GENOTYPING OF GEORGIAN ISOLATES OF ENTOMOPATHOGENIC FUNGI BEAUVERIA SPP.

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Abstract- Fungal entomopathogens are known to infect a wide range of insect species from many different orders and are readily isolated from soils in various ecosystems they are a producents of toxins and biological active materials which can cause high mortality in different species of pests The genotyping isolates of entomopathogenic fungi have been studied in the various region of Georgia within our research. As a result of actuality of biopesticideproduction around the world, based on molecular characterization of strains, for the first time in Georgia have been studied Beauveria spp. local strains and carried out amplification of several fragments of DNA such as: ITS region (the rRNA gene cluster), EF1 (the Elongation Factor 1-alpha) and (the intergenic) Bloc region. Also, results have been compared by using of computer programs (Mega6, Mafft, Blast,). The regional differences between strains were identified through phylogenetic tree. Base on conducted research was confirmed that the samples taken from the same region was located at the same clades on phylogenic tree. sequence analysis of Georgian DNA samples have been compared with gene bank data In accordance with our study, based on comparison process our strains have been located in A, and C cades.

Keywords- Beauveria, Entomopathogens, DNA, Sequence..

I. INTRODUCTION

Entomopathogenic fungi are distributed in a wide range of habitats including aquatic forest, agricultural, pasture, desert, and urban habitats (Vega, et al, 2009, Chandler et al.2000). Such fungi have the ability to regulate invertebrate pest populations and have an important role in biological control of various harmful insects and mites (Ingles et.al. 2001; Keller2003). Beauveria bassiana (Hypocreales: Cordycipitacea) is an entomopathogenic fungus, grows naturally in all continents, except Antarctica, and hasa cosmopolitan distribution. The fungus has been isolated from more than 700 insect species belonging to nine orders. Lepidoptera and Coleoptera are the most prevalent hosts (Wegensteiner et.al.,1996; Lacey et, al., 2015; Vega, et al., 2012). There are several benefits of using Beauveria bassianato control invertebrate pest populations: it does not harm non-target organisms or the environment, it is easyto mass-produce and disseminate, it causes rapid host mortality, it has the potential to recycle in the environment, it is compatible with agricultural chemicals, and it is susceptible to genetic selection and DNA transformation (Shah and Pell, 2003; Brownbridge, 2006; Charnley and Collins, 2007; Wraight et al., 2009; Zimmermann, 2007; Glare et al., 2010; Goettel et al., 2010). Importantly, this fungusis highly effectivein controlling vectored human pathogens, specifically adult mosquitoes. (Blanford et al., 2009; Kikankie et al., 2010). Entomopathogenic fungi naturally residing in soil are an environmental reservoir of insect pest antagonists. Local strains of such fungi may be adapted to their environment, which renders them of particular interest for use in biological control. Georgia has a large diversity of such fungi, due to its varying altitudes, eco-systems, and cropping systems, which offers a unique opportunity to study local insectpathogens. The objective of this study was to explore diverse habitats as potential sources of local strains of the entomopathogenic fungus Beuveria bassiana,as well as its relative distribution in various geographical sites in Georgia. Fungal entomopathogens are known to infect a wide range of insect species belonging tomany different orders and are readily isolated from soils in various ecosystems (Roy et al.,2010). Beauveria spp. are widely used entomopathogenic fungi in agriculture. These fungi naturally produce biologically active toxins during their life cycle, which causesustained, significant death of harmful insects of various species without human intervention. Therefore, it is crucial to undertake molecular characterization of Beauveria spp. for developing an effective strategy of biocontrol. Traditional identification of Beauveriaspp. is principally based on conidial morphology.

However, molecular phylogenetic analyses have revealed that the Beauveria genus includes cryptic species (Rehner and Buckley, 2005). Studies are currently underway worldwide to address this gap in knowledge. Aside from Beauveria, other entomopathogenic fungi are commercially used, including Metarhizium spp., among others. Such fungi are widely used against pests in the agricultural sector, particularly in greenhouses and in the forest, but also in farms for producing ecologically pure products, “Organic Foods”. Such products are characterized by a high nutritional value and are in demand in the modern market (Rehner and Buckley,

2005; Rehner et al. 2006; Ghikas et al.2010). Georgia is an agrarian country that produces many agricultural products, whereproduct quality and mass production determine competitiveness at the market and thus business development. For example, suchpestsas Melolontha, Curculio nucum, Xileborus dispar and Palomena prasinahinder nut plantation yields cultivated in Georgia. The life cycle of Beauveria spp. occurs in soil. Conidial morphology is the traditional method for identification of Beauveria spp., but as molecular and phylogenetic research has shown, the order of Beauveria spp. includes unidentified species requiring characterization. For example, Beauveria bassiana sensu lato (s.l.) are a morphospecies that are not monophyletic and includes members that are located in various clades. Several studies propose that these fungi represent a different species (Meyling et al. 2012). The South Caucasus region, particularly Georgia, is distinguished by its diversity of climate zones and biological species. Thus, some level of genetic diversity ofentomophatogenic fungi in this region is expected due to the variability and uniqueness of local climates and host organisms. It is generally agreed that morphological characteristics are often not informative for distinguishing species of anamorphic fungi and that additional taxonomic characteristics are required. Many tools based on molecular markers have been extensively developed for Beauveria strain or species identification. (Robène-Soustrade,Jouen etal., 2015) Phylogenetic characterization of Beauveria isolates into specific clades can be achieved through sequencingthe internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene cluster of genomic DNA(White et al.,1990), the end region of Elongation Factor 1-alpha (EF1-a) (Ellen Buckley2005),(Bischoff et al. 2006, 2009),and the intergenic Bloc region (Rehner et al., 2006).Traditionally, the ITS, EF1-α, and intergenic Bloc regions are used, as they are more variable and thusmore informative. By this method, Beauveria spp. isolates can be divided into A, B, and C cladesandcan be further categorized by variability within clades (S A. Rehner, E, Buckley 2005) (Rehner et al., 2006).

II. MATERIALS AND METHODS

A. Fungal isolates and DNA extraction

Soil samples were obtained in 2007-2009 from four different geographical sites at different altitudes (600-1700 meters above sea level (MASL), with each representing unique agricultural and forest ecosystems of Georgia. Isolation of entomopathogenic fungi was performed using the Galleria bait method (Zimmermann 1986). In our study, Colorado Potato beetles, Leptinotarsa decemlineata, were used for baiting the soil samples. Individual insects presenting symptoms of entomopathogenic fungal infection were isolated from a population of Ips typographus adults. Fungi isolated from mosquitos were cultivated on PDA media and incubated at 23° ± 2 C for 12-15 days. Six isolates of Beauveria bassiana were obtained from various regions of Georgia.

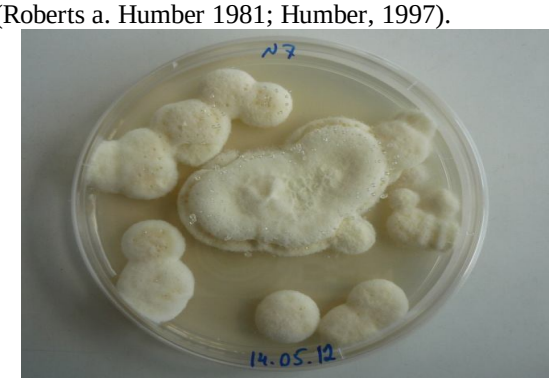
B. DNA Extraction and PCR amplification, sequencing

Genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, USA)(Robène-Soustrade et, al 2015). Extracted DNA was quantified after gel electrophoresis (Park,2001).PCR amplification of Beuveria spp. DNA was performed in 25 µl reaction volumes using primer pairs ITS region (ITS4-ITS5) of the rRNA gene cluster (Meyling et al.,2009.White et al., 1990, Robène-Soustrade et al.,2015). Elongation Factor 1-alpha (Bischoff et al., 2006, 2009) and the intergenic Bloc region (Meyling et al.,2009,Rehner et al., 2006).

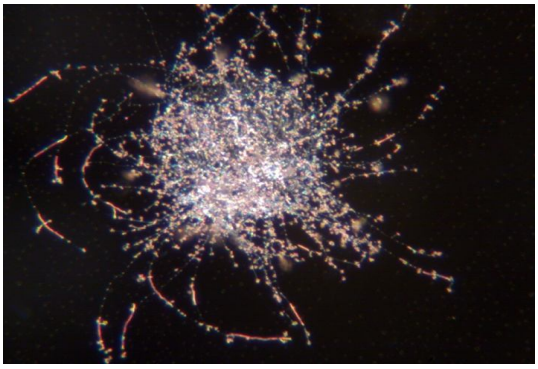
For the ITS region, PCR conditions included 3 min initial denaturation at 94 °C, 35 cycles of 1 min at 94 °C,1 min at 50 °C, and 1.5 min at 72 °C followed by final extension of 7 min at 72 °C. In the case of EF1-a and Bloc regions, 3 min initial denaturation at 94 °C, 35 cycles of 1 min at 94 °C,1 min at 55 °C, and 1 min at 72 °C followed by final extension of 10 min at 72 °C. Bands of expected size were run on a 1% agarose gel containing ethidium bromide in 1X TAE buffer and were visualized by UV illumination using a digital imaging system. Nucleic acid concentration and purity ratios were obtained using a NanoDrop. Purified PCR products were sequenced from both ends using the following primers Approximately 3-20 ngof DNA were used in sequencing reactions with the ABI Prism® BigDye® Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems, Foster City, CA). Cycle sequencing was performed on a GeneAmp® PCR System 9700 or 2720 Thermal Cycler (Applied Biosystems) according to the manufacturer's protocol (BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol, Rev A, Applied Biosystems).Dye terminators were removed from the cycle sequencing reactions using Multiscreen-HV plates (Millipore, Mississauga, ON) loaded with Sephadex G-50 superfine (Sigma, Oakville, ON). Clean reactions were analyzedon an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). A minimum read length of 700 bp was generated for each of the reactions. Chromatograms were analyzed using ABI Prism® DNA Sequencing Analysis Software Version 4 (Applied Biosystems) to generate quality target sequences within the software's clear confidence range. Single nucleotide polymorphism (SNP) analysis, Indels (insertion/deletion) and mutation detection,and phylogenetic tree construction were performed using the computer programs Mafft and Blast (Katoh etal., 2002;Altschul et al.,1990)(http://www.ncbi.nlm.nih.gov).

III. RESULTS

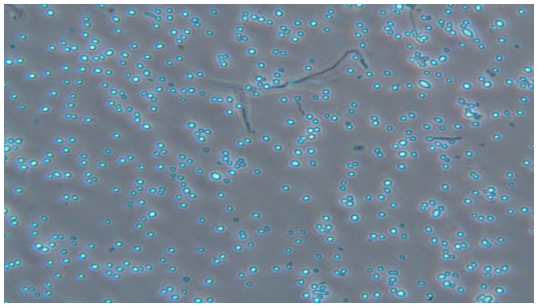
Adult beetles (Ips typographus) that were infected with entomopathogenic fungi were found in regions of Georgia under tree bark in the maternal gallery. The fungi were identified using microscopic preparations made directly from mycelia developing on beetles in dead bark. After morphological analysis of monocultures, individual isolates of Beauveria bassiana were collected. Conidia dimensions were between (1.5) 2.0 – 3.0 (4.0) x (1.5) 2.0 – 2.5 (- 3.0) µm (Roberts a. Humber 1981; Humber, 1997).



A

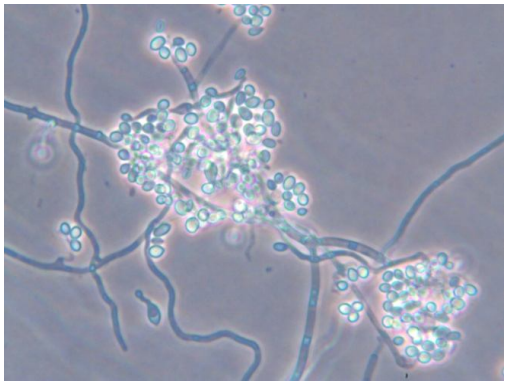


B



C

Fig.1 1Conidia of Beauveria bassiana sample B1. A - growing on PDA (Potato Dextrosa Agar) culture medium; B- colony (x75), C - pure spores (x300), D- conidia and conidiophores (x600)



D

In this study, we characterized genotypes of the entomopathogenic fungi Beauveria bassiana from various regions of Georgia.To the best of our knowledge, this report describes the first time that Beauveria spp. in Georgia have been characterized using molecular genetics. The following genetic markers for characterization of Beauveria were used in our analysis: the ITS region of the rRNA gene cluster, a region of EF1-α, and the intergenic Bloc region. Mega6, Mafft, Blast, and DNASTAR were used to analyze nucleic acid sequence data. Strains from different geographical regions were compared using phylogenetic analysis of sequences of multiple gene fragments. Samples obtained from the same region were grouped together as clades within the phylogenetic tree (Figs. 2 and3). Sequences of Georgian species were compared to other strains from outside of Georgia (Meyling, Eilenberg, 2006a). Georgian strains were found to belong to multiple clades, with the greatest homology in samples belonging to the A or C clade. Samples collected in this study were identified as Beauveria bassiana (99-100% coincidence) through comparison of sequences obtained from the ITS region of the ribosomal gene with those published in GenBank. ITS5 and ITS4 primers are verified reference sequences for PCR amplification of unknown species. Partial sequences of two nuclear loci, including EF1-α and the intergenic Bloc region, were also used in our analysis. EF1-α was selected for analysis because of its widespread use in phylogenetic studies of Beauveria and other fungi. (Rehner a. Buckley 2005; Sung et al. 2007). Bloc is a nuclear intergenic region initially developed for investigation of cryptic speciation in B. bassiana (Rehner et al. 2006a). In samples that were obtained from two different regions of Georgia, the following sequence differences were identified: two samples from the Borjomi and three samples from Shovi were identical to one another, respectively (Figs. 2 and 3).



Fig. 2 EF1- alpha region of samples 5S,4S,3S,2S (Shovi) and 6b,1b (Borjomi)

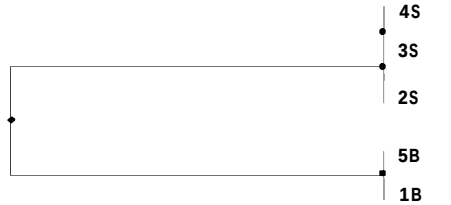


Fig. 3 ITS region 4S, 3S, 2S(Shovi) and 5B, 1B (Borjomi)

Georgian DNA samples were compared with literature data Georgian strains are located in diferent clades. The highest level of homology was detected between samples located in A and C clade. The EF1-alpha and the intergenic Bloc region were also compared (in contrast to ITS region - the rRNA gene cluster, EF1-alpha, Bloc region are more variable and contains more information) of Georgian samples with the samples taken from various country, located in the different clades (Rehner et, al2011). Samples from Borjomi belong to clade A, whereas samples from Shovi belong to clade C in the phylogenic tree (Pic. 2 EF1-alpha comparing with other samples).

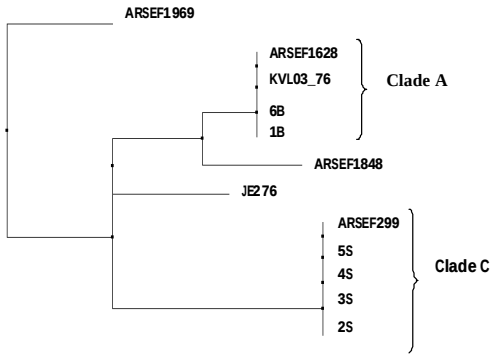


Fig. 4. Elongation factor-1a (EF1-alfa) phylogeny of Beauveriabassiana (B1, B6, S2, S3, S4, S5) ConstructedNeighbour Joining tree using PID (Waterhouse et al 2009). The GenBank accession used for the analyses are Beauveria bassiana samples: AY531896(ARSEF1628),EF193188(KVL03-76), AY531904(ARSEF1848),EF193181(KVL03-114), DQ376244(JE276), EF193192(KVL 03-91), AY531921, (ARSEF292)AY531923(ARSEF299), AY531907(ARSEF1969).

Only two samples were obtained for the Bloc region (Borjomi region). Both samples are identical and differ by one SNP from GenBank isolates. Comparison of KVL 03_76 (Denmark, clade A) (Meiling et al., 2011) with Georgian samples is described in Table.1

Samples	Nucleotide position	Nucleotide
KVL 03 76	1118	G
B1, B6	549	A

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