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Evaluating the Diversity of *Chit18-5* Gene Region across *Trichoderma* Species for Effective Biocontrol Strategies

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Abstract: *Trichoderma* species are widely used as biological agents to control plant diseases. Chitinases are crucial in mycoparasitism and defense against other fungi or arthropods. In this study, we evaluated 41 amino acid sequences related to the *Chit18-5* gene from four sections and 15 *Trichoderma* species. The conserved domains, motifs, and phylogenetic tree were analyzed using the InterProScan database, COBALT tool, MEME V5.5.1 software, ClustalW algorithm, and MEGA11 software. The results showed that the gene region under investigation can effectively distinguish different *Trichoderma* species and is an effective tool for optimizing biocontrol strategies. This study highlights the potential of exploring genetic diversity as a means of identifying new solutions for managing pests and diseases in agriculture. The putative motifs of chitinase proteins identified in this study may participate in *Trichoderma* antagonistic activities.

Keywords: *Trichoderma*, Chit18-5 gene region, biocontrol, phylogenetic tree, genetic diversity.

INTRODUCTION

Biological agents such as *Trichoderma* species are frequently employed to manage plant diseases (Mukherjee et al. 2022). By combining mycoparasitism, antibiosis, induced defense response (IDR), and competition, *Trichoderma* spp. aid in the disease's suppression (Sharma et al. 2017). The capacity of some species to parasitize other plant-pathogenic fungi sparked the initial investigation into the potential use of *Trichoderma* in biological control (Weindling 1932). Glucanases, chitinases, and proteases are recognized as important enzymes in mycoparasitism (Cortes et al. 1998, Vazquez-Garciduenas et al. 1998, Carsolio et al. 1999), because the cell wall of true fungi is made up of glucans, chitin, and proteins (Gow et al. 2017, Ruiz-Herrera & Ortiz-Castellanos 2019, Garcia-Rubio et al. 2020). Furthermore, β-1,4-linked N-acetyl-D-glucosamine (GlcNAc) monomers form the renewable polymer known as chitin, which is the second most prevalent polysaccharide in nature. It is an important part of the exoskeleton of arthropods and the cell walls of fungi. Enzymes called chitinases (EC 3.2.1.14) hydrolyze the bonds that hold GlcNAc residues together. According to Karlsson & Stenlid (2008), they are essential for a variety of biological processes, such as autolysis, hyphal development, branching, and cell wall remodeling during spore germination and constriction. In order to supply nutrients and engage in an aggressive pattern of competition and defense against other fungi or arthropods, they also break down exogenous chitin found in the hyphal cell wall or the exoskeleton of arthropods. They accomplish this by killing their fungal prey and then feeding on the contents of the dead cells (Karlsson & Stenlid, 2008, Seidl-Seiboth et al. 2014, Seidl 2008). Because chitinases are involved in defense responses against infections, previous research on them has mostly concentrated on gene cloning and transformation, particularly with regard to the ech-42 gene (Bolar et al. 2000, De La Cruz et al. 1992, Emani et al. 2003, Garcia et al. 1994, Gentile et al. 2007). However, recent advances in genomic analyses have expanded our understanding of chitinase genes and their classification models for filamentous fungi. For instance, Seidl et al. (2005) used the *T. reesei* QM6a genome as a reference to describe the first genome-wide investigation of fungal chitinase genes. They identified eighteen chitinases that were phylogenetically categorized into A, B, and C, each of which was further subdivided into many subgroups. Among fungal taxa, the size of the chitinase gene family varies significantly. For instance, while *Schizosaccharomyces pombe* has only one chitinase gene, *Trichoderma* virens has as many

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as 36 (Karlsson & Stenlid 2008, Kubicek et al. 2011). It is noteworthy that every fungal chitinase that has been identified solely falls under the glycoside hydrolase (GH) family 18 (Seidl et al. 2005). Each year, insect pests and plant-pathogenic fungi seriously harm crops. Fortunately, genera in the order Hypocreales, including *Beauveria*, *Metarhizium*, and *Trichoderma*, are effective mycoparasites that inhibit these pests (Shah & Pell 2003, Woo et al. 2014). Alternatives to fungicides that are safe, long-lasting, effective, and environmentally friendly are becoming more and more necessary in modern agriculture. In order to fulfill this requirement, these biological resources have been used as biocontrol agents in agriculture (Wang & Zhuang 2019, Wang & Zhuang 2020). Chitinases play a crucial role in the initial stage of mycoparasitic infections, which involves the lysis of the host cell wall (Herrera-Estrella & Chet 2003, Howell 2003, Druzhinina et al. 2011). Interesting concerns about the evolution of this significant gene family are raised by the expansion and contraction of the chitinase gene family as well as the molecular mechanisms underpinning these changes. Recent research has shown that mycoparasitic fungal species have higher levels of group B and group C chitinases, although their amino acid sequences have a low degree of conservation. According to these results, evolutionary paths should become more diverse (Seidl-Seiboth et al. 2014, Ihrmark et al. 2010). Through a bioinformatics analysis of the amino acid sequences of the Chit18-5 protein, this study intends to use genomic information to analyze the relationship between chitinases and the mycoparasitic lifestyle of *Trichoderma* species.

MATERIALS AND METHODS

In this study, were evaluated 41 amino acid sequences related to the *Chit18-5* gene from four sections and 15 *Trichoderma* species (Table 1S). Sequences of 100-400 amino acids were received in FASTA file format from the UniProtKB protein bank (Coudert et al. 2023). The conserved domains in this gene region were checked by using the InterProScan database and COBALT tool in the NCBI database (Papadopoulos & Agarwala 2007, Jones et al. 2014). Identifying protected motifs and determining the position of these motifs in domains, using MEME V5.5.1 software with default parameters, including choosing motifs regardless of the frequency of repetition, identifying a maximum of 10 motifs and the lowest and the analysis focused on the maximum length of motifs, which were between 6 and 50 amino acids long (Bailey et al. 2015). The amino acid sequences were aligned using the ClustalW algorithm and the Neighbor-Joining tree was drawn with MEGA11 software (Tamura et al. 2021). The strength of the internal branches of the resulting trees was tested with bootstrap (BS) analysis using 1000 replications (Felsenstein 1985). In addition, the amino acid sequences were translated into nucleotide sequences using the tblastn tool in the NCBI gene bank, and the nucleotide sequences with the highest percentage of similarity were received in FASTA file format. The nucleotide sequences were aligned using the ClustalW algorithm and the phylogeny tree was drawn as above mentioned. To root the phylogenetic tree, the amino acid and nucleotide sequence of *Trichoderma atroviride* related to the *Chit18-3* gene was used as the outgroup taxa.

RESULTS AND DISCUSSION

Assessing the gene region related to *Chit18-5* by using the InterProScan database showed that there are three to five conserved domains in the amino acid sequences associated with this gene region (Table 1), and these domains were confirmed in the sequences that were evaluated (Fig. 1). Furthermore, the presence of domains was confirmed using the COBALT tool in the NCBI database (Fig. 2). The results of the assessment of sequences with MEME V5.5.1 software revealed two to nine different motifs in all the amino acid sequences that were evaluated. The differences in the positions of these motifs led to the separation of the sequences. The identified motifs and their distribution in the amino acid sequences investigated in this research are shown in Fig.s 3 and 4. The abundance of each amino acid indicates its prevalence in the related motif. Our results reveal the presence of diverse domains and motifs within the gene region associated with chitinase enzymes across *Trichoderma* species, both intra- and inter-species.

Two phylogenetic trees were constructed using amino acid (Fig. 5) and nucleotide (Fig. 6) sequences. Examining of both trees demonstrated a clear separation of species, with those sharing the same name clustering (Fig. 5). Furthermore, the evaluated species in this study were classified into four sections of the *Trichoderma* genus, namely *Harzianum* and *Virens*, *Longibrachiatum*, *Pachybasium*, and *Trichoderma*, based on their placement in the respective sections. The findings suggest that the gene region under investigation can effectively distinguish these sections.

Trichoderma atroviride, related to the *Chit18-3* gene, was assigned as the outgroup taxa. The results showed that the separation was done correctly due to the difference between these two gene regions, and the tree was rooted accordingly. The alignment of chitinase proteins indicated that this gene region is highly conserved, particularly in its domains, and has a relatively similar structure across different *Trichoderma* species. Upon examining both phylogenetic trees, it was observed that, in most cases, the order of species placement was consistent between the two trees.

Trichoderma species are notoriously difficult to identify morphologically due to their high diversity. As a result, molecular methods have been employed in various studies for species identification

In this research, two phylogenetic trees were compared, revealing that chitinase proteins-specifically the *Chit18-5* gene region - exhibit greater inter-species diversity than intra-species diversity. This can be attributed to the presence of conserved domains within the protein. The results suggest that the gene region studied is an effective tool for distinguishing between different *Trichoderma* species.

The results of this study highlighting the diversity in amino acid sequences of the *Chit18-5* gene region among many *Trichoderma* species may have significant practical implications for the agricultural sector. One of the most significant biopolymers found in nature is chitin, which is mostly produced by nematodes, arthropods, and fungi. It serves as a scaffold in insects, supporting the peritrophic matrices that line the intestinal epithelium as well as the cuticles of the trachea and epidermis. Growth and morphogenesis in insects are exclusively dependent on their ability to reorganize structures that include chitin. In several tissues, insects continuously manufacture chitin synthases and chitinolytic enzymes for this reason. Strict control of the involved enzymes is necessary for the coordination of chitin synthesis and degradation during development (Merzendorfer & Zimoch 2003). In true fungi such as Ascomycota, Basidiomycota, and Chytridiomycota cell walls are usually based on glucans and chitin, and also the fibers are chitin microfibrils, i.e. bundles of linear b-(1,4)-linked N-acetylglucosamine chains (Webster & Weber 2007). There is strong evidence that wall-lytic enzymes like chitinases and glucanases have an impact on the softness of the apical cell wall (Fontaine et al. 1997, Horsch et al. 1997). It has been demonstrated that the wall of resting spores contains chitin and is notably thick (Moxham & Buczacki 1983). The enzyme EC 3.2.1.14 - chitinase binds to chitin. It randomly cleaves glycosidic linkages in chitin and chitodextrins in a non-processive mode, generating chitooligosaccharides and free ends on which exo-chitinases and exo-chitodextrinases can act (Rottloff et al. 2011). Not all *Trichoderma* strains can exhibit strong antagonistic effects against pathogens (Liu et al. 2022).

Table 1. The list of identified domains by using InterProScan

Domains	Accession number
Glycoside hydrolase family 18, catalytic domain	All sequences
Glycosyl hydrolases family 18 (GH18) domain profile	All sequences
Glycosyl hydrolases family 18	All sequences
Chitinase II	All sequences except E5F5N0
2g34	All sequences except E5F5N0

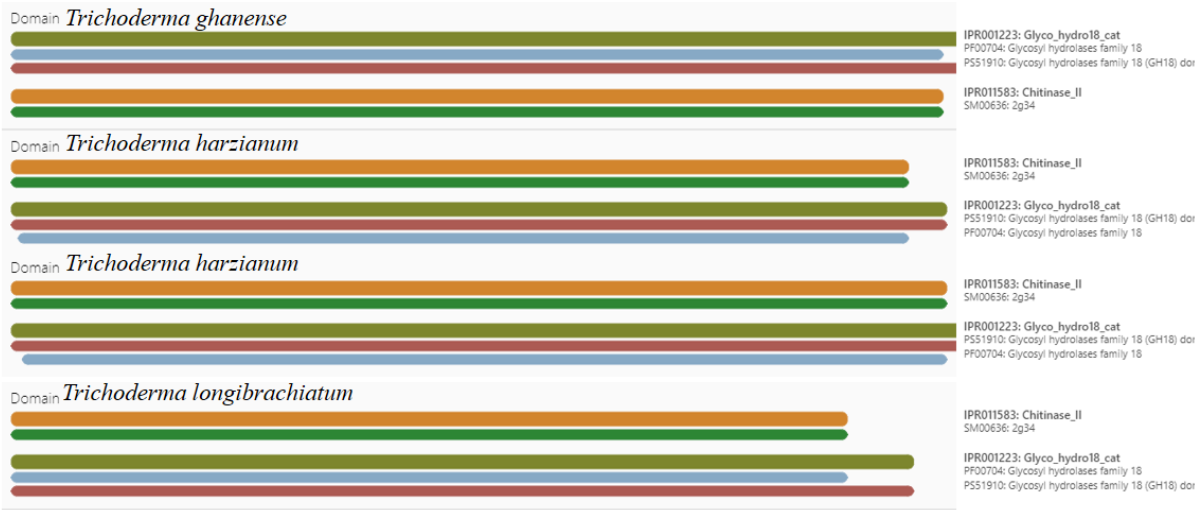

Fig. 1. The structure of identified domains by using InterProScan

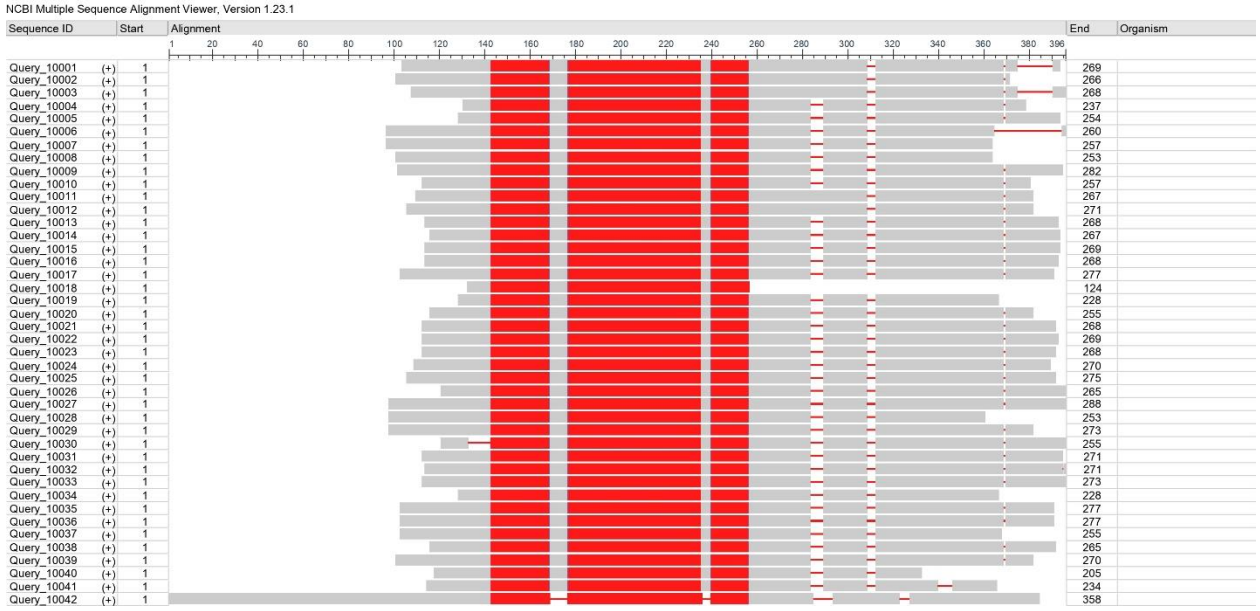

Fig. 2. The structure of identified domains by using the COBALT tool

Fig. 3. Motif logo in amino acid sequences by MEME software; the size of each amino acid in a motif indicates its relative abundance.

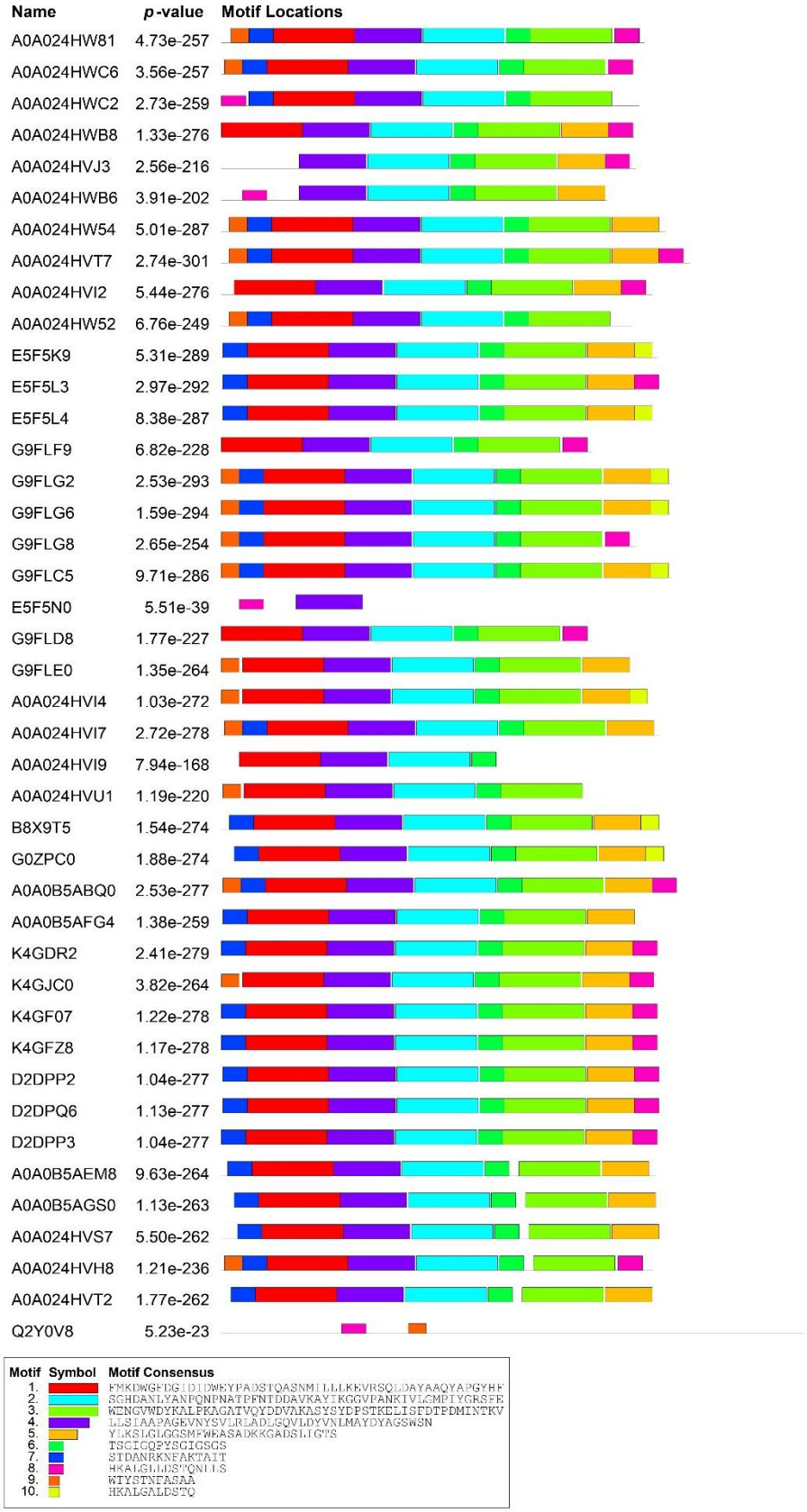

Fig. 4. Analysis of motifs in amino acid sequences and distribution of identified motifs by MEME software

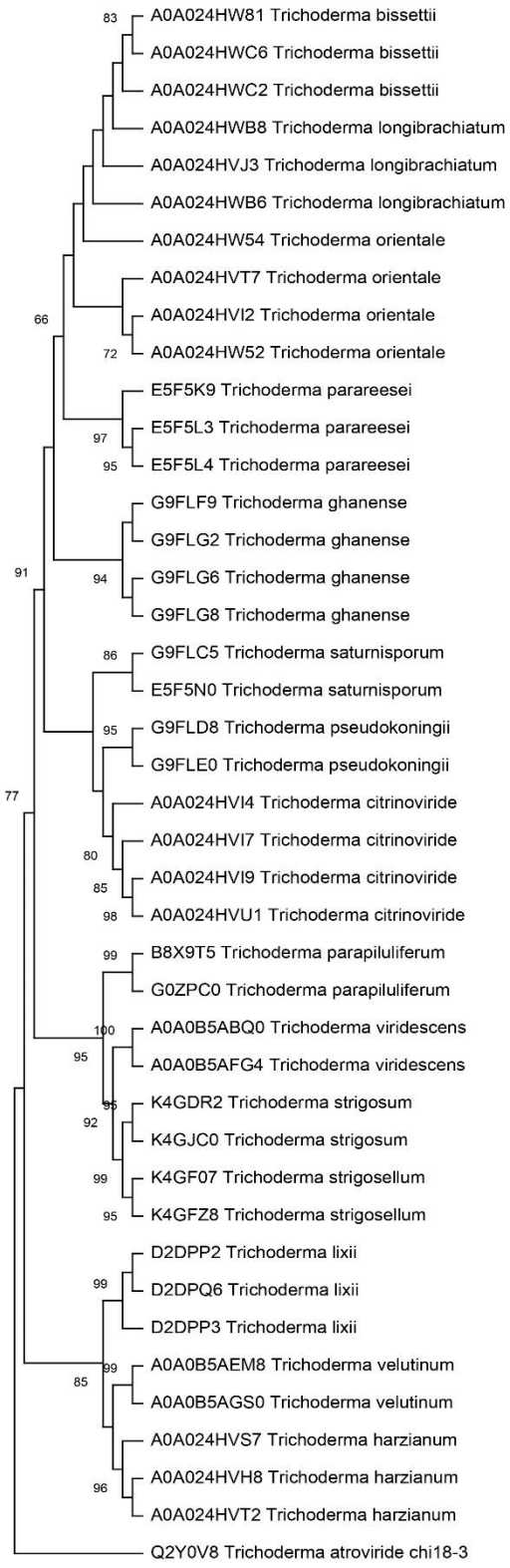

Fig. 5. Phylogenetic tree of 41 amino acid sequences in different *Trichoderma* species by using MEGA11 software, Neighbor-Joining method, and Bootstrap analysis with 1000 replications

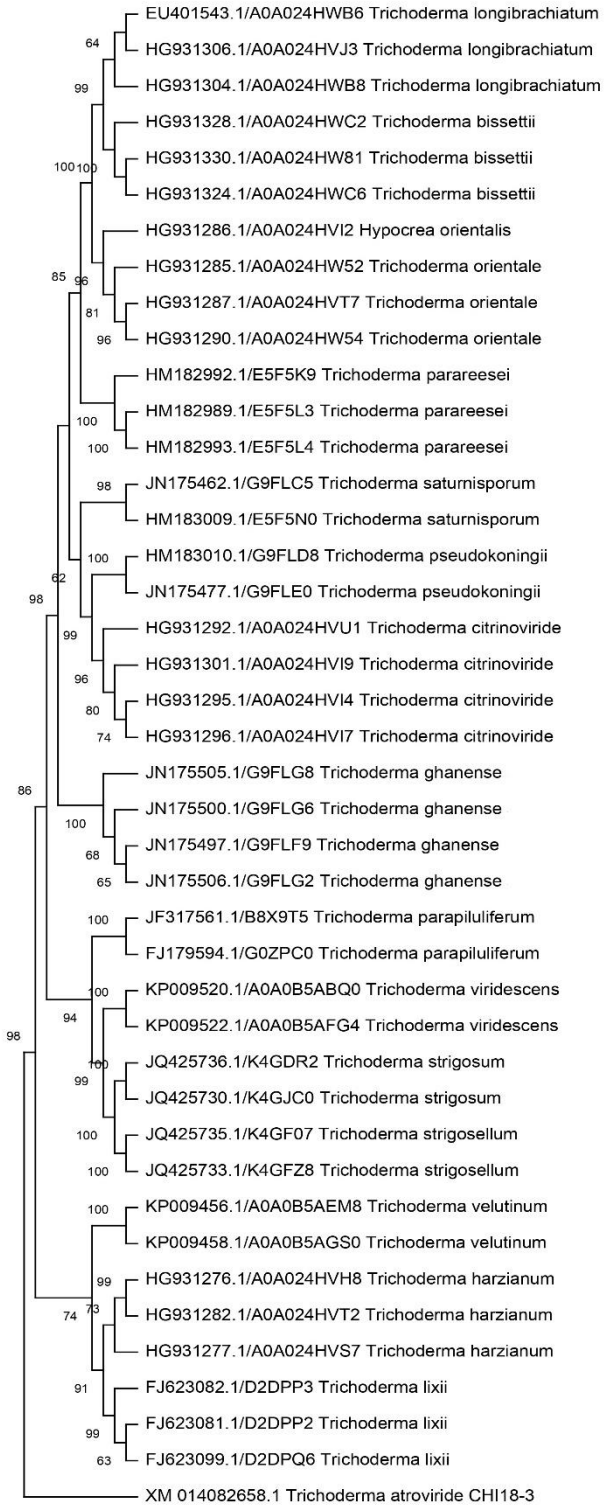

Fig. 6. Phylogenetic tree of 41 nucleotide sequences resulting from tblastn chitinase protein sequences in different *Trichoderma* spp. by using MEGA11 software, Neighbor-Joining method, and Bootstrap analysis with 1000 replications

The ability to distinguish between other *Trichoderma* species using this gene region could be a valuable tool for the identification of strong species optimizing biocontrol strategies and selecting the most effective *Trichoderma* strains for specific crop pests and diseases. Furthermore, these findings could also lead to further research exploring the evolutionary mechanisms behind the diversification of the *Chit18-5* gene family and their relationship with mycoparasitism and other biological processes. The putative motifs of chitinase proteins identified in this study may participate in *Trichoderma* antagonistic activities. It has been shown that some proteins participate in plant defense pathways related to *Trichoderma* effector functions. In maize and cotton, for example, reactive oxygen species buildup and pathogen-related gene expression responses are induced by cerato-platanin proteins Sm1 from *T. virens* and its homolog Epl1 from *T. atroviride* (Seidl et al. 2006). This could shed light on new approaches for developing more effective biocontrol agents against plant pathogens and insect pests. Overall, this study highlights the potential of exploring genetic diversity as a means of identifying new solutions for managing pests and diseases in agriculture. By understanding the genetic mechanisms behind biocontrol agents like *Trichoderma*, we can develop targeted and sustainable strategies for enhancing crop yields and protecting the environment by reducing the usage of chemical fungicides and pesticides. As stated by Bononi et al. (2020), the biological control strategy primarily consists of the synthesis of particular chemicals and the uptake of specific micronutrients to plants in order to enhance defense and improve plant growth.

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ارزیابی تنوع ناحیه ژن Chit18-5 در گونه‌های تریکودرما برای استراتژی‌های کنترل زیستی موثر

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چکیده: گونه‌های تریکودرما به عنوان عوامل زیستی برای کنترل بیماری‌های گیاهی، بطور گسترده مورد استفاده قرار می‌گیرند. کیتینازها در میکوپارازیتیسم و دفاع در برابر سایر قارچ‌ها و بندپایان، بسیار مهم هستند. در این مطالعه، ۴۱ توالی اسیدآمینه مربوط به ناحیه ژن *Chit18-5* از چهار بخش و ۱۵ گونه تریکودرما، بررسی شد. شناسایی و ارزیابی دومین‌ها، موتیف‌های حفاظت‌شده و درخت تبارزایی با استفاده از پایگاه داده InterProScan، ابزار COBALT، نرم‌افزار MEME V5.5.1، الگوریتم ClustalW و نرم‌افزار MEGA11، صورت گرفت. نتایج نشان داد که ناحیه ژنی مورد بررسی می‌تواند به طور موثر گونه‌های مختلف تریکودرما را از یکدیگر تفکیک نماید و ابزار موثری برای بهینه‌سازی استراتژی‌های کنترل زیستی است. این مطالعه، پتانسیل بررسی تنوع ژنتیکی را به عنوان ابزاری برای شناسایی راه حل‌های جدید برای مدیریت آفات و بیماری‌ها در کشاورزی، برجسته می‌نماید. موتیف‌های احتمالی پروتئین‌های کیتینازی شناسایی شده در این مطالعه ممکن است در فعالیت‌های آنتاگونیستی تریکودرما نقش داشته باشند.

کلمات کلیدی: تریکودرما، ناحیه ژن *Chit18-5*، کنترل زیستی، درخت تبارزایی، تنوع ژنتیکی

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