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NATURE

KUMULATIVE DISSERTATION

**RESOLVING THE CONUNDRUM OF *TRICHODERMA* TAXONOMY:
HOW ECOLOGICAL GENETICS COUPLED WITH EVOLUTIONARY ANALYSIS ENHANCES THE
POLYPHASIC SPECIES CONCEPT IN FUNGI**

ausgeführt zum Zweck der Erlangung des akademischen Grades eines
Doktor der Naturwissenschaften
unter der Leitung von

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“Nomina si nescis, perit et cognitio rerum”

Carl Linnaeus “Botanical Philosophy”, ed. 1, Stockholm & Amsterdam, 1751.”

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SUMMARY

Fungi comprise one of the most diverse groups of eukaryotes on Earth. Nearly two thousand new species are described each year, while the number of known fungal species - approximately 150,000, is estimated to hardly exceed only a few percent of the total diversity in this kingdom. The rapid growth of fungal taxonomy is escalated by the outstanding role that fungi play for human kind because new species may possess new properties. Fungi produce a rich spectrum of hydrolytic enzymes, surfactant proteins, and secondary metabolites that found their application in industry, pharmaceuticals, and agriculture. Many fungi are valuable for the food production; others are exploited as industrial cell factories to synthesize bioactive compounds or serve as models for cell biology research. Some biotrophic fungi can cause diseases in humans, and cattle or parasitize crops. Being ubiquitous in most ecosystems, fungi perform essential ecological processes as symbionts and decomposers. Fungi have a simple structure of their body that is either tubular (mycelium, or hyphae) or single-celled (spores, or yeasts). This morpho-anatomical organization frequently undergoes convergent evolution making the precise and accurate species identification required to predict their application or pathology-relevant properties challenging.

In the first part of this thesis, we focused on the diversity, species delimitation, and molecular identification of nearly four hundred species of the plant-beneficial and industrially-relevant filamentous fungi from the genus *Trichoderma* (Hypocreales, Ascomycota). We compiled a complete inventory of all *Trichoderma* species and DNA barcoding materials deposited in public databases. We have then developed an authoritative guideline for molecular identification of *Trichoderma* that requires analysis of the three DNA barcodes (ITS, *tef1*, and *rpb2*) and supported it by several online tools. We then used all the whole-genome sequenced (WGS) *Trichoderma* strains to provide versatile, practical examples of *Trichoderma* DNA Barcoding, reveal methodological and theoretical shortcomings, and discuss possible ambiguities. The work provides an in-depth discussion of species concepts applied in *Trichoderma* taxonomy. We conclude that these fungi are particularly suitable for implementing integrative taxonomy that fuses DNA Barcoding and the polyphasic phenotyping. Subsequently, we tested the applicability of the developed DNA Barcoding protocol on the collection of *Trichoderma*

spp. strains isolated from the emerging salt marches on the Yellow Sea coastal tidal flat zones. This study resulted in the discovery of *T. arenarium* sp. nov. and other fungal bioeffectors suitable for biosaline agriculture. The above taxonomic studies and the review of the ecological genomics of the most industrially-relevant species of *Trichoderma* - *T. reesei* revealed the critical shortage of species recognition criteria in the genus and highlighted the theoretical shortcomings of our understanding of the speciation process in fungi. To overcome it, we have proposed that speciation can be reflected in the evolution of genes relevant to fungal fitness (ecological genetics). For this purpose, in the second part of the thesis, we optimized the toolbox for the genetic recombination of *Trichoderma* and reviewed the strain improvement techniques available for these fungi. Our previous study discovered that the highly surface-active small secreted cysteine-rich proteins (saSSCPs) – hydrophobins (HFBs), strongly influence the fitness of the two sister species from the *Harzianum* Clade of *Trichoderma*. It showed that HFB evolution and function analysis could reveal distinct adaptations of sympatric species to microecological niches. Therefore, in the third part of the thesis, we studied the other family of saSSCPs that are massively secreted by *Trichoderma* – cerato-platanins (CPs). The *in silico* analysis of 283 CPs from 157 fungal genomes revealed the long evolutionary history of CPs in Dikarya fungi that have undergone several lateral gene transfer events and multiple gene duplications. Three genes were maintained in the core genome of *Trichoderma*, while some species have up to four CP-encoding genes. However, the functional analysis of CPs revealed that only EPL1 is active at all development stages but plays a minor role in interactions with other fungi and bacteria. The deletion of this gene resulted in increased colonization of rhizosphere by *Trichoderma* spp. Similarly, the biochemical tests of the heterologously produced EPL1 by *Pichia pastoris* support the above claims. Overall, this study pointed to the evolutionary and functional paradox of CPs in fungi. The high diversity and stabilizing selection suggest their importance for the speciation process.

In summary, the studies presented in this thesis provide the conceptual and methodological framework for establishing the integrative taxonomy of *Trichoderma* spp. that should rely on DNA Barcoding and consider the genetic background of ecological adaptations driving the evolution and speciation. We conclude that applying the polyphasic approach to species recognition in *Trichoderma* and other highly diverse genera of environmentally opportunistic fungi will finally resolve the conundrum of fungal taxonomy.

KURZFASSUNG

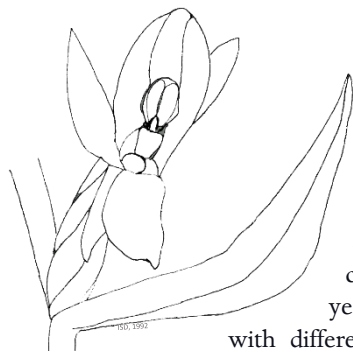
Pilze der Gattung *Trichoderma* (Hypocreales, Ascomycota) sind für den Menschen von besonderer Bedeutung aufgrund der Verwendung einzelner Arten in der Enzym produzierenden Biotechnologie, als biologische Pflanzenschutzmittel und Stimulatoren des Pflanzenwachstums in der Landwirtschaft, oder aber als Besiedler immunschwacher Menschen und Tiere. Gegenwärtig sind fast 400 Spezies bekannt, aber deren sichere Identifizierung ist schwierig. Ich habe mich daher in meiner Arbeit mit der Identifizierung der einzelnen Spezies sowie den möglichen Mechanismen der Artenbildung bei *Trichoderma* beschäftigt.

Als ersten Schritt hierzu habe ich eine auf DNA-Sequenzanalyse beruhende Methode („DNA Barcoding“) entwickelt: zu diesem Zweck habe ich die DNA-Sequenzen dreier Barcode-Marker (ITS, *tefi* und *rpb2*) aller Spezies verglichen, und eine bioinformatische Analysenmethode erarbeitet die eine sichere Speziesidentifizierung ermöglicht.

Die Methode wurde in der Folge anhand nicht identifizierter *Trichoderma* Isolate aus einem Salzmarsch-Ökosystem an der Küste des Gelben Meers getestet und verifiziert, was auch zur Identifizierung und Beschreibung einer neuen *Trichoderma* Art – *T. arenarium* sp. nov. - führte. Im Zuge dieser Untersuchung konnten – als sekundärer „Benefit“ - auch zahlreiche Isolate gefunden werden welche als Bioeffektoren für die Pflanzenzucht auf salzreichen Böden geeignet sind.

Die oben beschriebenen Ergebnisse zeigten aber auch dass die bei der Speziesbildung wirksamen Mechanismen in *Trichoderma* nur ungenügend bekannt sind. Ich habe daher in Weiterführung meiner Arbeit die Theorie aufgestellt dass die Speziesbildung in der Evolution von Genen für die Fitness im Ökosystem reflektiert sein müsste. Ich habe dies am Beispiel zweier Typen sogenannter “surface active, small secreted and cysteine rich” (saSSCPs) Proteine – der Hydrophobine (HFB) und der Ceratoplatanine (CPs) – analysiert. Meine Untersuchungen an *T. harzianum* und *T. guizhouense* zeigen dass die Funktion und Evolution der HFBs tatsächlich mit der Anpassung sympatrischer Spezies an ökologische Nischen korreliert. Im Falle der CPs konnte ich eine auf stabilisierender Selektion beruhende Evolution zeigen, was deren Rolle bei der Anpassung an das jeweilige Ökosystem verdeutlicht.

Meine Arbeit hat daher einen neuen Weg zur Identifizierung von *Trichoderma* Arten aufgezeigt und darüber hinaus Mechanismen der Artenbildung entdeckt. Diese Befunde sind nicht nur für *Trichoderma* bedeutsam sondern können auch als Vorlage für ähnliche Untersuchungen bei anderen Pilzgattungen dienen.



ACKNOWLEDGEMENTS

I was born in Zhuji, a small city surrounded by mountains in Zhejiang Province, southeast China. Given grown up there, I was always attracted by plants and forests, especially by orchids. I started my first orchid collection when I was 12. The collection grew fast as every year the boy went to the forests hunting for new “species” with different colors and flower shapes, what I now know are “phenotypes,” and those could probably be “populations”. The orchid collection stopped growing when I had to leave my hometown for college study at Nanjing Agricultural University (NAU), where I chose Plant Nutrition as my direction. The study was impressive; however, it somehow did not meet my goals for understanding the orchids. The books regarding orchids in the university library were read three times and daily checked for updating. I think something was attracting me there what maybe can be called “diversity”. However, I could not figure out at that moment. It was just that they are too many and too different. Without hesitation, I studied in this direction for a master and for a Ph.D. degree on which plant beneficial microbes became my primary research materials. In 2017, I completed the Ph.D. thesis in the direction of Plant Nutrition. For this, I would like to express my sincere gratitude to my supervisor Prof. Qirong Shen for his positive influence, and also for the support of my intention to continue my education in the TU Wien, Vienna, where I met another Ph.D. mentor, Irina S. Druzhinina, and her group.

During the first period of studying in TU WIEN, 2014-2017, I tried the best to adjust to the local environment and laboratory life, which were somewhat different from that of the other side of the globe. Luckily, a lady Günseli Bayram Akcapinar was in this group too. Being under the direct tuition by Günseli, I grabbed the lab skills of biotechnology quickly. Therefore, I would like to express my heartfelt thanks to Günseli, who first influenced me with her insightful ideas, practical advice, and feasible instructions. Working together with the HFB team members Agnieszka Przylucka, Komal Chenthamara, and Tatyana Yemelyanova, I noticed the value of being in a team and appreciate all their general helps, useful discussions, and supports, though ladies, your full names are still so hard for me. I would also express my deepest thanks to Marica Grujic (Maric), with whom we spent the most working deep nights together. Thanks to Carina Pretzer for her delightful lab sorting and general help. Furthermore, Komal again, by whom I got a glimpse of “phylogeny”, which turned out to be an essential word in my current research. Therefore, many thanks to the ladies Marica, Carina, and Komal for their support in work and their friendship, no matter local and the long distance.

Thanks to Civan Yagtu, Saliha Durak, Simger Seven, and Victor Lobanov, for working together with me as master students and more than that. Thanks to Mohammad Rahimi for delicious food and interesting chatting topics. Thanks to Youzhi Miao for working together on the CRISPR system through which I learned more than it. Thanks to Alexey Kopchinskiy for always helping solve imaging and IT-related problems. Thanks to Shadi Pourmehdi, Lea Atanasova, Melanie Grandis, Christina Ortner, and all MIBI group members with whom the lab is often joyful.

The completion of this dissertation would not be possible without the full support from my Ph.D. mentor Irina S. Druzhinina, who opened the “gate” of EVOLUTION and ECOLOGICAL GENETICS as well as TAXONOMY to me. Working with Irina for more than six years, I keep growing in these directions and on all other related topics. I appreciate her guidance over the last years for helping me digging and finding my genuine interest and offering me an opportunity to go to MBL to learn MOLECULAR EVOLUTION, which is the crucial term for my research interest in the future. Moreover, I think this is also the key term that I missed since a boy collecting different orchids. With the guidance and daily scientific debates with Irina, I finally found a person with whom I can communicate my research interests and whose influence helps me see myself more and more clearly.

I am incredibly thankful to my boss’s boss Christian Kubicek, for his long-distanced collaboration and full yet justified supports for my scientific arguments with Irina. Every face-to-face talk and email exchange was so bright and meaningful when it is with an abbreviation of CPK.

This is also a perfect time to thank Fakultät für Technische Chemie. A special thanks to the Head of the ICEBE, Robert Mach and Astrid Mach-Aigner, for organizing high-standard and bright labs over the years, allowing international students to feel more inclusive and qualified, and also for their collaboration for hosting me remotely during this COVID hard time. Thanks to Monika Schmoll, I learned the topic of signaling in fungi and scientific writing skills. The research project could not have been completed without the help of external collaborators: Erik Reimhult from BOKU, Hinrich Grothe, Bernhard Seiboth, Laura Felgitsch, Christian Derntl, and Thiago Machado Mello De Sousa, from TU WIEN, Igor V. Grigoriev, and Andrei S. Steindorff from JGI (USA).

I would also acknowledge the China Scholarship Council (CSC) for providing me with the personnel cost during my stay in Vienna. Furthermore, thanks to my local colleagues Zheng Zhao, Ren Wei Gao, Siqi Jiang, Mingyue Ding, and Guan Pang, with whose supports I could complete my second Ph.D. dissertation more sufficiently.

With a delightful but reluctant mood, I wrote down this acknowledgment, meaning that my TU WIEN Ph.D. student life is pending to end. I shall express my wishes to the people I mentioned above and those I could not list here good health and a bright future.

Thank you all!

INTRODUCTION

Fungi comprise one of the most diverse groups of eukaryotes on Earth. Nearly two thousand new species are described each year, while the number of known fungal species - approximately 150,000, is estimated to hardly exceed only a few percent of the total diversity in this kingdom (1). The rapid growth of fungal taxonomy is escalated by the outstanding role that fungi play for human kind because new species may possess new properties. Fungi produce a rich spectrum of hydrolytic enzymes, surfactant proteins, and secondary metabolites that found their application in industry, pharmaceuticals, and agriculture (2). Many fungi are valuable for the food production; others are exploited as industrial cell factories to synthesize bioactive compounds or serve as models for cell biology research. Some biotrophic fungi can cause diseases in humans, and cattle or parasitize crops. Being ubiquitous in most ecosystems, fungi perform essential ecological processes as symbionts and decomposers. Fungi have a simple structure of their body that is either tubular (mycelium, or hyphae) or single-celled (spores, or yeasts). This morpho-anatomical organization frequently undergoes convergent evolution making the precise and accurate species identification required to predict their applied or pathology-relevant properties challenging (2).

Ubiquitous mycotrophic and phytosaprotrophic fungi from the genus *Trichoderma* (syn. *Hypocrea*, Hypocreales) have been known to mycologists for more than 200 years that is tractable in the scientific literature (3-5). Similar to other common fungi, the last two decades sharply transformed *Trichoderma* to the species-rich genus (6-10) that made it comparable to such fungi as *Fusarium* (Hypocreales), *Aspergillus*, or *Penicillium* (Eurotiales) and left all sister hypocrealean or even the model genus for fungal biology *Neurospora* (Sordariales) far behind. The increase in the total number of *Trichoderma* species was not strongly influenced by the general mycological movement “One fungus – one name” (11), as the connection with the single *Hypocrea* teleomorph (with only a few exceptions) has been established earlier and considered in the first species counts (6, 9). The drastic increase in *Trichoderma* species number can be explained by the emerging importance of *Trichoderma* for humankind. Approximately 50 years ago, *T. reesei* was recognized as a highly efficient producer of plant biomass-degrading enzymes for biofuel and other industries (12). A couple of decades later, several other species (*T. atroviride*, *T. virens*, *T. harzianum*, and others) were proposed as potent bioeffectors for plant protection (*biofungicides*) and plant growth promotion (*biofertilizers*) (13), and they are now widely used for biological control of fungal pests in

sustainable agriculture (*biocontrol*). *Trichoderma* was also documented as the causative agent of the green mold disease on mushroom farms and as an opportunistic pathogen in humans. Although the applications are still restricted to a few species, the growth of species richness positively influences the *Trichoderma* science development as the number of *Trichoderma*-based publications grows proportionally to it. Another striking property of *Trichoderma* that makes it a useful model of taxonomic studies is the evident lack of hidden diversity or “dark *Trichoderma* species” (14), meaning that most or all species can be successfully cultivated *in vitro*. Therefore, *Trichoderma* spp. can potentially be extensively phenotypically and physiologically characterized along with taxonomic or nomenclatural acts. The possibility of the extended ecophysiological profiling paves the way for the introduction of the integrative (polyphasic) taxonomy for species delimitation, i.e., the combination of genealogy (phylogeny), phenotype (including autecology), and even possibly reproductive biology (when feasible) (2). The analysis of a relatively large number of whole-genome sequences (WGS) for *Trichoderma* spp. also provided insights into the evolutionary timeline of this genus (15, 16). Thus, *Trichoderma* can serve as a useful model for the observation of taxonomic development with an impact on the precision, accuracy, and ambiguity of species delimitation and subsequent identification.

Therefore, the aim of this thesis was the holistic evaluation of the whole-genus *Trichoderma* systematics from the perspective of modern integrative (polyphasic) taxonomy.

The structure of the resulting thesis is best presented by the three sections, each consisting of one to three publications (**Figure 1**).

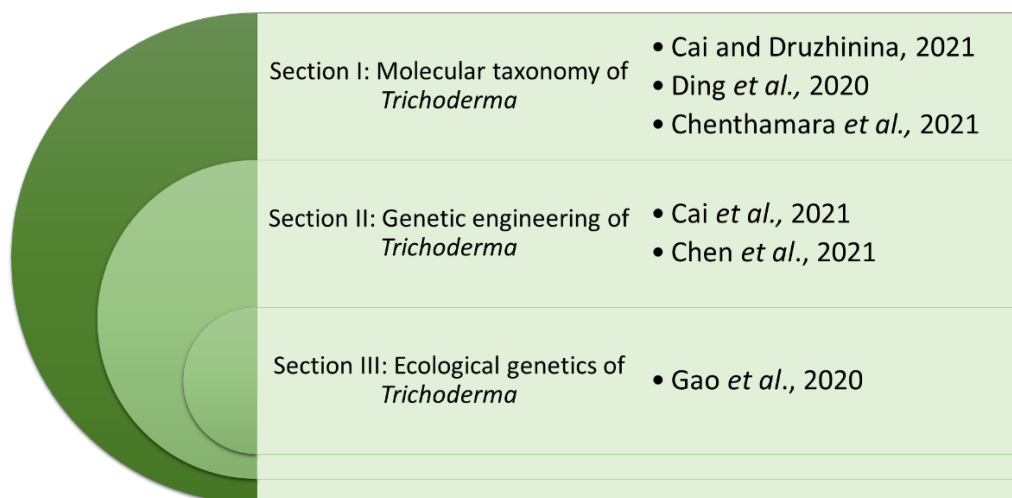


FIGURE 1. STRUCTURE OF THE THESIS

Section I

The first section of this thesis includes two research articles and a theoretical part summarized in the book chapter (**Figure 1**).

First, we focused on the diversity, species delimitation, and molecular identification of nearly four hundred species *Trichoderma*. We compiled a complete inventory of all *Trichoderma* species and DNA barcoding materials deposited in public databases. The results of this work were presented in the scientific peer-reviewed publication “**Cai, F., Druzhinina, I. S. 2020. In honor of John Bissett: Authoritative guidelines on molecular identification of *Trichoderma*. *Fungal Diversity*. DOI: 10.1007/s13225-020-00464-4**”. As specified in the title, the core of this work is the authoritative guideline for molecular identification of *Trichoderma* that requires analysis of the three DNA barcodes (ITS, *tef1*, and *rpb2*) and is supported by several online tools. We then used all the whole-genome sequenced (WGS) *Trichoderma* strains to provide versatile, practical examples of *Trichoderma* DNA Barcoding, reveal methodological and theoretical shortcomings, and discuss possible ambiguities. This work provides an in-depth discussion of species concepts applied in *Trichoderma* taxonomy. We conclude that these fungi are particularly suitable for implementing integrative taxonomy that fuses DNA Barcoding and the polyphasic phenotyping.

Methodology: DNA Barcoding of fungi, molecular evolutionary analysis, theoretical biology, fungal taxonomy, fungal genomics.

Own contribution: initialization of pairwise similarity threshold for fungal species delimitation, molecular phylogenetic analyses, development of DNA Barcoding protocol, involvement in manuscript writing and revision, preparation of figures and tables.

Subsequently, we tested the applicability of the developed DNA Barcoding protocol on the collection of *Trichoderma* spp. strains isolated from the emerging salt marshes on the Yellow Sea coastal tidal flat zones. This study resulted in the scientific peer-reviewed publication “**Ding, M., Chen, W., Ma, X., Lv, B., Gao, R., Jiang, S., Zhao, Z., Cai, F., Druzhinina, I. S. 2020. Emerging salt marshes as a source of *Trichoderma arenarium* sp. nov. and other fungal bioeffectors for biosaline agriculture. *Journal of Applied Microbiology*. DOI:10.1111/jam.14751**”. Our motivation was the urgent need of effective and safe *biofertilizers* and *biofungicides* for the sustainable agriculture. Natural ecosystems that closely resemble the conditions of *biosaline* agriculture may present a reservoir for fungal strains that can be used as novel *bioeffectors*. We isolated a library of fungi from the rhizosphere of three natural halotolerant plants grown in the emerging tidal salt marshes on the southeast coast of China. DNA barcoding of 116 isolates based on the rRNA ITS1 and 2 and other markers (*tef1* or *rpb2*) revealed 38 fungal species, including plant pathogenic (41%), saprotrophic (24%), and mycoparasitic (28%) taxa. The mycoparasitic fungi were mainly species from the hypocrealean genus *Trichoderma*, including at least four novel phylotypes. Two of them, representing the taxa *Trichoderma arenarium* sp. nov. (described in this publication) and *T. asperelloides*, showed effective antagonistic activity against five phytopathogenic fungi, and significant growth promotion on tomato seedlings under the conditions of saline agriculture. Thus, *Trichoderma* spp. of salt marshes play the role of natural biological control in young

soil ecosystems with a putatively premature microbiome. The saline soil microbiome is a rich source of halotolerant *bioeffectors* that can be used in *biosaline* agriculture.

Methodology: Bacterial metagenomic, soil property, field sampling, DNA Barcoding of fungi, molecular evolutionary analysis, ecophysiological characterization of fungi, fungal-plant interaction assay, dual confrontation assays of fungi, basic microbiology and molecular biology, light and electron microscopy.

Own contribution: specified in the publication; author for correspondence.

The theory on molecular evolution of *Trichoderma* was then summarized and critically reviewed in “Chenthamara, K., Rahimi, M., Grujic, M., Druzhinina, I. S., Cai, F. 2021. *Trichoderma reesei* – Methods and Protocols: Chapter 1 Ecological genomics and evolution of *Trichoderma reesei*, Mach-Aignar, A., and Martzy, R., eds. *Methods in Molecular Biology*, Springer Nature, pp 1-21. DOI: 10.1007/978-1-0716-1048-0_1” The filamentous fungus *Trichoderma reesei* (Hypocreales, Ascomycota) is an efficient industrial cell factory for the production of cellulolytic enzymes used for biofuel and other applications. Therefore, research addressing *T. reesei* is relatively advanced compared to other *Trichoderma* spp. because of the significant bulk of available knowledge, multiple genomic data, and gene manipulation techniques. However, the established role of *T. reesei* in industry has resulted in a frequently biased understanding of the biology of this fungus, where the valuable applied properties could be extrapolated to the environmental adaptations of the fungus. Thus, the recent studies unexpectedly show that the superior cellulolytic activity of *T. reesei* and other *Trichoderma* species evolved due to multiple lateral gene transfer events, while the innate ability to parasitize other fungi (mycoparasitism) was maintained in the genus, including *T. reesei*. In this chapter, we follow the concept of ecological genomics and describe the ecology, distribution, and evolution of *T. reesei*, as well as critically discuss several common misconceptions that originate from the success of this species in applied sciences and industry.

Methodology: DNA Barcoding of fungi, molecular evolutionary analysis, theoretical biology, fungal taxonomy, ecological genomics.

Own contribution: conceptualization and structuring, molecular phylogenetic analyses, participation in manuscript writing and revision, preparation of figures and tables.

The above taxonomic studies and the review of the ecological genomics of the most industrially-relevant species of *Trichoderma* - *T. reesei* revealed the critical shortage of species recognition criteria in the genus and highlighted the theoretical shortcomings of our understanding of the speciation process in fungi. To overcome it, we have proposed that speciation can be reflected in the evolution of genes relevant to fungal fitness (ecological genetics).

Section II

For this purpose, in the second section of the thesis, we summarized our previously obtained knowledge on the optimization of the toolbox for the genetic recombination of *Trichoderma*. It resulted in the publication of two book chapters, both are currently *in press*.

“Cai, F., Kubicek, C. P., Druzhinina, I. S. 2021. **Biofuels and Biodiesel: Genetic transformation of *Trichoderma* spp.** Chhandak B., ed. **Methods in Molecular Biology, Springer Nature ISBN 978-1-0716-1322-1**” The production of biofuels from plant biomass is dependent on the availability of enzymes that can hydrolyze the plant cell wall polysaccharides to their monosaccharides. These enzyme mixtures are formed by microorganisms but their native compositions and properties are often not ideal for application. Genetic engineering of these microorganisms is therefore necessary, in which introduction of DNA is an essential precondition. The filamentous fungus *Trichoderma reesei* – the main producer of plant-cell-wall-degrading enzymes for biofuels and other industries – has been subjected to intensive genetic engineering towards this goal and has become one of the iconic examples of the successful genetic improvement of fungi. However, the genetic manipulation of other enzyme-producing *Trichoderma* species is frequently less efficient and, therefore, rarely managed. In this chapter, we therefore describe the two potent methods of *Trichoderma* transformation mediated by either (i) polyethylene glycol (PEG) or (ii) *Agrobacterium*. The methods are optimized for *T. reesei* but can also be applied for such transformation-resilient species as *T. harzianum* and *T. guizhouense*, which are putative upcoming alternatives for *T. reesei* in this field.

Methodology: genetic transformation of fungi using polyethylene glycol (PEG) and *Agrobacterium*-based protocols.

Own contribution: development of the protocols, participation in writing and revision, preparation of figures and tables.

“Chen, P.J., Pang, G., Cai, F., Druzhinina, I. S. 2021. **Strain improvement and genetic engineering of *Trichoderma* for industrial applications.** Zaragoza O., and Casadevall A., eds. **Encyclopedia of Mycology, Elsevier ISBN: 9780128199909**”. The excellent abilities of cellulase production for biofuel and other industries and the plant-beneficial potential of *Trichoderma* spp. have led this fungus to being subjected to intensive genetic engineering and become one of the iconic examples for fungal genetics. Genetic manipulation to improve the strains is a highly effective means of meeting tailor-made applications. In this chapter, we review the technologies and methods that have been developed for *Trichoderma* strain improvement, including untargeted mutagenesis, targeted genetic recombination, RNA interference, promoter engineering, and the new promising genome editing technology-CRISPR/Cas9.

Methodology: the whole spectrum of genetic manipulation with fungi.

Own contribution: conceptualization and structuring, critical reviewing of the described approaches, participation in manuscript writing and revision, preparation of figures and tables.

Our previous study discovered that the highly surface-active small secreted cysteine-rich proteins (saSSCPs) – hydrophobins (HFBs), strongly influence the fitness of the two sister species from the *Harzianum* Clade of *Trichoderma*. The results presented in “Cai, F., Gao, R., Zhao, Z., Ding, M., Jiang, S., Yagtu, C., Zhu, H., Zhang, J., Ebner, T., Mayrhofer-Reinhartshuber, M., Kainz, P., Chenthamara, K., Bayram-Akcapinar, G., Shen, Q., Druzhinina, I. S. 2020. Evolutionary compromises in fungal fitness: hydrophobins hinder the adverse dispersal of spores and challenge their survival. *The ISME Journal*. 14:2610–2624. DOI: 10.1038/s41396-020-0709-0” (see *Appendix* to the Thesis) showed that HFB evolution and function analysis could reveal distinct adaptations of sympatric species to microecological niches.

Section III

Therefore, in the third section of the thesis, we studied the family of saSSCPs that are massively secreted by *Trichoderma* – cerato-platanins (CPs) in submerged conditions: “Gao, R., Ding, M., Jiang, S., Zhao, Z., Chenthamara, K., Shen, Q., Cai, F., Druzhinina I.S. 2020. The evolutionary and functional paradox of cerato-platanins in fungi. *Applied and Environmental Microbiology* 86:e00696-20. DOI: 10.1128/AEM.00696-20”.

Cerato-platanins (CPs) form a family of SSCP and are of particular interest not only because of their surface activity but also their abundant secretion by fungi. We performed an evolutionary analysis for 283 CPs from 157 fungal genomes with the focus on the opportunistic plant-beneficial and mycoparasitic fungus *Trichoderma*. Our results revealed the long evolutionary history of CPs in Dikarya fungi that have undergone several events of lateral gene transfer and gene duplication. Three genes were maintained in the core genome of *Trichoderma*, while some species have up to four CP-encoding genes. All *Trichoderma* CPs evolve under stabilizing natural selection pressure. The functional analysis of CPs in *T. guizhouense* and *T. harzianum* revealed that only EPL1 is active at all stages of the development but plays a minor role in interactions with other fungi and bacteria. The deletion of this gene results in increased colonization of tomato roots by *Trichoderma* spp. Similarly, the biochemical tests of the heterologously produced EPL1 by *Pichia pastoris* support the above claims. Based on the obtained results, we conclude that the function of CPs is probably linked to their surfactant properties and the ability to modify the hyphosphere of submerged mycelium and thus facilitate the nutritional versatility of fungi. The effector-like properties do not sufficiently describe the diversity and evolution of these proteins in fungi as they are also maintained, duplicated, or laterally transferred in the genomes of non-herbivore fungi.

Methodology: Fungal genomics, molecular evolutionary analysis, genetic transformation, heterologous production of proteins, ecophysiological characterization of fungi, fungal-plant interaction assay, plant immunity assays, dual confrontation assays of fungi, basic microbiology and molecular biology.

Own contribution: specified in the publication; author for correspondence.

In summary, the studies presented in this thesis provide the conceptual and methodological framework for establishing the integrative taxonomy of *Trichoderma* spp.

that should rely on DNA Barcoding and consider the genetic background of ecological adaptations driving the evolution and speciation (Figure 2). We conclude that applying the polyphasic approach to species recognition in *Trichoderma* and other highly diverse genera of environmentally opportunistic fungi will finally resolve the conundrum of fungal taxonomy.

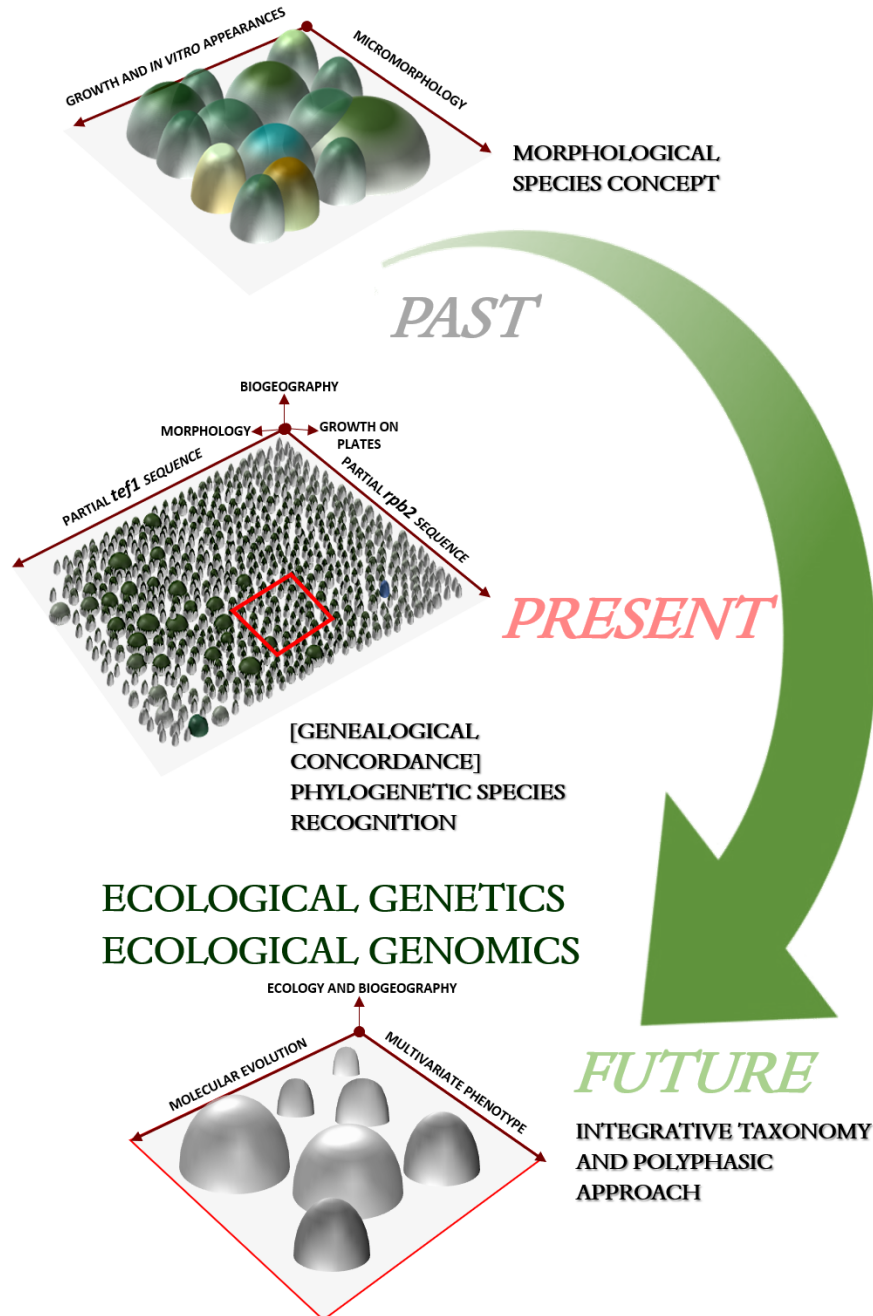


FIGURE 2. THE SCHEMATIC TIMELINE OF *TRICHODERMA* SPECIES RECOGNITION AS DESCRIBED IN CAI AND DRUZHININA, 2020, FUNGAL DIVERSITY.

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2. R. Lücking *et al.*, Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? *IMA Fungus* **11**, 14 (2020).
3. A. Y. Rossman *et al.*, Genera in Bionectriaceae, Hypocreaceae, and Nectriaceae (Hypocreales) proposed for acceptance or rejection. *IMA Fungus* **4**, 41-51 (2013).
4. W. M. Jaklitsch, H. Voglmayr, Biodiversity of *Trichoderma* (Hypocreaceae) in Southern Europe and Macaronesia. *Stud Mycol* **80**, 1-87 (2015).
5. W. M. Jaklitsch, H. Voglmayr, New combinations in *Trichoderma* (Hypocreaceae, Hypocreales). *Mycotaxon* **126**, 143-156 (2013).
6. I. S. Druzhinina, A. G. Kopchinskiy, C. P. Kubicek, The first 100 *Trichoderma* species characterized by molecular data. *Mycoscience* **47**, 55-64 (2006).
7. W. M. Jaklitsch, European species of *Hypocrea* Part I. The green-spored species. *Stud Mycol* **63**, 1-91 (2009).
8. W. M. Jaklitsch, European species of *Hypocrea* part II: species with hyaline ascospores. *Fungal Divers* **48**, 1-250 (2011).
9. L. Atanasova, I. S. Druzhinina, W. M. Jaklitsch, "Two hundred *Trichoderma* species recognized on the basis of molecular phylogeny" in *Trichoderma: biology and applications*, P. K. Mukherjee, B. A. Horwitz, U. S. Singh, M. Mukherjee, M. Schmoll, Eds. (CABI, Croydon, UK, 2013), 10.1079/9781780642475.0010 chap. 2, pp. 10-42.
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11. J. W. Taylor, One Fungus = One Name: DNA and fungal nomenclature twenty years after PCR. *IMA Fungus* **2**, 113-120 (2011).
12. I. S. Druzhinina, C. P. Kubicek, Familiar Stranger: Ecological genomics of the model saprotroph and industrial enzyme producer *Trichoderma reesei* breaks the stereotypes. *Adv Appl Microbiol* **95**, 69-147 (2016).
13. I. S. Druzhinina *et al.*, *Trichoderma*: the genomics of opportunistic success. *Nat Rev Microbiol* **9**, 749-759 (2011).
14. M. A. Friedl, I. S. Druzhinina, Taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species that regulate each other's development. *Microbiology* **158**, 69-83 (2012).
15. I. S. Druzhinina *et al.*, Massive lateral transfer of genes encoding plant cell wall-degrading enzymes to the mycoparasitic fungus *Trichoderma* from its plant-associated hosts. *PLoS Genet* **14**, e1007322 (2018).
16. C. P. Kubicek *et al.*, Evolution and comparative genomics of the most common *Trichoderma* species. *BMC Genomics* **20**, 485 (2019).

CURRICULUM VITAE

PERSONAL DATA

Name	Feng Cai, Ph. D
Address	Ziqizhongshan 2-417, Xuanwu Daodao 699-39, Nanjing, Jiangsu, 210000, China
Contact	Tel.: +86 13505199590 E-mail: czfscf@hotmail.com
Date and place of birth	23 Sep. 1988, Zhuji, Zhejiang, China
Web links	www.FungiG.org <u>ResearchGate:</u> https://www.researchgate.net/profile/feng_cai9 ORCID: 0000-0003-2032-6190

RESEARCH AND WORK EXPERIENCE

2017 - 2020 **Postdoc Fellow (current position)**

Fungal Genomics Group (FungiG), College of Resources and Environment,
Nanjing Agricultural University (NAU), Weigang 1, 210095, Nanjing, China

EDUCATION AND TRAININGS

2019 **Molecular Evolution Workshop**, Marine
Biological Laboratories (MBL), Woods Hole, MS, USA

2011 - 2017 **Joined Master and Ph.D. program** Molecular Biology and Plant-
Microbial Interaction, Nanjing Agricultural University, Nanjing, China

2014 - 2017 **Ph.D program in Fungal Genetics and Genomics** in the
Microbiology and Applied Genomics Group (Head. Prof. Dr. Irina S. Druzhinina),
Institute of Chemical, Environmental & Bioscience Engineering (ICEBE), Vienna
University of Technology (TU Wien), Vienna, Austria

2007 - 2011 **Bachelor study** in Plant Nutrition and Soil Science, Nanjing
Agricultural University, Nanjing, China

FUNDED RESEARCH PROJECTS

- 2019-2021 Functional genetics of surface-active protein HFBs in *Trichoderma* and their relationships with its root colonization, **National Science Foundation of China**, China (31801939), Project leader, 250,000 RMB
- 2018-2021 Characterization of small secreted cysteine-rich proteins from *Trichoderma* and their applications in agriculture, **Ministry of Science & Technology of Jiangsu Province**, China (BK20180533), Project leader, 200,000 RMB
- 2019-2020 Phylogenetic analysis and protein engineering of hydrophobins from *Trichoderma*, **China Postdoctoral Science Foundation**, China (2018M630567), Project leader, 100,000 RMB
- 2017-2019 Functional annotation of the polyketide synthase cluster genes in *Trichoderma*, **National Science Foundation of China**, China (31701992), Principle research associate, 250,000 RMB
- 2017-2019 Analysis of the biosynthesis pathway of a novel secondary metabolite, harzianolide, from *Trichoderma harzianum*, **Ministry of Science & Technology of Jiangsu Province**, China (BK20160726), Principle research associate, 200,000 RMB

EXPERTISE AND TEACHING EXPERIENCE

EXPERIMENTAL SKILLS

- Microbiological techniques and microscopy: axenic cultures, microbial diagnostics by DNA barcoding, fungal morphology, microbial cultivations, electron microscopy (TEM and SEM), *in situ* microscopy (LSCM, epifluorescent microscopy, stereofluorescent microscopy, fluorescent staining techniques) and phenotype microarrays
- The whole scope of molecular biological techniques for algae, fungi and bacteria (qualitative and quantitative nucleic acid manipulation techniques, microbial transformation, CRISPR/Cas9 gene edition)
- Proteomic techniques such as heterologous protein expression in prokaryotic/eukaryotic cell factories, SDS-PAGE and immunoblotting assays
- Microbial metabolomic techniques (HPLC, LC/MS, GC/MS and NMR)
- Physicochemical methods (QCM, DLS, IFT, WCA, CD and AFM)
- Fermentations of bacteria and fungi (research scale)
- Plant physiology and soil science (soil and plant biochemical assays)
- Lab management skills

IN SILICO SKILLS

Molecular evolutionary analyses such as multiloci phylogenetics, phylogenomics, lateral gene transfer tests (T-Rex, Notung), natural selection pressure analyses, DNA barcoding and protein modeling, fungal taxonomy.

Basic skills in genomics (sequence similarity search, manual gene annotation), basic skills in the analyses of NGS data (16S rRNA and ITS rRNA microbiomes, and eukaryotic transcriptomes)

Descriptive and multivariate exploratory statistical techniques, R packages

Bitmap and vector graphic skills

TEACHING EXPERIENCE

2019 – present Teaching assistant,
Graduate course “Ecological Genomics of Fungi”, Nanjing Agricultural
University (Course leader Irina S. Druzhinina)

Advisor and tutor for the student research programs (including ongoing)

	TU Wien, Austria	NAU, China
Ph. D. students	-	3
Master students	3	9
Bachelor students	1	3
Laboratory training	1	2
Total	5	17

PEER-REVIEW ACTIVITY

2021 – present **Applied and Environmental Microbiology** (ASM), Editorial Board member

2020 – present reviewer for **Bioresource Technology** (Elsevier), **mSystem** (ASM), reviewer, **Applied and Environmental Microbiology** (ASM), **Applied Soil Ecology** (Elsevier)

PRESENTATIONS ON THE INTERNATIONAL CONFERENCES

(**Talk**) “Never shall those born to crawl, learn to fly: Evolutionary compromises between spore hydrophobicity and fitness in *Trichoderma*”. The 15th European Conference on Fungal Genetics (ECFG15), February 2020. Rome, **Italy**.

(**Talk**) “Hydrophobins influence fungal fitness by modulating spore dispersal and survival”, Session “Fungal spore: development, dormancy and germination”. The 30th Fungal Genetics Conference, March 2019. Asilomar, CA, **USA**.

(**Poster**) “Unconventional secretion of hydrophobins by aerial hyphae resembles the autophagy and explains the conidiation landscape of *Trichoderma* colony”. The 30th Fungal Genetics Conference, March 2019. Asilomar, CA, **USA**.

(**Poster**) “Hydrophobins constitute the major part of the massive extracellular matrix of the conidiating *Trichoderma* colony and influence its fitness by modulating spore dispersal and survival”. The 30th Fungal Genetics Conference, March 2019. Asilomar, CA, **USA**.

(**Poster**) “Hydrophobins of *Trichoderma guizhouense* inhibit tomato defense system for successful colonization of rhizosphere”. The 13th European Conference on Fungal Genetics (ECFG13), April, 2016. Paris, **France**.

(**Poster**) “HFB8, the orphan-hydrophobin of *Trichoderma guizhouense*, is involved in mycoparasitism, surface growth and protects hyphae from fungicides”, The 14th European Conference on Fungal Genetics (ECFG14), February, 2018. Haifa, **Israel**.

LANGUAGE SKILLS

Chinese Native; English Fluent; German Basic

INTERESTS / HOBBIES

Natural history, biodiversity in particular flora, human evolution, science theory.
Personal development and efficient time management.

LIST OF PUBLICATIONS

FIRST- AND CORRESPONDING-AUTHORED ARTICLES

Cai, F., Druzhinina, I. S. **2020**. In honor of John Bissett: Authoritative guidelines on molecular identification of *Trichoderma*. *Fungal Diversity*. DOI: 10.1007/s13225-020-00464-4

Cai, F., Gao, R., Zhao, Z., Ding, M., Jiang, S., Yagtu, C., Zhu, H., Zhang, J., Ebner, T., Mayrhofer-Reinhartshuber, M., Kainz, P., Chenthamara, K., Bayram-Akcapinar, G., Shen, Q., Druzhinina, I. S. **2020**. Evolutionary compromises in fungal fitness: hydrophobins hinder the adverse dispersal of spores and challenge their survival. *The ISME Journal*. 14:2610–2624

Ding, M., Chen, W., Ma, X., Lv, B., Gao, R., Jiang, S., Zhao, Z., **Cai, F.**, Druzhinina, I. S. **2020**. Emerging salt marshes as a source of *Trichoderma arenerea* sp. nov. and other fungal bioeffectors for biosaline agriculture. *Journal of Applied Microbiology*. doi:10.1111/jam.14751

Gao, R., Ding, M., Jiang, S., Zhao, Z., Chenthamara, K., Shen, Q. **Cai, F.**, Druzhinina I.S. **2020**. The evolutionary and functional paradox of cerato-platanins in the mycoparasitic fungi. *Applied and Environmental Microbiology* 86:e00696-20

Cai, F., Pang, G., Li, R.-X., Li, R., Gu, X.-L., Shen, Q.-R., Chen, W. **2017a**. Bioorganic fertilizer maintains a more stable soil microbiome than chemical fertilizer for monocropping. *Biology and Fertility of Soils*. 53:861–872

Cai, F., Pang, G., Miao, Y., Li, R., Li, R., Shen, Q., and Chen, W. **2017b**. The nutrient preference of plants influences their rhizosphere microbiome. *Applied Soil Ecology*. 110:146–150

Cai, F., Chen, W., Wei, Z., Pang, G., Li, R., Ran, W., and Shen, Q. **2015**. Colonization of *Trichoderma harzianum* strain SQR-T037 on tomato roots and its relationship to plant growth, nutrient availability and soil microflora. *Plant and Soil*. 388:337–350

Li, R.-X., **Cai, F.**, Pang, G., Shen, Q.-R., Li, R., and Chen, W. **2015**. Solubilisation of phosphate and micronutrients by *Trichoderma harzianum* and its relationship with the promotion of tomato plant growth. *PLoS ONE*. 10:e0130081 (*shared first authorship*)

Cai, F., Yu, G., Wang, P., Wei, Z., Fu, L., Shen, Q., and Chen, W. **2013**. Harzianolide, a novel plant growth regulator and systemic resistance elicitor from *Trichoderma harzianum*. *Plant Physiology and Biochemistry*. 73:106–113

CO-AUTHORED ARTICLES

Perez-Llano, Y., Rodriguez-Pupo, E.C., Druzhinina, I.S., Chenthamara, K., **Cai, F.**, Gunde-Cimerman, N. et al. **2020**. Stress reshapes the physiological response of halophile fungi to salinity. *Cells* 9.

Zhang, Y., Wang, X., Pang, G., **Cai, F.**, Zhang, J., Shen, Z., Li, R., Shen, Q., **2019**. Two-step genomic sequence comparison strategy to design *Trichoderma* strain-specific primers for quantitative PCR. *AMB Express* 9: 179.

Zhang, J., Miao, Y., Rahimi, M.J., Zhu, H., Steindorff, A., Schiessler, S., **Cai, F.**, Pang, G., Chenthamara, K., Xu, Y., Kubicek, C.P., Shen, Q., Druzhinina, I.S., **2019**. Guttation capsules containing hydrogen peroxide: an evolutionarily conserved NADPH oxidase gains a role in wars between related fungi: The role of hydrogen peroxide in fungal wars. *Environmental Microbiology*.

Hatvani, L., Homa, M., Chenthamara, K., **Cai, F.**, Kocsubé, S., Atanasova, L., Mlinaric-Missoni, E., Manikandan, P., Revathi, R., Dóczy, I., Iványi, B., Bogáts, G., Narendran, V., Büchner, R., Vágvölgyi, C., Druzhinina, I.S., Kredics, L. **2019**. Agricultural systems as potential sources of emerging human mycoses caused by *Trichoderma* *FEMS Microbiology Letters*, in press

Jiang, S.-Q., Yu, Y.-N., Gao, R.-W., Wang, H., Zhang, J., Li, R., Long, X.-H., Shen, Q.-R., Chen, W., **Cai, F.** **2019**. High-throughput absolute quantification sequencing reveals the effect of different fertilizer applications on bacterial community in a tomato cultivated coastal saline soil. *Science of the Total Environment*. 687:601–609

Kubicek, C. P., Steindorff, A. S., Chenthamara, K., Manganiello, G., Henrissat, B., Zhang, J., **Cai, F.**, Kopchinskiy, A. G., Kubicek, E. M., Kuo, A., Baroncelli, R., Sarrocco, S., Noronha, E. F., Vannacci, G., Shen, Q., Grigoriev, I. V., Druzhinina, I. S. **2019**. Evolution and comparative genomics of the most common *Trichoderma* species. *BMC Genomics*. 20

Druzhinina, I. S., Chenthamara, K., Zhang, J., Atanasova, L., Yang, D., Miao, Y., Rahimi, M. J., Grujic, M., **Cai, F.**, Pourmehdi, S., Salim, K. A., Pretzer, C., Kopchinskiy, A. G., Henrissat, B., Kuo, A., Hundley, H., Wang, M., Aerts, A., Salamov, A., Lipzen, A., LaButti, K., Barry, K., Grigoriev, I. V., Shen, Q., Kubicek, C. P. **2018**. Massive lateral transfer of genes encoding plant cell wall-degrading enzymes to the mycoparasitic fungus *Trichoderma* from its plant-associated hosts. *PLOS Genetics*. 14:e1007322

Pang, G., **Cai, F.**, Li, R., Zhao, Z., Li, R., Gu, X., Shen, Q., Chen, W. **2017**. *Trichoderma*-enriched organic fertilizer can mitigate microbiome degeneration of monocropped soil to maintain better plant growth. *Plant and Soil*. 416:181–192

Przylucka, A., Akcapinar, G. B., Chenthamara, K., **Cai, F.**, Grujic, M., Karpenko, J., Livoi, M., Shen, Q., Kubicek, C. P., Druzhinina, I. S. **2017**. HFB7 – A novel orphan hydrophobin of the *Harzianum* and *Virens* clades of *Trichoderma*, is involved in response to biotic and abiotic stresses. *Fungal Genetics and Biology*. 102:63–76

BOOK CHAPTERS

Chenthamara, K., Rahimi, M., Grujic, M., Druzhinina, I. S., **Cai, F.** **2021**. *Trichoderma reesei* – Methods and Protocols: Chapter 1 Ecological genomics and evolution of *Trichoderma reesei*, Mach-Aignar, A., and Martzy, R., eds. *Methods in Molecular Biology*, Springer Nature, pp 1-21

Rahimi, M., **Cai, F.**, Grujic, M., Chenthamara, K., Druzhinina, I. S. **2021**. *Trichoderma reesei* – Methods and Protocols: Chapter 14 Molecular identification of *Trichoderma reesei*. Mach-Aignar, A. and Martzy, R., eds. *Methods in Molecular Biology*, Springer Nature, pp 157-175

Cai, F., Kubicek, C. P., Druzhinina, I. S. **2021**. Biofuels and Biodiesel: Genetic transformation of *Trichoderma* spp. Chhandak B., ed. *Methods in Molecular Biology*, Springer Nature (*in press*)

Chen, P.J., Pang, G., **Cai, F.**, Druzhinina, I. S. **2021**. Strain improvement and genetic engineering of *Trichoderma* for industrial applications. Zaragoza O., and Casadevall A., eds. *Encyclopedia of Mycology*, Elsevier (*in press*)