



Morphological and Molecular Characterization of Entomopathogenic Fungi, *Beauveria brongniartii* Associated with White Grub Populations of North Western Himalaya

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ABSTRACT

Background: In the Northwestern Himalayas, white grubs are a serious and persistent pest of several crops. Because they are more effective than chemical pesticides, microbial control techniques are crucial to integrated pest management. The entomopathogenic fungi used in the present investigation were obtained from white grub species prevalent in the northwestern Himalayan region of India.

Methods: The investigations during 2011- 2013 were carried out in CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur. Based on morphology and molecular traits, fungi were identified and characterized. *Beauveria brongniartii* (Sacc.) was determined to be the principal entomopathogenic fungus using inter-simple sequence repeats (ISSR) markers. Moreover, the sequences derived from these strains were confirmed by phylogenetic analysis to be within the same clade as the reference sequences of *B. brongniartii*.

Result: Five native strains were isolated with 3 identified as entomopathogenic fungus *Beauveria brongniartii*. These findings improve the understanding of the mycobiota of crops in the northwestern Himalayan region, which acts as a basis for the creation of technological products in the field of biological control.

Key words: *Beauveria*, Entomopathogenic fungi, Molecular analysis, White grubs.

INTRODUCTION

Among the most damaging soil pests are the larvae of the scarabaeid beetles, the most prevalent leaf chafers commonly known as white grubs (Gardner, 1935). Because of their lamellate antennae, scarabaeid beetles cause significant damage to agricultural crops. According to Misra and Chandel, (2003), white grubs feed on plant roots and decompose organic waste. Approximately 20 different species of white grubs attack crops like potatoes in India (Chandel *et al.*, 2015). As reported by Mehta *et al.*, (2010), these white grubs have characteristic C-shaped bodies, brown heads and a well-developed thoracic region.

The most prevalent way of managing white grubs involves chemical insecticides. However, their indiscriminate use poses substantial risks to both the environment and human health due to toxic residues (Bajja *et al.*, 2015). Consequently, alternative, environmentally friendly strategies like integrated pest management (IPM) using biopesticides are essential (Kawalekar, 2013).

Currently, a significant field in sustainable agriculture involves the use of biological control agents that have the innate ability to parasitize and eradicate plant pests (Dannon *et al.*, 2020). Among these organisms are entomopathogenic fungi that act as promising biocontrol controls because they naturally infect and disrupt pests. According to Coates *et al.* (2002) and McGuire *et al.* (2005), entomopathogenic fungi can infect pests at various life stages, have a wide host range and produce spectacular epizootics, making them potential for insect pest population

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control (Zimmermann, 1993). Additionally, they possess characteristics and modes of action that classify them as biopesticides (Mantzoukas *et al.*, 2022). Li *et al.* (2024) emphasized the safety and efficacy of biological control methods that rely on parasitoids, entomopathogenic fungus

(EPF) and natural predators as alternatives to chemical pesticides.

Since fungi are more prevalent than bacterial or viral infections, they are crucial for efficiently managing Coleoptera pests (Pradhan and Sahoo, 2024). One of the most studied fungi; *Beauveria* infects a wide range of insect species across the globe (Hajek and St. Leger, 1994). Several species of *Beauveria* are critically important both economically and ecologically, therefore serving as an important fungal resource (Wang *et al.*, 2022). They are often isolated from dead and mycosed insects (Devi *et al.*, 2001), are employed as an entomopathogenic agent in biological control programs (Dolci *et al.*, 2006) and are identified morphologically through microscopic and macroscopic structures (Piontelli, 2011). Characteristics like conidia size and shape are commonly used to identify *Beauveria* species (Humber, 2012). Nevertheless, phenotypic plasticity and genetic variability can complicate species-level identification (Paz *et al.*, 2011). Phenotypic characteristics, however, are insufficient to monitor the release of biocontrol agents into the field or to differentiate between several entomopathogenic isolates (Castrillo *et al.*, 2003).

Advancements in polymerase chain reaction (PCR)-based technologies have often significantly progressed the identification and characterization of entomopathogenic fungi. This is especially relevant for widely distributed taxa *Beauveria bassiana* and *Metarhizium anisopliae*. The study of the evolutionary relationships among entomopathogenic fungi is rendered feasible by the ribosomal DNA genes (rDNA) and the internal transcribed spacers (ITS) (Estrada *et al.*, 2007; Sevim *et al.*, 2010).

According to Kaur and Padmaja, (2008), molecular markers enable researchers to screen several isolates of a specific entomopathogen and determine the molecular phylogenetic relationships to virulent phenotypes. Chandel *et al.* (2015) highlighted the extraction of insect-pathogenic fungi from the North Western Himalayan region to identify native fungal biocontrol agents. They employed a combination of traditional and molecular approaches to identify fungal variability collected from various sites. Morphological and molecular identification of fungal strains is a critical first step in the selection of biological control agents (Boucias *et al.*, 2000). The present study focused

on isolating and identifying entomopathogenic fungal strains from various crops of agricultural relevance in the northwestern Himalayan region using both morphological and molecular approaches.

MATERIALS AND METHODS

The present investigations were carried out in the Department of Entomology, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur. The white grubs are active in the soil from June-November; therefore, most of the experiments were carried out between July-October 2011-2013. The work on molecular characterization of entomopathogenic fungi was carried out in Molecular Plant Pathology Laboratory, Department of Plant Pathology, CSK HPKV, Palampur.

Collection of white grubs from field

Soil was carefully scooped using a shovel to collect both healthy and diseased grubs that were present. In order to find the white grubs, a cubic foot of soil was scraped for each sample. Using a 1ft × 1ft quadrant that did not overlap two sample pits, sampling sites were selected at random. Shillaroo, Kharapathar, Keradhar, Barot and Bajaura were the locations from which the white grubs exhibiting entomopathogenic fungal infection were collected. Information was documented regarding the total population of grubs gathered and the population of mycosed grubs. Table 1 provides an overview of all the isolates utilized in this investigation along with information about where they came from. In order to isolate the fungus in laboratory conditions, the infected grubs that had white mycelial growth on their bodies were collected separately in the respective vials. The more healthy grubs were engulfed in plastic containers in unions of about 40-50 with soil taken from the same collection site and brought to the laboratory. The selected infected grubs were also brought to the laboratory in their individual plastic vials.

Fungal isolation

Fungi are isolated directly from infected grubs, exhibiting white mycelial growth on their bodies. The contaminated grubs were aseptically surface sterilized for two minutes in a 5% sodium hypochlorite solution, followed by rinsing

Table 1: Natural infection of *Beauveria brongniartii* in white grubs across various locations in Himachal Pradesh.

Location	Altitudem (amsl)	Species collected	Crop	No. of grubs collected	Mycosed grubs observed in Mycosis (%)			
					Field	Lab	Field	Lab
Bajaura, Kullu	1111	<i>B. coriacea</i> and <i>Holotrichia spp.</i>	Cabbage	42	1	0	2.38	0
Barot, Kangra	1847	<i>Melolontha spp.</i>	Potato, Cabbage	23	2	0	8.70	0
Kharapathar, Shimla	2728	<i>B. coriacea</i> and <i>Holotrichia sppd.</i>	Potato	21	3	6	14.29	28.57
Kheradhar, Sirmaur	2032	<i>B. coriacea</i>	Potato	171	20	25	11.70	14.62
Kheradhar, Sirmaur	2479	<i>B. coriacea</i>	Potato	255	16	42	6.28	16.47

in sterile distilled water. The sterilized grubs were dissected under sterile conditions using a sterilized blade and the fungal specimen was transferred aseptically on the slants of PDA and then incubated at approximately $26\pm1^{\circ}\text{C}$ for further growth. The streak plate method was employed to subculture actively growing fungal colonies until pure cultures were obtained.

Maintenance of cultures

Entomopathogenic fungal spores from infected cadavers were then cultured onto the potato dextrose agar that is supplemented with 0.2% extract of yeast (PDAY) and 40 mg/ml of tetracycline to inhibit bacterial growth. Pure cultures on the PDAY medium that do not contain antibiotics were maintained at about $23\pm2^{\circ}\text{C}$ in dark conditions with 75% relative humidity.

Morphological Identification

Based on macroscopic and microscopic characteristics, the fungal strains were identified morphologically. Macroscopic, externally visible characteristics such as colony color (upper and lower surface of the plates), shape, elevation, surface and edge were visible first to morphologically identify the fungal strains (Kirk *et al.*, 2008). Microscopic observations were based on the arrangement along with the size of conidiophores, conidia and hyphae to identify the entomopathogenic genera of interest of the fungus based on their morphology (Humber, 2012). The mean along with the standard values of deviation, 20-30 measured structures were imputed and indicated to determine the microscopic measurements of the reproductively important structures.

For preliminary identification of the fungal isolates, factors such as the presence of infected larvae, morphology and spore sizes and shape, along with colony size, were recorded at $23\pm2^{\circ}\text{C}$ under dark conditions with about 75% relative humidity. Surface cultures on PDA were used to compare *in vitro* growth and spore germination was assessed by counting 100 spores per plate (3 replicates per strain) using the stripe-plate technique. Fungal spores were inoculated (1 site/plate) and replicated three times for each isolate to determine the colony size using a sterile needle loop. The plates were examined at intervals of 16, 24 and 36 hours. Slide cultures for light microscopy were prepared by the procedures described by Goetteland and Inglis (1997). Spores and conidiophore sizes were measured

using a Nikon Eclipse 80I microscope and subsequently identified as per the key provided by Humber (1997).

Molecular Identification

The identity of the entomopathogenic fungus as *B. brongniartii* was further confirmed by rDNA analysis.

DNA sequencing and phylogenetic analysis

Fungal DNA was taken out using the Qiagen Plant DNeasy preparation kit. Partial rRNA areas (18S, ITS1, 5.8S, ITS2 and a partial 28S) were amplified using universal primers ITS1 and ITS4 (White *et al.*, 1990). PCR was conducted using 1 μl of genomic DNA, 0.5 μM of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM dNTPs and 1-2 U *Taq* polymerase in a 50 μl reaction. PCR was carried out in a thermal cycler under the standard conditions: 94°C for about 3 min.; 30 cycles of about 94°C for roughly 30 sec, 54°C for 40 sec and 72°C for 1 min.; and lastly, a final extension at 72°C of 7 min. was carried out. PCR products and others were analyzed using 1.5% agarose gel, cloned into pGEM®-T easy vector and sequenced using the Big Dye® Terminator cycle sequencing kit on an automated DNA sequencer. Sequences were analyzed with BLASTN (Zhang *et al.*, 2000). Phylogenetic and molecular evolutionary analyses were performed using MEGA 3.1 software (Kumar *et al.*, 2004; Patel *et al.*, 2023). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using the distance matrix from the alignment and distances were calculated by the Kimura 2-parameter (Kimura, 1980). The reliability of the tree was measured by bootstrap analysis with 1,000 replicates (Felsenstein, 1985).

Analysis of the genetic diversity

Out of the 50 ISSR primers screened, six polymorphic primers were selected to study genetic variability among *B. brongniartii* isolates. Bands were analyzed using the known GENE PROFILER V 6.0 software package (AlphaInfotech Corporation, USA) and binary matrices were analyzed by NTSYS-PC 2.0 software.

RESULTS AND DISCUSSION

Identification of the fungi

Using standard descriptive keys, the fungal isolates were identified as *Beauveria brongniartii* (Sacc.) based on

Table 2: Macroscopic characterization of *Beauveria brongniartii* isolates.

Isolate code	Location	Colony observation		
		Growth pattern	Colour	Conidia shape
Bb	Bajaura, Kullu	Disperse and dense	Whitish	Ellipsoid
BbR	Barot, Kangra	Disperse and dense	Whitish	Ellipsoid
KD	Kharapathar, Shimla	Disperse and dense	Whitish	Ellipsoid
KH I	Kheradhar, Sirmaur	Disperse and dense	Whitish	Ellipsoid
KH II	Kheradhar, Sirmaur	Disperse and dense	Whitish	Ellipsoid
SH I	Shillaroo, Shimla	Disperse and dense	Whitish	Ellipsoid
SH II	Shillaroo, Shimla	Disperse and dense	Whitish	Ellipsoid

morphological and cultural characteristics under a compound microscope.

Based on morphological and cultural traits

Initially, the isolates formed a loose, white mycelial mat with cushions or areas of conidial structures typically indicative of the fungal growth pattern on the diseased insect body (Table 2). The entire test isolate exhibited sympodial conidium development typical of the genus *Beauveria*. The ellipsoid conidia ($2-3 \times 1.5-2.5 \mu\text{m}$) rarely occur in clusters of conidiogenous cells.

Table 3: Studies on cultural characters of *Beauveria brongniartii* isolates.

Isolate	Mean radial growth (diameter in cm)	Conidial size (μm)
Bb	4.9 ^a (12.79)	2.0×1.5
BbR	4.8 ^a (12.72)	2.0×1.5
KD	5.0 ^a (12.92)	2.3×1.7
KH I	6.0 ^{ab} (14.18)	2.5×2.0
KH II	5.9 ^a (14.06)	2.5×2.1
SH I	7.5 ^b (15.89)	3.1×2.5
SH II	7.4 ^b (15.84)	3.0×2.5

Figures in parentheses are arc sine transformed values.

Means followed by a common letter are not significantly different at the 5% level by DMRT

Among the isolates, two from Shillaroo exhibited maximum radial growth (7.50 cm and 7.45 cm), while the Barot isolates (BbR) showed minimal radial growth (4.85 cm). Table 3 displays the growth measurements of KH I and KH II, the two isolates from Kheradhar, which were 6.00 cm and 5.90 cm, respectively. All isolates showed white mycelial growth on PDA medium. The morphological and ellipsoidal conidial shape matched the description of the test fungus *Beauveria brongniartii* provided by Samson (1981).

Jayaramaiah (1981) reported *B. brongniartii* as a highly virulent pathogen for white grubs in India. The infected grubs were usually completely engulfed with a white mat of mycelium. Colonies on PDA grew quickly and were usually white (Glare and Inwood, 1998). The morphological features observed in this study were consistent with descriptions of Mugnai *et al.* (1989) and Shimazu *et al.* (1988).

rDNA analyses

rDNA analysis targeting the ITS region-specific was performed to confirm entomopathogenic fungi identity. Amplicons of 569 bp were obtained and submitted to NCBI Gene Bank via accession numbers KC879265 to KC879271 (Table 4, Fig 1). BLASTN analysis of the sequences revealed 98-99 percent homology with *Beauveria brongniartii*, confirming the identity of the isolates.

To infer a relationship between *B. brongniartii* isolates infecting white grubs based on the region of ITS, an

Table 4: Identification of *Beauveria brongniartii* by partial sequencing of 18S-28S rRNA genes.

Code of sequence isolate	Matching organism in NCBI gene bank database with accession number	Identity of the sequenced <i>Beauveria</i> spp.,	Percentage similarity code	E-value	Accession number
Bb	<i>B. brongniartii</i> KX255641	<i>Beauveria brongniartii</i>	98-99	0.0	KC879266
BbR	<i>B. brongniartii</i> KX255641	<i>Beauveria brongniartii</i>	98-99	0.0	KC879269
KD	<i>B. brongniartii</i> KC879267	<i>Beauveria brongniartii</i>	98-99	0.0	KC879268
KH-I	<i>B. brongniartii</i> KX255641	<i>Beauveria brongniartii</i>	98-99	0.0	KC879271
KH-II	<i>B. brongniartii</i> KX255641	<i>Beauveria brongniartii</i>	98-99	0.0	KC879270
SH-I	<i>B. brongniartii</i> KC879267	<i>Beauveria brongniartii</i>	98-99	0.0	KC879267
SH-II	<i>B. brongniartii</i> KC879267	<i>Beauveria brongniartii</i>	98-99	0.0	KC879265

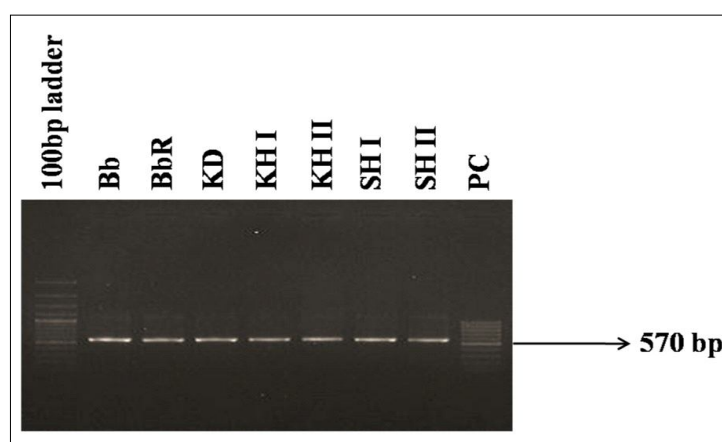


Fig 1: PCR amplification of *Beauveria brongniartii* isolates using ITS primer pair.

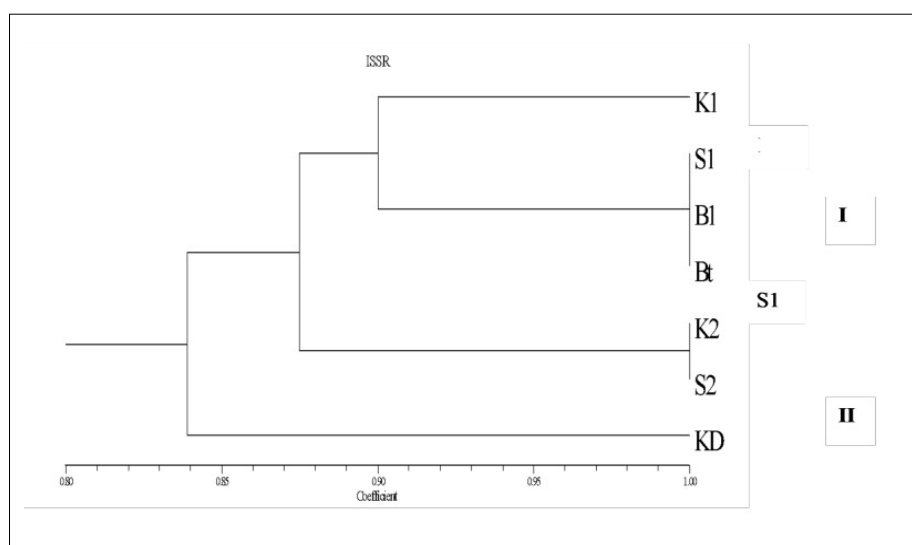


Fig 2: Dendrogram of seven isolates of entomopathogenic fungus *Beauveria brongniartii* generated by UPGMA analysis of DNA fingerprints obtained with ISSR markers.

approximately optimal tree was generated by utilizing the UPGMA method. The distances were computed with the help of the neighbor-joining method (NJM) and the maximum composite likelihood method (MCLM).

Genetic diversity analysis

Genetic diversity among *B. brongniartii* isolates was studied using ISSR markers. Seven isolates from different locations in Himachal Pradesh were analyzed using ISSR markers and the UPGMA-generated dendrogram classified the isolates into two major clusters (Fig 2). The cluster I contained 6 isolates, further divided into 3 subclades, while Cluster II comprised only an isolate from Kharapathar (KD). This indicates significant variability among the isolates present in Himachal Pradesh, supported by their distinct morpho-cultural traits and their growth pattern.

Previous studies (Takatsuka, 2007; Wang *et al.*, 2000; Muro *et al.*, 2005) demonstrated a similar application of ISSR markers to assess genetic diversity in *Beauveria* species. McGuire *et al.* (2005) emphasized the importance of combining morphological characters with molecular markers for accurate characterization of isolates. Thus it is best to first identify which population is present in the isolate collection and then characterize each population with markers. The present study revealed that all seven isolates of *B. brongniartii* from Himachal Pradesh formed a distinct group. In previous studies, correlations between geographic origin and genetic diversity have been studied by Poprawski *et al.* (1988).

The present investigations confirmed *B. brongniartii* as the predominant entomopathogenic fungus parasitizing white grubs in the northwestern Himalayas region of Himachal Pradesh. The rDNA analysis demonstrated that the isolates represent a distinct population of these entomopathogenic fungi compared to those reported in

other countries. Although ISSR analysis revealed high variability among isolates; geographic origin strongly influenced their genetic clustering. The distinctiveness of these isolates may indicate adaptations for enhanced pathogenicity towards host insects.

The results are per the findings of Sabbahi *et al.* (2012) who differentiated the *Beauveria* isolates by targeting 28S rDNA sequences as well as 18S rRNA, ITS regions and 5.8S of rDNA. De Muro *et al.*, (2012) observed a correlation between morphological traits and ITS sequence analysis, thus supporting the present findings. A careful evaluation of entomopathogenic fungi enables their effective use in biocontrol programs. These fungi are the most versatile agents due to their broad host range, ability to infect insects at all stages and ease of mass production methods. These attributes make it a suitable alternative to other conventional insect control methods (Ranadey *et al.*, 2023).

The study emphasis is on the potential of the *B. brongniartii* for managing white grubs such as *B. coriacea* in agricultural systems of the northwest Himalayas in India. Five native found strains of entomopathogenic fungi were extracted from the soil samples along with the parasitized insects, three of which corresponded to *B. brongniartii*. The sequencing and amplification of the ITS1-5.8S-ITS2-related region, molecular database comparisons and the phylogenetic analyses confirmed the taxonomic identity of these strains.

CONCLUSION

This study provides information on entomopathogenic fungi associated with agriculturally important crops in the northwest Himalayas of India. Five native strains were being isolated from the soil samples and parasitized insects. Three strains were identified as belonging to *Beauveria brongniartii* based on their macroscopic and microscopic characteristics. Their taxonomic identity was

further confirmed through ITS1-5.8S-ITS2 region sequencing, molecular database comparison and phylogenetic analyses.

Conflict of interest

On behalf of all authors of this work, the corresponding author hereby states that there is no conflict of interest.

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