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ORIGINAL ARTICLE

Assessment of morpho-cultural, genetic and pathological diversity of *Rhizoctonia solani* isolates obtained from different host plants

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Abstract

Rhizoctonia solani is a destructive soil borne plant pathogen that infects various crops including rice. A total of 35 isolates of *R. solani* from rice and other different hosts were collected and established. Morphological characterization was done on PDA media by analyzing radial growth, sclerotial pattern and colony colour. Pathogenicity test was conducted in a susceptible variety of rice Pusa Basmati 1. Maximum lesion height (86%) was observed for isolate TP3 and TP26 whereas, the minimum disease was observed for the isolate TP30 (29.2%). Molecular markers namely, internal transcribed spacer (ITS), universal rice primer (URP) and simple sequence repeats (SSR) were used to determine the genetic diversity of the pathogen. The phylogenetic tree obtained based on ITS region sequences grouped *R. solani* isolates in a single main cluster and 6 subclusters. Based on URP and SSR markers, isolates of *R. solani* were grouped into three and six clusters respectively. The structure analysis and AMOVA analysis revealed greater genetic variation within populations (91%) compared to among populations (9%). The results demonstrated that *R. solani* isolates evaluated were pathogenic to rice, irrespective of the host, however, variation exists at molecular level. The findings highlighted the diversity and complexity of the genetic background of *R. solani* which will be helpful to develop management strategies against the sheath blight disease of rice. Further, *R. solani* isolates from the host species finger millet (*Eleusine coracana*), lablab bean (*Lablab purpureus),* and ginger (*Zingiber officinale*) were first time characterized from the North-Eastern state through this study.

Keywords Rice · *Rhizoctonia solani* · Simple sequence repeats (SSR) · Universal rice primer (URP) · Internal transcribed spacer (ITS) · Genetic diversity

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Introduction

Rice (*Oryza sativa* L.) is one of the most important and cultivated crops in the world. It is a staple food crop which is consumed by nearly half of the global population. More than 3 billion people globally consume rice as a staple food, accounting for 80% of their calorie intake (Delseny et al. 2001). Rice cultivation is adversely affected by several biotic stresses, one of which is sheath blight disease in rice (Hobbs 2001). According to the Foreign Service Association of United States Department of Agriculture statistics, China and India have been ranked first and second in the production of rice (FSA/USDA 2011). The sheath blight disease in rice was first reported from Gurdaspur district of Punjab (Paracer and Chahal 1963) which later became a major production constrains in many states of India. Sheath blight disease caused by *Rhizoctonia solani* Kuhn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a devastating disease and is now reported from all rice-growing regions of the world. About 4–50% of yield losses due to sheath blight have been reported depending on crop stage at the time of infection, disease severity and environmental conditions (Singh et al. 2004; Zheng et al. 2013; Bhunkal et al. 2015). This pathogen is known to be highly diverse in terms of its morphological, cultural, pathological, and physiological characteristics (Ou 1985). *R. solani* is a destructive fungal pathogen which has a very wide host range and survives in the form of mycelia and sclerotia in the infected plant debris. The symptoms of the sheath blight include the formation of lesions on the sheath, which could also spread to the upper sheaths, leaves and even to the panicle. The infected sheath or leaves will get dried later and eventually die resulting in a reduction in canopy leaf area, leading to a reduced yield.

Mutation and heterokaryosis are the main reasons for the variability in *R. solani* affecting the cultural, morphological, pathogenic, and molecular characteristics of the *R. solani* population (Anderson 1982). Consequently, this affects the epidemiology of the disease. Knowledge about pathogen diversity is very important to understand host–pathogen interaction and disease epidemiology. Substantial variability in morphological and pathogenic characters in *R. solani* populations has also been reported earlier (Sunder et al. 2003). Problems related to understanding different diversity levels in *Rhizoctonia solani* are best discussed with the use of molecular genetic markers (Toda et al. 1999). Molecular markers are very helpful in understanding the development of species concept by providing information about the limit of the genetically isolated group in relation to patterns of morphological variation and behavior at the species level. At the population level, they also provide a base for identifying patterns, dispersal, and colonization in spatial and temporal distribution (Vilgayls and Cubeta 1994). *R. solani* isolates are widely classified based on anastomosis groups (Ogoshi 1987). However, several studies reported that anastomosis groups are not host-specific. Limited studies have been done on the characterization and grouping of host-specific isolates of *R. solani* (Prashantha et al. 2021). Molecular tools are very beneficial in evaluating the level of genetic diversity among the isolates and identifying the races of the pathogen. Internal transcribed spacer (ITS) region is widely utilized for the identification, anastomosis grouping and phylogenetic analysis of *R. solani* (Dubey et al. 2014; Prashantha et al. 2021). Universal rice primers (URPs) derived from Korean weedy rice have been used earlier to perform the fingerprinting of plants, microbes and animal genomes including very few fungi (Kang et al. 2002). Further, the usage of repetitive sequences derived from fungal genomes has not gained much attention in providing fingerprinting of fungal species.

The variability in the *R. solani* pathogen complex is considered a major problem by researchers in the development of resistant host genotypes and deploying tolerant varieties (Sandoval Regina Faye and Cumagun Christian Joseph 2019). Information about variability in a pathogen population and their virulence pattern in host plants will benefit researchers in breeding programs. Therefore, taking these points into consideration, the present study was targeted to evaluate the morphological, pathological, and molecular variability in the *R. solani* isolates collected from rice and other host plants (Banerjee et al. 2012).

Material and methods

Collection and maintenance of isolates

Rice (*Oryza sativa* L.) and other host plants including mucuna weed (*Mucuna pruriens*), pea (*Pisum sativum*), finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), turmeric (*Curcuma longa*), cabbage (*Brassica oleracea*), mustard (*Brassica juncea*), and ginger (*Zingiber officinale*) showing the appearance of sheath blight symptoms were collected from different rice-producing regions of Tripura (Table 1). The diseased portion of the plant sample was cut into square pieces and surface sterilized in 0.1% sodium hypochlorite solution for 1 min, followed by washing with sterile distilled water three times for 30 s. The leaf/sheath bits were dried on autoclaved filter paper and then subsequently placed on potato dextrose agar (PDA) and incubated in a BOD incubator (Sanco) for 4 days. Fungal colonies showing hyphal growth with right angle branching

Table 1 Morpho-cultural characteristics of different isolates of *R. solani* on PDA medium

Isolate code	Crop (host)	Radial growth (48 h)	Total sclerotia	Sclerotial weight*/ plate (g)	Colony colour*	Sclerotial pattern*	Geographical location (Lag. and long.)
TP1	Mucuna weed (*Mucuna pruriens*)	2.22^{d}	86.00^{ab}	0.21 ^{cd}	White	scattered	Lembuchara Latitude:23.91388° longitude:91.320643°
TP2	Rice (*Oryza sativa*)	6.68^{ab}	93.33^{a}	0.31^{bcd}	White	peripheral	Laxmi lunga Latitude: 23.933096° Longitude:91.294443°
TP3	Rice (*Oryza sativa*)	7.25^{ab}	21.33^{bcd}	0.08 ^{cd}	Light brown	peripheral	Laxmilunga Latitude: 23.936671° Longitude 91.292757°
TP4	Rice (*Oryza sativa*)	0.55^{e}	58^{abcd}	0.36^{abc}	White	middle	Lembucherra Latitude: 23.939500° longitude: 91.291414°
TP5	Rice (*Oryza sativa*)	0.30^{e}	42^{abcd}	0.53^{ab}	White	middle	Lembucherra Latitude: 23.911875° Longitude:91.316615°
TP6	Rice (*Oryza sativa*)	0.73^{e}	74.33^{abcd}	0.3^{bcd}	White	peripheral	Lembucherra Latitude: 23.912330° Longitude: 91.316545°
TP7	Rice (*Oryza sativa*)	8.00^{a}	40^{abcd}	0.64^{a}	Light brown	scattered	Lembucherra, Latitude: 23.912830° Longitude: 91.316281°
TP8	Rice (*Oryza sativa*)	7.37^{ab}	12.67 ^{cd}	0.15 ^{cd}	Cream	middle	Lembucherra Latitude: 23.913262° Longitude: 91.316493°
TP9	Rice (*Oryza sativa*)	7.60^{ab}	76^{abcd}	0.18 ^{cd}	Light brown	scattered	Lembucherra Latitude: 23.912388° Longitude:91.316301°
TP10	Rice (*Oryza sativa*)	8.55^{a}	62^{abcd}	0.17 ^{cd}	Light brown	peripheral	Mohanpur Latitude: 23.981062° Longitude:91.382622°
TP11	Rice (*Oryza sativa*)	6.90^{ab}	79.67^{abcd}	0.2 ^{cd}	Light brown	scattered	Batapur, khowai Latitude: 24.0672° Longitude: 91.6057°
TP12	Rice (*Oryza sativa*)	8.70^{a}	34.33^{abcd}	0.15 ^{cd}	Light brown	peripheral	Kakrabon, Mirza Latitude: 23.447106 Longitude: 91.410707
TP13	Rice (*Oryza sativa*)	8.70^{a}	21.67^{abcd}	0.13 ^{cd}	Light brown	central	Kakrabon, Mirza Latitude: 23.447106° Longitude: 91.410707°
TP14	Rice (*Oryza sativa*)	8.70^{a}	43.67^{abcd}	0.15 ^{cd}	Light brown	scattered	Khowai Latitude: 23.9021962° Longitude: 91.631362°
TP15	Rice (*Oryza sativa*)	8.60^{a}	64.33^{abcd}	0.17 ^{cd}	Dark brown	peripheral	Mohanpur Latitude:23.972912° longitude: 91.375591°

Table 1 (continued)

Isolate code	Crop (host)	Radial growth (48 h)	Total sclerotia	Sclerotial weight*/ plate (g)	Colony colour*	Sclerotial pattern*	Geographical location (Lag. and long.)
TP16	Rice (*Oryza sativa*)	8.58^{a}	61.33^{abcd}	0.32^{bcd}	Light brown	peripheral	Lembucherra Latitude: 23.914147° Longitude: 23.320929°
TP17	Rice (*Oryza sativa*)	0.42^{e}	84^{abc}	0.04^{d}	brown	central	Lembucherra Latitude:23,914,147° Longitude:23.320929°
TP18	Rice (*Oryza sativa*)	8.70^{a}	65.67^{abcd}	0.31^{bcd}	Light brown	peripheral	Khowai Latitude: 23.023936° Longitude: 91.632904°
TP19	Rice (*Oryza sativa*)	8.00^{a}	34.67^{abcd}	0.22 ^{cd}	Cream	peripheral	Kakrabon, Mirza Latitude: 23.447106° Longitude:91.410707°
TP20	Rice (*Oryza sativa*)	6.87^{ab}	38^{abcd}	0.54^{ab}	Light brown	middle	Kakrabon, Mirza Latitude: 23.447106° Longitude: 91.410707°
TP21	Rice (*Oryza sativa*)	4.92^{bcd}	87^{ab}	0.21 ^{cd}	White	scattered	Kakrabon, Mirza Latitude: 23.447106° Longitude: 91.410707°
TP22	Rice (*Oryza sativa*)	7.73^{ab}	82.33^{abcd}	0.33^{bcd}	White	peripheral	Kakrabon, Mirza Latitude: 23.447106° Longitude: 91.410707°
TP23	Rice (*Oryza sativa*)	3.10^{cde}	71.5^{abcd}	0.26^{bcd}	Light brown	peripheral	Kakrabon, Mirza Latitude: 23.447106° Longitude: 91.410707°
TP24	Rice (*Oryza sativa*)	8.70^{a}	50^{abcd}	0.21 ^{cd}	Light brown	central	Kakrabon, Mirza Latitude: 23.447106° Longitude: 91.410707°
TP25	Rice (*Oryza sativa*)	8.45^{a}	53^{abcd}	0.13 ^{cd}	Light brown	scattered	Lembucherra Latitude: 23,914,147° Longitude: 23.320929°
TP26	Lablab bean (*Lablab purpureus*)	7.80^{a}	17.33^{bcd}	0.24^{bcd}	White	scattered	Lembucherra Latitude: 23,911,080° Longitude:91.317615°
TP 27	Lablab bean (*Lablab purpureus*)	8.00^{a}	46.67^{abcd}	0.16 ^{cd}	Light brown	peripheral	Laxmi bill Latitude: 23,693,957° Longi-tude:23.298223°
TP28	Pea (*Pisum sativum*)	7.82^{a}	64.33^{abcd}	0.29^{bcd}	Light brown	scattered	Laxmi Lunga Latitude: 23,934,048° Longitude: 91.294524°
TP 29	Pea (*Pisum sativum*)	7.73^{ab}	45.33^{abcd}	0.27^{bcd}	Light brown	peripheral	Laxmi Lunga Latitude: 23,938,483° Longitude: 91.291715°

Table 1 (continued)

Isolate code	Crop (host)	Radial growth (48 h)	Total sclerotia	Sclerotial weight*/ plate (g)	Colony colour*	Sclerotial pattern*	Geographical location (Lag. and long.)
TP 30	Ginger *Zingiber officinale*	7.10^{ab}	53.33^{abcd}	0.38^{abc}	Light brown	middle	Khowai Latitude: 23.9021962° Longitude: 91.631362°
TP 31	Finger millet (*Eleusine coracana*)	8.00^{a}	40.67^{abcd}	0.3^{bcd}	White	scattered	Lembucherra Latitude: 23.914032° Longitude: 91.320966°
TP 32	Foxtail millet (*Setaria italic*)	1.95^{e}	60.67^{abcd}	0.21 ^{cd}	White	peripheral	Lembucherra Latitude: 23.913923° Longitude: 91.321114°
TP 33	Turmeric (*Curcuma longa*)	6.35^{ab}	10.67^{d}	0.05^{d}	Light brown	peripheral	Lembucherra Latitude:23,911,080° Longitude: 91.317615°
TP 34	Mustard (*Brassica juncea*)	8.70^{a}	75.33^{abcd}	0.16 ^{cd}	Brown	scattered	Lembucherra Latitude:23.913923° Longitude: 91.321114°
TP 35	Cabbage (*Brassica oleracea)*	8.70^{a}	70.67^{abcd}	0.18 ^{cd}	White	scattered	Mohanpur Latitude: 23.972912° Longitude:91.375591°

were transferred and maintained on PDA slants at 25 ± 2 °C. Further, isolates were purified using hyphal tip culture and stored in slants at 4 °C until further use.

Morpho-cultural characterization

The variation in cultural and morphological characteristics of *R. solani* isolates was determined based on observation of colony colour and growth rate. A mycelial disc having a diameter of 5 mm was cut from a freshly growing colony (42 h old culture) of each isolate and was placed aseptically on Potato dextrose agar (PDA) containing Petri plates in three replications per isolate and incubated at a temperature of 25 ± 1 °C. The colour of the fungal colony was recorded after 10 days of incubation. The growth rate (mm/hour) was determined by recording the mycelial growth.

Pathogenicity test

The pathogenicity test was conducted under net house conditions at ICAR-Indian Agricultural Research Institute, New Delhi in the month of July 2020 and 2021 to determine the pathogenic behaviour of *R. solani* isolates on the highly susceptible rice variety Pusa Basmati 1. The fungal inoculum of each isolate for the experiment was multiplied on typha (*Typha angustata*) stem pieces as described by Bhaktavatsalam et al. (1978). For which the stems of typha were cut into small pieces, washed properly, and soaked in the medium (sucrose 20 g, peptone 10 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.1 g, distilled water 1 Litre). The excess water was drained out and typha bits were loosely filled in 250 ml conical flasks. Flasks were autoclaved at 1.1 kg/cm^2 for 30 min repeatedly two times. Sterilized flasks were inoculated with 10-day-old fungal cultures of *R. solani* and incubated at 25 ± 1 °C. After 14 days of incubation colonized typha pieces were used as inoculum. The plants of cultivar PB-1 were inoculated with colonized typha pieces at the maximum tillering stage (Prashantha et al. 2021). Four typha stem pieces were inserted between the tillers in the rice plant above the water level. The water level (5–10 cm) in the pots was continuously maintained to ensure the required humidity 85–100% and temperature 28–32 °C for disease progression. Height of the lesion and total plant height were measured at 3 different time intervals i. e. 7 days, 14 days, and 21 days after inoculation and was used to calculate relative lesion height (RLH) (SES 1996). Mean lesion height of two years was taken for further analysis. The total number of lesions, number of lesions on each tiller and number of tillers per plant infected were also measured at 7 days after inoculation. Pathogenicity test was conducted in randomized block design and data obtained was statistically analyzed using SPSS software (version: SPSS 16.0.0).

Genomic DNA extraction

Pure cultures of *R. solani* isolates were multiplied in potato dextrose broth (10 g of potato dextrose broth, 100 ml of distilled water) at 27 ± 1 °C on an electric shaker (Lab Therm) for 7 days. The mycelial mat produced was collected by filtration method using filter paper (Whatman 1; diameter 150 mm). The genomic DNA was extracted by using Cetrimide, Tetradecyl Trimethyl Ammonium Bromide (CTAB) method given by Murray and Thompson (1980). Briefly, one gram of mycelial mat was crushed into fine powder in pre-chilled pestle mortar using liquid nitrogen. The powder was transferred into sterile centrifuge tubes having 10 ml of pre-heated (65 °C) CTAB DNA extraction buffer (5 M NaCl, 0.5 M EDTA, 1 M tris HCl, 2 g CTAB w/v). In the final step, the supernatant was discarded, and the pellet was washed with 70% ethanol twice and centrifuged at 10,000 rpm for 10 min. Isolated DNA quality and quantity were measured by recording absorbance at 260 and 280 nm wavelengths in spectrophotometer Nanodrop (Thermo Fisher). Extracted DNA was of good quality and the working concentration was 100 ng/ul.

ITS sequencing and phylogenetic analysis

The amplification of the ITS region was attained as mentioned by White et al. (1990) using primers ITS1 (TCCGTA GGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGA TATGC). Each 25 µl reaction mix consisted of PCR reaction consisted of 12.5 µl of Dream Taq Green PCR master mix (2 X) (Thermo Scientific), 1.5 µl of 100 ng of template DNA, 0.1 µM forward and reverse primer and 9 µl nuclease-free water. The DNA amplification was achieved in a Thermal Cycler (Bio-RAD, T100) under the following conditions: initial denaturation at 95 °C for 4 min followed by 34 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min with an elongation of 72 °C for 10 min. The amplified products were resolved by electrophoresis on 1.2% agarose gel in 1 X TAE buffer stained with ethidium bromide. The gel was photographed under UV illumination using the gel Doc system (UVITEC; Fire reader, V10). 1 Kb ladder (Thermo Scientific) was used as a marker. The amplified PCR products were sequenced and annotated using Bio edit v7.0.9 and the phylogenetic tree was prepared using MEGAX software following Unweighted Pair Group Method with Arithmetic Mean (UPGMA) at the bootstrap value of 1000 replications.

DNA fingerprinting of isolates using Universal Rice Primer-PCR (URP-PCR)

The URPs consist of 20-oligonucleotide, originally obtained from the repetitive sequences of weedy rice (Kang et al. 2002). A total of 11 URP primers (Table 3) were synthesized from the company Genuine Chemical Corporation (GCC), India. The PCR was performed in a Thermal Cycler (Bio-Rad, USA) and each PCR reaction of 25 µl consisted of 100 ng of genomic DNA, 0.1 µM primer and Dream Taq Green PCR master mix (2X). The thermal cycler conditions for the PCR reaction were initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min with a final extension of 72 °C for 7 min. The PCR products obtained were electrophoresed on 1.2% agarose gel stained with ethidium bromide containing TAE buffer (pH 8.0) along with a 1 Kb DNA ladder (Thermo Scientific). The electrophoresis was carried out at a constant voltage of 60 V for one hour and further, gel visualization was done under UV transilluminator and photographed in Gel Doc system (UVITEC; Fire reader, V10). The relation between the isolates was studied based on means of scorable DNA bands amplified from different URP markers. Each band was presumed as a character and was scored as either present (1) or absent (0). The similarity between isolates was predicted by calculation of Jaccard's similarity coefficient and cluster analysis was done using unweighted pair group method with arithmetic mean (UPGMA) using NTSYS.pc (v.2.02e) software (Rohlf 1998).

SSR (simple sequence repeats)

SSRs are 12–15 oligonucleotide short primer sequences. A total of 7 primers of *R. solani* (Ganeshamoorthi and Dubey 2013) were synthesized by the company Eurofins India. The PCR was performed in a Thermal Cycler (Bio-Rad, USA) and each PCR reaction consisted of 100 ng of genomic DNA. PCR reaction 25 µl was comprised of 0.1 µM primer and *Taq* DNA Polymerase and PCR master mix (Thermo Scientific) 12.5 µl. The thermal cycler conditions for PCR reaction were initial denaturation at 94 °C for 4 min followed by 39 cycles of denaturation at 94 °C for 1 min, annealing ranging between 55–52 °C depending on primer for 1 min, and extension at 72 °C for 1 min with a final extension of 72 °C for 10 min. The PCR products obtained were electrophoresed on 2% agarose gel stained with ethidium bromide containing TAE buffer (pH 8.0) along with a 100 bp DNA ladder. The electrophoresis was carried out at a constant voltage of 80 V for one hour and further, gel visualization was done under UV Transilluminator and photographed in Gel Doc (UVITEC; Fire reader, V10). The relation between the isolates was studied on the basis of means of scorable DNA bands amplified from different SSR markers. SSR was done three times and repeated bands were selected for scoring. Each band was presumed as a character and was scored as either present (1) or absent (0). Cluster analysis was conducted using NTSYS.pc (V2.02e) software as described above.

Population genetic analysis

Seven SSR markers' genotypic data were utilized to estimate genetic diversity. STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to test for the presence of genetic structure. It was run with K = 1 to K = 10 clusters, 10 independent replications for each cluster, a 50,000 burn in time, and 50,000 Markov chain Monte Carlo (MCMC) runs to estimate distributions. The peak value of ΔK was estimated using the method given by Evanno et al. (2005) and the STRUCTURE HARVESTER software (Earl and vonHoldt 2012) was used to find the ideal K value. The components of variance and Principal coordinate analysis (PCoA) between Rice and Non-Rice isolates were estimated by using the Analysis of molecular variance (AMOVA) procedure using GenAIEx v.6.502 (Peakall and Smouse 2012).

Results

Collection and isolation of *R. solani* isolates

A total of 35 isolates of *R. solani* were collected from rice and other different hosts (grown in the month of July to December, 2019) such as Mucuna weed (*Mucuna pruriens*), pea (*Pisum sativum*), finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), turmeric (*Curcuma longa*), cabbage (*Brassica oleracea*), mustard (*Brassica juncea),* lablab bean (*Lablab purpureus*), and ginger (*Zingiber officinale*) (Table 1). All the isolates cultured on potato dextrose agar showed typical characteristics of *R. solani, i. e.,* hyphae branching at an acute to a right angle, with a slight constriction at the point of branching and septum formation near the constriction point (Parmeter 1970). Hence, all isolates were identified as *R. solani.*

Morpho-cultural characterization

Based on the observation of colony colour in the Petri plates on the 10th day of inoculation, all isolates were divided into five colour groups: white, cream, light brown, brown and dark brown. Among them 11 isolates (TP1, TP2, TP4, TP5, TP6, TP21, TP22, TP26, TP31, TP32, TP35) were found to be white in colour, 2 isolates (TP 8, TP19) were cream coloured, 19 isolates (TP3, TP7, TP9, TP10, TP11, TP12, TP13, TP14, TP16, TP18, TP20, TP23, TP24, TP25, TP27, TP28, TP29, TP30, TP33) were light brown, 2 isolates (TP17, TP34) were brown and 1 isolate (TP 15) was dark brown. The growth rate of the isolates was determined by taking an observation of radial growth diameter. Significant differences were observed for the radial growth among the isolates after 48 h of inoculation. Isolates showing growth up to 3 cm (TP1, TP4, TP5, TP6, TP7, TP32) were classified as slow growing, 3 to 6 cm (TP21, TP23) were medium growing and 6 cm to 9 cm as fast growing. Out of 35 isolates TP34, TP35, TP24, TP13 and TP 12 exhibited maximum radial growth (Table 1). The sclerotial characteristics of all the isolates were also recorded after 10 days of incubation. Isolates were assigned to 4 groups based on sclerotial counts 0–25 as less, 26–50 as medium, 50–75 as good, and 75–100 as very good. Isolates TP3, TP8, TP13, TP26, and TP33 produced less no. number of sclerotia. Isolates TP5, TP7, TP8, TP12, TP14, TP19, TP20, TP24, TP27, TP29, TP31 were medium number of sclerotia producer. Isolates TP6, TP10, TP15, TP16, TP18, TP23, TP25, TP28, TP32, TP34, TP 35 were good sclerotia producers whereas, isolates TP1, TP2, TP9, TP11, TP21, TP22, TP34 were high sclerotia producers. Fifteen isolates (TP2, TP3, TP6, TP10, TP12, TP15, TP16, TP18, TP19, TP22, TP23, TP27, TP29, TP32, TP33) showed the peripheral type of sclerotial pattern, 3 isolates (TP13, TP17, TP24) formed sclerotia at the centre, 12 isolates (TP1, TP7, TP9, TP11, TP14, TP21, TP25, TP26, TP28, TP31, TP34, TP35) produced sclerotia thoroughly all over the plate and only 5 isolates (TP4, TP5, TP8, TP20, TP30) produced sclerotia in the middle of the plate (Table 1). Among 35 isolates, 17 (TP2, TP3, TP5, TP8, TP9, TP10, TP11, TP12, TP13, TP14, TP15, TP17, TP23, TP25, TP27, TP34, TP35) isolates has sclerotial weight in the range of 0–0.20 g 17 (TP1, TP4, TP6, TP16, TP18, TP19, TP20, TP21,TP22, TP24, TP26, TP28, TP29, TP30, TP31, TP32, TP33) isolates sclerotial weight in the range of 0.21–0.40 g and only single isolate (TP7) has sclerotial weight in the range of 0.61–0.80 g (Table 1).

Pathogenicity test

The relative lesion height (%) and AUDPC of *R. solani* isolates were evaluated on a highly susceptible PB-1 rice cultivar in the month of July 2020 and July 2021. Significant differences were observed among the isolates for the lesion height in the Pusa Basmati1 rice genotype at 21 days of inoculation (SupplementaryTable 1). Based on RLH, isolates were categorized as less virulent (20–30%), medium virulent (31–45%), virulent (46–75%) and highly virulent (75–100%). At 21 days after incubation, highest RLH was observed for isolate TP3 of rice (86.13%) followed by isolate TP26 (86.9%). Isolates TP4 (41.27%), TP8 (47.37%), TP9 (40.97%), TP31 (38.60%), and TP33 (40.17%). Isolates TP1, TP2, TP5, TP6, TP7, TP10, TP11, TP12, TP13, TP16, TP18, TP19, TP20, TP21, TP23, TP24, TP25, TP27, TP28, TP29, TP32 were virulent and isolates TP3, TP14, TP15, TP26 were highly virulent (Table 2). Isolate TP15 (rice isolate) infected the highest number of tillers (21) and isolate TP20 (rice isolate) infected the lowest number of tillers (10.33%). The total numbers of lesions produced by *R. solani* isolates were in the range from 2.78–6.67 at 7 days after inoculation. Similarly, a significant difference was observed among the isolates for the area under the disease progress

Table 2 The pathogenicity and molecular characterization of *R. solani* isolates collected from different hosts

Isolate code	Gen Bank Accession Number	Mean Relative lesion Height (RLH %)*	Area Under Disease Progress Curve (AUDPC)*
TP1	ON383500	60.83^{abcde}	495.56^{d}
TP2	ON383484	68.47^{abcd}	517.89^{e}
TP3	ON383492	83.13^{ab}	572.39^{f}
TP4	ON383496	41.27^{cde}	429.34^{b}
TP5	ON383506	67.37^{abcde}	522.09^{e}
TP6	ON383493	66.2^{abcde}	511.84^{e}
TP7	ON383501	70.37^{abcd}	564.48^{f}
TP8	ON383485	47.37^{bcde}	386.43^{a}
TP9	ON383505	40.97^{cde}	446.25^{c}
TP10	ON383508	45.07^{bcde}	442.33^{c}
TP11	ON383483	53.07^{abcde}	435.66^{c}
TP12	ON383495	74.17^{abcd}	564.90^{f}
TP13	ON383495	64.13^{abcde}	519.61^{e}
TP14	ON383490	77.93^{abc}	545.30^{e}
TP15	ON383494	76.70^{abcd}	564.41^{f}
TP16	ON383513	56.77^{abcde}	486.99^{d}
TP17	ON383482	61.10^{abcde}	459.44^{c}
TP18	ON383481	64.63^{abcde}	507.74^{e}
TP19	ON383511	72.73^{abcd}	535.71^{e}
TP20	ON383499	57.47^{abcde}	482.19^{d}
TP21	ON383486	56.00^{abcde}	434.56^{b}
TP22	ON383497	39.60^{cde}	430.64^{b}
TP-23	ON383498	72.73^{abcd}	543.20^{e}
TP24	ON383512	49.57^{abcde}	531.86^{e}
TP25	ON383503	63.63^{abcde}	475.19^{d}
TP26	ON383509	86.9^{a}	621.00^{h}
TP 27	ON383510	68.6^{abcd}	621.14^{h}
TP28	ON383489	63.97^{abcde}	530.88^{e}
TP 29	ON383487	52.83^{abcde}	468.16^{d}
TP 30	ON383507	29.23^{e}	375.27^{a}
TP 31	ON383516	38.60^{de}	394.76^{a}
TP 32	ON383488	55.30^{abcde}	451.53^{c}
TP 33	ON383502	40.17^{cde}	403.69^{a}
TP 34	ON383491	50.27^{abcde}	450.10^{c}
TP 35	ON383504	47.17^{bcde}	407.26^{b}

*Mean value of relative lesion height of 2020 and 2021

curve (AUDPC). High AUDPC was observed for isolates TP3 (654.16); TP7 (645.12); TP15 (645.04) of rice, TP26 (709.12) and TP27 (709.88) of lablab bean. Low AUDPC was observed in TP4 (490.68) and TP8 (441.64) of rice, TP30 (428.88) of ginger, TP33 (461.36) of turmeric, TP35 (465.44) of cabbage and TP30 (375.27) of ginger. Statistical analysis of the data revealed there was a significant difference among isolates for relative lesion height (RLH) ($P = 0.005$) whereas, a significant difference among isolates was not observed for the number of infected tillers and a total number of lesions ($P = 0.005$).

Amplification of ITS_1 and ITS_4 regions

The amplification of ITS regions with primers ITS_1 and ITS_4 generated a band size of approximately 650 bp. The ITS sequences were assembled using (Bioedit) and checked for sequence similarity using the BLAST tool of NCBI. The ITS sequences of the respective isolates showed 94–100% identity with *R. solani* (teleomorph: *Thanatephorus cucumeris*). The phylogenetic analysis of ITS sequences was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

using Mega X software. Based on phylogenetic analysis, all the *R. solani* isolates were grouped in a single cluster whereas, *Sclerotium hydrophylum* taken as an out-group was in another cluster. Further, *R. solani* isolates were divided into 6 subclusters. Cluster I comprised of *R. solani* isolates of different hosts, cluster II with isolate TP8, and cluster III with isolate TP18 and TP1. Isolate TP15, TP7 and TP4 were grouped in separate subclusters-IV, V and VI respectively (Fig. 1).

URP fingerprinting analysis

All the isolates of *R. solani* collected from different areas of Tripura were tested for polymorphism using 11 URP primers, out of which 6 primers could give amplification (Table 3). Bands produced with these 6 primers were scorable and reproducible. The bands expressing the same electrophoretic mobility were assumed to be identical fragments, irrespective of staining intensity. The total numbers of bands amplified from the 6 primers were 60, out of which most bands amplified were polymorphic and none of the band was found to be monomorphic resulting in an estimation of 100% polymorphism. The highest number of bands was amplified by primer URP-6R (13 bands), URP-2F (12 bands) followed by URP-2R (11 bands) (Fig. 1, Supplementary Figs. 2, and 3, respectively). The resulting data of the URP-PCR fingerprint pattern was used to construct a dendrogram based on the Unweighted air Group Method with Arithmetic Mean (UPGMA) using Jaccard's similarity coefficient. A dendrogram constructed from the fingerprint pattern of URP-2F primer grouped the isolates into three clusters. Most rice isolates were grouped in the first cluster while isolates collected from different hosts

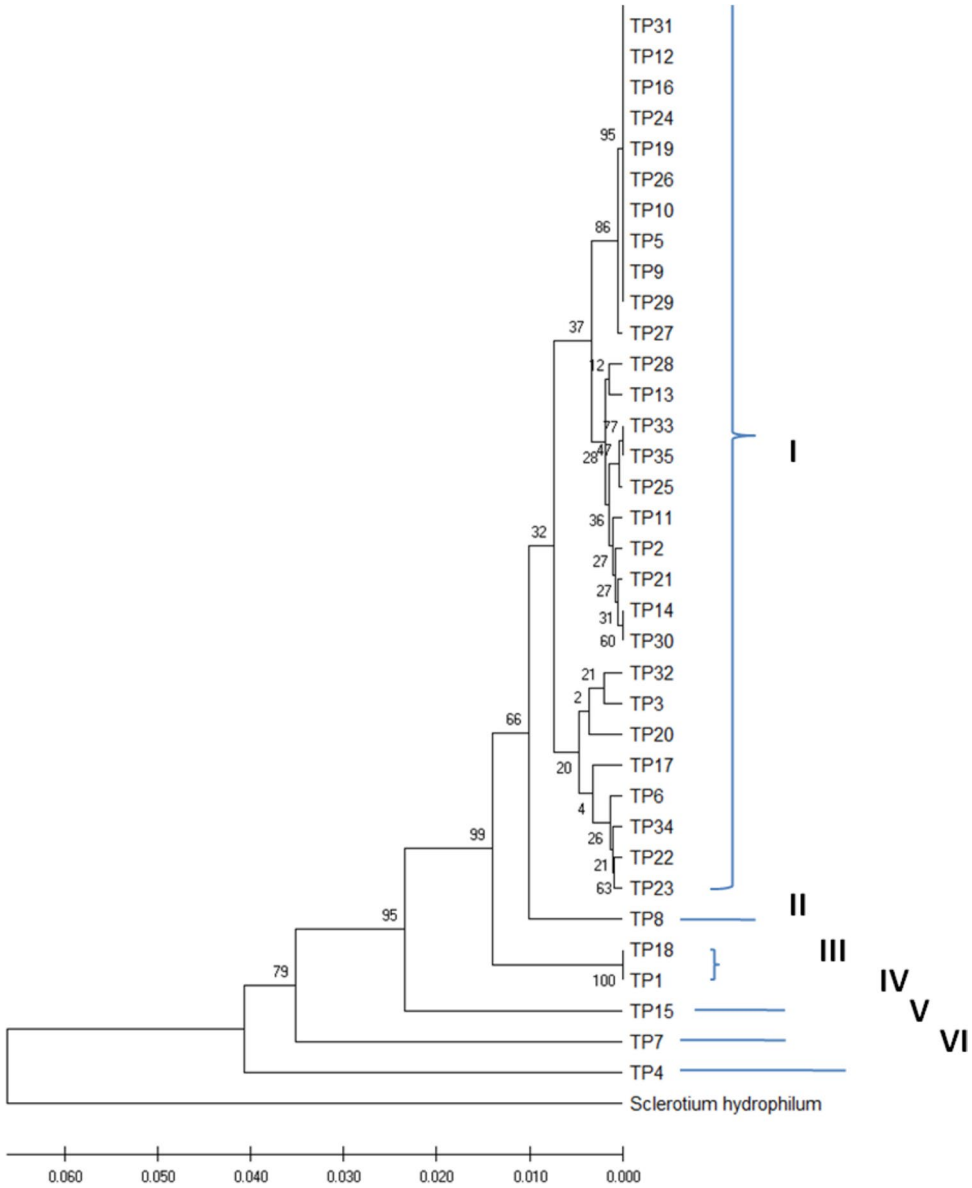

Fig. 1 Phylogenetic analysis of ITS sequences of *Rhizoctonia solani* isolates collected from different hosts using UPGMA method. *Sclerotium hydrophilum* was taken as outgroup

Table 3 Primer sequences of universal rice primers (URP), simple sequence repeats (SSR) and their polymorphism obtained in *Rhizoctonia solani* isolates.

Serial no.	Primer sequence (5'-3')	GC content (%)	Temp. (°C)	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	Percent polymorphism	Size range of bands (bp)
1	URP-2R CCCAGCAACTGATCGCACAC	60.00	61.40	11	11	0	100	2000–300
2	URP-6R GGCAAGCTGGTGGGAGGTAC	65.00	63.45	13	13	0	100	1800–200
3	URP-2F GTGTGCGATCAGTTGCTGGG	60.00	61.40	7	7	0	100	1500–500
4	URP-38F AAGAGGCATTCTACCACCAC	50.00	57.30	9	9	0	100	2700–200
5	URP-25F GATGTGTTCTTGGAGCCTGT	50.00	57.30	8	8	0	100	1700–500
6	URP-17R AATGTGGGCAAGCTGGTGGT	55.00	59.35	12	12	0	100	3000–370
7	SSR-1F CATCCTTTGCAGAGTTGCTG	50.00	57.30	37	37	0	100	450–550
	SSR-1R AGAGCACGAACACCTGGAC	55.00	59.35					
8	SSR-2F CAAGTCGATGCAGAAATGT	55.00	55.25	35	35	0	100	400–600
	SSR-2R CCGAGAGTGGGATCGAGTT	57.89	58.82					
9	SSR-3F CAGCGGGGCCTAAAAATAAT	50.00	55.25	35	35	0	100	300–500
	SSR-3R GGGCAAGCAAAGTAGTCTCG	55.00	59.35					
10	SSR-4F CGCATTTTCGCTTTCTTGAT	45.00	54.20	35	35	0	100	500–1000
	SSR-4R AGTGGCGGATATTACCGAGA	50.00	57.30					
	SSR-7R ACGAACACCTGGACTTAC CG	55.00	59.35					

were included in the second cluster. The ginger isolate TP30 was grouped separately. Moreover, a similar inference was drawn when another UPGMA based dendrogram was constructed based on the URP-PCR fingerprint pattern of all 6 URP primers (Fig. 2). Maximum nos. of rice isolates were grouped in the first cluster and ginger was grouped separately. The rice isolates were further sub grouped and most isolates from other host sub grouped separately. The genetic similarity observed between the isolates ranged from 33 to 92%. The maximum similarity was observed in isolates TP28 and TP35 and the minimum similarity was observed with TP12.

SSR

R. solani isolates collected from different hosts were tested for polymorphism using 8 SSR primers out of which 7 primers produced bands. The SSR primers produced a total of 147 bands out of which 142 were polymorphic bands ranging from 300 to 1000 bp for 4 primers (Table 3). A total of 38 alleles and 26 loci with an average of 1.5 alleles per locus were identified. The PIC value varied from 0.25 to 0.98 with an average of 0.93 which indicated that these loci contained a considerable amount of genetic information. SSR P1 clustered isolates into three groups; in one group, TP2-TP6, TP10, TP13 to TP25, TP 28 to TP 31, TP3, TP 34 and TP35 in the second group. This primer separated TP26 and TP27 into different group (SupplementaryFig. 6). SSRP2 clustered all isolates in 3 groups, *i.e.,* TP1, TP5 to TP10 (rice host) in first group; TP11, TP22, TP23, TP35 in second group and TP2 to TP4, TP12 to TP21, TP24, TP25 to TP34 in last group (Supplementary Fig. 7). SSRP3 also grouped isolates into 3 subgroups, *i.e.,* TP1, TP2 to TP7, TP13, TP20, TP20 to TP24, TP26 to TP29, TP32 in 1st cluster, TP8, TP12 in 2nd cluster and TP9, TP11, TP16 to TP18, TP30, TP31, TP33, TP35 in last cluster (Supplementary Fig. 8). SSRP4 grouped isolates into 2 groups, *i.e.,* TP1, TP32, TP33, TP35, TP31, TP34, TP12 in one group and TP2 to TP30 in another group (Supplementary Fig. 9).

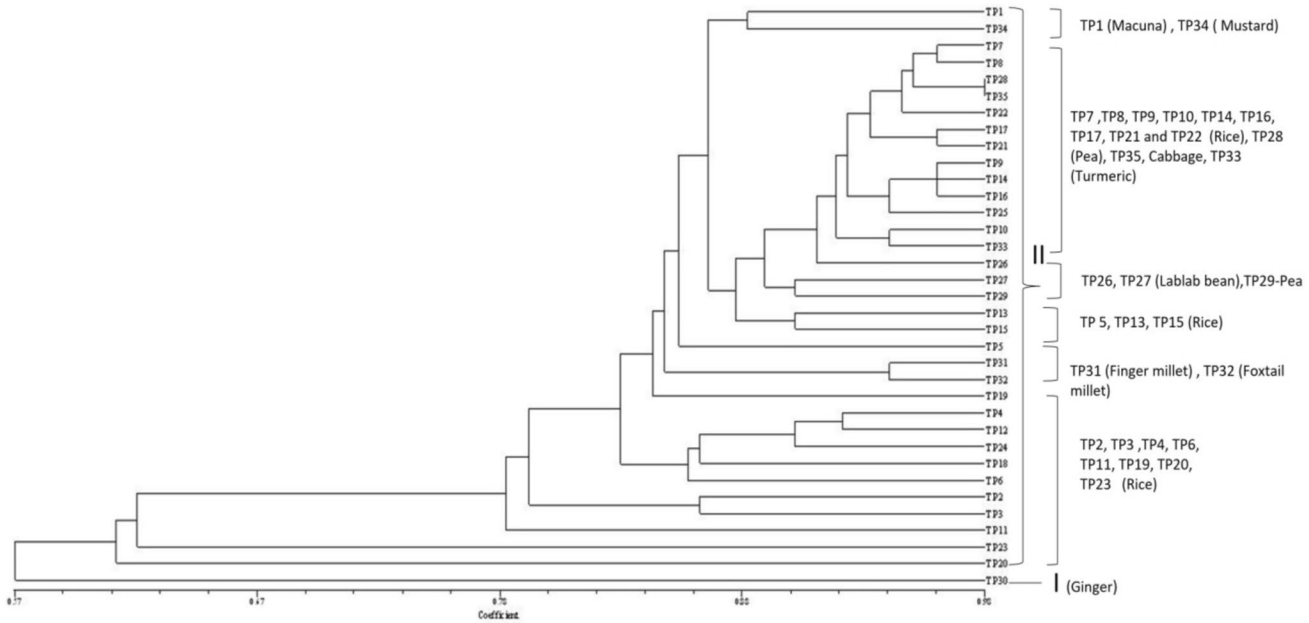

Fig. 2 URP based-dendrogram of 35 isolates of *R. solani* obtained with total 6 URP primers constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

Based on 7 SSR primers all the isolates of *R. solani* were grouped in two different clusters. Isolate TP13 and TP14 were grouped in cluster I, whereas, other *R. solani* isolates were grouped in cluster II. Cluster II is further subdivided into two subclusters cluster II a and cluster II b. Subcluster II a comprised of the rice isolates except TP30 and subcluster II b grouped isolates of rice and other hosts (Fig. 3).

Population genetic structure and PCoA

The allelic variations in 7 SSR regions were used to analyze population genetic structure. Genetic differences among isolates within a population were analyzed by STRUCTURE v 2.3.4. According to the principle of maximum likelihood value, the appropriate K value (5) was selected as the number of populations (Fig. 4). All isolates were divided into five groups as per their genetic structure.

The scatter plot from the PCoA of *R. solani* isolates from Tripura revealed that the second and third principal axis account for 26.51% and 19.07%, respectively, and all three axes together explained 72.82% cumulative variation (Fig. 5).

AMOVA

The AMOVA was carried out by segregating the total variation among populations (within the group) among isolates (within the population). We grouped isolates into two populations, *i.e.*, rice and non-rice based on their host. Higher variance (91%) was determined within the population and less variance (9%) was observed among populations (Fig. 6 and SupplementaryTable 2).

Discussion

The pathogen *Rhizoctonia solani* has been shown to affect many agricultural and horticulture crops and is considered as one of the most important soil-borne plant pathogens (Ogoshi et al. 1987). In India, the estimated losses due to sheath blight disease have been reported up to 54.3%. (Chahal et al. 2003). The fungal pathogen displays huge diversity in terms of cultural, morphological, physiological, and pathological characteristics (Ou 1985). Tripura is a hilly region where moderate warm and cold temperatures are expected in summer and winter, respectively. The important crop of this state is rice but with the least productivity. The state covers irrigated and rainfed regions, having 0.25 million ha under rice cultivation. The current average productivity of rice is about 2.3 tones/ha (DRR 2006–2010). The current investigation aimed to determine the phenotypic and genotypic diversity of *R. solani* isolates collected from different regions of Tripura, India. The results clearly confirmed the existence of variability in morphological, pathological, and genetic characters of *R. solani* isolates (Parmeter and Whitney 1970; Sharma et al. 2005). Morphological characteristics viz., colony colour, growth and sclerotial characteristics showed a high degree of variation among isolates with respect to environmental and genetic factors. These features help to predict the virulence and aggressiveness of the

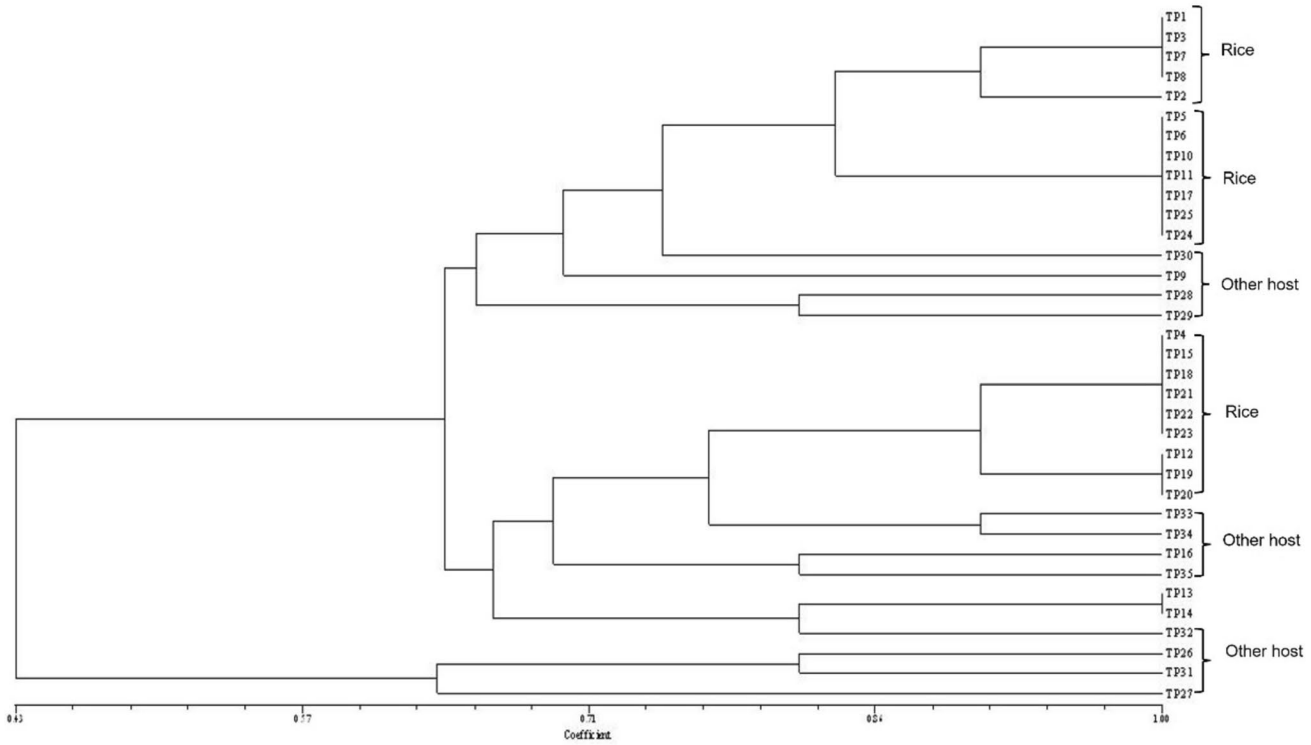

Fig. 3 SSR based-dendrogram of 35 isolates of *R. solani* obtained with total 7 SSR primers. Constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

pathogen. Variability is a general phenomenon observed both in plants and pathogens, isolates of *R. solani* collected from maize and rice were different in hyphal width, sclerotia size and colour (Madhavi et al. 2015).

The identity of the *R. solani* isolates was confirmed based on the ITS region amplification. The phylogenetic tree of the ITS region nucleotide sequences was constructed using the UPGMA method. Results confirmed all isolates belong to the *R. solani* species indicating high level of genetic similarity for this region. The internal spacer regions are of prime importance as they are highly conserved regions, used for elucidating the relationships among species within a single genus or among intraspecific populations. The internal transcribed spacer region, consisting of ITS1 and ITS2, evolves faster and can differentiate between species and the sequences of genes often have been used for the molecular identification of fungi (Mirmajlessi et al. 2012).

The pathogenicity of the *R. solani* isolates was examined by conducting an experiment in a net house facility at IARI, New Delhi on the highly susceptible rice cultivar, Pusa Basmati-1. Plants of PB-1 were inoculated with *R. solani* isolates at the tillering stage to measure the disease development both horizontally and vertically. Disease parameters such as relative lesion height, number of infected tillers and number of lesions produced were recorded for each isolate. Light brown/ white/ greyish colored lesions were observed on the stem of tillers indicating the presence of sheath blight symptoms. All isolates were found to be virulent with respect to pathogenic characters. This fact should be highlighted that an increasing trend of relative lesion height (RLH) from tillering stage to the panicle initiation stage was observed among all isolates. The number of tillers infected by the 35 isolates ranges from 10–21 while the number of lesions produced by the *R. solani* isolates ranges from 2.78–6.67 per tiller. Earlier studies by Lal et al. (2012) reported similar results, where the pathogenicity of 25 isolates of *R. solani* was determined on the highly susceptible genotype, PB-1. In another study reported by Susheela and Reddy (2013) where pathogenicity of 35 isolates of *R. solani* was determined on the susceptible cultivar IR-50. Out of 35 isolates, 17 isolates were highly virulent (> 70% DI), 14 were virulent (60–70% DI), 2 isolates were moderately virulent (50–60% DI) and the remaining isolates were least virulent (< 50% DI) group. Lal et al. (2014) evaluated 25 isolates and observed that 12 isolates were highly virulent whereas, 13 isolates were moderately virulent. From these studies, it could be summarized that three points are important from the perspective of pathogenic variation. Firstly, isolates may cause a different type of symptoms and disease. Secondly, isolates may vary from avirulent to highly virulent state. Thirdly, the host range

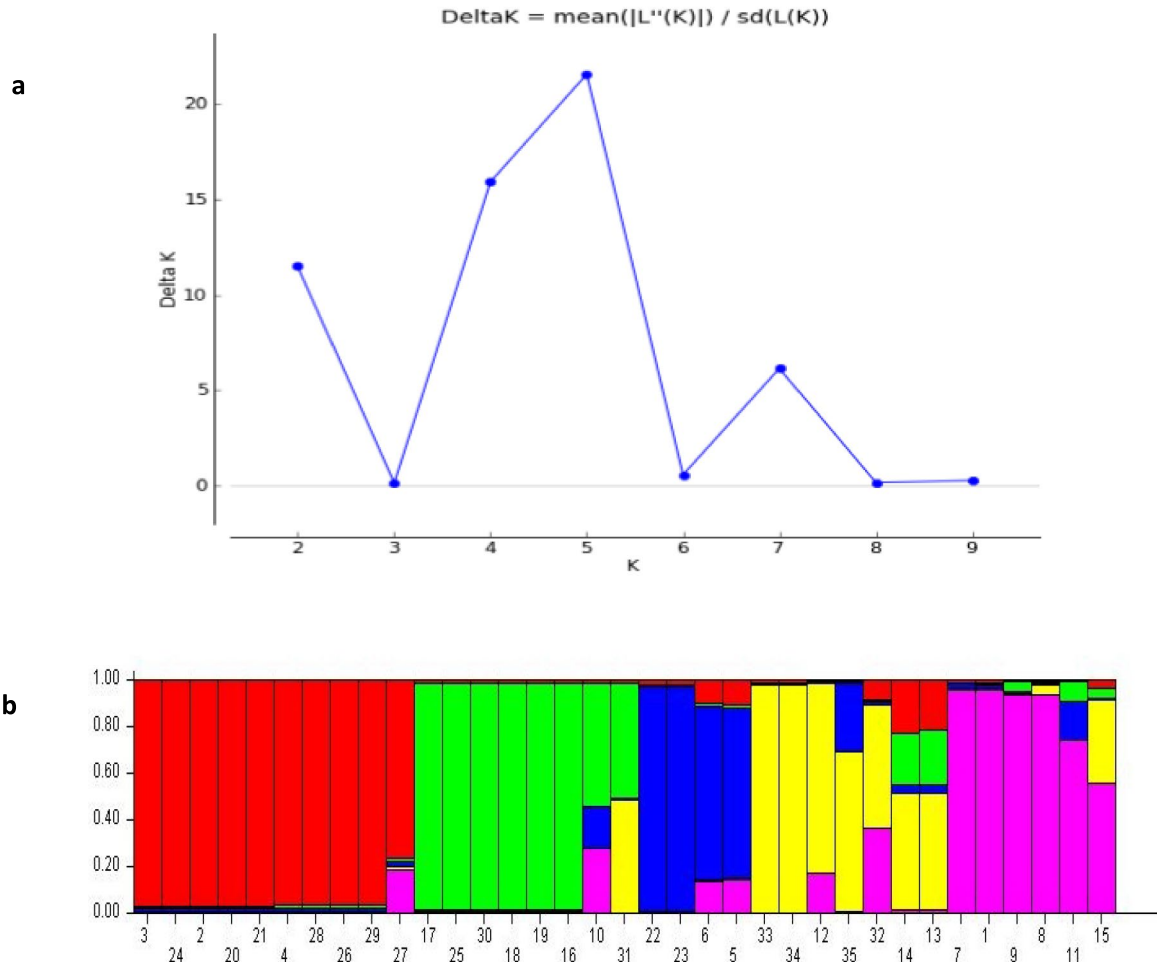

Fig. 4 Population clustering of *R. solani* isolates at estimated membership fraction for K = 5 using STRUCTURE. **a** The relationship between ΔK and K **b** the five major clusters

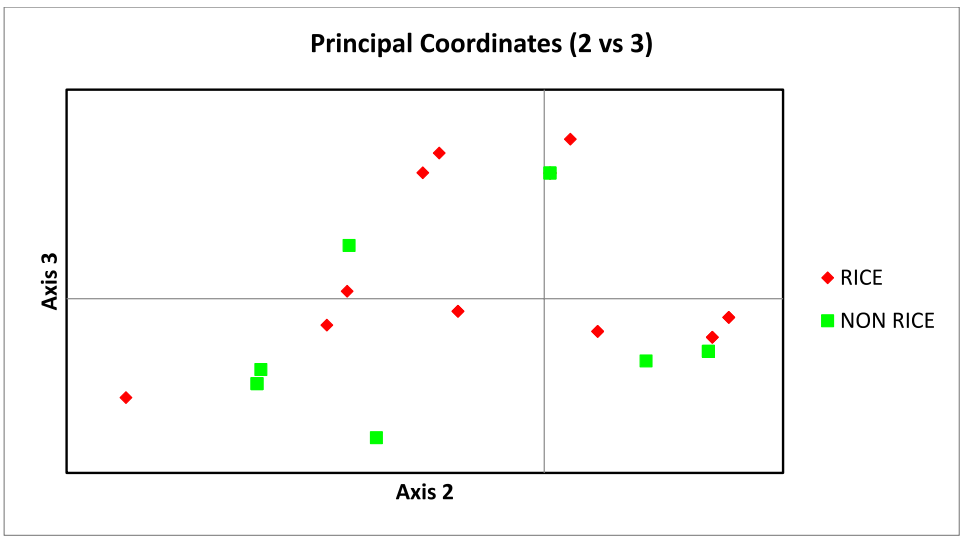

Fig. 5 Principal coordinate analysis (PCoA) of *R. solani* isolates from Tripura. The second and third principal coordinates account for 26.51% and 19.07%

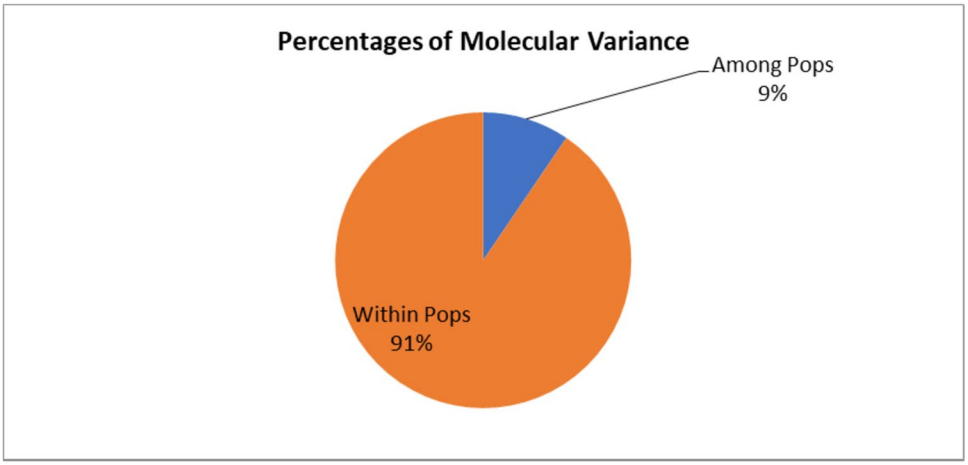

Fig. 6 Analysis of molecular variance (AMOVA) among rice and non- rice *R. solani* isolates

among isolates may vary from extremely wide to limited. Hence, comprehensive, and conclusive studies are required to understand the underlying differences among isolates in lesion progression and mode of pathogenicity under identical conditions.

In the present study, two sets of markers namely, URP and SSR were used to analyze the diversity of isolates of *R. solani,* and they produced 94.6% to 96.5% polymorphism. The genetic similarity observed between the isolates ranged from 33 to 92%. A similar result was also obtained from the dendrogram constructed based on the URP-PCR fingerprint pattern of all the 6 URP primers. The genetic similarity observed between the isolates ranged from 33 to 92%. The maximum similarity was observed in isolate TP28 (Pea isolate) and TP35 (cabbage isolate) and minimum similarity was observed in TP12 (rice isolate).

In SSR analysis, *R. solani* isolated groups were partially associated with host origin. UPGMA analysis of SSR primer set 1 and 4 revealed that isolates TP 31, TP33, TP35, TP 32, TP27, and TP 1 were different from other isolates. Dendrogram obtained from all 7 primers also showed that the above isolates were different from isolates obtained from rice. It is evident from these results that there could be some existence of genetic variability among these isolates as the *R. solani* is infecting a wide variety of hosts. It was observed that the isolates of *R. solani* obtained from the same type of hosts and same geographical regions showed similarity in DNA fingerprint profiles barring few exceptions. The molecular analysis was useful in assessing the intra- and inter-species specific diversity. The clusters formed in the present study correspond to their host origin. So, this study helps in the development of host specific markers and identification of the pathogen.

In the future it will be useful for integrated disease management and to understand the evolution of the pathogen better. Such work that has helped thus far described *R. solani* infecting mungbean and were characterized using SSR, RAPD (Dubey et al. 2012; Sharma et al. 2005). Another study looked at isolates of *R. solani* from maize, rice and evaluated genotypic variability using URP 9 (Mishra et al. 2015).

The 35 *R. solani* isolates are classified into five distinct genetic groups by the tool STRUCTURE and all the isolates were found to be admixtures of varying ranges. Moreover, the results of population structure were basically consistent with the factorial analysis of *R. solani* isolates. A similar study indicated the population structure of *R. solani* isolates from three different countries were found to be admixtures, Western, Eastern and Central China were the populations with the higher admixture followed by Japan isolates and no significant admixture was detected in the isolates of the Philippines (Cumagun et al. 2020). The analysis of molecular variance (AMOVA) indicated that most of the genetic diversity occurred within rice isolates (91%). Taheri et al. (2007) partitioned 29.68% of genetic variation among the two subgroups based on geographical region, and 70.32% within subgroups. Shu et al. (2014) also demonstrated that there was a relatively high level (81.93%) of genetic variation within subpopulations. This comprehensive understanding of the population structure and diversity of *R. solani* isolates will provide useful information for the comprehensive control of rice sheath blight disease.

Conclusion

Thirty-five isolates of *Rhizoctonia solani* obtained from rice and other host were variable in morphological characters like hyphal width, growth rate, number, size, and pattern of sclerotia formation. All 35 isolates were found to be virulent in pathogenicity testing, ranging from virulent, to moderately virulent, to least virulent based on RLH. The isolates were grouped into 12 subgroups based on ITS phylogenetic tree. The ITS analysis did not show host wide differentiation among the isolates of *R. solani.* The morphological characters did not correspond to the molecular

groups generated in the present study. URP and SSR markers were used for genetic diversity analysis of the isolates and based on UPGMA analysis of URP markers together; the isolates grouped into three clusters. In SSR UPGMA of all 7 primers grouped isolate in 6 groups, most rice isolates grouped together and isolates from the other hosts grouped separately. *Rhizoctonia solani* is a very destructive pathogenic fungus responsible for losses of rice productivity. Morphological and genotypic variability was evident among all *R. solani* isolates of rice. From the current investigation, it is evident that there is diversity among *R. solani* isolates obtained from rice and other host plants. This information will be helpful in future studies for understanding the phylogenetic diversity of the different populations of *R. solani* at the molecular level.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42161-023-01515-w.

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Accession numbers: The ITS sequence data has been deposited at GenBank (Accession no's: ON383481 to ON383516).

Data availability All data generated or analysed during this study are included in this published article and the raw datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest in the publication.

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