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To cite this article: M. M. Emam Ahmed, M. Ashik Iqbal Khan, Md. Shahjahan Kabir, Yoshimichi Fukuta & Mitsuhiro Obara (2021) Genetic variations of root development traits under different concentrations and forms of nitrogen in Bangladeshi rice (*Oryza sativa* L.) accessions, *Soil Science and Plant Nutrition*, 67:1, 31-40, DOI: [10.1080/00380768.2020.1843370](https://doi.org/10.1080/00380768.2020.1843370)

To link to this article: <https://doi.org/10.1080/00380768.2020.1843370>



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Published online: 25 Nov 2020.



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## Genetic variations of root development traits under different concentrations and forms of nitrogen in Bangladeshi rice (*Oryza sativa* L.) accessions

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### ABSTRACT

Genetic variations in the root traits of rice remain unexplored in relation to varietal groups and diverse ecosystems. Therefore, we evaluated 257 Bangladeshi accessions, which included landraces and improved varieties belonging to four ecotypes (Aus, Aman, Boro and Jhum), to understand genetic variations in the total root length (TRL), maximum root length and root number under different concentrations of ammonium and nitrate nitrogen in hydroponic culture. There were large variations in all traits across all nitrogen levels. Active elongation of individual roots of the landrace type across all nitrogen conditions suggested a higher potential for the uptake of nutrients by extending roots distribution in any nitrogen environment, while the active development of primary root primordia in the improved type in the presence of nitrate suggested a higher potential for the uptake of nitrate from the soil surface by extending roots distribution around soil surface. Cluster analysis classified the examined accessions into two groups according to their TRL: cluster I with a longer TRL and cluster II with a shorter TRL across all nitrogen conditions. Each of these groups was further divided into two sub-clusters: sub-cluster Ia, in which the longer TRL resulted from active development of primary root primordia, sub-cluster Ib, in which the longer TRL resulted from active elongation of individual roots, sub-cluster IIa, in which the shorter TRL resulted from severe reduction of the elongation of individual roots, and sub-cluster IIb, in which the shorter TRL resulted from a moderate reduction in the elongation of individual roots. Further investigation revealed a difference in the composition of these four sub-clusters among the four ecotypes, indicating that Aman was the most diverse ecotype, followed by Aus. The root traits of these promising accessions could be exploited in the future to understand genetic and physiological mechanism(s) of adaptation to diverse ecosystems.

### ARTICLE HISTORY

Received 15 June 2020  
Accepted 26 October 2020

### KEY WORDS

Accession; diversity; ecotype; root development; varietal group

## 1. Introduction

Rice (*Oryza sativa* L.) has been mainly classified into three groups: the indica group, japonica group and tropical japonica group. Sequence analyses recently revealed that the indica group originated from a wide range of ancestors and spread toward South-East and South Asia, allowing it to maintain a broad diversity (Huang et al. 2012). Consequently, rice accessions from these Asian countries have been extensively used in genetic diversity studies on major traits associated with abiotic stress tolerance, resulting in the identification of candidate donors for tolerance to submergence (Bailey-Serres et al. 2010), salinity (Thomson et al. 2010) and phosphorus starvation (Wissuwa, Yano, and Ae 1998). Generally, landraces are defined as dynamic population(s) of a cultivated species that has a historic origin and distinct identity that was locally adapted for cultivation in specific areas (Dwivedi et al. 2016). For rice, improved cultivars have been developed through targeted breeding programs since introduction of semi-dwarf gene

which have numerous benefits over landrace varieties. Thus, they have been cultivated and adapted in wider areas for better yield and other suitable agronomic characters.

Bangladesh is a South Asian country that has a tropical climate with marked variation in rainfall and seasonal temperatures (Shelley et al. 2016). A total of 30 agro-ecological zones have been identified in Bangladesh based on land formation, inundation types, soil properties, and climatic conditions (Brammer et al. 1988), indicating the existence of diverse ecosystems for crop production. Moreover, Bangladeshi rice accessions have been divided into four ecotypes (Aus, Aman, Boro and Jhum) according to their cultivation zone and season (Khan et al. 2017), although the relationships between these ecotypes and ecosystems have not yet been fully defined. In general, the Aus ecotype is mainly grown in upland areas and to a lesser degree in lowland areas under rainfed conditions from the middle of the dry season to the rainy season, and the Jhum ecotype is grown on the hill slopes under rainfed conditions over a similar period. By contrast, the Aman ecotype is mainly grown in diverse lowland areas and some upland areas under

rained conditions from the middle of the rainy season to the early dry season, and the Boro ecotype is grown in lowland areas that retain sufficient water during the dry season (Khan et al. 2017; Sattar 2000; Shelley et al. 2016). These Bangladeshi rice ecotypes are unique in nature and have been studied from various research angles, such as the genetic improvement of rice and crop management practices for different seasons or ecotypes (Hossain et al. 2013; Shelley et al. 2016).

Nitrogen is one of the major soil nutrients that contribute to plant growth and grain yield. Inorganic nitrogen is available to rice plants in two forms in the soil: ammonium, which is a reduced form of nitrogen that usually occurs in flooded paddy fields, and nitrate, which is an oxidized form of nitrogen that usually occurs in upland fields (Yamaya and Oaks 2004). It is considered that landraces with a broader adaptability to the local soil nitrogen conditions have been selected under natural conditions. For intensive farming with improved types, the concentration of each of these forms of nitrogen changes drastically for breeding selection in rice-growing fields through the broadcast application of additional fertilizers, water management, and the influx of nitrogen from exogenous environments (Schneiders and Scherer 1998; Tsuji and Takei 2011). Thus, the adaptations to nitrogen conditions would be integrated in agronomical and physiological traits among rice accessions.

Roots are of particular importance for examining genetic variation in the adaptation to cultivated zones because the root is the only organ that interacts with the soil. Roots are complex trait and hence dissected genetically to components, length, number, thickness, and so on. Unlike top portion, it is difficult even for dissected traits to evaluate precisely since roots remain hidden under soil. Establishment of reliable system for evaluating the traits at seedlings enable to reveal clear genetic variations and identify a regulatory gene that function at field conditions as target to drought (Uga et al. 2013; Uga, Okuno, and Yano 2011) and phosphorus deficiency (Gamuyao et al. 2012). Our previous studies clearly demonstrated a high heritability of root development traits in plants that were grown hydroponically in environments that contained deficient or sufficient concentrations of the two forms of inorganic nitrogen (Obara, Fukuta, and Yanagihara 2019; Obara et al. 2014, 2011, 2010). Furthermore, this reliable growth system enabled us to reveal genetic variations in the root development traits and to identify constitutive and responsive quantitative trait loci (QTLs) for root elongation in the presence of different forms and concentrations of nitrogen. Thus, the root development traits of populations grown under hydroponic conditions would be useful for understanding genetic variation in the

adaptation of rice to the cultivation zones and breeding histories under different selection environments.

An understanding of the nature and extent of genotypic differences in root development traits in landrace or improved type rice varieties and their relationship with different ecotypes could help us to understand the basis of genetic variation in root development traits of rice plants under different nitrogen conditions in the soil. However, few studies have used large number of diverse rice accessions to investigate genetic variations in root development traits under different forms and concentrations of nitrogen. Therefore, the aim of the present study was to reveal the levels of genetic variation in the root development traits of a diverse set of Bangladeshi rice accessions grown hydroponically with different forms and concentrations of nitrogen and to characterize the respective varietal groups (landrace type and improved type) and ecotypes (Aus, Aman, Boro and Jhum) based on these genotypic differences. We use our findings to discuss possible relationships how these genetic variations are related to the selection environments and existing cultivation zones in Bangladesh.

## 2. Materials and methods

### 2.1. Plant materials

A total of 257 Bangladeshi rice accessions and two controls (IR 64 and Kasalath) were used in this study to investigate the levels of genetic variation in root development traits under hydroponic culture. The rice accessions were provided by the Rice Gene Bank of the Bangladesh Rice Research Institute (BRRI), Gazipur, Bangladesh (Khan et al. 2017), and were categorized based on available information about the varietal groups (landrace and improved types) and ecotypes (Aus, Aman, Boro and Jhum) in Bangladesh (Table 1). The varieties IR 64 and Kasalath were used as controls for hydroponic culture (Obara et al. 2014, 2010).

### 2.2. Hydroponic growth conditions

In this study, 8-day-old rice seedlings were used to measure three root development traits with three spatial replications: total root length (TRL), maximum root length (MRL) and root number (RN). Well-filled seeds were germinated at 30°C for 2 days following two-step seed stabilization, which involved soaking the seeds for 10 minutes in water at 60°C and then for 20 minutes in a 2% (v/v) sodium hydrochloride solution, as described previously (Obara et al. 2014). The germinated seeds were then sown on a nylon net (160 × 220 mm) (three seeds per accession; eight nets for each nitrogen application).

**Table 1.** Details of the 257 rice accessions that were included in this study.

Ecotype	No. of accessions (%) <sup>†</sup>		
	Landrace	Improved	Total
Aus	59 (89.4)	7 (10.6)	66 (100.0)
Aman	93 (80.9)	22 (19.1)	115 (100.0)
Boro	30 (63.8)	17 (36.2)	47 (100.0)
Jhum	29 (100.0)	0 (0.0)	29 (100.0)
Total	211 (82.1)	46 (17.9)	257 (100.0)

<sup>†</sup>Numbers in parentheses indicate the percentage of the total rice accessions in each group.

After sowing, a set with the four nylon nets with germinated seeds were kept afloat in a plastic container (50 × 37 × 27 cm) containing 40 L of distilled water mixed with basal nutrient solution. The same basal nutrient solution was used as described previously (Obara et al. 2014), and 5 mM 2-(N-morpholino) ethanesulphonic acid was added as a buffer to maintain the pH of the nutrient solution at approximately 5.5 to 5.8 during the experimental period. The seedlings were raised under natural light conditions in a greenhouse located at Tsukuba, Ibaraki, Japan (36°05'N, 140°09'E, 25 m above sea level), that was maintained at approximately 28°C.

To characterize the genetic variations in root development traits under different form and concentration of nitrogen that was available (ammonium or nitrate), plants were grown under four different conditions: in the presence of NH<sub>4</sub>Cl or KNO<sub>3</sub> at concentrations of 5 µM (nitrogen deficient) or 500 µM (nitrogen sufficient, representing the soil nitrogen concentration in paddy fields). The hydroponic medium was renewed at 4 and 6 days after seed sowing.

### 2.3. Evaluation of the root development traits

The root development traits TRL, MRL, and RN were evaluated to investigate genetic variation in the 257 Bangladeshi rice accessions. First, the longest root length of each 8-day-old seedling was measured using a ruler and defined as MRL. The seedlings were then placed in a plastic bag and stored at -30°C until measurement of the individual root length and number of roots. Following storage, the seedlings were placed outside for 30 min to bring them up to air temperature, following which the roots were separated from the seedlings by cutting the seedlings at the base. The primary roots length of each seedling was then measured to calculate TRL and the number of primary roots was counted to obtain RN. To estimate contribution of dissected traits MRL and RN in TRL, the measured value for MRL was used as an indicator for root elongation, while the value for RN was used as an indicator for development of primary root primordia in the whole manuscript. Broad-sense heritability ( $h_b^2$ ) was estimated from one-way analysis of variance (ANOVA) with the following formula:  $h_b^2 = \sigma_g^2 / \{(\sigma_e^2/r) + \sigma_g^2\}$ , where  $\sigma_g^2$  is the genetic variance,  $\sigma_e^2$  is the environmental variance, and  $r$  is the number of data employed (Kobayashi and Koyama 2002).

### 2.4. Cluster analysis

To classify the 257 rice accessions and two control varieties based on their root development traits under different form and concentration of nitrogen that was available, cluster analysis was performed using Ward's hierarchical clustering method (Ward and Joe 1963) with the program JMP 11.2.0 for Windows (SAS Institute, Inc., Cary, NC, USA). Differences in the mean value between the two groups were statistically analyzed using the unpaired t-test (two-tailed), and differences among multiple groups were compared using the Tukey-Kramer test (probability < 0.01).

## 3. Results

### 3.1. Genetic variations in root development traits

A total of 257 rice accessions consisting of 211 landraces and 46 improved varieties (Table 1) were evaluated to determine the levels of genetic variation in three root development traits: TRL, MRL, and RN. To examine this, seedlings were grown hydroponically in medium containing ammonium or nitrate nitrogen at concentrations of 5 µM or 500 µM, representing nitrogen-deficient and nitrogen-sufficient conditions, respectively. The third leaf was developing in almost all accessions in all applications (Supplemental fig. 1). Broad-sense heritability ( $h_b^2$ ) values that is index of genotypic effect on phenotypic variance was estimated for the root development traits in each application (Supplemental table 1). The  $h_b^2$  values were greater than 0.960 for TRL, 0.980 for MRL, and 0.850 for RN under all application, indicating that current data set is reliable to discuss genetic variation in root development traits in Bangladeshi rice accessions.

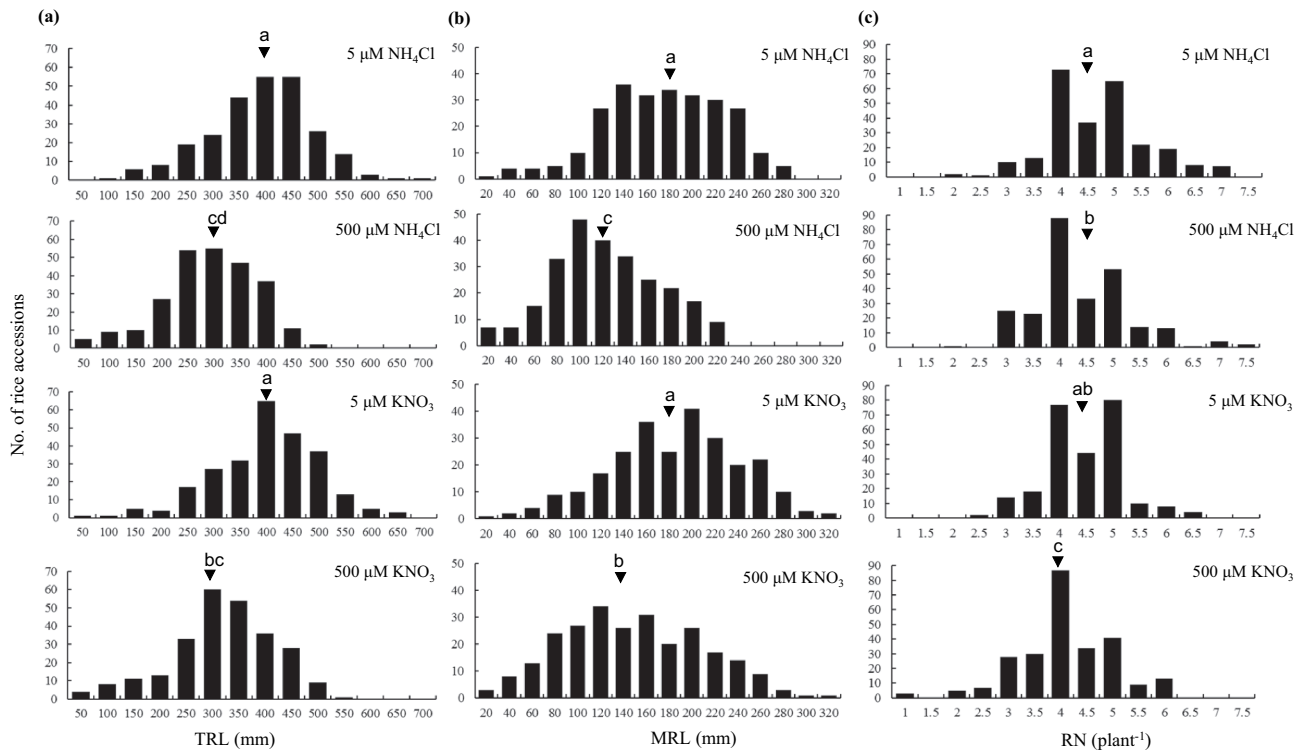
There was large variation in TRL among the 257 rice accessions, with values ranging from 111 mm to 708 mm (mean = 414.9 mm) for 5 µM NH<sub>4</sub>Cl, from 59 mm to 539 mm (mean = 318.3 mm) for 500 µM NH<sub>4</sub>Cl, from 94 mm to 695 mm (mean = 426.4 mm) for 5 µM KNO<sub>3</sub> and from 60 mm to 591 mm (mean = 344.7 mm) for 500 µM KNO<sub>3</sub> (Figure 1a). The distribution of TRL values differed between the two concentrations for both forms of nitrogen, with TRL being significantly higher with 5 µM NH<sub>4</sub>Cl or KNO<sub>3</sub> than with 500 µM NH<sub>4</sub>Cl or KNO<sub>3</sub>, respectively.

Large variation was also observed in MRL under the tested nitrogen conditions, with values ranging from 38 mm to 294 mm (mean = 184.1 mm) for 5 µM NH<sub>4</sub>Cl, from 26 mm to 235 mm (mean = 132.2 mm) for 500 µM NH<sub>4</sub>Cl, from 34 mm to 334 mm (mean = 194.7 mm) for 5 µM KNO<sub>3</sub> and from 24 mm to 326 mm (mean = 157.7 mm) for 500 µM KNO<sub>3</sub> (Figure 1b). Accessions grown in 5 µM NH<sub>4</sub>Cl or KNO<sub>3</sub> had larger MRLs than those grown in 500 µM NH<sub>4</sub>Cl or KNO<sub>3</sub>, respectively. In addition, accessions grown in KNO<sub>3</sub> had larger MRLs than those grown in NH<sub>4</sub>Cl, with a significant difference at a concentration of 500 µM.

RN also showed large variation when grown under different concentrations of the two forms of nitrogen, ranging from 2.3 to 7.3 (mean = 4.8) for 5 µM NH<sub>4</sub>Cl, from 2.3 to 7.7 (mean = 4.6) for 500 µM NH<sub>4</sub>Cl, from 2.5 to 6.7 (mean = 4.6) for 5 µM KNO<sub>3</sub> and from 1.0 to 6.3 (mean = 4.2) for 500 µM KNO<sub>3</sub> (Figure 1c). Accessions grown in 5 µM NH<sub>4</sub>Cl or KNO<sub>3</sub> had significantly higher RNs than those grown in 500 µM NH<sub>4</sub>Cl or KNO<sub>3</sub>, respectively. Furthermore, accessions grown in NH<sub>4</sub>Cl had significantly higher RNs than those grown in KNO<sub>3</sub> at a concentration of 500 µM but not at 5 µM.

### 3.2. Classification of the rice accessions

Cluster analysis classified the 257 Bangladeshi rice accessions and two indica group controls (IR 64 and Kasalath) into two major cluster groups (cluster I, n = 128 accessions; and cluster II, n = 129 accessions) based on genetic variations in the three root development traits (TRL, MRL, and RN) when grown in 5 µM or 500 µM NH<sub>4</sub>Cl or KNO<sub>3</sub> (Supplementary Fig. 2 and Supplemental Table 2). Each of these clusters was further divided into two sub-clusters (sub-cluster Ia, n = 57 accessions;



**Figure 1.** Frequency distributions of TRL (a), MRL (b) and RN (c) for 257 rice accessions grown in hydroponic culture medium containing  $\text{NH}_4\text{Cl}$  or  $\text{KNO}_3$  at concentrations of  $5 \mu\text{M}$  (nitrogen deficient) or  $500 \mu\text{M}$  (nitrogen sufficient). Arrows indicate the groups containing the mean values for each root trait under each nitrogen condition. Different lower-case letters above the arrows indicate significant differences among the nitrogen applications (Tukey-Kramer test,  $P < 0.01$ ). TRL: total root length; MRL: maximum root length; RN: root number.

sub-cluster Ib,  $n = 71$  accessions; sub-cluster IIa,  $n = 27$  accessions; and sub-cluster IIb,  $n = 102$  accessions) (Table 2 and Supplementary Fig. 2). The controls IR 64 and Kasalath were classified into sub-clusters Ia and Ib, respectively.

These four sub-clusters showed different TRLs in the presence of deficient and sufficient levels of the two forms of nitrogen (Table 2). TRL was always highest in sub-cluster Ia and lowest in sub-cluster IIa. Sub-cluster Ia had significantly higher TRL compared to sub-cluster Ib, in both  $5 \mu\text{M}$   $\text{NH}_4\text{Cl}$  and  $\text{KNO}_3$  but similar between these sub-clusters for plants grown

in  $500 \mu\text{M}$   $\text{NH}_4\text{Cl}$  and  $\text{KNO}_3$ . Like sub-cluster IIa, sub-cluster IIb had a significantly lower TRL than sub-clusters Ia and Ib across all of the tested nitrogen conditions.

By contrast, the MRLs of the four sub-clusters varied in a consistent manner across all of the nitrogen conditions (Table 2). MRL was always highest in sub-cluster Ib and lowest in sub-cluster IIa. Furthermore, sub-clusters Ia and IIa had significantly lower MRLs than sub-clusters Ib and IIb, respectively. In addition, sub-cluster IIb had a significantly lower MRL than sub-clusters Ia and Ib, irrespective of the form and concentration of nitrogen.

**Table 2.** Classification of rice accessions based on their responses to different forms and concentrations of nitrogen using cluster analysis.

Sub-cluster	Trait	No. of accessions	Nitrogen application			
			$5 \mu\text{M}$ $\text{NH}_4\text{Cl}$	$500 \mu\text{M}$ $\text{NH}_4\text{Cl}$	$5 \mu\text{M}$ $\text{KNO}_3$	$500 \mu\text{M}$ $\text{KNO}_3$
Ia	TRL (mm)	58	$501.4 \pm 72.5$ a	$389.4 \pm 57.4$ a	$509.6 \pm 61.1$ a	$409.7 \pm 89.7$ a
Ib		72	$453.6 \pm 69.2$ b	$374.0 \pm 58.5$ a	$468.9 \pm 74.8$ b	$381.3 \pm 73.1$ a
IIa	MRL (mm)	27	$242.2 \pm 54.5$ d	$148.5 \pm 48.4$ c	$247.0 \pm 61.3$ d	$169.5 \pm 59.7$ c
IIb		102	$384.6 \pm 58.9$ c	$283.2 \pm 44.2$ b	$397.3 \pm 58.0$ c	$328.0 \pm 56.0$ b
Ia	RN (plant <sup>-1</sup> )	58	$194.6 \pm 34.4$ b	$133.8 \pm 29.5$ b	$210.5 \pm 38.3$ b	$159.6 \pm 43.5$ b
Ib		72	$240.5 \pm 25.2$ a	$185.4 \pm 27.6$ a	$251.3 \pm 33.6$ a	$227.5 \pm 38.8$ a
IIa	MRL (mm)	27	$94.4 \pm 31.0$ d	$60.0 \pm 21.8$ d	$97.3 \pm 29.2$ d	$64.5 \pm 22.5$ d
IIb		102	$162.2 \pm 27.1$ c	$113.1 \pm 25.3$ c	$172.0 \pm 30.8$ c	$132.2 \pm 32.7$ c
Ia	RN (plant <sup>-1</sup> )	58	$5.7 \pm 0.8$ a	$5.5 \pm 0.9$ a	$5.2 \pm 0.6$ a	$4.6 \pm 1.0$ a
Ib		72	$4.5 \pm 0.7$ b	$4.5 \pm 0.8$ b	$4.4 \pm 0.6$ b	$3.6 \pm 0.7$ c
IIa	MRL (mm)	27	$4.5 \pm 0.7$ b	$3.8 \pm 0.5$ c	$4.2 \pm 0.6$ c	$4.1 \pm 0.5$ b
IIb		102	$4.7 \pm 0.8$ b	$4.3 \pm 0.7$ b	$4.6 \pm 0.7$ b	$4.5 \pm 0.8$ a

A total of 257 rice accessions from Bangladesh and two control varieties (IR 64 and Kasalath) were used.

IR 64 and Kasalath were categorized into sub-clusters Ia and Ib, respectively.

Three root development traits were investigated in 8-day-old seedlings that had been grown hydroponically: total root length (TRL), maximum root length (MRL) and root number (RN). Values are means  $\pm$  s.d (three spatial replications).

Different lower-case letters within the same column for each trait indicate significant differences among the sub-clusters under the same nitrogen application (Tukey-Kramer test,  $P < 0.01$ ).

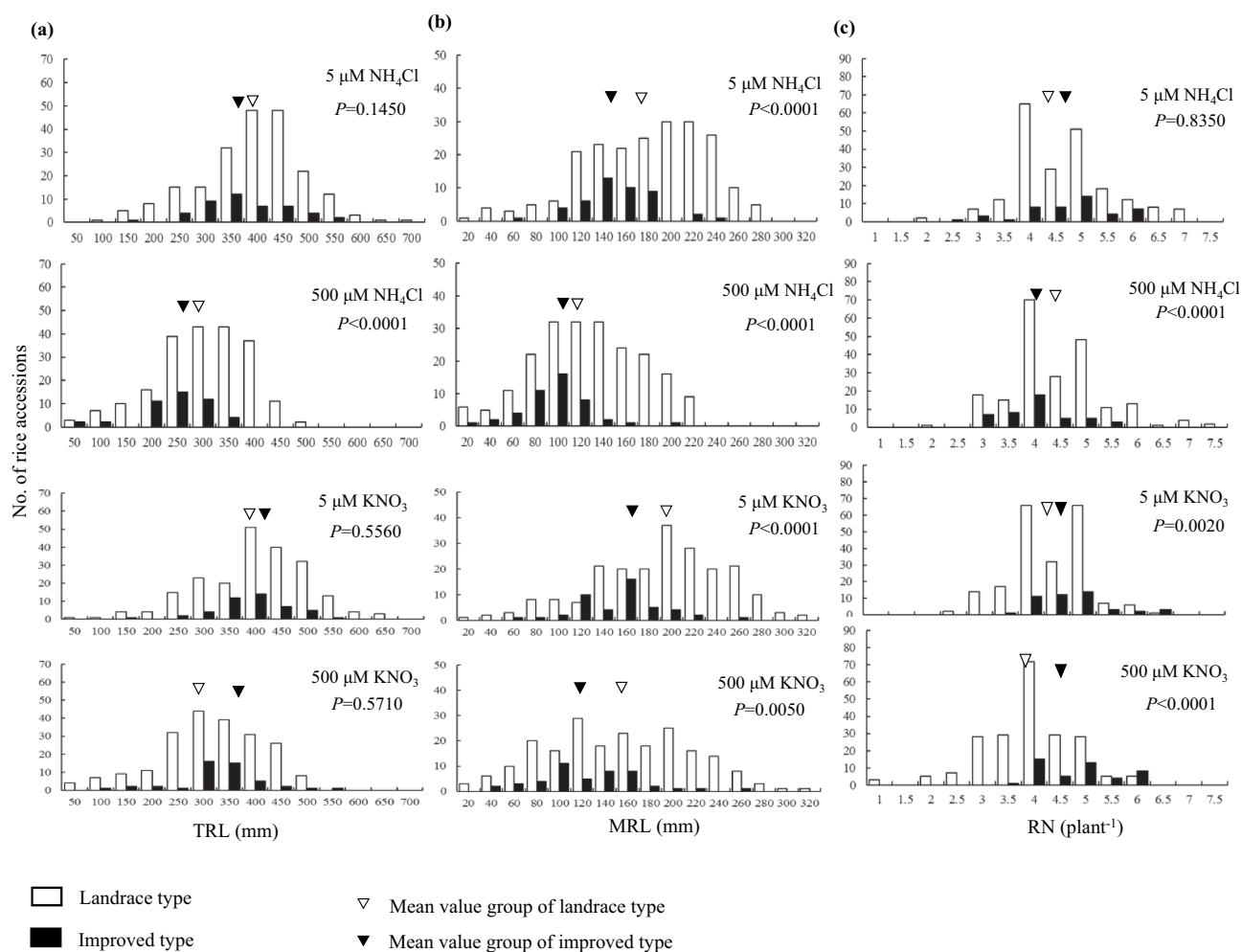
Different patterns of RN were observed in three of the four sub-clusters according to the nitrogen condition (Table 2), with only sub-cluster 1a showing a consistent pattern across all nitrogen conditions. Sub-cluster 1a had a higher RN than the other three sub-clusters across all nitrogen conditions. Sub-cluster 1b had a significantly lower RN than sub-cluster 1a, while sub-clusters 11b had significantly higher RNs than sub-cluster 11a, except in 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ . Sub-cluster 1b had a higher RN than sub-cluster 11a in 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$  and 5  $\mu\text{M}$   $\text{KNO}_3$  but a lower RN than sub-cluster 11a in 500  $\mu\text{M}$   $\text{KNO}_3$ . Like sub-cluster 1a, sub-cluster 11b had a higher RN than sub-clusters 1b and 11a only in 500  $\mu\text{M}$   $\text{KNO}_3$ .

### 3.3. Characterization of varietal groups based on root development traits

Large variation was observed in TRL in both varietal groups (landrace type and improved type) when grown in different forms and concentrations of nitrogen (Figure 2a). For the landrace type, TRL ranged from 111 mm to 708 mm (mean = 419.2 mm) for 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 59 mm to 539 mm (mean = 328.7 mm) for 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 94 mm to 695 mm (mean = 427.8 mm) for 5  $\mu\text{M}$   $\text{KNO}_3$  and from 60 mm to 523 mm (mean = 343.0 mm) for 500  $\mu\text{M}$

$\text{KNO}_3$ . By contrast, for the improved type, TRL ranged from 160 mm to 585 mm (mean = 394.9 mm) for 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 74 mm to 398 mm (mean = 270.3 mm) for 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 178 mm to 635 mm (mean = 419.5 mm) for 5  $\mu\text{M}$   $\text{KNO}_3$  and from 101 mm to 591 mm (mean = 352.3 mm) for 500  $\mu\text{M}$   $\text{KNO}_3$ . The landrace type had a significantly higher TRL than the improved type in 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$  only.

Both the landrace and improved types also showed large variation in MRL of each nitrogen condition (Figure 2b). For the landrace type, MRL ranged from 38 mm to 294 mm (mean = 189.1 mm) for 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 26 mm to 235 mm (mean = 137.8 mm) for 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 34 mm to 334 mm (mean = 201.3 mm) for 5  $\mu\text{M}$   $\text{KNO}_3$  and from 24 mm to 326 mm (mean = 162.7 mm) for 500  $\mu\text{M}$   $\text{KNO}_3$ . By contrast, for the improved type, MRL ranged from 66 mm to 247 mm (mean = 159.1 mm) for 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 37 mm to 215 mm (mean = 106.6 mm) for 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 75 mm to 268 mm (mean = 164.5 mm) for 5  $\mu\text{M}$   $\text{KNO}_3$  and from 48 mm to 265 mm (mean = 134.8 mm) for 500  $\mu\text{M}$   $\text{KNO}_3$ . The landrace type had a significantly higher MRL than the improved type across all nitrogen conditions.



**Figure 2.** Frequency distributions of TRL (a), MRL (b) and RN (c) for 211 landraces and 46 improved varieties grown in hydroponic culture medium containing  $\text{NH}_4\text{Cl}$  or  $\text{KNO}_3$  at concentrations of 5  $\mu\text{M}$  (nitrogen deficient) or 500  $\mu\text{M}$  (nitrogen sufficient). Arrows indicate the groups containing the mean values of the landrace and improved types for each root trait under each nitrogen condition. TRL: total root length; MRL: maximum root length; RN: root number.

Large variation in RN was also observed in the landrace and improved types (Figure 2c). For the landrace type, RN ranged from 2.3 to 7.3 (mean = 4.8) for 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 2.3 to 7.7 (mean = 4.6) for 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 2.5 to 6.7 (mean = 4.5) for 5  $\mu\text{M}$   $\text{KNO}_3$  and from 1.0 to 6.0 (mean = 4.1) for 500  $\mu\text{M}$   $\text{KNO}_3$ . For the improved type, RN varied from 2.7 to 6.3 (mean = 4.9) for 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 3.0 to 5.7 (mean = 4.2) for 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , from 3.7 to 6.7 (mean = 4.9) for 5  $\mu\text{M}$   $\text{KNO}_3$  and from 3.7 to 6.3 (mean = 4.9) for 500  $\mu\text{M}$   $\text{KNO}_3$  (Figure 2c). There were significant differences in RN between the landrace type and improved type across all nitrogen conditions except 5  $\mu\text{M}$   $\text{NH}_4\text{Cl}$ , with the landrace type having a greater RN in 500  $\mu\text{M}$   $\text{NH}_4\text{Cl}$  and the improved type having a greater RN in both 5  $\mu\text{M}$  and 500  $\mu\text{M}$   $\text{KNO}_3$ .

### 3.4. Sub-cluster compositions of the two varietal groups

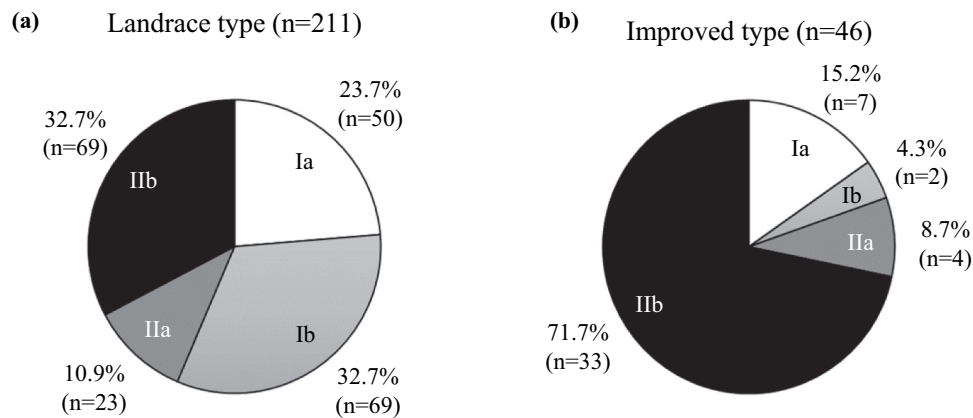
Sub-cluster compositions of each varietal group were compared to further characterization of the root development traits because there was large gap of the number of

accessions between the groups used in this study (Table 1). There were marked differences in the sub-cluster compositions of the two varietal groups (Figure 3). Most of the landraces were grouped into sub-clusters Ib and IIb, with equal proportions in each (32.7%), while the remainder was included in sub-clusters Ia (23.7%) and IIa (10.9%) (Figure 3a). By contrast, more than 70% of the improved varieties were included in sub-cluster IIb, while the remainders were grouped into sub-clusters Ia (15.2%), Ib (4.4%), and IIa (8.7%) (Figure 3b).

### 3.5. Sub-cluster compositions of the four ecotypes

We observed a common tendency for TRL and MRL in two of the four ecotypes examined, with these root development traits nearly always having the highest values for Aus and lowest values for Jhum (Table 3). However, we were unable to identify any typical patterns for the other two ecotypes, Aman and Boro.

Sub-cluster compositions of four ecosystems were compared to further characterization of the root development



**Figure 3.** Composition of rice accessions in landrace type (a) and improved type (b) when categorized into four cluster groups based on genetic variation in three root development traits against 5  $\mu\text{M}$  as deficient and 500  $\mu\text{M}$  as sufficient conditions of two forms of nitrogen,  $\text{NH}_4\text{Cl}$  and  $\text{KNO}_3$ .

**Table 3.** Variations in the root development traits of 8-day-old-seedlings of four ecotypes (Aus, Aman, Boro, and Jhum) grown hydroponically under four nitrogen conditions.

Trait	Application	Ecotype <sup>†</sup>			
		Aus (66)	Aman (115)	Boro (47)	Jhum (27)
TRL (mm)	5 $\mu\text{M}$ $\text{NH}_4\text{Cl}$	423.6 $\pm$ 91.3a	423.7 $\pm$ 105.8 a	419.0 $\pm$ 93.1 a	353.1 $\pm$ 77.0 b
	500 $\mu\text{M}$ $\text{NH}_4\text{Cl}$	341.9 $\pm$ 81.3 a	325.5 $\pm$ 104.0 ab	306.2 $\pm$ 68.2 b	255.4 $\pm$ 49.3 c
	5 $\mu\text{M}$ $\text{KNO}_3$	432.3 $\pm$ 87.6 a	441.7 $\pm$ 107.7 a	435.7 $\pm$ 83.4 a	337.0 $\pm$ 67.0 b
	500 $\mu\text{M}$ $\text{KNO}_3$	360.2 $\pm$ 81.2 a	347.5 $\pm$ 115.2 a	352.5 $\pm$ 76.2 a	285.6 $\pm$ 63.0 b
MRL (mm)	5 $\mu\text{M}$ $\text{NH}_4\text{Cl}$	200.5 $\pm$ 48.5 a	180.6 $\pm$ 55.5 b	188.6 $\pm$ 49.8 ab	152.9 $\pm$ 36.1 c
	500 $\mu\text{M}$ $\text{NH}_4\text{Cl}$	151.6 $\pm$ 49.3 a	126.4 $\pm$ 44.3 bc	133.4 $\pm$ 45.0 b	109.5 $\pm$ 37.2 c
	5 $\mu\text{M}$ $\text{KNO}_3$	208.0 $\pm$ 52.6 a	196.2 $\pm$ 57.1 a	198.3 $\pm$ 57.0 a	153.3 $\pm$ 46.5 b
	500 $\mu\text{M}$ $\text{KNO}_3$	171.3 $\pm$ 57.0 a	157.4 $\pm$ 63.2 a	161.2 $\pm$ 65.1 a	122.2 $\pm$ 47.8 b
RN (plant <sup>-1</sup> )	5 $\mu\text{M}$ $\text{NH}_4\text{Cl}$	4.7 $\pm$ 1.0 a	5.0 $\pm$ 0.9 a	4.9 $\pm$ 0.9 a	4.3 $\pm$ 0.8 b
	500 $\mu\text{M}$ $\text{NH}_4\text{Cl}$	4.5 $\pm$ 0.9 ab	4.8 $\pm$ 1.0 a	4.4 $\pm$ 0.7 b	3.9 $\pm$ 0.7 c
	5 $\mu\text{M}$ $\text{KNO}_3$	4.4 $\pm$ 0.8 b	4.8 $\pm$ 0.7 a	4.8 $\pm$ 0.7 a	4.0 $\pm$ 0.6 c
	500 $\mu\text{M}$ $\text{KNO}_3$	4.1 $\pm$ 0.9 ns	4.2 $\pm$ 0.9 ns	4.5 $\pm$ 1.1 ns	4.4 $\pm$ 0.7 ns

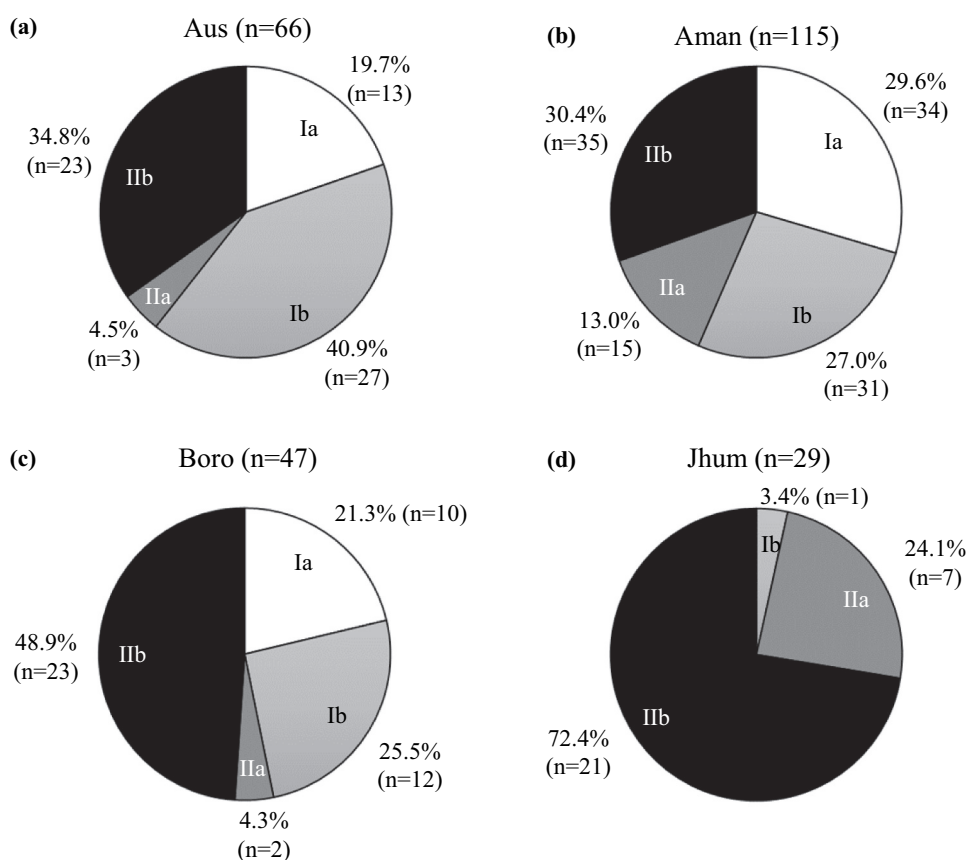
<sup>†</sup>Numbers in parentheses indicate the number of rice accessions in each ecotype.

Three root development traits were investigated in 8-day-old seedlings that had been grown hydroponically: total root length (TRL), maximum root length (MRL) and root number (RN).

Values are means  $\pm$  s.d (three spatial replications).

Different lower-case letters within the same column for each trait indicate significant differences among the sub-clusters under the same nitrogen application (Tukey-Kramer test,  $P < 0.01$ ).

ns: non-significant.



**Figure 4.** Composition of rice accessions among Aus (a), Aman (b), Boro (c) and Jhum (d) ecotypes when categorized into four cluster groups based on genetic variation in three root development traits against 5  $\mu\text{M}$  as deficient and 500  $\mu\text{M}$  as sufficient conditions of two forms of nitrogen,  $\text{NH}_4\text{Cl}$  and  $\text{KNO}_3$ .

traits because there was large gap of the number of accessions between the groups used in this study (Table 1). The rice accessions from the Aus, Aman and Boro ecotypes were distributed across all four sub-clusters based on genetic variations in their root traits under different nitrogen conditions, while Jhum was categorized into three sub-clusters (Figure 4). Marked differences were observed in the distributions of the sub-clusters among these ecotypes. Rice accessions of the Aus ecotype were mainly grouped into sub-clusters Ib (40.9%) and IIb (34.9%). Accessions of the Aman ecotype were assigned to sub-clusters Ia, Ib, and IIb, with similar proportions in each (27.0%–30.4%). The majority of accessions of the Boro ecotype were placed in sub-cluster IIb (48.9%), while the remainder were categorized into sub-clusters Ia and Ib in similar proportions (21.3% and 25.5%, respectively), as well as sub-cluster IIa (4.3%). Likewise, the majority of accessions of the Jhum ecotype were assigned to sub-cluster IIb (72.4%), with the remainder being placed in sub-clusters IIa (24.1%) and Ib (3.4%). None of the accessions in the Jhum ecotype were included in sub-cluster Ia.

#### 4. Discussions

In the present study, considerable genetic variations were observed in three root development traits (TRL, MRL, and RN) among 257 Bangladeshi rice accessions of seedlings grown under different nitrogen conditions. Below, we discuss

the nature and extent of these genetic variations in terms of cluster groups, varietal groups, ecotypes and then combine these to discuss how these genetic variations are related to the selection environments of varietal groups and existing ecotypes with major cultivation zones of these rice accessions.

##### 4.1. Genetic variations in root development traits under different nitrogen conditions

The rice accessions examined in the present study that evolved or were selected for cultivation in these diverse ecological conditions or ecotypes could provide insight into the extent of genetic variation in rice roots in adaptation to the varietal groups, ecotypes, and cultivation zones across highly variable ecosystems. In Bangladesh, the rice-growing environments differ markedly in terms of climate, water, and soil conditions. In most cases, both landrace and improved types differ across these ecotypes, which may have allowed them to develop novel root systems that are adapted to a particular cultivation zone. In fact, the 257 accessions that were examined in the present study showed at least 10-fold variation in TRL, 11-fold variation in MRL and 2.6-fold variation in RN (Figure 1). By contrast, 18 New Rice for Africa (NERICA) cultivars and their four parental varieties exhibited only two-fold variation (Obara, Fukuta, and Yanagihara 2019).



#### 4.2. Classification of the 257 rice accessions based on their root development traits under different nitrogen conditions

The nature of each sub-cluster based on the root development traits under different nitrogen conditions could support to better understand the root development traits of the varietal groups and ecotypes. Cluster analysis classified the 257 rice accessions into two major clusters: cluster I, which was characterized by a longer TRL, and cluster II, which was characterized by a shorter TRL. Each of these clusters was then further divided into two sub-clusters: Ia, Ib, IIa, and IIb (Supplementary Fig. 2). The higher TRL for sub-cluster Ia suggested active development of primary root primordia, so this sub-cluster was characterized by a longer TRL as a result of active primordia development of primary roots. By contrast, the higher MRL for sub-cluster Ib across all nitrogen conditions suggested consistent active root elongation, so this sub-cluster was characterized by a longer TRL as a result of active elongation of individual roots across all nitrogen conditions. Similarly, we observed a reduction in root length for the shorter-TRL sub-clusters IIa and IIb across all nitrogen conditions, with sub-cluster IIa exhibiting a severe reduction in MRL and sub-cluster IIb exhibiting a moderate reduction in MRL. Thus, sub-cluster IIa was characterized by a shorter TRL as a result of a severe reduction in the elongation of individual roots, while sub-cluster IIb was characterized by a shorter TRL as a result of a moderate reduction in the elongation of individual roots across all nitrogen conditions.

#### 4.3. Characterization of the varietal groups based on their root development traits under different nitrogen conditions

The selection environments of landrace and improved types have partially contributed to their novel root systems, which can be explained by the nature of root development adapting to the selection, breeding histories, and cultivation zones. Two of the major root development traits, MRL and RN, were found to differ between these two varietal groups in the present study, strongly suggesting that not only root elongation in any nitrogen conditions but also root number were the major factors contributing to the differences in root development traits between the landrace and improved types.

This difference in root elongation between the landrace and improved types may result from the fertilizer conditions of the fields. In terms of MRL, the improved type exhibited inactive root elongation of the longest root regardless of the form and concentration of nitrogen that was available (Figure 2b), whereas the landrace type had a significantly larger MRL under all nitrogen conditions. Root elongation has previously been identified as being beneficial for nitrogen uptake under both deficient and sufficient conditions of the two forms of nitrogen (Obara, Fukuta, and Yanagihara 2019; Obara et al. 2010). The landrace type was cultivated with or without organic fertilizer, which contains fewer and more slowly released nutrients than inorganic fertilizers (EPBS 1958), whereas the improved type was selected for growth in well-fertilized paddy fields in research stations that were supplied with split

applications of nitrogen fertilizer at different doses or rates depending on crop growth and soil conditions. Another explanation for why the majority of improved varieties fell in sub-cluster IIb is because few donor parents with longer TRLs in Bangladesh breeding program. In fact, now, landraces have been used directly only in 11 cases among 85 improved varieties developed and released (Bangladesh Rice Knowledge Bank 2020).

Improved type would have a higher potential to uptake abundant nitrate, which is likely to occur in the soil surface layers when nitrogenous fertilizers are supplied, which occurred during the selection process for advanced breeding lines (Chen et al. 2016). Because, in terms of RN, we observed opposite patterns for the two varietal groups depending on the form of nitrogen that was available, with the improved type having a greater RN than the landrace type in sufficient concentrations of nitrate nitrogen, but the reverse being the case for ammonium nitrogen (Figure 2c). For example, the phosphorus-starvation tolerance 1 (*PSTOL1*) gene was identified at the phosphorus-deficiency tolerance (*Pup1*) locus in the Aus landrace Kasalath and is a strong candidate for enhancing the uptake of phosphorus by increasing RN (Gamuyao et al. 2012). The observation of increased RNs under both deficient and sufficient nitrate conditions in the improved varieties supports this suggestion of enhanced nutrient uptake through the production of higher RNs like *PSTOL1* regulation.

The combined analysis of the varietal and sub-cluster groups also supported the two attributes mentioned above. Although both varietal groups included all of the sub-clusters, different sub-cluster compositions were found between the landrace and improved types, particularly corresponding to sub-clusters Ib and IIb (Figure 3). Sub-cluster IIb showed lower root elongation in terms of TRL and MRL across all nitrogen conditions compared with sub-cluster Ib, as well as a greater RN with a sufficient concentration of nitrate (Table 2).

#### 4.4. Characterization of the ecotypes based on their root development traits under different nitrogen conditions

Marked differences in major- and sub-cluster compositions among ecotypes (Figure 4) might be influenced by ecosystems and cultivation zones. The rice accessions belonging to cluster I (Ia and Ib) are likely to have been grown in upland areas, while those to cluster II (IIa and IIb) are likely to have been grown in lowland areas. Since extending roots via active root elongation and development of primary root primordia is one of the critical traits allowing to obtain nutrients from deeper layers of the soil in upland (Loresto et al. 1983). Conversely, there is less of a requirement to capture nutrients in lowland areas under rainfed and irrigated conditions depending on the native soil moisture conditions in Bangladeshi accessions. However, it has not been defined fully the relationship between the ecotypes (Aus, Aman, Boro, and Jhum) and corresponding to their ecosystems, meaning that rice is grown on different ecosystems, upland and lowland fields, even in same ecotype (Khan et al. 2017). Therefore, we considered the compositions of the sub-clusters to understand the nature of the root development traits of the Aus, Aman, Boro, and Jhum ecotypes and the

relationships between these ecotypes and the cultivation zones of Bangladesh.

Rice accessions of the Aus ecotype were mainly classified into sub-clusters Ib (40.9%) and IIb (34.9%) (Figure 4a). In Bangladesh, Aus rice is grown under rainfed conditions in both upland and lowland areas, with seeds being sown in the middle of the dry season and harvested in the middle of the wet season (Hossain et al. 2013; Parsons, Newbury, and Jackson 1999; Shelley et al. 2016). Upland areas are exposed to severe drought stress, the rice accessions that clustered into sub-cluster Ib are likely to have been grown in upland areas. Conversely, the rice accessions that clustered into sub-cluster IIb were likely grown in these lowland areas. These two contrasting moisture stress situations will have affected the nutrient availabilities in accessions of the Aus ecotype.

The majority of accessions of the Aman ecotype were placed in three sub-clusters: Ia (29.6%), Ib (27.0%), and IIb (30.4%) (Figure 4b). These results clearly indicate that rice accessions of the Aman ecotype have high diversity in the root development traits. Wide diversity of Aman was also supported by DNA marker typing analyses, demonstrating that Aman rice accessions have greater diversity than other ecotypes in Bangladesh (Khan et al. 2017). Aman is the most widespread ecotype in Bangladesh, where it is grown under rainfed condition from the middle of the wet season to the early dry season in a diverse range of lowland areas with favorable conditions as well as in areas that are prone to abiotic stresses, such as salinity and submergence and a smaller number of upland areas (Sattar 2000; Shelley et al. 2016).

Nearly all of the rice accessions of the Boro ecotype were grouped into three sub-clusters: Ia (21.3%), Ib (25.5%), and IIb (48.9%) (Figure 4c). The majority of these accessions were classified into sub-cluster IIb. These results can be explained by the water availability situations for nutrient uptake in Boro rice cultivation areas. In Bangladesh, Boro rice is grown in lowland areas that retain sufficient water throughout the growing period to support crop growth during the dry season (Khan et al. 2017; Parsons, Newbury, and Jackson 1999; Shelley et al. 2016). Traditional Boro rice was cultivated and selected in the lowlands, including typical low-lying areas that have a haor ecosystem (Saha et al. 2016), which remain under water for more than 6 months of the year. During the dry season, these lowland areas exhibit two main water availability situations: well-watered fields and water-limited fields with or without irrigation (Fujita 2010).

Unlike the other ecotypes, the rice cultivation of Jhum ecotype is limited to growth in hill regions using the so-called slash and burn method, which is also known as swidden or shifting cultivation (Chakma and Ando 2008). Almost all of the rice accessions of this ecotype were included in sub-clusters IIa and IIb (Figure 4d and Table 3). This result suggests that the evaluated Jhum rice accessions were selected under favorable conditions with sufficient nutrients in the soils as lowland. However, Jhum rice is actually grown on hill slopes under unfavorable conditions in terms of rainfall and soil nutrient levels (Chakma and Ando 2008). One possible explanation for this discrepancy is that the evaluated Jhum rice accessions were shifted from the fertile lowlands to the hill slopes. In fact, a recent survey of 247 farmers from the hill regions

revealed that farmers cultivate rice in both the fertile lowlands, where they grow rice mainly of Aman ecotypes, and on hill slopes, where they grow rice of Jhum ecotype (Chakma and Ando 2008).

## 5. Conclusions

The aim of this study was to reveal the levels of genetic variation in hydroponically grown root development traits under different nitrogen conditions in the landrace and improved type rice varieties of Bangladesh, as well as in the four rice ecotypes Aus, Aman, Boro, and Jhum. We observed large variations in all nitrogen conditions, allowing them to be classified into four sub-cluster groups (Ia, Ib, IIa, and IIb) according to TRL, MRL, and RN. The compositions of these four sub-clusters differed among the four ecotypes, indicating a possible relationship with cultivation zone. Aus was mainly grouped into sub-clusters Ib and IIb, Boro and Jhum were mainly placed in sub-cluster IIb, and Aman was distributed across sub-clusters Ia, Ib, and IIb in almost equal proportions, indicating that Aman accessions have conserved the most variation, followed by Aus. Further research using specific rice accessions from the speculated cultivation zones would confirm the nature of the root traits for each ecotype. In addition, a large number of landraces from these ecotypes were characterized by the presence of longer TRLs resulting from active development of primary root primordia and active elongation of the individual roots, allowing nomination of landraces as donor parent which were adapted to native conditions in breeding program of Bangladesh. Therefore, further physiological and genetic studies on relationship between rice ecotypes and cultivation zones could improve our understanding of genetic and physiological mechanism(s) of adaptation to diverse ecosystems.

## Acknowledgments

We thank the Rice Gene Bank, Genetic Resources and Seed Division, Bangladesh Rice Research Institute, Gazipur, Bangladesh for providing the Bangladesh rice accessions.

## Disclosure statement

No conflicts of interest declared.

## Funding

This work was supported by the Japan International Research Center for Agricultural Sciences (JIRCAS) under a research project, 'Environmental Stress-Tolerant Crops' (2016 onward); and under JIRCAS visiting research fellowship program (2018 fiscal year).

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