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Molecular Characterization of Native Isolates of *Metarhizium rileyi* (Farlow) Samson (Ascomycetes: Hypocreales) from Major Cropping Ecosystems of Northeastern Karnataka, India

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Authors' contributions

This work was carried out in collaboration among all authors. Author AH designed the research, conducted the study and MM performed statistical analysis. Author ADS contributed in morphological identification of M. rileyi. Author VNG contributed in molecular characterization and phylogenetic tree construction. Authors HSG, B and PG contributed in conducting the research. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In India, commercial biopesticides have been recommended against different insect pests on different crops, but their application has shown only a reduced efficiency attributed to differences in susceptibility of target insect pests or non-adaptability to Indian agro-climatic conditions. Therefore, there is a need to isolate region-specific biopesticide strains to enhance the efficacy of such biopesticides for pest management. The current study aimed to explore the new isolates of the entomopathogenic fungus, *Metarhizium rileyi* (Farlow) Samson species across the major cropping ecosystems of kalyana Karnataka. *M. rileyi* is an important entomopathogenic fungus of more than 30 species of Lepidoptera larvae. The aim of this research was to characterize the isolate of *M. rileyi* from Northeastern Karnataka by using morphological and molecular approaches.

Methodology: The fungus was isolated from the naturally infected cadavers collected from different cropping ecosystems and it was cultured on Sabouraud's Maltose Yeast Extract Agar (SMAY) media for morphological characterization, SMY media used for molecular characterization. The vegetative hyphae of fungi were 2-3 µm in diameter and hyaline, septate. The conidia ellipsoidal, smooth, and pale green were observed under the optical microscope. The DNA was isolated using the CTAB method and an internal transcribed spacer (ITS1, ITS4) was used as the primers for the amplification. The amplified products of 650 bp were purified and sequenced at ClustaL W in both directions using the above primers. A consensus sequence was obtained by alignment of the forward and reverse sequences for this region and deposited in GenBank (UASRBC Mr-24, UASRBC Mr-3, UASRBC Mr-20, UASRBC Mr-29, UASRBC Mr-7, UASRBC Mr-18).

Result: A BLAST search of the sequence in GenBank revealed a 50-100 percent identity with several strains of *M. rileyi* (Eg. JQ686668, MN907775 and KJ725726 *etc*.,).

Conclusion: The phylogenetic relationship was derived from comparisons of the ITS gene sequences and a Dendrogram was constructed. The homology and variations based on amino acid sequences among the isolates showed 71 per cent (OK184897) and 51 per cent similarity with four isolates (OK184899, OK177835, OK178862 and OK184898).

Keywords: Metarhizium rileyi; entomopathogenic fungi; biological control; Lepidoptera.

LIST OF ABBREVIATIONS

AFLP	:	Amplified Length Polymorphism
CD	:	Coefficient of Deviation
CTAB	:	Cetyltrimethylammonium bromide
DMRT	:	Duncan's Multiple Range Test
DNA	:	De-oxyribose Nucliec Acid
EDTA	:	Etheylenediaminetetraacetic acid
FAW	:	Fall armyworm
EPF	:	Entomopathogenic fungi
ITS	:	Internal Transcribed Spacer
FAO	:	Food and Agriculture Organisation
IPM	:	Integrated Pest Management
NrSf-1	:	*Nomuraea rileyi Spodoptera frugiperda*
PCR	:	Polymerase Chain Reaction
RAPD	:	Random Amplified Polymorphic DNA
NBAIR	:	National Bureau of Agricultural Insect Resources
NCBI	:	National Centre for Biotechnology Information
NDTP	:	De-oxynucleotide triphosphate
SE	:	Standard Error of Mean
SMAY	:	Sabouraud Maltose Yeast Extract Agar Medium
UASRBC Mr	:	University of Agricultural Sciences Raichur Biocontrol lab Metarhizium rileyi
UV	:	Ultraviolet rays

1. INTRODUCTION

"Metarhizium rileyi is a cosmopolitan species of entomopathogenic fungi of the family Clavicipitaceae (Hypocreales, Ascomycota) with extensive literature published under its synonym *Nomuraea rileyi"* [1]. "The main susceptible species of insects, which are key pests of crops such as cotton and soybean, belong to the lepidopteran families Noctuidae, Erebidae, and Nymphalidae" [2,3]. *M rileyi* usually presents high genetic variability [4], which has been closely related to the host species from which it is isolated. "Unlike other most common fungal entomopathogens with the greatest known epizootic potentials, such as *Metarhizium anisopliae* and *Beauveria bassiana*, *M. rileyi* has a narrow spectrum of hosts" [5]. Because of its high selectivity and effective control under natural or agricultural conditions, *M. rileyi* is an attractive biocontrol agent with potential for development as a bioinsecticide [2,3] or for prospecting potential biologically active compounds with many possible uses. The genome data of *M. rileyi* strains were obtained, to provide additional insights into fungal diversity and interactions with the host.

As an IPM practice biocontrol agents of Lepidopteran pests include the use of parasitoids, predators, entomopathogenic bacteria, viruses, and fungi for sustainable management of insect pests which is "of late" gaining importance because of environmental safety. Fungal bioagents infecting insects can provide an effective alternative for ecofriendly management of insect pests.

"M. rileyi extensive literature published under its synonym *Nomuraea rileyi* is a dimorphic, ubiquitous fungus with yeast-like hyphal bodies and true mycelial filaments and named initially as *Botrytis rileyi* (Farlow) and later as *Spicaria rileyi* (Farlow) Charles. The fungus was re-described and placed in the genus", *Nomuraea* by Kish et al. [6]. "Based on molecular analysis, using RAPD, internal transcribed spacer (ITS) sequence analysis, amplified length polymorphism (AFLP) and telomeric fingerprinting methods indicated that *N. rileyi* isolates were more closely related to *Metarhizium anisopliae* and *M. flavoviride* than to *N. atypicola* and *N. anemonoide"s* [7]. "Recently, *N. rileyi* has been changed to *M. rileyi* based on its morphological and molecular characterization" by Kepler *et al*. [8].

"In addition to the phenotypic analysis, several laboratories have initiated genotypic studies. For example, randomly amplified polymorphic DNA (RAPD) analysis using a series of 10-mer primers (Operon Corp., Alameda, CA) was shown to be able to discriminate among the *N. rileyi* pathotypes" [9]. "They were able to select 6 different oligonucleotides (arbitrary sequence primers) that were capable of differentiating DNA samples extracted from geographically distinct *N. rileyi* pathotypes. Cluster analysis of this preliminary data set showed that *N. rileyi* isolates were separated into two phenetic groups. Group 1, having 72% similarity, included all isolates from *S. frugiperda*, whereas members in Group 2, clustering with more than 68% similarity, were isolated from *A. gemmatalis*. RAPD analysis, although serving as a fast and simple technique for examining polymorphisms, can yield nonreproducible results. An alternative PCR-based strategy uses amplified fragment length polymorphism (AFLP) analysis that uses a high temperature, a low number of amplification cycles, and precision primers" [10,11]. The objective of this study was to examine the genotypic properties of a series of Northeastern Karnataka *N. rileyi* isolates. The Genomic DNA of *Nomuraea* was subjected to multiple analysis, including the sequencing of the ITS1-5.8s-ITS2 region of the rDNA, to examine the interspecific variation within *M. rileyi*.

2. MATERIALS AND METHODS

2.1 Cadavers collection and isolation of EPF

The roving survey was carried out during the cropping period in North Eastern region of Karnataka such as Raichur, Kalaburagi, Bidar, Yadgiri, Koppal, and the Ballari districts for the natural incidence of *M. rileyi* infected cadavers. To record the number of lepidopteran larvae one meter square area was marked and it was considered as a unit and such five units were randomly observed in each crop. The total number of larvae in each unit was critically observed and the cadavers on plants were collected and brought to the laboratory for further studies.

"The cadavers which were brought to the laboratory were identified based on the following characters. The larvae became hard, mummified, and adhered to the leaves and other plant parts with their prolegs with raised head and anterior parts of the body. White mycelial growth covered

the entire body of the infected caterpillar except for the head capsule. The fungus on infected caterpillars sporulated profusely in the field itself and such cadavers were seen covered with fine light green spores all over the body" [12].

"The cadavers were collected in sterilized vials and kept in a Petri plate on moistened filter paper for the development of fungus. Similarly, the prevailing weather conditions across the survey were recorded" [12].

"The dead cadavers were surface sterilized by immersing in 0.1 percent sodium hypochlorite solution for two minutes followed by rinsing in three changes of sterilized distilled water. The surface sterilized diseased specimens were cut in a sterile watch glass and a small portion of the infected tissue was transferred to a sterile culture plate containing Sabouraud Maltose Yeast Extract Agar Medium (SMAY) method followed by Bell [13]. The plates were incubated at room temperature 25 ± 2 °C with 80 to 85 percent relative humidity for 9-14 days and the colonies were further purified by repeated subculture on SMAY medium and pure cultures were maintained in slants" [12]. The various observations on morphological and growth characteristics of individual isolates of *M. rileyi* were observed after 14 days of incubation at 25 ± 2 °C.

2.2 Molecular Characterization of EPF Isolate

2.2.1 DNA extraction

100 milligrams of each isolate were ground in one ml of pre-warmed CTAB (5M NaCl, 0.5 M EDTA and 1M Tris Base) extraction buffer and added 200 µl of β- mercaptaethanol and incubated at 65 ºC in water bath for 60 to 90 minutes with occasional stirring, 500 µl of chloroform and isoamylalcohol (24:1 ratio) mixture is added to the sample then centrifuged at 10,000 revolutions per minute (rpm) at 20 ºC for five minutes, for aqueous layer 5 µl of RNAase (10 mg/ml) was added and incubated for 30 minutes at room temperature. Ice-cold isopropanol is added to each tube equal to the volume of the sample. DNA pellets were dissolved in 50 microlitre of elution buffer (10mM Tris - HCl + 0.1mM EDTA at pH 8.0) [14]. The quality and quantity of DNA were analyzed by running 5 µl of each sample in 0.8 per cent agarose gel. An alternative method is by nanodrop spectrophotometer at 260 nm (ND-1000, USA). This method was based on measuring the amount of ultraviolet (UV) irradiation that is absorbed by the bases.

2.2.2 PCR amplification

DNA Amplification and Sequencing [15] done by using 10X Assay Buffer (5 µl), $MgCl_2$ (3 µl), dNTP mix (2 µl), forward primer, reverse primer, Taq DNA polymerase and DNA sample. After the preparation of the reaction mixture, it was used for amplification through PCR. The amplification of the reaction mixture takes place at different stages [15]. PCR program involved 35 cycles of 94 ^{0}C for one min., 52^0 C for one min., 72 °C for one min. and the amplified products were sent for genome sequencing. The ITS1 - 5.8S - ITS4 amplified products (about 650 bp) were purified with the PCR and Gel Band Purification kit and sequenced in an automated system.

2.2.3 Phylogenetic tree construction

The sequences were aligned using the ClustalW (http://www.ebi.ac.uk/clustalw) program and compared those with available data in the GenBank database for *M. rileyi* [15].

3. RESULTS AND DISCUSSION

3.1 Abundance of *M. rileyi* in North Eastern Karnataka

The abundance of *M. rileyi* from different districts indicated that the highest percent was in Ballari (29.87 %) with five isolates followed by Koppal (21.02 %) which was on par with Raichur (20.32 %) with three and 20 isolates, respectively. Lowest abundance was recorded from Bidar (8.47 %) (Table 1). There was no isolate found from Yadgiri and Kalaburagi region which all depends on the stage of the pests and prevailing weather conditions.

The pearson's correlation studies for the natural incidence of *M. rileyi* in North Eastern region Karnataka found a significant and positive correlation between relative humidity and rainfall (Table 2).

The correlation studies on natural incidence of *M. rileyi* on various lepidopteran pests in different districts with weather parameters indicated that relative humidity had a positive and significant correlation in Koppal (0.39), Raichur (0.23) and Ballari (0.20) with the incidence of *M. rileyi* and negative correlation with maximum and minimum

temperature. From the survey data the investigation was supported by Ochoa et al. (2003), Firake and Behere [16] and Gebremariam et al. (2021) they suggested that the difference between the infection could be attributed to variability in cropping patterns, types of natural vegetation, weather parameters, undisturbed habitats, shading area protects the fungi from UV-radiation, free from chemical pesticide application. The low incidence of *M. rileyi* in Bidar, Yadgir and Kalaburagi may be due to the fact that the frequency of the survey and the time of the survey may not have coincided with the incidence.

The present study revealed that highest cadavers of *S. frugieprda* were noticed in maize growing districts *viz*., Raichur, Ballari and Koppal while in Bidar, Kalaburagi and Yadgir districts there was no natural incidence of *M. rileyi* which indicated that in traditional maize growing areas, the incidence of *M. rileyi* was high and the present findings are in line with Mallapur et al. [17] who also observed "in traditional maize-growing areas (Dharwad) had highest incidence (18.30 %) while in non traditional areas like Vijaypur had low incidence of *M. rileyi"*. The present findings are also corroborates with Sharanabasappa et al. [18] who opined that

Table 1. Prevalence of *M. rileyi* from different districts

Sl. No.	Districts	No. of isolates	Per cent of abundance
1	Raichur	20.00 (4.53)	20.32 (26.79)
2	Yadgiri	0.00 (0.71)	0.00 (0.52)
3	Ballari	5.00 (2.35)	29.87 (33.13)
4	Bidar	1.00 (1.22)	8.47 (16.92)
5	Kalburgi	0.00 (0.71)	0.00 (0.52)
6	Koppal	3.00 (1.87)	21.02 (27.29)
SEm±		0.38	0.25
CD @ 5%		1.14	0.75

Figures in the parenthesis indicate $\sqrt{x+0.5}$ *transformed values*

Table 2. Correlation of weather parameters with natural incidence of *M. rileyi* in North Eastern region of Karnataka

Districts	Natural incidence of mycosis (%)	Maximum Temperature	Minimum Temperature	Rainfall	Relative humidity
Raichur	0.61**	-0.09	0.10	-0.01	0.23*
Ballari	1.00**	-0.18	0.12	0.27	0.20*
Koppal	0.67**	-0.13	-0.32	0.37*	0.39*

***. Correlation is significant at the 0.05 level (2-tailed)*
**. Correlation is significant at the 0.05 level (2-tailed)*

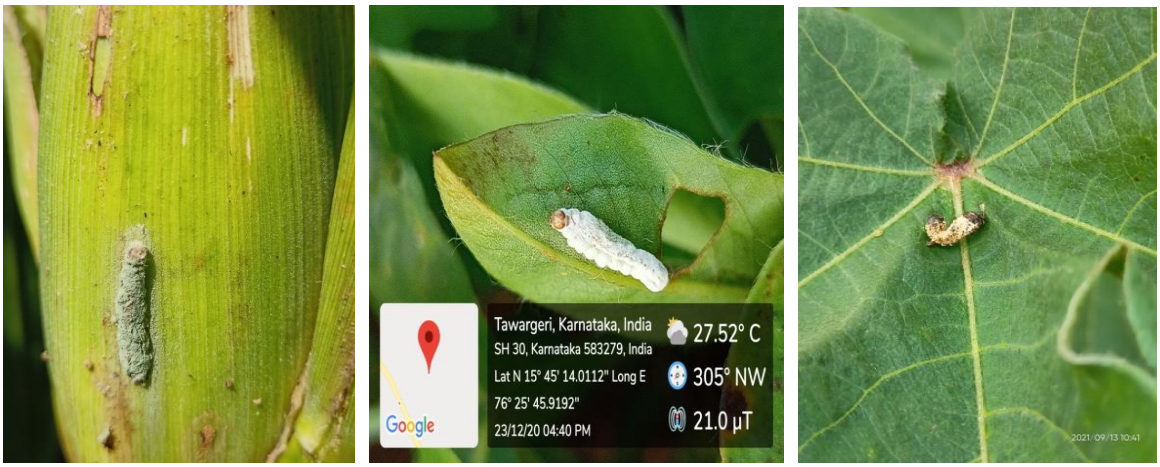

Plate 1. Mycosed cadavers of *S. frugiperda* collected in different crop ecosystems

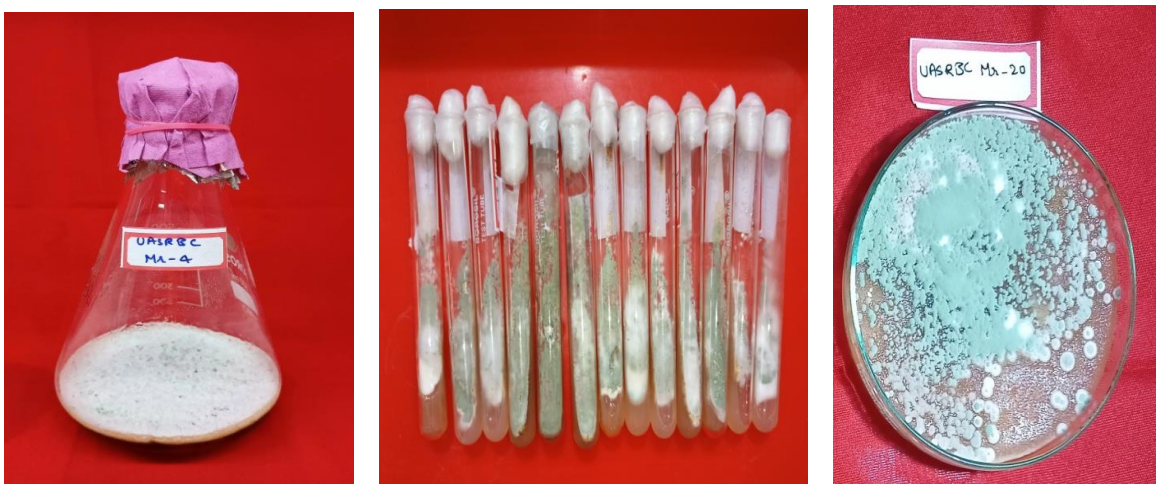

Plate 2. A) Mycelial mat for DNA isolation. B) Sub cultures of native isolates of *M. rileyi* collected from North Eastern Karnataka C) Pure culture of *M. rileyi*

"natural incidence of *M. rileyi* mainly depends on the availability of the host for the perpetuation of the disease". Similarly, Shylesh et al. [19] stated that "*M. rileyi* was predominant in maize growing areas" and also Visalakshi et al. [20] opined that "natural incidence of *M. rileyi* also depends on the availability of *S. frugiperda* varied with the cropping pattern in Andhra Pradesh".

3.2 Morphological Characterization

After the collection of cadavers from the survey was carried to the laboratory and some cadavers were kept for profuse sporulation and further purified by repeated subculture on SMAY medium. Initially, the colour of the colony was pale green to pale turtle green, then changing into olive to malachite green. Vegetative hyphae, 2-3 µm in mean diameter which is smooth, hyaline, and septate. Branches near a septum develop in whorls each giving rise to 2-4 phialides. The branches were usually cylindrical occasionally with a swollen base. The conidia in dry divergent chains are ellipsoidal, smooth, and pale green [21,22].

Plate 3. Sporulation of *M. rileyi* on cadavers

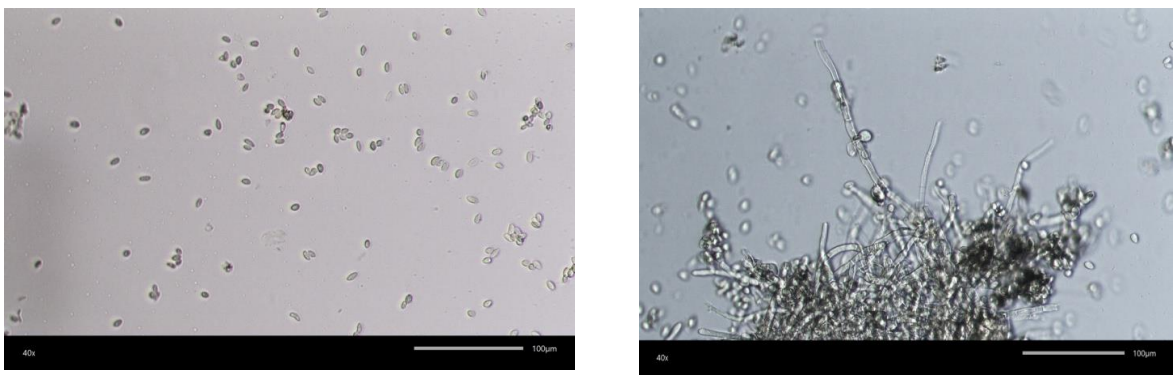

Plate 4. Conidial structures of *M. rileyi* under phase contrast microscope

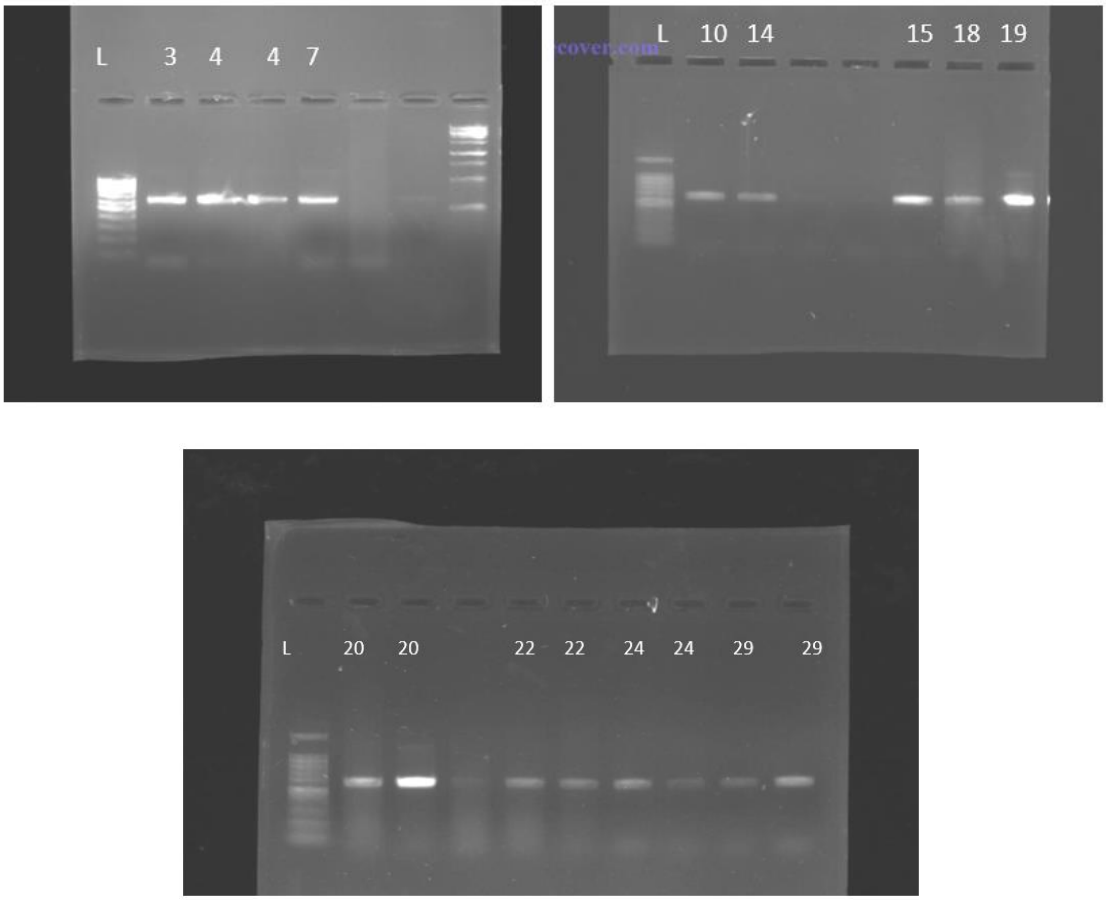

Plate 5. PCR amplified gel image for identification of base pairs of genomic DNA of M. rileyi isolates with ladder DNA (100 base pairs) (L : Ladder DNA) (The numbers represent the isolate number)

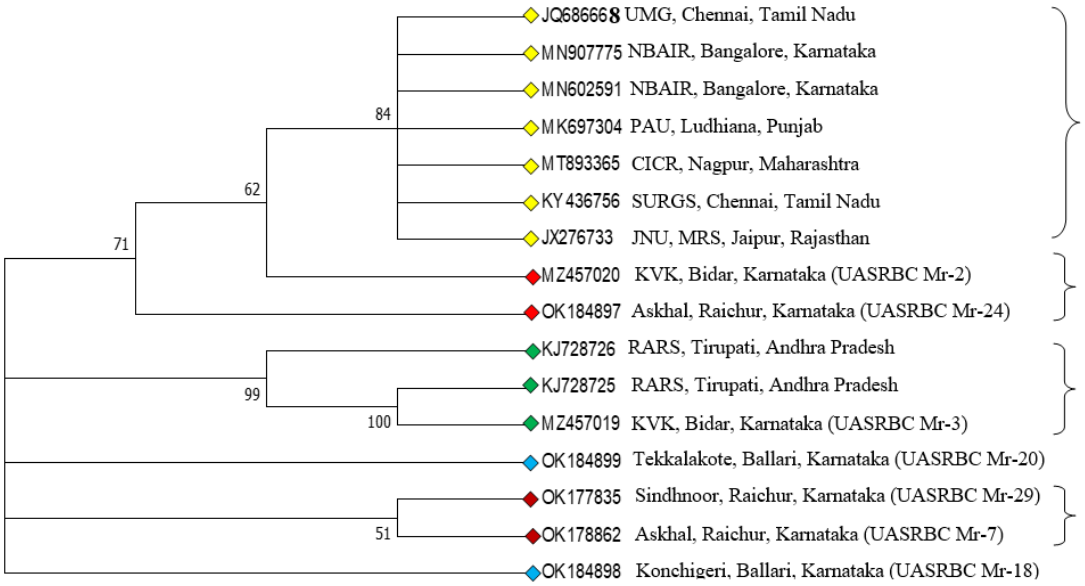

Fig. 1. Phylogenetic tree based on the nucleotide sequences of ITS region of isolated fungi with relevant sequences of *M. rileyi* from NCBI database

Table 3. Details of accession number for *M. rileyi* isolates isolated from lepidopteran cadavers collected from North Eastern Karnataka region

Sl. No.	Isolate number	Accession number	Crop	Host	Latitude and longitude	Location	Date of collection
1	UASRBC Mr -3	-	Maize	*S. frugiperda*	16.19639 77.32708	Askhal, Raichur	2-10-2020
2	UASRBC Mr -4	-	Groundnut	*S. litura*	16.19893 77.32715	UAS Raichur	19-12-2020
3	UASRBC Mr -7	OK178862	Redgram	*S. litura*	16.19936 77.32708	Askhal, Raichur	9-10-2020
4	UASRBC Mr -10	-	Groundnut	*A.modicella*	16.19893 77.32715	UAS Raichur	20-10-2020
5	UASRBC Mr 14	OK184900	Redgram	*S. litura*	16.198889, 77.216222	Kalmal, Raichur	28-10-2020
6	UASRBC Mr -15	-	Maize	*S. frugiperda*	15.28551 76.14853	Gondbal, Koppal	13-10-2020
7	UASRBC Mr -18	OK184898	Maize	*S. frugiperda*	15.419964 76.875882	Konchigeri, Raichur	17-10-2020
9	UASRBC Mr -20	OK184899	Maize	*S. frugiperda*	15.5248, 76.8793	Tekkalakote, Raichur	4-12-2020
11	UASRBC Mr -24	OK184897	Groundnut	*S. litura*	15.3485, 76.17812	Askhal, Raichur	9-10-2020
12	UASRBC Mr- 29	OK177835	Groundnut	*A.modicella*	15.764867, 76.538698	Kardchalami, Raichur	22-02-2021

3.3 Molecular Characterization of Native Isolates of *Metarhizium rileyi*

The fungus was identified as *M. rileyi* based on morphology and was further confirmed by amplification of the ITS region using PCR. PCR specific ITS regions of the EPF as *M. rileyi* were further confirmed by the NCBI-BLAST analysis of partial genome sequence amplified by ITS1 and ITS4 (Plate 5). The ITS generated sequences were deposited in the NCBI Gene Bank database and an accession number was obtained (Table 3) [23,24]. Using Neighbor joining method in MEGA software (Ver. 10.0), a phylogenetic tree was constructed by considering most reference sequences from the NCBI along with sequences of *M. rileyi* to study the evolutionary relationship between different isolates. The phylogeny is depicted in Fig. 1.

3.4 Genetic Similarity among *M. rileyi* Isolates

The phylogenetic tree analysis showed that the isolates were grouped into five major clusters. The isolates belonging to Tekkalakote Ballari, Konchigeri Ballari, formed a separate cluster and the rest of the species formed a cluster including our isolates. Two isolates of RARS, Tirupati, Andhra Pradesh; KVK, Bidar, Karnataka (UASRBC Mr-3) one subgroup with bootstrap value 99-100 percent indicates they are strongly supported. The isolates of UMG, Chennai, Tamil Nadu; NBAIR, Bangalore, Karnataka; PAU, Ludhiana, Punjab; Maharashtra, Chennai, Tamil Nadu; JNU, MRS, Jaipur, Rajasthan; KVK, Bidar, Karnataka(UASRBC Mr-2); and Askihal, Raichur, Karnataka (UASRBC Mr-24); formed one subgroup with bootstrap value 71 - 84 percent indicates they are well supported [25]. The Sindhnoor in Raichur Karnataka (UASRBC Mr-29), Askihal on pigeonpea in Raichur, Karnataka (UASRBC Mr-7) into a separate subgroup and Tekkalakote, Ballari, Karnataka (UASRBC Mr-20) and Konchigeri Ballari on maize in Karnataka (UASRBC Mr-18) formed two separate subgroups with a bootstrap value of 50-60 per cent indicates they are weakly supported [26,27,28].

From molecular analysis data, Lee et al. [29] used the ITS region along with β-tubulin and EF-1α for molecular identification of *M. rileyi*. Among the three regions, the ITS region has recorded the best resolution for the molecular identification of the *M. rileyi* in comparison to β-tubulin and EF-1α. Further, to study the evolutionary pattern of *M. rileyi*, a phylogenetic tree was constructed using the most relevant sequences of NCBI of closely related genera using neighbor-joining method. In their study, they found that all the *Nomuraea* grouped together. These results were highly concurrent and matched with the present study. Similarly, Visalakshi [20] reported that the fungus was identified as *M. rileyi* based on ITS sequence homology and the strain was designated as AKP-Nr-1.

In contrast to the ITS region, analysis of the partial beta-tubulin gene based on neighbor-joining and 1000 bootstrap, *N. atypicola* failed to form a monophyletic grouping with the other two species of *Nomuraea* [30].

This probably suggests that one isolate (UASRBC Mr-24) of *M. rileyi* from this study might have evolved from the same ecological niche as that of KVK, Bidar Karnataka (UASRBC Mr-2), and the remaining four isolates *viz*., UASRBC Mr-20, UASRBC Mr-29, UASRBC Mr-7 and UASRBC Mr-18) acts as a base for the future isolates of *M. rileyi*.

This strongly suggests that ITS was the effective region for species identification and grouping as well as to studying the evolutionary pattern of the *Nomuraea* spp.

4. CONCLUSION

Molecular characterization done by PCR based DNA sequencing analysis using ITS1 and ITS4 primers. The amplified DNA sequences of the ITS region (Internal Transcribed Spacer 1, 2, and 5.8S subunit) of the 6 isolates *viz*., UASRBC Mr-7, UASRBC Mr-14, UASRBC Mr-18, UASRBC Mr-20, UASRBC Mr-24 and UASRBC Mr-29 were submitted to NCBI Genebank under the Accession numbers OK178862, OK184900, OK184898, OK184899, OK184897 and OK177835 respectively. The phylogenetic relationship was derived from comparisons of the ITS gene sequences and a Dendrogram was constructed. The homology and variations based on amino acid sequences among the isolates showed 71 percent (OK184897) and 51 percent similarity with four isolates (OK184899, OK177835, OK178862 and OK184898). The molecular characters helpful to identify the variation in the different strains at its genome level.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models

(ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ETHICS APPROVAL

All experimental works were approved by University of Agricultural Sciences, Raichur.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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