International Journal of Pure and Applied Zoology
Volume 5, Issue 1, pp: 11-16, 2017
http://www.ijpaz.com

ISSN (Print) : 2320-9577
ISSN (Online): 2320-9585

Research Article

MOLECULAR IDENTIFICATION OF ROTYLENCHULUS RENIFORMIS (NEMATODA) BY USING ITS REGION OF RDNA

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Article History: Received 18th November 2016; Accepted 28th December 2016; Published 10th January 2017

ABSTRACT

Background: Reniform nematode (Rotylenchulus reniformis) is widely distributed in all cotton (Gossypium spp.) grown regions of India. In Reniform nemotode infection, the growth of cotton roots and shoots was reduced at level of more than 8 and 1 nematode per g soil, respectively. DNA-based Molecular identification of Plant parasitic nematode species has been determined by Internal Transcribed spacer (ITS) gene with PCR Techniques. The ribosomal DNA (rDNA) is a novel genetic marker and it is a multi-gene family consisting of many copies (100-500 in animals) of genes encoding for three ribosomal components 28S, 5.8S and 18S. Phylogeny is the history of organism lineage as they change through time. It implies that different species arise from previous forms via decent and that all organisms from the smallest microbes to the largest plants and vertebrates were connected by the passage by genes along the branches of the phylogenetic tree that links all the life form. In this study we have evaluated the diagnostic utility of the ITS1 and ITS2 region for constructing phylogenetic trees and determining taxonomic identity of Plant parasitic nematode species.

Results: Rotylenchulus reniformis was collected from soil and root samples of Cotton crop and from Rotylenchulus reniformis (PPN) the ITS region of rDNA sequence is isolated. Phylogenetic analyses of Neighbor Joining (NJ) was distance based method, in this our sequence Rotylenchulus reniformis and Rotylenchulus reniformis of USA country were formed as one clade. Rotylenchulus reniformis of *ITS gene* sequence depicting high congruence with Rotylenchulus reniformis of USA country by 100 bootstrap replicate values.

Conclusions: The ITS region becomes an important taxonomic feature in nematode identification. ITS versatility, specificity, effort of experimental manipulation, and growing ITS databases should accelerate its application in nematology. Most interesting finding in our studies with reference to plant parasitic nematode and its host plant suggested that *ITS gene* sequence helps in identifying nematode pest status and host plant susceptibility. Thus, these studies will help us to develop the Integrated Pest Management (IPM) package for a specific host plant and its variety. At the same time ITS supports Quarantine technology with reference to agro product export and import.

Keywords: Internal transcribed spacer (ITS); Phytonematode; Rotylenchulus reniformis; Ribosomal DNA (rDNA)

INTRODUCTION

Reniform nematode (*Rotylenchulus reniformis*) is widely distributed in all cotton (*Gossypium* spp.) grown regions of India. Traditionally, morphological and morphometric characters have been used to discriminate between nematode taxa, both for diagnostic purposes and to clarify the evolutionary relationships between them (Maggenti, 1991). These diagnostic methods can be time consuming, requiring a lot of skill and expertise (Subbotin *et al.,* 2000). In addition, many taxa can be diagnosed only from adult male or female specific structures, or from population measures of relative morphological characters. In such cases, larvae, individuals of the "wrong" sex or individual specimens may not be identifiable. For many studies, identifications are only made at the generic level, not to named species (Mai and Mullin, 1996; Floyd *et al.,* 2002). Also the nematode control by resistant plants is far more effective than any other alternative. Recently, it has been confirmed that precise genetic identification of plant cultivars and pathogens on both theoretical and practical levels offers a solid scientific platform when measuring host suitability for pathogens. It enables precise understanding of plant/pathogen relationships.

The nucleotide sequences of fragments of rRNA genes have been obtained in various species of plant parasitic nematodes, yielding a proper platform for both identification and taxonomic approaches (Giorgi *et al.,* 2002). Molecular methods for diversity assessment have already aided in the understanding of other groups of organisms that are difficult or impossible to study by any other means (Floyd *et al.,* 2002). There is currently much interest in the use of DNA sequences as markers for taxonomic identification and biodiversity surveys, an approach also known as DNA bar coding (Hebert *et al.,* 2003). The techniques that use

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universal primers to target microbial genes have significant advantages, e.g., convenience, high-throughput, and considerable savings in time (Puitika *et al.,* 2007); it is a quicker and more efficient way of studying nematode diversity than traditional taxonomic methods, which depend on morphological criteria (Floyd *et al.,* 2002). Also, knowledge of genetic diversity in the case of plant-parasitic nematodes is essential to effective resistance breeding programs for the host (Hahn *et al.,* 1994; Roberts, 2002). Earlier researchers worked on nematodes identification based on ITS sequences, DNA based approaches have been successful used for the molecular diagnostics of *Rotylenchulus sp* (Vovlas *et al.,* 2008; Atighi *et al.,* 2011; Cantalapiedra-Navarrete *et al.,* 2012). Yet, to the best of our knowledge, there is not a single nematode species that has failed to provide an amplification product of the ITS region when amplified with "universal" PCR primer sets. Universal amplification coupled with the ability to amplify ITS from individual nematodes suggests that any species, population, or ecological community of nematodes can be analyzed using a molecular approach based on the rDNA ITS region (Vrain and McNamara, 1994). Only few sequences available in the primary nucleotide databases span the entire rDNA array, although in several cases phylogenetic relationships within different species of plant-parasitic nematodes have been obtained even when only fragments of ribosomal genes were used (Banna *et al.,* 1997, Subbotin *et al.,* 2001).

In this study we have evaluated the diagnostic utility of the ITS1 and ITS2 region for molecular identification and constructing phylogenetic trees and to see evolutionary relationship of plant parasitic nematodes from selected crops.

MATERIALS AND METHODS

Selection of nematode collection sites and crops

This study was conducted in Muthireddy gudem of Ramannapet Mandal, Nalgonda District in Andhra Pradesh State during 2008-2011. Cotton crop field selected for the present study and sampling was done from each crop separately. Soil and plant samples were brought to the laboratory, in order to identify nematodes and maintained in laboratory conditions for extraction, counting, fixation and preservation of the phytonematodes. The nematodes were extracted with Cobb's sieving and decanting gravity method (Cobb, 1918), fixed in FAA, processed to glycerin and mounted on slides for compound microscopic studies.

DNA isolation, PCR amplification and sequencing

Identification of nematodes to the species level often requires detailed morphological analysis, growth of the nematode on different host plants, or DNA analysis. The samples were electrophoresed on 1% Agarose gel using buffer 1X TAE. For reference λ-Hind III Digest was loaded along the sides of the samples. For analysis 2 µl of DNA sample was taken and 6 µl of 6x Gel loading dye was added and loaded to the Agarose gel. The concentration of 10-12 ng/µl is realized through above methods using standard concentration of λ DNA which is realized for further process such as PCR and sequencing. The entire genome DNA extracted from nematodes was directly amplified. The ITS containing region spanning from the 3´end of the 18S rDNA to the 5´end of the 28S rDNA was amplified using Forward primer 18S/rDNA1 (5'TTGATTACGTCCCTGCCCTTT3') and Reverse primer 26S/rDNA2 (5'TTTCACTCGCCGTTACTAAGG3'). PCR amplification was carried out in 2 µl containing 2.5 mM of each dNTP, 1µl (10 pm/µl) of each primer and 1 unit of Taq DNA polymerase (1U/1 µl). PCR cycles consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 50 sec at 95°C (denaturation), 1 min at 55 °C (annealing), 1 min at 72°C (elongation) and a final step at 72°C for 7 min.

The size of amplification products was determined by comparison with the molecular weight marker Ladder 100 bp (Fermentas , St. Leon-Rot Germany) following electrophoresis of 10 µl on a 1% agarose gel. Ten µl of each PCR product was directly digested with the restriction enzymes: λ-Hind III (according manufacturer's instructions in a total volume of 20 µl). The digestions were conducted overnight or for 4hrs at 37°C. The resulting DNA fragments were separated by gel electrophoresis in a 1% agarose gel. The gels were then observed using UV light. The amplified fragments from nematodes were isolated from agarose gel using mdi Micro GEL extraction kit. The quality of the sequences produced was checked using Sequence Navigator Software (Applied Bio-systems, Warrington, UK).

Phylogenetic analysis were conducted using MEGA 4.0 (Molecular Evolutionary Genetics Analysis) Software, taking above Clustal X2 Alignment File, Convert into MEGA Format, Then calculate distances and finally Predict Bootstrap of Phylogeny tree of Algorithms like Neighbour Joining. Neighbor Joining (NJ) this is a method often used to construct phylogenetic trees (Li, 1981; Faith, 1985). Neighbor joining is a special example of star decomposition method. In this method, the phylogenetic tree is constructed from a star-like tree by grouping OTUs with shortest distance of branch length together. This method is very suitable with dataset consisting descendants with largely varying rates of evolution. This method was discovered by Saitou and Nei (Saitou and Nei ,1987). The bootstrap analysis strongly supports the relationship between the organisms used in the study.

RESULTS

Reniform nematode (*Rotylenchulus reniformis*) is widely distributed in all cotton (*Gossypium* spp.) grown regions of India. In Reniform nemotode infection, the growth of cotton roots and shoots was reduced at level of more than 8 and 1 nematode per g soil, respectively.

Rotylenchulus reniformis (Cotton) ITS1 and ITS2 gene sequence studies

Rotylenchulus reniformis is a phytonematode and

a pest on Cotton crop. We isolated ITS1 and ITS2 gene sequence of rDNA. The sequence length was 882bp and the number of Adenine's were 186, Cytosine's were 258, Guanine's were 260 and Thymidin's were 178. The rDNA gene sequence annotation is as follows (Figure 1).

The amplification of the ITS region using primer pair Nem_18S_F/rDNA1, Nem_26S_R /rDNA2 yielded one distinct amplicon which was approximately 882 bp in size for the plant parasitic nematode (PPN) *Rotylenchulus reniformis.* The amplified PCR product of rDNA region included the size of 5.8S rDNA ranged 577 to 601bp, the size of ITS2 ranged from 602 to 832 bp and the size of 28S rDNA ranged from 833 to 857 bp. The average nucleotide composition of ITS region was 186 A's, 258 C's, 260 G's and 178 U/T's.

Phylogenetic analyses

Rotylenchulus reniformis was collected from soil and root samples of Cotton crop and from *Rotylenchulus reniformis* (PPN) the ITS region of rDNA sequence is isolated (Figure 2).

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          10          20          30          40          50
GATTGATGGT  CCGGTGAGTG  TTCGGATCGC  GGCGACGTGG  GCGGTTCGCT
          60          70          80          90         100
GCCCGCGACG  TCGCGAGAAG  TCCACTGAAC  CTTATCATTT  AGAGGAAGGA
         110         120         130         140         150
GAAGTCGTAA  CAAGGTTTCC  GTAGGTGAAC  CTGCGGAAGG  ATCATTGTCG
         160         170         180         190         200
AAACCTGCCT  AGCAGAACGA  CCCGCGAACG  CGTTGCAAA AACACCGGAG
         210         220         230         240         250
GTGGTGCGGG  TGCATCCTCG  CCTCTCGCCA  CCCCCGTGTC  TCGGAGCGGC
         260         270         280         290         300
CAGTCTCGTC  GTCCCTTTGC  CCGTCGGGTG  GGGTGAGATG  CCGGGATCAA
         310         320         330         340         350
CCTCTTCGAG  GCAAAGCGAA  CAAACCCCCG  GCGCGAATCGCGCCAAGGAA
         360         370         380         390         400
TCGAAACGAA  AGAAGGGGCA  CGTCTTCTGT  CGCCGCACCG  TTCGCGGTGT
         410         420         430         440         450
CGATGCTTCA  GTGATGTTGT  TCTCTTGTCG  CAAAATATAC  AGAACGACTC
         460         470         480         490         500
TCGGCAACGG  ATATCTCGGC  TCTCGCATCG  ATGAAGAACG  TAGCGAAATG
         510         520         530         540         550
CGATACTTGG  TGTGAATTGC  AGAATCCCGT  GAACCATCGA  GTCTTTGAAC
         560         570         580         590         600
GCAAGTTGCG  CCCCAAGCCA  TTAGGCCGAG  GGCACGTCTG  CCTGGGTGTC
         610         620         630         640         650
ACGCATCGTC  GCCCCCATCC  AACCATGAGC  CCTCGAGCCT  CGGTTGGACC
         660         670         680         690         700
GCGGGCGGAA  ATTGGCCTCC  CGTGCGCTCA  CAGCCAGCGG  TTGGCCTAAA
         710         720         730         740         750
TTCGAGTCCT  CGACGACATC  ATCGTCGCGA  CGATCGGTGG  TAATGCTGCA
         760         770         780         790         800
AGCAACCTCG  TTCGGAGTCG  TGCGCGTCCG  TCGATCGAGA CCCTTGAACC
         810         820         830         840         850
CTTTCGGCAT  CGCAAGGCCG  GTGCTTGCGT  CGCGACCCCA GGTCAGGCAG
         860         870         880  882
GATTACCCGC  TGAGCTTAAG  CATATCAATA  AG
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Figure 1: *Rotylenchulus reniformis* ITS2 gene Annotation

Figure 2: Multiple sequence alignment of *ITS gene* sequences of *Rotylenchulus reniformis* plant parasitic nematodes with similar plant parasitic nematode of *ITS gene* sequences using Clustal X2 ***** shows conserved nucleotides of all sequences.

Neighbor joining (NJ)

Neighbor joining is distance based method. Phylogenetic analyses of *Rotylenchulus reniformis* ITS region of rDNA sequence is belongs to the Rotylenchulidae family. This sequence depicted similarity with same family of nematodes such as Rotylenchulidae. In this NJ tree mainly two clades were formed. In the first clade *Paratrichodorus sp* of China country and *Radopholus similis* of Malaysia country were formed and in this *Paratrichodorus poruses, Paratrichodorus renifer* and *Paratrichodorus minor* were formed as sister nodes. In the second clade our sequence *Rotylenchulus reniformis* and *Rotylenchulus reniformis* of USA country were formed. *Rotylenchulus reniformis* of *ITS gene* sequence depicting high congruence with *Rotylenchulus reniformis* of USA country by 100 bootstrap replicate values (Figure 3).

DISCUSSION

Rotylenchulus reniformis is widely distributed in all cotton grown regions of India (Gulsar Banu, 2007). *Rotylenchulus reniformis* was first described from Hawaii, USA and is widespread in the tropics and subtropics (Stephen *et al.,* 1991). About 19 Genera of plant parasitic nematodes are reported in cotton crop and in world until today (Nandini Gokte- Narkhedkar *et a.l,* 2002). Of these, the most important generic species in Indian context is *Rotylenchulus reniformis* (Reniform nematode), *Meloidogyne incognita, Hoplolaimus sp*. and *Pratylenchus sp.* The *Rotylenchulus reniformis* (Reniform nematode) has been recorded to be the key nematode species on cotton in Central and Southern India while in Northern cotton-growing areas, the *Meloidogyne incognita* (Root knot nematode) is dominant (Nandini Gokte *et al.,* 2002).

In any organism the nucleotide base sequence is the primary source of biological variation (Powers *et al.,* 1997). DNA bar coding method DNA sequences as markers for taxonomic identification and helps in biodiversity surveys (Hebert *et al.,* 2003). The techniques what we used especially universal primers are fundamentally target microbial genes have significant advantages, e.g., convenience, high-throughput, and considerable savings in time (Puitika *et al.,* 2007); It is a quicker and more efficient way of studying nematode diversity than traditional taxonomic methods, which depend on morphological criteria (Floyd *et al.,* 2002). Knowledge of genetic diversity in the case of plant-parasitic nematodes is essential in breeding plants with high resistance in host plants an ecologically very important method of pest nematode control (Hahn *et al.,* 1994; Roberts, 2002).

In this, we were used a single universal forward primer and reverse primers set Nem_18S_F/rDNA1, Nem_26S_R / rDNA2 to amplify all the target species of single nematode in a single tube, which was formed successful PCR bands on the agarose gel for the tested nematode samples. The use of multiplex reactions intensity bands in the PCR products helped to detect species of nematodes. Similarly, Floyd et al. (Floyd *et al.,* 2005) also noticed and reported that the sensitivity of universal (Forward and Reverse) primer set gives it the ability to identify a mixed nematode population when only one nematode individual is taken for study.

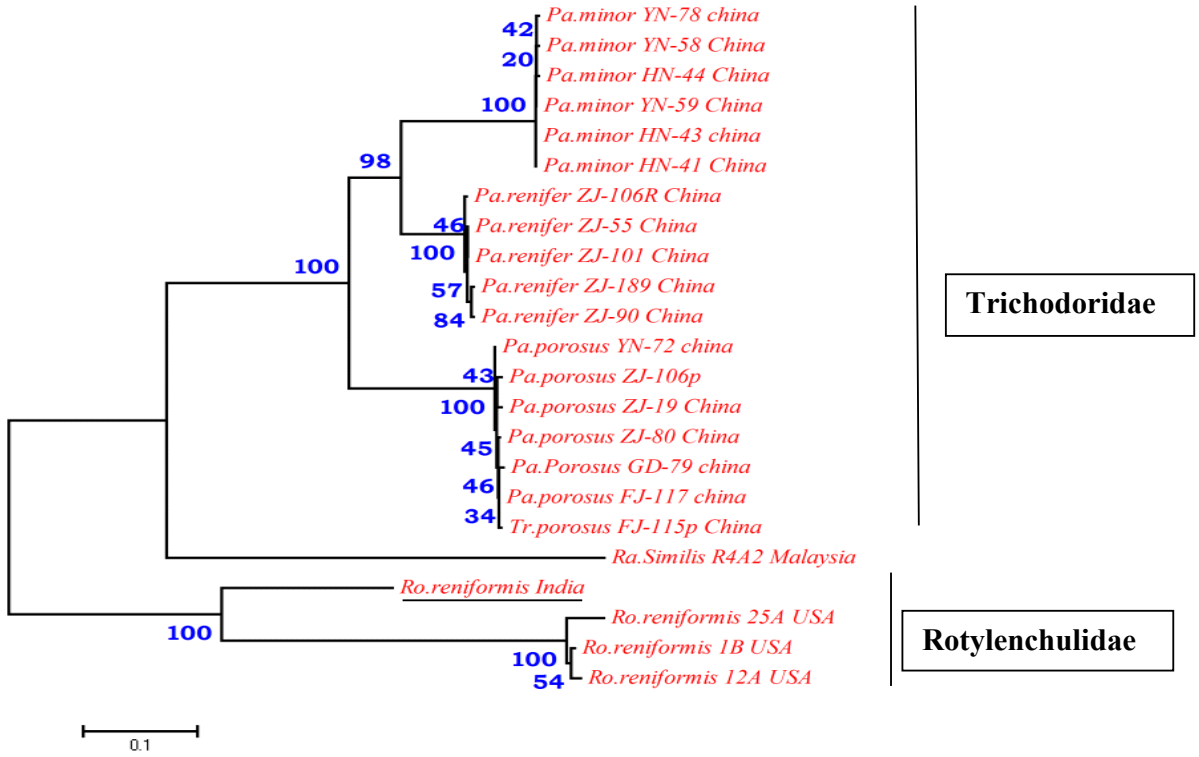

Figure 3: Phylograms depicting the degree of relationship of rDNA region of *ITS gene* sequence of *Rotylenchulus reniformis* with other nematode *ITS gene* sequences using Algorithms of Neighbor Joining.

By using our experimental genetic data we determined the molecular phylogeny of different nematode species based on sequence of ribosomal internal transcribed spacer (ITS) region (Strimmer and Haeseler, 1996; Ronquist and Huelsenbeck 2003; Kumar *et al.,* 2004). Phylogeny is the history of organism lineage as they change through time. It implies that different species arise from previous forms via decent and that all organisms from the smallest microbes to the largest plants and vertebrates were connected by the passage by genes along the branches of the phylogenetic tree that links all the life form.

Plant parasitic nematode *Rotylenchulus reniformis* was isolated from Cotton field and it's ITS sequence was obtained. In phylogenetic analyses *Rotylenchulus reniformis* showed similarities with *Rotylenchulus reniformis* (GI 398025668) of USA. In these analyses our sequence formed a clade with *Rotylenchulus reniformis* of USA. These species belongs to the same family Rotylenchulidae. It showed high similarity in these analyses of Neighbor Joining and Maximum parsimony and ITS sequence had formed a clade with different bootstrap replicate values (Figure 3).

Overall, the ITS region becomes an important taxonomic feature in nematode identification. ITS versatility, specificity, effort of experimental manipulation, and growing ITS databases should accelerate its application in nematology. Its usefulness, however, will hinge on a careful evaluation of the relationship between *ITS gene*tic variation and traditional taxonomic positions in the coming days. Moreover, even minor variation that one noticed at *ITS gene* sequence may help in detecting ecotypes or the future evolutionary predictions with reference to that particular nematode species.

Most interesting finding in our studies with reference to plant parasitic nematode and its host plant suggested that *ITS gene* sequence helps in identifying nematode pest status and host plant susceptibility. Thus, these studies will help us to develop the Integrated Pest Management (IPM) package for a specific host plant and its variety. At the same time ITS supports Quarantine technology with reference to agro product export and import.

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