled starchy foods like rice | Gelatinized starch; Retrograde starch |
| RS4 | Chemically modified starch | Cross-linked starch (acetylated, hydroxylated, octenyl succinate starch etc.) | Cross linkages (acetylated, hydroxylated etc.) |
| RS5 | Amylose-lipid complex | Steric acid-complexed high-amylose starch | Amylose-lipid complex |

## Allelic diversity in starch biosynthesis

Starches with higher amylose content or with longer-branched amylopectin have been reported to have higher tendency towards crystalline packing which rapidly retrograde and thus slow down the enzymatic degradation in the digestive track (RS2 & RS3). The ratio of amylose to amylopectin being a critical parameter contributing towards RS, various enzymes have been contributing in this process (Figure 3).

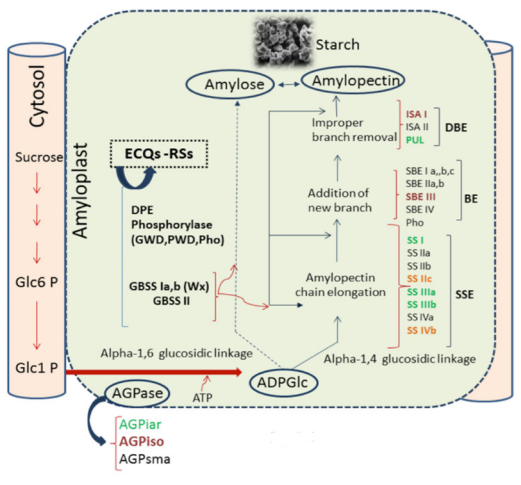



**Figure 3. Integration of starch biosynthesis pathway genes (alleles & isoforms) to starch packaging (ECQ-RS) involved in or plays distinct roles in different steps of starch synthesis.**

Dotted lines shows amylose synthesis pathway genes, solid lines shows amylopectin synthesis genes, genes in different colour codes and bold letters are involved in rice starch grain ECQs and RS types (AC -green, GC -purple, and GT- orange, and GP-black). ECQ, eating and cooking quality; RS, resistant starch; AC, amylose content, GC, gel consistency; GT, gelatinization temperature; GB- granular packaging; AGP ,ADP-glucose pyrophosphorylase; AGPlar, AGP large subunit; AGPiso, AGP large subunit isoform; AGPsma, AGP small subunit; GBSS, granule-bound starch synthase; Wx, waxy gene; SSE, soluble starch synthase enzymes; SBE, starch branching enzyme; Pho, plastidial phosphorylase; belong to branching enzymes (BE) as well to GP; ISA, isoamylase; PUL, pullulanase; ISA and PUL belong to starch debranching enzyme (DBE); GWD, glucan water dikinase; PWD, phosphoglucan water dikinase; DPE, Dis-proportionating enzyme belongs to ECQs and RSs.

Amylose is synthesized by ADP glucose pyrophosphorylase (AGPase) and granule-bound starch synthase I (GBSSI), whereas amylopectin is synthesized by concerted reactions catalyzed by AGPase, soluble starch synthase (SS), starch-branching enzyme (BE), and starch-debranching enzyme (DBE). AGPase catalyzes the first reaction in starch synthesis, producing the activated glucosyl donor ADP-glucose(Glc). GBSSI and SSs act specifically to elongate amylose and amylopectin, respectively. Higher GBSS activity contributes for RS3, as higher amylose content retrograde faster. The enzyme GBSS has also been reported to play role in synthesizing 'super-long' chains in amylopectin (Hanashiro *et al.,* 2008).

Branching enzymes (BEs) and DBEs, which extend and trims the glucose strings critically determines the chain length as well as the packing of granules generating RS2. BEs are involved in generating alpha-1,6 glycoside bonds by cleaving internal alpha-1,4 glycoside bond and transferring the released reducing ends to C6 hydroxyls, thereby forming a new branched chain. Degree of polymerisation (DP); DP>33 – Long chains; 13<DP<33 – Intermediate chains; <13 – Short chains produced by BEs along with DBEs like isoamylase (ISO) and pullulanase (PUL), play role in efficient tight packed structure. Simultaneously, starch degradation is initiated by the addition of phosphate groups at the C6-position and C3-position of individual glucosyl residues that act to disrupt the packing of the glucans at the granule surface. Two enzymes, glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD) respectively, catalyze these phosphate additions. The hydrolysis of the

resulting glucan and phosphoglucan chains is carried out by a set of enzymes including the phosphoglucan phosphatases , β-amylases, DBE; ISA3, α-amylase (AMY3), α-glucanphosphorylase and the disproportionating enzyme 1 (D-enzyme 1; an α-1,4-glucanotransferase). In the cytosol glucose is converted to substrates for either sucrose synthesis, glycolysis or the oxidative pentose phosphate pathway by a number of enzymes including the disproportionating enzyme 2 (D-enzyme 2; an α-1,4-glucanotransferase), α-glucanphosphorylase, hexokinase and phosphoglucomutase. DBEs hydrolyze 1,6-glycoside bonds and play an essential role in the formation of amylopectin. Additionally, a-glucanphosphorylase (Pho) is involved in storage starch synthesis. In rice, the loss of plastidialphosphorylase (Pho1) causes smaller sized starch granules to accumulate and modifies the amylopectin structure, resulting in abnormal endosperm phenotypes, such as white core, shrunken, and pseudonormal endosperms (Satoh *et al.,* 2008). Pho1 may play an important role in the glucan initiation process by synthesizing glucan primers from short-chain malto oligosaccharides (MOSs; Hwang *et al.,* 2010).

Dis-proportionating enzyme (DPE) is an α-1,4-glucanotransferase that catalyzes the cleavage of α-1,4-glucosidic bonds of glucans, transferring the glucosyl groups to the non reducing end of another glucan or free glucose(Glc) and releasing Glc or aglucan chain, depending on the cleavage site. Different isoforms of starch-synthesizing enzymes control amylose and amylopectin content in rice, which in turn greatly influence rice cooking, eating and textural quality. Activity of one or more isoforms of starch synthesizing enzymes results in various forms of starch structure based on the amylopectin chain length and average external, internal and core chain length distribution and hence results in varying physicochemical and cooking quality (Pandey *et al.,* 2012). Genome of rice consists of at least 27 genes encoding starch-biosynthesizing enzymes dispersed in 7 groups based on their isoforms, six for AGPase, two for GBSSI (Waxy gene), eight for SS, three for BE, four for DBE, two for Pho, and two for D-enzyme (Ohdan *et al.,* 2005). Among them, the functions of the former six groups have been well studied; however, not much is known about the exact role of DPE1 in starch metabolism in developing rice endosperm, although endosperm-specific over-expression or suppression of DPE1 affected the amylose content, starch structure, and morphological and physicochemical p roperties of starch granules. The activities of other major starch synthesizing enzymes were not found changed in DPE1-overexpressed or suppressed seeds. DPE1 and a-1, 4-D-glucanotransferase, has been thought to be involved in storage starch synthesis in cereal crops. However, the precise function of DPE1 remains to be established. DPE1 overexpression decreased amylose content and resulted in small and tightly packed starch granules, whereas DPE1 suppression increased amylose content and formed heterogeneous-sized, spherical, and loosely packed starch granules (Dong *et al.,* 2015).

The reduction of SBEIIb activity is another route to increase amylose content. However, much higher amylose contents have been obtained in rice by targeting SBEIIa, SBEIIb and SBEI, (Fasahat et al.2014) as well amylopectin-synthesizing enzymes, such as SSIIIa, SSIIa, SSIVb, BEI, BEIIb, and PUL have also been found to target amylopectin synthesis simultaneously (Ordonioa and Matsuokab, 2016). Branching enzyme proteins consist of three common characteristic domains, carbohydrate-binding, catalytic amylase and a-amylase C-terminal domain (Pfister and Zeeman, 2016). These genes/enzymes both ways, either directly or indirectly (via synthesis of amylase, amylopectin and grain packing) contributes to the variation in the granular structure of starch grain through modulations in their RS content (Fig.2).

A link between starch synthesis genes and starch digestion properties is well established. GBSSI (waxy) is primarily responsible for the linear chains of glucose molecules i.e amylose content. A number of SNPs in the rice waxy gene are found to impact starch cooking quality and starch grain texture (Kharabian-Masouleh *et al.,*2012). There are two types of cultivated Asian rice-*indica* and *japonica*. Grains of *indica* rice generally contain higher amylose than *japonica* and this makes a good basis for distinguishing them from each other. The type of Waxy gene that sets them apart is, *indica* rice has the fully active wild-type allele (Wxa), whereas *japonica* rice has the intermediate one (Wxb). Zhou et al. (2016) found that the RS level is different between *indica* and *japonica* and such difference depends on their Wx alleles. Some studies also reported that higher amylose content increases RS level, and thus it is not surprising for the Wxb allele in *japonica* to cause lower RS content relative to that in *indica* rice. According to Fujita et al. (2007) ssIIIa mutation causes 1.4- to 1.8-fold increase in GBSS1 and 1.3- fold increase in the amylose content of *japonica* rice, which is good in terms of being able to increase RS in *japonica*. Crofts et al. (2012) introduced the *indica*Wxa allele into *japonica* rice carrying either SSIIIa or ssIIIa. The amount of GBSS1 has been found to be increased in the *japonica* rice containing

SSIIIa allele. In *japonica* rice containing ssIIIa allele, the amylose content was higher but not much difference in Wx expression, probably because the GBSS1 level was already maximal. Zhou et al. (2016) suggested that the interaction between SSIIIa and GBSS1 is likely to occur at the post translational stage. Considering the nutraceutical properties of phenolics present in pigmented rice varieties, three QTLs viz., qPC1.1, qPC11.1, and qPC11.2 were associated with phenolic content (PC) of brown rice (Qin et al. 2009). Zhong et al. (2011) reported two consistent QTLs for PC in milled rice as qPr1 and qPr7 on chromosome 1 and 7 respectively. Rc and Rd locus regulates pigmentation where Rc encodes for a regulatory BHLH protein that allows accumulation of proanthocyanidins (Sweeney *et al.,* 2006; Furukawa *et al.,* 2007); Rd codes for DFR (Dihydroflavanol reductase) which encodes anthocyanins. Understanding of the role of these genes led to manipulating this complex pathway for better quality starch.

## Manipulation of starch metabolism for improving RS content – Breeding to Genome Editing

Until now, classical breeding had a significant impact on improving the starch quality of rice cultivars by making crosses, backcrosses and selection of high amylose or high RS cultivars. For identifying this desired property, two general approaches have been followed. Either directly screening the phenotype, which requires a rapid screening strategy to identify variants or through identifying mutations in the genes that are known to influence RS content. The amount of amylose content (AC), waxy haplotype and the digestibility of rice has been significantly correlated (Kharabian-Masouleh *et al.,* 2012) and hence an initial step in this direction is to screen the variation in AC content. Analyses of the ACs of a set of germplasm collected by the International Rice Research Institute showed that AC in wild and cultivated rice ranges from 0 to 30% depending on the rice variety (Butardo *et al.,* 2008). Apparent AC is primarily controlled by the Waxy gene, which codes for GBSS (Chen et al. 2008a). Even though GBSSI synthesizes amylose and high amylose content is correlated to RS, over-production of GBSS-I by mutations did not result with high RS. GBSS has also known to contribute towards starch retrogradation properties, which yield type3RS. Other genes that have reported to contribute in retrogradation were glucose-6-phosphate translocator 1, SSI, SBEI and SSIIIa. Chen et al (2008a, b) reported that the waxy gene showed 4 haplotypes - viz., In1T-Ex6A, In1GEx6C,In1G-Ex6A and In1T-Ex6C used for the classification of AC in rice. Angwara et al. (2014) characterized 26 Thai rice varieties for RAG and Waxy haplotype (In1-Ex6) as Glycemic index indicators. Zeng *et al.,* 2016 identified QTLs of RS for rice (qRS7-1, qRS7-2) on chromosome 7. Other than altering amylose content, it is also possible that alterations in amylopectin structure, for example the production of highly branched molecules that inhibit the access of alpha amylase to its 1, 4-linked substrate might increase RS but this has not yet been demonstrated. Many mutants with elevated RS content have been identified in rice, including Goami 2, RS111,and Jiangtangdao 1 (Yang *et al.,* 2012). Discovery of QTLs associated with RS will assist for future fine mapping or pinpointing more functional genes, which can be manipulated using reverse genetic approaches.

Lower activity of ADP-Glc pyro phosphorylase and SBEs, and higher activity of SS and SDBEs has observed in high-RS rice, which might be responsible for the formation of small, irregular starch granules with large spaces (Shu and Rasmussen, 2014). However, morphologically different, starches have been identified in some rice mutants due to gradual decrease in enzymatic expression of SBE responsible for the formation of heterogenous starch granules (Wang *et al.,* 2018).Considering the immense role of starch biosynthetic enzymes; they have been manipulated for increasing RS content using various genomic strategies. SSI mutants, produced by the insertion of the Tos-17 retro transposon into the gene for rice SSI has been described in rice. Mutants with an altered structure where the proportion of short chains (DP6–7) and long chains (DP16–19) were increased and the chains of DP8–DP15 were reduced have been observed. A map based cloning of a RS locus in *indica* rice identified a defective soluble starch synthase gene (SSIIIa) responsible for RS production in b10 mutant (Zhou *et al.,* 2016). Teqing resistant starch (TRS) is another high amylose and RS transgenic line developed by modifying antisense RNA inhibition for SBE in rice. In high-amylose TRS rice, the C-type starch, which might result from the combination of both A-type and B-type starch, was observed and subsequently confirmed by multiple physical techniques, including X-ray powder diffraction, solid-state nuclear magnetic resonance, and Fourier transform infrared.

Induced mutations in SBEII were phenotyped with high amylose and with increased RS content (Hazard *et al.,* 2012). In rice, high amylose sbeIIb mutants were generated by means of chemical treatment or radiation (Shu *et al.,* 2006), or through hairpin RNA (hp-RNA) mediated RNA interference (RNAi) (Butardo *et al.,* 2011). Putative gene

sbe3-rs for RS mutated from SBE3 for Starch Branching Enzyme in Rice (*Oryza sativa* L.) has been reported as a marker gene for RS content (Yang *et al.,* 2012). In rice, both the sugary1 (sug1) locus and the ISA1 gene are located on chromosome 8 (Fujita *et al.,* 1999), while the PUL gene is located on chromosome 4. The reduction of ISA1 activity to about only 6% by using antisense technology resulted in modified amylopectin with more abundant short side chains and increased accumulation of soluble a-glucans (Fujita *et al.,* 2003). In transgenic rice generated by the introduction of the wheat ISA1 gene into sug1 rice, phytoglycogen synthesis was substantially replaced by starch synthesis in the endosperm (Kubo *et al.,* 2005). These reports strongly suggest that sugary1 mutations in maize and rice are caused by ISA1 deficiency and ISA1 plays a crucial role in amylopectin biosynthesis. Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of SBEI and SBEIIb reported with 25% increase in AC and 9.8% RS content (Sun *et al.,* 2017). Recently a novel class of regulators of starch metabolism have been described. These proteins, known as water di-kinases, are involved in starch degradation through control of phosphorylation of C3 and C6 positions of glucose in the leaves of the model plant Arabidopsis and lead to the starch excess phenotype in leaves (Ritte *et al.,* 2004). Modulation of granule micro-structure achieved by decreasing starch branching and increasing starch-bound phosphate content in the barley caryopsis starch by RNAi suppression of all three Starch Branching Enzyme (SBE) isoforms or overexpression of potato GlucanWater Dikinase (GWD) resulted Amylose-Only (AO) and Hyper Phosphorylated (HP) starch chemotypes. Such AO lines explained how re-direction of carbon partitioning occurs in starch grain (Shaik *et al.,* 2016).

Wild pigmented rice has been found to be effective in improving abnormal glucose metabolism by having higher proportion of digestion RS as well as due to the presence of antioxidants like anthocyanins (Han and Huang, 2013). Previous studies have demonstrated that food derived active ingredients such as phenolic compounds can control blood glucose level (Thondre *et al.,* 2013). In addition, fortification with anthocyanins from plant sources effectively reduced the digestion rate of bread (Sui *et al.,* 2016). Bae *et al.,* (2017) reported the role of anthocyanins in inhibiting digestive enzymes and thus suppressing starch hydrolysis under *in vitro* conditions. Anti-glucosidase activity of anthocyanins has been reported (McDougall *et al.,* 2005). Yao et al. (2010) found that anthocyanins isolated from black rice inhibited alpha-glucosidase. In addition, in an animal model of T2DM, treatment with an anthocyanin (cyanidin 3-glucoside) significantly reduced blood glucose concentration and improved insulin sensitivity after an insulin tolerance test in male mice. Through classical genetic approaches, Yoshimura et al (1997) identified pb and pp loci on chromosome 4 and 1 for pericarp pigmentation in black rice. Further, by association mapping Shao *et al.,* 2011 reported RM339 and RM316 as common markers for antioxidants, flavonoids and phenolics. These can assist breeders in developing neutraceutically rich varieties with improved RS content.

## Conclusion

Digestion-resistant starches are type of functional starch with immense health benefits but its process of synthesis and organization is still a mystery. Environmental and genetic factors that affect starch resistance in crops are being identified, including using biotechnology to control starch digestibility. A myriad of enzymes, during granule biogenesis as well as packing will assist in RS formation. Starch pathway engineering using classical tool to genome editing has revealed potential candidates. Future integrative research that addresses all of these issues will help expand the potential uses for digestion-RS in health promotion. Starch granule initiation and formation, enzyme complexes involved in starch metabolism and control of flux in starch synthesis critically contribute towards functional aspects of starch. Careful manoeuvring the key regulatory enzymes using genetic tools will help in developing the desired configurations of starch molecules.

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# Characterization of genetic diversity among wild rice accessions using genome specific In-Del markers

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## Abstract

The genus *Oryza* contains 22 wild species and two cultivated species namely *O. sativa* and *O. glaberrima.* In the present study, 17 accessions belonging to 17 different wild species of rice characterized using 21 wild rice genome specific In-Del markers. A total of 115 fragments/alleles were detected among the 17 wild species. The overall number of alleles per locus ranged from three to 10 with an average of 5.47 indicating the existence of high level of diversity among the accessions. The size of the detected alleles produced using the In-Del primer sets ranged from 105 bp with respect to ch10-044G to 545 bp in ch03-363w. The differences in molecular size between the smallest and the largest allele for a given In-Del locus varied from 25 bp (ch02-342w) to 375 bp (ch05-109G). The PIC values for 21 In-Del loci varied from 0.4 (ch04-276G) to 0.85 (ch05-109G) with an average of 0.68. The overall *He* values ranged from 0.54 (ch02-342w) to 0.87 (ch05-109G) with an average of 0.72. The cluster analysis grouped the 17 accessions into three main clusters. Cluster I consisted of two subclusters IA with *O. glumaepatula, O. longistaminata*, *O. rufipogon* and subcluster IB consist of *O. meridionalis*, *O. barthii*, and *O. nivara*. Cluster II consist of *O. rhizomatis*, *O. officinalis* and *O. eichingeri* which originated in same officinalis complex in rice evolution. Cluster III was subdivided into two sub-clusters with IIIA consisting of *O. punctata*, *O.minuta*, *O. ridleyi* and *O. longiglumis* and IIIB consisting of *O. latifolia*, *O. australiensis*, *O. alta* and *O. grandiglumis*. The results of the study reveal that genetic diversity among the accessions from different wild species considered in the present study is moderate. The In-Del markers utilized in this study were able to distinguish 10 out of the 17 species of *Oryza*. Based on the results obtained it can be concluded that In-Del marker based analysis in consonance with morphological marker data would be more appropriate to ascertain the identity of wild rice.

**Key words:** Genetic diversity, In-Del markers, wild rice species.

## Introduction

Rice is the staple food for more than one third of the global population (Chakravarthi and Naraveni, 2006). The genus *Oryza* contains 24 species, of which 22 are wild species and two are cultivated species namely *O. sativa* and *O. glaberrima* (Brar and Khush, 2003). The wild species are either diploid or tetraploid with 2n=24 or 48 respectively. The species of the genus *Oryza* are broadly classified into four complexes (Vaughan, 1994) *viz*., *Sativa*, *Officinalis*, *Ridley* and *Meyeriana* complexes (Table 1).

**Table1. List of wild species of rice used in the study are given**

| Sl. No | Species | IRGC No. | Ploidy | Genome | Chromosome number |
|---|---|---|---|---|---|
| 1 | *Oryza nivara* | 80435 | Diploid | AA | 24 |
| 2 | *Oryza rufipogon* | 81900 | Diploid | AA | 24 |
| 3 | *Oryza barthii* | 104290 | Diploid | AA | 24 |
| 4 | *Oryza longistaminata* | 103560 | Diploid | AA | 24 |
| 5 | *Oryza meridionalis* | 105283 | Diploid | AA | 24 |
| 6 | *Oryza glumaepatula* | 100969 | Diploid | AA | 24 |
| 7 | *Oryza punctuate* | 88824 | Diploid & Tetraploid | BB, BBCC | 24,48 |
| 8 | *Oryza minuta* | 93257 | Tetraploid | BBCC | 48 |
| 9 | *Oryza officinalis* | 80733 | Diploid | CC | 24 |
| 10 | *Oryzarhizomatis* | 103410 | Diploid | CC | 24 |
| 11 | *Oryza eichingeri* | 81804 | Diploid | CC | 24 |
| 12 | *Oryza latifolia* | 99580 | Tetraploid | CCDD | 48 |
| 13 | *Oryza alta* | 100952 | Tetraploid | CCDD | 48 |
| 14 | *Oryza grandiglumis* | 105157 | Tetraploid | CCDD | 48 |
| 15 | *Oryza australiensis* | 100882 | Diploid | EE | 24 |
| 16 | *Oryza ridleyi* | 100877 | Tetraploid | HHJJ | 48 |
| 17 | *Oryza longiglumis* | 100974 | Tetraploid | HHJJ | 48 |

A systematic study analysis of genetic diversity of wild rice accession is essential to exploit the inherent variability of wild rice for adding novel traits and to broaden the genetic base of rice cultivars. Several studies have advocated use of molecular markers for precise and reliable characterization of diversity of wild rice germplasm (Karkousis *et al*., 2003). Allelic diversity in the germplasm is assessed by polymorphic information content (PIC). The PIC is reflection of allelic diversity and frequency among the germplasm lines (Krupa *et al.,* 2017). The extent of genetic diversity in the population is assessed by expected heterozygosity ($H_e$). The $H_e$ is the probability that, at a single locus, any two alleles, chosen at random from the population, are different to each other (Nei, 1987).

Molecular markers namely RAPDs, AFLPs, SSRs, have been employed in molecular characterisation of wild species of rice (Lu *et al.,* 2009). Insertions/deletions (In-Dels) markers have also been employed in several studies (Varshney *et al.,* 2007). In-Dels are easy to use, PCR-based, co-dominant, highly polymorphic and widely distributed throughout the genome (Pacurar 2012; Lv *et al*., 2013; Yamaki *et al*., 2013; Wu *et al*., 2014). Short sequence and homo nucleotide repeats in genome tend to accumulate In-Dels due to polymerase slippage during replication while frame shift In-Dels in coding regions can result in loss of function or non-sense mutation (Rockah-Shmuel *et al*., 2013). Yamaki *et al.,* (2013) have developed 22 In-Del markers specific to genomes of wild species of rice. These markers were suggested to be useful in characterization and identification of different wild species of rice. In the present study 17 wild species of rice species were characterized with the 21 In-Del markers developed by Yamaki *et al*. (2013) to ascertain the species identity and for molecular diversity analysis.

**Materials and methods:**

***Plant materials:*** A total of 17 wild rice accessions each one represents different rice species genome (Table 1), available at wild rice garden, ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad

***Genomic DNA isolation and quantification:*** Total genomic DNA was isolated from 15 days old seedlings of wild rice plants following CTAB (Cetyl Try Methyl Ammonium Bromide) methodas described by Zheng *et al.* (1991). The total genomic DNA was quantified using 0.8% agarose gel electrophoresis lamda (λ) Hind III DNA as standard. The DNA samples were normalized in $T_{10}E_1$ buffer (10 mMTris-HCl, 1 mM EDTA, pH 8.0) to get final concentration of 50 ng/ µl for PCR amplification.

***In-Del markers****:* Oligonucleotide sequence of 21 In-Del markers (Yamaki *et al*., 2013) accessed at on the integrated

rice science database, Oryzabase (http://www.shigen.nig.ac/oryzabase) and the primers were custom synthesised (IDT, USA) for further studies.

***Polymerase chain reaction (PCR):*** The PCR reaction was performed as described in Yamaki *et al*. (2013) in a total reaction volume of 10 µl containing 2.5mM dNTP mix, 10 pmol each of forward and reverse IN-DEL primers, 10x optimized Taq buffer and 1unit of *Taq* DNA polymerase. PCR was performed under conditions of 94$^0$c for 2min and a subsequent 35 cycles of 94 $^0$C for 1min, 56 $^0$C for 1min and 72 $^0$C for 1min, followed by 72 $^0$C for 2min. The amplified PCR products were mixed with bromophenol blue and resolved by 3% Agarose gel prepared in 1X TAE buffer stained with ethidium bromide at 37$^0$C (10mg/ml) along with 100bp ladder. The gel electrophoresis was carried out for three hours at 100 volts. The gel was viewed under UV trans-illuminator in a gel documentation system (Medicare, GELSTAN, USA). Gels were analyzed for status of the genotype by In-Del markers using the software of the gel documentation system. Total bands present in each cultivar was detected from the gels and scored manually. Based on the molecular weights of the bands, 1, 2, 3… scoring was subjected for statistical analysis.

***Data Analysis:*** Molecular profiles of wild species obtained from the present study were compared with findings of Yamaki *et al*. (2013) to ascertain species identity. The genotypic data generated from 17 different wild species, 21 In-Del markers were utilized for cluster analysis and a Dendrogram was generated using DARwin v 6.0 (Perrier and Jacquemoud-Collet, 2006) based on unweighted neighbour joining (UNJ) method followed by boost strap analysis with 1000 per mutations for total cultivars. To measure the informativeness of the markers, polymorphism information content (PIC) for each of the In-Del markers was computed according to the formula: PIC =1− Σ $pi^2$, Where pi is the frequency of the $i^{th}$ allele of each locus (Botstein *et al.*, 1980). Genetic diversity parameters *viz.,* number of alleles (*Na*) and expected Heterozygosity ($H_e$) (Nei *et al.,* 1973) were evaluated using POP-GENE version 1.31 (http://www.ualberta.ca/,fyeh).

**Results and Discussion**

Wild relative of crop plants possess enormous genetic diversity for various biotic, abiotic and yield enhance traits. Utilisation of wild relative of crop plants through conventional and molecular breeding approach is imperative to broadening the genetic base of crop cultivars. Molecular characterisation and genetic diversity analysis is important tools to understand how the species have evolved, their relatedness and distinct genomic signatures among the various accessions of wild species. The Genus *Oryza* consists of 22 wild relatives and two cultivated species constituting the genetically most diverse species of model crop plants. In the present study, 17 wild species of rice (Table 1) were characterised with 21 genome specific In-Del markers (Table 2) to assess the molecular diversity among the wild species of rice.

***Characterisation of wild species***

Molecular characterisation of wild species of rice using 21 In-Del markers revealed that, out of 17 species studies, the amplicon size of In-Del markers in ten species namely, *O. rufipogon* (IRGC 80435) varied from 122bp (ch02-308w) to 455bp (ch03-363w), Similarly for *O. barthii* (IRGC-104290) from 122 bp (ch10-044G) to 444 bp (ch-03-363), in *O. glumaepatula* (IRGC-100969) from 122bp (ch-10-44W) to 450bp (ch-03-363W), in *O. punctata*(IRGC-88824) from 117bp (ch-10-44G) to 433bp (ch-03-363W), in *O. officinalis* (IRGC-80733) from 110 bp (ch-10-44G) to 525 bp(ch-03-363w), in *O. rhizomatis* (IRGC-103410) from 125bp (ch-10-44G) to 545bp (ch-03-363W), in *O. alta* (IRGC-100952) from 128 bp (ch-10-44G) to 434bp (ch-03-363W), in *O. grandiglumis* (IRGC-105157) from 130bp (ch-10-044G) to 437 bp (ch-03-363W), in *O. australiensis*, (IRGC-100882) from 126bp (ch-10-44G) to 450bp (ch03-363w) and in *O. meridionalis* (IRGC-105283) from 115 bp (ch-02-044G) to 459 bp (ch-03-276G) with one allele per marker loci in all these 10 species studied. The molecular profiles of the ten species were similar to the findings of Yamaki*et al.* (2013) indicating the identity of species and utility of the In-Del markers in identification of ten wild species of rice.

Interestingly, the molecular profile of six species namelym O. *longistaminata, O. longiglumis, O. minuta, O. eichingeri, O. latifolia,* differed from the report of Yamaki *et al.* (2013) with respect to either amplicon size or number of alleles per markers (Table 2). The findings from the present study need to be further confirmed with morphological data to ascertain the species identity the six wild species. Importantly, this study also characterized, molecular profiling of *O. nivara* (IRGC-80435) accession with the In-Del markers, which was not characterized by Yamaki *et al*. (2013). Further, this information will be useful for identification of the *O. nivara* species.

***Genetic diversity analysis***

The amplicon size of the detected alleles of 21 In-Del markers among the 17 wild species of rice ranged from 105 bp with respect to the marker ch10-044G to 545 bp in the marker ch03-363w indicating the presence of

large insertions and/deletions in the wild rice genome of different species. Similar kinds of results were reported earlier with a large difference in the number of repeats between the different alleles for SSR primers by Adegbaju *et al.* (2015). A total of 115 alleles were amplified by the 21 In-Del markers on in different wild species of rice. The number of alleles per marker ranged from three to 10 alleles per allele with an average of 5.47 alleles (Table 2) indicating the presence of higher allelic diversity of In-Del markers among the accessions of wild species studied. This was comparable to the report of Rani *et al.* (2016) who reported average allele number of 4.3 per locus as high level of allelic diversity.

**Table2. The list of 21 In-Del primers used in the estimation of genetic diversity of 17 wild rice accessions, showing variation for number of alleles (AN), amplicon size (bp), PIC (polymorphism Information Content) and $H_e$ (Genetic Diversity) values**

| S. No. | Primer Name | amplicon size (bp) | No. of Alleles | PIC | $H_e$ Values Genetic diversity index |
|---|---|---|---|---|---|
| 1 | ch01-301w | 347 – 421 | 6 | 0.74 | 0.78 |
| 2 | ch02-308w | 115 – 189 | 3 | 0.53 | 0.6 |
| 3 | ch02-342w | 200 – 225 | 3 | 0.49 | 0.54 |
| 4 | ch02-343w | 128 – 388 | 7 | 0.75 | 0.78 |
| 5 | ch03-128w | 271 – 324 | 4 | 0.66 | 0.71 |
| 6 | ch03-173w | 114 – 374 | 6 | 0.73 | 0.77 |
| 7 | ch03-363w | 427 – 545 | 6 | 0.77 | 0.8 |
| 8 | ch04-276G | 115 – 148 | 3 | 0.48 | 0.55 |
| 9 | ch04-312w | 148 – 203 | 4 | 0.68 | 0.73 |
| 10 | ch05-067w | 118 – 235 | 6 | 0.71 | 0.75 |
| 11 | ch05-070w | 234 – 378 | 6 | 0.77 | 0.8 |
| 12 | ch05-109G | 151 – 526 | 10 | 0.85 | 0.87 |
| 13 | ch05-202w | 127 – 280 | 4 | 0.59 | 0.65 |
| 14 | ch05-277w | 288 – 345 | 5 | 0.68 | 0.72 |
| 15 | ch06-269w | 259 – 394 | 8 | 0.79 | 0.81 |
| 16 | ch06-300w | 150 – 196 | 5 | 0.69 | 0.73 |
| 17 | ch06-306w | 106 – 200 | 7 | 0.77 | 0.79 |
| 18 | ch07-233w | 168 – 327 | 9 | 0.8 | 0.82 |
| 19 | ch08-006w | 228 – 295 | 5 | 0.76 | 0.79 |
| 20 | ch09-037G | 116 – 188 | 5 | 0.65 | 0.7 |
| 21 | ch10-044G | 105 - 135 | 3 | 0.55 | 0.62 |
| **Total** | | | **115** | **14.44** | **15.31** |
| **Mean** | | | **5.47** | **0.68** | **0.72** |

The marker ch05-109G showed maximum number of alleles (10 alleles) with the highest PIC value (0.85) and $H_e$ index (0.87) followed by the marker ch07-233w with nine alleles, 0.80 PIC value and 0.82 $H_e$ index. The Polymorphism information content (PIC) values provides an estimates of discriminating power of a marker based on the number of alleles at a locus and relative frequencies of these alleles (Chen *et al*., 2017). The PIC values of 21 In-Del markers in our study varied from 0.4 (ch04-276G) to 0.85 (ch05-109G) with an average of 0.68 while more than 40 per cent markers were in the range of 0.7 – 0.8 (Figure 1) indicating wide distribution of polymorphism among the markers. Hence these In-Del markers could be considered as highly informative and useful for genetic diversity studies. Nei's genetic diversity estimated by average expected heterozygosity ($H_e$). It is the probability that, at a single locus, any two alleles, chosen at random from the population, are different to each other Nei (1973). The overall $H_e$ values ranged from 0.54 (ch02-342w) to 0.87 (ch05-109G) (table 2) with an average of 0.72 while more than 50 per cent of the markers were in the frequency of 0.7-0.8 (Figure 1). The average $H_e$ over all loci is an estimate of the extent of genetic variability in the population. $H_e$ value will be maximum, when there is maximum number of alleles per locus. The average $H_e$ in the study was 0.72 indicating the presence of high genetic diversity. The un-weighted neighbour joining (UNJ) dendrogram was constructed based on polymorphism data of the 21 In-Del markers using DARwin 6.0. The dendrogram classified 17 accessions into three main clusters (Figure 2). Cluster I consist of AA genome species of *Oryza* complex. The Cluster I was further classified into two sub-clusters IA with *O. glumaepatula, O. longistaminata*, *O. rufipogon* and sub-cluster IB consist of *O. meridionalis*, *O. barthii*, and *O. nivara*. Cluster II consist of *O. rhizomatis* (CC), *O. Officinalis* (CC) and *O. eichingeri* (CC) which belongs to *officinalis* complex with CC genome. Cluster III sub-divided into two with IIIA consist of *O. punctata (BBCC)*, *O. minuta (BBCC)*, and also *O. ridleyi (HHJJ)*, *O. longiglumis (HHJJ)* while IIIB consist of *O. latifolia (CCDD)*, *O. alta (CCDD), O. grandiglumis (CCDD)* and also *O. australiensis* (EE). Maximum dissimilarity (Table 3) was observed between *O. rufipogon* and *O. grandiglumis* (0.732) possibly due to distant ancestry of the two wild species. Similar kind of results were observed by Kundur *et al.* (2015) who reported 37% of genetic variation for target region amplification polymorphism (TRAP) markers.

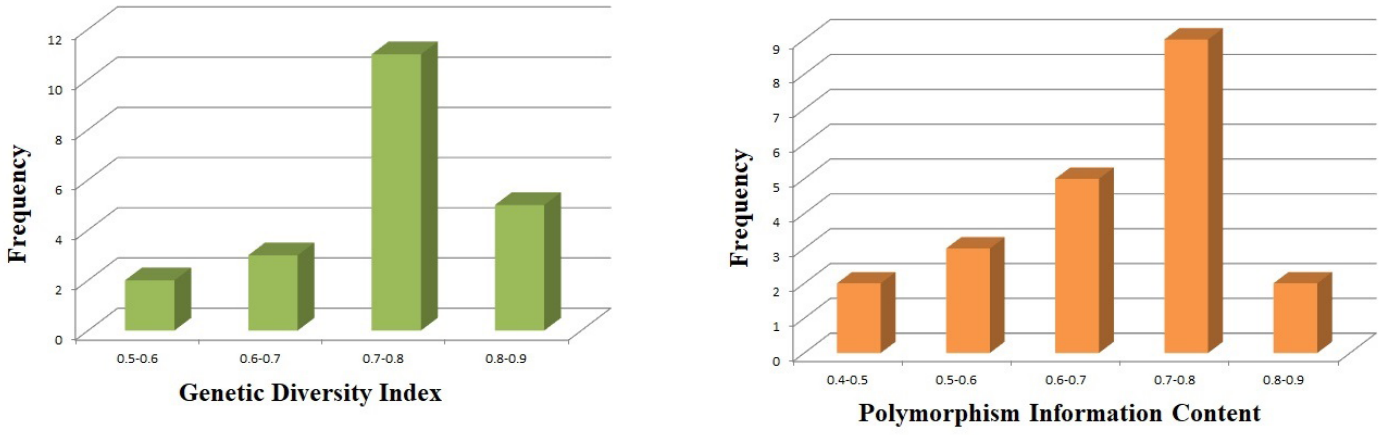


**Figure 1. (a) Frequency distribution of Genetic Diversity Index and (b) Polymorphism Information Contents**

**Table 3. Jaccard Dissimilarity Matrix as obtained based on 21 In-Del markers**

| Genotypes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.95 | | | | | | | | | | | | | | | |
| 3 | 0.975 | 0.975 | | | | | | | | | | | | | | |
| 4 | 0.95 | 1 | 1 | | | | | | | | | | | | | |
| 5 | 0.975 | 1 | 0.975 | 0.95 | | | | | | | | | | | | |
| 6 | 0.975 | 0.785 | 0.978 | 0.933 | 0.978 | | | | | | | | | | | |
| 7 | 0.981 | 0.976 | 1 | 1 | 0.785 | 0.957 | | | | | | | | | | |
| 8 | 1 | 0.978 | 1 | 0.795 | 0.885 | 0.847 | 0.978 | | | | | | | | | |
| 9 | 1 | 1 | 0.933 | 1 | 1 | 0.978 | 0.976 | 0.884 | | | | | | | | |
| 10 | 0.753 | 0.966 | 0.975 | 0.884 | 0.785 | 1 | 1 | 1 | 0.975 | | | | | | | |
| 11 | 1 | 0.973 | 1 | 0.978 | 0.945 | 1 | 1 | 0.732 | 0.977 | 1 | | | | | | |
| 12 | 1 | 0.845 | 1 | 0.951 | 1 | 1 | 1 | 1 | 1 | 0.976 | 0.978 | | | | | |
| 13 | 1 | 1 | 0.975 | 1 | 0.975 | 0.978 | 0.973 | 0.978 | 1 | 1 | 1 | 1 | | | | |
| 14 | 0.975 | 0.975 | 0.952 | 1 | 1 | 0.845 | 0.981 | 0.978 | 0.975 | 1 | 1 | 0.878 | 0.95 | | | |
| 15 | 1 | 1 | 0.975 | 1 | 1 | 0.978 | 0.951 | 1 | 0.878 | 0.966 | 0.977 | 0.983 | 1 | 1 | | |
| 16 | 1 | 0.878 | 0.967 | 1 | 0.945 | 0.883 | 0.963 | 0.978 | 0.993 | 0.976 | 1 | 1 | 1 | 1 | 1 | |
| 17 | 1 | 1 | 0.975 | 1 | 1 | 0993 | 1 | 1 | 0.971 | 0.884 | 1 | 0.963 | 1 | 0.975 | 0.975 | 1 |

**1.***O. punctata*, **2.***O. minuta***3.***O. rhizomatis*, **4.***O. ridleyi*, **5.***O. longiglumis*, **6.***O. nivara*, **7.***O. barthii*, **8.***O.grandiglumis*, **9.***O. meridionalis*, **10.***O. longistaminata*, **11.***O. rufipogon*, **12.***O. glumaepatula*, **13.***O. latifolia*, **14.***O. australiensis*, **15.***O. eichingeri*, **16.***O. alta*, **17.***O. officinalis*

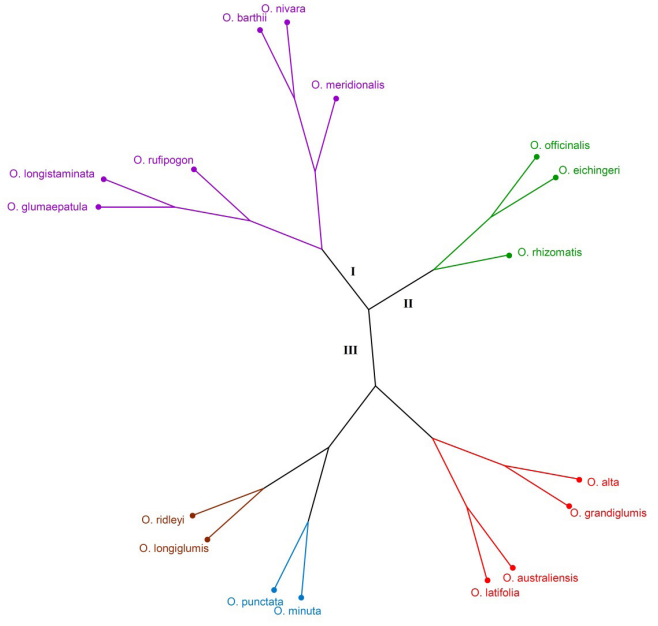


**Figure 2. Un-weighted Neighbour Joining (UNJ) tree showing genetic relationship among 17 wild species for 21 In-Del markers**

The In-Del markers used in the study were useful in distinguishing the most of wild species of rice. The molecular profile from In-Del markers along with morphological data should be used for discriminating and classifying the wild rice. In-Del markers were also useful in assessing the genetic diversity of wild rice. Nearly 11 species of wild rice genomes have been sequenced (www.Gramene.org). Using genome sequence information of wild species of rice, it is essential to identify unique genome signature to each genome of wild rice to further develop the robust and non-ambiguous DNA markers to distinguish the different species of wild rice without any perplexity.

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