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Molecular characterization of *Beauveria* isolates from Punjab based on ITS Region

GEETIKA BANTA[1] and VIKAS JINDAL[2]

Punjab Agricultural University, Ludhiana, Punjab 141 004, India

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ABSTRACT

Three local isolates of *Beauveria* (BbR1, BbR2 and BbR3), isolated from Punjab soils along with one standard strain MTCC 2028 (BbM1) were selected for molecular identification. All the isolates showed maximum growth on potato dextrose agar (PDA) for an incubation period of seven days at 28±2°C. Morphologically the shape of colonies was round, lightly raised with white powdery surface and smooth walled hyphae. The mycelia showed white powdery translucent radial growth. Molecular characterization was done based on internal transcribed spacer (ITS) region, a fungal barcoding region for species level identification. The ITS region amplified by PCR with specific primers resulted in 603bp amplicon in all examined *Beauveria* isolates. The BLAST analysis showed that amplified gene has 99-100% homology with *B.bassiana* thus confirming all isolates as *B.bassiana*. The phylogenetic tree exhibited no sequence diversity among Punjab isolates. However, Punjab isolates showed genetic variation of 0.63–1.47% from other Indian isolates based on ITS region. The phylogenetic tree developed, including isolates from other countries, resulted in two main groups.The first group includes all the three local isolates of Punjab, one isolate from Chandigarh, standard BbM1 and 13 from other countries. The second group wasformed by 10 isolates from different states of India and 6 from other countries. The analyses indicated inter and intraspecific variations among *B. bassiana* isolates associated with diverse geographical origins.

Key words: *Beauveria,* Entomopathogenic fungus, ITS region, Molecular characterization, Phylogenetic analysis.

Entomopathogenic fungi, *Beauveria* (Balsamo-Crivelli) Vuill. are ubiquitous in nature and has the largest host range among the fungi imperfecti making itthe principal components of bioinsecticides (Xu *et al.* 2007). *Beauveria* are cosmopolitan, soil-borne insect pathogens, and are easily distinguishable at genus level, however, species identification often remains complicated because of the lack of distinctive morphological characters and taxonomic keys which are limited to cultural features, conidial shapes and sizes (Wang *et al.* 2005*,* Robene-Soustrade *et al.* 2015). Xiao *et al.*(2012) reported that genomic analysis of *B. bassiana*, as one of the best-studied and most widely used insect biocontrol agents. Imoulan *et al.* (2016) reported 10 species of *Beauveria* representing divergence among species based on fungal barcode targeting ITS region. In India, a few studies have been reported on molecular taxonomy of genus and phylogenetic relationships of Indian isolates. Agrawal *et al.* (2014) reported ITS based phylogenetic relationships of *B. bassiana* and *Beauveria*-like isolates of Indian origin and Bala Naik *et al.* (2015) identified five local *B. bassiana* isolates from Rayalaseema region of Andhra Pradesh.

The International Sub-commission on Fungal Barcoding proposed the ITS region as the prime fungal barcode region for species identification (http://www.allfungi.com/its-barcode.php). In addition, the recognition of ITS as the official DNA barcode marker for fungal identification has been established (Schoch *et al.* 2012,Raja *et al.* 2017). The entire ITS region of nuclear DNA (nrDNA) can be easily amplified using primers among all lineages of fungi and can be used to elucidate the phylogenetic relationships of fungal taxon (Rehner *et al.* 2011,Koljalg *et al.* 2013). There is no report on molecular identification of *Beauveria* species from Punjab, based on internal transcribed spacer (ITS) region. So, present study was planned to characterize *Beauveria* isolates and evaluate their sequence variability with available database.

MATERIALS AND METHODS

Fungal Isolates: Three isolates of *Beauveria* (BbR1, BbR2 and BbR3), isolated from Punjab soils along with one standard culture of *B. bassiana* (BbM1) (MTCC 2028) procured from Microbial Type Culture Collection, Chandigarh (IMTECH) were used for present studies. The

Present address: [1]Principal Investigator (geetikabanta1@gmail.com), DBT-BioCARe; [2]Assistant Entomologist (vikas_ento@pau.edu), Department of Entomology, Punjab Agricultural University, Ludhiana-141 004, Punjab, India.

study was conducted at Punjab Agricultural University, Ludhiana (2016-17). For growth of the fungus in liquid culture, 50 ml of potato dextrose broth was inoculated with culture inoculum (preserved on slants) and incubated at 28±2°C in a BOD incubator (Dhar *et al.* 2016). Abit of mycelial growth with sporulating mass was surface plated on potato dextrose agar (PDA) and was incubated at 28±2ºC. The conidial suspension was prepared by scrapping the surface conidia from plates wetted with 0.3% of Tween 20 using sterile loop. The conidial suspension was then filtered using muslin cloth to remove mycelial bits. The spore counts were taken using a haemocytometer and 10^7conidia/ml was prepared for each isolate for virulence assay.

Assessing virulence of Beauveria culture: The virulence potential of local isolates was evaluated by exposing the second instar larvae of the wax moth, *Galleria mellonella*, to the conidial suspensions of isolates of *Beauveria* (BbR1, BbR2 and BbR3) (in Petriplates). The *Galleria mellonella* larvae were collected from spent combs of honey bee (*Apis mellifera*) infected with wax moth. Fifty individual larvae (20-25 mg weight, 1 cm length) were given heat treatment to prevent webbing. Five larvae were exposed to fungal infection by dipping the larvae in conidial suspension (10^8 conidiophores/ml) for 30-40 sec. The treated larvae were kept in petriplates containing moist filter paper (Whatman No.1) with little ventilation holes and incubated at 28±2ºC in dark and observations were taken. Small bits of fungal growth emerging out of dead larvae were again inoculated onto PDA and were used for present study.

Morphological characterization: For morphological characterization freshly grown bits of mycelia with sporulating mass were suspended in 3.0 ml of sterile water by vortexing and 50µl of the same was plated on PDA media plates. The plates were incubated at 28±2ºC to allow growth of fungal colonies from single spores. The shape and pattern of colonies was observed under microscope.

Molecular characterization: The mycelial biomass was harvested and dried on filter paper. Total genomic DNA was extracted from the mycelial biomass (100mg) grounded to powdered in liquid nitrogen for each isolate using CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle 1987). The isolated DNA was run on (1.0%) agarose gel. The concentration of DNA was estimated using spectrophotometer at 260 nm (Eppendorf Bio Spectrometer).

Cloning of ITS Gene: To establish the molecular identity of isolates, internal transcribed spacer (ITS) region for each *Beauveria* isolate (BbR1, BbR2, BbR3, BbM1 (MTCC 2028) was amplified using primer set, bbITS-F/ SR6RandbbITS-R/LR1 (Table 1). The PCR reaction mixtures consisted of 4µl of genomic DNA in 50µl reaction volume containing 5µL 10x PCR buffer, 10µL of 1mMdNTP, 2µL each of forward and reverse primes (10µM), 2µL of 3U Taq DNA Polymerase. The PCR reaction was performed with initial denaturation for 4 min at 94ºC, followed by amplification for 35 cycles with following conditions: 1 min at 94ºC, 1 min at 55ºC, 1 min at 72ºC and a final 10 min extension at 72ºC. PCR product was detected on 1% agarose gel. The amplified PCR product was purified using 'QIAquick Gel Extraction Kit' (Qiagen) and cloned in pGEM-T easy vector using Promega kit. The recombinant clones were reconfirmed through restriction with EcoR1 and again PCR amplified with specific primers and universal M13 primer. Two clones for each isolate were submitted to Xcelris, Ahmedabad for both directional nucleotide sequencing.

Table 1 Primer sequences for amplification of ITS region in *Beauveria*

Name of primer	Nucleotide Sequence 5'⟶3'	Source	Reference
bb-ITS/ SR6R	AAGTATAAGTCGTA-ACAAGG	Small subunit RNA (SR)	Lane *et al.* 1985, Vilgalys and Hester 1990
bb-ITS/LR1	GGTTG-GTTTCTTTCCT	Large subunit RNA (LR)	

Analysis of nucleotide sequence: Nucleotide sequences of the clones (plasmids) were compared with known sequences of isolates already present in GenBank. The sequences obtained were initially edited using DNA software CLC Sequence Viewer 6.5.4 (CLC bio A/S) andsubmitted to National Centre for Biotechnology Information (NCBI). Further the sequences were subjected to BLAST analysis to identify our sequences and compare the sequence data with known sequences already present in the NCBI database. Homology searches were performed using the GenBank server of the (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the BLASTn algorithm. The ITS sequences of *Beauveria bassiana* available at NCBI GenBank database from different countries were downloaded and the phylogenetic tree was developed using maximum likelihood method based on the Tamura-3 parameter model using MEGA6 programme.

RESULTS AND DISCUSSION

The BbR1, BbR2 and BbR3 isolates showed 86.67, 93.33 and 86.67% mortality of *G. mellonella* larvae after seven days of inoculation, respectively. These results confirmed the pathogenecity of the isolates which was reported by Dhar *et al.* (2019). The profusely growing white cottony fungal mass wholly entrapping the dead larval mass was observed. *B. bassiana* isolates (BbR1, BbR2 and BbR3) when grown on potato dextrose agar (PDA) media showed characteristic like colonies were white in colour, with round shape, lightly raised powdery surface and the mycelia showed white powdery translucent radial growth. The conidiospores appear as densely clustered in whorls, smooth having globosely flask-like base. The colony of *B. bassiana* was swollen bowl-shaped lateral cells with smooth walled hyphae. It has been reported by Imoulan *et al.*(2017) that morphological features like shape and size of conidia get altered after culturing resulted in extensive

overlap in conidial features as well as misidentification of species. Even though Glare & Inwood (1998) could distinguish *Beauveria* species using spore shape and size.The invention of molecular techniques provided strong evidence that the *Beauveria* species consist of a number of cryptic species that are independent lineages (Rehner *et al.* 2011, Uztan *et al.* 2016). Barcoding the ITS region in fungi has been extensively used in ecological and phylogenetic studies because it gives a high level of interspecific divergence. PCR amplification of ITS region in BbR1, BbR2, and BbR3 gave the desired amplicon of 603bp as observed on agarose gel electrophoresis and further obtained from sequencing. Same amplification was observed in all three isolates which is concordant to 603bp of standard culture of *B. bassiana* (BbM1) (MTCC 2028).

The results of BLAST analysis showed 99–100% homology to ITS region of *B. bassiana* already available in NCBI database, which confirmed that our isolates were *B.bassiana.* All the sequences were submitted to Barcode of Life database and GenBank, and assigned accession numbers MG670098, MG670100, MG670102 for BbR1, BbR2, and BbR3, respectively. The nucleotide sequences alignment revealed that all the isolates from present study displayed sequence similarity with each other as most of the isolates were collected from the same geographical area.

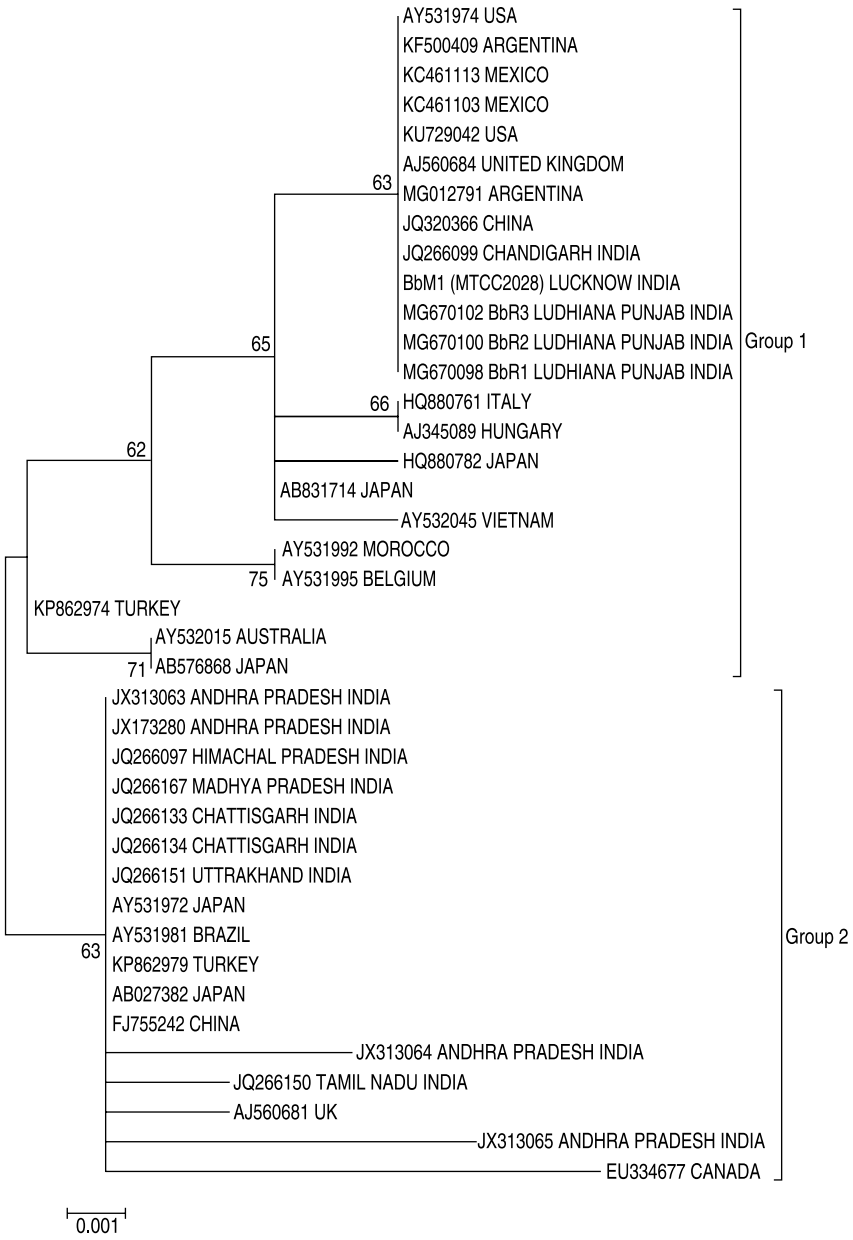

Fig 1 Phylogenetic relationship among the three local isolates of *Beauveriabassiana* (BbR1, BbR2, and BbR3) inferred from maximum likelihood method for diversity analysis. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 476 positions in the final dataset.

In order to derive genetic relatedness amongst all the different isolates under study, multiple alignment data was used to derive phylogenetic tree for isolates from India and other countries (Fig 1). It was found that the Indian isolates form two major groups indicating high sequence diversity among Indian isolates. First group is formed by Punjab local isolates BbR1, BbR2, BbR3, standard BbM1, one from Chandigarh and isolates from Japan, Mexico, USA, Argentina, China, UK, Italy, Hungary, Vietnam, Morocco, Belgium, Turkey, Australia. The second main group formed by isolates from Himachal Pradesh, Madhya Pradesh, Chhattisgarh,Uttarakhand from India and isolates from Brazil, Turkey, Japan, China, UK and Canada. The genetic distance between the isolates from Punjab and from other state of India ranged from 0.63–1.47% with maximum (1.47 and 1.26%) from Andhra Pradesh isolate followed by Tamil Nadu, Himachal Pradesh, Madhya Pradesh, Chhattisgarh and Uttarakhand (Fig 1). This genetic variation can be justified by the fact that *Beauveria* spp. is a haploid fungus with a predominant asexual reproduction, so most of its genetic variation is due to mutation as described by Castrillo *et al.* (1999). A high genetic similarity among the isolates from different climatic zones and hosts was observed by Devi *et al.* (2001) and Gaitan *et al.* (2002). We identified local isolates from Punjab as *Beauveria bassiana* using barcoding. The genetic variation among the isolates from different geographical regions showed that more local isolates should be isolated

and evaluated for their efficacy against insect pests to find out some potential isolates of *B. bassiana*.

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