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Evolutionary Explanation for *Beauveria bassiana* Being a Potent Biological Control Agent Against Agricultural Pests

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Beauveria bassiana (Cordycipitaceae, Hypocreales, Ascomycota) is an anamorphic fungus having a potential to be used as a biological control agent because it parasitizes a wide range of arthropod hosts including termites, aphids, beetles and many other insects. A number of bioactive secondary metabolites (SMs) have been isolated from *B. bassiana* and functionally verified. Among them, beauvericin and bassianolide are cyclic depsipeptides with antibiotic and insecticidal effects belonging to the enniatin family. Non-ribosomal peptide synthetases (NRPSs) play a crucial role in the synthesis of these secondary metabolites. NRPSs are modularly organized multienzyme complexes in which each module is responsible for the elongation of proteinogenic and non-protein amino acids, as well as carboxyl and hydroxyacids. A minimum of three domains are necessary for one NRPS elongation module: an adenylation (A) domain for substrate recognition and activation; a tholation (T) domain that tethers the growing peptide chain and the incoming aminoacyl unit; and a condensation (C) domain to catalyze peptide bond formation. Some of the optional domains include epimerization (E), heterocyclization (Cy) and oxidation (Ox) domains, which may modify the enzyme-bound precursors or intermediates.

In the present study, we analyzed genomes of *B. bassiana* and its allied species in Hypocreales to verify the distribution of NRPS-encoding genes involving biosynthesis of beauvericin and bassianolide, and to unveil the evolutionary processes of the gene clusters. Initially, we retrieved completely or partially assembled genomic sequences of fungal species belonging to Hypocreales from public databases. SM biosynthesizing genes were predicted from the selected genomes using antiSMASH program. Adenylation (A) domains were extracted from the predicted NRPS, NRPS-like and NRPS-PKS hybrid genes, and used them to construct a phylogenetic tree. Based on the preliminary results of SM biosynthetic gene prediction in *B. bassiana*, we analyzed the conserved gene orders of beauvericin and bassianolide biosynthetic gene clusters among the hypocrealean fungi. Reciprocal best blast hit (RBH) approach was performed to identify the regions orthologous to the biosynthetic gene cluster in the selected fungal genomes.

A clear recombination pattern was recognized in the inferred A-domain tree in which A-domains in the 1st and 2nd modules of beauvericin and bassianolide synthetases were grouped in CYCLO and EAS clades, respectively, suggesting that two modules of each synthetase have evolved independently. In addition, inferred topologies were congruent with the species phylogeny of Cordycipitaceae, indicating that the gene fusion event have occurred before the species divergence. Beauvericin and bassianolide synthetases turned out to possess identical domain organization as C-A-T-C-A-NM-T-T-C. We also predicted precursors of beauvericin and bassianolide synthetases based on the extracted signature residues in A-domain core motifs. The result showed that the A-domains in the 1st module of both synthetases select D-2-hydroxyisovalerate (D-Hiv), while A-domains in the 2nd modules specifically activate L-phenylalanine (Phe) in beauvericin synthetase and leucine (Leu) in bassianolide synthetase. antiSMASH ver. 2.0 predicted 15 genes in the beauvericin biosynthetic gene cluster of the *B. bassiana* genome dispersed across a total length of approximately 50kb. The beauvericin biosynthetic gene cluster contains beauvericin synthetase as well as *kivr* gene encoding NADPH-dependent ketoisovalerate reductase which is necessary to convert 2-ketoisovalarate to D-Hiv and a gene encoding a putative Gal4-like transcriptional regulator. Our syntenic comparison showed that species in Cordycipitaceae have almost conserved beauvericin biosynthetic gene cluster although the gene order and direction were sometimes variable. It is intriguing that there is no region orthologous to beauvericin synthetase gene in *Cordyceps militaris* genome. It is likely that beauvericin synthetase was present in common ancestor of Cordycipitaceae but selective gene loss has occurred in several species including *C. militaris*. Putative bassianolide biosynthetic gene cluster

consisted of 16 genes including bassianolide synthetase, cytochrome P450 monooxygenase, and putative Gal4-like transcriptional regulator genes. Our synteny analysis found that only *B. bassiana* possessed a bassianolide synthetase gene among the studied fungi. This result is consistent with the groupings in A-domain tree in which bassianolide synthetase gene found in *B. bassiana* was not grouped with NRPS genes predicted in other species. We hypothesized that bassianolide biosynthesizing cluster genes in *B. bassiana* are possibly acquired by horizontal gene transfer (HGT) from distantly related fungi. The present study showed that *B. bassiana* is the only species capable of producing both beauvericin and bassianolide. This property led to *B. bassiana* infect multiple hosts and to be a potential biological control agent against agricultural pests.