RESEARCH ARTICLE

Assessment on variation of performance indicators under phosphorus-starved conditions in a core-set of rice cultivars and their diversity in *pup1*-linked DNA marker-haplotypes

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Abstract: The development of phosphorous deficiency tolerance (PDT) in rice cultivars is important for sustainable rice production. If the performances (PDT indicators) of the rice cultivars can be correlated with *Pup1* (major QTL linked to PDT in rice) haplotypes, marker assisted breeding (MAB) programmes could be launched to produce phosphorous deficiency tolerant rice varieties. In the present study, 27 rice cultivars (nine landraces and 18 improved varieties) were screened under P-deficient soil conditions in two seasons (*Yala* and *Maha*, the major cropping seasons in Sri Lanka) based on shoot dry weight and P utilisation efficiency. To establish the basis for MAB of PDT, the *Pup1* haplotypes were assessed using 17 *Pup1* linked DNA markers and sequence polymorphism of the two marker loci, *K29* and *RM28102*. The PDT screening results revealed that the performance of the rice cultivars is season-dependent. The landrace *Madathawalu* and the old-improved variety H-4 showed the highest trait values for yield, shoot dry weight, and P utilisation efficiency in both *Yala* and *Maha* seasons. Remarkably, *Madathawalu* and H-4 share a common *Pup1* haplotype demonstrating that MAB for PDT is possible using these two rice cultivars as parents. The *Pup1* linked marker haplotypes and variation of the PDT indicators of the studied cultivars can be used to conduct MAB programmes to develop improved rice varieties.

Keywords: Marker assisted breeding, phosphorus efficient rice, phosphorus fertiliser crisis, *Pup1* QTL, rice landraces.

INTRODUCTION

Phosphorus (P) is one of the most important macronutrients for the growth and development of rice plant (Halford, 1997). The problem associated with P as a macronutrient is the limited availability in soil for the plants due to the fixation by Fe_2O_3 , Al_2O_3 , CaCO_3 , MgCO_3 , carboxyl ions, and humic substances (Wang *et al.*, 2013). However, farmers do not recognise the fixation of P ions within the soil, and they add higher concentrations of P fertiliser for better growth and development of the plants. The applications of artificial P fertiliser cause two problems in rice farming (Bulluck & Ristaino, 2002; O'neil *et al.*, 2012). Sri Lanka spends 0.3 billion US dollars annually (1.5 % of GDP) on importing P fertiliser to Sri Lanka, and farmers struggle to purchase these fertilisers due to their higher price (Aluwihare *et al.*, 2016). Therefore, the government has to provide P fertiliser under a subsidised rate to the farmers creating an extra burden to the national economy. The additional P fertiliser incorporated to the soil would end up in runoff water; ultimately polluting the water bodies causing eutrophication and health hazards (Bulluck &

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Ristaino, 2002; O'neil *et al.*, 2012). In addition to the higher price and environmental pollution caused by the heavy dependence on P fertiliser, the currently available P reserves in the world are depleting rapidly and likely to cause a massive fertiliser crisis in the near future (Steen, 1998; Cordell *et al.*, 2009; FAO, 2015). Farmers need large amounts of P fertiliser in every season; however, P reserves are rapidly depleting which will place the future rice farming in a futile situation.

The only sustainable solution to face the indisputable P fertiliser crisis of the future rice farming is to produce P deficiency tolerant rice varieties through DNA marker assisted breeding (MAB) (i.e., molecular breeding). The genetics of P deficiency tolerance (PDT) has been studied in detail using worldwide rice germplasm (Ni *et al.*, 1998; Wissuwa *et al.*, 1998). Majumder *et al.* (1989) first reported that PDT is a quantitatively inherited trait. The major QTL underlying the PDT in rice has been identified and named *Phosphorus Uptake 1* (*Pup1*) by using a segregating progeny made by crossing P deficiency tolerant *Kasalath* and P deficiency sensitive *Nipponbare* as the parents (Ni *et al.*, 1998; Wissuwa *et al.*, 1998). Although some other minor QTLs were detected, *Pup1* was further validated as the major QTL with 80 % effect on the trait PDT. The effect of *Pup1* was further verified by Chin *et al.* (2010) and Wissuwa *et al.* (2002) by incorporating the *Pup1* allele of *Kasalath* to *Nipponbare* through backcrossing, which demonstrated the increment of P uptake by 170 % and grain yield by 250 %.

Subsequently, the *Pup1* locus of *Kasalath* was molecularly characterised with respect to the counterpart allele in *Nipponbare*. The total length of the *Pup1* QTL in *Kasalath* is 423 kb, whereas *Nipponbare* has 293 kb allele difference due to the presence of a large deletion of about 130 kb (Heuer *et al.*, 2009). The molecular markers within *Pup1*, the candidate genes and their relative

physical and mapping distances were characterised and are publicly available (Chin *et al.*, 2010; 2011). One of the drawbacks in *Pup1* characterisation studies is the underutilisation of country-specific improved varieties and traditional landraces. It has been observed that such locally adapted rice varieties perform impressively in infertile soils. The local varieties may contain additional QTLs to *Pup1* or hitherto unknown haplotypes of *Pup1*.

The old improved rice cultivars and landraces in Sri Lanka possess novel *Pup1* haplotypes compared to the *Kasalath* type *Pup1* haplotype (Aluwihare *et al.*, 2016; 2018). This observation highlights the significance of studying the important local rice landraces and varieties (i.e., cultivars) for PDT and then detecting their *Pup1* haplotypes with respect to the association with PDT conferring traits. Thus, MAB can be swiftly carried out within the regional rice breeding programmes because of the availability of locally present PDT promoting haplotypes. They would provide a strong basis for selecting more efficient rice varieties for local needs rather than just examining for the presence of *Pup1* haplotype in the lines to be selected. Therefore, the present study, was aimed at screening a set of rice landraces and improved rice varieties in Sri Lanka for PDT, identify their maker haplotypes of *Pup1* and characterise two key co-dominant sequence tagged sites (STS) within *Pup1* locus to facilitate MAB of rice for PDT.

METHODOLOGY

Growing seasons

Based on the two seasons of monsoonal rains in Sri Lanka, rice farming is conducted under two major cropping seasons, namely, *Yala* and *Maha*. The growing season *Yala* runs from early April to late August with

Table 1: Climatic parameters of the field location during the two seasons

Location and global positioning system (GPS) coordinates	Agro-ecological zone (AEZ) ^a	Season	Temperature (°C)	Total rainfall (mm) of the season	Sunshine hours (h/day)
Bathalagoda (N7°31'49.21"; E80°26'25.49")	IL1a	<i>Maha</i> (Sep-Mar) 2016/2017	21.3 – 32.4	479.6	7.6 – 8.0
		<i>Yala</i> (Apr -Aug) 2017	24.3 – 32.2	408.4	6.2 – 8.0

^a AEZ classification was obtained from the Department of Agriculture, Sri Lanka (<http://www.agridept.gov.lk/>). IL: Intermediate low

the Southwest monsoonal rains and the growing season. *Maha* runs from late September to early March with the Northeast monsoonal rains (DOA, 2006). The growing

and climatic conditions of the field where the trial was conducted are given in Table 1. The trials were conducted in *Maha* and *Yala* seasons of the year 2016/2017.

Table 2: Rice genotypes screened for phosphorous deficiency tolerance

Name of the genotype	Type of cultivar	Naturally preferred growing condition	Key characteristics
<i>Herath banda</i>	L	UL	Red pericarp, high amylose content
<i>Kuruluthudu</i>	L	UL	Red pericarp, good cooking quality, rich in protein and fibre, enhance male sexual potency, improved bladder function
<i>Madathawalu</i>	L	UL	Red pericarp, comparatively low glycemic index, recommended in Ayurvedic treatments
<i>Masuran</i>	L	UL	Red pericarp, bold grain type
<i>Pachchapermal</i>	L	UL	Red pericarp, rich in protein, recommended for diabetes and cardiovascular diseases
<i>Rath suwandel</i>	L	UL	Red pericarp
<i>Rathdel</i>	L	UL	Recommended for cirrhosis, prevent formation of stones in the bladder and gall bladder
<i>Sulai</i>	L	UL	Red pericarp, good cooking quality, resistant to brown plant hopper
<i>Thatu vee</i>	L	UL	Red pericarp
At308	NI	LL	Suitable to grow under rain fed and irrigated farming conditions
At309	NI	LL	White pericarp, moderately resistant and resistant to blast and moderately resistant to brown plant hopper
At311	NI	LL	Red elongated pericarp
At354	NI	LL	White pericarp
At373	NI	LL	White pericarp, short round grain, good cooking quality, resistance to gall midge
Bg310	NI	LL	White pericarp, high amylose content, resistant to blast, gall midge and brown plant hopper
Bg359	NI	LL	Resistance to gall midge 1 and 2, rice blast disease and bacterial leaf blight, moderately tolerant to brown plant hopper
Bg366	NI	LL	White pericarp, non-glutinous endosperm, resistant to bacterial leaf blight
Bg369	NI	LL	White pericarp, long grain shape, recommended for saline affected areas
Bw363	NI	LL	White pericarp, moderately tolerant to brown plant hopper, blast and gall midge
Bw367	NI	LL	Short round grain type, moderately tolerant to ion toxicity, tolerant for lodging
Bw452	NI	LL	Red pericarp, little tall, tolerant to bronzing and submergence in low country wet zone
Ld356	NI	LL	Red pericarp, short round grains
Ld365	NI	LL	White pericarp
Ld371	NI	LL	Tolerant to seed discolourations and neck blast, resistant/moderately resistant to blast, gall midge and brown plant hopper
H-10	OI	UL	Red pericarp, less photoperiod sensitivity
H-4	OI	UL	Resistant to rice blast disease
H-7	OI	UL	Better grain quality, higher response to nitrogen

Source: Rice Research and Development Institute, Bathalagoda, Sri Lanka

L: landrace; NI: newly improved; OI: old improved (during 1950's); UL: upland; LL: lowland

Rice cultivars, plant establishment and maintenance

The breeder seeds of 27 rice cultivars [landraces (L), old-improved (OI) and newly-improved (NI) varieties] were obtained from the Rice Research and Development Institute (RRDI) Bathalagoda, Sri Lanka (Table 2). Seeds were germinated and established in non-fertilised soil at RRDI, where a separate block of the field is maintained as non-fertilised soil for the last 45 years (Kumaragamage & Indraratne, 2011; Sirisena & Wanninayake, 2014).

Data collection

Growth and yield parameters

The plant height (PLH), number of tillers (NT), flag leaf length (FLL), flag leaf width (FLW) and yield per plant were collected for both seasons. PLH was measured from the base of the plant to the tip of the top leaf in centimeters. These measurements were taken at the flowering stage. NT was also recorded at the flowering stage for all the cultivars. FLL and FLW were measured after the flowering stage. The FLL was measured from the bottom of the leaf to the tip in centimeters. Maximum width of the leaf was taken as the FLW and measured in millimeters. Aerial parts of the plant were used to obtain the shoot dry weight (SDW). The plants were harvested at the flowering stage and the shoot parts were washed with distilled water and oven dried at 60 °C to obtain a constant dry weight (grams).

PDT indicators

The plants from each cultivar were collected at the harvesting stage, roots were removed, and oven dried until a constant weight was obtained. Weight of the oven dried samples were measured and taken as the SDW in grams. Oven dried shoots were ground into fine powder and a total of 0.5 g of powdered shoot material was taken for the digestion. The process of digestion was carried out using an acid mixture of conc. HNO₃ and conc. HClO₄ in a ratio of 4:1, respectively. The shoot P concentration (SPC) was obtained as the amount of P (mg) in 1 g of shoot dry matter by following the phosphovanadate method described in Hanson (1950). The shoot phosphorous uptake (SPU) was calculated using the formula described in Fageria *et al.* (1988) and Gunes *et al.* (2006). SPU was calculated for each variety in mg per plant.

Phosphorus utilisation efficiency (PUE) was calculated in grams per milligrams, in other words biomass produced per unit P accumulated in shoot (Rose & Wissuwa, 2012).

Establishment of DNA marker haplotypes for the *Pup1* locus

DNA extraction and PCR

Immature leaf samples of rice cultivars (Table 2) were collected and subjected to DNA isolation using Dneasy® plant mini kit (Qiagen, Solna, Sweden). The isolated DNA of each cultivar was subjected to PCR amplification with the markers developed for *Pup1* QTL (Table 3). The PCR conditions were provided in the Thermal Cycler (Takara, Japan) as follows; initial denaturation at 94 °C for 5 min, then 35 cycles at 94 °C for 30 s for denaturation, primer annealing temperature (Ta) (Table 3) for 90 s, and 72 °C for 2 min, finally extension at 72 °C for 10 min. The PCR products were size separated using ethidium bromide stained 2.5 % agarose gel electrophoresis.

Detection of the sequence polymorphism in *K29* and *RM28102*

The PCR products of *K29* and *RM28102* were purified using QIAquick® PCR Purification Kit (Catalog No: 28104, Qiagen, Hilden, Germany). The purified PCR products of the markers were subjected to 3× Sanger sequencing in Macrogen Inc., South Korea.

Data analysis

Growth, yield and PDT parameters

All the tested parameters were subjected to general linear model procedure (GLM) and Duncan's mean separation in SAS Version 9.4 (SAS Institute Cary, NC, USA).

Pup1 DNA haplotypes

Based on the binary data for DNA marker polymorphism, a dissimilarity matrix was constructed using the unweighted pair group method with arithmetic means (UPGMA) (Nei & Kumar, 2000) in Phylip package v3.697 (Felsenstein, 2005) and the tree was visualised using FigTree v1.4.3. (Rambaut, 2018).

Detection of *K29* and *RM28102* haplotypes

Two separate alignments were constructed for the markers *RM28102* and *K29* using MEGA v7 software (Kumar *et al.*, 2016) and the dendrograms were constructed using UPGMA algorithm. The obtained sequences were aligned using the MEGA software with a reference sequence obtained from the GenBank (www.ncbi.nlm.nih.gov; https://www.ncbi.nlm.nih.gov/assembly/GCA_009829375.1). The SNPs and the

INDELs were identified from the generated multiple sequence alignment. The generated *RM28102* and *K29* sequences were stored under GenBank Accession Nos.

MK890248-MK890274 and MK890275-MK890301, respectively (PopSet IDs 1789811496 and 1789811549, respectively).

Table 3: *Pup1* linked markers assessed in the present study

Marker	Forward and reverse primer sequences	Band size (bp)	Ta (°C)	Reference
<i>K20</i>	TCAGGTGATGGGAATCATTG TGTTCACCAACAACCTG	240, 243	55	Chin <i>et al.</i> (2010)
<i>K29</i>	CCATAGTAGCACAAGAAACCGACA GCTTCAATGAGCCCAGATTACGAA	850, 491	55	
<i>K41</i>	TGATGAATCCATAGGACAGCGT TCAGGTGGTGCTTCGTTGGTA	382	57	
<i>K42</i>	CCCGAGAGTTCATCAGAAGGA AGTGAGTGGCGTTTGCGAT	918	57	
<i>K43</i>	AGGAGGATGAGCCTGAAGAGA TCGCACTAACAGCAGCAGATT	912	57	
<i>K46</i>	TGAGATAGCCGTCAAGATGCT AAGGACCACCATTCCATAGC	523	57	
<i>K48</i>	CAGCATTCAGCAAGACAACAG ATCCGTGTGGAGCAACTCATC	847	57	
<i>K52</i>	ACCGTCCCCAACAGATTCCAT CCCGTAATAGCAACAACCCAA	505, 700	57	
<i>K59</i>	GGACACGGATTCAAGGAGGA TGCTTTCCATTGCGGCTC	550	57	
<i>RM28102</i>	CACTAATTCTTCGCTCCACTTAGG GTGGAAGCTCCGAGAAAGTGC	168	55	
<i>RM28073</i>	GTGTTGGTGGTGATGAAGCAAGG GGACGAAGGATGTATGTGTCTGTACC	656	55	Pariasca-Tanaka <i>et al.</i> (2014)
<i>K46-K1</i>	TGAGATAGCCGTCAAGATGCT TGAGCCAGTAGAATGTTTGAGG	342	57	
<i>K46-K2</i>	CTGAAGTGAAAAGAATGACTAA TGAGCCAGTAGAATGTTTGAGG	110, 433	57	
<i>K46-3</i>	TCCAAAGATCTCTGATTTGGC GCTTTCCAACATCTCAAGGACT	400	57	
<i>K46-CG1</i>	CTAGAGTATCTCCACAGTCGTT AAGGACCACCATTCCATAGC	258	57	
<i>K46-CG2</i>	CCGAAGTAAGAAGATGACGGA TGATCCAGGAGAATGTTTGTGG	130, 433	57	
<i>Ba76H14_7154</i>	GAAACGGGGTCAAATAAGC GGGTTTCGTCCAACAGGAGTA	292, 259	55	Heuer <i>et al.</i> (2009)

RESULTS AND DISCUSSION

Selection of cultivars

The identification of P deficiency tolerant rice cultivars is important to use as parents in breeding programmes and to recommend as varieties for organic rice farming.

Even in conventional rice farming, P efficient rice cultivars are important to reduce the cost of production and environmental pollution, and as answers to the P fertiliser crisis in the future (Wissuwa & Ae, 2001; Cordell *et al.*, 2009; O'neil *et al.*, 2012). Worldwide rice genetics and breeding programmes have screened diverse germplasm for PDT. In Sri Lanka, Aluwihare

et al. (2016) have screened 30 rice cultivars for PDT. In the present study, 27 cultivars were assessed including a few varieties (Rathdel, H-4, H-7, H-10, At354, Ld356) from Aluwihare *et al.* (2016) to further identify useful P deficiency tolerant parent/cultivars for rice breeding and production in Sri Lanka. Nine landraces were incorporated into the present study because they have untapped genetic resources for various resistance or tolerance traits (Herath *et al.*, 1982; Fageria & Santos, 2002; Chin *et al.*, 2010). Fifteen newly improved varieties were also incorporated to check the PDT. As indicated in Table 2 and the rice varietal descriptions in RRDI Sri Lanka, the selected rice cultivars in the present study

are identified as important parents for rice breeding and varieties for production in Sri Lanka. As explained by Majumder *et al.* (1989) and in subsequent studies, PDT is a quantitative trait (Wissuwa *et al.*, 1998; 2002; Ni *et al.*, 1998; Wissuwa & Ae, 2001) and heavily dependent on the *Pup1* haplotype present in the cultivar, environment and the environment $\times$ QTL interaction.

Plant height and number of tillers

In the *Maha* season, mean PLH at flowering stage was significantly higher in *Kuruluthudu* and *Rathdel* compared to the rest of the cultivars. Whereas in *Yala*

Table 4: Variation of the plant height (PLH) and no. of tillers (NT) of the rice cultivars under P starved field condition

Type	Cultivar	PLH (cm)		NT	
		Maha	Yala	Maha	Yala
Landrace	<i>Herath banda</i>	79.0 ^{bcde}	93.7 ^{ef}	2.4 ^h	3.4 ^{bcde}
	<i>Kuruluthuda</i>	120.3 ^a	-	2.6 ^{gh}	-
	<i>Madathawalu</i>	79.6 ^{bcd}	67.3 ^{klm}	5.0 ^{abcd}	2.0 ^{ghij}
	<i>Masuran</i>	83.9 ^{bc}	94.5 ^{ef}	3.9 ^{cdefgh}	3.0 ^{cdefg}
	<i>Pachchaperumal</i>	73.3 ^{cdef}	115.2 ^b	3.6 ^{cdefgh}	3.6 ^{abcd}
	<i>Rathdel</i>	111.6 ^a	-	2.5 ^h	-
	<i>Rath suwandel</i>	56.9 ^{hij}	107.2 ^{cd}	4.5 ^{abcdef}	2.5 ^{efgh}
	<i>Sulai</i>	78.6 ^{bcde}	87.7 ^{ghi}	2.6 ^{gh}	1.5 ^{hij}
	<i>Thatuvee</i>	91.8 ^b	84.6 ^{hi}	2.3 ^h	1.5 ^{hij}
Newly Improved	At308	54.4 ^{ij}	65.1 ^{lmn}	3.5 ^{cdefgh}	2.1 ^{fghij}
	At309	59.2 ^{ghij}	61.8 ^{no}	5.9 ^{ab}	2.8 ^{defg}
	At311	61.1 ^{fghi}	57.8 ^o	5.3 ^{abc}	3.5 ^{bcde}
	At354	69.3 ^{defgh}	71.1 ^{jk}	4.9 ^{abcde}	3.8 ^{abcd}
	At373	51.9 ^{ij}	64.3 ^{mn}	5.8 ^{ab}	2.3 ^{fghi}
	Bg310	46.0 ^j	69.4 ^{ikl}	2.8 ^{fgh}	2.8 ^{defg}
	Bg359	86.3 ^{bc}	62.0 ^{no}	3.5 ^{cdefgh}	3.0 ^{cdefg}
	Bg366	65.3 ^{efghi}	73.7 ^j	3.0 ^{fgh}	3.1 ^{bcdef}
	Bg369	72.0 ^{cdefg}	70.9 ^j	3.1 ^{efgh}	2.0 ^{ghij}
	Bw363	81.8 ^{bcd}	83.4 ⁱ	3.9 ^{cdefgh}	3.0 ^{cdefg}
	Bw367	68.4 ^{defgh}	97.4 ^e	3.4 ^{defgh}	1.1 ^j
	Bw452	53.5 ^{ij}	72.7 ^j	2.9 ^{fgh}	2.3 ^{fghi}
	Ld356	74.0 ^{cdef}	147.6 ^a	4.0 ^{cdefgh}	1.4 ^{ij}
	Ld365	63.3 ^{fghi}	108.7 ^c	4.4 ^{bcddefg}	3.9 ^{abc}
	Ld371	81.4 ^{bcd}	88.9 ^{gh}	6.1 ^a	4.6 ^a
Old Improved	H-10	86.4 ^{bc}	71.3 ^{jk}	4.9 ^{abcde}	4.2 ^{ab}
	H-4	82.3 ^{bcd}	91.2 ^{fg}	4.0 ^{cdefgh}	2.8 ^{defg}
	H-7	82.5 ^{bcd}	103.1 ^d	5.1 ^{abcd}	4.0 ^{abc}

Means denoted by same letters within each column are not significantly different at $p < 0.05$

season, the significantly higher PLH was reported in LD356. Mean NT was significantly higher in LD371 in both *Yala* and *Maha* seasons ($p < 0.05$) (Table 4). The landraces *Kuruluthudu* and *Rathdel* did not flower in *Yala* season because of their photoperiod sensitivity and remained in the vegetative phase; thus, their PLH and NT were not recorded and included in the analysis. The parameters PLH and NT have shown high variability and cultivar specificity indicating that they are less sensitive to PD imposed (Table 4).

Flag leaf size and yield

The landrace *Herath banda* and the newly improved variety BW452 took at least 8–10 weeks to flower and produce panicles in *Yala* season. By that time all the other plots were harvested, and therefore the plots of *Herath banda* and BW452 were heavily exposed to birds, pests, and diseases. Thereby we were unable to collect the yield data for these two cultivars in *Yala* season (Table 5).

Table 5: Variation of the size of flag leaf [flag leaf length (FLL) and width (FLW)] and yield under P starved field condition

Origin	Cultivar	FLL (cm)		FLW (cm)		Yield (g/plant)	
		<i>Maha</i>	<i>Yala</i>	<i>Maha</i>	<i>Yala</i>	<i>Maha</i>	<i>Yala</i>
Landrace	<i>Herath banda</i>	24.06 ^{de}	40.62 ^a	7.94 ^{cdef}	12.63 ^{bc}	3.40 ^{op}	-**
	<i>Kuruluthudu</i>	37.37 ^a	-*	11.44 ^{bc}	-*	8.98 ^{bc}	-*
	<i>Maathawalu</i>	26.08 ^{cd}	38.02 ^{ab}	8.81 ^{cdef}	12.50 ^{bc}	7.97 ^{cd}	11.11 ^b
	<i>Masuran</i>	23.92 ^{de}	33.69 ^c	8.13 ^{cdef}	10.63 ^{cde}	2.08 ^{rs}	6.84 ^d
	<i>Pachchaperumal</i>	19.55 ^{efg}	30.34 ^{de}	6.38 ^{defgh}	8.20 ^{hi}	9.43 ^{bc}	11.76 ^b
	<i>Rath suwandel</i>	20.13 ^{efg}	30.46 ^{de}	6.13 ^{defgh}	8.50 ^g	1.38 ^{tu}	5.56 ^{ef}
	<i>Rathel</i>	30.81 ^b	-*	9.63 ^{cde}	-*	8.08 ^{cd}	-*
	<i>Sulai</i>	22.78 ^{def}	23.04 ^{gh}	8.88 ^{cdef}	10.63 ^{cde}	4.36 ^{kl}	7.55 ^c
	<i>Thatu vee</i>	28.92 ^{bc}	32.27 ^{cd}	7.88 ^{cdef}	10.63 ^{cde}	3.54 ^{no}	6.25 ^{de}
Newly Improved	At308	16.88 ^{figh}	19.67 ^{hi}	8.38 ^{cdef}	10.00 ^{cde}	3.16 ^{opq}	19.53 ^a
	At309	16.26 ^{figh}	19.22 ^{hi}	9.19 ^{cdef}	7.88 ^{hij}	7.22 ^e	4.17 ^j
	At311	17.89 ^{figh}	21.69 ^{ghi}	7.31 ^{cdefg}	7.88 ^{hij}	5.05 ^j	3.55 ^k
	At354	22.58 ^{def}	26.23 ^f	10.5 ^{bcd}	11.13 ^{bcd}	11.91 ^a	7.81 ^c
	At373	15.22 ^{figh}	18.35 ^{hi}	9.25 ^{cde}	11.00 ^{bcd}	4.46 ^{kl}	5.56 ^{ef}
	Bg310	16.69 ^{figh}	20.48 ^{hi}	8.75 ^{cdef}	10.38 ^{cde}	0.84 ^{uv}	11.22 ^b
	Bg359	23.60 ^{def}	19.84 ^{hi}	8.93 ^{cdef}	10.13 ^{cde}	5.35 ^{ij}	5.21 ^{gh}
	Bg366	22.71 ^{def}	26.23 ^f	11.31 ^{bc}	13.25 ^{ab}	4.22 ^{lm}	4.81 ^{ghi}
	Bg369	22.26 ^{def}	26.68 ^f	8.50 ^{cdef}	10.13 ^{cde}	9.26 ^{bc}	7.81 ^c
	Bw363	17.58 ^{figh}	33.11 ^{cd}	5.86 ^{defgh}	7.50 ^{hij}	0.92 ^{uv}	4.17 ^j
	Bw367	17.99 ^{figh}	21.94 ^{ghi}	13.00 ^a	13.63 ^{ab}	3.54 ^{no}	5.85 ^{ef}
	Bw452	17.58 ^{figh}	20.59 ^{hi}	8.63 ^{cdef}	12.00 ^{bc}	1.79 st	-**
	Ld356	21.94 ^{def}	31.62 ^{cd}	8.06 ^{cdef}	10.00 ^{cde}	1.05 ^{uv}	5.21 ^{gh}
	Ld365	19.16 ^{efg}	19.95 ^{hi}	9.44 ^{cde}	11.63 ^{bcd}	9.70 ^{bc}	4.17 ^j
	Ld371	22.32 ^{def}	33.69 ^{cd}	7.25 ^{defgh}	7.63 ^{hij}	1.70 st	4.17 ^j
	H-10	22.78 ^{def}	31.53 ^{cd}	7.56 ^{cdef}	9.75 ^{def}	6.06 ^{figh}	6.51 ^{de}
	H-4	26.68 ^{cd}	30.90 ^{de}	10.44 ^{bcd}	10.50 ^{cde}	6.26 ^{fg}	6.25 ^{de}
	H-7	22.39 ^{def}	32.92 ^{cd}	5.50 ^{efgh}	7.50 ^{hij}	3.34 ^{op}	5.21 ^{gh}

* Data were not collected as these landraces did not flower in *Yala* season.

** Unable to collect data since the seeds were exposed to birds, pests and diseases.

Means denoted by same letters within each column are not significantly different at $p < 0.05$.

The highest FLL was reported for *Kuruluthudu* in *Maha* season and *Herath banda* in *Yala* season; however, the widest flag leaf was observed in BW367 in both *Yala* and *Maha* seasons. Significantly higher yields were reported by newly improved cultivars under *Yala* and *Maha*

seasons where AT354 reported 11.91 g per plant in *Yala* season and AT306 reported 19.53 g per plant in *Maha* season (Table 5). Similar to PLH; FLL and the FLW followed the varietal specific pattern and seem to be less affected by PD conditions (Table 5).

Figure 1: The scatter plot drawn between the mean yield of the cultivars assessed in *Maha* and *Yala* seasons. Six grams per plant is considered as the threshold for identifying the high yielding cultivars under P starved conditions. Dark gray area represents the high yielding cultivars in both seasons and light gray area represent the low yielding cultivars in both seasons (For BW452, *Herath banda*, *Rathdel* and *Kuruluthudu*, data were only available in *Maha* season).

The yield per plant is an ideal trait to identify P deficiency tolerant cultivars under P starved conditions. The grand mean of the yield reported for a cultivar in both seasons was six grams per plant. When this yield level was considered as the expected threshold to declare the P deficiency tolerant cultivars, *Pachchaperumal*, *Madathavalu*, Bg369, AT354, H-10 and H-4 provided higher yield than six grams per plant, thereby can be considered as promising cultivars providing the above

average yield under P starved conditions (Figure 1). A total of one OI, one landrace and seven NI provided a mean yield of less than six grams per plant in both seasons, showing the negative effect of P limitation on the yield (Table 5; Figure 1). The yield under P limited conditions is especially important as a selection parameter in breeding and screening. However, only six cultivars were identified as providing above average yield under P sparse conditions.

Table 6: Variation of phosphorus deficiency tolerance (PDT) indicators under P starved field condition

Type	Cultivar	SDW (g/plant)		SPC (mg/g)		SPU (mg/plant)		PUE (g/mg)	
		<i>Maha</i>	<i>Yala</i>	<i>Maha</i>	<i>Yala</i>	<i>Maha</i>	<i>Yala</i>	<i>Maha</i>	<i>Yala</i>
Landrace	<i>Herath banda</i>	3.34 ^{efg}	29.17 ^a	1.61 ^{ab}	1.49 ^{bcde}	5.37 ^{ab}	41.69 ^a	0.62 ^{ab}	0.67 ^{bcdef}
	<i>Kuruluthudu</i>	16.78 ^a	-*	1.12 ^b	-*	18.62 ^{ab}	-*	0.89 ^{ab}	-*
	<i>Madathawalu</i>	15.66 ^a	14.13 ^{bcd}	2.02 ^{ab}	1.48 ^{bcde}	30.90 ^{ab}	20.65 ^{abcd}	0.50 ^b	0.68 ^{bcdef}
	<i>Masuran</i>	7.16 ^{abcdef}	6.76 ^{hijk}	1.42 ^{ab}	1.38 ^{bcde}	10.47 ^{ab}	22.91 ^{abc}	0.71 ^{ab}	0.72 ^{bcdef}
	<i>Pachchaperumal</i>	3.84 ^{cdefg}	5.89 ^{ijkl}	0.59 ^b	1.73 ^{abcd}	2.29 ^b	10.00 ^{cdefghi}	1.69 ^a	0.58 ^{cdef}
	<i>Rathdel</i>	15.66 ^a	-*	2.10 ^{ab}	-*	35.48 ^a	-*	0.48 ^b	-*
	<i>Rath suwandel</i>	2.88 ^{fg}	3.85 ^l	0.93 ^b	1.12 ^{cde}	2.66 ^{ab}	9.77 ^{cdefghi}	1.07 ^{ab}	0.89 ^{bcde}
	<i>Sulai</i>	6.76 ^{abcdefg}	7.08 ^{ghijk}	1.64 ^a	0.88 ^{de}	11.22 ^{ab}	10.35 ^{cdefghi}	0.61 ^{ab}	1.14 ^{bcd}
	<i>Thatuveal</i>	6.45 ^{abcdefg}	9.12 ^{defghi}	0.86 ^b	0.89 ^{de}	5.31 ^{ab}	14.29 ^{bcdef}	1.16 ^{ab}	1.13 ^{bc}
Newly Improved	At308	3.54 ^{defg}	4.17 ^l	1.10 ^a	1.83 ^{abcd}	12.59 ^{ab}	7.59 ^{efghi}	0.91 ^b	0.55 ^{def}
	At309	4.73 ^{bcdefg}	5.62 ^{ikl}	1.40 ^{ab}	1.32 ^{bcde}	6.68 ^{ab}	7.50 ^{efghi}	0.71 ^{ab}	0.76 ^{bcdef}
	At311	11.61 ^{ab}	12.88 ^{cde}	2.34 ^{ab}	1.41 ^{bcde}	26.61 ^{ab}	5.43 ^{hi}	0.43 ^b	0.71 ^{bcdef}
	At354	7.49 ^{abcdef}	10.47 ^{defgh}	2.19 ^{ab}	1.81 ^{abcd}	16.22 ^{ab}	15.85 ^{bcdef}	0.46 ^b	0.55 ^{def}
	At373	8.81 ^{abcde}	8.71 ^{efghij}	1.69 ^{ab}	1.74 ^{abcd}	14.45 ^{ab}	18.20 ^{abcde}	0.59 ^{ab}	0.57 ^{cdef}
	Bg310	2.37 ^g	4.17 ^l	1.04 ^b	1.73 ^{abcd}	2.34 ^b	7.16 ^{efghi}	0.96 ^{ab}	0.58 ^{cdef}
	Bg359	4.73 ^{bcdefg}	8.32 ^{efghij}	2.72 ^{ab}	0.84 ^{de}	12.88 ^{ab}	6.84 ^{efghi}	0.37 ^b	1.20 ^b
	Bg366	8.03 ^{abcdef}	7.85 ^{efghijk}	1.70 ^{ab}	1.74 ^{abcd}	13.49 ^{ab}	13.65 ^{bcdefg}	0.59 ^{ab}	0.57 ^{cdef}
	Bg369	5.12 ^{bcdefg}	6.10 ^{ijkl}	1.10 ^b	0.92 ^{de}	5.43 ^{ab}	5.62 ^{ghi}	0.91 ^{ab}	1.09 ^{bcde}
	Bw363	4.78 ^{bcdefg}	7.00 ^{ghijk}	1.31 ^{ab}	1.81 ^{abcd}	5.96 ^{ab}	12.59 ^{bcdefgh}	0.76 ^{ab}	0.55 ^{def}
	Bw367	11.22 ^{abc}	11.48 ^{cdef}	1.99 ^{ab}	1.44 ^{bcde}	22.13 ^{ab}	8.32 ^{defghi}	0.50 ^b	0.70 ^{bcdef}
	Bw452	5.68 ^{abcdefg}	5.19 ^{kl}	1.78 ^{ab}	2.12 ^{abc}	10.00 ^{ab}	15.85 ^{bcdef}	0.56 ^{ab}	0.47 ^{ef}
	Ld356	7.07 ^{abcdef}	10.47 ^{defgh}	1.22 ^b	0.92 ^{de}	8.04 ^{ab}	4.95 ⁱ	0.82 ^{ab}	1.09 ^{bcde}
	Ld365	8.31 ^{abcdef}	10.72 ^{defgh}	1.60 ^{ab}	2.60 ^a	16.41 ^{ab}	27.23 ^{ab}	0.62 ^{ab}	0.39 ^f
	Ld371	6.60 ^{abcdefg}	10.00 ^{defgh}	1.31 ^{ab}	0.99 ^{de}	8.71 ^{ab}	10.23 ^{cdefghi}	0.76 ^{ab}	1.02 ^{bcde}
Old Improved	H-10	10.83 ^{abc}	17.18 ^{bc}	1.84 ^{ab}	2.17 ^{ab}	19.28 ^{ab}	22.91 ^{abc}	0.54 ^{ab}	0.46 ^f
	H-4	10.47 ^{abcd}	20.18 ^{ab}	1.55 ^{ab}	1.09 ^{de}	16.22 ^{ab}	21.88 ^{abc}	0.65 ^{ab}	0.92 ^{bcdef}
	H-7	6.16 ^{abcdefg}	8.41 ^{efghij}	1.61 ^{ab}	0.52 ^e	9.89 ^{ab}	4.37 ⁱ	0.62 ^{ab}	1.92 ^a

* Data were not collected from these landraces as they did not flower in *Yala* season.

SDW: shoot dry weight; SPC: shoot P concentration; SPU: shoot P uptake; PUE: P utilization efficiency.

Means denoted by same letters within each column are not significantly different at $p < 0.05$.

Figure 2: The scatter plot drawn between shoot dry weight (SDW) and p utilisation efficiency (PUE) of the rice cultivars in A: *Maha* season; B: *Yala* season. Two threshold lines are drawn at 7 g/plant of SDW and 0.5 g/mg of PUE. The P deficiency tolerant cultivars are indicated in dark gray box and the PD sensitive cultivars are indicated in light gray box.

Variation on the PDT indicators

The significantly higher SDW was identified in *Rathdel* in *Maha* season and *Herath banda* in *Yala* season. However, under the Duncan's grouping of means, many other cultivars also showed significantly higher mean SDW values with overlapping groups (Table 6). The variation of SPC, SPU and PUE indicated that the studied rice cultivars are variable and mostly seasonal-specific in responding to P limited soil conditions. The inverse values of SPC is defined as PUE. Higher SPC or SPU means that the plants accumulate a higher amount of P without partitioning into required tissues. Therefore, the relationship between SDW (amount of biomass generated under P limited conditions in the context of present study) and PUE would provide the basis to identify PDT of the rice cultivars. As given in previous studies, 7 g of SDW per plant and 0.5 g/mg of PUE were

used as the two thresholds (i.e., independent culling levels) (Vandamme *et al.*, 2016; Chankaew *et al.*, 2019) to identify the P deficiency tolerant and P deficiency sensitive cultivars (Figure 2).

In the *Maha* season, *Kuruluthudu*, *Madathawalu*, *Masuran*, and seven improved cultivars showed SDW and PUE above the thresholds indicating that they were PD tolerant. Similarly, in *Yala* season, *Madathawalu*, *Thatu vee*, *Sulai*, and ten improved cultivars were PD tolerant. The landrace *Madathawalu*, OI cultivar H-4 and NI cultivars Bg366, Ld359, At373, Bw367 and Bg366 were PD tolerant in both seasons (Figures 2A and 2B). Bg359 in *Maha* season, and Bw452 in *Yala* season were found to be PD sensitive. Other cultivars got moved back and forth in two moderately P deficiency tolerant classes ($PUE > 0.5$ g/mg, $SDW < 7$ g or $PUE < 0.5$ g/mg and $SDW > 7$ g) in *Maha* and *Yala* seasons (Figures 2A and 2B).

Figure 3: The clustergram constructed for the 27 rice cultivars based on the *Pup1* linked marker haplotypes. The dissimilarity matrix was constructed using the unweighted pair group method with arithmetic means (UPGMA) (Nei & Kumar, 2000).

Many studies reported that both SDW and PUE must be considered to identify the level of PDT of a given rice cultivar (Wissuwa *et al.*, 1998; Aluwihare *et al.*, 2016). The breeders must target for higher levels of SDW and PUE in developing improved rice varieties. In the present study, P deficiency tolerant cultivars for *Maha*, and *Yala* seasons (Figure 2A and 2B) were identified. The results are in line with the results presented by Aluwihare *et al.* (2016) as both studies identified H-4 as PDT.

SDW is an ideal parameter for selection because it expresses the amount of biomass produced under P sparse conditions, and PUE is also ideal as it reflects the partitioning of P into sink tissues under low P conditions. However, it is important to select simultaneously based on the yield and PDT indicators for more robust

screening outcomes (Wissuwa & Ae, 2001; Fageria & Santos, 2002; Fageria & Knupp, 2013). In both *Maha* and *Yala* seasons, if yield and PDT indicators are considered simultaneously, H-4 and *Madathawalu* can be considered as the stable P deficiency tolerant rice cultivars (Figures 1 and 2).

Diversity of *Pup1* linked DNA marker haplotypes

The UPGMA cluster analysis generated 12 clusters (*Pup1*-C1 to *Pup1*-C12 in Figure 3). Cluster *Pup1*-C1 only contains two Sri Lankan landraces, which are moderately tolerant or tolerant to P deficiency. *Pup1*-C2 contains two P deficiency tolerant rice cultivars, *Madathawalu* and H-4 and the rest of the two are moderately tolerant cultivars (At 373 and *Rath Suwandel*). Cluster *Pup1*-C3 contains

Figure 4: The clustergram constructed for rice cultivars based on the sequence polymorphism at K29 locus. The cluster labels are indicated at each cluster.

only moderately tolerant rice genotype. Within *Pup1*-C4 moderately tolerant Bg359 and At354 grouped distantly from the sensitive cultivar At309. *Pup1*-C11 contains the most genetically distinct P deficiency tolerant genotypes (At311 and Bw367). Within the *Pup1*-C8, Bw363 which is a PD sensitive cultivar showed a clear genetic distance from the rest of the cultivars within the cluster (Figure 3).

***K29* and *RM 28102* based sequence diversity**

The sequence polymorphism at *K29* locus generated eight clusters; *K29*-C1 contains the P deficiency tolerant cultivar H-4. However, the overall clustering structure does not coincide with the PDT variation shown in Figure 2 for *Maha* and *Yala* seasons (Figure 4). The sequence polymorphism at *RM 28102* generated eight clusters and a big paraphyletic group in *RM28102*-C1 (Figure 5). There, *Madathawalu* and *Rath suwadal* are apparent as unique haplotypes. However, the overall clustering structure is not correlated with PDT diversity structure shown in Figure 2.

The haplotype analysis at *Pup1* QTL for rice cultivars and the sequence polymorphism-based diversity analysis for two linked markers revealed variable diversity structures. This kind of higher diversity within *Pup1* QTL is reported in many studies (Heuer *et al.*, 2009; Chin *et al.*, 2010; 2011) and has also been linked to the very high transposon activity going on near and within *Pup1* QTL (Heuer *et al.*, 2009). The haplotype and sequence diversity observed in the present study also proved that the rice germplasm in Sri Lanka is very highly diverse at this locus (Aluwihare *et al.* 2016) and implied the possibility of mining novel haplotypes for PDT. For MAB of rice for PDT, it is interesting to note that the stable P deficiency tolerant H-4 and *Madathawalu* (as indicated by yield and PDT indicators) are positioned within the *Pup1*-C2 cluster. Aluwihare *et al.* (2016) reported that H-4 bears a different *Pup1* QTL haplotype to *Kasalath*. The tolerant haplotype in H-4 was inherited from one of its parents, *Murungakayan*, another P deficiency tolerant landrace in Sri Lanka (Aluwihare *et al.*, 2016). Therefore, it is logical to assume that *Madathawalu* also shares

Figure 5: The cluster diagram constructed for rice cultivars based on the sequence polymorphism at *RM 28102* locus. The cluster labels are indicated at each cluster.

the same haplotype as in H-4 and *Murungakayan*. The present study thereby proves the utmost importance of screening locally available landraces and cultivars and subsequent correlation with *Pup1* haplotype to lay a platform for MAB of rice (Chin *et al.*, 2010; Aluwihare *et al.*, 2017; 2018).

CONCLUSIONS

The present study identified that the rice cultivars *Kuruluthudu*, *Madathawalu*, *Masuran*, At373, Bg366, Bw367, H-4, H-10, Ld356, and Ld365 are phosphorus deficiency tolerant in *Maha* season while *Madathawalu*, *Sulai*, *Thatuvee*, At-311, At354, At373, Bg359, Bg366, Bw363, Bw367, H-4, H-7, Ld356, and Ld371 are phosphorus deficiency tolerant in *Yala* season. The landrace *Madathawalu* and old improved variety H-4 are phosphorus deficiency tolerant and provided significantly higher yield performance in both *Maha* and *Yala* seasons. Also, *Madathawalu* and H-4 got clustered together in *Pup1* linked DNA marker-based haplotype analysis. Thus, *Madathawalu* and H-4 can be used as breeding parents in marker assisted breeding programmes to introgress phosphorus deficiency tolerance to new rice varieties. *Madathawalu* is one of the frequently grown landraces in organic rice farming in Sri Lanka. According to the phosphorus deficiency screening results of the present study, *Madathawalu* can be recommended as a promising landrace to grow under zero or minimum phosphorus fertiliser-application regimes.

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