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RESEARCH ARTICLE

SEQUENCING AND IDENTIFICATION OF POTENTIAL NATIVE STRAINS OF *BEAUVERIA* SPP. THROUGH INTERNAL TRANSCRIBED SPACER R DNA (RDNA-ITS)

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ABSTRACT

Native strains of *Beauveria* spp. were evaluated against *Spodoptera litura* using leaf spray method. Twelve native and one reference strain of *B. bassiana* were assayed at 1×10^{10} spores ml^{-1} to determine LT_{50} by probit analysis. The least LT_{50} values of 135.84 and 179.96 hours were recorded in native strains of SGb and MKb followed by Hb, Kb and Bb i.e. LT_{50} value of 195.49, 201.20 and 235.30. The highest LT_{50} values of 500.88 and 336.90 hours were recorded in RARST-b and NBAII-b strains. Five effective and promising *B. bassiana* strains (SGb, MKb, Kb, Bb and Hb) were characterized using rDNA-ITS sequences. All the five strains of *B. bassiana* showed 100% homology with already submitted *B. bassiana* strain in NCBI GenBank. Phylogenic dendrogram exhibited *B. bassiana* strains Bb and Kb as distinct cluster compared to others, where as *B. bassiana* strains SGb, MKb and Hb formed one cluster, however SGb grouped into same clade with AB 027382 (IFO 4848) and FJ 755242 (CZ 590) which were isolated from China and Japan, respectively. Gene sequences of five native strains were deposited in NCBI Gene Bank, Bethesda, USA with the accession numbers: JX173280.1 (SGb, Bb1), JX313063.1 (MKb, Bb2), JX313064.1 (Kb, Bb3), JX313065.1 (Bb, Bb4) and JX313066.1 (Hb, Bb5).

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INTRODUCTION

The tobacco cutworm *Spodoptera litura* (Fab.) is regarded as a most destructive pest of subtropical and tropical agricultural crops. *S. litura* (Fab.) (Lepidoptera: Noctuidae), the common cutworm, is an economically serious and polyphagous pest in India. Control currently relies mainly on the application of various classes of chemical insecticides including carbamates, pyrerhroids and organophosphates (Liburd *et al*., 2000). It is recognized that widespread continuous use of these chemical insecticides causes environmental problems and leads to the development of insect resistance, Microbial insecticides such as entomopathogenic fungus can provide as alternative, more environmentally friendly option to control this insect pest. The indiscriminate use of chemical pesticides is assuming a serious cause of concern to human health and environment safety. A viable alternative to chemical pesticides is integrated pest management.

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Biological control of pests with entomopathogenic fungi is an attractive alternative to the use of conventional pesticides, mainly because these fungi are safer for plants, animals and the environment (Khetan, 2001). Entomopathogenic fungi are important in the natural regulation of many insect populations, and they cause often widespread epizootics under ideal environmental conditions (Lacey and Goettel, 1995). There are many commercial products available worldwide based on entomopathogenic fungi. The most widely used species available commercially is *B. bassiana* (Goettel *et al*., 2005). Products based on this species are available for use against a very wide variety of insect pests, from banana weevils (Cosmopolites sordidus) in Brazil to pine caterpillars (*Dendrolimus* spp.) in China (Goettel *et al*., 2005; Alves *et al*., 2003; Feng *et al*., 1994). The entomopathogenic fungus *B. bassiana* is a promising and extensively researched biological control agent that can suppress a variety of economically important insect pests (Coates *et al*., 2002: McGuire *et al*., 2005). Sporadic occurrence of *B. bassiana* has been observed (Uma Devi *et al*., 2003).

B. bassiana has a wide host range, but difference in both host specificity and virulence among isolates has been reported (Ferron *et al*., 1991). Ribosomal genes and their ITS and IGS spacer regions have been widely used for the identification and differentiation of species (Fouly *et al.*, 1997) as well as in taxonomic (Driver *et al.*, 2000), phylogenetic (Rakotonirainy *et al.*, 1994) and genetic diversity (Anderson *et al.,* 2001; Uetake *et al*., 2002) studies, with ITS sequences have been reported as being useful for discriminating between different species of fungi (Neuveglise *et al.*, 1994; Fouly *et al.*, 1997; Jensen *et al.*, 2001; Anderson *et al.*, 2001; Thomsen and Jensen, 2002). The RAPD primers have so far provided differentiation at the intraspecific level, allowing differentiation of closely related isolates within single species (Bridge *et al.,* 1997). The internal transcribed spacers of the ribosomal DNA (rDNA-ITS) sequencing (Coates *et al*., 2002; Gaitan *et al*., 2002; Muro *et al*., 2005; Wada *et al*., 2003) have been successfully employed to assess the genetic variability of *Beauveria* spp.

MATERIALS AND METHODS

Fungal cultures

The thirteen isolates of B. bassiana used in the present study included 10 isolates from silkworms, one from S. litura, one from soil sample and one collected from NBAII-Bangalore i.e. reference strain. The fungus was isolated from insect cadavers obtained from Rayalaseema region (Chittoor, Anantapur and Kurnool) of Andhra Pradesh (PAb, Kb, PMb, Gb, PYb, Bb, Vb, Hb, MKb, MTb, SGb and RARSTb). Pure cultures were maintained on Sabouraud dextrose yeast agar (Dextrose - 4%; peptone - 1% and yeast extract - 1%) slants and plates. The cultures were maintained at 25^0C and 14/10 h day/night regime. Conidia from 14-days-old fungal cultures were used in the laboratory bioassays on insect larvae. To obtain mycelia for DNA extraction, the cultures were grown in liquid medium (Sabourauds dextrose broth with 1% peptone and 2% dextrose) according to the method described by Pfeifer and Khachatourians (1993). The mycelium was harvested by filtration, washed three times with distilled water, freeze-dried and stored aseptically at -70^0 C until needed.

Insect cultures

The second instar larvae of S. litura were obtained by breeding the field-collected insects in the laboratory. Female moths lay up to 300 eggs in mass, which hatch within 3 - 5 days. The egg mass laid on castor leaves by the moths were carefully transferred onto fresh castor leaves for the eggs to hatch. The neonate larvae were later transferred to and maintained on fresh castor leaves until they reached the second instar stage. Bioassays were done with these larvae and a homogenous population was maintained.

Bioassays

Virulence of *B. bassiana* isolates were tested at second instar stage of the pest. Fifteen larvae were taken for each treatment in a plastic container of 8 cm in diameter and 10 cm in height, lined with moistened filter paper groundnut leaves and two 2 ml of conidial suspension at a concentrations of 1 x 10^{10} spores/ml was sprayed using a hand sprayer. Three replicates were maintained for treatment with each isolate. A control was maintained with a spray of 0.02% Tween-80 solution in sterile distilled water. The treated larvae were kept in the plastic containers and fed with groundnut leaves during incubation at 25 ± 2°C. During the incubation period the relative humidity was maintained at about 95%. The dead larvae before 24 h were removed from the experiment. The larval mortality was recorded at 24 hrs interval. The mummified larvae, if any, were kept for reisolation of the fungus in Petri plates lined with moistened filter paper.

DNA extraction

The DNA was extracted according to the method of Lee and Taylor (1990) with minor modifications. One gram of newly harvested fungal mycelia of *B. bassiana* isolates were homogenized by using pre-sterilized pestle and mortar in liquid nitrogen, and transferred to sterile eppendorf tubes and suspended in 1000 µl of lysis buffer (containing 0.15 M NaCl, 50 mM Tris HCL, 50 mM EDTA, 3% SDS and 1% β-mercapto ethanol). The homogenates were mixed by vortexing for few minutes and incubated for one hour at 65°C in water bath. And then the samples were centrifuged for 10 minutes at 10,000 rpm. Supernatant was collected in a separate tube and equal volumes of phenol : chloroform (1:1) were added and again the samples were kept for centrifugation for 10 minutes at 10,000 rpm, supernatant collected and to this equal volume of chloroform: isoamyl alcohol (24:1) was added and spun at 10,000 rpm for 10 minutes. To the supernatant, 0.6 volume (e.g. 300 µl then 0.6 x 300 = 180 µl) of ice cold isopropanol and 0.1 volume of Sodium acetate (3M) (pH 5.2) were added and incubated at -20°C for 30 min for precipitation of DNA. After incubation, the tubes were spun at 13,000 rpm for 20 minutes at 4°C and the supernatant was discarded. Later the pellet was washed with 70 per cent ethanol (100 µl) and centrifuged at 13,000 rpm for 10 minutes at 4°C. The pellet was allowed to air dry. Then the dried pellet was dissolved in 100 µl sterile distilled water and 1µl RNAse (10 mg/ml) was added then eppendorf tubes were stored at -20°C for further use. The concentration and quality of DNA was estimated using nano drop spectrophotometer at 260 nm (ND-1000, USA).

Sequencing potential isolates through internal transcription spacer rDNA (ITS- rDNA)

The effective and potential native isolates of *B. bassiana* strains based on lab bioassay studies against *S. litura* were selected for sequencing.

PCR amplification of ITS region

For ITS reaction primers ITS1 (5′-TCCGTAGGTGAAC CTGCGG-3′) and ITS2 (5′-TCCCTTTCAACAATTTCACG-3′) that amplify the internal transcribed spacers (ITS1 and ITS2), the complete 5.8S rRNA gene and the 5′ end of the 28S rRNA gene were evaluated. The PCR mixture comprised 0.25 mM each of primer pair, 0.25 mM dNTP, 1.5 mM $MgCl_2$, 2 U of Taq DNA polymerase and 1X PCR buffer mix. To this mixture 50-80 ng (2.0µl) of the DNA template was added. The

control tube was added with 2µl deionized water in place of DNA sample and the reaction mixture in the tubes were made up to 25µl volume using deionized water. This was replicated three times for each isolate. The reaction was amplified in a Master cycler gradient (Eppendorf, Germany) using 0.5µl tubes. The PCR conditions were, initial denaturation at 95°C for 2 min., 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 sec, extension step at 72°C for 2 min and a final extension at 70°C for 7 min (White *et al*., 1990). Each of amplification products was checked by electrophoresis of 5µl on 1% Agarose gel containing Ethidium bromide. To verify the size of product a 1 Kb ladder was run along with the sample. After confirmation of size of the amplicon, checked the quantity and quality of nucleic acid of PCR product. This product was sent for DNA sequencing at Eurofins Genomics India Pvt Ltd, Bangalore. The sequence analysis was done using Bioedit software.

Alignment of DNA sequence data with known DNA sequences in NCBI GenBank

Once the nucleotide sequence of the PCR product had been derived, it was compared with known sequences of strains already deposited in GenBank. Sequence alignment was performed using the NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST) (http://130.14.29.110/BLAST/) to compare the sequence data with known sequences submitted in the NCBI database. The sequences obtained were analyzed using the multiple sequence alignment program clustalX 1.81 in order to determine homology within the sequences as well as to determine their evolutionary lineage. Phylogenetic trees were constructed using the Neighbour Joining (NJ) and bootstrap tree methods of the clustalX program.

Statistical analysis

The data on larval mortality was subjected to probit analysis for determining LT50 value with help of XLSTAT-2012.

RESULTS

Pathogenicity

Pathogenicity test of *B. bassiana* isolates carried out on II instar larvae of *S. litura*, showed difference in mortality rate among the 13 isolates at different days after treatment. The least LT_{50} values of 135.84 and 179.96 hours were recorded in native strains of SGb and MKb followed by Hb, Kb and Bb i.e. LT_{50} value of 195.49, 201.20 and 235.30. The highest LT_{50} values of 500.88 and 336.90 hours were recorded in RARST-b and NBAII-b strains. Regression equation, slope, fiducial limits and R^2 values were presented in Table 1. Chi Square test showed no heterogeneity among the population tested. So the potential strains of *B. bassiana* were SGb, MKb, Hb, Kb and Bb. The results of present study are in conformity with Kaur and Padmaja (2008) who categorized 24 isolates of *B. bassiana* based on LT_{50} value. Three isolates showed least LT_{50} value *i.e.,* 0-130 hours which were categorized as highly virulent. 11 isolates showing a range of LT_{50} value from 130-140 hours were categorized as moderately virulent and remaining 10 isolates showing a range of LT_{50} value from 141-154 hours were categorized as less virulent. According to Samuels *et al*. (1989) the LT_{50} more than 14 days (336 hours) indicated low pathogenicity of *B. bassiana*. Vijayavani *et al*. (2009) reported the pathogenicity of 2 isolates of *B. bassiana* (SBT#11and SBT#16) against *S. litura* larvae and recorded the LT_{50} value of 5.1 and 6.0 days in laboratory for SBT#11 and SBT#16 respectively. Moorthi *et al*. (2011) reported the LT_{50} value for Bb_{02} and Bb_{09} isolates of *B. bassiana* was 4.8 days, whereas it was 4.0 days for Bb_{10} @ 1×10^8 spores ml^{-1}. Pandey (2008) reported the higher pathogenicity of *B. bassiana* to *S. obliqua* larvae at low temperature (20^0C) compared to higher temperature (30^0C). The LT_{50} value of *B. bassiana* at 20^0C and 30^0C were 155.4 and 167.9 hours, 159.0 and 168.9 hours, 162.9 and 169.8 hours against second, third and fourth instar larvae respectively.

Sequencing of potential isolates through rDNA-ITS

For ITS1-5.8-ITS2 sequencing the five effective native strains of *B. bassiana* (SGb, MKb, Kb, Hb and Bb) based on bioassay in lab studies were selected and amplified in PCR using the primers, ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (TCCCTTTCAACAATTTCACG) shows no polymorphism in length of the amplified rDNA region was observed among the selected native strains of *B. bassiana*, since a fragment of 930 bp was amplified for all the five strains (Plate 1). Five strains of *Beauveria* spp. (SGb, MKb, Kb, Hb and Bb) were identified as *B. bassiana* by their rDNA sequence; 779 nucleotide sequences were obtained for five strains of *B. bassiana*. After complete alignment (Fig. 1), of sequence of nucleotides for five *B. bassiana* strains was submitted to BLAST search and found 100% homology with already submitted *B. bassiana* strains in NCBI GenBank (Table 2). Gene sequences of five strains were deposited in NCBI GenBank, Bethesda, USA with the accession number: JX173280.1 (SGb, Bb1), JX313063.1 (MKb, Bb2), JX313064.1 (Kb, Bb3), JX313065.1 (Bb, Bb4) and JX313066.1 (Hb, Bb5).

The bootstrap tree is a better option making assessments of evolutionary lineage (Creevey *et al*., 2004). When phylogenic dendrogram was constructed with 12 *B. bassiana* strains already available in NCBI GenBank (Table 2) and for five native strains of *B. bassiana* shown that SGb, MKb and Hb form one cluster with AB 027382 and FJ 755242 and the other two native strains Bb and Kb did not pair with any cluster when compared to 3 native strains and 12 other strains. SGb strain showed 100% genetic similarity with AB027382 (IFO 4848) and FJ 755242 (CZ590) (Table 3). The IFO 4848 and CZ590 strains belongs to China and Japan, respectively (Table 2) and SGb strain showed 100% amino acid similarity with AB 027382, FJ 755242, AB 576868 and EF 026006 (Table 4). The data obtained from the rDNA nucleotide sequencing provides strong support that the five strains isolated were indeed *B. bassiana*. The results of the present investigation revealed that five native strains of *Beauveria* spp. (SGb, MKB, Bb, Kb and Hb) in PCR amplification of ITS1-5.8S-ITS2 rDNA yielded uniform fragment of approximately 930 bp and this strains of *Beauveria* spp. (SGb, MKb, Kb, Hb and Bb) were identified as *B. bassiana* by their rDNA sequence. In phylogenetic dendrogram the *B. bassiana* strain Bb and Kb showed separate cluster compared to other.

Table 1. LT_{50} values (in hours) of different isolates of *Beauveria bassiana* against 2nd instar larvae of *Spodoptera litura* at 1×10^{10} spores/ml

Name of Isolates	2nd instar					
	Regression equation (Y=)	LT_{50} values(Hours)	Fiducial limits(Hours)		Slope (b)	R^2
			Lower	Upper		
PAb	-1.135+0.703x	286.28	234.67	379.73	0.703	0.847
Kb	-1.132+0.687x	201.20	175.11	234.96	0.687	0.786
PMb	-0.812+0.498x	263.61	215.90	343.66	0.498	0.825
Gb	-0.964+0.589x	231.23	196.57	280.31	0.589	0.823
SGb	-1.738+1.059x	135.84	123.50	148.43	1.059	0.816
Bb	-0.970+0.583x	235.30	201.97	283.86	0.583	0.782
Vb	-0.913+0.547x	249.59	213.10	305.31	0.547	0.763
Hb	-1.182+0.715x	195.49	171.84	225.92	0.715	0.784
MKb	-1.191+0.741x	179.96	159.44	204.45	0.941	0.855
MTb	-0.671+0.403x	312.99	250.78	439.86	0.403	0.754
PYb	-0.879+0.544x	241.38	198.99	306.71	0.544	0.852
NBAII-b	-0.610+0.363x	336.90	271.02	490.50	0.363	0.718
RARST-b	-0.368+0.218x	500.88	347.13	1108.45	0.218	0.708

Fiducial limits are calculated by using equivalent deviate at (P=0.05) level with the help of XLSTAT-2012
χ^2 test is not significant.

Table 2. Details of *Beauveria bassiana* strains their origin, isolation source and NCBI accession numbers used for comparison of 5 native strains *B. bassiana* i.e. SGb, Kb, Bb, MKb and Hb

S. No.	Beauveria spp.	Abbreviation	Geographical origin	Host species	Accession number
1	*Beauveria bassiana*	IFO 4848	Japan	-	AB027382
2	*Beauveria bassiana*	CZ590	China	Silkworm	FJ755242
3	*Beauveria bassiana*	GHA	Japan	Biological insecticide	AB576868
4	*Cordyceps bassiana*	ATCC 26854	Korea	-	EF026006
5	*Cordyceps* sp.	97005	Japan	-	AB044636
6	*Beauveria bassiana*	DAOM195005	Canada	*Choristoneura fumiferana* (Budworm)	EU334677
7	*Beauveria bassiana*	INRS-IP	Canada	*Lygus* sp.	EU334675
8	*Beauveria bassiana*	INRS-CFL	Canada	*Tomicus piniperda* (pine shoot beetle)	EU334674
9	*Beauveria bassiana*	DAOM216540	Canada	*Reticulitermes flavipes*	EU334679
10	*Beauveria bassiana*	DAOM210087	Canada	*Leptinotarsa decemlineata*	EU334678
11	*Beauveria bassiana*	ARSEF2991	Canada	*Leptinotarsa decemlineata*	EU334676
12	*Cordyceps bassiana*	F760	Japan	-	AB100038

Table 3. Nucleotide similarity (%) for five native strains and twelve strains from NCBI of *Beauveria bassiana* based on sequencing

Isolates	B1 (SGb)	B2 (Kb)	B3 (MKb)	B4 (Bb)	B5 (Hb)	AB02 7382	FJ75 5242	AB57 6868	EF02 6006	AB04 4636	EU33 4677	EU33 4675	EU33 4674	EU33 4679	EU33 4678	EU33 4676	AB10 0038
B1 (SGb)	100.0																
B2 (Kb)	99.2	100.0															
B3 (MKb)	99.7	98.9	100.0														
B4 (Bb)	99.6	98.8	99.3	100.0													
B5 (Hb)	98.9	98.1	98.7	98.5	100.0												
AB027382	100.0	99.2	99.7	99.6	98.9	100.0											
FJ755242	100.0	99.2	99.7	99.6	98.9	100.0	100.0										
AB576868	99.7	98.9	99.4	99.3	98.7	99.7	99.7	100.0									
EF026006	99.7	98.9	99.4	99.3	98.7	99.7	99.7	99.7	100.0								
AB044636	99.6	98.8	99.3	99.2	98.5	99.6	99.6	99.6	99.6	100.0							
EU334677	98.5	97.8	98.3	98.2	97.5	98.5	98.5	98.5	98.5	98.7	100.0						
EU334675	98.5	97.8	98.3	98.2	97.5	98.5	98.5	98.5	98.5	98.7	100.0	100.0					
EU334674	98.5	97.8	98.3	98.2	97.5	98.5	98.5	98.5	98.5	98.7	100.0	100.0	100.0				
EU334679	98.3	97.5	98.0	97.9	97.3	98.3	98.3	98.3	98.3	98.7	99.4	99.4	99.4	100.0			
EU334678	98.3	97.5	98.0	97.9	97.3	98.3	98.3	98.3	98.3	98.7	99.4	99.4	99.4	99.4	100.0		
EU334676	98.0	97.3	97.8	97.6	97.0	98.0	98.0	98.0	98.0	98.4	99.2	99.2	99.2	99.4	99.2	100.0	
AB100038	97.9	97.2	97.6	97.9	96.8	97.9	97.9	97.6	97.6	97.5	96.5	96.5	96.5	96.5	96.2	96.0	100.0

Table 4. Amino acids similarity (%) for five native strains and twelve strains from NCBI of *Beauveria bassiana* based on sequencing

Isolates	B1 (SGb)	B2 (Kb)	B3 (MKb)	B4 (Bb)	B5 (Hb)	AB02 7382	FJ75 5242	AB57 6868	EF02 6006	AB04 4636	EU33 4677	EU33 4675	EU33 4674	EU33 4679	EU33 4678	EU33 4676	AB10 0038
B1 (SGb)	100																
B2 (Kb)	97.5	100															
B3 (MKb)	99.5	97.1	100														
B4 (Bb)	99.1	96.7	98.7	100													
B5 (Hb)	97.6	95.2	97.2	96.8	100												
AB027382	100	97.5	99.5	99.1	97.6	100											
FJ755242	100	97.5	99.5	99.1	97.6	100	100										
AB576868	100	97.5	99.5	99.1	97.6	100	100	100									
EF026006	100	97.5	99.5	99.1	97.6	100	100	100	100								
AB044636	99.5	97.1	99.1	98.7	97.2	99.5	99.5	99.5	99.5	100							
EU334677	98.0	95.6	97.6	97.2	95.6	98.0	98.0	98.0	98.0	97.6	100						
EU334675	98.0	95.6	97.6	97.2	95.6	98.0	98.0	98.0	98.0	97.6	100	100					
EU334674	98.0	95.6	97.6	97.2	95.6	98.0	98.0	98.0	98.0	97.6	100	100	100				
EU334679	97.6	95.2	97.2	96.8	95.2	97.6	97.6	97.6	97.6	98.0	98.8	98.8	98.8	100			
EU334678	97.2	94.8	96.8	96.4	94.8	97.2	97.2	97.2	97.2	97.6	98.4	98.4	98.4	98.8	100		
EU334676	96.8	94.4	96.4	96.0	94.4	96.8	96.8	96.8	96.8	97.2	98.0	98.0	98.0	99.2	98.0	100	
AB100038	95.9	93.9	95.5	95.9	93.6	95.9	95.9	95.9	95.9	95.5	94.0	94.0	94.0	93.6	93.2	92.8	100

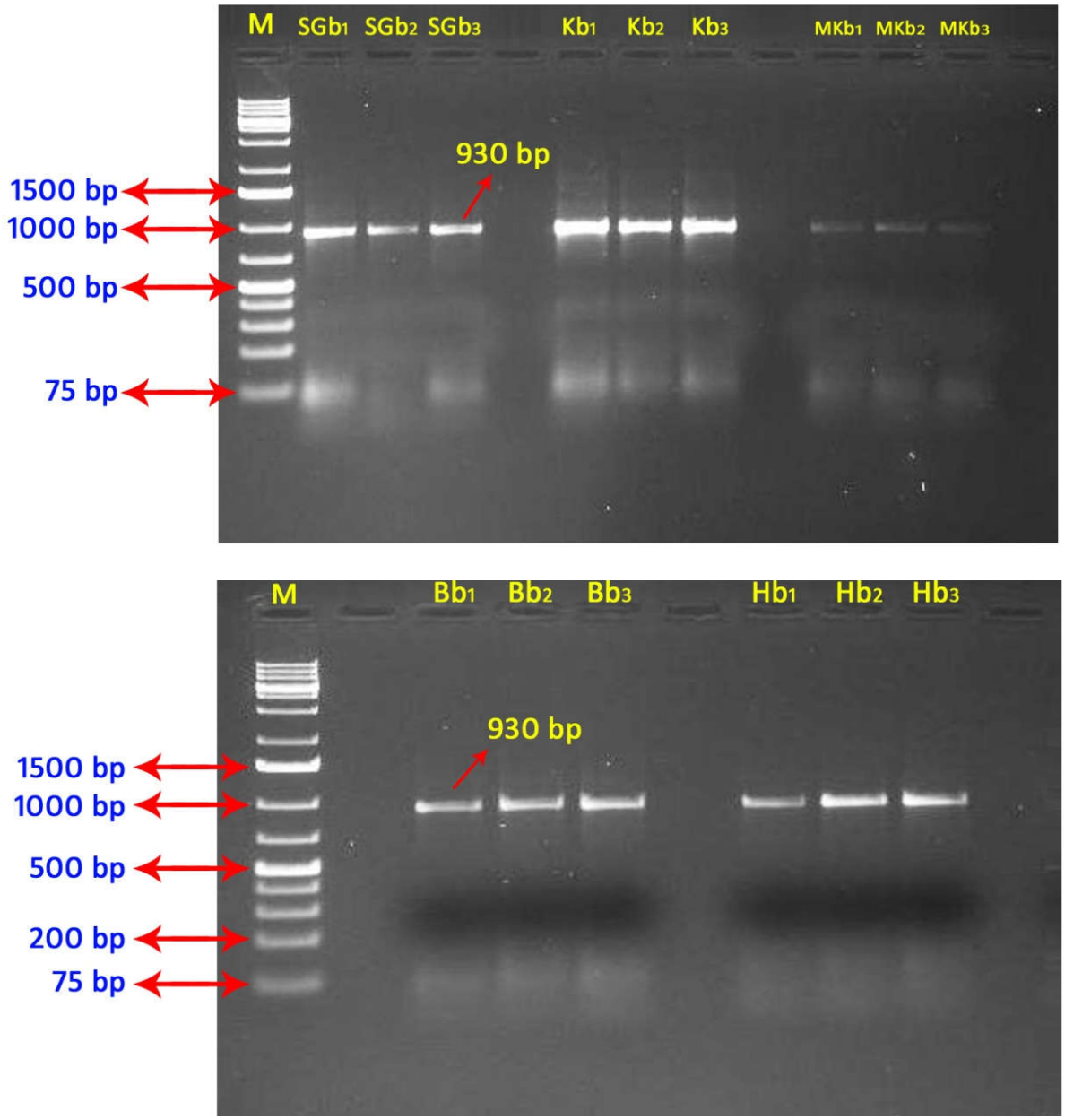
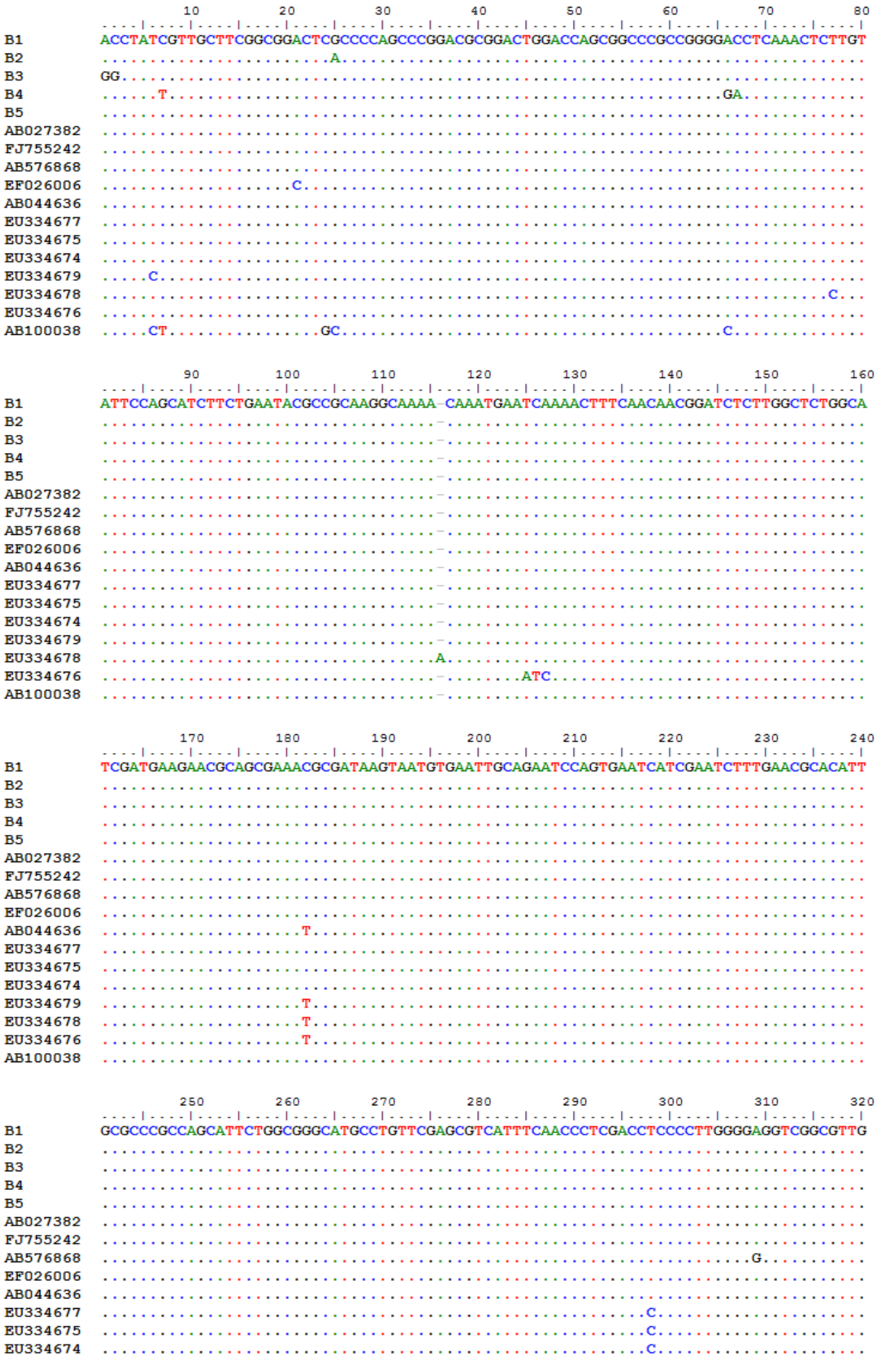
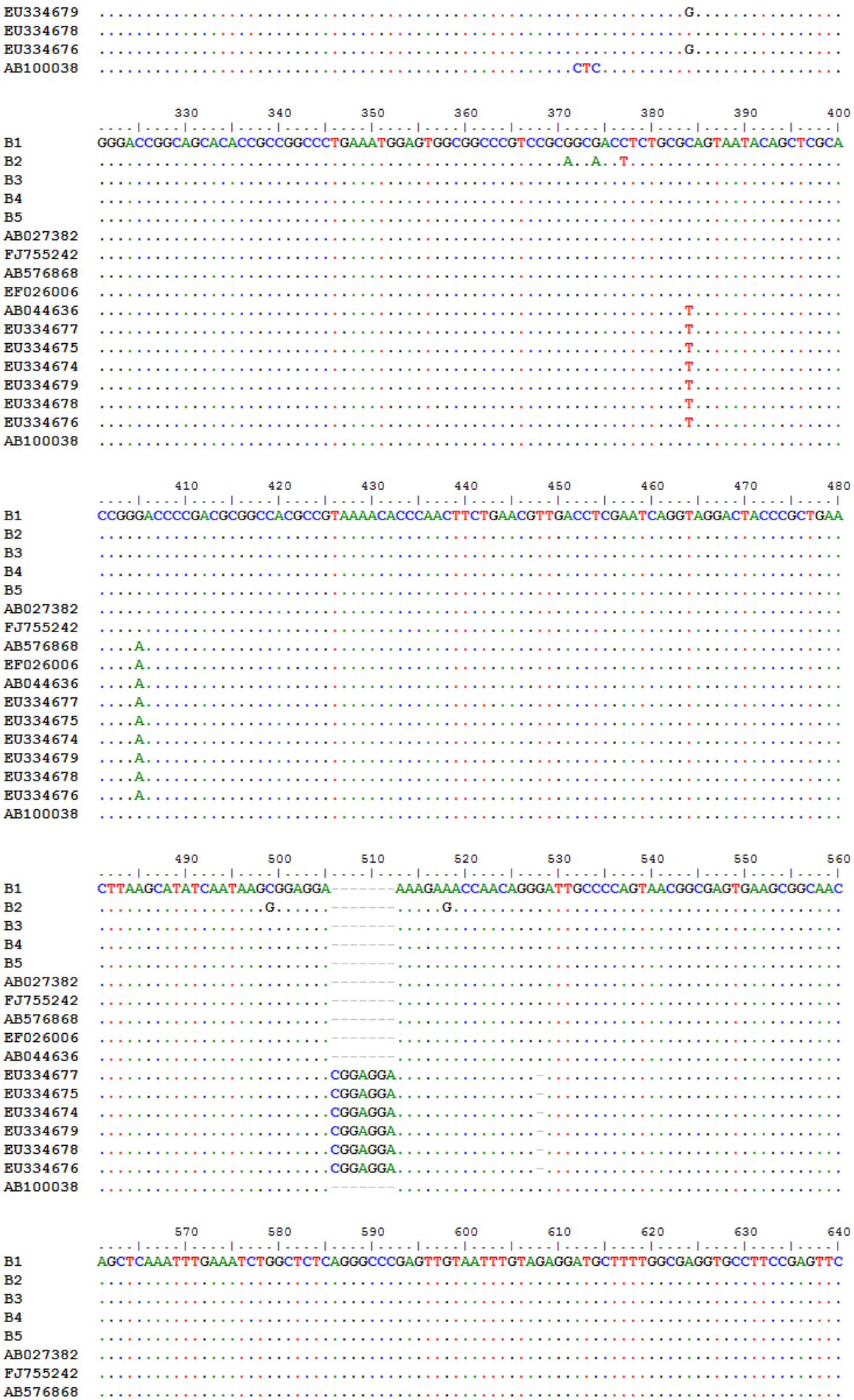


Plate 1. PCR amplification of native strains of *B. bassiana*using ITS1 and ITS2 primers

Three strains of *B. bassiana* SGb, MKb and Hb form one cluster (Fig. 2). Similar results were obtained by Carneiro *et al.* (2008) who have observed no polymorphism in length of the amplified rDNA region among the *Beauveria* isolates. Since a fragment of 570 bp was amplified for all the 24 isolates. Sequencing of these fragments was compared with data from the GenBank, and the isolates were characterized as *B. bassiana* or *B. brongniartii* with high degree of identity. A low level of sequence variation was detected within the ITS region. Neuveglise *et al.* (1994) found clear differences between 28 *B. brongniartii* strains and two *B. bassiana* strains on restriction analyses of the ITS regions. Travis and Alison (1998) reported the genetic variation and classification of New Zealand isolates of *Beauveria* spp. and other country strains based on the ITS regions. Robert *et al.* (2010) used PCR primers and DNA sequencing to genetically characterize 14 isolates of *B. bassiana* and twelve of the 14 Hawaiian isolates were unique and the GHA strain was not among those isolated from the wild. The PCR amplification of ITS1-5.8S-ITS2 rDNA yielded a unique fragment of approximately 540 bp for *M. anisopliae* variety *anisopliae* strains E9, B/Vi and C (isolated in Brazil), 600 bp for *M. anisopliae* variety *anisopliae* strain 14 (isolated in Australia), 650 bp for the *M. album* and 600 bp for *M. flavoviride* strains (Ricardo *et al.,* 2004).

Results of present investigation showed that *B. bassiana* was genetically diverse over small spatial scales. For example, 3 strains of *B. bassiana* (SGb, Kb and Bb) collected during January, 2012 from Palamaner division (3 villages, located 5-14 km apart) shows genetic diversity in ITS sequencing. High genetic diversity of *B. bassiana* over small spatial and temporal scales has been found by other researchers.


10 20 30 40 50 60 70 80
B1 ACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCAGCGGCCCGCCGGGGACCTCAAACTCTTGT
B2 .....A.....
B3 GG.....
B4 .....T.....GA.....
B5
AB027382
FJ755242
AB576868
EF026006 .....C.....
AB044636
EU334677
EU334675
EU334674
EU334679 .....C.....
EU334678 .....C...
EU334676
AB100038 .....CT.....GC.....C.....
90 100 110 120 130 140 150 160
B1 ATTCCAGCATCTTCTGAATACGCCGCAAGGCAAAA-CAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGCA
B2 .....-.....
B3 .....-.....
B4 .....-.....
B5 .....-.....
AB027382 .....-.....
FJ755242 .....-.....
AB576868 .....-.....
EF026006 .....-.....
AB044636 .....-.....
EU334677 .....-.....
EU334675 .....-.....
EU334674 .....-.....
EU334679 .....-.....
EU334678 .....A.....
EU334676 .....-.....ATC.....
AB100038 .....-.....
170 180 190 200 210 220 230 240
B1 TCGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATT
B2
B3
B4
B5
AB027382
FJ755242
AB576868
EF026006
AB044636 .....T.....
EU334677
EU334675
EU334674
EU334679 .....T.....
EU334678 .....T.....
EU334676 .....T.....
AB100038
250 260 270 280 290 300 310 320
B1 GCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTG
B2
B3
B4
B5
AB027382
FJ755242
AB576868 .....G.....
EF026006
AB044636
EU334677 .....C.....
EU334675 .....C.....
EU334674 .....C.....


EU334679 ..... G.....
EU334678 .....
EU334676 ..... G.....
AB100038 ..... CTC.....
330 340 350 360 370 380 390 400
B1 GGGACCGGCAGCACACCGCCGGCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTAATACAGCTCGCA
B2 ..... A..A..T.....
B3 .....
B4 .....
B5 .....
AB027382 .....
FJ755242 .....
AB576868 .....
EF026006 .....
AB044636 ..... T.....
EU334677 ..... T.....
EU334675 ..... T.....
EU334674 ..... T.....
EU334679 ..... T.....
EU334678 ..... T.....
EU334676 ..... T.....
AB100038 .....
410 420 430 440 450 460 470 480
B1 CCGGGACCCCGACGCGGCCACGCCGTAAAACACCCAACTTCTGAACGTTGACCTCGAATCAGGTAGGACTACCCGCTGAA
B2 .....
B3 .....
B4 .....
B5 .....
AB027382 .....
FJ755242 .....
AB576868 ....A.....
EF026006 ....A.....
AB044636 ....A.....
EU334677 ....A.....
EU334675 ....A.....
EU334674 ....A.....
EU334679 ....A.....
EU334678 ....A.....
EU334676 ....A.....
AB100038 .....
490 500 510 520 530 540 550 560
B1 CTTAAGCATATCAATAAGCGGAGGA-------AAAGAAACCAACAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAAC
B2 .....G.....-------.....G.....
B3 .....-------.....
B4 .....-------.....
B5 .....-------.....
AB027382 .....-------.....
FJ755242 .....-------.....
AB576868 .....-------.....
EF026006 .....-------.....
AB044636 .....-------.....
EU334677 .....CGGAGGA.....-.....
EU334675 .....CGGAGGA.....-.....
EU334674 .....CGGAGGA.....-.....
EU334679 .....CGGAGGA.....-.....
EU334678 .....CGGAGGA.....-.....
EU334676 .....CGGAGGA.....-.....
AB100038 .....-------.....
570 580 590 600 610 620 630 640
B1 AGCTCAAATTTGAAATCTGGCTCTCAGGGCCCGAGTTGTAATTTGTAGAGGATGCTTTTGGCGAGGTGCCTTCCGAGTTC
B2 .....
B3 .....
B4 .....
B5 .....
AB027382 .....
FJ755242 .....
AB576868 .....

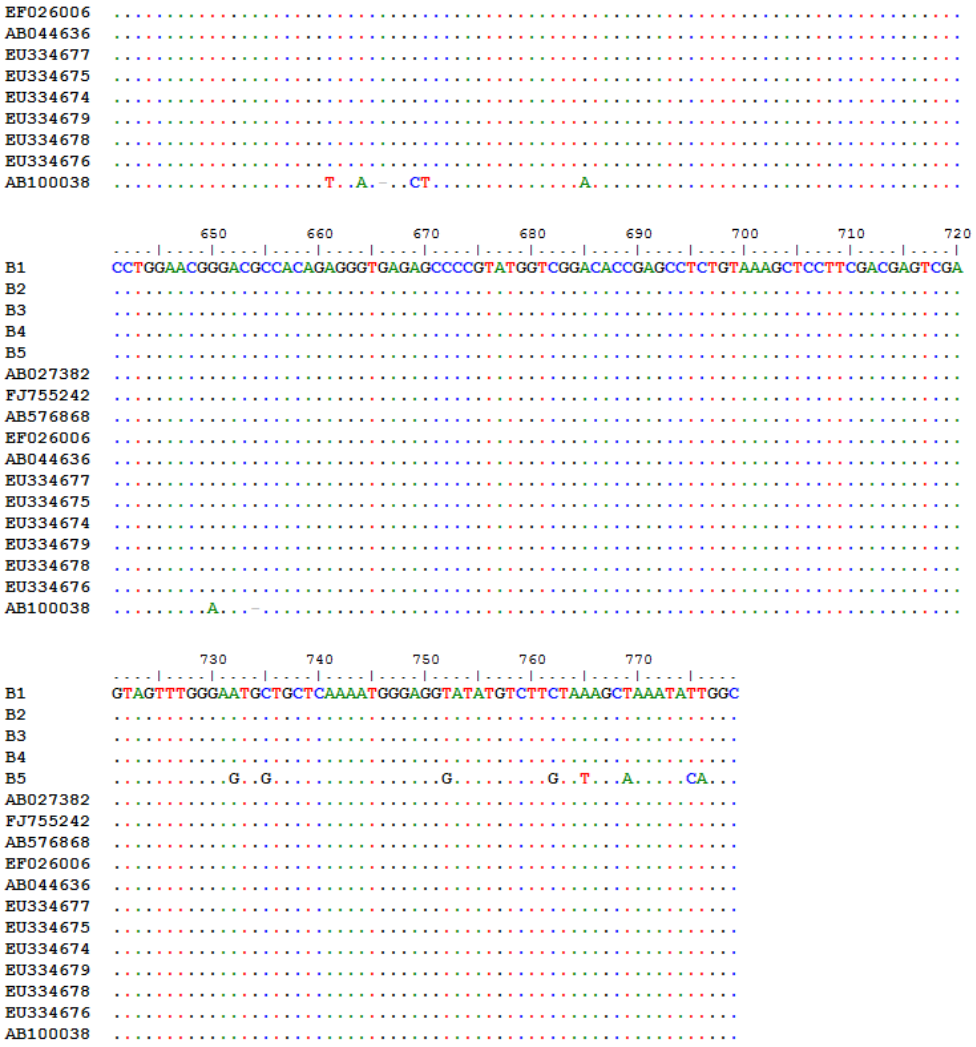
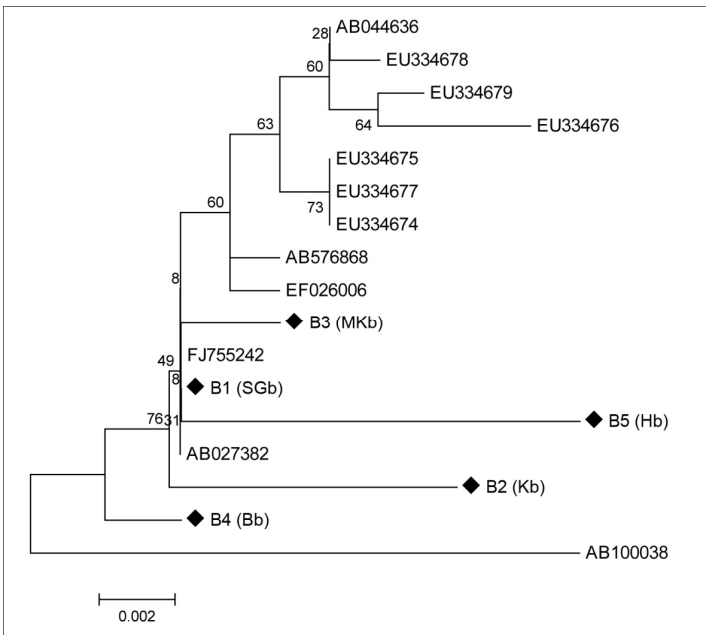


Fig. 1. BLAST alignment of the nucleic acid sequence derived from five native strains of *B. bassiana* (SGb (B1), Kb (B2), Mb (B3), Bb (B4) and Hb (B5)) and that of known*B. bassiana* 5.8s rDNA gene sequence found on the NCBI database



Dendrogram of *Beauveria bassiana* ITS sequencing

Fig. 2. Phylogenic dendrogram for samples derived from the native strains SGb, Kb, Bb, MKb and Hb and that of known strains of *B. bassiana* rDNA gene sequence found on the NCBI database

Meyling and Eilenberg (2006) recovered 13 strains of *B. bassiana* from leaves of hedgerow plants in two villages in Denmark, including six strains from one village and seven from the other. Four strains were common to both villages, located 7.6 km apart. Castrillo *et al.* (2007) reported the variation in 16 different strains of *B. bassiana* infecting greenhouse shore flies (*Scatella tenuicosta* Collin) sampled from a laboratory colony over a 5 months period in Ithaca, NY (USA). Similar results were recorded by Robert *et al*. (2010) in isolates of *B. bassiana* collected from Hawaii.

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