Genome sequencing and phylogenetic analyses of common and dwarf bunt of wheat provide insights into their genomic diversity and species boundaries, and enable the development of a detection assay for *Tilletia controversa*

Dissertation

to attain the doctoral degree Dr. rer. nat.

of the Faculty of Agricultural Sciences

Georg-August-Universität Göttingen

Submitted by

Somayyeh Sedaghatjoo

born in Mashhad, Iran

Göttingen, July 2021

1st Referee: Prof. Dr. Petr Karlovsky

2nd Referee: Dr. Wolfgang Maier

3rd Referee: Prof. Dr. Andreas von Tiedemann

Date of oral examination: Sep 20th, 2021

Success is not measured by what you accomplish, but by the opposition you have encountered, and the courage with which you have maintained the struggle against overwhelming odds.

Dr. Orison Swett Marden
(1848–1924)

Table of Contents

3

4

5

List of Tables

List of Figures

List of Additional Tables

List of Additional Figures

Chapter 1 – General introduction

1. 1. Genus *Tilletia*

The subphylum Ustilaginomycotina (next to Pucciniomycotina and Agaricomycotina) is one of the three subphyla of the Basidiomycota that accommodates most smut fungi. It contains the classes of Ustilaginomycetes, Exobasidiomycetes (Begerow et al., 1997; Bauer et al., 2006; Hibbett et al., 2013), and recently the Moniliellomycetes and the Malasseziomycetes (Wang et al., 2015b; Riess et al., 2016). Tilletiales is one of the six orders within Exobasidiomycetes (Begerow et al., 1997; Wang et al., 2015b). The genus Tilletia was named in the honor of the French botanist M. Mathieu Tillet's work (Tillet, 1755) by the Tulasne brothers in 1847. Tillet was the first who showed that the cause of wheat bunt was the blackish powder on the contaminated wheat kernels. At the moment, the genus Tilletia comprises nearly some 200 described species (Vánky, 2012; Denchev and Denchev, 2013; Li et al., 2014; Denchev et al., 2018; Denchev and Denchev, 2018b; Denchev and Denchev, 2018a) (Accessed 20.08.2019). The genus is Poaceae parasite and is characterized by the formation of dark-colored teliospores with reticulated ornamentations (Castlebury et al., 2005). Species are mainly classified based on morphological features of the teliospores ornamentation and host specificity. The majority of Tilletia species cause locally infecting bunt diseases and only a few of them cause systemic infections (Carris et al., 2006). Teliospores germinate to produce basidiospores at the terminal of aseptate basidium. Basidiospores almost immediately conjugate (Goates, 1996) and give rise to infectious intercellular hyphae which have capless dolipore septa (Roberson and Luttrell, 1989; Bauer et al., 2006). The cereal-infecting Tilletia species, which produce teliospores in the ovary of the host plant are called bunt fungi (Carris et al., 2006) instead of smut.

1. 2. Wheat bunts; causal agents, epidemiology, and distribution

Four *Tilletia* species are reported to infect wheat species (*Triticum spp.*) and triticale (× *Triticosecale*) namely, *T. caries, T. controversa*, *T. laevis*, and *T. indica*. The type species of the genus is *T. caries* (DC.) Tul. (syn. *T. tritici*). This species as well as *T. laevis* Kühn (syn. *T. foetide*) causes common bunt of wheat in both spring and winter wheat. *Tilletia controversa* Kühn causes dwarf bunt and is restricted to winter wheat (Carris, 2010) and finally *T. indica* Mitra (syn. *Neovossia indica*) is the causal agent of karnal bunt. Four species can be morphologically differentiated based on their teliospores ornaments and sizes. *Tilletia laevis* has smooth teliospores walls of 14 - 17 × 16 - 24 μm diam, while

teliospores of *T. caries* are reticulately ornamented and (14 -)16 - 20(-25) μm in diameter with muri height of 0.5 - 1.5 μm. Both species lack a gelatinous sheath. Teliospores of *T. controversa* are 17 - 21 × 18 - 23 μm diam with muri height of 1.5 - 3 μm. The ornament shape is similar to that of *T. caries,* but the exospore is relatively deeper (Vánky, 2012). Hyaline sheath is reported from absent to prominent in *T. controversa* species (Hess, 1986). Teliospores of *T. indica* can range from 26 - 54 μm in diameter (average of 35 μm) and are tuberculate with cerebriform exospore ornamentation (Mathur and Cunfer, 1993). This makes the species easily distinguishable from other wheat bunts, while the differentiation of *T. caries* from *T. controversa* teliospores is especially difficult due to their ornamentation similarity and presence of great morphological variability within two species (Holton, 1954).

The epidemiology and life cycle of the wheat bunts are distinct from each other. Common bunt (*T. caries* and *T. laevis*) teliospores germinate within a week at the temperature of 15 °C and do not require light. Dwarf bunt (*T. controversa*) teliospores require the optimum temperature of 5 °C and germinate between 6 - 8 weeks. Illumination is essential for the successful germination of teliospores (Carris, 2010). Teliospores of *T. indica* germinate after three weeks of incubation in continuous illumination at 15 - 20 °C (Morris R. Bonde, 1977). Common bunt is soil and seed-borne diseases whereas the source of inoculum in *T. controversa* is mostly teliospores that remain in the soil (Goates and Peterson, 1999). Additionally, common bunt infection occurs shortly after germination of wheat seed whereas *T. controversa* infects seedling during coleoptile emergence (Purdy et al., 1963). In both cases, pathogens remain latent until clum elongation stage. Infection of *T. indica* is via airborne inoculum meaning that primary sporidia on the soil surface are the main inoculum source. The fungus attacks wheat plants while flowering (Morris R. Bonde, 1977; Goates, 1996) and unlike common and dwarf bunt, *T. indica* does not replace the entire kernels of a wheat spike with sori. Infected wheat has a fetid, fishy smell due to the production of trimethylamine in all four species (Hanna et al., 1932; Nielsen, 1963).

Common bunt is distributed worldwide and can be found almost everywhere in wheat cultivating regions. It was reckoned as one of the destructive diseases of wheat in Europe (Strickland, 2008) and the Pacific Northwest of the USA before the 1950s (Hoffmann, 1982). Later, the disease could be controlled by using effective systemic fungicides (Sitton et al., 1993; Goates, 1996). Common bunt is however re-emerging especially in European organic farming due to the lack of resistant cultivars and exclusion of synthetic chemical seed treatments (Borgen and Davanlou, 2001; Matanguihan et al., 2011; Zupunski et al., 2012; Dumalasová et al., 2014). Unfortunately, most of the widely grown local wheat cultivars in Europe are susceptible to common bunt (Waldow and Jahn,

2007; Matanguihan et al., 2011; Aydoğdu and Kaya, 2020) and consequently to dwarf bunt, because the wheat genes confer the common bunt resistance are also responsible in dwarf bunt resistance (Hoffmann and Metzger, 1976). Therefore, seed contamination threshold limitation for control of common and dwarf bunt is practiced in the European Union and other countries. For instance, only one teliospore per wheat kernel in Scotland (Cockerell and McNeil, 2004) and maximum 20 teliospores per wheat kernel in Germany (Spiess and Dutschke, 1991) is accepted to be sown without seed treatments in organic wheat production.

Dwarf bunt is more restricted to higher altitudes and regions with prolonged low temperatures in winter, favorably with clay soil (Conners, 1954; Goates, 1996), which is suitable for its teliospore germination. The disease caused substantial yield loss mostly in the 1990s where fields with 95% infected wheat were reported (Mathre, 1996). The current distribution, as well as loss caused by *T. controversa* in recent years, are largely unknown. One of the recent surveys showed that *T. controversa* plays practically no role in the production of conventional winter wheat in Germany (Rudloff et al., 2020). The same study also reported the expanse of disease to lowland regions in Germany. Altogether, fifteen countries including Algeria, Brazil, Canada, Chile, People's Republic of China, Czech Republic, India, Macedonia, Morocco, New Zealand, Paraguay, Poland, South Africa, Tunisia, and Turkey have documented regulatory restrictions against the importation of *T. controversa*–infested wheat (Peterson et al., 2009). This is done to either prevent the establishment of the fungus (ten countries) or limit the distribution of the disease within the country (five countries).

Tilletia indica is geographically more restricted compared to the other wheat bunt pathogens and its presence is limited to small areas in the United States, Afghanistan, India, Iran, Iraq, Mexico, Nepal, Syria, and South Africa (Royer and Rytter, 1985; Crous et al., 2001; Rush et al., 2005; Jones, 2008). The pathogen has not yet been reported from Europe, and it is therefore treated as A1 quarantine pathogen (OEPP/EPPO, 2018). Common bunt is seldom under phytosanitary regulation for wheat importation. One such country is Mexico where *T. caries* is considered a regulated pathogen (IPPC, 2020).

1. 3. Phylogenetic relationship of common and dwarf bunt

The first phylogenetic analysis of *Tilletia* genus utilizing a part of the nuclear large subunit (nLSU) rDNA gene was published by Castlebury et al. (2005). Analysis based on nLSU showed that reticulate-spored species within *Tilletia* genus formed a well-supported clade from the rest of the taxa. Before this study, the internal transcribed spacer (ITS) rDNA and Polymerase Chain Reaction-

Restriction Fragment Length Polymorphism (RFLP) was employed to differentiate *T. walkeri* (ryegrass bunt) and *T. indica* on the limited number of *Tilletia* species by Levy et al. (2001). Since then, the new species descriptions have been mostly based on ITS in combination with nLSU region, next to morphological analyses and host affiliation (Shivas and McTaggart, 2009; McTaggart et al., 2012; Li et al., 2014). However, a few numbers of taxa (max. 18 species) have been analyzed by molecular phylogenetic analyses for the description of the new *Tilletia* species in those studies. A broader taxa screening based on these two regions was done by Jayawardena et al. (2019). These regions were not variable enough to separate Pooid-infecting species within the genus. Another study based on phenotypic analysis of teliospores combined analysis of DNA sequences of the ITS, translation elongation factor 1 alpha (*EF1α)*, and the second largest subunit of RNA polymerase II *(RPB2)* provided strongly supported clades representing species with a narrow host range on Pooid grass hosts (Carris et al., 2007). The same analyses could not resolve the relationship between the three species of *T. caries, T. controversa*, and *T. laevis*, unequivocally. Exploring variability of other loci such as encoding the sixth subunit of ATP synthase (*ATP6*), Beta-tubulin, Cyclooxygenase-3 (*COX3*), intergenic spacer I (IGS1), and the largest subunit of RNA polymerase II gene *(RPB1)* has been reported by Carpenter-Boggs et al. (2003). They reported lack of sufficient variability in the sequenced loci. To the best of my knowledge, it is unknown if the phylogenetic relation between common and dwarf bunt has been resolved utilizing those loci.

1. 4. Host specificity in common and dwarf bunt

Besides wheat species and triticale, common and dwarf bunt also occur on various grasses belonging to Poaceae (Hardison et al., 1959; Durán and Fischer, 1961; Vánky, 2012). The host spectra of *T. controversa* was extended by Durán and Fischer (1956) in a critical study of the comparative morphology of many hundreds of collections of the genus *Tilletia* from all over the world. In this study, several species such as *T. calospora, T. elymicola, T. hordei, T. hordeina, T. lolii*, *T. pancicii, T. pancicii*, *T. secalis*, *T. trabutii,* and *T. tritici* on different hosts were synonymized to *T. controversa* because distinguishable morphological differences between these samples and those of *T. controversa* were hard to define. At the moment the host spectra for common and dwarf bunt species includes some 60 different hosts (Purdy et al., 1963). Many of the hosts are determined after successful artificial inoculation under laboratory conditions. Most of the grasses by using an injection of sporidia into the plant boot stage turn susceptible under laboratory conditions, whereas only a small fraction of them naturally occur (Hardison and Corden, 1952; Hardison et al., 1959; Goates, 1996). This method

therefore may not represent natural conditions unequivocally and consequently, it is unknown whether such hosts play any role in the dissemination of fungus in nature (Purdy et al., 1963). The recognition of *T. controversa* especially affected species concept of *T. brevifaciens* which is treated differently and remains under debate until now (Fischer, 1952; Della Torre, 1962; Carris et al., 2007; Vánky, 2012).

Begerow et al. (2004) showed that out of 600 studied smuts, 55% of the species are reported to only occur on a single host suggesting that the smut species are generally highly host-specific. In line with this finding, the recent phylogenetic studies of several broad host range smuts and anther smut revealed that they mostly represent some host-specific species (Kemler et al., 2009; Piątek et al., 2013; Savchenko et al., 2014; Ziegler et al., 2018; Kruse et al., 2018). The majority of *Tilletia* species, like other smuts, have also a relatively narrow host range which is usually restricted to one genus or even a single host (Vánky, 2012). However, sorting hosts of multi-species parasites within the *Tilletia* genus, such as *T. caries* and *T. controversa,* using molecular phylogenetic analyses have yet to be completed. For instance, the study of species similar to *T. controversa* on *Thinopyrum intermedium* (intermediate wheatgrass), *Hordeum murinum* (false barley), and *Secale cereale* (rye) showed that the samples were distinct not only phylogenetically but also physiologically from *T. controversa* on wheat (Carris et al., 2007). They therefore were distinguished or re-distinguished as three distinct species; *T. brevifaciens* (syn *T. controversa* by (Fischer, 1952; Conners, 1954; Vánky, 1994)); *T. trabutii*; and *T. secalis* respectively (Carris et al., 2007). Sorting especially *T. controversa* hosts based on molecular analyses to delineate species boundaries is of great importance because *T. controversa* is a quarantine pathogen in several countries and needs to be accurately and specifically identified.

1. 5. Molecular detection of *T. controversa,* causal agent of dwarf bunt

The current international diagnostic protocol for detection and quantification of *T. controversa* in wheat seeds is based on morphological features of teliospores by a filtration method according to the International Seed Testing Association (ISTA) handbook (1984). In this method, a filtration apparatus is used to collect the spore suspension that is washed from a subsample of wheat seeds on a filter membrane. A qualified person examines the filter membrane using a light microscope for the presence of dwarf bunt teliospores. This morphology-based diagnostic method has obvious limitations. The resolution of the microscopical picture is dramatically disturbed when using a filter membrane, compared to spores in water. Moreover, several *Tilletia* species have overlaps in the morphological features of teliospores with *T. controversa* (compare teliospores morphometrics in

(Carris, 2008)). Especially, "virtually every characteristic used in the separation of dwarf bunt (*T. controversa*) from common bunt (*T. caries*) is one of degree only" stated by Holton and Kendrick (1956). All together identification based on a single teliospore, where average teliospore sizes cannot be measured and the host is unknown, is difficult to impossible. To eliminate such uncertainty and accelerate the identification process, a robust, sensitive, and reliable molecular method for the detection of *T. controversa* is required.

In recent years, several studies have addressed the detection of wheat bunt pathogens using different DNA-based detection methods as an alternative for the time-consuming and laborious traditional microscopic method. Some of the developed markers were designed in a way that the assay could not differentiate between common and dwarf bunt (Mulholland and McEwan, 2000; Josefsen and Christiansen, 2002; Kochanova et al., 2004; McNeil et al., 2004; Eibel et al., 2005; Kochanová et al., 2006; Zouhar et al., 2010; Zgraja et al., 2016; Pieczul et al., 2018; Yao et al., 2019). For instance, Mulholland and McEwan (2000) used 25s rRNA region, which is a component of the LSU rDNA region, and reported the developed PCR assay as genus-specific. Similarly, Pieczul et al. (2018) used part of rDNA IGS 2 (intergenic spacers II) to develop an assay for the common detection of common and dwarf bunt. The lack of phylogenetic resolution with respect to *T. caries*, *T. controversa*, and *T. laevis* employing *EF1α*, ITS, and *RPB2* (Mulholland and McEwan, 2000; Levy et al., 2001; Carris et al., 2007; Bao, 2010; Jayawardena et al., 2019) suggested that these regions were not suitable for the development of species-specific detection assays. Therefore, alternative DNA regions needed to be explored for the development of species-specific markers.

Finding suitable polymorphic regions for species-specific marker development among closely related species is challenging. Several approaches have been developed to facilitate the findings of such regions for species-specific assay development. These segments can be driven from approaches based on PCR amplification namely; inter-simple sequence repeats (ISSR) (Stewart et al., 2013; Gao et al., 2014; Priyanka et al., 2014), start codon targeted (SCoT)(Mulpuri et al., 2013; Hao et al., 2018), inter-retrotransposon amplified polymorphism (IRAP) (Pasquali et al., 2007; Su et al., 2008; Shimada et al., 2009; Xiao et al., 2011; Abdollahi Mandoulakani et al., 2015) and intron length polymorphisms (ILP)(Shimada et al., 2009). However, these methods are sensitive to PCR amplification conditions and altogether, fewer candidate regions are identified. Anonymous loci specific to *T. controversa* were identified in several studies using different approaches such as Amplified fragment length polymorphism (AFLP) and ISSR (Liu et al., 2009; Gao et al., 2010; Gao et al., 2011; Gao et al., 2014; Liu et al., 2020). However, the developed assays based on these loci were tested only on a limited number of samples, and the close relatedness of common and dwarf bunt (Russell and Mills, 1993;

Russel, 1993; Russell, 1994) was not taken into account. By using the genomic data, we could demonstrate that for example, the DNA fragment used for marker development by Liu et al. (2020) identified by the inter-simple sequence repeat (ISSR) technique could not be specific to *T. controversa* because of having homologous in common bunt genome (Accessed 01.02.2020). The same was demonstrated for *T. laevis*-specific loci identified by Yao et al. (2019), which has identical homologous loci in *T. caries* whole-genome sequences (Accessed 01.12.2019). Additionally, the markers developed by (Gao et al., 2014) depicted some false-positive amplification of *T. caries* (collected from Europe) samples in our laboratory (unpublished data). In the end, comparative genomic remains a promising approach to find a substantial number of potential DNA regions unique to *T. controversa* (or other fungi) for the species-specific assay development. Genome comparison for finding such loci is becoming more and more common in selective assay development (Moolhuijzen et al., 2009; Behr et al., 2016; Burbank and Ortega, 2018). Nguyen et al. (2019) were the first to apply a comparative genomic approach for finding candidate gene regions specific to each of common and dwarf bunt species. The study however lacks the wet lab validation of the suggested primers and the selection criteria are limited to single-copy genes.

1. 6. Genome resources and comparative genomics of *Tilletia* spp.

At the initiation of this work and until 2016, no genomic data was available for common and dwarf bunt fungi, while two other *Tilletia* species namely *T. horrida*, the causal agent of rice kernel smut (Wang et al., 2015a), and two *T. indica* (Sharma et al., 2016) whole genomes were sequenced. *Tilletia indica* and *T. horrida* are nonsystemic fungi that are only distantly related to systemically infecting common and dwarf bunt (Carris et al., 2006). Soon after, one *T. caries* and one *T. controversa* genome sequence were released in GenBank (released on 16.05.2019), the publication though was lacking until the end of 2019 (released on 30.10.2019) when the initially submitted assembly versions were superseded (Nguyen et al., 2019). At the moment 18 genome assemblies of *Tilletia* isolates belonging to two *T. caries* (GCA_001645005.2, GCA_004334575.1), two *T. controversa* (GCA_001645045.2, GCA_009428265.1), one *T. horrida (GCA_001006505.1),* eight *T. indica* (GCA_009428345.1, GCA_009428365.1, GCA_001645015.2, GCA_001689995.1, GCA_001689945.1, GCA_002220835.1, GCA_002997305.1) (genome assembly accession of GCA_002997305.1 is the improved version of GCA_003054935.1 and both belong to a single isolate), two *T. laevis* (GCA_009428275.1, GCA_009428285.1), and two *T. walkeri* (GCA_001645055.2, GCA_009428295.1) are available in GenBank (accessed on 08.09.2020), which

were released during this project (Wang et al., 2015a; Sharma et al., 2016; Kumar et al., 2017; Kumar et al., 2018; Gurjar et al., 2019; Nguyen et al., 2019). Additionally, one isolate of *T. horrida* (Wang et al., 2018) and seven further (Wang et al., 2019) are sequenced for which only raw reads became publicly available (accessed on 09.08.2020).

The basic genomic features of common and dwarf bunt agents comprise of structural annotations were published by Nguyen et al. (2019) through whole-genome sequencing of one strain of *T. caries*, two strains of *T. controversa*, and two strains of *T. laevis* beside three strains of *T. indica* and two strains of *T. walkeri*. The draft genomes of common and dwarf bunt strains were assembled from 28.1 to 29.9 Mb and predicted to code for 9649 to 9952 genes. These genome sizes were bigger than the largest genome size proposed for *T. horrida* (23.2 Mb (Wang et al., 2018)) and smaller compared to that of *T. indica* drafted up to 33.7 Mb (Gurjar et al., 2019). Nguyen et al (2019) compared single-copy orthologous protein-coding genes of ten *Tilletia* isolates and identified 72 unique proteins to *T. caries*, two to *T. controversa*, and one to *T. laevis*. The putative functions of the identified proteins remained unknown. Lack of genome sequences not only delayed the development of species-specific markers for closely related common and dwarf bunt fungal agents but also their functional genomics, genomic structure, and genomic diversity among and between them remained unknown.

1. 7. Phylogenomic analysis of *Tilletia* spp.

Tilletia species were absent from the phylogenomic studies of the broad Ustilaginomycotina members published by Kijpornyongpan et al. (2018). This has changed in the last year by the availability of the whole-genome sequencing data to study the phylogenetic relationships of *Tilletia* species employing more loci. The first phylogenomic report was published by Mishra et al. (2019) using seven *Tilletia* genomes. The only representative of each *T. caries* and *T. controversa* species were placed in one clade together with *T. walkeri*, while the samples of *T. indica* were in a separated clade. Several studies have shown that *T. walkeri*, the causal agent of ryegrass bunt (Castlebury and Carris, 1999) is closely related to *T. indica* (Pimentel et al., 1998; Levy et al., 2001; Tan and Murray, 2006). The placement of *T. walkeri*, in one cluster together with common and dwarf bunt fungi and *T. indica* samples in another is contradictory to previous studies. We speculate that not trimming the poorly aligned regions and divergent regions, which may have been saturated by multiple substitutions might have caused artifacts (Castresana, 2000; Portik and Wiens, 2020). Soon after, using the same genomes and analyzing 3751 orthologous genes Gurjar et al. (2019) reported one clade containing wheat bunts where common and dwarf bunt clustered together and separated from *T. indica* isolates. The latest

phylogenomic study of *Tilletia* species by using 4896 single-copy orthologous genes of 10 *Tilletia* strains (one *T. caries* strain, two *T. controversa* strains, two *T. laevis* strains, three *T. indica* strains, and two *T. walkeri* strains) was done by Nguyen et al. (2019). Using these loci, the five species clustered into separated well-supported clades. Due to the lack of the corresponding annotation from the other six publicly available whole genomes (one *T. horrida* isolate, five *T. indica* strains), the inclusion of all 16 genomes available by that time was not possible and the study was limited to their sequenced genomes which were mostly collected from North USA.

1. 8. Objectives of the thesis

Investigation of inter- and intraspecies variation in genomes of *Tilletia caries*, *T. controversa* and *T. laevis* – Chapter 2

Although there is an increasing concern about the threat of common and dwarf bunt, especially in organic farming, less is known about their genomic structure, their gene content, and what set these three species apart.

Objectives of this study were to 1) sequence genomes of four *T. caries*, five *T. controversa*, and two *T. laevis* isolates which except for one are collected from recent European populations; 2) structurally and functionally annotate their genomes to gain first insight into their genomic features; 3) *in silico* compare common and dwarf bunt isolates for inter- and intraspecies genomic variation with special focus on important modulators such as secretomes and effectors, carbohydrate-active enzymes, and secondary metabolite biosynthesis gene clusters; 4) find species-specific protein-coding genes that may explain the three species differences in their teliospores physiology and infection biology.

Development of a loop-mediated isothermal amplification assay for the detection of *Tilletia controversa* based on genome comparison – Chapter 3

For over 38 years, seed testing organizations in the European Union are using the filtering method established by the International Seed Testing Association (ISTA) (1984), followed by microscopic examination of teliospores, to detect and quantify *T. controversa* teliospores in wheat seed samples. The method is however time-consuming and needs experts. Therefore, a robust, sensitive, and quick DNA-based detection assay is urgently needed. The sequenced loci, mainly routinely used loci for the phylogenetic studies, lack suitable polymorphism to be used for the species-specific assay development.

The objective of this study was to develop a loop-mediated isothermal amplification (LAMP) assay to detect *T. controversa* species using 21 genomic data of six *Tilletia* species in a genome comparison approach. The developed assay was validated for its reproducibility in an interlaboratory test performance study that included five national seed testing organizations and plant protection agencies.

Species delimitation of *Tilletia* controversa using molecular phylogenetic and phylogenomic approaches – Chapter 4

Despite different physiological and morphological features of teliospores between common bunt causal agents (*T. caries* and *T. laevis*) and dwarf bunt (*T. controversa*), multilocus phylogenetic analysis incorporating sequencing data of three loci including elongation factor 1α (*EF1a*), partial internal transcribed spacer (ITS) rDNA, and partial the second largest subunit of RNA polymerase II *(RPB2*) could not resolve the phylogenetic relationship corresponding to each species. Moreover, common and dwarf bunts are reported to infect more than 60 grasses species of Poaceae besides wheat species and triticale. The phylogenetic relationships of such samples to those collected from wheat hosts have remained largely unknown.

The objectives of this work were to 1) test whether *T. controversa* collected from different hosts were conspecific with those obtained from wheat species by employing multilocus (*EF1a*, ITS, *RPB2*) phylogeny approach, 3) phylogenomically infer the relation of common and dwarf bunt that could not be resolved using multi-locus phylogenetic study. For this, the eleven newly sequenced genomes of common and dwarf bunt which mostly have European origin are complimented with two *T. caries*, two *T. controversa*, one *T. horrida*, two *T. laevis*, seven *T. indica*, and two *T. walkeri* whole-genomes which became available during the course of this work.

1. 9. References

Abdollahi Mandoulakani, B., Yaniv, E., Kalendar, R., Raats, D., Bariana, H. S., Bihamta, M. R., & Schulman, A. H. (2015). Development of IRAP- and REMAP-derived SCAR markers for marker-assisted selection of the stripe rust resistance gene Yr15 derived from wild emmer wheat. *Theor Appl Genet*, *128*(2), 211-219. https://doi.org/10.1007/s00122-014-2422-8

Aydoğdu, M., & Kaya, Y. (2020). Reactions of spring wheat varieties to common bunt (*Tilletia laevis*) in Turkey. *Cereal Research Communications*, *48*(3), 333-339. https://doi.org/10.1007/s42976-020-00040-1

Bao, X. (2010). Host specificity and phylogenetic relationships among *Tilletia* species infecting wheat and other cool season grasses. PhD thesis, Department of Plant Pathology, Washington State University.

Bauer, R., Begerow, D., Sampaio, J. P., Weiβ, M., & Oberwinkler, F. (2006). The simple-septate basidiomycetes: a synopsis. *Mycological Progress*, *5*(1), 41-66. https://doi.org/10.1007/s11557-006-0502-0

Begerow, D., Bauer, R., & Oberwinkler, F. (1997). Phylogenetic studies on nuclear large subunit ribosomal DNA sequences of smut fungi and related taxa. *Canadian Journal of Botany*, *75*(12), 2045-2056. https://doi.org/10.1139/b97-916

Begerow, D., Göker, M., Lutz, M., & Stoll, M. (2004). On the evolution of smut fungi and their hosts (M. P. Reinhard Agerer , Paul Blanz, Ed.). IHW-Verlag & Verlagsbuchhandlung.

Behr, J., Geissler, A. J., Schmid, J., Zehe, A., & Vogel, R. F. (2016). The identification of novel diagnostic marker genes for the detection of beer spoiling *Pediococcus damnosus* strains using the BlAst diagnostic gene findEr. *PLoS One*, *11*(3), e0152747. https://doi.org/10.1371/journal.pone.0152747

Borgen, A., & Davanlou, M. (2001). Biological control of common bunt (*Tilletia tritici*). *Journal of Crop Production*, *3*(1), 157-171. https://doi.org/10.1300/J144v03n01_14

Burbank, L. P., & Ortega, B. C. (2018). Novel amplification targets for rapid detection and differentiation of Xylella fastidiosa subspecies fastidiosa and multiplex in plant and insect tissues. *J Microbiol Methods*, *155*, 8-18. https://doi.org/10.1016/j.mimet.2018.11.002

Carpenter-Boggs, L., Carris, L. M., & Castlebury, L. (2003). Multi-locus phylogenetic analysis of *Tilletia* species infecting hosts in grass subfamily Pooideae. Inoculum, USA.

Carris, L. (2008). Is rye bunt, *Tilletia secalis*, present in North America? *North American Fungi*, *3*, 147-159. https://doi.org/10.2509/naf2008.003.0078

Carris, L. M. (2010). smuts. In W. W. Bockus, R. L. Bowden, R. M. Hunger, W. L. Morrill, & T. D. Murray (Eds.), *Compendium of Wheat Diseases and Pests* (60-65). American Phytopathological Society.

Carris, L. M., Castlebury, L. A., & Goates, B. J. (2006). Nonsystemic bunt fungi *Tilletia indica* and *T. horrida:* a review of history, systematics, and biology. *Annu Rev Phytopathol, 44,* 113-133. https://doi.org/10.1146/annurev.phyto.44.070505.143402

Carris, L. M., Castlebury, L. A., Huang, G., Alderman, S. C., Luo, J., & Bao, X. (2007). *Tilletia vankyi*, a new species of reticulate-spored bunt fungus with non-conjugating basidiospores infecting species of *Festuca* and *Lolium*. *Mycological research*, *111*(Pt 12), 1386-1398. https://doi.org/10.1016/j.mycres.2007.09.008

Castlebury, L. A., & Carris, L. M. (1999). *Tilletia walkeri*, a New Species on *Lolium multiflorum* and *L. perenne*. *Mycologia*, *91*(1), 121-131. https://doi.org/10.2307/3761200

Castlebury, L. A., Carris, L. M., & Vinky, K. (2005). Phylogenetic analysis of *Tilletia* and allied genera in order Tilletiales (Ustilaginomycetes; Exobasidiomycetidae) based on large subunit nuclear rDNA sequences. *Mycologia*, *97*(4), 888-900. https://doi.org/10.3852/mycologia.97.4.888

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol*, *17*(4), 540-552. https://doi.org/10.1093/oxfordjournals.molbev.a026334

Cockerell, V., & McNeil, M. (2004). Using seed health testing results to make balanced seed management decisions. Proceedings Crop Protection in Northern Britain 2004, Dundee.

Conners, I. L. (1954). The Organism causing dwarf bunt of wheat. *Canadian Journal of Botany*, *32*(3), 426-431. https://doi.org/10.1139/b54-038

Crous, P. W., Van Jaarsveld, A. B., Castlebury, L. A., Carris, L. M., Frederick, R. D., & Pretorius, Z. A. (2001). Karnal bunt of wheat newly reported from the african continent. *Plant Dis*, *85*(5), 561. https://doi.org/10.1094/PDIS.2001.85.5.561B

Della Torre, B. (1962). *Tilletia brevifaciens*, a specilized strain of *T. controversa. Rivista di Patologia Vegetale, 2*(3), 271-273. http://www.jstor.org/stable/42567744

Denchev, C., & Denchev, T. (2013). Erratomycetaceae, fam. nov., and validation of some names of smut fungi recently described from India. *Mycobiota*, *1*, 63-70. https://doi.org/10.12664/mycobiota.2013.01.07

Denchev, T. T., & Denchev, C. M. (2018a). *Tilletia tripogonellae* (Tilletiaceae), a new smut fungus on *Tripogonella spicata* (Poaceae) from Argentina. *Annales Botanici Fennici*, *55*(4-6), 273-277, 275. https://doi.org/10.5735/085.055.0407

Denchev, T. T., & Denchev, C. M. (2018b). Two new smut fungi on *Ventenata Poaceae*: *Tilletia elizabethae* from Slovakia and *T. ventenatae* from Turkey. *Willdenowia*, *48*(2), 177-183, 177. https://doi.org/10.3372/wi.48.48201

Denchev, T. T., Van Der Zon, A. P. M., & Denchev, C. M. (2018). *Tilletia triraphidis* (Tilletiaceae), a new smut fungus on *Triraphis purpurea* (Poaceae) from Namibia. *Phytotaxa*, *375*(2), 182-186. https://doi.org/10.11646/phytotaxa.375.2.5

Dumalasová, V., Leišová-Svobodová, L., & Bartoš, P. (2014). Common bunt resistance of Czech and European winter wheat cultivars and breeder lines. *Czech J Genet Plant Breed* 50: 201-207

and European winter wheat cultivars and breeder lines. *Czech J. Genet. Plant Breed.*, *50*(No. 3), 201-207. https://doi.org/10.17221/241/2013-cjgpb

Durán, R., & Fischer, G. W. (1956). Further studies on the synonymy and host range of the dwarf bunt fungus. *Research Studies of State Collge Washington*, *XXIV*(4), 8.

Durán, R., & Fischer, G. W. (1961). *The genus Tilletia.* Pullman, WA. Washington State University.

Eibel, P., Wolf, G. A., & Koch, E. (2005). Detection of *Tilletia caries*, causal agent of common bunt of wheat, by ELISA and PCR. *J. Phytopathol.*, *153*(5), 297-306. https://doi.org/10.1111/j.1439-0434.2005.00973.x

Fischer, G. W. (1952). *Tilletia brevifaciens* sp. nov., causing dwarf bunt of wheat and certain grasses. *Research Studies. Washington State University*, *20*(1), 11-14.

Gao, L., Chen, W., & Liu, T. (2011). An ISSR-based approach for the molecular detection and diagnosis of dwarf bunt of wheat, caused by *Tilletia controversa* Kühn. *J. Phytopathol.*, *159*(3), 155-158. https://doi.org/10.1111/j.1439-0434.2010.01735.x

Gao, L., Chen, W. Q., & Liu, T. G. (2010). Development of a SCAR marker by inter-simple sequence repeat for diagnosis of dwarf bunt of wheat and detection of *Tilletia controversa* KuHN. *Folia Microbiol (Praha)*, *55*(3), 258-264. https://doi.org/10.1007/s12223-010-0038-1

Gao, L., Yu, H., Han, W., Gao, F., Liu, T., Liu, B., Kang, X., Gao, J., & Chen, W. (2014). Development of a SCAR marker for molecular detection and diagnosis of *Tilletia controversa* Kuhn, the causal fungus of wheat dwarf bunt. *World J Microbiol Biotechnol*, *30*(12), 3185-3195. https://doi.org/10.1007/s11274-014-1746-5

Goates, B. J. (1996). common bunt and dwarf bunt. In R. D. Wilcoxson & E. E. Saari (Eds.), *Bunt and smut diseases of wheat; concepts and methods of disease management*. CIMMYT.

Goates, B. J., & Peterson, G. L. (1999). Relationship between soilborne and seedborne inoculum density and the incidence of dwarf bunt of wheat. *Plant Dis*, *83*(9), 819-824. https://doi.org/10.1094/PDIS.1999.83.9.819

Gurjar, M. S., Aggarwal, R., Jogawat, A., Kulshreshtha, D., Sharma, S., Solanke, A. U., Dubey, H., & Jain, R. K. (2019). *De novo* genome sequencing and secretome analysis of *Tilletia indica* inciting Karnal bunt of wheat provides pathogenesis-related genes. *3 Biotech*, *9*(6), 219. https://doi.org/10.1007/s13205-019-1743-3

Hanna, W. F., Vickery, H. B., & Pucher, G. W. (1932). The isolation of trimethylamine from spores of *Tilletia levis*, the stinking smut of wheat. *Journal of Biological Chemistry*, *97*(2), 351-358. http://www.jbc.org/content/97/2/351.short

Hao, J., Jiao, K., Yu, C., Guo, H., Zhu, Y., Yang, X., Zhang, S., Zhang, L., Feng, S., Song, Y., Dong, M., Wang, H., & Shen, C. (2018). Development of SCoT-based SCAR marker for rapid authentication of taxus media. *Biochem Genet*, *56*(3), 255-266. https://doi.org/10.1007/s10528-018-9842-0

Hardison, J. R., & Corden, M. E. (1952). A serious outbreak of dwarf bunt in tall oat grass in Oregon. *Plant disease report*, *36*, 2.

Hardison, J. R., Meiners, J. P., Hoffmann, J. A., & Waldher, J. T. (1959). Susceptibility of gramineae to *Tilletia contraversa*. *Mycologia*, *51*(5), 656-664. https://doi.org/10.2307/3755895

Hess, W. M. (1986). Use of electron microscopy to characterize teliospores of *Tilletia caries* and *T. controversa*. *Plant Disease*, *70*(5), 458. https://doi.org/10.1094/pd-70-458

Hibbett, D. S., Stajich, J. E., & Spatafora, J. W. (2013). Toward genome-enabled mycology. *Mycologia*, *105*(6), 1339-1349. https://doi.org/10.3852/13-196

Hoffmann, J. A., & Metzger, R. J. (1976). Current status of virulence genes and pathogenic races of the wheat bunt fungi in the northwestern USA. *Phytopathology*, 66, 4.

Hoffmann, J. A. (1982). Bunt of wheat. *Plant Disease*, *66*(1), 979. https://doi.org/10.1094/pd-66-979

Holton, C. S. (1954). Natural hybridization between common and dwarf bunt as related to the problem of delimitation of species of *Tilletia* occurring on wheat. *Phytopathology*, *44*(9), 493-493.

Holton, C. S., & Kendrick, E. L. (1956). Problems in the delimitation of species of *Tilletia* occurring on wheat. *Research Studies of the State College of Washington*, *24*, 318 - 325.

International Seed Testing Association (ISTA). (1984). International seed testing association (hrsg.), working sheet No. 53, *Triticum aestivum*, *Tilletia contraversa* Kühn, *Tilletia caries* (DC) Tul., *Tilletia foetida* (Wallr.) Liro. In *ISTA Handbook on Seed Health Testing*. Zurich, Switzerland.

International Plant Protection Convention (IPPC). List of regulated pests from Mexico. Retrieved Jun 22. 2019 from www.ippc.int/en/countries/mexico/reportingobligation/3

Jayawardena, R. S., Hyde, K. D., Jeewon, R., Ghobad-Nejhad, M., Wanasinghe, D. N., Liu, N., Phillips, A. J. L., Oliveira-Filho, J. R. C., da Silva, G. A., Gibertoni, T. B., Abeywikrama, P., Carris, L. M., Chethana, K. W. T., Dissanayake, A. J., Hongsanan, S., Jayasiri, S. C., McTaggart, A. R., Perera, R. H., Phutthacharoen, K., Savchenko, K. G., Shivas, R. G., Thongklang, N., Dong, W., Wei, D., Wijayawardena, N. N., & Kang, J.-C. (2019). One stop shop II: taxonomic update with molecular phylogeny for important phytopathogenic genera: 26–50 (2019). *Fungal Diversity*, *94*(1), 41-129. https://doi.org/10.1007/s13225-019-00418-5

Jones, D. R. (2008). Towards a more reasoned assessment of the threat to wheat crops from *Tilletia indica*, the cause of Karnal bunt disease. *European Journal of Plant Pathology*, *123*(3), 247-259. https://doi.org/10.1007/s10658-008-9364-4

Josefsen, L., & Christiansen, S. K. (2002). PCR as a tool for the early detection and diagnosis of common bunt in wheat, caused by *Tilletia tritici*. *Mycological research*, *106*(11), 1287-1292. https://doi.org/10.1017/s0953756202006603

Kemler, M., Lutz, M., Göker, M., Oberwinkler, F., & Begerow, D. (2009). Hidden diversity in the non-caryophyllaceous plant-parasitic members of *Microbotryum* (Pucciniomycotina: Microbotryales). *Systematics and Biodiversity*, *7*(3), 297-306. https://doi.org/10.1017/s1477200009990028

Kijpornyongpan, T., Mondo, S. J., Barry, K., Sandor, L., Lee, J., Lipzen, A., Pangilinan, J., LaButti, K., Hainaut, M., Henrissat, B., Grigoriev, I. V., Spatafora, J. W., & Aime, M. C. (2018). Broad genomic sampling reveals a smut pathogenic ancestry of the fungal clade Ustilaginomycotina. *Mol Biol Evol*, *35*(8), 1840-1854. https://doi.org/10.1093/molbev/msy072

Kochanová, M., Prokinová, E., & Ryšánek, P. (2006). Laboratory diagnostics of common bunt and dwarf bunt. *Czech J. Genet. Plant Breed.*

Kochanová, M., Zouhar, M., Prokinová, E., & Ryšánek, P. (2011). Detection of *Tilletia controversa* and *Tilletia caries* in wheat by PCR method. *Plant, Soil and Environment*, *50*(No. 2), 75-77. https://doi.org/10.17221/3684-pse

Kruse, J., Pia Tek, M., Lutz, M., & Thines, M. (2018). Broad host range species in specialised pathogen groups should be treated with suspicion - a case study on *Entyloma* infecting *Ranunculus*. *Persoonia*, *41*, 175-201. https://doi.org/10.3767/persoonia.2018.41.09

Kumar, A., Mishra, P., Maurya, R., Mishra, A. K., Gupta, V. K., Ramteke, P. W., & Marla, S. S. (2018). Improved draft genome sequence of a monoteliosporic culture of the karnal bunt (*Tilletia indica*) pathogen of wheat. *Genome Announc*, *6*(20), e00015-00018. https://doi.org/10.1128/genomeA.00015-18

Kumar, A., Pandey, V., Singh, M., Pandey, D., Saharan, M. S., & Marla, S. S. (2017). Draft genome sequence of Karnal bunt pathogen (*Tilletia indica*) of wheat provides insights into the pathogenic mechanisms of quarantined fungus. *PLoS One*, *12*(2), e0171323. https://doi.org/10.1371/journal.pone.0171323

Levy, L., Castlebury, L. A., Carris, L. M., Meyer, R. J., & Pimentel, G. (2001). Internal transcribed spacer sequence-based phylogeny and polymerase chain reaction-restriction fragment length polymorphism differentiation of *Tilletia walkeri* and *T. indica*. *Phytopathology*, *91*(10), 935-940. https://doi.org/10.1094/PHYTO.2001.91.10.935

Li, Y.-M., Shivas, R. G., & Cai, L. (2014). Three new species of *Tilletia* on Eriachne from north-western Australia. *Mycoscience*, *55*(5), 361-366. https://doi.org/10.1016/j.myc.2013.12.003

Liu, J., Li, C., Muhae-Ud-Din, G., Liu, T., Chen, W., Zhang, J., & Gao, L. (2020). Development of the droplet digital pcr to detect the teliospores of *tilletia controversa* Kuhn in the soil with greatly enhanced sensitivity. *Front Microbiol*, *11*(4), 4. https://doi.org/10.3389/fmicb.2020.00004

Liu, J. H., Gao, L., Liu, T. G., & Chen, W. Q. (2009). Development of a sequence-characterized amplified region marker for diagnosis of dwarf bunt of wheat and detection of *Tilletia controversa* Kuhn. *Letters in applied microbiology*, *49*(2), 235-240. https://doi.org/10.1111/j.1472-765X.2009.02645.x

Matanguihan, J. B., Murphy, K. M., & Jones, S. S. (2011). Control of common bunt in organic wheat. *Plant Dis*, *95*(2), 92-103. https://doi.org/10.1094/PDIS-09-10-0620

Mathre, D. E. (1996). Dwarf bunt: politics, identification, and biology. *Annu Rev Phytopathol*, *34*, 67-85. https://doi.org/10.1146/annurev.phyto.34.1.67

Mathur, S. B., & Cunfer, B. M. (1993). Karnal bunt. In *Seed-borne diseases and seed health testing of wheat* (31). Danish Government Institute of Seed Pathology for Developing Countries.

McNeil, M., Roberts, A. M. I., Cockerell, V., & Mulholland, V. (2004). Real-time PCR assay for quantification of *Tilletia caries* contamination of UK wheat seed. *Plant Pathology*, *53*(6), 741-750. https://doi.org/10.1111/j.1365-3059.2004.01094.x

McTaggart, A. R., Shivas, R. G., Geering, A. D., Callaghan, B., Vanky, K., & Scharaschkin, T. (2012). Soral synapomorphies are significant for the systematics of the *Ustilago-Sporisorium-Macalpinomyces* complex (Ustilaginaceae). *Persoonia*, *29*, 63-77. https://doi.org/10.3767/003158512X660562

Mishra, P., Maurya, R., Gupta, V. K., Ramteke, P. W., Marla, S. S., & Kumar, A. (2019). Comparative genomic analysis of monosporidial and monoteliosporic cultures for unraveling the complexity of molecular pathogenesis of *Tilletia indica* pathogen of wheat. *Sci Rep*, *9*(1), 8185. https://doi.org/10.1038/s41598-019-44464-0

Moolhuijzen, P. M., Lew-Tabor, A. E., Wlodek, B. M., Aguero, F. G., Comerci, D. J., Ugalde, R. A., Sanchez, D. O., Appels, R., & Bellgard, M. (2009). Genomic analysis of *Campylobacter fetus* subspecies: identification of candidate virulence determinants and diagnostic assay targets. *BMC Microbiol*, *9*(1), 86. https://doi.org/10.1186/1471-2180-9-86

Morris R. Bonde, G. L. P., and Norman W. Schaad. (1977). Karnal bunt of wheat. *Plant Disease*, *81*(12).

Mulholland, V., & McEwan, M. (2000). PCR-based diagnostics of *Microdochium nivale* and *Tilletia tritici* infecting winter wheat seeds. *EPPO Bulletin*, *30*(3-4), 543-547. https://doi.org/10.1111/j.1365-2338.2000.tb00944.x

Mulpuri, S., Muddanuru, T., & Francis, G. (2013). Start codon targeted (SCoT) polymorphism in toxic and non-toxic accessions of *Jatropha curcas* L. and development of a codominant SCAR marker. *Plant Sci*, *207*, 117-127. https://doi.org/10.1016/j.plantsci.2013.02.013

Nguyen, H. D. T., Sultana, T., Kesanakurti, P., & Hambleton, S. (2019). Genome sequencing and comparison of five *Tilletia* species to identify candidate genes for the detection of regulated species infecting wheat. *IMA Fungus*, *10*(1), 11, https://doi.org/10.1186/s43008-019-0011-9

Nielsen, J. (1963). Trimethylammonium compounds in *Tilletia* Spp. *Canadian Journal of Botany*, *41*(3), 335-339. https://doi.org/10.1139/b63-032

OEPP/EPPO. (2018). *Tilletia indica. EPPO Bulletin,* 48: 7– 31. https://doi.org/10.1111/epp.12452

Pasquali, M., Dematheis, F., Gullino, M. L., & Garibaldi, A. (2007). Identification of race 1 of *Fusarium oxysporum* f. sp. *lactucae* on lettuce by inter-retrotransposon sequence-characterized amplified region technique. *Phytopathology*, *97*(8), 987-996. https://doi.org/10.1094/PHYTO-97-8-0987

Peterson, G. L., Whitaker, T. B., Stefanski, R. J., Podleckis, E. V., Phillips, J. G., Wu, J. S., & Martinez, W. H. (2009). A risk assessment model for importation of united states milling wheat containing *Tilletia contraversa*. *Plant Dis*, *93*(6), 560-573. https://doi.org/10.1094/PDIS-93-6-0560

Piatek, M., Lutz, M., & Chater, A. O. (2013). Cryptic diversity in the *Antherospora vaillantii* complex on *Muscari* species. *IMA Fungus*, *4*(1), 5-19. https://doi.org/10.5598/imafungus.2013.04.01.02

Pieczul, K., Perek, A., & Kubiak, K. (2018). Detection of *Tilletia caries*, *Tilletia laevis* and *Tilletia controversa* wheat grain contamination using loop-mediated isothermal DNA amplification (LAMP). *J Microbiol Methods*, *154*, 141-146. https://doi.org/10.1016/j.mimet.2018.10.018

Pimentel, G., Carris, L. M., Levy, L., & Meyer, R. J. (2018). Genetic variability among isolates of *Tilletia barclayana*, *T. indica* and allied species. *Mycologia*, *90*(6), 1017-1027. https://doi.org/10.1080/00275514.1998.12027000

Portik, D. M., & Wiens, J. J. (2021). Do alignment and trimming methods matter for phylogenomic (UCE) analyses? *Syst Biol*, *70*(3), 440-462. https://doi.org/10.1093/sysbio/syaa064

Priyanka, S. R., Uppalapati, S. R., Kingston, J. J., Murali, H. S., & Batra, H. V. (2014). Development of ISSR-derived SCAR marker-targeted PCR for identification of *Aspergillus* section *Flavi* members. *Letters in applied microbiology*, *58*(5), 414-422. https://doi.org/10.1111/lam.12207

Purdy, L. H., Kendrick, E. L., Hoffmann, J. A., & Holton, C. S. (1963). Dwarf bunt of wheat. *Annu. Rev. Microbiol.*, *17*(1), 199-222. https://doi.org/10.1146/annurev.mi.17.100163.001215

Riess, K., Schon, M. E., Lutz, M., Butin, H., Oberwinkler, F., & Garnica, S. (2016). On the evolutionary history of *Uleiella chilensis,* a smut fungus parasite of *Araucaria araucana* in south america: Uleiellales ord. nov. in Ustilaginomycetes. *PLoS One*, *11*(1), e0147107. https://doi.org/10.1371/journal.pone.0147107

Roberson, R. W., & Luttrell, E. S. (2018). Dolipore septa in *Tilletia*. *Mycologia*, *81*(4), 650-652. https://doi.org/10.1080/00275514.1989.12025800

Royer, M. H., & Rytter, J. L. (1985). Artificial inoculation of wheat with *Tilletia indica* from Mexico and India. *Plant Dis*. 69, 317-319. 10.1094/PD-69-317

Rudloff, J. E., Bauer, R., Büttner, P., Sedaghtjoo, S., Kirsch, N., & Maier, W. (2020). Monitoring zum Vorkommen von *Tilletia controversa* (Zwergsteinbrand) an konventionell erzeugtem Winterweizen in den Bundesländern Brandenburg, Mecklenburg-Vorpommern, Niedersachsen, Nordrhein-Westfalen, Sachsen, Sachsen-Anhalt und Schleswig-Holstein [Monitoring of the occurrence of *Tilletia controversa* on conventionally produced winter wheat in Northern Germany]. *Kulturpflanzen*, *72*(8), 7. https://doi.org/10.5073/JfK.2020.08.16

Rush, C. M., Stein, J. M., Bowden, R. L., Riemenschneider, R., Boratynski, T., & Royer, M. H. (2005). Status of Karnal bunt of wheat in the United States 1996 to 2004. *Plant Dis*, *89*(3), 212-223. https://doi.org/10.1094/PD-89-0212

Russel, B. W. (1993). Electrophoretic karyotypes and molecular genetic analysis of *Tilletia caries* and *T. controversa*. MSc thesis. Oregon State University

Russell, B. W. (1994). Morphological, physiological, and genetic evidence in support of a conspecific status for *Tilletia caries, T. controversa*, and *T. foetida*. *Phytopathology*, *84*(6), 576-582. https://doi.org/10.1094/Phyto-84-576

Russell, B. W., & Mills, D. (1993). Electrophoretic karyotypes of *Tilletia caries*, *T. controversa*, and their F1 progeny: further evidence for conspecific status. *Molecular plant-microbe interactions : MPMI*, *6*(1), 66-74. https://doi.org/10.1094/mpmi-6-066

Savchenko, K. G., Carris, L. M., Castlebury, L. A., Heluta, V. P., Wasser, S. P., & Nevo, E. (2014). Stripe smuts of grasses: one lineage or high levels of polyphyly? *Persoonia*, *33*, 169-181. https://doi.org/10.3767/003158514X685202

Sharma, P., Tiwari, R., Saharan, M. S., Sharma, I., Kumar, J., Mishra, S., Muthusamy, S. K., Gupta, R. K., Jaiswal, S., Iquebal, M. A., Angadi, U. B., Kumar, N., Fatma, S., Rai, A., & Kumar, D. (2016). Draft genome sequence of two monosporidial lines of the karnal bunt fungus *Tilletia indica* Mitra (PSWKBGH-1 and PSWKBGH-2). *Genome Announc*, *4*(5), e00928-00916. https://doi.org/10.1128/genomeA.00928-16

Shimada, N., Nakatsuka, T., Nakano, Y., Kakizaki, Y., Abe, Y., Hikage, T., & Nishihara, M. (2009). Identification of gentian cultivars using SCAR markers based on intron-length polymorphisms of flavonoid biosynthetic genes. *Scientia Horticulturae*, *119*(3), 292-296. https://doi.org/10.1016/j.scienta.2008.08.001

Shivas, R. G., & McTaggart, A. R. (2009). Three new species of *Tilletia* on native grasses from northern Australia. *Australasian Plant Pathology*, *38*(2), 128-131. https://doi.org/10.1071/ap08089

Sitton, J. W. (1993). Difenoconazole seed treatment for control of dwarf bunt of winter wheat. *Plant Disease*, *77*(11), 1148. https://doi.org/10.1094/pd-77-1148

Spiess, h., & Dutschke, h. (1991). Bekämpfung des Weizenstein-brandes (*Tilletia caries*) im Biologisch-Dynamischen Landbau unter experimentellen und praktischen Bedingungen. *Gesunde Pflanzen*, *43*, 7.

Stewart, J. E., Andrew, M., Bao, X., Chilvers, M. I., Carris, L. M., & Peever, T. L. (2013). Development of sequence characterized amplified genomic regions (SCAR) for fungal systematics: proof of principle using *Alternaria*, *Ascochyta* and *Tilletia*. *Mycologia*, *105*(4), 1077-1086. https://doi.org/10.3852/12-287

Strickland, A. H. (2008). Plant protection and world crop production By H. H. Cramer Leverkusen: 'Bayer' Pflanzenschutz (1967), 524. *Experimental Agriculture*, *5*(1), 82-83. https://doi.org/10.1017/s0014479700010036

Su, H., Wang, L., Liu, L., Chi, X., & Zhang, Y. (2008). Use of inter-simple sequence repeat markers to develop strain-specific SCAR markers for *Flammulina velutipes*. *J Appl Genet*, *49*(3), 233-235. https://doi.org/10.1007/BF03195619

Tan, M. K., & Murray, G. M. (2006). A molecular protocol using quenched FRET probes for the quarantine surveillance of *Tilletia indica*, the causal agent of Karnal bunt of wheat. *Mycological research*, *110*(2), 203-210. https://doi.org/10.1016/j.mycres.2005.08.006

Tillet, M. (1755). Dissertation on the cause of the corruption and smutting of the kernels of wheat in the head and the means of preventing these untoward circumstances. Phytopathology Classics 5.

Vánky, K. (1994). *European Smut fungi*. Gustav Fischer.

Vánky, K. (2012). *Smut fungi of the world* American Phytopathological Society.

Waldow, F., & Jahn, M. (2016). Investigations in the regulation of common bunt (*Tilletia tritici*) of winter wheat with regard to threshold values, cultivar susceptibility and non-chemical protection measures. *Journal of Plant Diseases and Protection*, *114*(6), 269-275. https://doi.org/10.1007/bf03356228

Wang, A., Pang, L., Wang, N., Ai, P., Yin, D., Li, S., Deng, Q., Zhu, J., Liang, Y., Zhu, J., Li, P., & Zheng, A. (2018). The pathogenic mechanisms of *Tilletia horrida* as revealed by comparative and functional genomics. *Sci Rep*, *8*(1), 15413. https://doi.org/10.1038/s41598-018-33752-w

Wang, A., Shu, X., Niu, X., Yi, X., & Zheng, A. (2019). Transcriptome analysis and whole genome re-sequencing provide insights on rice kernel smut (*Tilletia horrida*) pathogenicity. *Journal of Plant Pathology*, *102*(1), 155-167. https://doi.org/10.1007/s42161-019-00401-8

Wang, N., Ai, P., Tang, Y., Zhang, J., Dai, X., Li, P., & Zheng, A. (2015). Draft genome sequence of the rice kernel smut *Tilletia horrida* Strain QB-1. *Genome Announc*, *3*(3), e00621-00615. https://doi.org/10.1128/genomeA.00621-15

Wang, Q. M., Begerow, D., Groenewald, M., Liu, X. Z., Theelen, B., Bai, F. Y., & Boekhout, T. (2015). Multigene phylogeny and taxonomic revision of yeasts and related fungi in the Ustilaginomycotina. *Stud Mycol*, *81*, 55-83. https://doi.org/10.1016/j.simyco.2015.10.004

Xiao, Y., Dai, Y., Lu, Y., Liu, W., Wang, Z., & Bian, Y. (2010). Development of IRAP-SCAR marker for strain identification in *Lentinula edodes*. *World Journal of Microbiology and Biotechnology*, *27*(7), 1731-1734. https://doi.org/10.1007/s11274-010-0626-x

Yao, Z., Qin, D., Chen, D., Liu, C., Chen, W., Liu, T., Liu, B., & Gao, L. (2019). Development of ISSR-derived SCAR marker and SYBR Green I Real-time PCR method for detection of teliospores of *tilletia laevis* Kuhn. *Sci Rep*, *9*(1), 17651. https://doi.org/10.1038/s41598-019-54163-5

Zdobnov, E. M., Tegenfeldt, F., Kuznetsov, D., Waterhouse, R. M., Simao, F. A., Ioannidis, P., Seppey, M., Loetscher, A., & Kriventseva, E. V. (2017). OrthoDB v9.1: cataloging evolutionary and functional annotations for animal, fungal, plant, archaeal, bacterial and viral orthologs. *Nucleic acids research*, *45*(D1), D744-D749. https://doi.org/10.1093/nar/gkw1119

Zgraja, G., Krämer, S., Jonitz, A., & Wagner, W. (2016). Identification and quantification of *Tilletia caries* and *T. controversa* in seed samples and discrimination of the two species. *Research & Reviews: Journal of Botanical Sciences*, *5*(2), 7.

Ziegler, R., Lutz, M., Piatek, J., & Piatek, M. (2018). Dismantling a complex of anther smuts (*Microbotryum*) on carnivorous plants in the genus *Pinguicula*. *Mycologia*, *110*(2), 361-374. https://doi.org/10.1080/00275514.2018.1451697

Zouhar, M., Mazáková, J., Prokinová, E., Váňová, M., & Ryšánek, P. (2010). Quantification of *Tilletia caries* and *Tilletia controversa* mycelium in wheat apical meristem by real-time PCR. *Plant Protection Science*, *46*(3), 107-115.

Župunski, V., Jevtić, R., Ignjatović-Micić, D., Stanković, S., Lević, J., Ivanović, D., & Nikolić, A. (2012). Incidence of *Tilletia* species in non-processed seed of *Triticum aestivum* in Vojvodina, Serbia. *Seed Science and Technology*, *40*(3), 320-332. https://doi.org/10.15258/sst.2012.40.3.04

Chapter 2 – *In silico* investigations reveal low levels of inter- and intraspecies variation in genome sequences of *Tilletia caries*, *T. controversa*, and *T. laevis*, causal agents of wheat bunts

Somayyeh Sedaghatjoo[1], Bagdevi Mishra[2], Monika K. Forster[3], Yvonne Becker[1], Jens Keilwagen[4], Berta Killermann[3], Marco Thines[5], Petr Karlovsky[6*], Wolfgang Maier[1*]

Email of the corresponding author: s.sedaghatjoo@gmail.com

Manuscript under review (IMA Fungus)

[1] Julius Kühn Institute (JKI) – Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

[2] Senckenberg Biodiversity and Climate Research Centre, Senckenberganlage 25, 60325 Frankfurt am Main, Germany

[3] Bavarian State Research Center for Agriculture, Institute for Crop Science and Plant Breeding, Vöttinger Straße 38, 85354 Freising, Germany

[4] Julius Kühn Institute (JKI) – Federal Research Centre for Cultivated Plants, Institute for Biosafety in Plant Biotechnology, Erwin-Baur-Str. 27, 06484 Quedlinburg, Germany

[5] Goethe University, Faculty of Biological Sciences, Institute of Ecology, Evolution and Diversity, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

[6] Molecular Phytopathology and Mycotoxin Research, University of Goettingen, Grisebachstrasse 6, 37077 Goettingen, Germany

* joint senior authors, listed in alphabetical order.

2. 1. Abstract

Tilletia caries and *T. laevis*, which are the causal agents of common bunt, as well as *T. controversa,* which causes dwarf bunt of wheat, threaten especially organic wheat farming. The three closely related fungal species differ in their teliospore morphology and partially in their physiology and infection biology. Intraspecies variation in these species and the genetic basis of their separation is unknown.

We sequenced four *T. caries*, five *T. controversa*, and two *T. laevis* genomes. We extended this dataset with five publicly available genomes. The genomes of the three species displayed microsynteny with up to 94.3% pairwise aligned regions excluding repetitive regions. Overall, 75% of the total identified protein-coding genes were conserved and shared across all 16 isolates, comprising 84% of the total predicted carbohydrate utilizing enzymes, 72.5% putatively secreted proteins, and 47.4% of effector-like proteins. Most of the functionally characterized genes involved in pathogenicity, life cycle, and infection of corn smut, *Ustilago maydis,* were absent or poorly conserved in the draft genomes. We predicted nine highly identical secondary metabolite biosynthesis gene clusters comprising in total 62 genes in all species. The biosynthetic pathway for trimethylamine in *Tilletia* spp. was found to be different from bacteria. Less than 0.1% of the protein-coding genes were species-specific and their function remained mostly unknown. Excluding repetitive regions, *T. controversa* had the highest inter- and intraspecies genetic variation, followed by *T. caries* and the lowest in *T. laevis.*

Although the genomes of the three species are very similar, *T. controversa* differs from common bunt fungi by higher genetic diversity. Despite the conspicuously different teliospore ornamentation of *T. caries* and *T. laevis*, a high degree of genomic identity and the lack of species-specific genes indicate that the two species could either be conspecific or separated only recently.

Keywords

comparative genomics, fungal pangenomes, functional genomics, trimethylamine biosynthesis, Basidiomycota

2. 2. Introduction

The basidiomycete genus *Tilletia* (Tilletiales, Exobasidiomycetes, Ustilaginomycotina) comprises about 186 described species causing smut disease on *Poaceae* (Vánky, 2012; Denchev and Denchev, 2013; Li *et al.*, 2014; Denchev and Denchev, 2018b; a; Denchev *et al.*, 2018). *Tilletia* species are biotrophs that do not develop specialized cellular infection structures but form so-called local interaction zones in the host tissue (Begerow *et al.*, 2014). The term bunt is used for cereal-infecting species of *Tilletia* that produce teliospores in the ovary of the host plant (Carris *et al.*, 2006). The infection of cereal crops by bunt species remains asymptomatic up to culm elongation (Purdy *et al.*, 1963). The infected seeds smell like fish due to the production of trimethylamine (Hanna *et al.*, 1932; Nielsen, 1963). Contaminated seeds are not suitable for human and animal consumption at a certain infection level and must be treated according to their infection level for use as seeds in organic and conventional farming.

Three kinds of bunt diseases are known from wheat species (*Triticum* spp.). Common, dwarf, and karnal bunt. Only common and dwarf bunt affect wheat production in Central Europe, where they are under phytosanitary regulation for seed certification in organic and conventional farming. *Tilletia caries* [syn. *T. tritici*] and *T. laevis* [syn. *T. foetida*] cause common bunt of wheat (Woolma and Humphrey, 1924; Vánky, 2012), a disease that occurs in wheat-growing areas worldwide (Hoffmann, 1982; Goates, 1996). Dwarf bunt is caused by *T. controversa,* which is reported to be limited to higher elevations (Goates, 1996) or regions with prolonged cooler temperatures (Carris, 2010). However, in recent years the disease has also been observed to extend to lowland regions in Germany (Rudloff *et al.*, 2020). *Tilletia controversa* is economically important for international seed trading because it is a quarantine pathogen in several countries (Mathre, 1996; Whitaker *et al.*, 2001; Peterson *et al.*, 2009; Jia *et al.*, 2013).

Tilletia caries, *T. controversa*, and *T. laevis* differ in several biological and physiological features. Firstly, the morphology of teliospores varies from smooth in *T. laevis* to deep and broadened reticulations in *T. controversa* and an intermediate form in *T. caries*. Secondly, the teliospores of *T. caries* and *T. laevis* germinate within a week at 12 to 15 °C under illumination or in dark, while germination of *T. controversa* teliospores requires up to eight weeks at the optimum temperature of 3 to 5 °C and light is essential for germination (Purdy *et al.*, 1963). Furthermore, the infection of wheat by common bunt pathogens occurs before the emergence of the coleoptile, whereas *T. controversa* attacks the same organ after emergence (Carris, 2010). Also, disease symptoms differ moderately between common and dwarf bunt. Substantial wheat stunting and enhanced tillering occur in dwarf bunt and its severity

varies among wheat cultivars (Goates, 1996; Carris, 2010), while stunting in common bunt diseased wheat is not readily distinguishable. Despite the different morphological and physiological features, molecular phylogenetic analysis based on three loci could not resolve the three species unequivocally (Carris *et al.*, 2007). However, a phylogenomic study based on 4,896 single-copy orthologous genes analyzing ten *Tilletia* isolates (one *T. caries*, two *T. controversa*, two *T. laevis*, three *T. indica* (wheat karnal bunt), two *T. walkeri* (ryegrass bunt) isolates) suggested that the three species are distinct (Nguyen *et al.*, 2019).

Recent studies reported transcriptomic analyses of wheat spikes infected by *T. controversa* (Ren *et al.*, 2020) and characterization of the wheat resistance response against *T. controversa* (Muhae-Ud-Din *et al.*, 2020). Despite the growing concern about common and dwarf bunt as major threat to especially organic wheat production due to a limited number of durably resistant cultivars (Ruzgas and Liatukas, 2008; Matanguihan *et al.*, 2011; Aydoğdu and Kaya, 2020), and the fact that *T. controversa* is a quarantine pathogen, the genomic structure and gene contents of the three species has so far not been studied. Recent studies reported however genomic and transcriptomic analyses of *T. indica,* (Sharma *et al.*, 2016; Kumar *et al.*, 2017; Kumar *et al.*, 2018; Gurjar *et al.*, 2019; Mishra *et al.*, 2019; Pandey *et al.*, 2019; Singh *et al.*, 2019; Singh *et al.*, 2020), and *T. horrida*, the rice kernel smut (Wang *et al.*, 2015; Wang *et al.*, 2018; Wang *et al.*, 2019a; Wang *et al.*, 2019b; Wang *et al.*, 2020). Within the genus these two species are only distantly related to common and dwarf bunt and also differ in their infection biology as they are not systemically but locally infecting species (Carris *et al.*, 2006).

Recently, genome sequencing data for one *T. caries* isolate, two isolates of *T. controversa,* and two isolates of *T. laevis*, mainly collected from North America, as well as *T. indica* and *T. walkeri* were published and used for the identification of species-specific DNA markers as well as their phylogenetic relation (Nguyen et al., 2019) The study has however not addressed the differences among the sequenced genomes where sampling size per common and dwarf bunt species was small. Here, we report draft genome sequences of four *T. caries* isolates, five *T. controversa* isolates, and two isolates of *T. laevis,* obtained from single teliospore cultures that except for one isolate of *T. laevis*, originated from recent European populations. These genome sequences were analyzed together with five published *Tilletia* spp. genomes (Nguyen *et al.*, 2019) to provide a first insight into the genomic diversity within and between these three important pathogens.

2. 3. Methods

2. 3. 1. Isolates, single teliospore cultures, and genomes

Isolates of four *T. caries*, five *T. controversa*, and two *T. laevis* were whole-genome sequenced in this study (Table 1). To obtain DNA for genome sequencing, cultures of *T. caries*, *T. controversa,* and *T. laevis* were grown from single teliospores. For the production of single teliospore cultures, teliospores were surface-sterilized as described by Castlebury *et al.* (2005). Briefly, bunt balls were crushed using a pair of sterile fine-point forceps and the wheat ovary tissue was carefully removed. The teliospores were immersed in 0.26% (v/v) NaClO (Carl Roth, Karlsruhe, Germany) for 30 s, pelleted by centrifugation for 10 s and rinsed twice with sterile, distilled water. For germination, surface-sterilized teliospores were streaked on 1.5% water-agar and incubated either at 5 °C under constant light (*T. controversa* germination) or at 15 °C in darkness (*T. caries* and *T. laevis* germination). A single germinated teliospore of each specimen was then transferred to M-19 agar medium (Trione, 1964) using a sterile needle. Cultures on M-19 were maintained at 15 °C in the dark to establish colonies for nucleic acid extraction. The medium was supplemented with penicillin G (240 mg/L) and streptomycin sulfate (200 mg/L). The mycelium was freeze-dried (Christ ALPHA1-4 LSC, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) at -40 °C for 48 h and afterward kept at 4 °C until use. Duplicates of the single teliospore cultures obtained in this study were deposited at Westerdijk Fungal Biodiversity Institute (CBS-KNAW, Utrecht, The Netherlands). Additionally, the genome assembly and annotation files of one *T. caries*, two *T. controversa*, and two *T. laevis* isolates were retrieved from the National Center for Biotechnology Information (NCBI) repository. In total, 16 genomes comprising five *T. caries*, seven *T. controversa*, and four *T. laevis* isolates were used in this study (Table 2-1).

Table 2-1 Analyzed *Tilletia* isolates in this study

Taxon	Isolate (Accession number[1])	Collection year	Geographical origin	Host	BioProject accession	Sequencing technology	Reference
T. caries	AA11 (CBS 144825)	2015	Austria	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. caries	AI (CBS 145171)	2015	Italy	*T. durum*	PRJEB40624	Illumina HiSeq 4000	This work
T. caries	AO (CBS 145172)	2014	Germany	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. caries	AZH3 (CBS 145166)	2015	Switzerland	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. caries	DAOMC 238032	1996	USA	*Triticum* sp.	PRJNA317434	Illumina HiSeq	(Nguyen et al., 2019)
T. controversa	DAOMC 236426	1998	Canada	*Triticum* sp.	PRJNA317433	Illumina HiSeq, MiSeq, PacBio	(Nguyen et al., 2019)
T. controversa	DAOMC 238052	1997	Canada	*Triticum* sp.	PRJNA393324	Illumina MiSeq	(Nguyen et al., 2019)
T. controversa	OA2 (CBS 145169)	2015	Austria	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. controversa	OL14 (CBS 145167)	2014	Germany	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. controversa	OR (CBS 144827)	2013	Germany	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000, PacBio RS II	This work
T. controversa	OV (CBS 145170)	2011	Germany	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. controversa	OW (CBS 145168)	2013	Germany	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. laevis	ATCC 42080	-	USA	*Triticum* sp.	PRJNA393337	Illumina MiSeq	(Nguyen et al., 2019)
T. laevis	DAOMC 238040	1997	Australia	*Triricum* sp.	PRJNA393335	Illumina MiSeq	(Nguyen et al., 2019)
T. laevis	LLFL (CBS 144826)	2015	Germany	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work
T. laevis	L-19 (CBS 145173)	-	-	*T. aestivum*	PRJEB40624	Illumina HiSeq 4000	This work

[1] Westerdijk Fungal Biodiversity Institute (CBS-KNAW; Utrecht, The Netherlands)

2. 3. 2. High molecular weight nucleic acid extraction and whole-genome sequencing

Total genomic DNA from freeze-dried mycelium was extracted using a modified CTAB-based method (Brandfass and Karlovsky, 2008). We prepared pulverized 30 – 50 mg lyophilized mycelium in 2 ml reaction tubes with four 4 mm sterile tungsten carbide beads at 22 Hz for 50 s using a tissue lyser (Qiagen Tissuelyser II, Qiagen, Hilden, Germany). The bead-beating step was repeated twice. The reaction tubes were shaken vigorously between the two disruption steps to loosen the mycelium from the bottom of the tubes after bead beating.

The CTAB-based protocol was modified as follows: the ultrasonic bath step was omitted, the CTAB buffer was additionally supplemented with 400 µg RNase (Carl Roth, Karlsruhe, Germany) and β-mercaptoethanol was increased to 5 µL. Both chemicals were added to the buffer shortly before incubation. The samples were incubated for 60 min at 65 ºC and 400 rpm. DNA was additionally purified by adding one volume of phenol/chloroform/isoamylalcohol (25:24:1 v/v/v) (Carl Roth, Karlsruhe, Germany) and then precipitated by using 0.6 volume of isopropanol (Merck, Darmstadt, Germany). The DNA was finally dissolved in 500 µL commercially available elution buffer (10 mM Tris-Cl, pH 8.5) (Qiagen, Hilden, Germany) at room temperature.

To digest the remaining RNA, 100 µg RNase (Carl Roth, Karlsruhe, Germany) were added to the extracted gDNA, the mixture was inverted several times and incubated for 1 h at 42 °C at 100 rpm. After 30 min 7 µg proteinase K (Carl Roth, Karlsruhe, Germany) were also added. RNA and proteins were removed by adding one volume of phenol/chloroform/isoamylalcohol (25:24:1 v/v/v), and DNA was precipitated by isopropanol (1:1 v/v) (Merck, Darmstadt, Germany). Polar fractions were retrieved through 13,000 ×*g* centrifugation. The obtained DNA pellet was washed twice with 70% (v/v) ethanol and resuspended in the elution buffer. DNA from different extraction replicates was pooled. The quality and quantity of the isolated DNA was measured with a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Darmstadt, Germany) and stored at -20 ºC until shipping.

For whole-genome sequencing, the DNA from single teliospore cultures of eleven isolates was shipped to GATC biotech AG (GATC Biotech AG, Konstanz, Germany) for fragmentation, library preparation, and sequencing on an Illumina HiSeq 4000 platform (125 bp, paired-end reads). Whole-genome shotgun sequencing of one isolate (*T. controversa* OR) was additionally performed using a PacBio RS II instrument P6-C4 chemistry and a total of seven Single Molecule Real-Time (SMRT) cells were sequenced for this isolate.

2. 3. 3. DNA sequence assembly

Trimmomatic v0.36 (Bolger *et al.*, 2014) was used to trim adapters and low quality reads from Illumina HiSeq data from 11 *Tilletia* species (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:70 AVGQUAL:25). High-quality reads with minimum lengths of 70 bp for both reads and >25 average quality were retained for further processing. PacBio reads of *T. controversa* (OR) were corrected using the cleaned Illumina HiSeq reads from the same species using Proovread v2.12 (Hackl *et al.*, 2014). The prooveread-corrected untrimmed PacBio reads were further corrected and trimmed using self-correction and trimming method implemented in Canu v1.6 assembler (Koren *et al.*, 2017). A draft assembly was constructed using corrected-trimmed PacBio reads using Canu assembler, which was further scaffolded using Illumina paired reads (SSpace-Standard V3.0 (Boetzer *et al.*, 2011)) and with Illumina-corrected-trimmed PacBio reads (SSpace-LongRead (Boetzer and Pirovano, 2014)). The assembly statistics were generated using assemblathon_stats.pl (Author: Keith Bradnam, Genome Center, UC Davis) and CEGMA v2.5 (Parra *et al.*, 2007) to assess genome completeness. In a separate approach, all the 11 *Tilletia* isolates were assembled, using the remaining Illumina reads employing Velvet assembler v1.2.10 (Zerbino and Birney, 2008) (-scaffolding on). Several assemblies were generated for all the species at different *k*-mers. Assembly statistics and CEGMA completeness of all the assemblies were tabulated and for individual species the best assembly in terms of statistics and CEGMA completeness was manually chosen.

2. 3. 4. Identification of repetitive regions, simple sequence repeats, and transposable elements

Draft genome sequences were used to identify SSRs, also known as microsatellites by using the tool MIcroSAtellite identification (MISA) (Beier *et al.*, 2017). The search criteria were at least ten repeat units for mononucleotide, six repeats for dinucleotide, and five for tri-, tetra-, penta-, and hexanucleotide motifs. SSRs with less than 100 bp distance from each other were considered as compound microsatellite. The relative abundance for each SSR type was calculated by the number of repeats per Megabyte of genome.

Transposable elements (TE)s were identified computing TransposonPSI (Haas, 2010) with default settings. The program employs PSI-BLAST search (Altschul *et al.*, 1997) against a database of various collections of TE families to identify matching regions in the genome. Additionally, we used RepeatModeler version 1.0.11 (Smit *et al.*, 2008-2019) to create a library comprising *de novo* identified

repetitive elements. RepeatModeler employs three *de novo* repeat finders, RECON (Bao and Eddy, 2002), RepeatScout (Price *et al.*, 2005), and Tandem Repeats Finder (Benson, 1999). The number of identified TEs and SRRs (see above) was subtracted from the total number of repeats identified by RepeatModeler as unclassified repetitive elements.

The resulting library of RepeatModeler was used to mask respective elements in the target genome sequences using RepeatMasker 4.0.9 (Smit *et al.*, 2013-2015). For different purposes (see below), we used both soft-masking (repeats replaced by lowercase letters) and hard-masking (repeats replaced by N).

2. 3. 5. Detection of single nucleotide polymorphisms (SNPs) and small insertions and deletions (indels)

For pairwise SNP and indel identification, we used hard-masked genomes generated by RepeatMasker. For each genome pair, SNPs and the total length of indels in the aligned regions were counted using dnadiff wrapper from MUMmer 3.0 (Kurtz *et al.*, 2004) package (show-snps -C). Average nucleotide identities of one-to-one alignments were also obtained from dnadiff output.

2. 3. 6. Gene model prediction

Genes in the newly sequenced genomes (n = 11) were predicted from the soft-masked assemblies while for the publicly available genomes we used the existing gene annotations. We used a combination of *ab initio* and homology-based approach for gene model prediction. Gene models were created first by the incorporation of multiple sources of evidence using Gene Model Mapper (GeMoMa pipeline: V1.6.2 beta) (Keilwagen *et al.*, 2016; Keilwagen *et al.*, 2018). GeMoMa is a homology-based gene prediction program and uses RNA-Seq data to incorporate evidence for splice site prediction. Afterwards, BRAKER2 (Brůna *et al.*, 2020), which utilizes the *ab initio* gene predictor Augustus 3.3.3 (Stanke *et al.*, 2006) and GeneMark-ET 4.33 (Lomsadze *et al.*, 2014) self-training algorithms were applied. To do so, publicly available genome sequences and structural annotations of six Exobasidiomycetes isolates (Kijpornyongpan *et al.*, 2018) and additionally the smut model organism *U. maydis* (Kamper *et al.*, 2006) were downloaded from GenBank (references are given in Additional Table 2-1). Additionally, three RNA-Seq datasets were derived from two different *T. caries* isolates (DAOMC 238032 and WSP 72095/517) and one *T. controversa* isolate (DAOMC 236426) were downloaded from GenBank (Additional Table 2-2).

For adapter clipping and read trimming of the RNA-Seq data, the utility program Trim Galore version 0.4.0 (Krueger, 2012-2019) was employed (qval >= PHRED 30, minimal read length of

50 bp). Trimmed reads were mapped to the assembled genome sequences using STAR version 2.4.0d-2 (Dobin *et al.*, 2012) with default parameters. The two RNA-Seq datasets of *T. caries* (SRR2513861 and SRR3337311) were mapped to *T. laevis* assemblies because no RNA-Seq data was available for *T. laevis* and the two species are closely related.

Protein-coding exons were extracted from the seven reference genomes by GeMoMa module Extractor (part of GeMoMaPipeline) using the default parameters. The GeMoMa Extract RNA-Seq evidence (ERE) was used to extract intron boundaries of each target genome by utilizing RNA-Seq data (coverage = true). We permitted alternative transcripts. The rest of the parameters were set as follows: maximum intron length = 2500, tBLASTn = false, ambiguity = ambiguous, score = ReAlign, rename = no. Filtered predictions (start = 'M' and stop = '*' sorting = score/AA >= 0.50) file for each genome generated by GeMoMa was used with align2hints command to produce hint file for BRAKER2. The corresponding softmasked genome, the STAR RNA-Seq mapped file, and the GeMoMa hint file were used to run BRAKER2 (UTR = on) for each genome. The generate gene model by BRAKER2 was used for further analysis. To predict the coding regions of transfer RNA (tRNAs), tRNAscan-SE 2.0 (Lowe and Chan, 2016) was retrieved with eukaryotic sequence source in the default search mode.

To estimate the completeness of the gene model predictions, BUSCO V. 3.0.2 (Benchmarking Universal Single-Copy Orthologs) program (Simao *et al.*, 2015) was used. BUSCO utilizes sets of core genes in taxon-specific databases to evaluate the relative completeness of a given annotation. We used the lineage dataset for Fungi-OrthoDB9 (Zdobnov *et al.*, 2016) in the proteome mode.

2. 3. 7. Functional annotation of the predicted genes

Genome-wide annotation was done to relate putative biological functions to the predicted genes. To make functional annotation comparable between all draft genomes, we analyzed all 16 genomes used in this study. The putative functions were assigned to the predicted proteins through one-to-one orthology assignments by eggNOG-Mapper 5.0.0 (Huerta-Cepas *et al.*, 2018) (one-to-one ortholog, auto taxonomic adjust mode). Only functional annotations derived from Eukaryote or fungal sequence sources were accepted. Functional descriptions of Gene Ontology (Go) terms (Ashburner *et al.*, 2000; Gene Ontology, 2015), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and modules (Kanehisa *et al.*, 2013), and COG/KOG functional categories (Levasseur *et al.*, 2013; Galperin *et al.*, 2014), and SMART/PFAM domains (Letunic and Bork, 2018) were obtained using eggNOG-Mapper.

2. 3. 8. Prediction of encoded carbohydrate-active enzymes, secreted proteins, and secondary metabolites

Carbohydrate-active enzymes derived from the draft genomes were predicted using the HMM-based-dbCAN server (HMMdb v8.0) with a cut-off E-value < $1e^{-17}$ (suggested for fungi) and coverage > 0.50 (Zhang *et al.*, 2018). Out of this prediction, the potential plant cell wall degrading enzymes were classified for their substrate according to Kijpornyongpan *et al.* (2018).and Benevenuto *et al.* (2018).

Putatively secreted proteins (referred to secretome in their totality) were identified by the presence of a signal peptide and absence of transmembrane domains in the predicted proteomes of each genome according to the suggestions of (Min, 2010). Briefly, the proteome of each draft genome was first screened by SignalP 5.0 (Almagro Armenteros *et al.*, 2019). To check whether the prediction belonged to an integral membrane protein, transmembrane α-helix predictor TMHMM v. 2.0 (Krogh *et al.*, 2001) in tandem was employed. Those signal-peptide-like proteins showing any transmembrane helix topology were filtered out. Additionally, the signal peptides were predicted using Phobius (Kall *et al.*, 2007) webserver accessed on Sep. 2019 with default parameters. In the end, only those putative proteins containing signal peptides that had been predicted by both independent approaches were annotated as secretome. To predict the effector repertoire from the predicted secretome of each genome, EffectorP 2.0 (Sperschneider *et al.*, 2018) accessed on Sep. 2019 was used.

The draft genome sequences were searched for secondary metabolites and biosynthetic gene clusters using the fungal version of antiSMASH 5.0 (antibiotics and Secondary Metabolite Analysis Shell) (Medema *et al.*, 2011). Identified gene clusters were grouped based on their similarity (>80% identity). Since genes of a cluster may be dispersed on different contigs, the presence, the completeness, and the order of each gene cluster was validated by aligning Illumina reads of each isolate to a reference sequence from each gene cluster group according to a mapping approach described by Weber *et al.* (2019). Briefly, the reference sequence was selected for each gene cluster group based on either length or high sequence conservation among the different isolates. Illumina reads of each isolate (Additional Table 2 6) were trimmed using Trimmomatic version 0.36 (Bolger *et al.*, 2014) with a 4:15 sliding window. The trimmed reads were aligned to the different genes clusters references using Bowtie v2.4.1 (Langmead and Salzberg, 2012). SAMtools v1.10 (Li *et al.*, 2009) was used for file conversion to bam, validation of read pairing information (fixmate), removal of reading duplicates (rmdup), removal of mapped singleton version 3.5.1 (R Development Core Team, 2013).

2. 3. 9. Orthologous gene identification and clustering

The OrthoMCL pipeline v 2.0 (Li *et al.*, 2003) was used to identify clusters of orthologous genes among all 16 isolates of the three species (inflate = 1.8 and E-value = $1e^{-10}$). Input translated protein sequences of all predicted genes contained also alternative transcripts per gene. The OrthoMCL program applies all-against-all BLASTp to estimate similarities between proteins and identifies groups using Markov clustering algorithm. The output of the program was parsed by using a custom-made phyton script to define; (i.) orthologous genes that were present in all isolates referred to shared genes at the interspecies level and core genome at intraspecies level; (ii.) orthologues genes shared between all isolates of a species and absent in the others (species-specific genes); (iii.) the accessory (at intraspecies level) or variable (at interspecies level) genes which were dispensable and not present in all genomes; (iv.) singletons presented only in a single isolate (isolate-specific genes). Putative functional prediction of an orthology cluster was reported only when at least 75% of the genes within shared an identical annotation.

2. 4. Results

2. 4. 1. Whole-genome sequencing and genome annotation of *T. caries*, *T. controversa*, and *T. laevis*

The assembly of ten draft genomes based only on Illumina reads (four *T. caries*, four *T. controversa*, and two *T. laevis* isolates) resulted in assembled genome sizes of 30.3 to 31.7 Mb (*T. caries*), 29.4 to 31.9 Mb (*T. controversa*), and 30.8 Mb (*T. laevis*) with GC contents between 56.5 to 56.7% (Figure 2-1). N50 values varied between 9.3 and 17.8 kb. The hybrid assembly of the *T. controversa* isolate OR, which was sequenced using both Pacific Biosciences (PacBio) and Illumina reads, resulted in a draft genome size of 49.3 Mb (scaffold N50 = 137 kb) distributed in 985 scaffolds with the GC content of 55.7% (Figure 2-1).

A combination of *de novo* and order-specific gene model data was used after assessing annotation completeness for each annotation approach separately employing a genome (Additional Table 2-4). In total, 9,807 to 9,943 protein-coding genes were annotated in the *T. caries* genomes, 9,679 to 10,459 in the *T. controversa* genomes, and 10,160 and 10,203 in the two *T. laevis* genomes (Table 2-2). Coding sequences (CDS) consisted of 3.5 exons on average. Alternative splicing forms were predicted for up to 1.4% of the total CDS (Table 2-2). Genomes contained 110 to 178 genes encoding transfer RNAs (tRNAs) (Table 2-2). The specificity of these tRNAs covered up to 48 of 61 possible anticodons and the codon usage was identical in all three species. To check whether the

tRNA genes were clustered, we examined the location of tRNA genes in the most contiguous genome (OR isolate). A total of 178 putative tRNA genes were distributed over 102 scaffolds. The maximum number of 14 tRNA genes plus 9 pseudogenes spanned a 151,829 bp long region on scaffold number OR-9 (accession number CAJHJB010000889).

The completeness of the genomes annotation was evaluated using the fungi database (OrthoDB v9 (Zdobnov *et al.*, 2016)) as a reference. From the 290 total BUSCO (Benchmarking Universal Single-Copy Orthologs) (Simao *et al.*, 2015) groups of the database, 91.1 to 96.9% were recovered in the draft genomes (Table 2-2). For the comparative analyses, we added five publicly available draft genomes (see Table 2-1) to the 11 genomes obtained in this study, resulting in five genomes of *T. caries*, seven genomes of *T. controversa*, and four genomes of *T. laevis*.

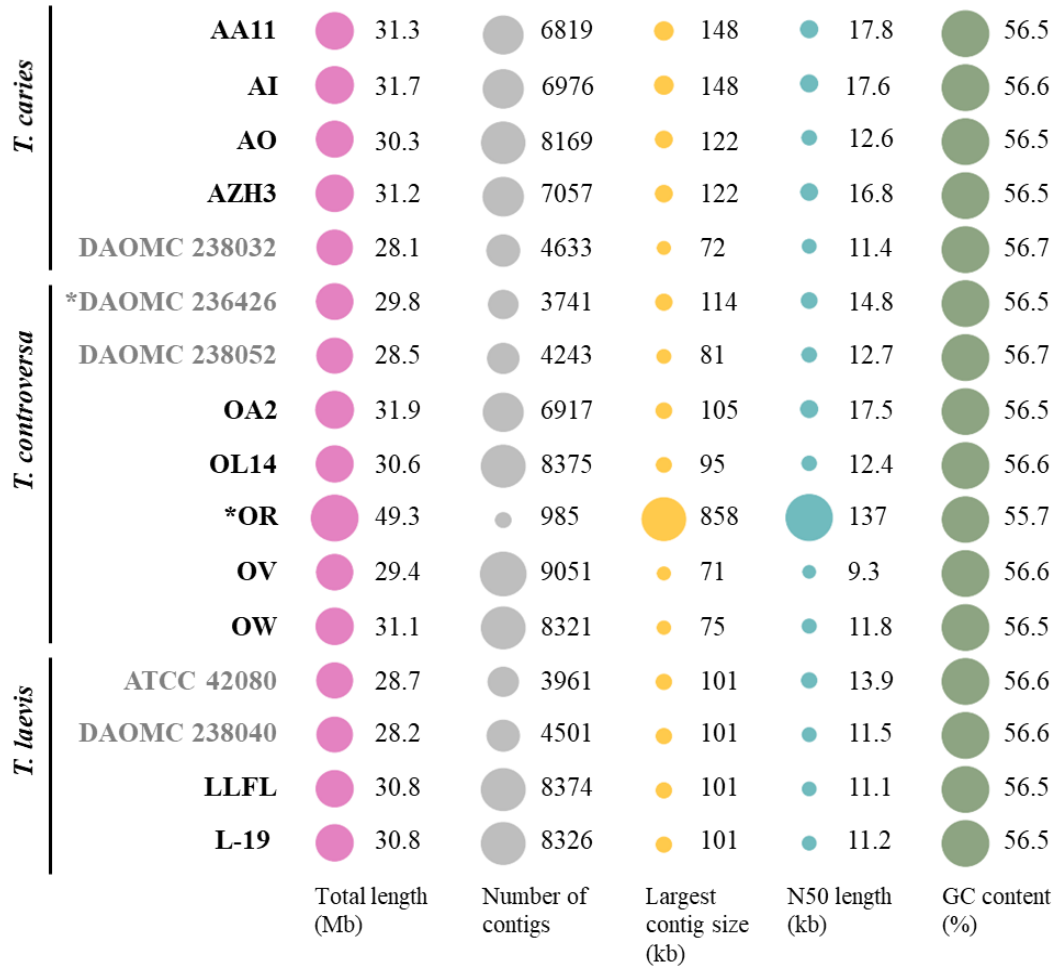


Figure 2-1 Bubble plots of descriptive numbers for each genome. The bubble sizes are scaled only within categories. The genome assemblies done in this work are presented in black color. Two *T. controversa* isolates OR and DAOMC 236426 were sequenced both on Illumina and PacBio platforms and marked with asterisk.

Table 2-2 Summary of gene model prediction results

	T. caries				*T. controversa*					*T. laevis*	
Annotation statistics	**AA11**	**AI**	**AO**	**AZH3**	**OA2**	**OL14**	**OR***	**OV**	**OW**	**LLFL**	**L-19**
Gene model	9913	9943	9807	9930	9679	9956	10459	9680	9797	10203	10160
tRNA	119	128	114	123	119	116	178	110	118	116	116
Average gene length (bp)	1790	1734	1756	1766	1813	1706	1931	1720	1755	1671	1674
Total CDS length (Mb)	14.9	14.8	14.5	14.8	14.8	14.6	16.9	14.1	14.5	14.6	14.6
Number of exons	36009	35788	35117	35601	35266	34951	40922	33870	34789	35521	35439
Exons per gene	3.63	3.6	3.58	3.58	3.64	3.51	3.91	3.49	3.55	3.48	3.48
Number of introns	26443	26440	25784	26103	25944	25832	30399	24833	25549	26258	26216
Introns per gene	2.66	2.65	2.62	2.62	2.68	2.59	2.9	2.56	2.6	2.57	2.58
% of genome covered by genes	56.3	54.1	56.5	55.8	54.7	55	40.5	56.3	55	55.03	54.86
Transcripts	10052	10069	9930	10053	9800	10066	10585	9801	9914	10334	10292
Annotation completeness	96.6	95.8	93.8	95.1	95.5	94.5	96.9	91.1	93.8	93.1	94.1

* Reference genome

2. 4. 2. Structural genomics

2. 4. 2. 1. Repetitive sequences and transposable elements

In eukaryotic genomes, repetitive elements are widespread. Although they are generally regarded as genome parasites or remnants of molecular evolution, some repetitive sequences were shown to play diverse roles in environmental adaptation and genome evolution (Wöstemeyer and Kreibich, 2002). The genome fraction assigned to repetitive elements in *Tilletia* species ranged from 7.8 to 13.7% for *T. caries,* 8.9 to 13.6% for *T. controversa*, and 9.1 to 11.8% for *T. laevis* (Additional Table 2-5) and overall, a higher proportion of repetitive elements was found in the newly sequenced genomes. Exceptionally, roughly four times higher repetitive elements (37%) in the genome sequence of *T. controversa* isolate OR and its 49.3 Mb assembled genome size was revealed. Transposition is one of the causes of genomic plasticity and plays an important role in pathogenicity and adaptive evolution (Casacuberta and González, 2013; Muszewska *et al.*, 2019; Razali *et al.*, 2019). Transposable elements (TEs) made up to 3.7% of all repetitive elements in the studied genomes. The values were very similar for all three species. Transposable elements can move or copy from one locus to another, are classified based on their mode of dispersion (Levin and Moran, 2011). The detected TEs were classified into one of 15 superfamilies (), of which DDE-1, *gypsy*, hAT, helitronORF, Itr-Roo, Line, mariner-ant1, MuDR-A-B, and TY1-*copia* were more prevalent in the genomes of European isolates compared to the North American genomes. In all isolates, regardless of the sequencing platform used, the *gypsy*-like and TY1-*copia*-like superfamilies were the most common, accounting for more than half of the total TEs in each genome (Figure 2-2).

Additionally, we classified a total of 6,564 to 10,031 repetitive elements as simple sequence repeats (SSRs), accounting for 0.53 to 0.63% of the entire genomes. Trinucleotides SSRs (35.2 to 42.8% of all SSRs) were the most abundant.

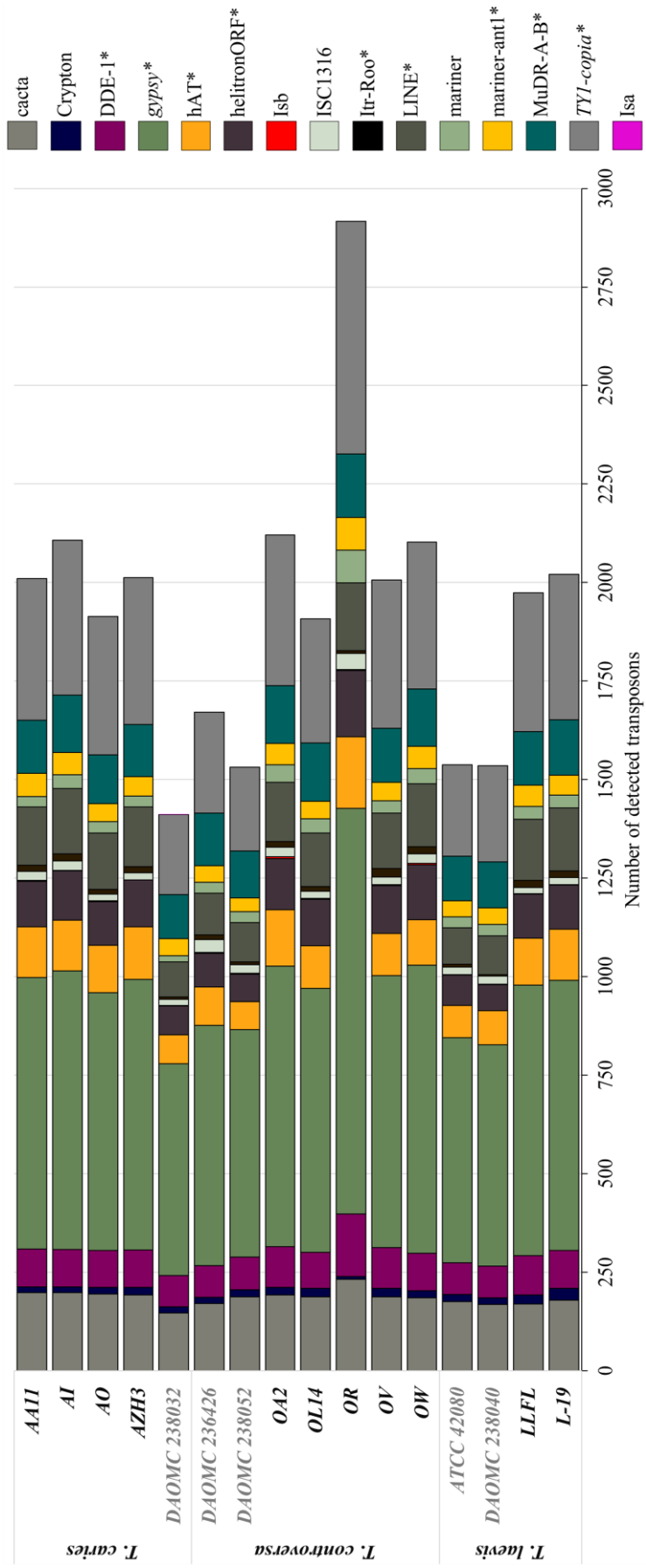


Figure 2-2 Number of detected transposable elements and classification of them, in 16 isolates of common and dwarf bunt. The significant differences in the total number of detected transposable elements between sequenced genomes of this study (black color) and publicly available ones are marked with * (Tukey HSD, $\alpha = 0.001$).

2. 4. 2. 2. Genomic synteny and genome-wide diversity

Overall, 82.7 to 94.3% of the genomes could be aligned pairwisely with an average nucleotide identity between 98.7 to 99.6% in one-to-one aligned regions, excluding repetitive sequences (Additional Table 2-7). Based on the number of single nucleotide polymorphisms (SNPs) and the total length of small insertion or deletions (indels) within species, *T. laevis* isolates, with max. 0.52 SNPs/kb and 1.09 bp indels/kb, were the most homogeneous, while *T. controversa* showed the greatest degree of nucleotide diversity (max. 1.47 SNPs/kb and 2.48 indels bp/kb) (Figure 2-3). On the interspecies level, low nucleotide variation was observed between *T. caries* and *T. laevis* species while all isolates of these two species exhibited a greater distance to the isolates of *T. controversa* (Figure 2-3). No correlation between the sequencing platform and genomic diversity was observed.

2. 4. 3. Functional genomics

Functional information was assigned to gene products based on protein sequence homology (reference database: eggNOG v5.0) (Huerta-Cepas *et al.*, 2018). To ensure comparability, functional annotation was performed for the protein-coding genes of all genomes including new functional annotation of published genomes. At least 55.5% of all coding sequences in each genome were functionally annotated (Figure 2-4A). In general, the identified biological pathways and functional categories were remarkably similar across the three species.

To overcome plant defense systems for successful colonization, plant pathogens employ plant cell wall-degrading enzymes (PCWDE) that are part of carbohydrate-active enzymes (CAZymes), effectors which are a subgroup of secreted proteins, and secondary metabolites (Kimura *et al.*, 2001; Chisholm *et al.*, 2006). We searched the predicted proteomes for these modulators as described below.

	T. caries					*T. controversa*							*T. laevis*			
Isolates	**AA11**	**AI**	**AO**	**AZH3**	DAOMC 238032	DAOMC 236426	DAOMC 238052	**OA2**	**OL14**	**OR**	**OV**	**OW**	ATCC 42080	DAOMC 238040	**LLFL**	**L-19**
AA11		0.49	0.45	0.37	0.49	1.98	2.00	1.81	1.81	0.99	1.97	1.91	0.62	0.54	0.42	0.52
AI	1.53		0.47	0.42	0.53	1.90	1.90	1.81	1.79	0.97	2.06	1.83	0.61	0.52	0.52	0.51
AO	1.42	1.35		0.40	0.53	2.01	2.01	1.86	1.87	0.99	2.06	1.94	0.51	0.40	0.35	0.37
AZH3	1.44	1.50	1.41		0.45	1.95	1.98	1.82	1.78	0.97	1.97	1.86	0.59	0.54	0.42	0.48
DAOMC 238032	0.94	0.96	0.95	0.95		1.98	2.03	1.82	1.82	0.95	2.05	1.92	0.60	0.57	0.50	0.55
DAOMC 236426	2.45	2.39	2.47	2.44	2.17		0.62	1.39	1.39	0.70	1.35	1.35	2.05	2.11	1.89	1.87
DAOMC 238052	2.44	2.39	2.44	2.45	2.22	0.53		1.30	1.41	0.75	1.45	1.37	1.98	2.07	1.83	1.78
OA2	2.95	2.89	2.91	2.91	2.71	2.11	2.10		1.35	0.76	1.31	1.40	2.07	2.10	1.87	1.94
OL14	2.85	2.76	2.81	2.83	2.57	1.98	2.10	2.29		0.66	1.37	1.47	1.93	1.97	1.76	1.77
OR	2.43	2.50	2.36	2.50	1.99	1.38	1.53	2.11	1.92		1.11	1.23	1.65	1.64	1.57	1.58
OV	2.95	2.94	3.00	2.99	2.89	2.14	2.34	2.38	2.42	1.21		1.39	2.13	2.11	1.87	1.91
OW	2.61	2.58	2.65	2.62	2.40	1.80	1.87	2.14	2.20	1.04	2.48		2.11	2.15	1.95	1.94
ATCC 42080	1.09	1.07	1.00	1.10	0.66	2.07	2.16	2.47	2.42	1.16	2.89	2.26		0.52	0.46	0.42
DAOMC 238040	0.84	0.82	0.81	0.83	0.59	2.06	2.20	2.35	2.36	1.12	2.81	2.20	0.56		0.45	0.44
LLFL	1.29	1.25	1.17	1.30	0.82	2.31	2.37	2.58	2.56	1.34	2.94	2.48	0.79	0.80		0.38
L-19	1.35	1.29	1.24	1.33	0.91	2.33	2.36	2.66	2.55	1.34	2.92	2.49	0.84	0.80	1.09	

Figure 2-3 The number of SNPs per kb (upper triangle) and the total length of indels per kb (lower triangle) in the repeat masked genomes of 16 isolates of common and dwarf bunt using NUCmer. The isolates and species are given as row and column labels where the sequenced genomes in this study are presented in black. Density of the SNPs and indels are represented by two color scales.

Figure 2-5 The gene organization of the nine putative secondary metabolite gene clusters found in all 16 *T. caries*, *T. controversa*, and *T. laevis* isolates.

2. 4. 3. 5. Genomic insight into trimethylamine synthesis in *Tilletia* spp.

Fishy smell of grains infected with smut is caused by trimethylamine (TMA), which was isolated already in 1887 from ergot (*Claviceps purpurea*) and *Ustilago* sp. (Diehl, 1887) and in 1932 from *T. laevis*-infected wheat (Hanna *et al.*, 1932). It is not known how fungi synthesize TMA. The biosynthesis of TMA in bacteria was recently unraveled (Craciun and Balskus, 2012). The precursor of TMA in bacteria is choline and the reaction is catalyzed by choline trimethylamine-lyase CutC, which is activated by activating protein CutD. The sequences of both proteins are highly conserved (Martínez-del Campo *et al.*, 2015). Based on the report that TMA in ergot also originates from choline

(Brieger, 1887), we assumed that TMA biosynthesis in bacteria and fungi follow a convergent path or the pathway was transferred from bacteria to fungi, as was reported for other genes (Jaramillo *et al.*, 2015; Navarro-Muñoz and Collemare, 2020). We therefore searched for homologs of *cut*C in *Tilletia* spp. No such protein was found in the proteome of common and dwarf bunt proteome, indicating that *Tilletia* spp. do not possess choline trimethylamine-lyase. Search for proteins similar to activating protein CutD failed, too. Both genes were also missing from the genomes of *Ustilago* and *Claviceps*, indicating that the biosynthesis of TMA in smut and ascomycetes fungi is different from bacteria. Ethanolamine is a structural analog of choline. In bacteria, the degradation of ethanolamine to ammonia is catalyzed by vitamin B_{12}-dependent ethanolamine ammonia-lyase EutBC (Garsin, 2010). This enzyme inspired the search for choline degradation pathway that eventually led to the discovery of CutC/CutD (Craciun and Balskus, 2012). We searched *Tilletia* genomes for genes similar to *eut*BC, too, but no such gene was found, indicating that the synthesis of TMA in fungi does proceed by the removal of the hydroxyethyl group from choline by an enzyme related to ethanolamine ammonia-lyase.

2. 4. 4. Inter- and intraspecies variation of protein-coding genes

To compare protein-coding genes within and among the three species, all 159,834 predicted CDS were grouped into orthology clusters based on the sequence homology of their products using OrhoMCL (Li *et al.*, 2003). From the total of the CDS, 97.6% were grouped into 11,463 orthology clusters; the remaining 2.4% genes were singletons that did not group to any orthology cluster (Figure 2-6). A total of 5,919 orthology clusters (comprising 75.4% of total CDS) were shared by all 16 isolates. Many of them (4,167) were single-copy genes that did not have any paralog in any isolate. Additional 3,203 orthology clusters were shared among all species by at least one but not all isolates per species, indicating that these genes were neither essential nor species-specific.

Interestingly, 84% of the total predicted CAZymes (Figure 2-7), 72.5% of the total secretome (Figure 2-4B), and 47.4% of the genes encoding effectors (Figure 2-4B) were among the 5,919 orthology clusters shared and conserved across all species. The number of species-specific orthology clusters defined by CDS that were present in all isolates of the target species but not in any isolate of the other species varied between 1 (*T. caries*), 21 (*T. controversa*), and 3 (*T. laevis*) (Additional Table 2-11). With a more relaxed definition, allowing the gene of an orthology cluster to be missing in the maximum one isolate of the target species, the numbers increased to 7, 39, and 10 in *T. caries*, *T. controversa*, and *T. laevis*, respectively. We were also interested in the genes that were present in both

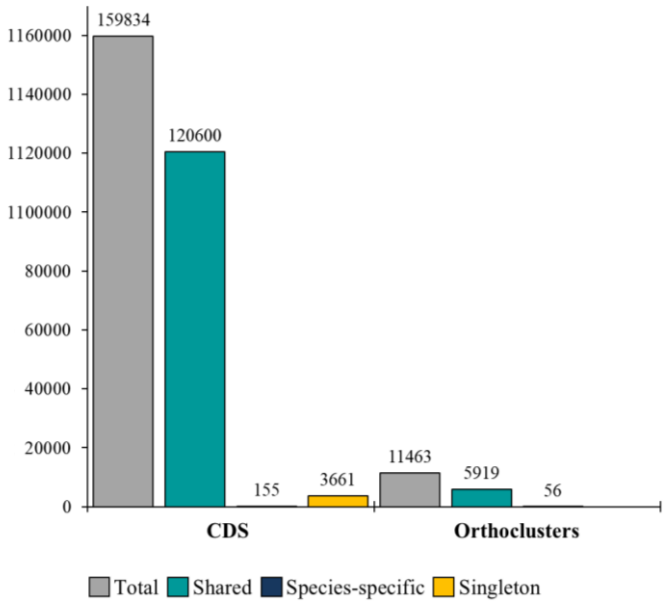


Figure 2-6 Distribution of orthologues clusters and CDS among 16 isolates of *T. caries*, *T. controversa*, and *T. laevis* species. Out of 159,834 total CDS, the majority (120,600) which clustered in 5,919 orthology clusters were among the shared between all the 16 isolates.

causal agents of common bunt (*T. caries* and *T. laevis*) only. Using the strict definition, we found 19 common bunt-specific orthology clusters. Under relaxed criteria, allowing an orthology cluster to be missing at most two isolates, we found 40 common bunt-specific orthology clusters. Putative functions were assigned to only 16 out of 96 total common and dwarf bunt-specific orthology clusters (relaxed and strict) (Additional Table 2-11). In total, species-specific orthology clusters comprised only 0.09% of the total CDS (Not a single effector nor CAZyme was detected among the species-specific genes. However, two orthology clusters comprised genes that putatively encode secreted proteins. One of them was specific for *T. controversa* and the other for the common bunt species *T. caries* and *T. laevis*.

Based on the orthology clusters, we assigned CDS to core genomes (present in all isolates of a species) and pan-genomes (all CDS present in at least one isolate of a species) of each species individually. The largest core genome belonged to *T. laevis* (95.4% of the pangenome). *Tilletia controversa* had the largest accessory genome (6.1% of the pangenome) (Table 2-4).

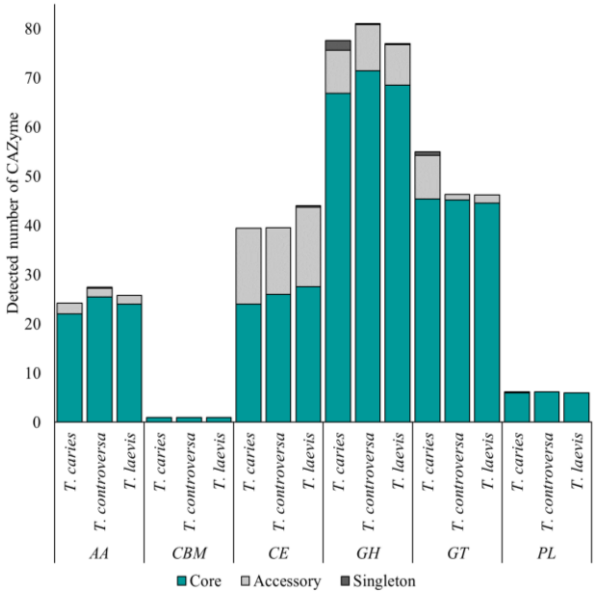


Figure 2-7 Distribution of putative CAZyme in the shared and variable orthology clusters, and singleton genes between five isolates of *T. caries*, seven isolates of *T. controversa*, and four isolates of *T. laevis*. Majority of the CAZymes are conserved and shared between the three species. Glycoside Hydrolases (GHs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs), Glycosyltransferases (GTs), Auxiliary Activities (AAs), and Carbohydrate-Binding Modules (CBMs).

2. 5. Discussion

In this study, the results of the whole-genome sequencing, assembly, structural and functional annotation of 11 isolates of *T. caries*, *T. controversa,* and *T. laevis* were performed. We additionally included the assembled genome of five isolates which were publicly available (Nguyen *et al.*, 2019) to assess the inter- and intraspecific genetic variation of these important wheat pathogens.

The size of the assembled genomes of ten *Tilletia* isolates using only Illumina reads ranged from 29.4 to 31.9 Mb, which were in line with the size of previously published genomes (Nguyen *et al.*, 2019). However, these ten sequenced genomes were more fragmented compared to the published genomes due to lower coverage. However, the gene-space seemed to be adequately covered as revealed by similar BUSCO results. The genome of one isolate of *T. controversa* (OR) was assembled using PacBio and Illumina reads, resulted in the fewest scaffolds (985 scaffolds) and an increase of N50 to 137 kb. In addition, as more repetitive DNA could be resolved using the long PacBio reads, the assembled genome size increased significantly. This higher sequencing depth also resulted in almost 50% more tRNA identification compared to the other isolates.

The proportion of repetitive regions can differ significantly between closely related species. For instance, *Fusarium oxysporum* and *F. graminearum*, two closely related species differ in their proportion of repetitive elements with 16.83 Mb and 0.24 Mb respectively (Ma *et al.*, 2010). In our study, the percentage of repetitive regions was variable among the 15 isolates (7.8 to 13.7%) without resulting in a difference between the three species. However, the true proportion of repetitive regions can be expected to be higher as exemplified by the number of 37% found in the long read-based genomes of isolate OR, suggesting that a variable number of repeat elements might have been collapsed in the assemblies based on short reads due to their high sequence similarity. The transposon types *Gypsy* followed by *Copia*, both belonging to the long terminal direct repeats (LTR) class of retrotransposons, were the most abundant in all three bunt species. Similarly, *Gypsys* were the most frequent TE reported in *T. indica* (Gurjar *et al.*, 2019; Mishra *et al.*, 2019) as well as *T. horrida* (Wang *et al.*, 2018). *Gypsy*s are the most successful group of TEs in fungi (Gorinsek *et al.*, 2004) and plants (Sabot and Schulman, 2006) that can increase their number by autonomous transposition (Elliott and Gregory, 2015).

2. 5. 1. Genomic synteny and genome-wide diversity

The genomes of the three *Tilletia* species appeared to be largely syntenic as up to 94% of non-repetitive DNA regions could be aligned in a pairwise manner. Furthermore, we detected more than 98.7% average nucleotide identity in one-to-one aligned DNA regions (data not shown), which was in agreement with Nguyen et al. 2019 (Nguyen *et al.*, 2019). Genomic macrosynteny among closely related species has been reported for some *Fusarium* species (De Vos *et al.*, 2014) and within Dothideomycetes (Ohm *et al.*, 2012). The microsynteny observed between the three species can be explained by their close phylogenetic relationship (Russell and Mills, 1993; Russell, 1994; Carris *et al.*, 2007; Vánky, 2012).

Genome-wide average diversity was least between two species of *T. caries* and *T. laevis* with 0.51 SNPs/kb and 1.04 indels bp/kb on average. At the same time, both species showed almost equal distance to *T. controversa* correlating with the fact that both are identical in teliospore physiological features and infection biology, but different from *T. controversa*. This is especially remarkable because the common bunts isolates' geographic origins were more distant to each other (Austria, Italy, Germany, Switzerland, and USA) than those of the dwarf bunt isolates, which were mostly collected from Germany.

At species level, up to three times higher nucleotide polymorphisms were observed among the seven isolates of *T. controversa* (max. 1.47 SNPs/kb) compared to the five isolates of *T. caries* (max.

0.53 SNPs/kb) and four isolates of *T. laevis* (max. 0.52 SNPs/kb). However, the number of SNPs were generally lower for all three bunt species compared to the reports for other species of Basidiomycota. For instance, different genotypes of *Heterobasidion irregulare* had 4 SNPs/kb (Sillo *et al.*, 2015) and *Melamspora larici-populina* 6 SNPs/kb (Persoons *et al.*, 2014), respectively. The especially low genetic diversity observed within the common bunt species and still low, and the somewhat higher genetic diversity of *T. controversa*, could be the consequence of different mating systems. Both common and dwarf bunt display bipolar mating behaviors meaning that selfing is the dominant reproduction form, which happens by quick mating of the compatible basidiospores of the same basidium (Goates, 1996) and limiting the chances of outcrossing. However, the mating system is biallelic in common bunt (Holton, 1951; Holton and Kendrick, 1957) and multiallelic in dwarf bunt (Hoffmann and Kendrick, 1969) leading to a higher chance of occasional outcrossing and consequently a higher degree of diversity in *T. controversa* (Pimentel *et al.*, 2000).

2. 5. 2. Inter- and intraspecies diversification

We compared the inventory of the protein-coding genes of the three closely related *Tilletia* species by clustering ortholog use to define variation across 16 isolates. A total of 97.7% of the total predicted CDS were clustered to an orthologous group and 75.4% of all predicted CDS were shared across all 16 isolates. The shared fraction of genes between the three species of *T. caries*, *T. controversa*, and *T. laevis* together was only 5% lower than the minimum core genome size reported within single fungal species.

For instance, McCarthy and Fitzpatrick (2019) reported that 80% to 90% of the total genes represented the core genomes at species level studying four different fungal species (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans var. grubii* and *Saccharomyces cerevisiae*). Moreover, the size of the core genome of six *Aspergillus niger* isolates was also 80% of the pan-genome (Vesth *et al.*, 2018) while they mostly have asexual reproduction form. The high degree of gene conservation between the three *Tilletia* species is an indication that *T. caries*, *T. controversa*, and *T. laevis* share a common ancestor and further raise the question whether the morphological and physiological

Table 2-4 Pan-genomes of *T. caries*, *T. controversa*, and *T. laevis*

		Pan-genome		**Core genome**			**Accessory genome**			**Singleton**	
Species	**Isolates**	**CDs**	**Orthology cluster**	**CDs**	**Orthology cluster**	**%**	**CDs**	**Orthology cluster**	**%**	**CDs**	**%**
T. caries	5	50056	9187	46492	7020	92.8	2238	2167	4.5	1284	2.5
T. controversa	7	69702	9732	63728	6238	91.4	4297	3494	6.1	1677	2.4
T. laevis	4	40076	9153	38264	7200	95.4	1187	1953	2.9	625	1.5

differences the species definitions are based on are sufficient in the light of their high genomic similarity.

In this study a large core-genome and a small accessory genome for each species. Accessory genes are frequently involved in pathogenicity, virulence, antimicrobial resistance, and adaptation to the environment (Sheppard *et al.*, 2018; Lee and Andam, 2019). The fraction of accessory genome was highest in the pan-genome of *T. controversa* (6.1%) followed by *T. caries* (4.5%) and was the lowest for *T. laevis* (2.9%). All these values were significantly lower than the accessory genomes reported for other fungal species, which ranged from 9 to 20% of their pan-genomes (McCarthy and Fitzpatrick, 2019). We found only very few species-specific genes to *T. laevis* and *T. caries* and a high percentage of shared orthology clusters between them. This corresponded with the low numbers of SNPs and indels and may suggest that the two species could be two morphotypes of one species so-called pseudomorphospecies (Vanky, 2008) or have just diverged recently. Altogether, the genomic similarities and differences found in this study correlated better with the delineation of the diseases common bunt (*T. caries* and *T. laevis*) versus dwarf bunt (*T. controversa*) than with the circumscription of the three species.

2. 5. 3. Carbon utilization and establishment of fungal biotrophy

As other biotrophic pathogens, *T. caries*, *T. controversa*, and *T. laevis* encode for a relatively low number of CAZymes (Zhao *et al.*, 2013; Lyu *et al.*, 2015). Moreover, the three species were quite similar in the diversity and abundance of identified CAZyme families. Plant-parasitic fungi secrete a variety of CAZymes, which may play a role in pathogenicity and virulence and are needed to successfully degrade plant cell walls and to complete host invasion (Annis and Goodwin, 1997; Gibson *et al.*, 2011; Kubicek *et al.*, 2014). Based on the *in silico* analyses, all 16 isolates had two putative secreted CAZymes, one belonged to one chitin deacetylases of the family CE4 and one to the GH152 family. CE4 is suggested to play role in the modification of the fungal cell walls for masking hyphae to escape from enzymatic hydrolysis by host chitinases through de-N-acetylation of chitin (El Gueddari *et al.*, 2002; Boneca *et al.*, 2007). The enzyme β-1,3-glucanase (GH152) is suggested to play a role in cell wall softening during morphogenesis in *Aspergillus fumigatus* (Mouyna *et al.*, 2013).

Interestingly, common and dwarf bunt, similar to *T. indica* (Gurjar *et al.*, 2019) have genes encoding for GH8 (broad activity hydrolase), which was suggested to be present in all Ustilaginomycotina (Kijpornyongpan *et al.*, 2018). In addition, *Tilletia* spp. harbored families coding for PL14 and AA2 enzymes that are involved in lignin decomposition, which were completely absent in other studied Ustilagoinmycotina, but present in Agaricomycotina (Kijpornyongpan *et al.*, 2018).

Putative genes encoding for PL14 and AA2 are also reported from *T. indica* CAZyme analyses (Gurjar *et al.*, 2019).

Almost half of the putative genes encoding for effectors in the multi-species comparison of *T. caries, T. controversa,* and *T. laevis* were among the variable genes (dispensable and not present in all genomes), which is in agreement with their ability to undergo rapid diversification including duplication, deletions, and point mutations (Oliver and Solomon, 2010; Rouxel *et al.*, 2011). Interestingly, the functionally characterized genes which had orthologous in the genome of the three species were suggested to be either essential for establishing biotrophy in smuts such as Pep1 or important virulence factor such as Srt1 (Hemetsberger *et al.*, 2015; Kijpornyongpan *et al.*, 2018; Lanver *et al.*, 2018).

2. 5. 4. Secondary metabolites pathways and trimethylamine synthesis

The putative secondary metabolite gene clusters in *T. caries*, *T. controversa*, and *T. laevis* were predicted in this study Secondary metabolites are known as virulence factors (Oide *et al.*, 2006), toxins, inhibitors (Shwab and Keller, 2008), and antifeedants or deterrents (Tanaka *et al.*, 2005; Xu *et al.*, 2019). NRPS gene clusters are often repetitive in their internal structures and the identification of a higher number of them in the PacBio sequenced genome can be explained by a higher coverage of repetitive regions. The *in silico* analysis of putative secondary metabolites gene clusters revealed that the three species were nearly identical.

Production of TMA gave the stinking smut its name. The biological function of TMA in *Tilletia* spp. (Ettel and Halbsguth, 1963; Singh and Trione, 1969) as well as in *Geotrichum candidum* (Robinson *et al.*, 1989) is the autoinhibition of spore germination. Other metabolites of *Tilletia* were shown to inhibit spore germination *in vitro* (Trione and Ross, 1988), but they are not volatile and therefore cannot fulfill the function of autoinhibitors. The precursor of TMA in smut fungi and bacteria is choline, but the lack of homologous genes in smut fungi indicates that the biosynthetic pathway is different. The biosynthesis of TMA in smut fungi also appears unrelated to ethylamine degradation by bacteria. We therefore hypothesize that smut fungi possess a different biosynthetic route to TMA, which has yet to be discovered.

2. 6. Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Adherence to national and international regulations

Not applicable

Availability of data and material

The datasets generated during the current study (raw reads, assemblies, and structural annotations) are available in the European Nucleotide Archive database repository (http://www.ebi.ac.uk/ena) and can be accessed under the project accession number of PRJEB40624.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by two grants (numbers 2812NA128 and 2812NA017) from the German Federal Ministry of Food and Agriculture (BMEL) based on a decision of the German Federal Parliament.

2. 7. Acknowledgments

The authors acknowledge the critical reviews and helpful comments by Dr. Stephan Fuchs. Dr. Anne Fiebig's support for the submission of genomes is acknowledged. Many thanks are extended to A. E. Müllner, Veronica Weyermann, Robert Bauer, Dr. Blair Goates, and Prof. David Hole who freely shared samples with us. Anke Brißke-Rode's and Thomas Berner's excellent technical assistance are greatly appreciated.

2. 8. References

Ali, S., Laurie, J. D., Linning, R., Cervantes-Chavez, J. A., Gaudet, D., & Bakkeren, G. (2014). An immunity-triggering effector from the Barley smut fungus *Ustilago hordei* resides in an Ustilaginaceae-specific cluster bearing signs of transposable element-assisted evolution. *PLoS Pathog*, *10*(7), e1004223. https://doi.org/10.1371/journal.ppat.1004223

Almagro Armenteros, J. J., Tsirigos, K. D., Sonderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat Biotechnol*, *37*(4), 420-423. https://doi.org/10.1038/s41587-019-0036-z

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research*, *25*(17), 3389-3402. https://doi.org/10.1093/nar/25.17.3389

Annis, S. L., & Goodwin, P. H. (1997). Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *European Journal of Plant Pathology*, *103*(1), 1-14. https://doi.org/10.1023/a:1008656013255

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., & Sherlock, G. (2000). Gene Ontology: tool for the unification of biology. *Nature Genetics*, *25*(1), 25-29. https://doi.org/10.1038/75556

Aydoğdu, M., & Kaya, Y. (2020). Reactions of spring wheat varieties to common bunt (*Tilletia laevis*) in Turkey. *Cereal Research Communications*, *48*(3), 333-339. https://doi.org/10.1007/s42976-020-00040-1

Bao, Z., & Eddy, S. R. (2002). Automated de novo identification of repeat sequence families in sequenced genomes. *Genome research*, *12*(8), 1269-1276. https://doi.org/10.1101/gr.88502

Basse, C. W., Stumpferl, S., & Kahmann, R. (2000). Characterization of a *Ustilago maydis* gene specifically induced during the biotrophic phase: evidence for negative as well as positive regulation. *Mol Cell Biol*, *20*(1), 329-339. https://doi.org/10.1128/mcb.20.1.329-339.2000

Begerow, D., Schäfer, A. M., Kellner, R., Yurkov, A., Kemler, M., Oberwinkler, F., & Bauer, R. (2014). Ustilaginomycotina. In D. J. McLaughlin & J. W. Spatafora (Eds.), *Systematics and Evolution: Part A* (295-329). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-55318-9_11

Beier, S., Thiel, T., Münch, T., Scholz, U., & Mascher, M. (2017). MISA-web: a web server for microsatellite prediction. *Bioinformatics*, *33*(16), 2583-2585. https://doi.org/10.1093/bioinformatics/btx198

Benevenuto, J., Teixeira-Silva, N. S., Kuramae, E. E., Croll, D., & Monteiro-Vitorello, C. B. (2018). comparative genomics of smut pathogens: insights from orphans and positively selected genes into host specialization. *Front Microbiol*, *9*(660), 660. https://doi.org/10.3389/fmicb.2018.00660

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic acids research*, *27*(2), 573-580. https://doi.org/10.1093/nar/27.2.573

Boetzer, M., Henkel, C. V., Jansen, H. J., Butler, D., & Pirovano, W. (2011). Scaffolding pre-assembled contigs using SSPACE. *Bioinformatics*, *27*(4), 578-579. https://doi.org/10.1093/bioinformatics/btq683

Boetzer, M., & Pirovano, W. (2014). SSPACE-LongRead: scaffolding bacterial draft genomes using long read sequence information. *BMC Bioinformatics*, *15*(1), 211. https://doi.org/10.1186/1471-2105-15-211

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114-2120. https://doi.org/10.1093/bioinformatics/btu170

Boneca, I. G., Dussurget, O., Cabanes, D., Nahori, M.-A., Sousa, S., Lecuit, M., Psylinakis, E., Bouriotis, V., Hugot, J.-P., Giovannini, M., Coyle, A., Bertin, J., Namane, A., Rousselle, J.-C., Cayet, N., Prévost, M.-C., Balloy, V., Chignard, M., Philpott, D. J., Cossart, P., & Girardin, S. E. (2007). A critical role for peptidoglycan N-deacetylation in *Listeria evasion* from the host innate immune system. *Proceedings of the National Academy of Sciences*, *104*(3), 997-1002. https://doi.org/10.1073/pnas.0609672104

Brandfass, C., & Karlovsky, P. (2008). Upscaled CTAB-based DNA extraction and real-time PCR assays for *Fusarium culmorum* and *F. graminearum* DNA in plant material with reduced sampling error. *Int J Mol Sci*, *9*(11), 2306-2321. https://doi.org/10.3390/ijms9112306

Brefort, T., Tanaka, S., Neidig, N., Doehlemann, G., Vincon, V., & Kahmann, R. (2014a). Characterization of the largest effector gene cluster of *Ustilago maydis*. *PLoS pathogens*, *10*(7), e1003866-e1003866. https://doi.org/10.1371/journal.ppat.1003866

Brieger, L. (1887). Die Quelle des Trimethylamins im Mutterkorn. *Zeitschrift für Physiologische Chemie*, *11*, 2.

Brůna, T., Hoff, K. J., Lomsadze, A., Stanke, M., & Borodovsky, M. (2020). BRAKER2: automatic eukaryotic genome annotation with GeneMark-EP+ and AUGUSTUS supported by a protein database. *bioRxiv*, 2020.2008.2010.245134. https://doi.org/10.1101/2020.08.10.245134

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., & Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic acids research*, *37*(Database issue), D233-238. https://doi.org/10.1093/nar/gkn663

Carris, L. M. (2010). smuts. In W. W. Bockus, R. L. Bowden, R. M. Hunger, W. L. Morrill, & T. D. Murray (Eds.), *Compendium of Wheat Diseases and Pests* (60-65). American Phytopathological Society.

Carris, L. M., Castlebury, L. A., & Goates, B. J. (2006). Nonsystemic bunt fungi *Tilletia indica* and *T. horrida*: a review of history, systematics, and biology. *Annu Rev Phytopathol*, *44*, 113-133. https://doi.org/10.1146/annurev.phyto.44.070505.143402

Carris, L. M., Castlebury, L. A., Huang, G., Alderman, S. C., Luo, J., & Bao, X. (2007). *Tilletia vankyi*, a new species of reticulate-spored bunt fungus with non-conjugating basidiospores infecting species of *Festuca* and *Lolium*. *Mycological research*, *111*(Pt 12), 1386-1398. https://doi.org/10.1016/j.mycres.2007.09.008

Casacuberta, E., & González, J. (2013). The impact of transposable elements in environmental adaptation. *Mol Ecol*, *22*(6), 1503-1517. https://doi.org/10.1111/mec.12170

Castlebury, L. A., Carris, L. M., & Vinky, K. (2005). Phylogenetic analysis of *Tilletia* and allied genera in order Tilletiales (Ustilaginomycetes; Exobasidiomycetidae) based on large subunit nuclear rDNA sequences. *Mycologia*, *97*(4), 888-900. https://doi.org/10.3852/mycologia.97.4.888

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, *124*(4), 803-814. https://doi.org/10.1016/j.cell.2006.02.008

Craciun, S., & Balskus, E. P. (2012). Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(52), 21307-21312. https://doi.org/10.1073/pnas.1215689109

De Vos, L., Steenkamp, E. T., Martin, S. H., Santana, Q. C., Fourie, G., van der Merwe, N. A., Wingfield, M. J., & Wingfield, B. D. (2014). Genome-wide macrosynteny among *Fusarium* species in the *Gibberella fujikuroi* complex revealed by amplified fragment length polymorphisms. *PLOS ONE*, *9*(12), e114682. https://doi.org/10.1371/journal.pone.0114682

Delaunois, B., Jeandet, P., Clement, C., Baillieul, F., Dorey, S., & Cordelier, S. (2014). Uncovering plant-pathogen crosstalk through apoplastic proteomic studies. *Front Plant Sci*, *5*, 249. https://doi.org/10.3389/fpls.2014.00249

Denchev, C., & Denchev, T. (2013). Erratomycetaceae, fam. nov., and validation of some names of smut fungi recently described from India. *Mycobiota*, *1*, 63-70. https://doi.org/10.12664/mycobiota.2013.01.07

Denchev, T. T., & Denchev, C. M. (2018a). *Tilletia tripogonellae* (Tilletiaceae), a new smut fungus on *Tripogonella spicata* (Poaceae) from Argentina. *Annales Botanici Fennici*, *55*(4-6), 273-277, 275. https://doi.org/10.5735/085.055.0407

Denchev, T. T., & Denchev, C. M. (2018b). Two new smut fungi on *Ventenata Poaceae*: *Tilletia elizabethae* from Slovakia and *T. ventenatae* from Turkey. *Willdenowia*, *48*(2), 177-183, 177. https://doi.org/10.3372/wi.48.48201

Denchev, T. T., Van Der Zon, A. P. M., & Denchev, C. M. (2018). *Tilletia triraphidis* (Tilletiaceae), a new smut fungus on *Triraphis purpurea* (Poaceae) from Namibia. *Phytotaxa*, *375*(2), 182-186. https://doi.org/10.11646/phytotaxa.375.2.5

Diehl, L. (1887). Report on the progress of pharmacy. *Proceedings of the American Pharmaceutical Association*, *35*, 372.

Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahnt, J., Osorio, S., Tohge, T., Fernie, A. R., Feussner, I., Feussner, K., Meinicke, P., Stierhof, Y.-D., Schwarz, H., Macek, B., Mann, M., & Kahmann, R. (2011). Metabolic priming by a secreted fungal effector. *Nature*, *478*(7369), 395-398. https://doi.org/10.1038/nature10454

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2012). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15-21. https://doi.org/10.1093/bioinformatics/bts635

Dodds, P. N., Rafiqi, M., Gan, P. H. P., Hardham, A. R., Jones, D. A., & Ellis, J. G. (2009). Effectors of biotrophic fungi and oomycetes: pathogenicity factors and triggers of host resistance. *New Phytol.*, *183*(4), 993-1000. https://doi.org/10.1111/j.1469-8137.2009.02922.x

Doehlemann, G., Reissmann, S., Aßmann, D., Fleckenstein, M., & Kahmann, R. (2011). Two linked genes encoding a secreted effector and a membrane protein are essential for *Ustilago maydis*-induced tumour formation. *Molecular microbiology*, *81*(3), 751-766. https://doi.org/10.1111/j.1365-2958.2011.07728.x

Doehlemann, G., van der Linde, K., Aßmann, D., Schwammbach, D., Hof, A., Mohanty, A., Jackson, D., & Kahmann, R. (2009). Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. *PLOS Pathogens*, *5*(2), e1000290. https://doi.org/10.1371/journal.ppat.1000290

El Gueddari, N. E., Rauchhaus, U., Moerschbacher, B. M., & Deising, H. B. (2002). Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi. *New Phytol.*, *156*(1), 103-112. https://doi.org/10.1046/j.1469-8137.2002.00487.x

Elliott, T. A., & Gregory, T. R. (2015). Do larger genomes contain more diverse transposable elements? *BMC Evolutionary Biology*, *15*(1), 69. https://doi.org/10.1186/s12862-015-0339-8

Ettel, G., & Halbsguth, W. (1963). The effect of trimethylamine, calcium nitrate and light on the germination of bunt spores of *Tilletia tritici*. *Beiträge zur Biologie der Pflanzen*, *39*, 37.

Galperin, M. Y., Makarova, K. S., Wolf, Y. I., & Koonin, E. V. (2014). Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Research*, *43*(D1), D261-D269. https://doi.org/10.1093/nar/gku1223

Garsin, D. A. (2010). Ethanolamine utilization in bacterial pathogens: roles and regulation. *Nature reviews. Microbiology*, *8*(4), 290-295. https://doi.org/10.1038/nrmicro2334

Gene Ontology, C. (2015). Gene Ontology Consortium: going forward. *Nucleic acids research*, *43*, D1049-1056. https://doi.org/10.1093/nar/gku1179

Ghareeb, H., Drechsler, F., Löfke, C., Teichmann, T., & Schirawski, J. (2015). Suppressor of apical dominance1 of *Sporisorium reilianum* modulates inflorescence branching architecture in maize and arabidopsis. *Plant Physiol*, *169*(4), 2789-2804. https://doi.org/10.1104/pp.15.01347

Gibson, D. M., King, B. C., Hayes, M. L., & Bergstrom, G. C. (2011). Plant pathogens as a source of diverse enzymes for lignocellulose digestion. *Curr Opin Microbiol*, *14*(3), 264-270. https://doi.org/10.1016/j.mib.2011.04.002

Girard, V., Dieryckx, C., Job, C., & Job, D. (2013). Secretomes: the fungal strike force. *Proteomics*, *13*(3-4), 597-608. https://doi.org/10.1002/pmic.201200282

Goates, B. J. (1996). Common Bunt and Dwarf Bunt. In R. D. Wilcoxson & E. E. Saari (Eds.), *Bunt and smut diseases of wheat; concepts and methods of disease management.* CIMMYT.

Gorinsek, B., Gubensek, F., & Kordis, D. (2004). Evolutionary genomics of chromoviruses in eukaryotes. *Mol Biol Evol*, *21*(5), 781-798. https://doi.org/10.1093/molbev/msh057

Gurjar, M. S., Aggarwal, R., Jogawat, A., Kulshreshtha, D., Sharma, S., Solanke, A. U., Dubey, H., & Jain, R. K. (2019). De novo genome sequencing and secretome analysis of *Tilletia indica* inciting Karnal bunt of wheat provides pathogenesis-related genes. *3 Biotech*, *9*(6), 219. https://doi.org/10.1007/s13205-019-1743-3

Haas, B. (2010, 2010-08-22). TransposonPSI: an application of psi-blast to mine (retro-)transposon orf homologies. Retrieved 20 Sep 2019 from http://transposonpsi.sourceforge.net/

Hackl, T., Hedrich, R., Schultz, J., & Förster, F. (2014). proovread : large-scale high-accuracy PacBio correction through iterative short read consensus. *Bioinformatics*, *30*(21), 3004-3011. https://doi.org/10.1093/bioinformatics/btu392

Hanna, W. F., Vickery, H. B., & Pucher, G. W. (1932). The isolation of trimethylamine from spores of *Tilletia levis*, the stinking smut of wheat. *Journal of Biological Chemistry*, *97*(2), 351-358. http://www.jbc.org/content/97/2/351.short

Heimel, K., Scherer, M., Vranes, M., Wahl, R., Pothiratana, C., Schuler, D., Vincon, V., Finkernagel, F., Flor-Parra, I., & Kämper, J. (2010). The transcription factor Rbf1 is the master regulator for b-mating type controlled pathogenic development in *Ustilago maydis*. *PLOS Pathogens*, *6*(8), e1001035. https://doi.org/10.1371/journal.ppat.1001035

Hemetsberger, C., Herrberger, C., Zechmann, B., Hillmer, M., & Doehlemann, G. (2012). The *Ustilago maydis* effector Pep1 suppresses plant immunity by inhibition of host peroxidase activity. *PLOS Pathogens*, *8*(5), e1002684. https://doi.org/10.1371/journal.ppat.1002684

Hemetsberger, C., Mueller, A. N., Matei, A., Herrberger, C., Hensel, G., Kumlehn, J., Mishra, B., Sharma, R., Thines, M., Huckelhoven, R., & Doehlemann, G. (2015). The fungal core effector Pep1 is conserved across smuts of dicots and monocots. *New Phytol*, *206*(3), 1116-1126. https://doi.org/10.1111/nph.13304

Hoffmann, J. A. (1982). Bunt of wheat. *Plant Disease*, *66*(1), 979. https://doi.org/10.1094/pd-66-979

Hoffmann, J. A., & Kendrick, E. (1969). Genetic control of compatibility in *Tilletia controversa*. *Phytopathology*, *59*, 4.

Hogenhout, S. A., Van der Hoorn, R. A., Terauchi, R., & Kamoun, S. (2009). Emerging concepts in effector biology of plant-associated organisms. *Molecular plant-microbe interactions : MPMI*, *22*(2), 115-122. https://doi.org/10.1094/MPMI-22-2-0115

Holton, C. S. (1951). Methods and results of studies on heterothallism and hybridization in *Tilletia caries*. *Phytopathology*, *41*, 11.

Holton, C. S., & Kendrick, E. L. (1957). Fusion between secondary sporidia in culture as a valid index of sex compatibility in *Tilletia caries*. *Phytopathology*, *47*, 2.

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., Mende, D. R., Letunic, I., Rattei, T., Jensen, Lars J., von Mering, C., & Bork, P. (2018). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Research*, *47*(D1), D309-D314. https://doi.org/10.1093/nar/gky1085

Jaramillo, V. D. A., Sukno, S. A., & Thon, M. R. (2015). Identification of horizontally transferred genes in the genus *Colletotrichum* reveals a steady tempo of bacterial to fungal gene transfer. *BMC Genomics*, *16*(1), 2. https://doi.org/10.1186/1471-2164-16-2

Jia, W. M., Zhou, Y. L., Duan, X. Y., Luo, Y., Ding, S. L., Cao, X. R., & Fitt, B. D. L. (2013). Assessment of risk of establishment of wheat dwarf bunt (*Tilletia controversa*) in China. *Journal of Integrative Agriculture*, *12*(1), 87-94. https://doi.org/10.1016/S2095-3119(13)60208-7

Kall, L., Krogh, A., & Sonnhammer, E. L. (2007). Advantages of combined transmembrane topology and signal peptide prediction--the Phobius web server. *Nucleic acids research*, *35*(Web Server issue), W429-432. https://doi.org/10.1093/nar/gkm256

Kamoun, S. (2006). A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology*, *44*(1), 41-60. https://doi.org/10.1146/annurev.phyto.44.070505.143436

Kamoun, S. (2007). Groovy times: filamentous pathogen effectors revealed. *Curr Opin Plant Biol*, *10*(4), 358-365. https://doi.org/10.1016/j.pbi.2007.04.017

Kamper, J., Kahmann, R., Bolker, M., Ma, L. J., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Muller, O., Perlin, M. H., Wosten, H. A., de Vries, R., Ruiz-Herrera, J., Reynaga-Pena, C. G., Snetselaar, K., McCann, M., Perez-Martin, J., Feldbrugge, M., Basse, C. W., Steinberg, G., Ibeas, J. I., Holloman, W., Guzman, P., Farman, M., Stajich, J. E., Sentandreu, R., Gonzalez-Prieto, J. M., Kennell, J. C., Molina, L., Schirawski, J., Mendoza-Mendoza, A., Greilinger, D., Munch, K., Rossel, N., Scherer, M., Vranes, M., Ladendorf, O., Vincon, V., Fuchs, U., Sandrock, B., Meng, S., Ho, E. C., Cahill, M. J., Boyce, K. J., Klose, J., Klosterman, S. J., Deelstra, H. J., Ortiz-Castellanos, L., Li, W., Sanchez-Alonso, P., Schreier, P. H., Hauser-Hahn, I., Vaupel, M., Koopmann, E., Friedrich, G., Voss, H., Schluter, T., Margolis, J., Platt, D., Swimmer, C., Gnirke, A., Chen, F., Vysotskaia, V., Mannhaupt, G., Guldener, U., Munsterkotter, M., Haase, D., Oesterheld, M., Mewes, H. W., Mauceli, E. W., DeCaprio, D., Wade, C. M., Butler, J., Young, S., Jaffe, D. B., Calvo, S., Nusbaum, C., Galagan, J., & Birren, B. W. (2006). Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature*, *444*(7115), 97-101. https://doi.org/10.1038/nature05248

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2013). Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Research*, *42*(D1), D199-D205. https://doi.org/10.1093/nar/gkt1076

Keilwagen, J., Hartung, F., Paulini, M., Twardziok, S. O., & Grau, J. (2018). Combining RNA-seq data and homology-based gene prediction for plants, animals and fungi. *BMC Bioinformatics*, *19*(1), 189. https://doi.org/10.1186/s12859-018-2203-5

Keilwagen, J., Wenk, M., Erickson, J. L., Schattat, M. H., Grau, J., & Hartung, F. (2016). Using intron position conservation for homology-based gene prediction. *Nucleic acids research*, *44*(9), e89. https://doi.org/10.1093/nar/gkw092

Khrunyk, Y., Münch, K., Schipper, K., Lupas, A. N., & Kahmann, R. (2010). The use of FLP-mediated recombination for the functional analysis of an effector gene family in the biotrophic smut fungus *Ustilago maydis*. *New Phytol*, *187*(4), 957-968. https://doi.org/10.1111/j.1469-8137.2010.03413.x

Kijpornyongpan, T., Mondo, S. J., Barry, K., Sandor, L., Lee, J., Lipzen, A., Pangilinan, J., LaButti, K., Hainaut, M., Henrissat, B., Grigoriev, I. V., Spatafora, J. W., & Aime, M. C. (2018). Broad genomic sampling reveals a smut pathogenic ancestry of the fungal clade Ustilaginomycotina. *Mol Biol Evol*, *35*(8), 1840-1854. https://doi.org/10.1093/molbev/msy072

Kim, K.-T., Jeon, J., Choi, J., Cheong, K., Song, H., Choi, G., Kang, S., & Lee, Y.-H. (2016). Kingdom-wide analysis of fungal small secreted proteins (SSPs) reveals their potential role in host association. *Front Plant Sci*, *7*, 186-186. https://doi.org/10.3389/fpls.2016.00186

Kimura, M., Anzai, H., & Yamaguchi, I. (2001). Microbial toxins in plant-pathogen interactions: Biosynthesis, resistance mechanisms, and significance. *The Journal of general and applied microbiology*, *47*(4), 149-160. https://doi.org/10.2323/jgam.47.149

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., & Phillippy, A. M. (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome research*, *27*(5), 722-736. https://doi.org/10.1101/gr.215087.116

Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol*, *305*(3), 567-580. https://doi.org/10.1006/jmbi.2000.4315

Krombach, S., Reissmann, S., Kreibich, S., Bochen, F., & Kahmann, R. (2018). Virulence function of the *Ustilago maydis* sterol carrier protein 2. *New Phytol.*, *220*(2), 553-566. https://doi.org/10.1111/nph.15268

Krueger, F. (2012-2019). *Babraham Bioinformatics - Trim Galore!* Retrieved 20 Jun 2019 from https://www.bioinformatics.babraham.ac.uk/projects/trim_galore

Kubicek, C. P., Starr, T. L., & Glass, N. L. (2014). Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi. *Annual Review of Phytopathology, Vol 52*, *52*, 427-451. https://doi.org/10.1146/annurev-phyto-102313-045831

Kumar, A., Mishra, P., Maurya, R., Mishra, A. K., Gupta, V. K., Ramteke, P. W., & Marla, S. S. (2018). Improved draft genome sequence of a monoteliosporic culture of the karnal bunt (*Tilletia indica*) pathogen of wheat. *Genome Announc*, *6*(20), e00015-00018. https://doi.org/10.1128/genomeA.00015-18

Kumar, A., Pandey, V., Singh, M., Pandey, D., Saharan, M. S., & Marla, S. S. (2017). Draft genome sequence of Karnal bunt pathogen (*Tilletia indica*) of wheat provides insights into the pathogenic mechanisms of quarantined fungus. *PLoS One*, *12*(2), e0171323. https://doi.org/10.1371/journal.pone.0171323

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., & Salzberg, S. L. (2004). Versatile and open software for comparing large genomes. *Genome Biol*, *5*(2), R12. https://doi.org/10.1186/gb-2004-5-2-r12

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357-359. https://doi.org/10.1038/nmeth.1923

Lanver, D., Müller, A. N., Happel, P., Schweizer, G., Haas, F. B., Franitza, M., Pellegrin, C., Reissmann, S., Altmüller, J., Rensing, S. A., & Kahmann, R. (2018). The biotrophic development of *Ustilago maydis* studied by RNA-Seq analysis. *Plant Cell*, *30*(2), 300-323. https://doi.org/10.1105/tpc.17.00764

Lee, I. P. A., & Andam, C. P. (2019). Pan-genome diversification and recombination in *Cronobacter sakazakii*, an opportunistic pathogen in neonates, and insights to its xerotolerant lifestyle. *BMC Microbiology*, *19*(1), 306. https://doi.org/10.1186/s12866-019-1664-7

Letunic, I., & Bork, P. (2018). 20 years of the SMART protein domain annotation resource. *Nucleic acids research*, *46*(D1), D493-d496. https://doi.org/10.1093/nar/gkx922

Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M., & Henrissat, B. (2013). Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol Biofuels*, *6*(1), 41. https://doi.org/10.1186/1754-6834-6-41

Levin, H. L., & Moran, J. V. (2011). Dynamic interactions between transposable elements and their hosts. *Nature Reviews Genetics*, *12*(9), 615-627. https://doi.org/10.1038/nrg3030

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., & Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078-2079. https://doi.org/10.1093/bioinformatics/btp352

Li, L., Stoeckert, C. J., Jr., & Roos, D. S. (2003). OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome research*, *13*(9), 2178-2189. https://doi.org/10.1101/gr.1224503

Li, Y.-M., Shivas, R. G., & Cai, L. (2014). Three new species of *Tilletia* on Eriachne from north-western Australia. *Mycoscience*, *55*(5), 361-366. https://doi.org/10.1016/j.myc.2013.12.003

Lomsadze, A., Burns, P. D., & Borodovsky, M. (2014). Integration of mapped RNA-Seq reads into automatic training of eukaryotic gene finding algorithm. *Nucleic Acids Research*, *42*(15), e119-e119. https://doi.org/10.1093/nar/gku557

Lowe, T. M., & Chan, P. P. (2016). tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. *Nucleic acids research*, *44*(W1), W54-57. https://doi.org/10.1093/nar/gkw413

Lyu, X., Shen, C., Fu, Y., Xie, J., Jiang, D., Li, G., & Cheng, J. (2015). Comparative genomic and transcriptional analyses of the carbohydrate-active enzymes and secretomes of phytopathogenic fungi reveal their significant roles during infection and development. *Scientific Reports*, *5*(1), 15565. https://doi.org/10.1038/srep15565

Ma, L.-J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M.-J., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P. M., Kang, S., Shim, W.-B., Woloshuk, C., Xie, X., Xu, J.-R., Antoniw, J., Baker, S. E., Bluhm, B. H., Breakspear, A., Brown, D. W., Butchko, R. A. E., Chapman, S., Coulson, R., Coutinho, P. M., Danchin, E. G. J., Diener, A., Gale, L. R., Gardiner, D. M., Goff, S., Hammond-Kosack, K. E., Hilburn, K., Hua-Van, A., Jonkers, W., Kazan, K., Kodira, C. D., Koehrsen, M., Kumar, L., Lee, Y.-H., Li, L., Manners, J. M., Miranda-Saavedra, D., Mukherjee, M., Park, G., Park, J., Park, S.-Y., Proctor, R. H., Regev, A., Ruiz-Roldan, M. C., Sain, D., Sakthikumar, S., Sykes, S., Schwartz, D. C., Turgeon, B. G., Wapinski, I., Yoder, O., Young, S., Zeng, Q., Zhou, S., Galagan, J., Cuomo, C. A., Kistler, H. C., & Rep, M. (2010). Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature*, *464*(7287), 367-373. https://doi.org/10.1038/nature08850

Ma, L.-S., Wang, L., Trippel, C., Mendoza-Mendoza, A., Ullmann, S., Moretti, M., Carsten, A., Kahnt, J., Reissmann, S., Zechmann, B., Bange, G., & Kahmann, R. (2018). The *Ustilago maydis* repetitive effector Rsp3 blocks the antifungal activity of mannose-binding maize proteins. *Nature Communications*, *9*(1), 1711. https://doi.org/10.1038/s41467-018-04149-0

Macheleidt, J., Mattern, D. J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V., & Brakhage, A. A. (2016). Regulation and role of fungal secondary metabolites. *Annual Review of Genetics*, *50*(1), 371-392. https://doi.org/10.1146/annurev-genet-120215-035203

Martínez-del Campo, A., Bodea, S., Hamer, H. A., Marks, J. A., Haiser, H. J., Turnbaugh, P. J., & Balskus, E. P. (2015). Characterization and detection of a widely distributed gene cluster that predicts anaerobic choline utilization by human gut bacteria. *mBio*, *6*(2). https://doi.org/10.1128/mBio.00042-15

Matanguihan, J. B., Murphy, K. M., & Jones, S. S. (2011). Control of common bunt in organic wheat. *Plant Dis*, *95*(2), 92-103. https://doi.org/10.1094/PDIS-09-10-0620

Mathre, D. E. (1996). Dwarf bunt: politics, identification, and biology. *Annu Rev Phytopathol*, *34*, 67-85. https://doi.org/10.1146/annurev.phyto.34.1.67

McCarthy, C. G. P., & Fitzpatrick, D. A. (2019). Pan-genome analyses of model fungal species. *Microb Genom*, *5*(2), e000243. https://doi.org/10.1099/mgen.0.000243

Medema, M. H., Blin, K., Cimermancic, P., de Jager, V., Zakrzewski, P., Fischbach, M. A., Weber, T., Takano, E., & Breitling, R. (2011). antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic acids research*, *39*, W339-346. https://doi.org/10.1093/nar/gkr466

Min, X. J. (2010). Evaluation of computational methods for secreted protein prediction in different eukaryotes. *Journal of Proteomics & Bioinformatics*, *3*(5), 143-147. https://doi.org/10.4172/jpb.1000133

Mishra, P., Maurya, R., Gupta, V. K., Ramteke, P. W., Marla, S. S., & Kumar, A. (2019). Comparative genomic analysis of monosporidial and monoteliosporic cultures for unraveling the complexity of molecular pathogenesis of *Tilletia indica* pathogen of wheat. *Sci Rep*, *9*(1), 8185. https://doi.org/10.1038/s41598-019-44464-0

Mouyna, I., Hartl, L., & Latge, J. P. (2013). Beta-1,3-glucan modifying enzymes in *Aspergillus fumigatus*. *Front Microbiol*, *4*, 81. https://doi.org/10.3389/fmicb.2013.00081

Muhae-Ud-Din, G., Chen, D., Liu, T., Chen, W., & Gao, L. (2020). Characterization of the wheat cultivars against *Tilletia controversa* Kühn, causal agent of wheat dwarf bunt. *Scientific Reports*, *10*(1), 9029. https://doi.org/10.1038/s41598-020-65748-w

Müller, O., Schreier, P. H., & Uhrig, J. F. (2008). Identification and characterization of secreted and pathogenesis-related proteins in *Ustilago maydis*. *Molecular Genetics and Genomics*, *279*(1), 27-39. https://doi.org/10.1007/s00438-007-0291-4

Muszewska, A., Steczkiewicz, K., Stepniewska-Dziubinska, M., & Ginalski, K. (2019). Transposable elements contribute to fungal genes and impact fungal lifestyle. *Scientific Reports*, *9*(1), 4307. https://doi.org/10.1038/s41598-019-40965-0

Navarro-Muñoz, J. C., & Collemare, J. (2020). Evolutionary histories of type III Polyketide Synthases in Fungi. *Frontiers in Microbiology*, *10*(3018). https://doi.org/10.3389/fmicb.2019.03018

Nguyen, H. D. T., Sultana, T., Kesanakurti, P., & Hambleton, S. (2019). Genome sequencing and comparison of five *Tilletia* species to identify candidate genes for the detection of regulated species infecting wheat. *IMA Fungus*, *10*(1), 11. https://doi.org/10.1186/s43008-019-0011-9

Nielsen, J. (1963). Trimethylammonium compounds in *Tilletia* Spp. *Canadian Journal of Botany*, *41*(3), 335-339. https://doi.org/10.1139/b63-032

Ohm, R. A., Feau, N., Henrissat, B., Schoch, C. L., Horwitz, B. A., Barry, K. W., Condon, B. J., Copeland, A. C., Dhillon, B., Glaser, F., Hesse, C. N., Kosti, I., LaButti, K., Lindquist, E. A., Lucas, S., Salamov, A. A., Bradshaw, R. E., Ciuffetti, L., Hamelin, R. C., Kema, G. H. J., Lawrence, C., Scott, J. A., Spatafora, J. W., Turgeon, B. G., de Wit, P. J. G. M., Zhong, S., Goodwin, S. B., & Grigoriev, I. V. (2012). Diverse lifestyles and strategies of plant pathogenesis encoded in the genomes of eighteen Dothideomycetes fungi. *PLOS Pathogens*, *8*(12), e1003037. https://doi.org/10.1371/journal.ppat.1003037

Oide, S., Moeder, W., Krasnoff, S., Gibson, D., Haas, H., Yoshioka, K., & Turgeon, B. G. (2006). NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. *Plant Cell*, *18*(10), 2836-2853. https://doi.org/10.1105/tpc.106.045633

Ökmen, B., Kemmerich, B., Hilbig, D., Wemhöner, R., Aschenbroich, J., Perrar, A., Huesgen, P. F., Schipper, K., & Doehlemann, G. (2018). Dual function of a secreted fungalysin metalloprotease in Ustilago maydis. *New Phytol.*, *220*(1), 249-261. https://doi.org/10.1111/nph.15265

Oliver, R. P., & Solomon, P. S. (2010). New developments in pathogenicity and virulence of necrotrophs. *Curr Opin Plant Biol*, *13*(4), 415-419. https://doi.org/10.1016/j.pbi.2010.05.003

Pandey, V., Gupta, A. K., Singh, M., Pandey, D., & Kumar, A. (2019). Complementary proteomics, genomics approaches identifies potential pathogenicity/virulence factors in *Tilletia indica* induced under the influence of host factor. *Sci Rep*, *9*(1), 553. https://doi.org/10.1038/s41598-018-37810-1

Parra, G., Bradnam, K., & Korf, I. (2007). CEGMA: a pipeline to accurately annotate core genes in eukaryotic genomes. *Bioinformatics*, *23*(9), 1061-1067. https://doi.org/10.1093/bioinformatics/btm071

Persoons, A., Morin, E., Delaruelle, C., Payen, T., Halkett, F., Frey, P., De Mita, S., & Duplessis, S. (2014). Patterns of genomic variation in the poplar rust fungus *Melampsora larici-populina* identify pathogenesis-related factors. *Front Plant Sci*, *5*(450). https://doi.org/10.3389/fpls.2014.00450

Peterson, G. L., Whitaker, T. B., Stefanski, R. J., Podleckis, E. V., Phillips, J. G., Wu, J. S., & Martinez, W. H. (2009). A risk assessment model for importation of United States milling wheat containing *Tilletia contraversa*. *Plant Dis*, *93*(6), 560-573. https://doi.org/10.1094/PDIS-93-6-0560

Pimentel, G., Peever, T. L., & Carris, L. M. (2000). Genetic variation among natural populations of *Tilletia controversa* and *T. bromi*. *Phytopathology*, *90*(4), 376-383. https://doi.org/10.1094/PHYTO.2000.90.4.376

Price, A. L., Jones, N. C., & Pevzner, P. A. (2005). *De novo* identification of repeat families in large genomes. *Bioinformatics*, *21 Suppl 1*, i351-358. https://doi.org/10.1093/bioinformatics/bti1018

Purdy, L. H., Kendrick, E. L., Hoffmann, J. A., & Holton, C. S. (1963). Dwarf bunt of wheat. *Annu. Rev. Microbiol.*, *17*(1), 199-222. https://doi.org/10.1146/annurev.mi.17.100163.001215

Pusztahelyi, T., Holb, I., & Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. *Front Plant Sci*, *6*(573). https://doi.org/10.3389/fpls.2015.00573

R Development Core Team. (2013). *R:* A Language and Environment for Statistical Computing. In R foundation for Statistical Computing. http://www.R-project.org/

Razali, N. M., Cheah, B. H., & Nadarajah, K. (2019). Transposable elements adaptive role in genome plasticity, pathogenicity and evolution in fungal phytopathogens. *International Journal of Molecular Sciences*, *20*(14). https://doi.org/10.3390/ijms20143597

Redkar, A., Hoser, R., Schilling, L., Zechmann, B., Krzymowska, M., Walbot, V., & Doehlemann, G. (2015). A secreted effector protein of *Ustilago maydis* guides maize leaf cells to form tumors. *Plant Cell*, *27*(4), 1332-1351. https://doi.org/10.1105/tpc.114.131086

Redkar, A., Villajuana-Bonequi, M., & Doehlemann, G. (2015). Conservation of the *Ustilago maydis* effector See1 in related smuts. *Plant Signal Behav*, *10*(12), e1086855-e1086855. https://doi.org/10.1080/15592324.2015.1086855

Ren, Z., Liu, J., Din, G. M. U., Zhang, H., Du, Z., Chen, W., Liu, T., Zhang, J., Zhao, S., & Gao, L. (2020). Transcriptome analysis of wheat spikes in response to *Tilletia controversa* Kühn which cause wheat dwarf bunt. *Scientific Reports*, *10*(1), 21567. https://doi.org/10.1038/s41598-020-78628-0

Robinson, P. M., McKee, N. D., Thompson, L. A. A., Harper, D. B., & Hamilton, J. T. G. (1989). Autoinhibition of germination and growth in *Geotrichum candidum*. *Mycological research*, *93*(2), 214-222. https://doi.org/10.1016/s0953-7562(89)80120-0

Röttig, M., Medema, M. H., Blin, K., Weber, T., Rausch, C., & Kohlbacher, O. (2011). NRPSpredictor2—a web server for predicting NRPS adenylation domain specificity. *Nucleic Acids Research*, *39*(suppl_2), W362-W367. https://doi.org/10.1093/nar/gkr323

Rouxel, T., Grandaubert, J., Hane, J. K., Hoede, C., van de Wouw, A. P., Couloux, A., Dominguez, V., Anthouard, V., Bally, P., Bourras, S., Cozijnsen, A. J., Ciuffetti, L. M., Degrave, A., Dilmaghani, A., Duret, L., Fudal, I., Goodwin, S. B., Gout, L., Glaser, N., Linglin, J., Kema, G. H. J., Lapalu, N., Lawrence, C. B., May, K., Meyer, M., Ollivier, B., Poulain, J., Schoch, C. L., Simon, A., Spatafora, J. W., Stachowiak, A., Turgeon, B. G., Tyler, B. M., Vincent, D., Weissenbach, J., Amselem, J., Quesneville, H., Oliver, R. P., Wincker, P., Balesdent, M.-H., & Howlett, B. J. (2011). Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by Repeat-Induced Point mutations. *Nature Communications*, *2*(1), 202. https://doi.org/10.1038/ncomms1189

Rudloff, J. E., Bauer, R., Büttner, P., Sedaghtjoo, S., Kirsch, N., & Maier, W. (2020). Monitoring zum Vorkommen von *Tilletia controversa* (Zwergsteinbrand) an konventionell erzeugtem Winterweizen in den Bundesländern Brandenburg, Mecklenburg-Vorpommern, Niedersachsen, Nordrhein-Westfalen, Sachsen, Sachsen-Anhalt und Schleswig-Holstein [Monitoring of the occurrence of *Tilletia controversa* on conventionally produced winter wheat in Northern Germany]. *Kulturpflanzen*, *72*(8), 7. https://doi.org/10.5073/JfK.2020.08.16

Russell, B. W. (1994). Morphological, physiological, and genetic evidence in support of a conspecific status for *Tilletia caries, T. controversa*, and *T. foetida*. *Phytopathology*, *84*(6), 576-582. https://doi.org/10.1094/Phyto-84-576

Russell, B. W., & Mills, D. (1993). Electrophoretic karyotypes of *Tilletia caries*, *T. controversa*, and their F1 progeny: further evidence for conspecific status. *Molecular plant-microbe interactions : MPMI*, *6*(1), 66-74. https://doi.org/10.1094/mpmi-6-066

Ruzgas, V., & Liatukas, Ž. (2008). Resistance genes and sources for the control of wheat common bunt (*Tilletia tritici* (DC.) Tul.). *Biologija*, *54*(4), 274-278. https://doi.org/10.2478/v10054-008-0056-y

Sabot, F., & Schulman, A. H. (2006). Parasitism and the retrotransposon life cycle in plants: a hitchhiker's guide to the genome. *Heredity (Edinb)*, *97*(6), 381-388. https://doi.org/10.1038/sj.hdy.6800903

Scherer, M., Heimel, K., Starke, V., & Kämper, J. (2006). The Clp1 protein is required for clamp formation and pathogenic development of *Ustilago maydis*. *Plant Cell*, *18*(9), 2388-2401. https://doi.org/10.1105/tpc.106.043521

Schipper, K. (2009). Charakterisierung eines *Ustilago maydis* Genclusters, das für drei neuartige sekretierte Effektoren kodiert. Philipps-Universität Marburg . Marburg.

Seitner, D., Uhse, S., Gallei, M., & Djamei, A. (2018). The core effector Cce1 is required for early infection of maize by *Ustilago maydis*. *Molecular Plant Pathology*, *19*(10), 2277-2287. https://doi.org/10.1111/mpp.12698

Sharma, P., Tiwari, R., Saharan, M. S., Sharma, I., Kumar, J., Mishra, S., Muthusamy, S. K., Gupta, R. K., Jaiswal, S., Iquebal, M. A., Angadi, U. B., Kumar, N., Fatma, S., Rai, A., & Kumar, D. (2016). Draft genome sequence of two monosporidial lines of the karnal bunt fungus *Tilletia indica* Mitra (PSWKBGH-1 and PSWKBGH-2). *Genome Announc*, *4*(5), e00928-00916. https://doi.org/10.1128/genomeA.00928-16

Sharma, R., Mishra, B., Runge, F., & Thines, M. (2014). Gene loss rather than gene gain is associated with a host jump from monocots to dicots in the smut fungus *Melanopsichium pennsylvanicum*. *Genome Biology and Evolution*, *6*(8), 2034-2049. https://doi.org/10.1093/gbe/evu148

Sharma, R., Ökmen, B., Doehlemann, G., & Thines, M. (2018). *Pseudozyma* saprotrophic yeasts have retained a large effector arsenal, including functional Pep1 orthologs. *bio*R*xiv*, 489690. https://doi.org/10.1101/489690

Sheppard, S. K., Guttman, D. S., & Fitzgerald, J. R. (2018). Population genomics of bacterial host adaptation. *Nature Reviews Genetics*, *19*(9), 549-565. https://doi.org/10.1038/s41576-018-0032-z

Shwab, E. K., & Keller, N. P. (2008). Regulation of secondary metabolite production in filamentous ascomycetes. *Mycological research*, *112*(Pt 2), 225-230. https://doi.org/10.1016/j.mycres.2007.08.021

Sillo, F., Garbelotto, M., Friedman, M., & Gonthier, P. (2015). Comparative genomics of sibling fungal pathogenic taxa identifies adaptive evolution without divergence in pathogenicity genes or genomic structure. *Genome Biology and Evolution*, *7*(12), 3190-3206. https://doi.org/10.1093/gbe/evv209

Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210-3212. https://doi.org/10.1093/bioinformatics/btv351

Singh, J., Aggarwal, R., Gurjar, M. S., Sharma, S., Jain, S., & Saharan, M. S. (2020). Identification and expression analysis of pathogenicity-related genes in *Tilletia indica* inciting Karnal bunt of wheat. *Australasian Plant Pathology*. https://doi.org/10.1007/s13313-020-00711-x

Singh, J., Aggarwal, R., Gurjar, M. S., Sharma, S., & Saharan, M. S. (2019). Identification of carbohydrate active enzymes from whole genome sequence of *Tilletia indica* and sporulation analysis. *Indian Journal of Agricultural Sciences*, *89*(6), 1023-1026.

Singh, J., & Trione, E. (1969). Self inhibition of the germination of bunt teliospores of *Tilletia caries* and of *Tilletia controversa*. *Phytopathology*, *59*, 15.

Skibbe, D. S., Doehlemann, G., Fernandes, J., & Walbot, V. (2010). Maize tumors caused by *Ustilago maydis*; require organ-specific genes in host and pathogen. *Science*, *328*(5974), 89. https://doi.org/10.1126/science.1185775

Smit, A., Hubley, R., & Green, P. (2008-2019). *RepeatModeler*. Retrieved 05 Sep 2019 from http://www.repeatmasker.org/RepeatModeler/

Smit, A., Hubley, R., & Green, P. (2013-2015). *RepeatMasker Open-4.0.9*. Retrieved 05 Sep 2019 from http://www.repeatmasker.org/

Soberanes-Gutiérrez, C. V., Juárez-Montiel, M., Olguín-Rodríguez, O., Hernández-Rodríguez, C., Ruiz-Herrera, J., & Villa-Tanaca, L. (2015). The pep4 gene encoding proteinase A is involved in dimorphism and pathogenesis of *Ustilago maydis*. *Molecular Plant Pathology*, *16*(8), 837-846. https://doi.org/10.1111/mpp.12240

Sperschneider, J., Dodds, P. N., Gardiner, D. M., Singh, K. B., & Taylor, J. M. (2018). Improved prediction of fungal effector proteins from secretomes with EffectorP 2.0. *Mol Plant Pathol*, *19*(9), 2094-2110. https://doi.org/10.1111/mpp.12682

Stachelhaus, T., Mootz, H. D., & Marahiel, M. A. (1999). The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chemistry & biology*, *6*(8), 493-505. https://doi.org/10.1016/s1074-5521(99)80082-9

Stanke, M., Schöffmann, O., Morgenstern, B., & Waack, S. (2006). Gene prediction in eukaryotes with a generalized hidden Markov model that uses hints from external sources. *BMC Bioinformatics*, *7*(1), 62. https://doi.org/10.1186/1471-2105-7-62

Stirnberg, A., & Djamei, A. (2016). Characterization of ApB73, a virulence factor important for colonization of *Zea mays* by the smut *Ustilago maydis*. *Molecular Plant Pathology*, *17*(9), 1467-1479. https://doi.org/10.1111/mpp.12442

Tanaka, A., Tapper, B. A., Popay, A., Parker, E. J., & Scott, B. (2005). A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. *Molecular microbiology*, *57*(4), 1036-1050. https://doi.org/10.1111/j.1365-2958.2005.04747.x

Teertstra, W. R., van der Velden, G. J., de Jong, J. F., Kruijtzer, J. A. W., Liskamp, R. M. J., Kroon-Batenburg, L. M. J., Müller, W. H., Gebbink, M. F. B. G., & Wösten, H. A. B. (2009). The Filament-specific Rep1-1 Repellent of the Phytopathogen *Ustilago maydis* Forms Functional Surface-active Amyloid-like Fibrils [10.1074/jbc.M900095200]. *Journal of Biological Chemistry*, *284*(14), 9153-9159.

Trione, E. J. (1964). Isolation and in vitro culture of the wheat bunt fungi *Tilletia caries* and *Tilletia controversa*. *Phytopathology*, *54*, 592_596.

Trione, E. J., & Ross, W. D. (1988). Lipids as bioregulators of teliospore germination and sporidial formation in the wheat bunt fungi, *Tilletia* species. *Mycologia*, *80*(1), 38-45. https://doi.org/10.1080/00275514.1988.12025495

Vanky, K. (2008). Smut fungi (*Basidiomycota* p.p., *Ascomycota* p.p.) of the world. novelties, selected examples, trends. *Acta Microbiol Immunol Hung*, *55*(2), 91-109. https://doi.org/10.1556/AMicr.55.2008.2.2

Vánky, K. (2012). *Smut fungi of the world* American Phytopathological Society.

Vesth, T. C., Nybo, J. L., Theobald, S., Frisvad, J. C., Larsen, T. O., Nielsen, K. F., Hoof, J. B., Brandl, J., Salamov, A., Riley, R., Gladden, J. M., Phatale, P., Nielsen, M. T., Lyhne, E. K., Kogle, M. E., Strasser, K., McDonnell, E., Barry, K., Clum, A., Chen, C., LaButti, K., Haridas, S., Nolan, M., Sandor, L., Kuo, A., Lipzen, A., Hainaut, M., Drula, E., Tsang, A., Magnuson, J. K., Henrissat, B., Wiebenga, A., Simmons, B. A., Mäkelä, M. R., de Vries, R. P., Grigoriev, I. V., Mortensen, U. H., Baker, S. E., & Andersen, M. R. (2018). Investigation of inter- and intraspecies variation through genome sequencing of *Aspergillus* section *Nigri*. *Nature Genetics*, *50*(12), 1688-1695. https://doi.org/10.1038/s41588-018-0246-1

Wahl, R., Wippel, K., Goos, S., Kämper, J., & Sauer, N. (2010). A Novel High-Affinity Sucrose Transporter Is Required for Virulence of the Plant Pathogen *Ustilago maydis*. *PLOS Biology*, *8*(2), e1000303. https://doi.org/10.1371/journal.pbio.1000303

Wang, A., Pan, L., Niu, X., Shu, X., Yi, X., Yamamoto, N., Li, S., Deng, Q., Zhu, J., Liang, Y., Wang, L., Li, P., & Zheng, A. (2019). Comparative secretome analysis of different smut fungi and identification of plant cell death-inducing secreted proteins from *Tilletia horrida*. *BMC Plant Biol*, *19*(1), 360. https://doi.org/10.1186/s12870-019-1924-6

Wang, A., Pang, L., Wang, N., Ai, P., Yin, D., Li, S., Deng, Q., Zhu, J., Liang, Y., Zhu, J., Li, P., & Zheng, A. (2018). The pathogenic mechanisms of *Tilletia horrida* as revealed by comparative and functional genomics. *Sci Rep*, *8*(1), 15413. https://doi.org/10.1038/s41598-018-33752-w

Wang, A., Shu, X., Niu, X., Yi, X., & Zheng, A. (2019). Transcriptome analysis and whole genome re-sequencing provide insights on rice kernel smut (*Tilletia horrida*) pathogenicity. *Journal of Plant Pathology*, *102*(1), 155-167. https://doi.org/10.1007/s42161-019-00401-8

Wang, A., Zha, Z., Yin, D., Shu, X., Ma, L., Wang, L., Li, P., & Zheng, A. (2020). Comparative transcriptome analysis of *Tilletia horrida* infection in resistant and susceptible rice (*Oryza sativa* L.) male sterile lines reveals potential candidate genes and resistance mechanisms. *Genomics*, *112*(6), 5214-5226. https://doi.org/10.1016/j.ygeno.2020.09.036

Wang, N., Ai, P., Tang, Y., Zhang, J., Dai, X., Li, P., & Zheng, A. (2015). Draft genome sequence of the rice kernel smut *tilletia horrida* strain QB-1. *Genome Announc*, *3*(3), e00621-00615. https://doi.org/10.1128/genomeA.00621-15

Weber, R. E., Pietsch, M., Frühauf, A., Pfeifer, Y., Martin, M., Luft, D., Gatermann, S., Pfennigwerth, N., Kaase, M., Werner, G., & Fuchs, S. (2019). IS26-mediated transfer of blaNDM–1 as the main route of resistance transmission during a polyclonal, multispecies outbreak in a german hospital. *Frontiers in Microbiology*, *10*(2817). https://doi.org/10.3389/fmicb.2019.02817

Whitaker, T. B., Wu, J., Peterson, G. L., Giesbrecht, F. G., & Johansson, A. S. (2001). Variability associated with the official USDA sampling plan used to inspect export wheat shipments for *Tilletia controversa* spores. *Plant Pathology*, *50*(6), 755-760. https://doi.org/DOI 10.1046/j.1365-3059.2001.00640.x

Woolma, N., H. M., & Humphrey, H. B. (1924). Summary of literature on bunt or stinking smut of Wheat. *Bulletin. United States Department of Agriculture*(1210), 44

Wöstemeyer, J., & Kreibich, A. (2002). Repetitive DNA elements in fungi (Mycota): impact on genomic architecture and evolution. *Current Genetics*, *41*(4), 189-198. https://doi.org/10.1007/s00294-002-0306-y

Wösten, H. A., Bohlmann, R., Eckerskorn, C., Lottspeich, F., Bölker, M., & Kahmann, R. (1996). A novel class of small amphipathic peptides affect aerial hyphal growth and surface hydrophobicity in *Ustilago maydis*. *EMBO J*, *15*(16), 4274-4281

Xu, Y., Vinas, M., Alsarrag, A., Su, L., Pfohl, K., Rohlfs, M., Schäfer, W., Chen, W., & Karlovsky, P. (2019). Bis-naphthopyrone pigments protect filamentous ascomycetes from a wide range of predators. *Nature Communications*, *10*(1), 3579. https://doi.org/10.1038/s41467-019-11377-5

Zdobnov, E. M., Tegenfeldt, F., Kuznetsov, D., Waterhouse, R. M., Simao, F. A., Ioannidis, P., Seppey, M., Loetscher, A., & Kriventseva, E. V. (2017). OrthoDB v9.1: cataloging evolutionary and functional annotations for animal, fungal, plant, archaeal, bacterial and viral orthologs. *Nucleic acids research*, *45*(D1), D744-D749. https://doi.org/10.1093/nar/gkw1119

Zerbino, D. R., & Birney, E. (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome research*, *18*(5), 821-829. https://doi.org/10.1101/gr.074492.107

Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., Busk, P. K., Xu, Y., & Yin, Y. (2018). dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Research*, *46*(W1), W95-W101. https://doi.org/10.1093/nar/gky418

Zhao, Z., Liu, H., Wang, C., & Xu, J.-R. (2013). Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics*, *14*(1), 274. https://doi.org/10.1186/1471-2164-14-274

Additional Table 2-4 Assessment of the genome annotation completeness when different approaches (homology-based, *ab initio* and a combination) were used

Tools	**Genes**	**Transcripts**	**Fungi DB**	**Basidiomycota DB**
GeMoMa+RNA-Seq	9182	14114	C:92.1%[S:30.7%,D:61.4%],F:6.6%,M:1.3%	C:85.9%[S:34.4%,D:51.5%],F:9.4%,M:4.7%
Braker+RNA-Seq	11124	11517	C:95.5%[S:91.4%,D:4.1%],F:4.5%,M:-0.0%	C:90.9%[S:88.5%,D:2.4%],F:6.0%,M:3.1%
Braker+RNA-Seq+GeMoMa+RNA-Seq	9913	10053	C:96.6%[S:94.5%,D:2.1%],F:3.1%,M:0.3%	C:91.7%[S:89.5%,D:2.2%],F:5.5%,M:2.8%

Tilletia caries isolate AA11 was used for the comparison of different structural annotation. The proportions classified as complete (C), complete single-copy (S), complete duplicated (D), fragmented (F), and missing (M). The total number of tested genes were 290 for Fungi-odb and 1335 for Basidiomycota-odb. The annotation completeness was improved using a combination of GeMoMa and BRAKER2 gene model prediction tools.

Additional Table 2-5 Percentage of repetitive regions in five isolates of *T. caries*, seven isolates of *T. controversa* and four isolates of *T. laevis*

	T. caries							***T. controversa***						***T. laevis***		
	AA11	AI	AO	AZH3	DAOMC238032	DAOMC236426	DAOMC238052	OA2	OL14	OR	OV	OW	ATCC42080	DAOMC238040	LLFL	L-19
Short sequence repeat (%)	0.58	0.57	0.60	0.58	0.60	0.58	0.63	0.55	0.55	0.53	0.53	0.54	0.62	0.61	0.53	0.53
Transposon elements (%)	3.62	3.74	3.45	3.62	3.23	3.64	3.31	3.78	3.56	5.24	3.47	3.70	3.40	3.40	3.60	3.64
Non-classified (%)	8.09	9.43	6.40	7.72	4.00	8.43	5.59	9.30	6.86	31.93	4.94	8.02	6.27	5.11	7.50	7.64
Total of bases masked (%)	12.29	13.7	10.4	11.92	7.83	12.64	9.53	13.6	10.97	37.70	8.94	12.2	10.30	9.11	11.63	11.81

Additional Table 2-6 Number and relative abundance of total SSRs identified in the *T. caries*, *T. controversa*, and *T. laevis* genomes

			Mono nucleotide	**Din ucleotide**	**Tri nucleotide**	**Tetra nucleotide**	**Penta nucleotide**	**Hexa nucleotide**	**Compound**	**Total**
T. caries	AA11	#	1936	1342	2908	280	104	419	559	7548
		RA	61.7	42.8	92.7	8.9	3.3	13.4	17.8	
	AI	#	1920	1345	2911	276	101	425	557	7535
		RA	60.5	42.4	91.8	8.7	3.2	13.4	17.6	
	AO	#	1677	1319	2871	265	87	412	518	7149
		RA	55.3	43.5	94.7	8.7	2.9	13.6	17.1	
	AZH3	#	1882	1339	2911	278	97	435	561	7503
		RA	60.2	42.8	93.2	8.9	3.1	13.9	18.0	
	DAOMC 238032	#	1636	1262	2740	253	84	412	547	6934
		RA	58.1	44.8	97.4	9.0	3.0	14.6	19.4	
T. controversa	DAOMC 236426	#	1640	1272	2767	266	91	408	573	7017
		RA	54.9	42.6	92.6	8.9	3.0	13.7	19.2	
	DAOMC 238052	#	1967	1282	2780	258	92	415	602	7396
		RA	68.9	44.9	97.3	9.0	3.2	14.5	21.1	
	OA2	#	1722	1366	2912	276	95	428	550	7349
		RA	54.0	42.8	91.3	8.7	3.0	13.4	17.2	
	OL14	#	1568	1343	2867	273	89	430	519	7089
		RA	51.1	43.8	93.5	8.9	2.9	14.0	16.9	

Additional Table 2-6 *(continued)*

			Mono nucleotide	Din ucleotide	Tri nucleotide	Tetra nucleotide	Penta nucleotide	Hexa nucleotide	Compound	Total
T. controversa	OR	#	2819	1748	3537	358	143	595	831	10031
		RA	57.1	35.4	71.6	7.2	2.9	12.0	16.8	
	OV	#	1230.0	1293.0	2810.0	261.0	81.0	416.0	473.0	6564
		RA	41.8	44.0	95.6	8.9	2.8	14.1	16.1	
	OW	#	1498.0	1335.0	2869.0	251.0	95.0	426.0	530.0	7004
		RA	48.2	42.9	92.3	8.1	3.1	13.7	17.0	
T. laevis	ATCC 42080	#	1968	1283	2814	256	90	409	602	7422
		RA	68.4	44.6	97.8	8.9	3.1	14.2	20.9	
	DAOMC 238040	#	1749	1256	2778	252	84	409	563	7091
		RA	61.9	44.4	98.3	8.9	3.0	14.5	19.9	
	LLFL	#	1456	1327	2858	251	87	399	505	6883
		RA	47.2	43.0	92.6	8.1	2.8	12.9	16.4	
	L-19	#	1431	1326	2888	250	92	419	512	6918
		RA	46.4	43.0	93.6	8.1	3.0	13.6	16.6	

SSRs (short sequence repeats) identified with MISA. Compound SSRs are defined when two SSRs are separated by no more than 100 bp and the number of SSRs per Mbp is calculated as relative abundance R=Relative abundance.

Additional Table 2-7 Percentage of the maximum aligned bases in non-repetitive regions

		T. caries					*T. controversa*							*T. laevis*		
	Isolates	AA11	AI	AO	AZH3	DAOMC 238032	DAOMC 236426	DAOMC 238052	OA2	OL14	OR	OV	OW	ATCC 42080	DAOMC 238040	LLFL
T. caries	AI	93.2														
	AO	92.4	90.8													
	AZH3	92.5	92.2	92.5												
	DAOMC 23803	90.2	89.4	90.4	90.6											
T. controversa	DAOMC 236426	94.1	93.6	93.9	94.0	91.9										
	DAOMC 238052	93.4	92.8	93.7	93.0	92.8	88.2									
	OA2	93.4	93.0	93.1	93.3	90.4	93.2	92.1								
	OL14	93.1	92.1	93.1	93.3	91.4	94.1	93.5	92.0							
	OR	89.5	90.0	89.4	89.6	82.7	84.8	84.2	90.5	90.4						
	OV	92.8	92.1	93.0	92.8	91.2	94.3	93.9	91.8	93.3	83.4					
	OW	93.2	92.5	93.1	93.0	90.3	93.7	92.4	92.0	93.3	83.3	93.1				
T. laevis	ATCC 42080	92.3	91.6	92.7	92.5	89.4	93.2	93.1	93.2	93.9	84.9	94.0	93.1			
	DAOMC 238040	91.5	90.6	91.9	92.2	89.3	91.8	92.4	92.4	93.2	84.1	93.1	92.4	90.3		
	LLFL	92.2	91.3	92.4	91.9	90.0	93.7	93.0	93.1	92.8	89.7	92.6	92.6	92.2	91.3	
	L-19	92.5	91.7	92.8	92.2	90.0	93.8	93.0	92.8	93.1	89.9	92.8	92.8	92.9	92.1	92.0

The isolates and species are given in row and column labels. Colour intensity changes from white (94.3%) to yellow (82.7%) of alignment coverage.

Additional Table 2-8 The number of putative enzymes in each CAZyme family in different isolates and their substrate

Families	***T. caries***					***T. controversa***							***T. laevis***				**Substrate**
	AA11	AI	AO	AZH 3	DAO MC 238032	DAO MC 236426	DAO MC 238052	OA2	OL14	OR	OV	OW	ATC C 42080	DAO MC 238040	LLFL	L-19	
AA1_2	2	2	2	3	2	1	2	3	3	2	2	3	2	2	2	2	laccase
AA1_3	1	2	2	1	1	1	1	1	1	1	1	1	1	1	2	2	laccase
AA12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
AA2	2	2	2	2	2	2	2	3	3	3	3	3	2	2	2	2	lignin
AA3	2	1	1	1	2	2	2	1	1	1	1	1	2	2	1	1	cellulose
AA3_2	10	11	10	11	10	11	11	12	12	18	10	14	9	9	11	11	
AA5_1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
AA6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	biodegradation of aromatic compounds and in the protection of fungal cells from reactive quinone compounds
AA7	3	3	2	2	9	8	9	3	3	3	3	3	9	9	3	2	oxidize the reducing end glycosyl residues
CBM21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	granular starch-binding
CE1	1	1	1	1	1		1	1	1	1	1	1	1	1	1	1	arabinoxylan, pectin
CE10	11	12	10	11	22	25	24	11	11	13	11	10	25	25	10	10	esterases acting on non-carbohydrate substrates
CE16	4	3	5	4	2	4	4	4	4	4	4	4	4	4	4	4	

Additional Table 2-8 *(continued)*

Families	***T. caries***					***T. controversa***							***T. laevis***				**Substrate**
	AA11	AI	AO	AZH 3	DAO MC 238032	DAO MC 236426	DAO MC 238052	OA2	OL14	OR	OV	OW	ATC C 42080	DAO MC 238040	LLFL	L-19	
CE4	9	8	9	8	7	7	6	8	11	6	8	9	6	6	9	10	chitin
CE5	14	13	13	13	14	14	14	14	14		13	14	13	13	16	13	cutin,arabinoxylan
GH102					1												starch
GH105	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	pectin
GH128	6	6	6	6	6	5	6	6	6	7	6	6	6	6	6	6	fungal cell wall
GH13_10					1												starch
GH13_25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	starch
GH13_40	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	starch
GH13_8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	starch
GH13_9					1												starch
GH15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	starch
GH152	2	1	1	1	1	1	1	1	1	4	1	1	1	1	1	1	
GH16	1	2	1	2	1	2	2	2	3	3	1	3	2	2	2	2	fungal cell wall
GH17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	fungal cell wall
GH18	3	5	3	3	4	4	4	5	3	5	3	5	4	4	3	3	chitin

Additional Table 2-8 *(continued)*

Families	***T. caries***					***T. controversa***							***T. laevis***				**Substrate**
	AA11	AI	AO	AZH 3	DAO MC 238032	DAO MC 236426	DAO MC 238052	OA2	OL14	OR	OV	OW	ATC C 42080	DAO MC 238040	LLFL	L-19	
GH2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	galactomannan, pectin
GH20	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	chitin
GH23					1												bacterial peptidoglycan
GH25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	bacterial peptidoglycan
GH3	2	2	3	2	2	2	2	2	2	3	2	2	2	2	2	2	cellulose, xylan
GH30_3	1		1			2		2	2	1	2	2			2	2	fungal cell wall
GH31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	xyloglucan
GH32	2	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	sucrose/inulin
GH35	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	galactomannan, xyloglucan
GH37	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	trehalose
GH38	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
GH43_30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	hemicellulose/pectin
GH45	11	9	9	10	8	10	5	10	8	12	8	10	6	7	9	11	cellulose
GH47	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	protein glycosilation
GH5_12	2	2	2	2	1	2	2	2	2	2	1	2	2	2	1	2	cellulose, galactomannan

Additional Table 2-8 *(continued)*

Families	***T. caries***					***T. controversa***							***T. laevis***				**Substrate**
	AA11	AI	AO	AZH 3	DAO MC 238032	DAO MC 236426	DAO MC 238052	OA2	OL14	OR	OV	OW	ATC C 42080	DAO MC 238040	LLFL	L-19	
GH5_50	2	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	cellulose, galactomannan
GH5_51											1						cellulose, galactomannan
GH5_9	15	15	14	14	15	16	16	15	14	20	15	15	16	18	14	15	cellulose, galactomannan
GH55	2	2	2	2	2	2	2	2	2		2	2	2	2	2	2	fungal cell wall, callose
GH63					1	1	1						1	1			protein glycosilation
GH65	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	trehalase
GH79	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
GH8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	cell wall
GH85	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	fungal cell wall
GH9	1	1	1	2	2	2	1	1			1	1	1	2			cellulose
GH92	2	2	2	1	1	2	1	4	1	5	2	1	2	1	1	2	galactomannan
GT1	4	4	4	4	5	5	5	4	4	3	4	4	6	6	4	4	phenols, alcohols, amines and fatty acids
GT17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	glycoprotein synthesis
GT2_Chitin_ synth_1	6	6	6	6	5	5	5	5	5	5	5	5	5	5	6	6	chitin synthesis

Additional Table 2-8 *(continued)*

Families	***T. caries***					***T. controversa***							***T. laevis***				**Substrate**
	AA11	AI	AO	AZH 3	DAO MC 238032	DAO MC 236426	DAO MC 238052	OA2	OL14	OR	OV	OW	ATC C 42080	DAO MC 238040	LLFL	L-19	
GT2_Chitin_ synth_2	4	4	4	4	4	4	4	4	4	2	4	4	4	4	4	4	chitin synthesis
GT2_Glyco_t ranf_2_3	2	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	chitin synthesis
GT2_Glyco_t rans_2_3	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	chitin synthesis
GT2_Glycos _transf_2	2	2	2	2	3	2	2	2	2	2	2	2	2	2	2	2	chitin synthesis
GT20	5	4	4	3	3	3	2	4	4	4	2	5	2	2	2	3	biosynthesis of trehalose
GT21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
GT22	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	lyzozyma peptideoglican
GT24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	chitinase
GT3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	glycogen
GT31									1								xylan
GT32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	sucrose/invertase
GT33	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	hydrolysis of glyco substrates
GT39	3	3	3	3	3	3	3	3	3	4	3	3	3	3	3	3	
GT4	2	2	2	2	3	2	2	2	2	2	3	2	2	2	2	2	hydrolysis of polysaccharides

Additional Table 2-8 *(continued)*

Families	***T. caries***					***T. controversa***							***T. laevis***				**Substrate**
	AA11	AI	AO	AZH 3	DAO MC 238032	DAO MC 236426	DAO MC 238052	OA2	OL14	OR	OV	OW	ATC C 42080	DAO MC 238040	LLFL	L-19	
GT48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	chitinase
GT50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	agarase
GT57	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	amilase
GT58	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	endosialidase
GT59	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	hydrolyse α-L-arabinosides
GT66	1	1	1	1	1	1	1	1	1	4	1	1	1	1	2	2	amylose
GT69	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	PL16
GT70					1												phenols, alcohols, amines and fatty acids
GT76	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	mannosidase
GT8	2	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2	cellulase
GT83					1												hydrolysis of glyco substrates
PL1_3					1												pectin
PL1_4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	pectin
PL14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	bacterial exopolysaccharides
PL14_4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	bacterial exopolysaccharides

Additional Table 2-8 *(continued)*

Families	***T. caries***					***T. controversa***							***T. laevis***				**Substrate**
	AA11	AI	AO	AZH 3	DAO MC 238032	DAO MC 236426	DAO MC 238052	OA2	OL14	OR	OV	OW	ATC C 42080	DAO MC 238040	LLFL	L-19	
PL14_5	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	bacterial exopolysaccharides
PL35	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	

The number of putative enzymes in each CAZyme family in different isolates and their substrate Enzyme families comprise of 1) Auxiliary Activities (AAs): redox enzymes that act in conjunction with CAZymes, 2) Carbohydrate-Binding Modules (CBMs): adhesion to carbohydrates, 3) Carbohydrate Esterases (CEs): hydrolysis of carbohydrate esters, 4) Glycoside Hydrolases (GHs): hydrolysis and/or rearrangement of glycosidic bonds, 5) Glycosyl Transferases (GTs): formation of glycosidic bonds, and 6) Polysaccharide Lyases (PLs): nonhydrolytic cleavage of glycosidic bonds, mainly degrade glycosaminoglycans and pectins are represented by their family number according to the carbohydrate-active enzyme database. Abundance of the different enzymes within a family is represented by a color scale from 0 (white) to 25 (red) occurrences. Right side: known substrate of plant cell wall-decomposing enzymes (PCWDs) in green and families outlined in blue indicate CAZymes involving in starch/glycogen metabolism according to Kijpornyongpan et al. 2018 and Benevenuto et al. 2018.

Additional Table 2-9 Prediction of the secondary metabolite biosynthesis gene clusters

	T. caries					***T. controversa***							***T. laevis***				
	AA11	AI	AO	AZH3	DAOMC 238032	DAOMC 236426	DAOMC 238052	OA2	OL14	OR	OV	OW	ATCC 42080	DAOMC 238040	LLFL	L-19	% sequences identity
Indole	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	99.5
NRPS	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	99.4
NRPS-like I	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	98.1
NRPS-like II	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	98.8
NRPS-like III	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	99.2
NRPS-like IV	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	99.2
NRPS-like V	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	NA
Terpene I	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	99.8
Terpene II	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	98.7
t1PKS/NRPS-like	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	95.7

Additional Table 2-10 Analysis of adenylation domains of putative NRPS genes in *Tilletia* spp.

Domain	**Predicted AA substrate**	**Nearest neighbor score (%)**	**Extracted Stachelhaus-code**
NRPS			
A1	phg	50	DLMIIGLLIK
A2	orn	60	DVKAIGAIGK
A3	lys	50	DVIDAGLVYK
NRPS-like II			
A1	pro	90	DPRHFVMRAK
NRPS-like IV			
A1	phe	30	GGRYAASPI-

Additional Table 2-11 *In silico* functional prediction of the species-specific orthology clusters

	Criteria	Secreted	EC number	KEGG KO	KEGG pathway	KEGG module	KEGG reaction	KEGG class	BRITE	KOG cat	InterPro GO Names
T. caries											
Orthocluster_8896	S										
Orthocluster_8958	R										
Orthocluster_9357	R									L	F:hydrolase activity, acting on ester bonds
Orthocluster_9365	R										
Orthocluster_9374	R			ko:K03029	ko03050,ko05169,map03050,map05169	M00341			ko00000,ko00001,ko00002,ko03051	O	P:ubiquitin-dependent protein catabolic process; C:proteasome regulatory particle, base subcomplex
Orthocluster_9406	R										
Orthocluster_9442	R										
T. controversa											
Orthocluster_6819	S										
Orthocluster_7712	S	Secreted								S	
Orthocluster_8107	S										
Orthocluster_8152	S										
Orthocluster_8302	S										
Orthocluster_8303	S										
Orthocluster_8304	S										
Orthocluster_8307	S										
Orthocluster_8550	S										
Orthocluster_8551	S										
Orthocluster_8552	S			ko:K03029	ko03050,ko05169,map03050,map05169	M00341			ko00000,ko00001,ko00002,ko03051	O	P:ubiquitin-dependent protein catabolic process; C:proteasome regulatory particle, base subcomplex
Orthocluster_8553	S										

Additional Table 2-11 *(continued)*

	Criteria	Secreted	EC number	KEGG KO	KEGG pathway	KEGG module	KEGG reaction	KEGG class	BRITE	KOG cat	InterPro GO Names
Orthocluster_8554	S										
Orthocluster_8555	S										
Orthocluster_8557	S										
Orthocluster_8558	S										
Orthocluster_8559	S										
Orthocluster_8560	S										
Orthocluster_8562	S									T	F:calcium ion binding
Orthocluster_8565	S									Q	F:hydrolase activity
Orthocluster_8568*	S		2.4.1.15, 2.4.1.347	ko:K00697	ko00500,ko01100,map00500,map01100	0	R02737	RC00005,RC00049,RC02748	ko00000,ko00001,ko01000,ko01003	G	
Orthocluster_8531	R										
Orthocluster_8549	R										
Orthocluster_8561	R										
Orthocluster_8567	R									T	P:protein phosphorylation; F:protein kinase activity; F:ATP binding
Orthocluster_8569	R										
Orthocluster_8675	R										F:protein binding
Orthocluster_8750	R									S	
Orthocluster_8766	R										
Orthocluster_8804	R		3.6.5.5	ko:K17065	ko04139,ko04214,ko04217,ko04621,ko04668,map04139,map04214,map04217,map04621,map04668				ko00000,ko00001,ko01000,ko03029,ko04131,ko04147	U	F:GTP binding

Additional Table 2-11 *(continued)*

	Criteria	Secreted	EC number	KEGG KO	KEGG pathway	KEGG module	KEGG reaction	KEGG class	BRITE	KOG cat	InterPro GO Names
Orthocluster_8863	R										
Orthocluster_8864	R		2.5.1.1,2.5.1.10	ko:K00787	ko00900,ko01100,ko01110,ko01130,ko05164,ko05166,map00900,map01100,map01110,map01130,map05164,map05166	M00366,M00367	R01658,R02003	RC00279	ko00000,ko00001,ko00002,ko01000,ko01006	H	P:isoprenoid biosynthetic process; F:transferase activity, transferring alkyl or aryl (other than methyl) groups
Orthocluster_8867	R									S	
Orthocluster_8868	R										
Orthocluster_8869	R		3.6.5.5	ko:K17065	ko04139,ko04214,ko04217,ko04621,ko04668,map04139,map04214,map04217,map04621,map04668				ko00000,ko00001,ko01000,ko03029,ko04131,ko04147	U	F:GTPase activity; F:GTP binding
Orthocluster_8870	R										
Orthocluster_8871*	R									S	
Orthocluster_8873*	R		2.7.11.1	ko:K08850					ko00000,ko01000,ko01001,ko03036	T	
Orthocluster_8874	R										
T. laevis											
Orthocluster_9608	S										
Orthocluster_9627	S										
Orthocluster_9634	S										
Orthocluster_9861	R										

Additional Table 2-11 *(continued)*

	Criteria	Secreted	EC number	KEGG KO	KEGG pathway	KEGG module	KEGG reaction	KEGG class	BRITE	KOG cat	InterPro GO Names
Orthocluster_10118	R										
Orthocluster_10125	R										
Orthocluster_10130	R										
Orthocluster_10132	R										
Orthocluster_10135	R										
Orthocluster_10173	R										
T. caries/T. laevis (common bunt)											
Orthocluster_7863	S										
Orthocluster_7890	S										
Orthocluster_7893	S										
Orthocluster_7894	S										
Orthocluster_7895	S										
Orthocluster_7920	S		2.7.11.1	ko:K20872					ko00000,ko01000,ko01001,ko01009,ko03036	T	P:protein phosphorylation; F:protein kinase activity; F:ATP binding
Orthocluster_7939	S									L	F:nucleic acid binding
Orthocluster_7940	S			ko:K17260	ko04138,ko04530,map04138,map04530				ko00000,ko00001,ko04131,ko04147,ko04812	Z	
Orthocluster_7941	S		2.4.2.3	ko:K00757	ko00240,ko00983,ko01100,map00240,map00983,map01100		R01876,R02484,R08229	RC00063	ko00000,ko00001,ko01000	F	P:nucleoside metabolic process; F:catalytic activity
Orthocluster_7942	S										

Additional Table 2-11 *(continued)*

	Criteria	Secreted	EC number	KEGG KO	KEGG pathway	KEGG module	KEGG reaction	KEGG class	BRITE	KOG cat	InterPro GO Names
Orthocluster_7957	S									P	
Orthocluster_7966	S										
Orthocluster_7980	S										
Orthocluster_7993	S										
Orthocluster_8017	S										
Orthocluster_8020	S										
Orthocluster_8022	S										
Orthocluster_8026	S										
Orthocluster_8032	S										
Orthocluster_7899	R										
Orthocluster_7991*	R									L	
Orthocluster_8092	R										
Orthocluster_8120	R										
Orthocluster_8126	R										
Orthocluster_8134	R										
Orthocluster_8136	R										
Orthocluster_8149	R										
Orthocluster_8158	R			ko:K08637, ko:K08783					ko00000,ko01000,ko01002	O	P:proteolysis; F:metallocarboxypeptidase activity; F:zinc ion binding
Orthocluster_8166	R									L	P:DNA integration; F:nucleic acid binding
Orthocluster_8177	R										
Orthocluster_8178	R	Secreted									

Additional Table 2-11 *(continued)*

	Criteria	Secreted	EC number	KEGG KO	KEGG pathway	KEGG module	KEGG reaction	KEGG class	BRITE	KOG cat	InterPro GO Names
Orthocluster_8183	R		4.1.1.15	ko:K01580	ko00250,ko00410,ko00430,ko00650,ko01100,ko01110,ko01120,ko02024,ko04727,ko04940,map00250,map00410,map00430,map00650,map01100,map01110,map01120,map02024,map04727,map04940	M00027	R00261,R00489,R01682,R02466	RC00299	ko00000,ko00001,ko00002,ko01000	E	P:glutamate metabolic process; F:glutamate decarboxylase activity; F:pyridoxal phosphate binding
Orthocluster_8187	R										
Orthocluster_8194	R										
Orthocluster_8195	R										
Orthocluster_8196	R										
Orthocluster_8227	R										
Orthocluster_8243	R										
Orthocluster_8244	R										
Orthocluster_8263	R										

Those orthology clusters that the functional prediction was done only for one gene within the orthocluster are marked with *.EC is Enzyme Commission number and KEGG and BRITE identifiers are based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The orthology clusters selection criteria are defined strict (S) when all the isolates of the target species have at least one representative gene and the relaxed (R) is marked when a defined number of missing genes are allowed.

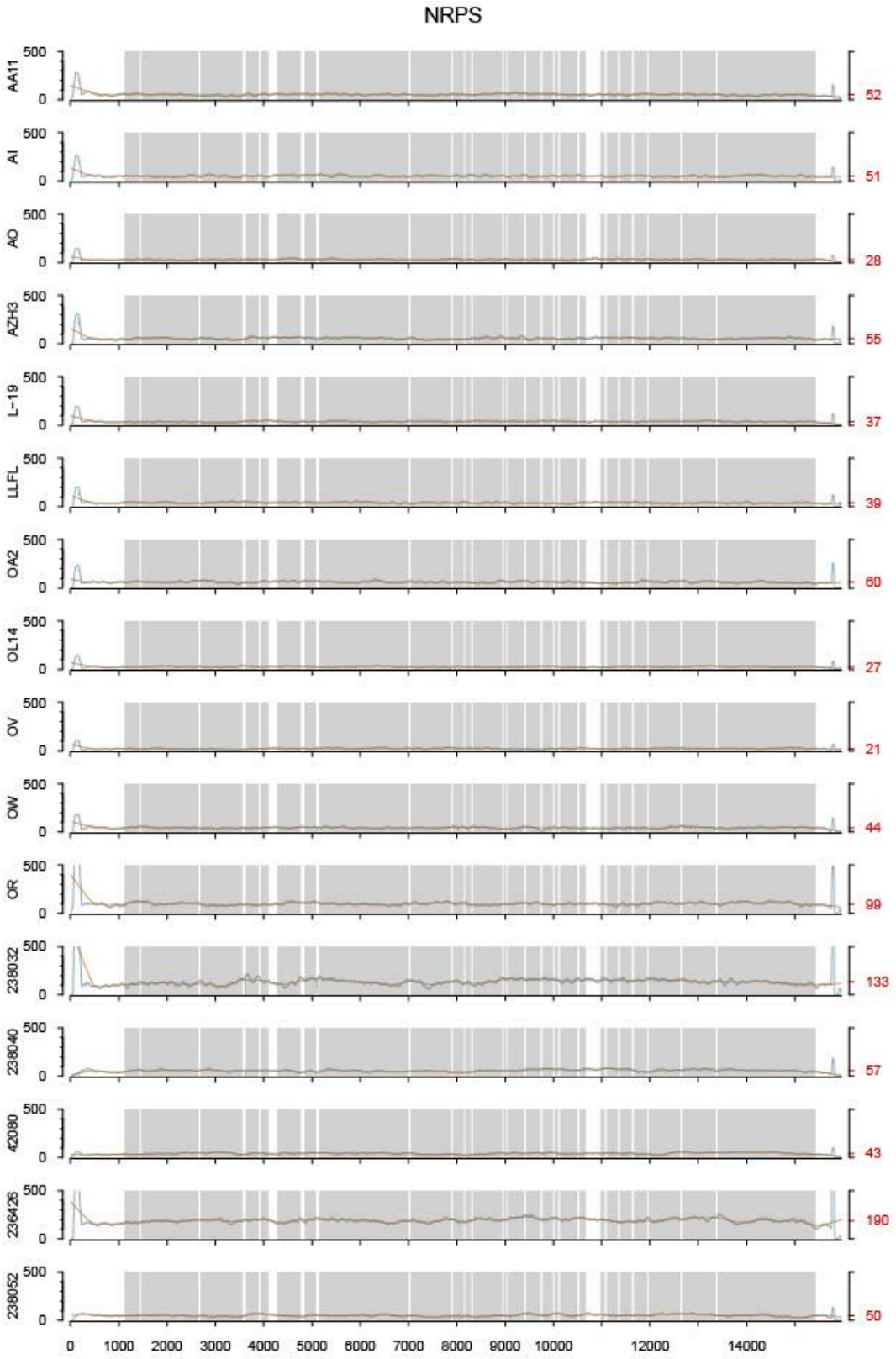
References cited in the additional files

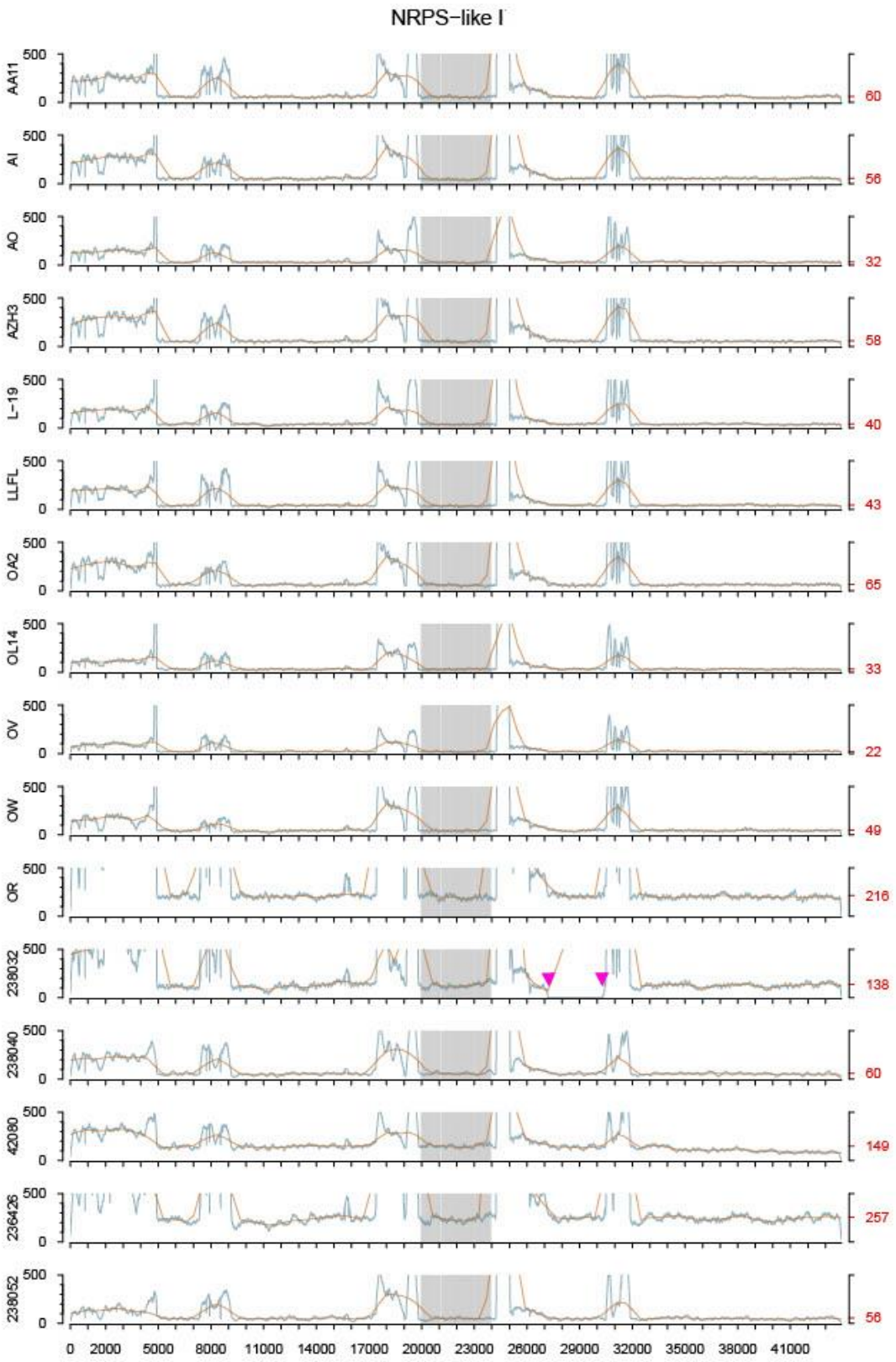
Benevenuto, J., Teixeira-Silva, N. S., Kuramae, E. E., Croll, D., & Monteiro-Vitorello, C. B. (2018). Comparative Genomics of Smut Pathogens: Insights From Orphans and Positively Selected Genes Into Host Specialization. *Front Microbiol*, *9*(660), 660. https://doi.org/10.3389/fmicb.2018.00660

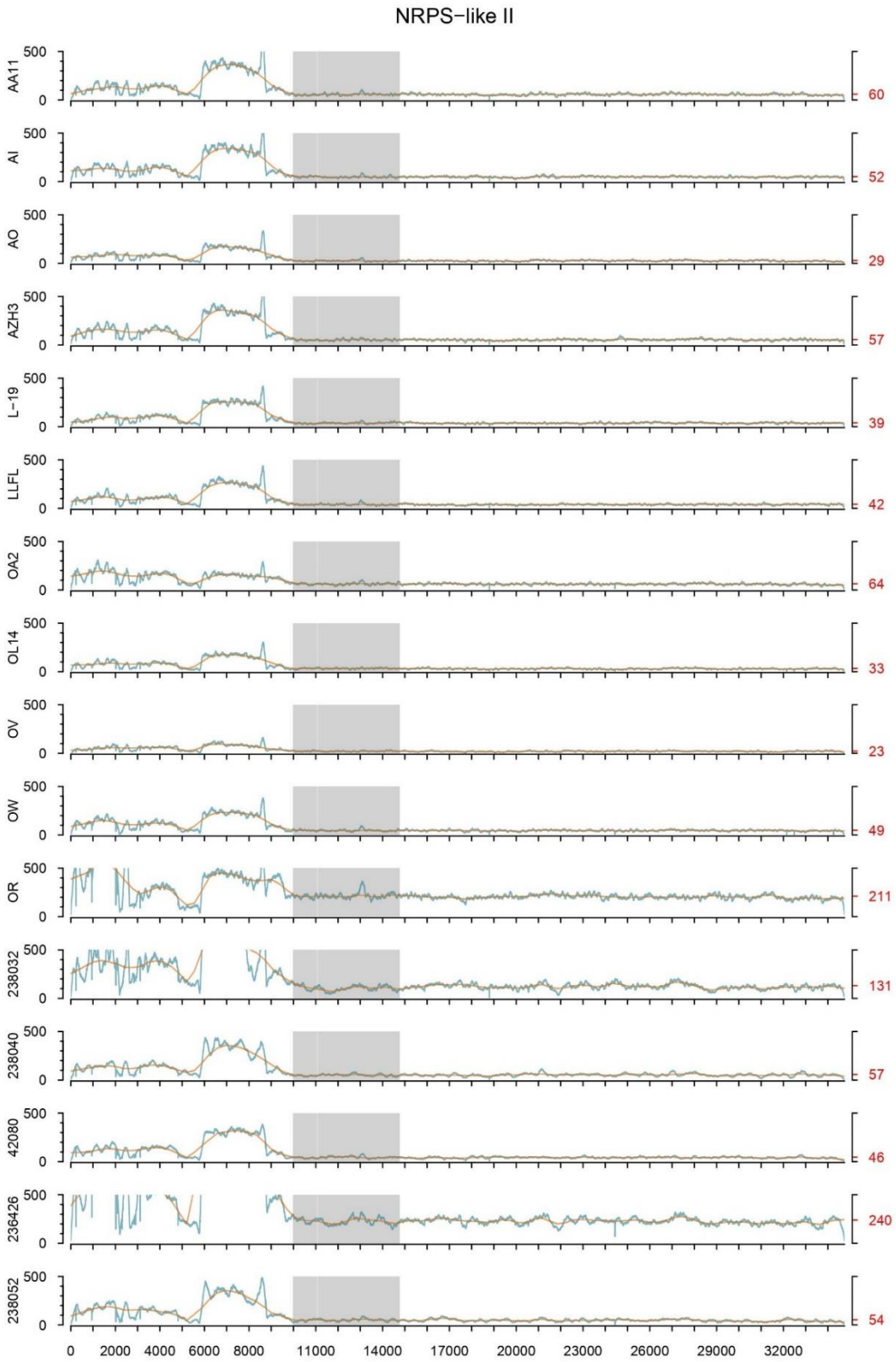
Kamper, J., Kahmann, R., Bolker, M., Ma, L. J., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Muller, O., Perlin, M. H., Wosten, H. A., de Vries, R., Ruiz-Herrera, J., Reynaga-Pena, C. G., Snetselaar, K., McCann, M., Perez-Martin, J., Feldbrugge, M., Basse, C. W., Steinberg, G., Ibeas, J. I., Holloman, W., Guzman, P., Farman, M., Stajich, J. E., Sentandreu, R., Gonzalez-Prieto, J. M., Kennell, J. C., Molina, L., Schirawski, J., Mendoza-Mendoza, A., Greilinger, D., Munch, K., Rossel, N., Scherer, M., Vranes, M., Ladendorf, O., Vincon, V., Fuchs, U., Sandrock, B., Meng, S., Ho, E. C., Cahill, M. J., Boyce, K. J., Klose, J., Klosterman, S. J., Deelstra, H. J., Ortiz-Castellanos, L., Li, W., Sanchez-Alonso, P., Schreier, P. H., Hauser-Hahn, I., Vaupel, M., Koopmann, E., Friedrich, G., Voss, H., Schluter, T., Margolis, J., Platt, D., Swimmer, C., Gnirke, A., Chen, F., Vysotskaia, V., Mannhaupt, G., Guldener, U., Munsterkotter, M., Haase, D., Oesterheld, M., Mewes, H. W., Mauceli, E. W., DeCaprio, D., Wade, C. M., Butler, J., Young, S., Jaffe, D. B., Calvo, S., Nusbaum, C., Galagan, J., & Birren, B. W. (2006). Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis* [10.1038/nature05248]. *Nature*, *444*(7115), 97-101. https://doi.org/10.1038/nature05248

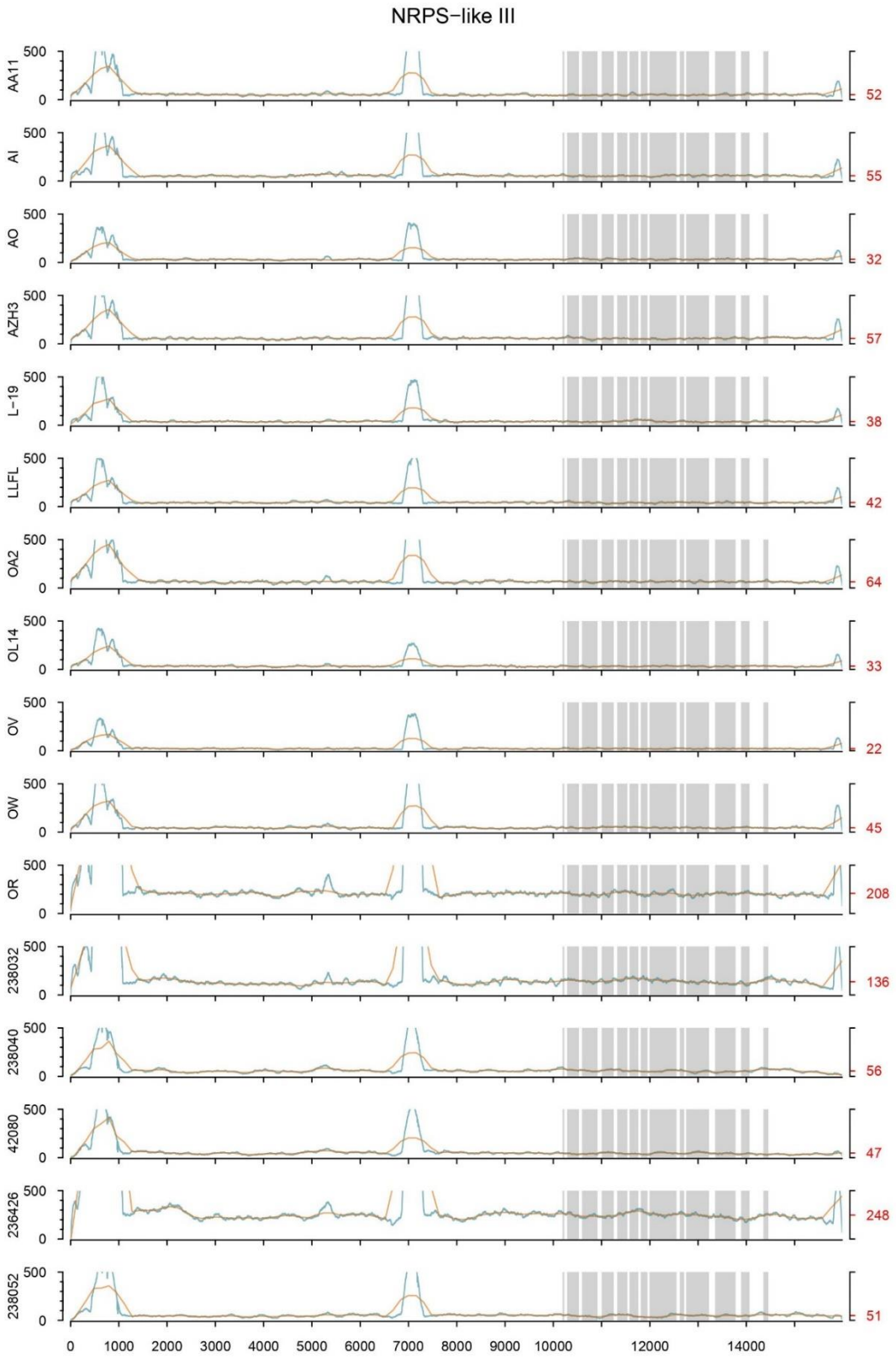
Kijpornyongpan, T., Mondo, S. J., Barry, K., Sandor, L., Lee, J., Lipzen, A., Pangilinan, J., LaButti, K., Hainaut, M., Henrissat, B., Grigoriev, I. V., Spatafora, J. W., & Aime, M. C. (2018). Broad Genomic Sampling Reveals a Smut Pathogenic Ancestry of the Fungal Clade Ustilaginomycotina. *Mol Biol Evol*, *35*(8), 1840-1854. https://doi.org/10.1093/molbev/msy072

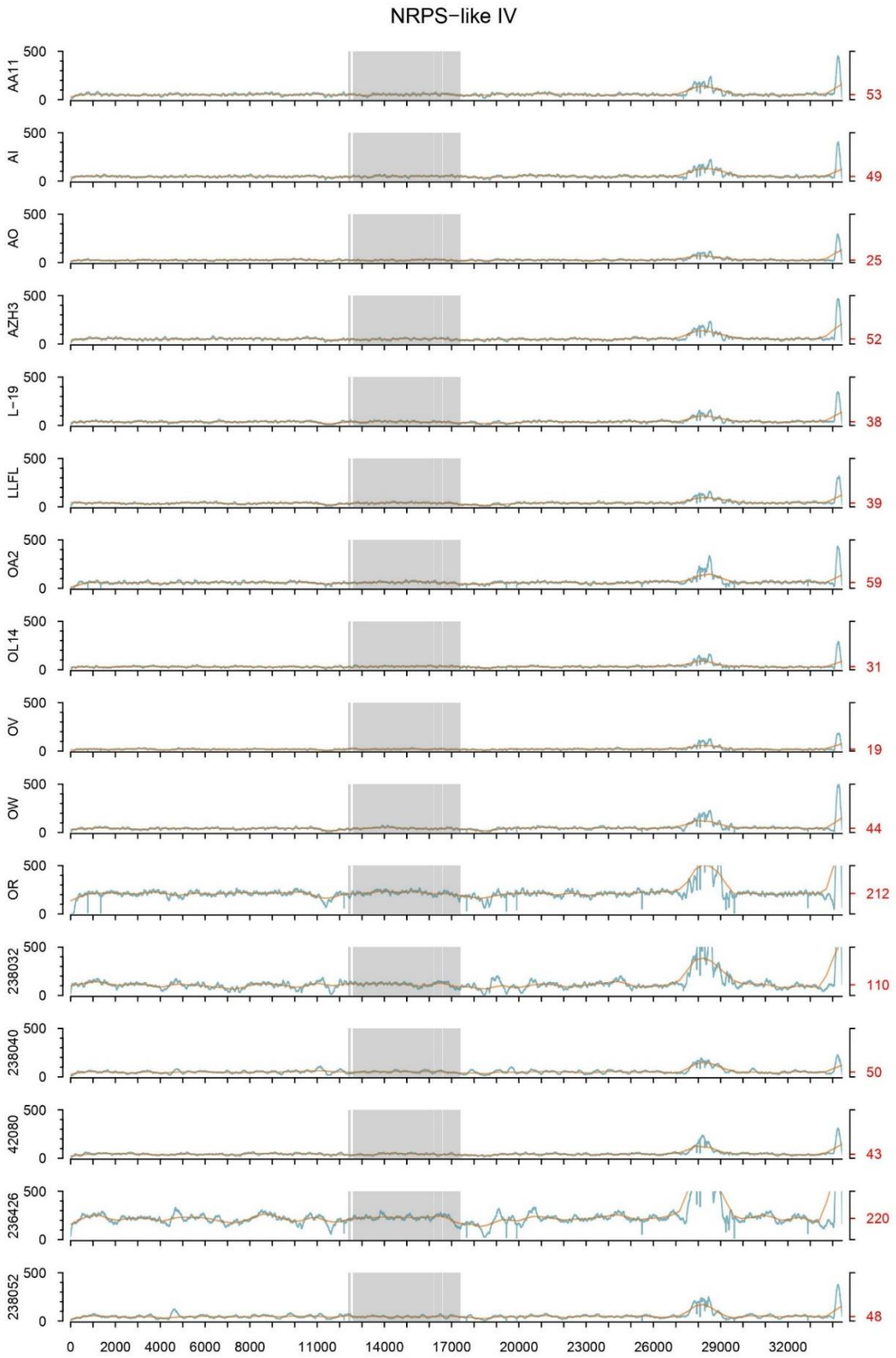
Nguyen, H. D. T., Sultana, T., Kesanakurti, P., & Hambleton, S. (2019). Genome sequencing and comparison of five *Tilletia* species to identify candidate genes for the detection of regulated species infecting wheat. *IMA Fungus*, *10*(1), 11. https://doi.org/10.1186/s43008-019-0011-

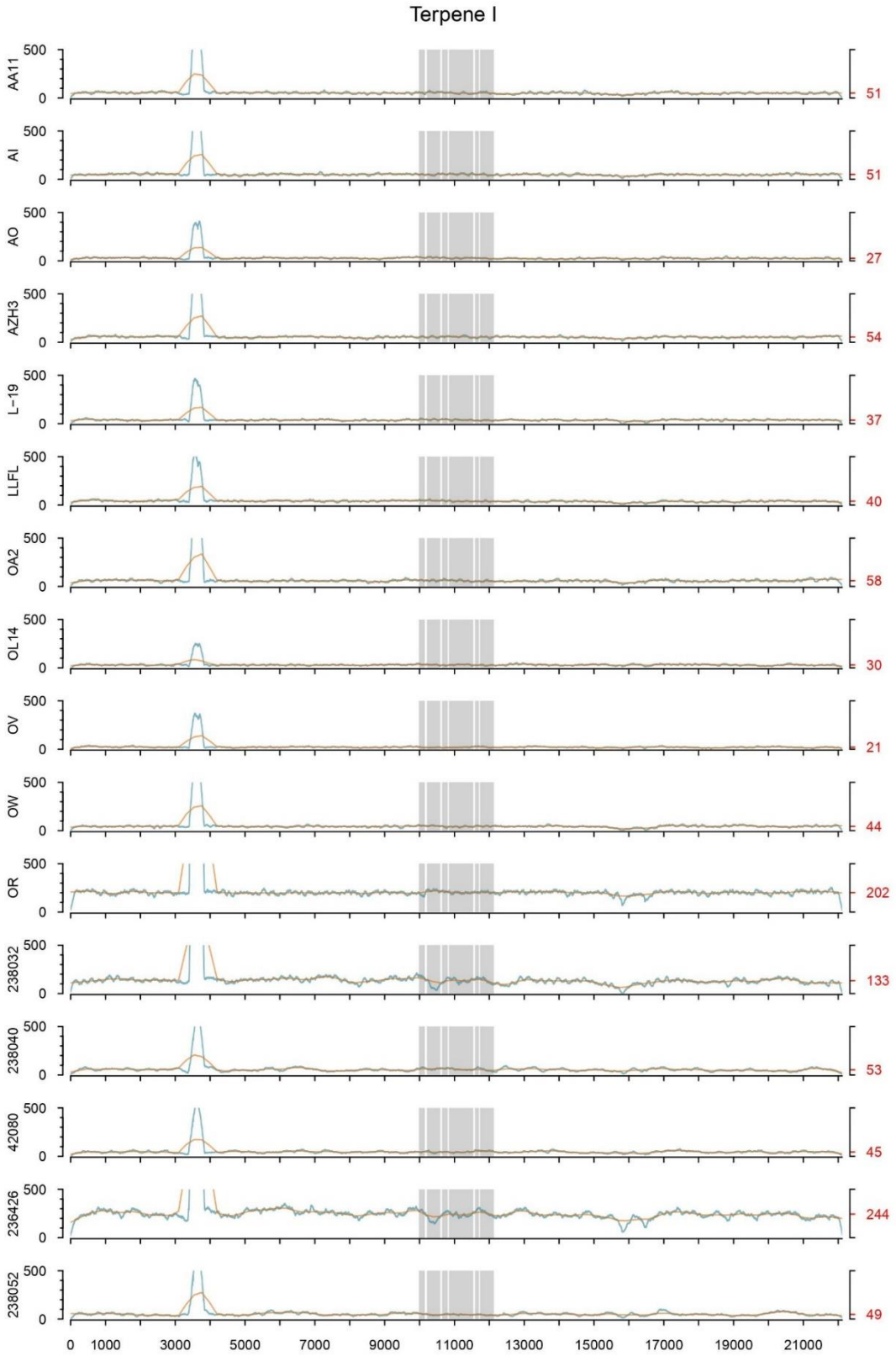

NRPS
AA11
AI
AO
AZH3
L-19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
52
51
28
55
37
39
60
27
21
44
99
133
57
43
190
50
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
12000
14000

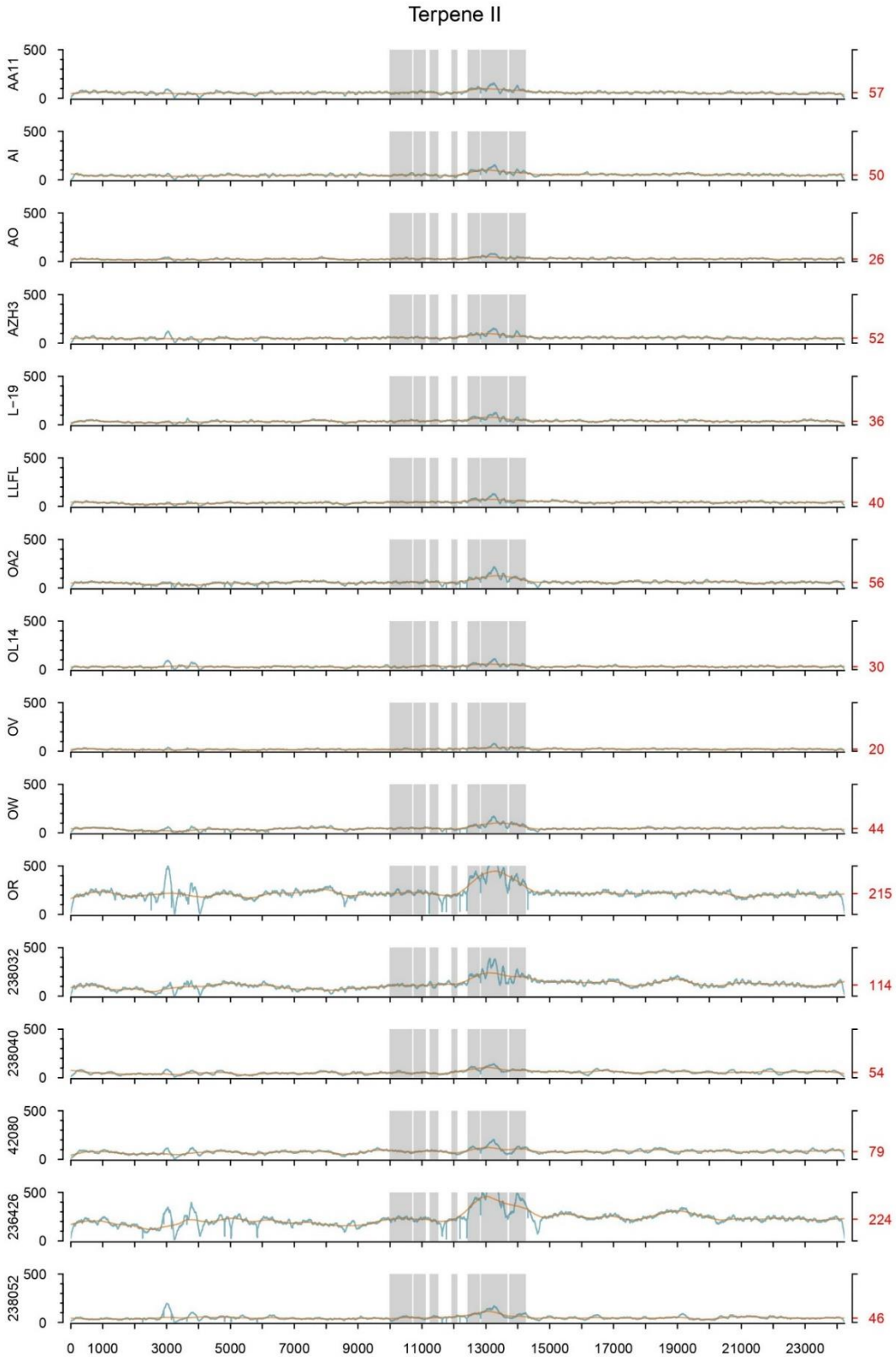

NRPS−like I
AA11
AI
AO
AZH3
L−19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
60
56
32
58
40
43
65
33
22
49
216
138
60
149
257
56
0 2000 5000 8000 11000 14000 17000 20000 23000 26000 29000 32000 35000 38000 41000

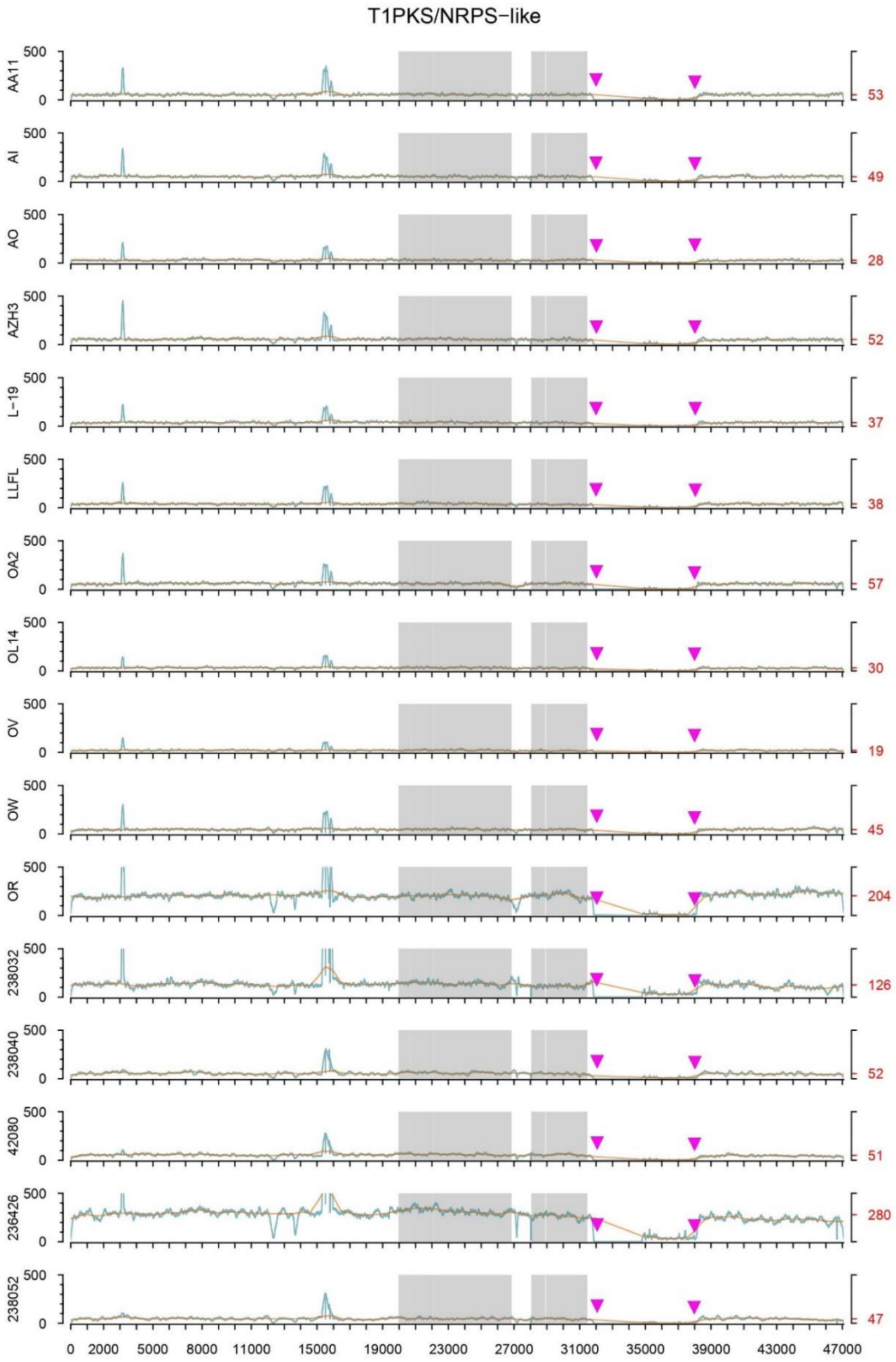

NRPS−like II
AA11
AI
AO
AZH3
L−19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
60
52
29
57
39
42
64
33
23
49
211
131
57
46
240
54
0
2000
4000
6000
8000
11000
14000
17000
20000
23000
26000
29000
32000


NRPS−like III
AA11
AI
AO
AZH3
L−19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
52
55
32
57
38
42
64
33
22
45
208
136
56
47
248
51
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
12000
14000


NRPS−like IV
AA11
AI
AO
AZH3
L−19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
53
49
25
52
38
39
59
31
19
44
212
110
50
43
220
48
0
2000
4000
6000
8000
11000
14000
17000
20000
23000
26000
29000
32000


Terpene I
AA11
AI
AO
AZH3
L−19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
51
51
27
54
37
40
58
30
21
44
202
133
53
45
244
49
0
1000
3000
5000
7000
9000
11000
13000
15000
17000
19000
21000


Terpene II
AA11
AI
AO
AZH3
L−19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
57
50
26
52
36
40
56
30
20
44
215
114
54
79
224
46
0 1000 3000 5000 7000 9000 11000 13000 15000 17000 19000 21000 23000


T1PKS/NRPS−like
AA11
AI
AO
AZH3
L−19
LLFL
OA2
OL14
OV
OW
OR
238032
238040
42080
236426
238052
500
0
53
49
28
52
37
38
57
30
19
45
204
126
52
51
280
47
0 2000 5000 8000 11000 15000 19000 23000 27000 31000 35000 39000 43000 47000

Additional Figure 2-1 Coverage of putative secondary metabolites and biosynthetic gene clusters. Trimmed Illumina reads of each isolate were aligned to the selected reference gene cluster sequence using default mappingparameters. The plot shows the read depth up to 500 (blue line) and the lowest regression (filter: 1/25; orange line). The median coverage is indicated on the right y-axis in red and the x-axes shows length of the predicted secondary metabolite gene cluster in bp. Regions which were annotated as core genes by antiSMASH are shadedin gray. The reference secondary metabolite gene clusters used for mappings are as follow: The predicted gene cluster of *T. controversa* strain OR for, Indole, NRPS-like I, NRPS-like II, T1PKS/NRPS-like, *T. caries* isolate AA11 for NRPS, Terpene I, Terpene II, *T. laevis* isolate ATCC 42080 for NRPS-like IV, and *T. controversa* isolate DAOMC 236426 for NRPS-like III. The region marked with arrow represent non-covered regions by reads. While the present and the order of nearly all gene clusters were also supported by Illumina reads, the observed drop in reads coverage in T1PKS/NRPS-like synthses was probably due to the present of long stretches of Ts and Ns nucleotides within this gene cluster in our selected reference. Part of the NRPS-like I genecluster in the *T. caries* isolate DOAMC 238032 could not be recovered by mapping.

Chapter 3 – Development of a loop-mediated isothermal amplification assay for the detection of *Tilletia controversa* based on genome comparison

Somayyeh Sedaghatjoo[1*], Monika K. Forster[2], Ludwig Niessen[3], Petr Karlovsky[4], Berta Killermann[2], Wolfgang Maier[1]

*Email of the corresponding author: s.sedaghatjoo@gmail.com

Published in Scientific Report (online first, 06 June 2021)

doi: https://doi.org/10.1038/s41598-021-91098-2

[1] Julius Kühn Institute (JKI) – Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

[2] Institute for Crop Science and Plant Breeding, Bavarian State Research Center for Agriculture, Vöttinger Straße 38, 85354 Freising, Germany

[3] Chair of Technical Microbiology, TUM School of Life Sciences, Technical University of Munich, Gregor-Mendel-Str. 4, 85454 Freising, Germany

[4] Molecular Phytopathology and Mycotoxin Research, University of Goettingen, Grisebachstrasse 6, 37077 Goettingen, Germany

3. 1. Abstract

Tilletia controversa causing dwarf bunt of wheat is a quarantine pathogen in several countries. Therefore, its specific detection is of great phytosanitary importance. Genomic regions routinely used for phylogenetic inferences lack suitable polymorphisms for the development of species-specific markers. We therefore compared 21 genomes of six *Tilletia* species to identify DNA regions that were unique and conserved in all *T. controversa* isolates and had no or limited homology to other *Tilletia* species. A loop-mediated isothermal amplification (LAMP) assay for *T. controversa* was developed based on one of these DNA regions. The specificity of the assay was verified using 223 fungal samples, comprising, 43 fungal species including 11 *Tilletia* species, in particular 39 specimens of *T. controversa*, 92 of *T. caries* and 40 of *T. laevis*, respectively. The assay specifically amplified genomic DNA of *T. controversa* from pure cultures and teliospores. Only *T. trabutii* generated false positive signals. The detection limit of the LAMP assay was 5 pg of genomic DNA per reaction. A test performance study that included five laboratories in Germany resulted in 100% sensitivity and 97.7% specificity of the assay. Genomic regions, specific to common bunt (*T. caries* and *T. laevis* together) are also provided.

Keywords

Bunts of wheat, Comparative genomics, Average nucleotide identity, Species-specific detection, Closely related phytopathogenic fungi

3. 2. Introduction

Wheat (*Triticum aestivum*) is the most widely cultivated crop worldwide with a production that reached 734 M tons in 2018 (FAOSTAT, 2018) and is still increasing. Several fungal pathogens reduce wheat yield by colonizing different organs of the plant; among them, causal agents of bunt diseases belong to the most important seed- and soil-borne pathogens (Carris, 2010; Matanguihan *et al.*, 2011), especially in organic farming. Disease symptoms appear at the heading stage and can be recognized by the formation of black, sooty masses of powdery spores, which replace mostly all grains of a kernel while the modified ovary coat is preserved. The infected grain breaks easily, causing the spread of millions of teliospores.

Common bunt of wheat is caused by *Tilletia caries* and *T. laevis*, dwarf bunt by *T. controversa*, and karnal bunt by *T. indica. Tilletia* belongs to the Exobasidiomycetes within the basidiomycetous smut fungi (Ustilaginomycotina). *Tilletia caries* [syn. *T. tritici*] and *T. laevis* [syn. *T. foetida*] are closely related species present throughout the wheat growing regions of the world (Goates, 1996; Vánky, 2012). Teliospores of common bunt germinate at 15 °C within one week even in the absence of light. *Tilletia controversa* causes dwarf bunt and is less widely distributed and reSed to certain regions of the Americas, Europe, and West Asia. For instance, the occurrence of the disease has not been reported from China and Australia. Dwarf bunt is distinguished from common bunt by requiring lower temperature (optimum at 5 °C) and light for the germination of teliospores (Lowther, 1948; Wade and Tyler, 1958). Germination of *T. controversa* typically takes 3 - 8 weeks. *Tilletia indica* [syn. *Neovossia indica*] requires temperatures between 15 - 25 °C for germination and takes 2 - 3 weeks (Singh, 1994). Karnal bunt is geographically reSed to a few countries Karnal bunt is geographically reSed to a few countries namely Afghanistan, India, Iran, Mexico, Nepal, Pakistan, South Africa, Syria, and USA and has not been reported from Europe (Fuentes-Davila, 1996) and has not been reported from Europe, where it is treated as an A1 quarantine pathogen by the European and Mediterranean Plant Protection Organization (EPPO) (2019, Sep).

Morphology of teliospores and sterile cells comprising their color and size, the size and height of muri, the number of meshes per teliospore diameter, and form of the sori (bunt balls), are traditionally used to distinguish the species of wheat bunt fungi (Vánky, 1994). Differentiation between *T. caries* and *T. controversa* requires extensive experience because of the variability of their teliospores morphology (Holton and Kendrick, 1956), however *T. laevis*, with its smooth teliospores, generally is easier to distinguish. (Pimentel *et al.*, 2000b; Carris *et al.*, 2007). Accurate distinction of dwarf bunt from common bunt and other *Tilletia* species, which are morphologically similar to dwarf

bunt, is of high importance. It is required for efficient disease management, as well as for regulatory reasons from a wheat trading perspective. Fifteen countries, including China and Brazil, implemented quarantine measures or reSions on the number of *T. controversa* teliospores per kernel in their wheat trade (Mathre, 1996; Whitaker *et al.*, 2001; Peterson *et al.*, 2009).

In recent years, several studies have attempted the detection of wheat bunt pathogens using different DNA-based methods. Some of these assays were not intended to differentiate between common and dwarf bunt (Mulholland and McEwan, 2000; Josefsen and Christiansen, 2002; Kochanova *et al.*, 2004; Zouhar *et al.*, 2010; Pieczul *et al.*, 2018). Assays designed to specifically detect *T. controversa* have been tested only against a limited number of samples (Liu *et al.*, 2009; Gao *et al.*, 2010; Gao *et al.*, 2011; Gao *et al.*, 2014; Liu *et al.*, 2020). Due to the lack of polymorphism in the genomic regions typically used for phylogenetic analyses (Mulholland and McEwan, 2000, Carris *et al.*, 2007; Bao *et al.*, 2010; Jayawardena *et al.*, 2019), alternative DNA regions had to be explored for the development of a species-specific assay. With the advent of new sequencing technologies, it has now become feasible to identify DNA regions for the development of a detection assay without prior knowledge regarding the function of the target sites (Lang *et al.*, 2010; Pieck *et al.*, 2017). Here, we employed a comparative genomics approach to detect DNA regions that are conserved in and unique to the *T. controversa* genome. These regions were then used to develop a loop-mediated isothermal amplification (LAMP) assay (Notomi *et al.*, 2000; Nagamine *et al.*, 2001; Tomita *et al.*, 2008) for the detection of *T. controversa* DNA in pure mycelia and teliospores (from bunt balls). The new assay was validated using a significant number of dwarf and common bunt specimens as well as other wheat pathogens and in an interlaboratory test performance study.

3. 3. Results

3. 3. 1. Genome comparison and primer design

Average nucleotide identity (ANI) analysis based on MUMmer(Kurtz *et al.*, 2004) alignment (ANIm) and a single linkage dendrogram were calculated among 20 genomes. For that we divided the genomes into two groups of closely related species, namely *T. caries, T. controversa,* and *T. laevis* together, and karnal bunt (*T. indica*) and ryegrass bunt (*T. walkeri*) in another group (Figure 3-1A and B; Additional Table 3-1). The alignment coverage is shown in Additional Table 3-2. In general, the higher the alignment coverage and the ANI values, the more identical are the genomes. The genomes of *T. caries*, *T. controversa*, and *T. laevis* (shown in dark red) shared >99% sequence identity with an

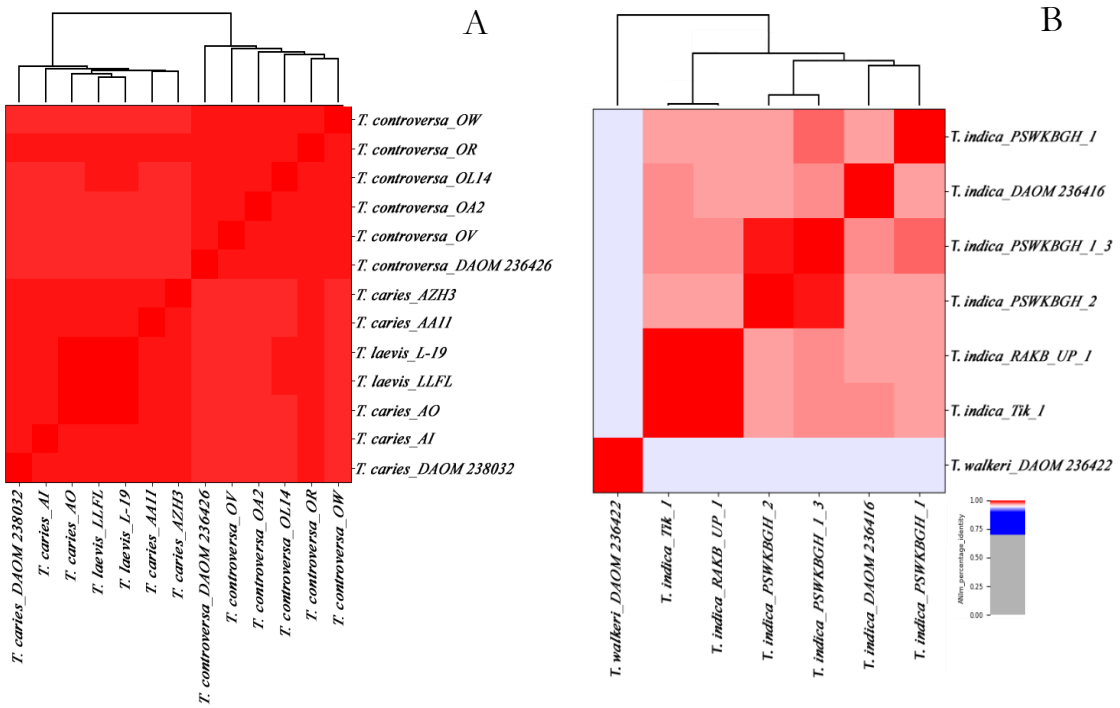


Figure 3-1 Heatmaps of ANIm percentage identity between genomes of *Tilletia* spp. Pairwise average nucleotide identity between two groups of *Tilletia* species (A: *T. caries, T. controversa*, and *T. laevis*, B: *T. indica* and *T. walkeri*) were determined by Pyani and used for the construction of a single linkage dendrogram. The isolates and species assignments are given as row and column labels. The value of the cophenetic correlation coefficient of the hierarchical clustering was 0.97 for (A) and 0.99 for (B).

average of 91% alignment coverage of the total genome length. In comparison, the identity within *T. indica* genomes was >97 % with an average of 79% alignment coverage.

The *T. indica* genomes shared on average 94% sequence identity to the single *T. walkeri* genome. In the first group, two clades corresponding to common bunt (*T. caries* and *T. laevis*) and dwarf bunt (*T. controversa*) were discriminated using single linkage cluster analysis (Figure 3-1A). *Tilletia caries* and *T. laevis* clustered together in a common clade, with little genetic distance from its neighboring clade comprising the genomes of *T. controversa***.** In the second group, *Tilletia indica* samples were separated from the single representative of *T. walkeri.*

The program rapid identification of PCR primers for unique core sequences (RUCS) (Thomsen *et al.*, 2017) was used to identify species-specific sequences in the genome of *T. controversa.* A total of 11,136 unique DNA segments (N50 = 61 bp) were obtained, of which 22 were longer than 1,500 bp. These sequences were used for the design of LAMP primers.

A total of 78 primer sets were designed and initially tested for their specificity against a preset comprising eight (three *T. caries*, three *T. controversa*, and two *T. laevis* isolates) selected cultured samples

(Additional Table 3-3) shows the list of cultured samples). The primer sets with no false detection and strong amplification as visualized on agarose gels were selected. The second round of testing was performed against the samples of common and dwarf bunt only. The primer sets were excluded when a false positive or false negative reaction occurred. The remaining primer sets were then tested against other *Tilletia* species and fungal pathogens. The primer set with the lowest false detection rate in this round was finally selected. We tested the selected primer set three times independently against the complete sample collection.

Table 3-1 provides the primer sequences and Figure 3-2 shows their location in the target sequence (*T. controversa* isolate OR, scaffold accession number CAJHJB010000001). The primer sequences did not show similarity to any relevant species when blasted against GenBank and this 210 bp intergenic region used for the development of the LAMP assay did not produce a BLAST hit when searched at the DNA level against GenBank's nucleotide collection.

Table 3-1 Primer sequences used in the LAMP assay

Primer name	**Nucleotide sequences 5′ - 3′**
O_8_2F3	GTGTATGAGCGTGAGTTCGA
O_8_2B3	CGACGCGTTTTGTGACATTC
O_8_2F2	CTCCCTTTKTCTTTGTGGCA
O_8_2B2	ATTTGAGCATCCTTGGAGCA
O_8_2FIP (F1c-F2)	GGCACACCAGGTAAGCAACGA_CTCCCTTTKTCTTTGTGGCA
O_8_2BIP (B1c-B2)	TTACCGCTGACGCTTGGA_ATTTGAGCATCCTTGGAGCA

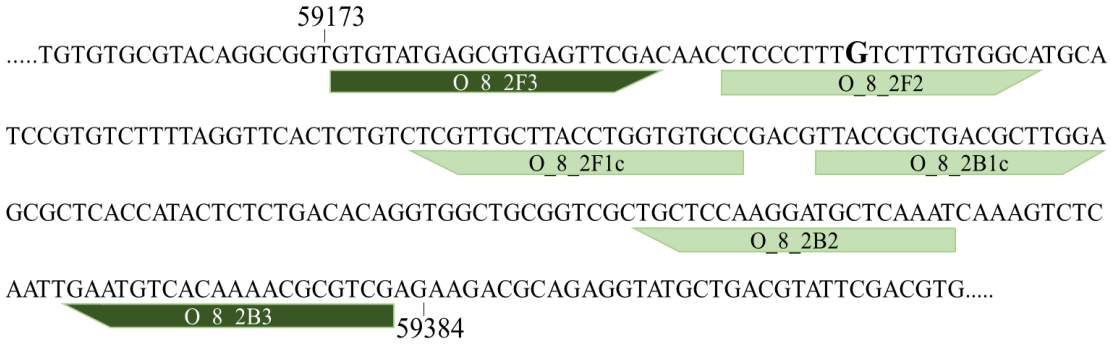


Figure 3-2 Position and orientation of the primer sequences in the scaffold (accession number CAJHJB010000001) that was used for the development of *T. controversa* LAMP assay. Binding sites for outer primers are shown in dark green, for inner primers in light green. Separation of the binding part is shown with the (_) in the primer sequences. The numbers show the position of nucleotides in the DNA segment. The nucleotide (G) shown in bold is changed to wobble position (K) in the primer sequence, after resequencing of the target region.

3. 3. 2. Sequence analysis of the species-specific DNA region used for the LAMP assay

PCR products of the predicted length (209 bp) were amplified from DNA of three samples of *T. controversa* from the culture collection (OA3, OR, and ORB isolates) using the LAMP primers O_8_2F3 and O_8_2B3 (Table 3-1). Sanger-sequencing of the obtained amplicons showed 100% sequence identity with the target DNA region derived from the genome analyses. Only one degenerate nucleotide (K) was introduced into one of the primers (Figure 3-2) because at this position a double signal (T/G) was observed in the sequencing chromatogram of an individual isolate. No PCR product was obtained when a subset of DNA obtained from *T. caries* or *T. laevis* samples were used (Figure 3-3).

3. 3. 3. The LAMP assay and DNA amplification

The LAMP assay detected DNA of *T. controversa* obtained from pure fungal cultures as well as from pure teliospores collected from bunt balls. Colorimetric detection of *T. controversa* was achieved by observing a color change of the reaction mixture from orange (no amplification) to pink (positive amplification). Figure 3-4 shows an example of the colorimetric visualization of the LAMP assay. The products of the reactions were also separated on a 2% agarose gel for confirmation. The typical ladder-like structure of different size amplicons produced in a positive LAMP reaction confirmed that the color change only happened when amplification occurred.

3. 3. 4. Verification of LAMP products

The amplification of the target DNA segment was confirmed with the DNA of six randomly selected *T. controversa* samples from the culture collection (OA2, OA3, OA6, OC1, OC2, and OMO isolates) as a template in the LAMP assay. Figure 3-5 shows the multiple alignments of 12 forward and reverse reads obtained from the shortest amplicon against the target DNA region (O_8_2F2 and O_8_2B2 primers). The sequences of the recovered amplicons (151 bp of 152 bp) were almost identical to the target region confirming that the target DNA segment was amplified during the LAMP assay

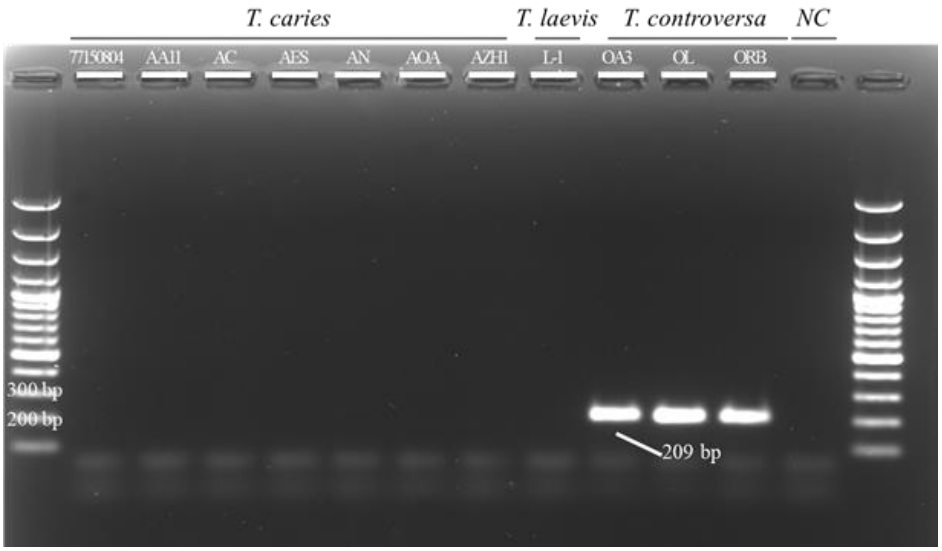


Figure 3-3 Polymerase chain reaction (PCR) using the outer LAMP primers to amplify the DNA region used for LAMP assay development. PCR products were separated on a 2% agarose gel and visualized using SYBR Safe gel staining. Ladder is a 100 bp Plus GeneRuler and NC is negative control. No amplicon was produced in the absence of *T. controversa* DNA.

.

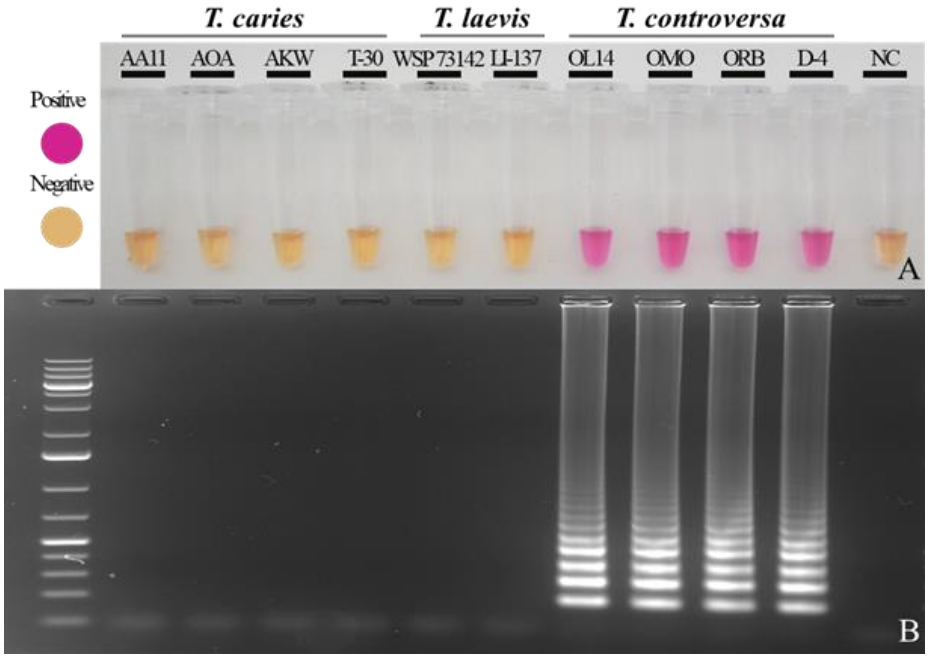


Figure 3-4 End-point detection of *T. controversa* using neutral red. The LAMP assay was performed at 65 °C for 45 min. A: Colorimetric detection under daylight conditions. Positive reactions appear in pink while negative reactions are light orange. NC is a water control. B: The same reactions separated on a 2% agarose gel and visualized using SYBR safe. A positive LAMP reaction is represented by a ladder-like fragments pattern. NC is the water control and Ladder is 1kb Plus DNA size marker. The assay detects only *T. controversa* gDNA.

3. 3. 5. Specificity and limit of detection of the LAMP assay

In total, we tested 223 fungal DNA samples of which 39 belonged to *T. controversa*. Pure cultures were produced for the development of the test. No false positive was observed with 92 *T. caries* and 40 *T. laevis* samples (Additional Table 3-3). Also, no cross-amplification of the assay was observed when testing 40 other fungal phytopathogens including other *Tilletia* species such as *T. cerebrina*, *T. holci*, *T. indica*, *T. lolioli*, *T. menieri,* and *T. olida*. However, *T. trabutii* and a taxonomically uncertainly identified *T. secalis* (GD 1707) were positive under the assay conditions. Wheat did not generate a positive signal when up to 5 ng DNA was used as template.

Figure 3-6 shows a series of LAMP assays with serial dilutions of pure *T. controversa* DNA. We estimated the LOD as the lowest DNA concentration at which all four repetitions displayed positive results. The assay gave positive results with all replicates at concentrations above 5 pg of DNA per reaction. Three out of four repetitions were amplified when 1 pg of the DNA was tested.

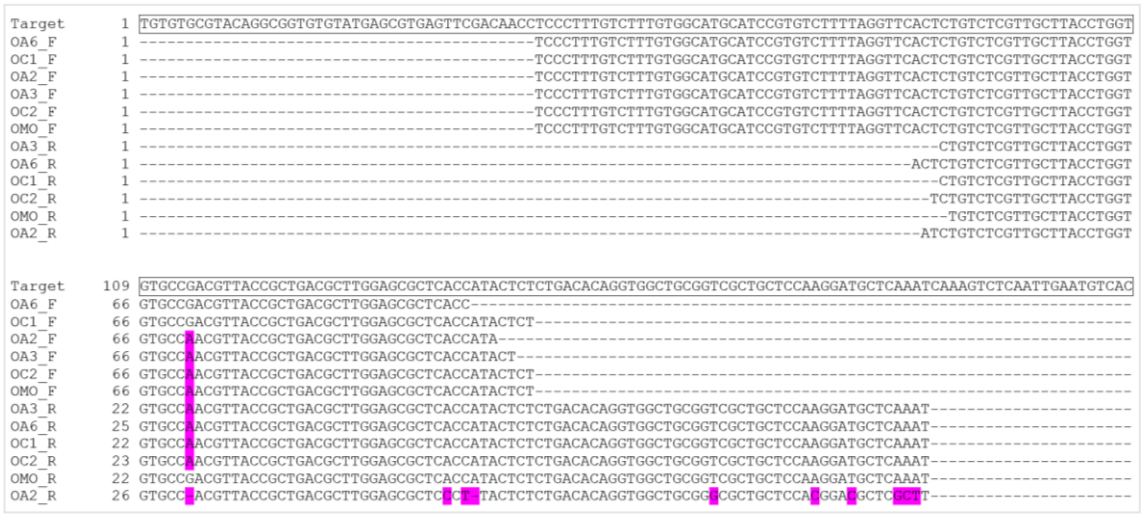


Figure 3-5 Sequence comparison of the shortest LAMP-product to the target region. The forward and reverse reads obtained from six randomly selected *T. controversa* samples aligned to the target region. The alignment is illustrated using BOXSHADE and non-matching nucleotides to the target region are highlighted in pink.

3. 3. 6. Reproducibility of the LAMP assay in an interlaboratory test performance

Testing five sets of LAMP test packages prior to sending them to different laboratories showed that all sets detected all *T. controversa* DNA above LOD after one week of storage of the reagents at -20 °C except for betaine, which was stored at +4 °C. From a total of 80 reactions (five participants testing 15 DNA samples and one negative control each), one false positive (FP), 35 true positives (TP), and 44 true negatives (TN) were reported. The performance parameters are presented in Table 3-2 and the photos provided by the participants are given in the Additional Figure 3-1. The positive predictive value (PPV) indicates how many of the test positives are true positives and negative predictive value (NPV) shows how many of the test negatives are true negatives. Both the sensitivity (the fraction of true positive samples that score positive) and the NPV of the assay were 100%. Specificity (the fraction of true negative samples that score negative) and the PPV were 97.7% and 96.5%, respectively.

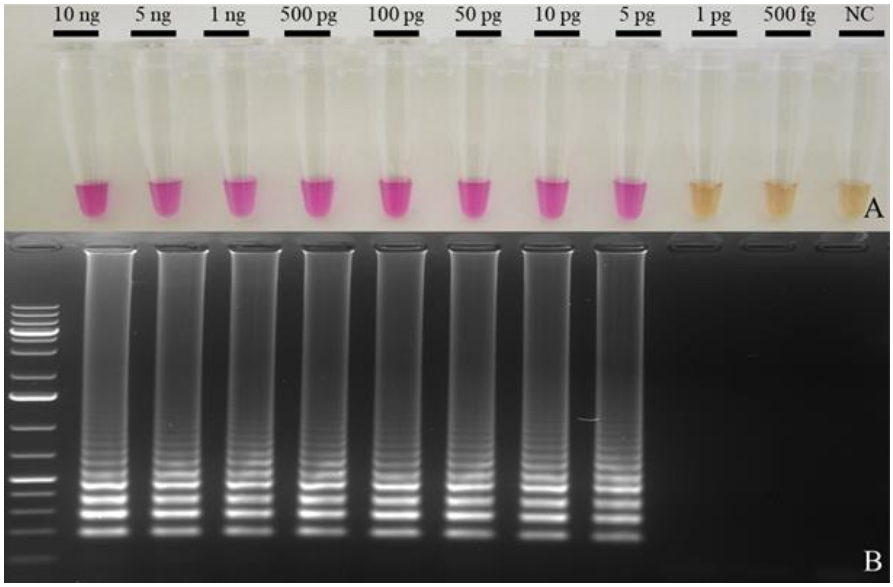


Figure 3-6 Determination of the LOD of the LAMP assay for *T. controversa*. A: The LAMP assay was carried out with serial dilutions of DNA of *T. controversa* isolate OL and the result was photographed under daylight condition. B: The products were separated on a 2% agarose gel and visualized under UV 360_{nm}. NC: water control; Ladder: size marker 1kb plus.

Table 3-2 Performance parameters of the LAMP assay performance

Evaluated parameter	**Value (%)**
True Positive Fraction (Sensitivity)	100
True Negative Fraction (Specificity)	97.7
Positive Predicted Value (PPV)	96.5
Negative Predicted Value (NPV)	100

3. 4. Discussion

So far, the lack of DNA polymorphisms between the very closely related causal agents of common and dwarf bunt has hampered the development of species-specific DNA-based diagnostic assays for *T. controversa*, as was also shown by other studies (Levy *et al.*, 2001; Carris *et al.*, 2007; Bao, 2010; Bao *et al.*, 2010; Stewart *et al.*, 2013; Jayawardena *et al.*, 2019). However, the recent availability of the whole-genome sequences of *Tilletia* species enabled us to develop a specific LAMP assay.

We calculated >99% average nucleotide identity (ANI) with a minimum alignment coverage of 88% between common and dwarf bunt if *T. controversa* isolate (OR) is excluded due to its significantly larger assembled genome size. This was in line with ANI values reported by Nguyen *et*

al. (2019), who used independent assemblies of DOAM isolates collection, and our previous study where we analyzed 16 common and dwarf bunt genomes while excluding repetitive regions (Sedaghatjoo et al. under review). The high ANI values indicate a remarkably high genetic similarity of common and dwarf bunt fungi. In comparison, the two closely related species of *T. indica* and *T. walkeri (Pimentel et al., 1998; Castlebury and Carris, 1999; Carris et al., 2006)* had ANI values of >94%. Despite the high similarity between the genomes of common and dwarf bunt fungi, nucleotide polymorphism in the genomes were sufficient to unambiguously separate six *T. controversa* samples from all *T. caries* and *T. laevis*. However, *T. caries* and *T. laevis* could not be differentiated. Interestingly, the ANI values within the sequenced isolates of *T. indica* were lower (>97%) compared to the ANI values between the three species of *T. caries*, *T. laevis,* and *T. controversa* together (>99%) indicating a very low genetic diversity between common and dwarf bunt and a relatively high genetic diversity within *T. indica*. Gurjar *et al.* (2020) also reported high genetic diversity within *T. indica* isolates analyzing single nucleotide polymorphisms (SNP)s and small deletions and insertions (indel)s. Because the percentage of aligned genomic regions between distantly related species was very low, the comparison of all 21 genomes in our ANI analysis was not possible.

The genomic regions of *T. controversa* shared with other *Tilletia* species, but not present in all the *T. controversa* isolates (n = 6) were excluded to be used for the LAMP assay development using RUCS (Thomsen *et al.*, 2017). RUCS employs *k*-mer comparisons to exclude regions shared between target and background genomes. Nguyen *et al.* (2019) used ten *Tilletia* genomes (one isolate of *T. caries*, two isolates of *T. controversa*, two isolates of *T. laevis*, three isolates of *T. indica*, and two isolates of *T. walkeri*) for the PCR primer design for species-specific detection of *T. controversa*. They limited their sequence comparison to a small number of single-copy protein-coding genes specific and unique to *T. controversa*. The use of RUCS in our study allowed us to include all publicly available sequences and genomes that were not structurally annotated (i.e., genes and their intron-exon locations were not predicted). This approach provided also a higher number of candidate genome regions because it also included intergenic regions. Since the comparison of *k*-mers by RUCS is independent of annotation, it also excludes errors due to annotation ambiguity.

In an attempt to specifically detect *T. caries*, we also searched for conserved and unique regions in their genomes by RUCS (Additional Table 3-4). In *T. caries*, 235 unique and specific regions were found (N50 = 39 bp), the longest spanning 116 bp. In *T. laevis,* we found 228 candidate regions (N50 = 39 bp), the longest of which spanned 215 bp. A minimum length of 200 bp is needed for LAMP because of the optimum distances between primer binding sites. Therefore, differentiation between *T. caries* and *T. laevis* by LAMP appears difficult. Species-specific real-time PCR assays based

on the regions identified by RUCS should however be theoretically possible. The number of unique and conserved regions dramatically increased when *T. caries* together with *T. laevis* were treated as a single target. This finding is well in line with the observation that *T. caries* and *T. laevis* could not be differentiated based on the ANI comparisons and clustered together in the single linkage dendrogram. RUCS identified 11,888 regions (N50 = 52 bp) with the longest contig length of 6,790 bp, suggesting that developing a common bunt-specific LAMP assay will be feasible.

The updated assembly of four out of 21 genomes (DAOM collection updated to DAOMC) used in this study as well as six additional *Tilletia* genomes has been published by Nguyen *et al.* (2019) at the time this manuscript was in preparation. We reconfirmed the specificity of the target region identified in this work by comparison of these genomes (see Additional Table 3-5 for the list of accession). The target region used in this study was present in both *T. controversa* genomes and absent from all eight genomes of the other *Tilletia* species. We also repeated the RUCS analysis on all 27 genomes (Additional Table 3-4). The number of extracted species-specific regions dropped sharply, which led to no remaining candidate region for *T. caries*. These results corroborated the pattern observed with 21 genomes. The positions of eleven top-ranked common bunt-specific DNA regions are provided in Additional Table 3-6.

In this study, the LAMP assay for the detection of *T. controversa* was optimized for 45 min at 65 °C using betaine and four salt-free primers with a colorimetric end-point detection. Adding betaine was essential for successful amplification even though the target region was not GC-rich (52.2% GC). This finding is in conflict with a previous report that betaine had no effect on the amplification in non-GC-rich target regions (Wang *et al.*, 2019). We suggest that the formation of secondary structures rather than mere GC content may account for the effect of betaine. Increasing the concentration of betaine above 0.5 M did not further improve amplification (data not shown). Here, the LAMP assay was optimized for primers that were not purified by HPLC. Tomita *et al.* (2008) suggested that HPLC-purification of primers were crucial for a successful LAMP. We compared both HPLC-purified and salt-free primers and found no difference. Although the region in general is long enough to design loop-primers (Nagamine *et al.*, 2002), we did not succeed in integrating them into the assay without compromising the test specificity. Therefore, and because they are generally not essential for the proper functioning of LAMP reactions, we did not include them in the assay. Recently swarm primers have been introduced, which can be added to a LAMP assay in order to improve its general performance (Martineau *et al.*, 2017). We manually designed and tested a set of swarm primers for the new LAMP assay (data not shown). However, also the addition of these primers neither

improved the sensitivity nor the specificity of the assay. Therefore, the final assay comprised only the four basic LAMP primers.

Colorimetric end-point detection of DNA amplification not only shortens the assay time but also reduces the risk of contamination by carryover of LAMP products. Apart from the pH-sensitive indicators neutral red and phenol red (Tanner *et al.*, 2015), two additional dyes have been widely used for the visualization of LAMP products: hydroxynaphthol blue, added before the reaction (Goto *et al.*, 2009) and SYBR Green (Tomita *et al.*, 2008), added after the completion of the reaction. Using SYBR Green increases the risk of cross-contamination due to the necessity to open the reaction vessels after the LAMP reaction. The color change in hydroxynaphthol blue, which is a metal-sensitive indicator, is occasionally difficult to distinguish (Tanner *et al.*, 2015). We therefore used neutral red as dye to ensure easy differentiation due to the high contrast between the pink color of a positive reaction and the light orange of a negative reaction.

In this study, the detection limit of the LAMP assay was estimated to be 5 pg total DNA (on average 142 genomes copy when (LOD*6.022x10^{23})/(genome length*1x10^{9}*650)) isolated from pure fungal cultures. This is similar to the sensitivity of the LAMP assay for *T. indica* with the LOD of 10 pg (265 genome copies), reported by Gao et al and (Gao *et al.*, 2016); Tan *et al.* (2016). It is however less sensitive than the reported LOD of 1 pg (22 genome copies on average) for the LAMP assay not differentiating among *T. caries*, *T. controversa,* and *T. laevis* published by Pieczul *et al.* (2018). The rough estimation of detection limits based on genome copy numbers should be taken cautiously since the exact genome sizes of the three species are unknown. The copy number of the target region and loop-primers (Nagamine *et al.*, 2002) may account for these differences. Higher sensitivity in the detection of *T. controversa* by LAMP could presumably be achieved by targeting a multi-copy region. Further investigation is needed to correlate the LAMP detection limit with the number of teliospores per kernel, which is of special interest for seed testing laboratories and farmers, because most of the practiced regulation is based on the number of teliospores per kernel (International Seed Testing Association (ISTA), 1984). But irrespective of this, the clear-cut results we obtained by applying LAMP to bunt samples suggest that it might play an important role in the future by differentiating *T. controversa* from *T. caries*, which can be a daunting task given their subtle morphological differences. These differences that can also show some overlaps are especially hard to distinguish if the teliospores are microscoped on filter paper as is done in the official seed testing method.

Broad geographical sampling is crucial for the validation of a species-specific assay, especially when information about the population diversity of the target organism is limited. Additionally, the extensive similarity between the genomes of common and dwarf bunt found here and in other studies

(Russel, 1993; Russell and Mills, 1993; Russell, 1994; Nguyen *et al.*, 2019) as well as the probable role of hybridization and recombination between these species (Flor, 1932; Holton, 1951; Holton, 1954) in nature makes testing of a geographically broad set of samples essential. One hundred sixty-eight samples of common bunt and dwarf bunt from a variety of geographical locations (Asia, Europe, and North America) were used in this study to evaluate the specificity of the developed LAMP assay using the broadest geographic sampling we could obtain. We made an effort to test both old herbarium samples (collected from 1920 onward) and more recently collected samples to test the independence of the LAMP results from sample age.

A *Tilletia* sample (GD 1707) from *Secale cereale* collected in Germany and identified as *T. secalis* tested positively in the LAMP assay developed in this study. *Tilletia controversa* and *T. secalis* can infect both wheat and rye (Dewey and Hoffmann, 1975; Carris, 2008; Vánky, 2012) and their differentiation based only on teliospores morphology is not possible (Niemann, 1954; Durán and Fischer, 1956; Durán and Fischer, 1961). Thus, the taxonomic identity of this sample remained ambiguous. Additionally, the assay could not differentiate between *T. controversa* and *T. trabutii*. *Tilletia trabutii* was reported from barley grasses (*Hordeum* spp.) and clustered as the sister group of *T. secalis* in a multilocus phylogenetic analysis (Carris *et al., 2007)*. It will be interesting to compare the phylogenetic relationship of *T. secalis* and *T. trabutii* to *T. controversa* on the whole-genome level. Furthermore, *T. controversa,* unlike the majority of smut fungi that are reSed to a single or few closely related host species (Begerow *et al.*, 2004), has been reported to infect not only wheat but also other members of the Poaceae family (Hardison *et al.*, 1959). We examined *T. controversa* collected from *Elymus repens* and *T. controversa* (Fischer, 1952; Conners, 1954; Vánky, 1994) (syn. *T. brevifaciens* (Carris *et al.,* 2007)) collected from *Thinopyrum intermedium* subsp. *intermedium* (syn *Elymus hispidus*) using our LAMP assay. All were positive. Additionally, *T. bromi* is morphologically similar to *T. controversa* and has similar teliospores germination requirements (Pimentel *et al.*, 2000b). These similarities make the distinction of those two species difficult. Although *T. bromi* and *T. controversa* are phylogenetically distinct, they are reproductively compatible under artificial condition (Pimentel *et al.*, 2000a; Pimentel *et al.*, 2000b). We did not have access to any *T. bromi* sample; therefore, the specificity of the LAMP assay toward this species could not be estimated.

The reproducibility of the LAMP assay was also examined in an interlaboratory test performance study including five laboratories, which used different equipments for the amplification. The assay LOD could be successfully reproduced in all the laboratories. We speculate that the most likely reason for one single false-positive result reported was cross-contamination. These results show

that the LAMP assay is robust. The assay has potential for several applications in seed testing laboratories, wheat export and import control as well as field applications.

3. 5. Methods

3. 5. 1. Sample collection and single teliospore cultures

Samples examined in this study are listed in Additional Table 3-3. Host names are listed according to Kew Royal Botanic Gardens online database (https://wcsp.science.kew.org/).

To produce single teliospore cultures, 54 viable samples were randomly selected (marked with * in Additional Table 3-3). Teliospores were surface-sterilized as described by Castlebury *et al.* (2005). Briefly, bunt balls were crushed using a pair of sterile fine-point forceps and wheat tissue was carefully removed. The teliospores were immersed in 0.26% v/v NaClO for 30 s, pelleted by centrifugation in a benchtop microcentrifuge for 10 s and rinsed twice with sterile, distilled water. Surface-sterilized teliospores were streaked on 1.5% water-agar and incubated either at 5 °C under constant light (*T. controversa* teliospores) or at 15 °C in darkness (*T. caries* and *T. laevis*). A single germinated teliospore of each sample was then transferred to M-19 agar medium (Trione, 1964) using a sterile needle and incubated at 15 °C in the dark. Medium was supplemented with penicillin G (240 mg/L) and streptomycin sulfate (200 mg/L). The developing mycelium was scraped from the medium using a flat blunt spatula, freeze-dried at -40 °C for 48 h and kept at +4 °C until use. Cultures of other fungal species were grown on PDA medium and stored at +4 °C.

3. 5. 2. Extraction of DNA from fungal mycelia and spores

Total DNA (gDNA) including mitochondrial DNA and mycoviruses was isolated from both mycelia and spores (cultured isolates) or only from spores (uncultured samples). For extraction of gDNA from fungal cultures, 10 - 30 mg of lyophilized mycelium were homogenized by shaking with four sterile tungsten carbide beads of 4 mm diameter in 2 ml reaction tubes at 22 Hz for 50 s using a tissue lyser. The bead beating step was repeated once. The tubes were shaken vigorously between the disruption steps to loosen mycelium from the bottom of the tubes.

For gDNA extraction directly from spores, 10 - 25 mg spores were surface-sterilized as described above and rinsing water was carefully removed. Four 1 mm and four 4 mm sterile tungsten carbide beads were added to each reaction tube. The tubes were then frozen in liquid nitrogen and the spores were disrupted in the tissue lyser at 22 Hz for 50 s. The procedure including cooling the

samples in liquid nitrogen was repeated twice. After tissue disruption, DNA was extracted using the Qiagen DNeasy Plant Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

3. 5. 3. Genome comparisons and identification of DNA segments specific to *T. controversa*

Twenty one genome sequences used in this study are listed in Table 3-3. Average nucleotide identity (ANI), the alignment coverage between genomes, and hierarchical clustering were calculated and reconstructed using Pyani (v 0.2.10) (Pritchard *et al.*, 2016) which employs MUMmer (ANIm mode) to align genomes, with default parameters (-m ANIm -g). The cophenetic correlation coefficient of the hierarchical clustering was calculated in RStudio (Version 1.1.463).

Conserved and unique DNA regions of *T. controversa* were extracted using RUCS (rapid identification of PCR primers for unique core sequences) v. 1.0 (Thomsen *et al.*, 2017) (https://cge.cbs.dtu.dk/services/RUCS/) with default parameters. The six target genomes (*T. controversa*) were defined and grouped as positive while the remaining 15 *Tilletia* genomes (*T. caries*, *T. horrida*, *T. indica*, *T. laevis*, and *T. walkeri*) were defined and grouped as negative data set or exclusion criteria. Extracted contigs found in the unique-core-sequences-contigs output file of RUCS that were longer than 1500 bp were selected as targets for LAMP development. To check these contigs for similarities with nontarget genomes, a custom BLAST database of all available *Tilletia* genomes (n = 15) excluding *T. controversa* genomes was constructed.

3. 5. 4. Primer design for the LAMP assay

A set of two inner and two outer primers were designed for the unique DNA contigs to *T. controversa* by PrimerExplorer V5 (http://primerexplorer.jp.) with default parameters. Since PrimerExplorer does not accept sequences longer than 2000 bp, we split sequences exceeding this limit. The designed primers were subjected to MegaBLAST against the non-redundant database 'nr/nt' of the National Centre for Biotechnology Information (NCBI) to examine their similarity with other relevant species.

Table 3-3 Genome sequences used in this study and their accession numbers

Species	Isolate (voucher number)	Assembly accession numbers	Genome size (Mb)	Reference
T. caries	AA11 (CBS 144825)	GCA_905072865.1	31.51	This study
T. caries	AI (CBS 145171)	GCA_905068135.1	31.84	This study
T. caries	AO (CBS 145172)	GCA_905071735.1	30.46	This study
T. caries	AZH3 (CBS 145166)	GCA_905071745.1	31.38	This study
T. caries	DAOM 238032	GCA_001645005.1	29.54	NA
T. controversa	DAOM 236426	GCA_001645045.1	28.84	NA
T. controversa	OA2 (CBS 145169)	GCA_905071725.1	32.05	This study
T. controversa	OL14 (CBS 145167)	GCA_905071785.1	30.83	This study
T. controversa	OR (CBS 144827)	GCA_905071765.1	49.87	This study
T. controversa	OV (CBS 145170)	GCA_905071775.1	29.54	This study
T. controversa	OW (CBS 145168)	GCA_905071705.1	31.24	This study
T. horrida	QB-1	GCA_001006505.1	20.10	Wang *et al.* (2015)
T. indica	DAOM 236416	GCA_001645015.1	30.38	NA
T. indica	PSWKBGD_1_3	GCA_001689965.1	43.73	NA
T. indica	PSWKBGH_1	GCA_001689995.1	37.46	Sharma *et al.* (2016)
T. indica	PSWKBGH_2	GCA_001689945.1	37.21	Sharma *et al.* (2016)
T. indica	RAKB_UP_1	GCA_002220835.1	33.77	Gurjar *et al.* (2019)
T. indica	Tik_1	GCA_002997305.1	31.83	Kumar *et al.* (2017); (Kumar *et al.*, 2018)
T. laevis	L-19 (CBS 145173)	GCA_905071715.1	31.00	This study
T. laevis	LLFL (CBS 144826)	GCA_905071755.1	30.98	This study
T. walkeri	DAOM 236422	GCA_001645055.1	24.34	NA

3. 5. 5. Sequence analysis of DNA segment used for the LAMP assay

To confirm the nucleotide sequence of the target region obtained by RUCS, we used the outer primers (F3 and B3) of LAMP as forward and reverse primers, respectively, in a conventional PCR and sequenced the obtained PCR product for a subset of samples. The PCR was conducted in 50 µL reaction mixtures containing 5 µL of 10x DreamTaq (Thermo Scientific, Vilnius, Lithuania), 0.2 mM of each of the four deoxynucleotide triphosphates (dNTPs, Thermo Scientific), 0.2 µM concentration of each forward and reverse primers, 1.25 U of *Taq* DNA polymerase (DreamTaq DNA polymerase,

Thermo Scientific, Vilnius, Lithuania), and 1 µL of DNA template (5 ng/µL). Initial denaturation was conducted at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 60 s. The final extension was performed at 72 °C for 10 min in a thermal cycler. Following PCR, 5 µl per reaction combined with 2 µL of 6x loading buffer (Thermo Scientific, Vilnius, Lithuania) were loaded onto a 2% agarose gel (w/v). The electrophoresis was run at 8 V cm^{-1} for 45 min in 1×TAE buffer (Maniatis *et al.*, 1975). PCR fragments were stained using SYBR® Safe DNA gel stain. The gel was visualized under UV 360_{nm} using a digital imaging system. PCR products were purified using the DNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions. Purified PCR products were Sanger-sequenced (Eurofins Genomics GmbH, Ebersberg, Germany) from both ends using PCR primers.

3. 5. 6. LAMP assay and verification of the LAMP products

The LAMP master mix (25 µL) contained 2.5 µL 10x amplification buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, pH 8.7), 2 µM of each inner primer and 0.2 µM for each outer primer (all salt-free), 8 mM $MgSO_4$, 1.4 mM concentration of each of the four deoxynucleoside triphosphates (dNTPs, Thermo Fisher), 0.5 M betaine (Sigma-Aldrich, Darmstadt, Germany), 8 U *Bst* DNA Polymerase 2.0 (New England Biolabs, Frankfurt, Germany), and 100 µM neutral red (Sigma-Aldrich, Darmstadt, Germany) prepared according to Niessen *et al.* (2018). One µL of DNA template was added per reaction. The reaction mixture was incubated at 65 °C for 45 min in a thermal cycler. The reaction was terminated by heating to 80 °C for 5 min. The tubes were photographed with a digital camera under daylight conditions. Gel electrophoresis of the LAMP products was performed as described above but the separation lasted 120 min. Either a 100 bp plus or 1kb Plus GeneRuler (Thermo Scientific, Vilnius, Lithuania) were used as DNA size markers in all electrophoretic gels.
To confirm that the amplification corresponded to the target DNA region, the shortest amplicon of six positive LAMP reactions was excised from a 2% agarose gel (w/v) (described previously) and recovered using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, USA) according to the manufacturer's instructions. The recovered amplicons were Sanger-sequenced using primers F2 and B2. Consensus sequences of all forward and reverse reads produced and trimmed using Sequencher™ 5.4.6 (Gene Codes Corporation, Ann Arbor, Michigan, USA) were pairwise aligned to the sequence of the target DNA region.

3. 5. 7. Determination of the specificity and limit of detection of the LAMP assay

The specificity of the LAMP assay was determined by applying the assay to DNA extracted from *Tilletia* cultures and teliospores, and from a range of phylogenetically distant fungal pathogens (Additional Table S3) under the described LAMP conditions. The following groups of pathogens were selected as negative controls: i) closely related species with a "sister group" phylogenetic relationship to the target pathogen (92 *T. caries* and 40 *T. laevis* samples); ii) further pathogens related to the target species (e.g. eight other *Tilletia* spp. and species of Ustilaginomycetes); iii) common wheat pathogens (e.g. *Fusarium* spp.); iv) fungi that are abundant in the environment due to their strong sporulation and airborne mode of distribution (e.g. *Penicillium* spp.). In addition, wheat DNA extracted from seedlings grown under sterile conditions was tested.

Limit of detection (LOD) was defined as the lowest amount of analyte detectable in a single reaction (Nutz *et al.*, 2011). DNA obtained from a pure culture of *T. controversa* isolate OL was quantified using Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Darmstadt, Germany) and used to prepare a dilution series at the concentrations of 10000, 5000, 1000, 500, 100, 50, 10, 5, 1, and 0.5 pg per assay.

3. 5. 8. Reproducibility of the LAMP assay in a test performance study

To evaluate the reproducibility and specificity of the LAMP assay, we conducted a test performance study with five German participants including plant protection agencies and seed testing laboratories. Total DNA of four *T. caries*, five *T. controversa*, and three *T. laevis* sample was extracted from teliospores as described above. The concentration of DNA stocks was determined using a Qubit® 3.0 Fluorometer and adjusted to 500 pg/µL. One of the *T. controversa* sample was additionally prepared in a serial dilution of 50, 5, and 0.5 pg/µL. All 15 DNA samples were coded, and aliquots were dispatched to the participating laboratories. Several batches of the LAMP master mix were prepared independently and assigned randomly to the participating laboratories. Homogeneity and stability testing were performed under described conditions with five randomly selected batches. The assays were performed on different days shortly before the chemicals and samples were distributed by an express delivery service while kept at -20 °C (except for betaine). The participants were asked to provide a photo of the reaction tubes and assign the samples as positive or negative according to the color of the reaction mixture after performing the assay. The results were evaluated according to Hajian-Tilaki (Hajian-Tilaki, 2013) using performance parameters shown in Table 3-4.

Table 3-4 Evaluation of the LAMP assay

Performance parameters	**Calculation**
True Positive Fraction (Sensitivity)	True Positive (TP) / (TP+False Negative (FN))
True Negative Fraction (Specificity)	True Negative (TN) / (TN+False Positive (FP))
Positive Predicted Value (PPV)	TP / (TP+FP)
Negative Predicted Value (NPV)	TN / (TN+FN)

3. 6. Declaration

Competing interests

The author(s) declare no competing interests.

Data availability

The datasets analyzed during the current study are publicly available as mentioned in the text.

3. 7. Acknowledgments

The Federal Ministry of Food and Agriculture of the Federal Republic of Germany funded this research project based on a decision of the German parliament (grant numbers 2812NA128 and 2812NA017). We sincerely thank colleagues who freely shared samples for our study. We especially thank Prof. Günter Deml for access to his personal herbarium samples deposited at Julius Kühn Institute (JKI). Andreas J. Geissler is greatly acknowledged for his initial genome analysis. The Participants of our reproducibility test and the technical assistance of Anke Brißke-Rode are gratefully acknowledged.

3. 8. References

Bao, X. (2010). Host specificity and phylogenetic relationships among *Tilletia species* infecting wheat and other cool season grasses, PhD thesis, Department of Plant Pathology, Washington State University.

Bao, X., Carris, L. M., Huang, G., Luo, J., Liu, Y., & Castlebury, L. A. (2010). *Tilletia puccinelliae*, a new species of reticulate-spored bunt fungus infecting *Puccinellia distans*. *Mycologia*, *102*(3), 613-623. https://doi.org/10.3852/09-135

Begerow, D., Göker, M., Lutz, M., & Stoll, M. (2004). *On the evolution of smut fungi and their hosts* (M. P. Reinhard Agerer , Paul Blanz, Ed.). IHW-Verlag & Verlagsbuchhandlung.

Carris, L. (2008). Is rye bunt, *Tilletia secalis*, present in North America? *North American Fungi*, *3*, 147-159. https://doi.org/10.2509/naf2008.003.0078

Carris, L. M. (2010). Smuts. In W. W. Bockus, R. L. Bowden, R. M. Hunger, W. L. Morrill, & T. D. Murray (Eds.), *Compendium of wheat diseases and pests* (60-65). American Phytopathological Society.

Carris, L. M., Castlebury, L. A., & Goates, B. J. (2006). Nonsystemic bunt fungi *Tilletia indica* and *T. horrida:* a review of history, systematics, and biology. *Annu Rev Phytopathol*, *44*, 113-133. https://doi.org/10.1146/annurev.phyto.44.070505.143402

Carris, L. M., Castlebury, L. A., Huang, G., Alderman, S. C., Luo, J., & Bao, X. (2007). *Tilletia vankyi*, a new species of reticulate-spored bunt fungus with non-conjugating basidiospores infecting species of *Festuca* and *Lolium*. *Mycological research*, *111*(Pt 12), 1386-1398. https://doi.org/10.1016/j.mycres.2007.09.008

Castlebury, L. A., & Carris, L. M. (1999). *Tilletia walkeri*, a New Species on *Lolium multiflorum* and *L. perenne*. *Mycologia*, *91*(1), 121-131. https://doi.org/10.2307/3761200

Castlebury, L. A., Carris, L. M., & Vinky, K. (2005). Phylogenetic analysis of *Tilletia* and allied genera in order Tilletiales (Ustilaginomycetes; Exobasidiomycetidae) based on large subunit nuclear rDNA sequences. *Mycologia*, *97*(4), 888-900. https://doi.org/10.3852/mycologia.97.4.888

Conners, I. L. (1954). The Organism Causing Dwarf Bunt of Wheat. *Canadian Journal of Botany*, *32*(3), 426-431. https://doi.org/10.1139/b54-038

Dewey, W. G., & Hoffmann, J. A. (1975). Susceptibility of Barly to *Tilletia controversa*. *Phytopathology*, *65*, 4.

Durán, R., & Fischer, G. W. (1956). Further studies on the synonymy and host range of the dwarf bunt fungus. *Research Studies of State Collge Washington*, *XXIV*(4), 8.

Durán, R., & Fischer, G. W. (1961). The genus *Tilletia*. Pullman, WA. Washington State University.

EPPO A1 List of pests recommended for regulation as quarantine pests. Retrieved 05. Oct, 2019 from https://www.eppo.int/ACTIVITIES/plant_quarantine/A1_list

FAOSTAT, S. D. (2018). Food and Agriculture Organization of the UN. Retrieved Nov 13th from http://faostat.fao.org

Fischer, G. W. (1952). *Tilletia brevifaciens* sp. nov., causing dwarf bunt of wheat and certain grasses. *Research Studies. Washington State University*, *20*(1), 11-14.

Flor, H. H. (1932). Heterothallism and hybridization in *Tilletia tritici* and *T. levis*. *Journal Of Agricultural Research*, *44*, 9.

Fuentes-Davila, G. (1996). Karnal bunt of wheat In R. D. Wilcoxson & E. E. Saari (Eds.), *Bunt and Smut Diseases of Wheat; Concepts and Methods of Disease managment*. CIMMYT.

Gao, L., Chen, W., & Liu, T. (2011). An ISSR-based Approach for the Molecular Detection and Diagnosis of Dwarf Bunt of Wheat, Caused by *Tilletia controversa* Kühn. *J. Phytopathol.*, *159*(3), 155-158. https://doi.org/10.1111/j.1439-0434.2010.01735.x

Gao, L., Chen, W. Q., & Liu, T. G. (2010). Development of a SCAR marker by inter-simple sequence repeat for diagnosis of dwarf bunt of wheat and detection of *Tilletia controversa* Kühn. *Folia Microbiol (Praha)*, *55*(3), 258-264. https://doi.org/10.1007/s12223-010-0038-1

Gao, L., Yu, H., Han, W., Gao, F., Liu, T., Liu, B., Kang, X., Gao, J., & Chen, W. (2014). Development of a SCAR marker for molecular detection and diagnosis of *Tilletia controversa* Kuhn, the causal fungus of wheat dwarf bunt. *World J Microbiol Biotechnol*, *30*(12), 3185-3195. https://doi.org/10.1007/s11274-014-1746-5

Gao, Y., Tan, M. K., & Zhu, Y. G. (2016). Rapid and specific detection of *Tilletia indica* using loop-mediated isothermal DNA amplification. *Australasian Plant Pathology*, *45*(4), 361-367. https://doi.org/10.1007/s13313-016-0422-7

Goates, B. J. (1996). Common Bunt and Dwarf Bunt. In R. D. Wilcoxson & E. E. Saari (Eds.), *Bunt and smut diseases of wheat; concepts and methods of disease management*. CIMMYT.

Goto, M., Honda, E., Ogura, A., Nomoto, A., & Hanaki, K. (2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques*, *46*(3), 167-172. https://doi.org/10.2144/000113072

Gurjar, M. S., Aggarwal, R., Jain, P., Aggarwal, S., Gupta, S., & Saharan, M. S. (2020). Comparative genome analysis of *Tilletia indica* inciting Karnal bunt of wheat reveals high genomic variation. *Indian Phytopathology*. https://doi.org/10.1007/s42360-020-00260-9

Gurjar, M. S., Aggarwal, R., Jogawat, A., Kulshreshtha, D., Sharma, S., Solanke, A. U., Dubey, H., & Jain, R. K. (2019). De novo genome sequencing and secretome analysis of *Tilletia indica* inciting Karnal bunt of wheat provides pathogenesis-related genes. *3 Biotech*, *9*(6), 219. https://doi.org/10.1007/s13205-019-1743-3

Hajian-Tilaki, K. (2013). Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation. *Caspian J Intern Med*, *4*(2), 627-635. https://www.ncbi.nlm.nih.gov/pubmed/24009950

Hardison, J. R., Meiners, J. P., Hoffmann, J. A., & Waldher, J. T. (1959). Susceptibility of Gramineae to *Tilletia contraversa*. *Mycologia*, *51*(5), 656-664. https://doi.org/10.2307/3755895

Holton, C. S. (1951). Methods and results of studies on heterothallism and hybridization in *Tilletia caries*. *Phytopathology*, *41*, 11.

Holton, C. S. (1954). Natural Hybridization between Common and Dwarf Bunt as Related to the Problem of Delimitation of Species of *Tilletia* Occurring on Wheat. *Phytopathology*, *44*(9), 493-493.

Holton, C. S., & Kendrick, E. L. (1956). Problems in the delimitation of species of *Tilletia* occurring on wheat. *Research Studies of the State College of Washington*, *24*, 318 - 325.

International Seed Testing Association (ISTA). (1984). *Triticum aestivum, Tilletia contraversa Kühn, Tilletia caries (DC) Tul., Tilletia foetida (Wallr.) Liro* (Working Sheet No. 53 ed.). International Seed Testing Association (Hrsg.). http://www.seedtest.org

Jayawardena, R. S., Hyde, K. D., Jeewon, R., Ghobad-Nejhad, M., Wanasinghe, D. N., Liu, N., Phillips, A. J. L., Oliveira-Filho, J. R. C., da Silva, G. A., Gibertoni, T. B., Abeywikrama, P., Carris, L. M., Chethana, K. W. T., Dissanayake, A. J., Hongsanan, S., Jayasiri, S. C., McTaggart, A. R., Perera, R. H., Phutthacharoen, K., Savchenko, K. G., Shivas, R. G., Thongklang, N., Dong, W., Wei, D., Wijayawardena, N. N., & Kang, J.-C. (2019). One stop shop II: taxonomic update with molecular phylogeny for important phytopathogenic genera: 26–50 (2019). *Fungal Diversity*, *94*(1), 41-129. https://doi.org/10.1007/s13225-019-00418-5

Josefsen, L., & Christiansen, S. K. (2002). PCR as a tool for the early detection and diagnosis of common bunt in wheat, caused by *Tilletia tritici*. *Mycological research*, *106*(11), 1287-1292. https://doi.org/10.1017/s0953756202006603

Kochanová, M., Zouhar, M., Prokinová, E., & Ryšánek, P. (2011). Detection of *Tilletia controversa* and *Tilletia caries* in wheat by PCR method. *Plant, Soil and Environment*, *50*(No. 2), 75-77. https://doi.org/10.17221/3684-pse

Kumar, A., Mishra, P., Maurya, R., Mishra, A. K., Gupta, V. K., Ramteke, P. W., & Marla, S. S. (2018). Improved Draft Genome Sequence of a Monoteliosporic Culture of the Karnal Bunt (*Tilletia indica*) Pathogen of Wheat. *Genome Announc*, *6*(20), e00015-00018. https://doi.org/10.1128/genomeA.00015-18

Kumar, A., Pandey, V., Singh, M., Pandey, D., Saharan, M. S., & Marla, S. S. (2017). Draft genome sequence of Karnal bunt pathogen (*Tilletia indica*) of wheat provides insights into the pathogenic mechanisms of quarantined fungus. *PLoS One*, *12*(2), e0171323. https://doi.org/10.1371/journal.pone.0171323

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., & Salzberg, S. L. (2004). Versatile and open software for comparing large genomes. *Genome Biol*, *5*(2), R12. https://doi.org/10.1186/gb-2004-5-2-r12

Lang, J. M., Hamilton, J. P., Diaz, M. G. Q., Van Sluys, M. A., Burgos, M. R. G., Vera Cruz, C. M., Buell, C. R., Tisserat, N. A., & Leach, J. E. (2010). Genomics-Based Diagnostic Marker Development for *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. *Plant Dis*, *94*(3), 311-319. https://doi.org/10.1094/PDIS-94-3-0311

Levy, L., Castlebury, L. A., Carris, L. M., Meyer, R. J., & Pimentel, G. (2001). Internal Transcribed Spacer Sequence-Based Phylogeny and Polymerase Chain Reaction-ReSion Fragment Length Polymorphism Differentiation of *Tilletia walkeri* and *T. indica*. *Phytopathology*, *91*(10), 935-940. https://doi.org/10.1094/PHYTO.2001.91.10.935

Liu, J., Li, C., Muhae-Ud-Din, G., Liu, T., Chen, W., Zhang, J., & Gao, L. (2020). Development of the Droplet Digital PCR to Detect the Teliospores of *Tilletia controversa* Kuhn in the Soil With Greatly Enhanced Sensitivity [Original Research]. *Front Microbiol*, *11*(4), 4. https://doi.org/10.3389/fmicb.2020.00004

Liu, J. H., Gao, L., Liu, T. G., & Chen, W. Q. (2009). Development of a sequence-characterized amplified region marker for diagnosis of dwarf bunt of wheat and detection of *Tilletia controversa* Kuhn. *Letters in applied microbiology*, *49*(2), 235-240. https://doi.org/10.1111/j.1472-765X.2009.02645.x

Lowther, C. V. (1948). Low temperature as a factor in the germination of dwarf bunt chlamydospores. *Phytopath*, *38*((4)), 309-310.

Maniatis, T., Jeffrey, A., & van deSande, H. (1975). Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochemistry*, *14*(17), 3787-3794. https://doi.org/10.1021/bi00688a010

Martineau, R. L., Murray, S. A., Ci, S., Gao, W., Chao, S. H., & Meldrum, D. R. (2017). Improved Performance of Loop-Mediated Isothermal Amplification Assays via Swarm Priming. *Anal Chem*, *89*(1), 625-632. https://doi.org/10.1021/acs.analchem.6b02578

Matanguihan, J. B., Murphy, K. M., & Jones, S. S. (2011). Control of Common Bunt in Organic Wheat. *Plant Dis*, *95*(2), 92-103. https://doi.org/10.1094/PDIS-09-10-0620

Mathre, D. E. (1996). DWARF BUNT: politics, identification, and biology. *Annu Rev Phytopathol*, *34*, 67-85. https://doi.org/10.1146/annurev.phyto.34.1.67

Mulholland, V., & McEwan, M. (2000). PCR-based diagnostics of *Microdochium nivale* and *Tilletia tritici* infecting winter wheat seeds. *EPPO Bulletin*, *30*(3-4), 543-547. https://doi.org/10.1111/j.1365-2338.2000.tb00944.x

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, *16*(3), 223-229. https://doi.org/10.1006/mcpr.2002.0415

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T., & Notomi, T. (2001). Loop-mediated isothermal amplification reaction using a nondenatured template. *Clinical Chemistry*, *47*(9), 1742-1743.

Nguyen, H. D. T., Sultana, T., Kesanakurti, P., & Hambleton, S. (2019). Genome sequencing and comparison of five *Tilletia* species to identify candidate genes for the detection of regulated species infecting wheat. *IMA Fungus*, *10*(1), 11. https://doi.org/10.1186/s43008-019-0011-9

Niemann, E. (1954). Zwergsteinbrand bei Roggen und Gerste nach kunstlicher Infektion mit verschiedenen *Tilletia*-Arten (Dwarf bunt of rye and barley with respect to artificial infection with various *Tilletia* species). *Zeitschrift fur Pflanzenbau und Pflanzenschutz 5*, 168-172.

Niessen, L., Bechtner, J., Fodil, S., Taniwaki, M. H., & Vogel, R. F. (2018). LAMP-based group specific detection of aflatoxin producers within *Aspergillus* section *Flavi* in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection. *Int J Food Microbiol*, *266*, 241-250. https://doi.org/10.1016/j.ijfoodmicro.2017.12.013

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids research*, *28*(12), E63. https://doi.org/10.1093/nar/28.12.e63

Nutz, S., Doll, K., & Karlovsky, P. (2011). Determination of the LOQ in real-time PCR by receiver operating characteristic curve analysis: application to qPCR assays for *Fusarium verticillioides* and *F. proliferatum*. *Anal Bioanal Chem*, *401*(2), 717-726. https://doi.org/10.1007/s00216-011-5089-x

Peterson, G. L., Whitaker, T. B., Stefanski, R. J., Podleckis, E. V., Phillips, J. G., Wu, J. S., & Martinez, W. H. (2009). A Risk Assessment Model for Importation of United States Milling Wheat Containing *Tilletia contraversa*. *Plant Dis*, *93*(6), 560-573. https://doi.org/10.1094/PDIS-93-6-0560

Pieck, M. L., Ruck, A., Farman, M. L., Peterson, G. L., Stack, J. P., Valent, B., & Pedley, K. F. (2017). Genomics-Based Marker Discovery and Diagnostic Assay Development for Wheat Blast. *Plant Dis*, *101*(1), 103-109. https://doi.org/10.1094/PDIS-04-16-0500-RE

Pieczul, K., Perek, A., & Kubiak, K. (2018). Detection of *Tilletia caries*, *Tilletia laevis* and *Tilletia controversa* wheat grain contamination using loop-mediated isothermal DNA amplification (LAMP). *J Microbiol Methods*, *154*, 141-146. https://doi.org/10.1016/j.mimet.2018.10.018

Pimentel, G., Carris, L. M., Levy, L., & Meyer, R. J. (2018). Genetic variability among isolates of *Tilletia barclayana*, *T. indica* and allied species. *Mycologia*, *90*(6), 1017-1027. https://doi.org/10.1080/00275514.1998.12027000

Pimentel, G., Carris, L. M., & Peever, T. L. (2000). Characterization of interspecific hybrids between *Tilletia controversa* and *T. bromi*. *Mycologia*, *92*(3), 411-420. https://doi.org/Doi 10.2307/3761499

Pimentel, G., Peever, T. L., & Carris, L. M. (2000). Genetic Variation Among Natural Populations of *Tilletia controversa* and *T. bromi*. *Phytopathology*, *90*(4), 376-383. https://doi.org/10.1094/PHYTO.2000.90.4.376

Pritchard, L., Glover, R. H., Humphris, S., Elphinstone, J. G., & Toth, I. K. (2016). Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Analytical Methods*, *8*(1), 12-24. https://doi.org/10.1039/c5ay02550h

Russel, B. W. (1993). Electrophoretic Karyotypes and Molecular Genetic Analysis *of Tilletia caries* and *T. controversa*. Origen State University

Russell, B. W. (1994). Morphological, Physiological, and Genetic Evidence in Support of a Conspecific Status for *Tilletia caries, T. controversa*, and *T. foetida*. *Phytopathology*, *84*(6), 576-582. https://doi.org/10.1094/Phyto-84-576

Russell, B. W., & Mills, D. (1993). Electrophoretic karyotypes of *Tilletia caries*, *T. controversa*, and their F1 progeny: further evidence for conspecific status. *Molecular plant-microbe interactions : MPMI*, *6*(1), 66-74. https://doi.org/10.1094/mpmi-6-066

Sharma, P., Tiwari, R., Saharan, M. S., Sharma, I., Kumar, J., Mishra, S., Muthusamy, S. K., Gupta, R. K., Jaiswal, S., Iquebal, M. A., Angadi, U. B., Kumar, N., Fatma, S., Rai, A., & Kumar, D. (2016). Draft Genome Sequence of Two Monosporidial Lines of the Karnal Bunt Fungus *Tilletia indica* Mitra (PSWKBGH-1 and PSWKBGH-2). *Genome Announc*, *4*(5), e00928-00916. https://doi.org/10.1128/genomeA.00928-16

Singh, A. (1994). Epidemiology and Management of Karnal Bunt Disease of Wheat. *Research Bulletin No. 127, G.B. Pant University of Agriculture and Technology, Pantnagar, India*.

Stewart, J. E., Andrew, M., Bao, X., Chilvers, M. I., Carris, L. M., & Peever, T. L. (2013). Development of sequence characterized amplified genomic regions (SCAR) for fungal systematics: proof of principle using *Alternaria*, *Ascochyta* and *Tilletia*. *Mycologia*, *105*(4), 1077-1086. https://doi.org/10.3852/12-287

Tan, M. K., Raman, H., Chambers, G., Sharma, I., Chen, Z., Deshpande, N., & Wilkins, M. R. (2016). Characterization of SNP and Structural Variations in the Mitochondrial Genomes of *Tilletia indica* and Its Closely Related Species Formed Basis for a Simple Diagnostic Assay. *PLoS One*, *11*(11), https://doi.org/10.1371/journal.pone.0166086

Tanner, N. A., Zhang, Y., & Evans, T. C., Jr. (2015). Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *Biotechniques*, *58*(2), 59-68. https://doi.org/10.2144/000114253

Team, R. (2016). *RStudio: Integrated Development Environment for R*. In http://www.rstudio.com/

Thomsen, M. C. F., Hasman, H., Westh, H., Kaya, H., & Lund, O. (2017). RUCS: rapid identification of PCR primers for unique core sequences. *Bioinformatics*, *33*(24), 3917-3921. https://doi.org/10.1093/bioinformatics/btx526

Tomita, N., Mori, Y., Kanda, H., & Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc*, *3*(5), 877-882. https://doi.org/10.1038/nprot.2008.57

Trione, E. J. (1964). Isolation and in Vitro culture of the wheat bunt fungi *Tilletia caries* and *Tilletia controversa*. *Phytopathology*, *54*, 592_596.

Vánky, K. (1994). *European Smut Fungi*. Gustav Fischer Verlag, Stuttgart. Jena, New York, 1994. ISBN 3–437–30745–2

Vánky, K. (2012). *Smut fungi of the world.* American Phytopathological Society.

Wade, G. D., & Tyler, L. J. (1958). Germination studies with spores of the dwarf bunt fungus. *Phytopath*, *48*(10).

Wang, A., Pan, L., Niu, X., Shu, X., Yi, X., Yamamoto, N., Li, S., Deng, Q., Zhu, J., Liang, Y., Wang, L., Li, P., & Zheng, A. (2019). Comparative secretome analysis of different smut fungi and identification of plant cell death-inducing secreted proteins from *Tilletia horrida*. *BMC Plant Biol*, *19*(1), 360. https://doi.org/10.1186/s12870-019-1924-6

Wang, N., Ai, P., Tang, Y., Zhang, J., Dai, X., Li, P., & Zheng, A. (2015). Draft Genome Sequence of the Rice Kernel Smut *Tilletia horrida* Strain QB-1. *Genome Announc*, *3*(3), e00621-00615. https://doi.org/10.1128/genomeA.00621-15

Whitaker, T. B., Wu, J., Peterson, G. L., Giesbrecht, F. G., & Johansson, A. S. (2001). Variability associated with the official USDA sampling plan used to inspect export wheat shipments for *Tilletia controversa* spores. *Plant Pathology*, *50*(6), 755-760. https://doi.org/DOI 10.1046/j.1365-3059.2001.00640.x

Zouhar, M., Mazáková, J., Prokinová, E., Váňová, M., & Ryšánek, P. (2010). Quantification of *Tilletia caries* and *Tilletia controversa* mycelium in wheat apical meristem by real-time PCR. *Plant Protection Science*, *46*(3), 107-115.

Additional Table 3-1 Heatmap of average nucleotide identity (ANI) among two closely related groups of *T. caries, T. controversa*, and *T. laevis* and two species of *T. indica* and *T. walkeri*

		Tilletia caries					*Tilletia controversa*						*Tilletia laevis*		*Tilleta indica*						*Tilletia walkeri*
		AA11	AI	AO	AZH3	DAOM 238032	DAOM 236426	OA2	OL14	OR	OV	OW	L-19	LLFL	DAOM 236416	PSWKBGH_1	PSWKBGD_1_3	PSWKBGH_2	RAKB_UP_1	TiK_1	DAOM 236422
Tilletia caries	AA11	100.0	99.5	99.6	99.6	99.5	99.1	99.1	99.2	99.2	99.1	99.2	99.6	99.6							
	AI	99.5	100.0	99.6	99.6	99.5	99.1	99.2	99.2	99.2	99.1	99.2	99.6	99.6							
	AO	99.6	99.6	100.0	99.6	99.5	99.1	99.1	99.2	99.2	99.1	99.2	99.6	99.6							
	AZH3	99.6	99.6	99.6	100.0	99.6	99.1	99.1	99.2	99.3	99.1	99.2	99.6	99.6							
	DAOM 238032	99.5	99.5	99.5	99.6	100.0	99.2	99.1	99.1	99.2	99.1	99.1	99.5	99.5							
Tilletia controversa	DAOM 236426	99.1	99.1	99.1	99.1	99.2	100.0	99.2	99.3	99.4	99.2	99.3	99.1	99.1							
	OA2	99.1	99.2	99.1	99.1	99.1	99.2	100.0	99.3	99.4	99.3	99.3	99.2	99.2							
	OL14	99.2	99.2	99.2	99.2	99.1	99.3	99.3	100.0	99.4	99.3	99.3	99.3	99.2							
	OR	99.2	99.2	99.2	99.3	99.2	99.4	99.4	99.4	100.0	99.4	99.4	99.3	99.3							
	OV	99.1	99.1	99.1	99.1	99.1	99.2	99.3	99.3	99.4	100.0	99.3	99.1	99.1							
	OW	99.2	99.2	99.2	99.2	99.1	99.3	99.3	99.3	99.4	99.3	100.0	99.2	99.2							
Tilletia laevis	L-19	99.6	99.6	99.6	99.6	99.5	99.1	99.2	99.3	99.3	99.1	99.2	100.0	99.7							
	LLFL	99.6	99.6	99.6	99.6	99.5	99.1	99.2	99.2	99.3	99.1	99.2	99.7	100.0							
Tilleta indica	DAOM 236416														100.0	96.6	96.9	96.6	96.7	96.9	94.3
	PSWKBGH_1														96.6	100.0	97.7	96.6	96.7	96.7	94.3
	PSWKBGD_1_3														96.9	97.7	100.0	99.6	97.0	97.0	94.5
	PSWKBGH_2														96.6	96.6	99.6	100.0	96.7	96.7	94.3
	RAKB_UP_1														96.7	96.7	97.0	96.7	100.0	100.0	94.4
	TiK_1														96.9	96.7	97.0	96.7	100.0	100.0	94.4
Tilletia walkeri	DAOM 236422														94.3	94.3	94.5	94.3	94.4	94.4	100.0

ANIm percentage among two group of *Tilletia* species calculated using Pyani with MUMmer aligner. The isolates and species are given as row and column labels. Colors change from 100% (green) to 99.1% (blue) in upper heatmap and from 100% (red) to 94.4% (green) among *T. indica* and *T. walkeri*.

Additional Table 3-2 Heatmap of alignment coverage (%) among two closely related groups of *T. caries, T. controversa*, and *T. laevis* and two species of *T. indica* and *T. walkeri*

		Tilletia caries					*Tilletia controversa*						*Tilletia laevis*		*Tilleta indica*						*illetia walker*
		AA11	AI	AO	AZH3	DAOM 238032	DAOM 236426	OA2	OL14	OR	OV	OW	L-19	LLFL	DAOM 236416	PSWKBGH_1	PSWKBGD_1_3	PSWKBGH_2	RAKB_UP_1	TiK_1	DAOM 236422
Tilletia caries	AA11	100.0	97.1	94.7	97.5	92.1	88.7	93.3	92.3	95.8	89.3	92.2	95.6	95.4							
	AI	96.0	100.0	93.5	95.9	90.8	88.0	92.8	91.1	95.0	88.0	91.6	94.2	94.1							
	AO	97.9	97.7	100.0	98.2	93.9	90.7	94.2	94.0	97.2	91.2	93.5	97.6	97.4							
	AZH3	97.9	97.3	95.4	100.0	92.1	88.7	93.3	92.3	95.4	89.5	92.5	95.5	95.7							
	DAOM 238032	98.2	97.9	96.9	97.9	100.0	93.8	95.5	94.9	98.8	92.5	94.4	96.7	96.8							
Tilletia controversa	DAOM 236426	97.0	97.2	95.8	96.5	96.1	100.0	98.4	97.3	100.0	96.2	98.3	96.2	96.1							
	OA2	91.7	92.2	89.6	91.4	88.0	88.5	100.0	91.4	96.3	88.8	92.2	90.3	90.4							
	OL14	94.4	94.1	92.9	94.0	90.9	91.0	95.0	100.0	96.8	92.6	94.6	93.6	93.5							
	OR	60.5	60.6	59.4	60.0	58.5	58.3	61.9	59.8	100.0	57.5	60.2	59.2	60.0							
	OV	95.2	94.8	94.1	95.1	92.4	93.9	96.4	96.6	97.2	100.0	96.2	94.5	94.6							
	OW	93.0	93.3	91.2	92.9	89.2	90.7	94.6	93.4	96.1	91.0	100.0	92.0	92.1							
Tilletia laevis	L-19	97.2	96.8	96.0	96.7	92.2	89.5	93.4	93.1	95.2	90.0	92.8	100.0	97.1							
	LLFL	97.0	96.7	95.8	96.9	92.3	89.5	93.5	93.1	96.6	90.2	92.9	97.1	100.0							
Tilleta indica	DAOM 236416														100	84.9	93.7	85.1	86	87.6	72.9
	PSWKBGH_1														68.8	100	83.4	72.3	71.2	70.6	59
	PSWKBGD_1_3														65.1	71.4	100	74.5	65.9	65.4	53.6
	PSWKBGH_2														69.5	72.8	87.5	100	70.5	70.3	57.9
	RAKB_UP_1														77.4	79	85.4	77.7	100	91.7	65.9
	TiK_1														83.6	83.1	89.9	82.2	97.2	100	69.6
Tilletia walkeri	DAOM 236422														91	90.8	96.3	88.5	91.5	91	100

Alignment coverage among two group of *Tilletia* species calculated using Pyani with MUMmer aligner. The isolates and species are given as row and column labels. Colors change from 100% (green) to 57.6% (blue) in upper heatmap and from 100% (red) to 53.7% (green) among *T. indica* and *T. walkeri*.

Additional Table 3-3 List of samples and isolates used in this study

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
T. caries	5150903	2015	*Triticum aestivum*	France	G. Orgeur [i]	
T. caries	18150622	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	32150804	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	35150921	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	41150831	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	43150918	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	49150621	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	49150722	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	58150915	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	61150720	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	61151016	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	77150804	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	79150803	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	89150818	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	89150903	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	26151001-1	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	26151001-2	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	26151001-4	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	26151001-5	2015	*Triticum aestivum*	France	G. Orgeur	
T. caries	AA10	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. caries	AA11	2015	*Triticum aestivum*	Austria	A. E. Müllner	*CBS 144825
T. caries	AA12	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. caries	AA7	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. caries	AA8	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. caries	AA9	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. caries	AC	2015	*Triticum aestivum*	Germany	H. Spieß	*
T. caries	AD	2014	*Triticum aestivum*	Germany	S. Schumann	*
T. caries	AD1	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AD2	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AD3	2017	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AD4	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AD5	-	*Triticum aestivum*	Denmark	A. Borgen	

Additional Table 3-3 *(continued)*

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
T. caries	AD10	2017	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AD119	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AD341N	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AD341R	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	ADG	2017	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	ADP	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	ADPS	-	*Triticum aestivum*	Denmark	A. Borgen	
T. caries	AER	2016	*Triticum aestivum*	Germany	S. Weller	*
T. caries	AES	2016	*Triticum aestivum*	Germany	S. Weller	*
T. caries	AEZO	2016	*Triticum spelta*	Germany	S. Weller	*
T. caries	AGW	2016	*Triticum aestivum*	Germany	B. Schwab	*
T. caries	AHW	2016	*Triticum aestivum*	Germany	S. Weller, H. Eichinger	*
T. caries	AI	2015	*Triticum durum*	Italy	V. Weyermann	*CBS 145171
T. caries	AKW	2016	*Triticum aestivum*	Germany	S. Weller	*
T. caries	AL	2010	*Triticum aestivum*	Germany	H. Mitterer	*
T. caries	AL14	2014	*Triticum aestivum*	Germany	H. Mitterer	*
T. caries	AL15	2015	*Triticum aestivum*	Germany	H. Mitterer	*
T. caries	AL17	2017	*Triticum aestivum*	Germany	M. K. Forster	
T. caries	ALA	2018	*Triticum aestivum*	Latvia	V. Strazdina	
T. caries	ALI1	-	*Triticum aestivum*	Lithuania	A. Borgen	
T. caries	ALI3	-	*Triticum aestivum*	Lithuania	A. Borgen	
T. caries	AM	2016	*Triticum aestivum*	Austria	M. Weinhappel	
T. caries	AN	2014	*Triticum aestivum*	Germany	R. Bauer	*
T. caries	AN15	2015	*Triticum aestivum*	Germany	R. Bauer	*
T. caries	AO	2014	*Triticum aestivum*	Germany	R. Bauer	*CBS 145172
T. caries	AOA	2016	*Triticum aestivum*	Germany	B. Schwab	*
T. caries	ARW	2016	*Triticum aestivum*	Germany	S. Weller	*
T. caries	AS	1977	*Triticum aestivum*	Switzerland	H. Zogg	
T. caries	AS11	2011	*Triticum aestivum*	Switzerland	I. Bänziger	
T. caries	AS14	2014	*Triticum aestivum*	Germany	B. Pölitz	
T. caries	ASR	2014	*Triticum aestivum*	Switzerland	I. Bänziger	
T. caries	ASW13	2013	*Triticum aestivum*	Switzerland	I. Bänziger	

Additional Table 3-3 *(continued)*

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
T. caries	AUN	-	*Triticum aestivum*	Germany	-	*
T. caries	AUO	-	*Triticum aestivum*	Germany	-	*
T. caries	AW	2014	*Triticum aestivum*	Germany	R. Bauer	*
T. caries	AZH1	2015	*Triticum aestivum*	Switzerland	V. Weyermann	*
T. caries	AZH2	2015	*Triticum aestivum*	Switzerland	V. Weyermann	*
T. caries	AZH3	2015	*Triticum aestivum*	Switzerland	V. Weyermann	*CBS 145166
T. caries	AZH4	2015	*Triticum aestivum*	Switzerland	V. Weyermann	*
T. caries	AZH5	2015	*Triticum aestivum*	Switzerland	V. Weyermann	*
T. caries	GD 1968	1998	*Triticum aestivum*	Romania	C. Gebhart	
T. caries	GD 404	1933	*Triticum aestivum*	Latvia	V. Tumss	
T. caries	GD 4420	1923	*Triticum aestivum*	Germany	H. Zillig	
T. caries	GD 4425	1922	*Triticum aestivum*	Germany	H. Zillig	
T. caries	GD 4421	1923	*Triticum aestivum*	Germany	H. Zillig	
T. caries	GD 4427	1922	*Triticum aestivum*	Germany	H. Zillig	
T. caries	GD 4431	1921	*Triticum aestivum*	-	M. Rotsweols	
T. caries	T-1	1984	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. caries	T-15	1978	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
	T-19	-	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. caries	T-2	1989	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. caries	T-30	-	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. caries	T-33	-	*Triticum aestivum*	USA	R. J. Metzger	
T. caries	T-34	-	*Triticum aestivum*	USA	R. J. Metzger	
T. caries	V117	2005	*Triticum aestivum*	Czech Republic	V. Dumalasova	
T. caries	V154	2012	*Triticum aestivum*	Czech Republic	V. Dumalasova	
T. caries	V155	2007	*Triticum aestivum*	Czech Republic	V. Dumalasova	
T. caries	V92	2003	*Triticum aestivum*	Czech Republic	V. Dumalasova	
T. caries	V94	2004	*Triticum aestivum*	Czech Republic	V. Dumalasova	

Additional Table 3-3 *(continued)*

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
T. caries/ T. laevis mix	RU11	-	*Triticum aestivum*	Czech Republic	V. Dumalasova	
T. cerebrina	GD 1713	1984	*Deschampsia caespitosa*	Finland	A. & W. Fuß	
T. controversa	Vánky 528	1984	*Triticum aestivum*	Germany	H. & I. Scholz	HUV 11761/ WSP 69062
T. controversa	D-12	-	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. controversa	D-13	-	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. controversa	D-17	1999	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. controversa	D-18	-	*Triticum aestivum*	USA	B. J. Goates, R. J. Metzger	
T. controversa	D-19	-	*Triticum aestivum*	Turkey	B. J. Goates, R. J. Metzger	
T. controversa	D-3	-	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. controversa	D-4	-	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. controversa	D-7	1999	*Triticum aestivum*	USA	R. J. Metzger, J. A. Hoffmann	
T. controversa	GD 1016	1980	*Triticum* sp.	Germany	-	
T. controversa	GD 1951	1997	*Triticum aestivum*	Germany	G. Deml	
T. controversa	GD 1952	1997	*Triticum aestivum*	Germany	G. Deml	
T. controversa	GD 1953	1997	*Triticum spelta*	Germany	G. Deml	
T. controversa	GD 1954	1997	*Triticum spelta*	Germany	G. Deml	
T. controversa	GD 1955	1997	*Triticum aestivum*	Germany	G. Deml	
T. controversa	OA1	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. controversa	OA2	2015	*Triticum aestivum*	Austria	A. E. Müllner	*CBS 145169
T. controversa	OA3	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. controversa	OA4	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. controversa	OA5	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. controversa	OA6	2015	*Triticum aestivum*	Austria	A. E. Müllner	*
T. controversa	OC1	2015	*Triticum aestivum*	Germany	H. Spieß	*
T. controversa	OC2	2015	*Triticum aestivum*	Germany	H. Spieß	*

Additional Table 3-3 *(continued)*

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
T. controversa	OL	2013	*Triticum aestivum*	Germany	H. Mitterer	*
T. controversa	OL13	2000& 2013	*Triticum aestivum*	Germany	M. K. Forster	
T. controversa	OL14	2014	*Triticum aestivum*	Germany	H. Mitterer	*CBS 145167
T. controversa	OL16	2016	*Triticum aestivum*	Germany	M. K. Forster	*
T. controversa	OMO	2016	*Triticum spelta*	Germany	R. Klügl	*
T. controversa	OR	2013	*Triticum aestivum*	Germany	R. Bauer	*CBS 144827
T. controversa	ORB	2016	*Triticum aestivum*	Germany	S. Weller	*
T. controversa	OST	2001	*Triticum aestivum*	Germany	W. Wenig	
T. controversa	OUN	-	*Triticum aestivum*	Germany	-	*
T. controversa	OUO	-	*Triticum aestivum*	Germany	-	*
T. controversa	OV	2011	*Triticum aestivum*	Germany	R. Bauer	*CBS 145170
T. controversa	OW	2013	*Triticum aestivum*	Germany	R. Bauer	*CBS 145168
T. controversa	OW15	2015	*Triticum aestivum*	Germany	R. Bauer	*
T. controversa	OZH	1998	*Triticum aestivum*	Switzerland	I. Bänziger	
T. controversa		1986	*Elymus repens*	Hungary	K. Vánky	HUV 12434
T. controversa	Vánky 2675	1982	*Thinopyrum intermedium* subsp. *Intermedium (Elymus hispidus)*	Hungary	M. Juhász, K. Vánky	HUV 11040
T. holci	Vánky 765	1990	*Holcus mollis*	New Zealand	E. H. C McKenzie, K. Vánky	HUV 15067
T. indica	II7	2007	*Triticum* sp.	India	P. Chhuneja	
T. indica	IM5	2005	*Triticum* sp.	Mexico	CIMMYT	
T. indica	IM6	2006	*Triticum* sp.	Mexico	CIMMYT	
T. laevis	5150826	2015	*Triticum aestivum*	France	G. Orgeur	
T. laevis	GD 4402	1935	*Triticum* sp.	USA	G. L. Zundel	
T. laevis	GD 683	1977	*Triticum aestivum*	Switzerland	H. Zogg	
T. laevis	L-1	1990	*Triticum aestivum*	-	R. J. Metzger, J. A. Hoffmann	
T. laevis	L-10	1990	*Triticum aestivum*	-	R. J. Metzger, J. A. Hoffmann	
T. laevis	L-16	1984	*Triticum aestivum*	-	R. J. Metzger, J. A. Hoffmann	
T. laevis	L-18	-	*Triticum aestivum*	-	R. J. Metzger	

Additional Table 3-3 *(continued)*

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
T. laevis	L-19	-	*Triticum aestivum*	-	R. J. Metzger	*CBS 145173
T. laevis	L-20	-	*Triticum aestivum*	Turkey	R. J. Metzger	
T. laevis	L-21	-	*Triticum aestivum*	USA	R. J. Metzger	*
T. laevis	LCR	-	*Triticum aestivum*	Czech Republic	A. Borgen	
T. laevis	LI-134	2018	*Triticum aestivum*	Iran	M. Kheirgoo	
T. laevis	LI-137	2018	*Triticum aestivum*	Iran	M. Kheirgoo	
T. laevis	LI-138	2018	*Triticum aestivum*	Iran	M. Kheirgoo	
T. laevis	LI-139	2018	*Triticum aestivum*	Iran	M. Kheirgoo	
T. laevis	LI-141	2018	*Triticum aestivum*	Iran	M. Kheirgoo	
T. laevis	LI-142	2018	*Triticum aestivum*	Iran	M. Kheirgoo	
T. laevis	LI-143	2018	*Triticum aestivum*	Iran	M. Kheirgoo	
T. laevis	LLFL	2015	*Triticum aestivum*	Germany	R. Bauer	*CBS 144826
T. laevis	LLI2	-	*Triticum aestivum*	Lithuania	A. Borgen	
T. laevis	LQ1	-	*Triticum aestivum*	Iraq	A. Borgen	
T. laevis	LQ2	-	*Triticum aestivum*	Iraq	A. Borgen	
T. laevis	LQ3	-	*Triticum aestivum*	Iraq	A. Borgen	
T. laevis	LSW	-	*Triticum aestivum*	Sweden	A. Borgen	
T. laevis	LT-1	-	*Triticum aestivum*	Turkey	A. Borgen	
T. laevis	LT-2	-	*Triticum aestivum*	Turkey	A. Borgen	
T. laevis	V61	1997	*Triticum aestivum*	Czech Republic	V. Dumalasova	
T. laevis	Vánky 766	1988	*Triticum aestivum*	Iran	B. Pourjam	HUV 15003/ WSP 71300
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73142
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73143
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73146
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73148
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73149
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73150
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73152
T. laevis		1979	*Triticum aestivum*	Turkey	R. J. Metzger	WSP 73155
T. laevis[ii]		1979	*Triticum aestivum*	Turkey	B. Metzger	WSP 73156
T. laevis		1979	*Triticum carthlicum*	Turkey	R. J. Metzger	WSP 73157
T. laevis		1979	*Triticum durum*	Turkey	R. J. Metzger	WSP 73145

Additional Table 3-3 *(continued)*

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
T. laevis		1979	*Triticum durum*	Turkey	R. J. Metzger	WSP 73153
T. lolioli	Vánky 763	1990	*Festuca orientalis (Loliolum subulatum)*	Iran	D. Ershad, M. Abbasi, T. & K. Vánky	HUV 15065/ WSP 71305
T. menieri	Vánky 581	1985	*Phalaris arundinacea*	Germany	H. & I. Scholz	HUV 12681
T. olida		1983	*Brachypodium pinnatum*	Italy	T. & k. Vánky	HUV 12682
T. olida		1985	*Brachypodium sylvaticum*	Germany	G. Hirsch	HUV 11766
T. olida		1987	*Brachypodium pinnatum*	Germany	K. Vánky	HUV 20601
T. secalis	GD 1707	1984	*Secale cereale*	Germany	G. Deml	
T. trabutii	Vánky 764	1990	*Hordeum murinum* ssp. *glaucum*	Iran	D. Ershad, H. Golzar, T. & K. Vánky	HUV 15036/ WSP 71299
Alternaria alternata		2017	*Hordeum vulgare*	Germany	P. Büttner	
A. alternata	69505	1995	*Triticum aestivum*	Germany	W. Radtke	
Alternaria sp.	72926	2016	*Secalse cereale*	Germany	P. Büttner	
Aspergillus niger	71709	2001	*Solanum tuberosum*	Germany	M. Götz	
Bipolaris sorokiniana	72924	2016	*Triticum aestivum*	Germany	P. Büttner	
Boeremia exigua var. *exigua*	62040	-	*Digitalis lanata*	Germany	-	
Botrytis cinerea	62086	-	*Triticum aestivum*	Germany	-	
B. cinerea	72325	2016	*Triticum aestivum*	Germany	P. Büttner	
Cladosporium fulvum	72927	2016	*Triticum aestivum*	Germany	P. Büttner	
Fusarium acumulatum		-	-	-	A. Sisic	
F. avenaceum		-	*Pisum sativum*	-	A. Sisic	
F. culmorum	65219	-	*Triticum durum*	Germany	C. Kling	
F. equiseti		-	*Prunus dulcis*	-	A. Sisic	
F. graminearum	64967	1987	*Triticum aestivum*	Germany	C. Kling	
F. oxysporum		-	*Prunus dulcis*	-	A. Sisic	
F. poae	73010	2017	*Hordeum vulgare*	Germany	P. Büttner	
F. sambusium		-	-	-	A. Sisic	
F. sporotrichioides	72922	2016	*Triticum aestivum*	Germany	P. Büttner	
F. tricinctum		-	*Pisum sativum*	-	A. Sisic	
Magnaporthe oryzae	IPP0685	2008	*Triticum aestivum*	Bolivia	M. Kohli	

Additional Table 3-3 *(continued)*

Taxon	Sample number	Year	Host	Geographic origin	Collector/ Source	Voucher No
Monographella nivali	72923	2016	*Triticum aestivum*	Germany	P. Büttner	
Penicillium expansum	67687	1993	-	Germany	-	
Penicillium sp.		2016	*Glycine max*	Germany	P. Büttner	
Puccinia graminis f. sp. tritici		-	*Triticum aestivum*	-	-	
P. recondita	77wxr	-	*Triticum aestivum*	-	-	
P. striiformis var. *striiformis*		-	*Triticum aestivum*	-	-	
Pyrenophora tritici-repentis	Asc-1	-	*Triticum aestivum*	Canada	L. Lamari	
P. tritici-repentis	Asc203	-	*Triticum aestivum*	-	-	
Rhizoctonia cerealis	64616	1985	*Triticum aestivum*	Germany	-	
Sclerotinia sclerotiorum	73011	2016	*Glycine max*	Germany	P. Büttner	
Septoria tritici	68366	-	*Triticum aestivum*	Germany	H. Mielke	
Sporisorium sorghi		2008	-	Egypt	M. Moharam	
Ustilago avenae		2012	*Avena sativa*	Scotland	M. McNeil	
U. hordei		2010	-	Germany	K. J. Müller	
U. maydis		-	-	-	-	
U. nuda	42874	2017	*Hordeum vulgare*	Germany	M. K. Forster	
U. nuda		2012	*Hordeum vulgare*	Switzerland	I. Bänziger	
U. tritici		2009	-	Germany	K. J. Müller	
U. tritici		2012	*Triticum aestivum*	Switzerland	I. Bänziger	
Urocystis occulta		2012	-	Germany	Syngenta AG	

CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; HUV (BRIP), Herbarium Ustilaginales Vánky, Queensland Plant Pathology Herbarium, Queensland, Australia; WSP, Washington State Plant Pathology Herbarium, Pullman, WA, USA
single teliospore cultures produced in this study
[i] the samples received from G. Orgeur are part of Groupe d'Etude et de contrôle des Variétés Et des Semences (Beaucouzé cedex, France) sample collection
[ii] the sample was originally defined as *T. caries* by B. Metzger

Additional Table 3-4 Unique and conserved DNA segments identified by RUCS, when 21 and 27 *Tilletia* genomes were used

RUCS output using 21 *Tilletia* genomes			
Target species	**No of extracted regions**	**N50 (bp)**	**Longest extracted region (bp)**
T. controversa	11,135	61	6,133
T. caries	235	39	116
T. laevis	228	39	215
T. caries/*T. laevis*	11,884	52	6,790
RUCS output using 27 *Tilletia* genomes			
Target species	**No of extracted regions**	**N50 (bp)**	**Longest extracted regions (bp)**
T. controversa	10,282	53	6,134
T. caries	16	39	95
T. laevis	43	39	215
T. caries/*T. laevis*	10,024	42	6,790

Additional Table 3-5 List of newly released and updated assemblies accession numbers

Taxon	Strain	Assembly accession numbers	Reference
T. caries	AA11 (CBS 144825)	GCA_905072865.1	This work
T. caries	AI (CBS 145171)	GCA_905068135.1	This work
T. caries	AO (CBS 145172)	GCA_905071735.1	This work
T. caries	AZH3 (CBS 145166)	GCA_905071745.1	This work
T. caries	DAOMC 238032	GCA_001645005.2*	Nguyen et al. 2019
T. controversa	DAOMC 236426	GCA_001645045.2*	Nguyen et al. 2019
T. controversa	DAOMC 238052	GCA_009428265.1	Nguyen et al. 2019
T. controversa	OA2 (CBS 145169)	GCA_905071725.1	This work
T. controversa	OL14 (CBS 145167)	GCA_905071785.1	This work
T. controversa	OR (CBS 144827)	GCA_905071765.1	This work
T. controversa	OV (CBS 145170)	GCA_905071775.1	This work
T. controversa	OW (CBS 145168)	GCA_905071705.1	This work
T. horrida	QB-1	GCA_001006505.1	Wang, et al. 2015
T. indica	DAOMC 236408	GCA_009428345.1	Nguyen et al. 2019
T. indica	DAOMC 236414	GCA_009428365.1	Nguyen et al. 2019
T. indica	DAOMC 236416	GCA_001645015.2*	Nguyen et al. 2019
T. indica	PSWKBGD_1_3	GCA_001689965.1	NA
T. indica	PSWKBGH_1	GCA_001689995.1	Sharma, et al. 2016
T. indica	PSWKBGH_2	GCA_001689945.1	Sharma, et al. 2016
T. indica	RAKB_UP_1	GCA_002220835.1	Gurjar, et al. 2019
T. indica	Tik_1	GCA_002997305.1	Kumar, et al. 2017, Kumar, et al. 2018
T. laevis	ATCC 42080	GCA_009428275.1	Nguyen et al. 2019
T. laevis	DAOMC 238040	GCA_009428285.1	Nguyen et al. 2019
T. laevis	L-19 (CBS 145173)	GCA_905071715.1	This work
T. laevis	LLFL (CBS 144826)	GCA_905071755.1	This work
T. walkeri	DAOMC 236422	GCA_001645055.2*	Nguyen et al. 2019
T. walkeri	DAOMC 238049	GCA_009428295.1	Nguyen et al. 2019

The assemblies marked with * are updated versions of the assemblies used initially in the LAMP assay development (Nguyen et al. 2019)

Additional Table 3-6 Common bunt-specific DNA segments identified by RUCS software and their predicted gene content

Number	Contig accession number	Start (bp)	End (bp)	Length (bp)	Gene ID	Position in the extracted DNA segment	Functional prediction
1	LWDD02000153.1	3904	10693	6790	A4X03_0g1772	Partially	Hypothetical protein
					A4X03_0g1770	Complete	Hypothetical protein
					A4X03_0g1771	Complete	Hypothetical protein
					A4X03_0g1769	Partially	Hypothetical protein
2	LWDD02000153.1	10751	16755	6005	A4X03_0g1772	Partially	Hypothetical protein
					A4X03_0g1773	Complete	Hypothetical protein
					A4X03_0g1774	Partially	Hypothetical protein
3	LWDD02000520.1	5494	9453	3960	A4X03_0g4074	Partially	Hypothetical protein
					A4X03_0g4076	Partially	Hypothetical protein
4	LWDD02000153.1	40	3902	3863	NA	Intergenic	NA
5	LWDD02002073.1	217	3686	3470	A4X03_0g7887	Complete	Hypothetical protein
					A4X03_0g7888	Partially	Hypothetical protein
6	LWDD02001289.1	812	4099	3288	A4X03_0g6555	Partially	Hypothetical protein
7	LWDD02001869.1	184	3133	2950	A4X03_0g7600	Complete	Hypothetical protein
8	LWDD02000790.1	371	3031	2661	A4X03_0g5184	Partially	Hypothetical protein
					A4X03_0g5185	Complete	Hypothetical protein
9	LWDD02002508.1	82	2523	2442	A4X03_0g8400	Complete	Hypothetical protein
10	LWDD02000253.1	37	2286	2250	A4X03_0g2549	Partially	Hypothetical protein
11	LWDD02001126.1	2678	4503	1826	A4X03_0g6179	Partially	Hypothetical protein

References cited in the additional files

Gurjar, M. S., Aggarwal, R., Jogawat, A., Kulshreshtha, D., Sharma, S., Solanke, A. U., Dubey, H., & Jain, R. K. (2019). De novo genome sequencing and secretome analysis of *Tilletia indica* inciting Karnal bunt of wheat provides pathogenesis-related genes. *3 Biotech*, *9*(6), 219. https://doi.org/10.1007/s13205-019-1743-3

Kumar, A., Mishra, P., Maurya, R., Mishra, A. K., Gupta, V. K., Ramteke, P. W., & Marla, S. S. (2018). Improved Draft Genome Sequence of a Monoteliosporic Culture of the Karnal Bunt (*Tilletia indica*) Pathogen of Wheat. *Genome Announc*, *6*(20), e00015-00018. https://doi.org/10.1128/genomeA.00015-18

Nguyen, H. D. T., Sultana, T., Kesanakurti, P., & Hambleton, S. (2019). Genome sequencing and comparison of five *Tilletia* species to identify candidate genes for the detection of regulated species infecting wheat. *IMA Fungus*, *10*(1), 11, https://doi.org/10.1186/s43008-019-0011-9

Sharma, P., Tiwari, R., Saharan, M. S., Sharma, I., Kumar, J., Mishra, S., Muthusamy, S. K., Gupta, R. K., Jaiswal, S., Iquebal, M. A., Angadi, U. B., Kumar, N., Fatma, S., Rai, A., & Kumar, D. (2016). Draft Genome Sequence of Two Monosporidial Lines of the Karnal Bunt Fungus *Tilletia indica* Mitra (PSWKBGH-1 and PSWKBGH-2). *Genome Announc*, *4*(5), e00928-00916. https://doi.org/10.1128/genomeA.00928-16

Wang, N., Ai, P., Tang, Y., Zhang, J., Dai, X., Li, P., & Zheng, A. (2015). Draft Genome Sequence of the Rice Kernel Smut *Tilletia horrida* Strain QB-1. *Genome Announc*, *3*(3), e00621-00615. https://doi.org/10.1128/genomeA.00621-15

Additional Figure 3-1 Photos of the interlaboratory test performance study of the developed LAMP assay**.** A: the reference photo taken by the organizer. B – F: photos that are received from the participants. The reaction color changes to pink when *T. controversa* is detected. The False Positive (FP) reported reaction is marked by (*). Photo F is color intensified by a photo-editing software (Adobe Photoshop version 6.0). The sensitivity and specificity of the test were 100 and 97.7%, respectively.

Chapter 4 – Species delimitation of *Tilletia controversa* using molecular phylogenetic and phylogenomic approaches

Somayyeh Sedaghatjoo[1,4], Monika K. Forster[2,3], Berta Killermann[2], Petr Karlovsky[4], Wolfgang Maier[1]

*Email of the corresponding author: s.sedaghatjoo@gmail.com

Manuscript in preparation

[1] Julius Kühn Institute (JKI) – Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

[2] Institute for Crop Science and Plant Breeding, Bavarian State Research Center for Agriculture, Vöttinger Straße 38, 85354 Freising, Germany

[3] Chair of Technical Microbiology, TUM School of Life Sciences, Technical University of Munich, Gregor-Mendel-Str. 4, 85454 Freising, Germany

[4] Molecular Phytopathology and Mycotoxin Research, University of Goettingen, Grisebachstrasse 6, 37077 Goettingen, Germany

4. 1. Abstract

The smut species of *Tilletia,* are of particular importance, because they parasite Poaceae family which contains a variety of important crop plants. Among the most economically important species are *T. caries*, *T. controversa*, and *T. laevis*, the causal agents of common and dwarf bunt of wheat. Unlike other *Tilletia* species which are proposed to be mostly host-specific, common and dwarf bunt are reported to have broad host range. To clarify whether they are indeed generalist species with a broad host range or rather represent complexes of cryptic species with narrow host ranges phylogenetic relationships of those species and close relatives by employing sequencing data of the internal transcribed spacer region rDNA (ITS), translation elongation factor 1 alpha (*EF1α*), and the second largest subunit of RNA polymerase II (*RPB2*). We additionally employed phylogenomic approach to investigate the relationship among ten common bunt and seven dwarf bunt isolates. In total 70 specimens of which 20 specimens were newly produced for this study. In general, the multi locus phylogenetic analysis resolved various species with narrow host ranges parasitizing wild grasses as distinct lineages such as *T. fusca* up to *T. olida* representing 12 species and *T. bromi* and *T. puccinelliae*. The situation is more complex in the case of *T. controversa*:, several small clusters of *T. controversa* from wild grasses (*Thinopyrum intermedium*, *Bromus marginatus*, *Agropyron cristatum*) and rye (*Secale cereale*), respectively, clustered as subgroups in a polytomous manner between different clusters of *T. caries*, *T. controversa*, and *T. laevis* on wheat. Interestingly, one group of *T. controversa* sequences obtained from *Elymus repens, Th. intermedium,* and *Agropyron* sp. clustered with high support values clearly separate from this polytomous group and together with *T. brevifaciens* isolates also obtained from *Th. intermedium*. These representatives of *T. controversa* with high likelihood represent at least one cryptic species restricted to these wild grasses as hosts and might potentially be conspecific with *T. brevifaciens*. Phylogenomic analysis based on 241 genes employing 27 genomes of seven *Tilletia* species supported distinction of *T. controversa* species from common bunt, however the analyses failed to support *T. caries* as phylogenetically distinct from *T. laevis* species. Therefore, it is suggested that common bunt fungi are either two pseudomorphs of a species or just recently separated.

Keywords

Tilletia, common and dwarf bunt of wheat, host specificity, multilocus phylogeny, phylogenomic

4. 2. Introduction

Tilletiales is one of the fungal orders in Exobasidiomycetes (Ustilaginomycetes, Basidiomycota) (Begerow et al., 1997; Begerow et al., 2006) that comprises of nearly 200 described species (Carris et al., 2007; Denchev and Denchev, 2013; Denchev and Denchev, 2018; Denchev et al., 2018; Li et al., 2014; Vánky, 2012). Genus *Tilletia* is characterized by the formation of usually reticulate ornamented teliospores, which replace mainly ovary tissues of Poaceae members (Castlebury et al., 2005; Vánky, 2012); the fifth largest family of flowering plants (Soreng et al., 2017). Teliospores germinate to form aseptate basidium (holobasidium) that bears terminal basidiospores. Basidiospores often conjugate and give rise to infectious intercellular hyphae which have dolipore septum without cap (Bauer et al., 2006; Bauer et al., 1997; Roberson and Luttrell, 1989).

Species delimitations within the genus are mainly based on teliospores morphology, host, and if available, the number and nuclear condition of primary basidiospores and ability of primary basidiospores to conjugate and form an infective dikaryon (Castlebury et al., 2005). Molecular phylogenetic analysis of *Tilletia* and allied taxa utilizing a part of the nuclear large subunit (nLSU) rDNA gene distinguished a well-supported lineage containing *Tilletia* species on the subfamily Pooideae (Castlebury et al., 2005). Neither this region, nor combined with internal transcribed spacer (ITS) (Jayawardena et al., 2019) were variable enough to separate pooid-infecting species within the lineage. However, a multilocus phylogenetic study based on combined ITS, translation elongation factor 1 alpha (*EF1a*), and the second largest subunit of RNA polymerase II (*RPB2*) provided strong support for individual, narrow host range species of *Tilletia* on pooid grass hosts (Carris et al., 2007). The three important species of wheat bunt (*T. caries, T. controversa*, and *T. laevis)* were shown to have a common origin with low genetic distances and could not be resolved according to the species boundaries as individual monophyletic lineages.

Common bunt of wheat is caused by *T. caries* and *T. laevis,* dwarf bunt by *T. controversa* which are wheat bunt causal agents reported in central Europe condition. *Tilletia controversa* was first reported on quackgrass (*Elymus repens*) in Germany by Kühn (1874) and is phylogenetically and genetically so close to *T. caries* and *T. laevis* that some studies have been questioned whether they can be regarded as distinct species (Carris et al., 2007; Holton, 1954; Holton and Kendrick, 1956; Russell, 1994; Russell and Mills, 1993). *Tilletia caries* and *T. controversa* are not only described from wheat species (*Triticum* spp.), but also on several different genera of Poaceae family (Goates, 1996; Hardison et al., 1959; Purdy et al., 1963; Schuhmann, 1960) and *T. controversa* is proposed to have a broad host range by Durán and Fischer (1961), while majority of the *Tilletia* species have a relatively narrow host range

usually reSed to one genus or even a single host (Begerow et al., 2004; Castlebury et al., 2005). So far, little is known about the molecular phylogenetic relation of *T. controversa* originated from different hosts. However, the phylogenetic study of several broad host range smut fungi has revealed that they are mostly representing species complexes comprising several species with narrow host specificities (Kruse et al., 2018; Piątek et al., 2013; Savchenko et al., 2014). Similar finding was also reported for the anther smuts of the genus *Microbotryum* belonging to Pucciniomycotina (Kemler et al., 2009; Ziegler et al., 2018).

After the recent publication of the first genome of one *T. caries* isolate and one *T. controversa* isolate, the first phylogenomic analyses that also included five *T. indica* (causal agent of wheat karnal bunt) isolates and one *T. horrida* (rice kernel smut) was published by Gurjar et al. (2019). This study showed that *T. caries* and *T. controversa* are very similar and closely related. A similar finding was made by Mishra et al. (2019) analyzing 3751 loci and including one *T. walkeri* (causal agent of ryegrass smut) isolate. The most recent study using 4896 single-copy orthologous genes of ten *Tilletia* isolates (one isolate of *T. caries*, two isolates of *T. controversa*, two isolates of *T. laevis*, three isolates of *T. indica*, and two isolates of *T. walkeri*) was done by Nguyen et al. (2019) resolved the five species into well-supported clades where only one *T. caries* isolates was included. Yet a phylogenomic analyses of all the available genomes is lacking while there are now 11 additional genomes of common bunt and dwarf bunt (four isolates of *T. caries*, five isolates of *T. controversa* and two isolates of *T. laevis*) available. The aim of this study was i) molecular phylogenetic analyses using three loci (*EF1a*, ITS, and *RPB2*), with broader taxon sampling, especially including specimens of *T. controversa* originated from grasses to obtain indications whether *T. controversa* represents a polyphagous species or rather several host specific species; ii) a phylogenomic analyses using several specimens of *T. caries, T. controversa*, and *T. laevis* from different geographic origins to test species status of these bunt fungi.

4. 3. Material and Methods

4. 3. 1. Fungal isolates and nucleic acid extraction

Collection of *Tilletia* spp. used in this study are listed in . Host taxonomy followed the Kew Royal Botanic Gardens online database (https://wcsp.science.kew.org/). Total genomic DNA was extracted from either single teliospore cultures or directly from teliospores when they were not viable anymore. Single teliospore cultures production and DNA extractions from both sources were done according to chapter 3.

4. 3. 2. PCR amplification and sequence analysis

Three nuclear DNA regions were amplified and sequenced: the elongation factor 1 alpha *(EF1a)*, the complete internal transcribed spacers 1 and 2 (ITS), and the second largest subunit of RNA polymerase II (*RPB2)* using a combination of the primers presented in Table 4-2.

All PCR reactions were performed in a final volume of 25 µL containing 1 µL template. We used different PCR mixes including; (i) 10× TrueStart, (NH4)2SO4 amended *Taq* buffer (Thermo Scientific, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs (Thermo Scientific, Vilnius, Lithuania), 0.4 µM of each primer, and 1 Unit *Taq* DNA polymerase (TrueStart Hot Start, Thermo Scientific, Vilnius, Lithuania), (ii) 20 µL ALLin™ Hot Strat Taq Mastermix (HighQu GmbH, Kraichtal, Germany), 0.4 µM of each primer, (iii) 10× DreamTaq Buffer, 0.2 mM each of dNTPs (Thermo Scientific, Vilnius, Lithuania), 1 µM of each primer and 1.25 Unit *Taq* DNA polymerase (DreamTaq, Thermo Scientific, Lithuania). Standard cycling parameters according to the manufacturers' manuals was used for each PCR mixture. For the amplification of *EF1a* 59 °C annealing temperature was used, 53 °C for ITS, and 60 °C for *RPB2*.

PCR products were purified using the DNA Clean and Concentrator™-5 kit (Zymo Research Corp., Irvine, California, USA) according to the manufacturer's instructions. The purified amplicons were sequenced by their respective PCR primers. A contig of the obtained forward and reverse sequences were produced, quality-checked edited and manually trimmed using Sequencher™ 5.4.6 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). The generated sequences and sequences downloaded from GenBank are summarized in Table 4-1. We did not include samples that were publicly available but lacked any of the three loci sequences, with two exceptions: one isolate of *T. togwateei* and *T. laguri*. These two samples were included even by lacking ITS sequences because they are only distantly related to *T. controversa.*

Table 4-1 *Tilletia* species analyzed in this study

No	Taxon	Sample/collection/ voucher number	Year	Host	Origin	Collector/ source	*EF1α*	ITS	*RPB2*	Ref.
1	*T. brevifaciens*	HUV 20802 (CBS 121948)	2004	*Thinopyrum intermedium*×*Elymus repens*	Poland	K. Vánky	EU257535	EU257565	EU257599	1
2	*T. brevifaciens*	WSP 68945 (Vánky 412)	1999	*Thinopyrum intermedium*	Austria	K. Vánky	EU257536	EU257566	EU257600	1
3	*T. bromi*	WSP 71272 (LMC 167, CBS 123002)	1992	*Bromus arvensis*	USA	L. Carris	EU257529	EU257556	EU257593	1
4	*T. bromi*	WSP 71273 (LMC 75, CBS 123001)	1991	*Bromus hordeaceus* ssp. *hordeaceus*	USA	L. Carris	EU257530	EU257557	EU257594	1
5	*T. bromi*	WSP 71315	2003	*Bromus sp.*	USA	G. Huang	EU257531	EU257558	EU257595	1
6	*T. bromi*	WSP 71271 (LMC 148)	1992	*Bromus tectorum*	USA	L. Carris	EU257528	EU257555	EU257592	1
7	*T. caries*	AL	2010	*Triticum aestivum*	Germany	H. Mitterer				5
8	*T. caries*	AN	2014	*Triticum aestivum*	Germany	R. Bauer				5
9	*T. caries*	AZH3 (CBS_145166)	2015	*Triticum aestivum*	Switzerland	V. Weyermann				5
10	*T. caries*	WSP 71303 (LMC J-19, CBS 121951)	1995	*Triticum aestivum*	Sweden	L. Johnsson	EU257533	EU257560	EU257597	1
11	*T. caries*	WSP 71304 (DAR 73302)	1997	*Triticum aestivum*	Australia	G. Murray	EU257532	EU257559	EU257596	1
12	*T. controversa*	D-19	-	*Triticum aestivum*	Turkey	B. J. Goates, R. J. Metzger				5
13	*T. controversa*	GD 1964	1997	*Alopecurus myosuroides*	Germany	M. Piepenbring				5
14	*T. controversa*	GD 1972	1999	*Alopecurus myosuroides*	Germany	M. Piepenbring				5
15	*T. controversa*	HUV 12434_O_E	1986	*Elymus repens*	Hungary	K. Vánky				5
16	*T. controversa*	OL14 (CBS 145167)	2014	*Triticum aestivum*	Germany	H. Mitterer	delete	delete	delete	5

Additional Table 4-1 *(continued)*

No	Taxon	Sample/collection/ voucher number	Year	Host	Origin	Collector/ source	*EF1α*	ITS	*RPB2*	Ref.
17	*T. controversa*	OR (CBS 144827)	2013	*Triticum aestivum*	Germany	R. Bauer				5
18	*T. controversa*	OUO	-	*Triticum aestivum*	Germany	-				5
19	*T. controversa*	WSP 63862	1954	*Triticum aestivum*[i]/ ***Thinopyrum intermedium*** *(Agropyron intermedium)*[ii]	Switzerland	H. Aebi	JQ245323	JQ245338	JQ245102	4
20	*T. controversa*	WSP 69062 (Vánky 528)	1984	*Triticum aestivum*	Germany	K. Vánky	EU257526	EU257562	EU257588	1
21	*T. controversa*	WSP 70123	1999	*Elymus repens*[i]/ ***Th. intermedium*** *(Elymus hispidus)*[ii]	Austria	Ch. Scheuer	JQ245324	JQ245339	JQ245434	4
22	*T. controversa*	WSP 71280 (LMC 282, CBS 121952)	1994	*Triticum aestivum*	USA	L. Carris	EU257534	EU257561	EU257598/JQ245109	1
23	*T. controversa*	WSP 71301 (LMC 94)	1991	*Bromus marginatus*	USA	L. Carris	EU257526	EU257563	EU257590	1
24	*T. controversa*	WSP 72054 (J-1)	1991	*Elymus repens*	Sweden	L. Johnsson	JQ245325	JQ245341	JQ245436	4
25	*T. controversa*	WSP 72055 (J-8)	1984	*Elymus repens*	Sweden	L. Johnsson	JQ245326	JQ245342	JQ245437	4
26	*T. controversa*	WSP 35784	1950	*Thinopyrum intermedium (Agropyron intermedium)*	USA	E. Horning	JQ245319	JQ245333	JQ245084	4
27	*T. controversa*	WSP 63665	1954	*Agropyron cristatum*	USA	J.P. Meiners	JQ245320	JQ245335	JQ245431	4
28	*T. controversa*	WSP 63680	-	*Bromus marginatus*	USA	J.P. Meiners	JQ245321	JQ245336	JQ245432	4
29	*T. controversa*	WSP 63687	1956	*Secale cereale*	USA	L.J. Tyler	JQ245322	JQ245337	JQ245433	4

Additional Table 4-1 *(continued)*

No	Taxon	Sample/collection/ voucher number	Year	Host	Origin	Collector/ source	*EF1α*	ITS	*RPB2*	Ref.
30	*T. elymi*	WSP 71274 (LMC 158, CBS 123000)	1992	*Elymus glaucus ssp. glaucus*	USA	L. Carris	EU257527	EU257564	EU257591	1
31	*T. fusca*	WSP 71275 (LMC 141, CBS 122991)	1971	*Vulpia microstachys var. microstachys*	USA	L. Carris	EU257537	EU257567	EU257601	1
32	*T. goloskokovii*	WSP 69687 (LMC 315, CBS 122995)	1995	*Apera interrupta*	USA	L. Carris	EU257539	EU257569	EU257603	1
33	*T. goloskokovii*	WSP 69688 (LMC 321)	1995	*Apera interrupta*	USA	L. Carris	EU257540	EU257570	EU257604	1
34	*T. goloskokovii*	WSP 71281 (LMC 238-2)	1993	*Apera interrupta*	USA	L. Carris	EU257538	EU257568	EU257602	1
35	*T. holci*	HUV 15067	1990	*Holcus mollis*	New Zealand	E. H. C McKenzie, K. Vánky				5
36	*T. laevis*	L-21	-	*Triticum aestivum*	USA	R. J. Metzger				5
37	*T. laevis*	LLFL (CBS 144826)	2015	*Triticum aestivum*	Germany	R. Bauer				5
38	*T. laevis*	WSP 71302 (LMC 98-194)	1998	*Triticum aestivum*	Australia	L. Carris	EU257542	EU257572	EU257606	1
39	*T. laevis*	WSP 72072 (TK126_2)	1979	*Triticum durum*	Turkey	-	JQ245331	JQ245346	JQ245089	2
40	*T. laguri*	HUV 16352	1992	*Lagurus ovatus*	Italy	K. Vánky		EU257574	EU257608	1
41	*T. lolii*	WSP 71298 (Vánky 767)	1990	*Lolium rigidum*	Iran	K. Vánky	EU257544	EU257575	EU257609	1
42	*T. lolioli*	WSP 71305 (Vánky 763)	1990	*Loliolum subulatum*	Iran	K. Vánky	EU257545	EU257576	EU257610	1
43	*T. menieri*	HUV 12681 (Vánky 581)	1985	*Phalaris arundinacea*	Germany	H. & I. Scholz				5
44	*T. olida*	GD 927	1980	*Brachypodium pinnatum*	Germany	G. Deml				5
45	*T. olida*	HUV 11766_530	1985	*Brachypodium sylvaticum*	Germany	G. Hirsch				5

Additional Table 4-1 *(continued)*

No	Taxon	Sample/collection/ voucher number	Year	Host	Origin	Collector/ source	*EF1α*	ITS	*RPB2*	Ref.
46	*T. olida*	HUV 20601_1866	1987	*Brachypodium pinnatum*	Germany	K. Vánky				5
47	*T. puccinelliae*	WSP 71465 (CBS 122994)	2004	*Puccinellia distans*	USA	L. Carris	EU910052	EU910058	EU910064	3
48	*T. puccinelliae*	WSP 71470 (CBS 122997)	2004	*Puccinellia distans*	USA	L. Carris	EU910054	EU910060	EU910066	3
49	*T. puccinelliae*	WSP 71470 (CBS 122998)	2004	*Puccinellia distans*	USA	L. Carris	EU910055	EU910061	EU910067	3
50	*T. puccinelliae*	WSP 71471 (CBS 122999)	2004	*Puccinellia distans*	USA	L. Carris	EU910050	EU910056	EU910062	3
51	*T. puccinelliae*	WSP 71472 (CBS 122996)	2004	*Puccinellia distans*	USA	L. Carris	EU910053b	EU910059	EU910065	3
52	*T. puccinelliae*	WSP 71469 (CBS 122993)	2004	*Puccinellia distans*	USA	L. Carris	EU910051	EU910057	EU910063	3
53	*T. secalis*	GD 1707	1984	*Secale cereale*	Germany	G. Deml				5
54	*T. secalis*	WSP 71279 (LMC 255)	1993	*Secale cereale*	USA	L. Carris	EU257525	EU257577	EU257589	1
55	*T. sphaerococca*	WSP 71314	2003	*Agrostis stolonifera*	USA	G. Huang	EU257546	EU257578	EU257611	1
56	*T. togwateei*	WSP 71276	1992	*Poa reflexa*	USA	L. Carris	-	EU257579	EU257612	1
57	*T. togwateei*	WSP 71277 (LMC 169)	1992	*Poa reflexa*	USA	L. Carris	EU257547	EU257580	EU257613	1
58	*T. trabutii*	VPRI 32106	2005	*Hordeum murinum* ssp. *leporinum*	Australia	I. Pascoe	EU257549	EU257582	EU257615	1
59	*T. trabutii*	GD 1980, O_HO	1988	*Hordeum sp.*	Morocco	J. Nielsen				5
60	*T. trabutii*	WSP 71299 (Vánky 764, CBS 122324)	1990	*Hordeum murinum* ssp. *glaucum*	Iran	K. Vánky	EU257548	EU257581	EU257614	1
61	*T. vankyi*	FF7_8	2005	*Festuca rubra* ssp. *fallax*	USA	S. Alderman	EU257553	EU257586	EU257619	1

Additional Table 4-1 *(continued)*

No	Taxon	Sample/collection/ voucher number	Year	Host	Origin	Collector/ source	*EF1α*	ITS	*RPB2*	Ref.
61	*T. vankyi*	FF7_8	2005	*Festuca rubra* ssp. *fallax*	USA	S. Alderman	EU257553	EU257586	EU257619	1
62	*T. vankyi*	LC 1326	2002	*Lolium perenne*	Australia	G. Huang	EU257551	EU257584	EU257617	1
63	*T. vankyi*	WSP 71270 (FF1-1, CBS 122323)	2005	*Festuca rubra* ssp. *fallax*	USa	S. Alderman	EU257552	EU257585	EU257618	1
64	*T. vankyi*	WSP 71316	2003	*Festuca rubra* ssp. *fallax*	USA	G. Huang	EU257550	EU257583	EU257616	1
65	*T. vankyi*	WSP 71266 (V21-713)	1997	*Lolium perenne*	Australia	L. Carris	EU257554	EU257587	EU257620	1
66	*Tilletia* sp.	WSP 73144	1979	*Agropyron* sp.	Turkey	R. J. Metzger/ Lori M. Carris				5
67	*Tilletia* sp.	WSP 73147	1979	*Aegilops cylindrica*	Turkey	R. J. Metzger				5
68	*Tilletia* sp.	WSP 73151	1979	*Aegilops cylindrica*	Turkey	R. J. Metzger				5
69	*Tilletia* sp.	WSP 73154	1979	*Aegilops biuncialis*	Turkey	R. J. Metzger				5
70	*Tilletia* sp.	WSP 73158	1979	*Agropyron* sp.	Turkey	R. J. Metzger				5

CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; HUV, Queensland Plant Pathology Herbarium (BRIP) Brisbane, Queensland, Australia, WSP, Washington State Plant Pathology Herbarium, Pullman, WA, USA, [i], submitted host in The National Center for Biotechnology Information (NCBI), [ii], submitted host according to WSU webpage, the correct host is presented in bold (reconfirmed host by herbarium). Reference numbers; 1) Carris et al., 2007, 2) Stewart et al., 2013, 3) Bao et al., 2010, 4) Bao, 2010, and 5) this study. Sample number 67 was originally identified as *T. caries*. It could not be confirmed by us.

Table 4-2 Primers used in this study

Locus	Primer	Sequence (5′ - 3′)	Reference
EF1a	EF1-526F*	GTCGTYGTYATYGGHCAYGT	Rehner, (DeepHypha web page)
	EF1-636F	TCAAGGTCGTYGTYATCGG	(Carris et al., 2007)
	EF1-1567R*	ACHGTRCCRATACCACCRATCTT	Rehner, (DeepHypha web page)
	EF1-2218R	ATGACACCRACRGCRACRGTYTG	Rehner, (DeepHypha web page)
ITS	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns, 1993)
	ITS2	GCTGCGTTCTTCATCGATGC	(White et al., 1990)
	ITS3	GCATCGATGAAGAACGCAGC	(White et al., 1990)
	ITS5*	GGAAGTAAAAGTCGTAACAAGG	(White et al., 1990)
	ITS4*	TCCTCCGCTTATTGATATGC	(White et al., 1990)
	ITS4-B	CAGGAGACTTGTACACGGTCCAG	(Gardes and Bruns, 1993)
RPB2	RPB2-740F	GATGGACGCGGTTTGTAATG	(Carris et al., 2007)
	RPB2-1365R	TCGAAGAGCYAACACTGAGACG	(Carris et al., 2007)

The primers that most of the samples were amplified with are marked with asterisks. Alternative primers were used when the sequencing results based on marked primers were not optimum, or the amplification failed.

4. 3. 3. Molecular phylogenetic reconstruction

The sequences of each locus were aligned independently using Mafft V. 7 (Katoh et al., 2017) adopting the iterative refinement algorithms L_INS_i. The leading and trailing gaps were manually trimmed in AliView v 1.26 (Larsson, 2014). The three loci were aligned individually and then concatenated into a single alignment. All the specimens were included in the concatenated alignment, with missing data for the two loci where sequences were lacking.

Phylogenetic relationships were inferred based on the concatenated alignment using Bayesian Metropolis coupled Markov chain Monte Carlo (MC^3) analyses (BPP), minimum evolutionary (ME), and maximum likelihood (ML). For Bayesian interference the program MrBayes v3.2. 7 (Larget and Simon, 1999; Ronquist et al., 2012) was used. To run MrBayes, the evolutionary model was first estimated using jModelTest 2.1.10 (Darriba et al., 2012) based on Bayesian Information Criteria (BIC). Two runs over 1,000,000 generations MC^3 sampling each consisting of 4 heated chains with a random start tree were then computed using the SYM+I+G model suggested by jModelTest (TrNef+I+G). Trees were sampled every 200^{th} generation and from these, the first 20% were discarded. The remaining trees were used to compute a 50% majority rule consensus tree to obtain estimates for the posterior probability. Balanced minimum evolution inference was done using

FastMe 2.0 (Lefort et al., 2015) using F84 (Felsenstein, 1984) as the nucleotide substitution model and gamma distributed rates across sites. Branch support values were estimated applying 1000 bootstrap replicates. The initial tree topology was optimized by the best of Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) method which generally finds better tree topologies. Maximum likelihood analysis was done employing the partitioned-marked concatenated alignment (Chernomor et al., 2016) using IQ-TREE 1.6.10 (Nguyen et al., 2015). The best-fitting models of evolution were estimated by ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE. For *EF1a* and ITS. The best fitting models were TNe+R2 and for *RPB2* HKY+F+G4, respectively. The tree was inferred using 1000 replicates of fast bootstrapping (Hoang et al., 2017). Only bootstrap support (BS) values with a minimum of 70% for ML and ME and 0.9 of Bayesian posterior probabilities (BPP) are given. The trees were visualized in the web interface iTOL v4 (Letunic and Bork, 2019).

To estimate if the differences in clade was supported by raw alignment data (Wägele and Mayer, 2007), we used SplitsTree4 v4.15.1 (Huson and Bryant, 2006) to compute Neighbor-Net analysis (Bryant and Moulton, 2004). This method provides a collection of possible resolutions through reticulation. We used the concatenated alignment with settings Variance = OrdinaryLeastSquares and uncorrected P-distance, performing 1000 bootstrap replicates.

4. 3. 4. Orthologue gene identification and species tree recognition

Genome sequences used in the phylogenomic analyses are listed in Table 4-3. For the phylogenomic study, orthologue genes were selected according to a modified approach described by Pizarro et al. (2018). Every genome was assessed for 303 single-copy genes of the eukaryote OrthoDB v9 (Waterhouse et al., 2013) using BUSCO (Benchmarking Universal Single-Copy Orthologs) version 3.0.2 (Simao et al., 2015) in the genomic mode. The putative gene regions identified by BUSCO were extracted. For the duplicated genes, we used the sequence with the higher similarity scored to its BUSCO reference. Each BUSCO gene recovered from each of the 27 genomes was aligned using MAFFT V. 7 adopting the iterative refinement algorithms L-INS-i (-local pair -maxiterate 1000 -adjustdirectionaccurately). In order to reduce the effects of missing data, alignments with more than 7% of missing data (lacking corresponding sequence in more than two isolates per loci) were removed. Ambiguous regions within each alignment were removed using Gblock v 0.91b (Castresana, 2000) with the default parameters (S).

The species tree inferences based on the multispecies coalescent model (Degnan and Rosenberg, 2006) was done because individual phylogenetic analyses based on individual genes can

result in different gene trees that differ from the true species tree (Rannala and Yang, 2003). First, we constructed approximately-maximum-likelihood phylogenetic trees for every single gene individually using FastTree 2.1.11 (Price et al., 2010) implemented in Geneious version 8.1.2 (Biomatters Limited, Auckland, New Zealand). We used settings of optimized gamma20 likelihood and Generalized Time-Reversible (GTR) model. The Accurate Species Tree Algorithm II (ASTRAL-II) (Mirarab and Warnow, 2015) was employed to summarize coalescent interferences resulting from all trees. Clade support was evaluated by computing the local posterior probability (LPP), generated by ASTRAL-II which is suggested to be more precise (Sayyari and Mirarab, 2016). The tree was visualized as described before.

Table 4-3 List of the genomes used in this study

No.	Species	Isolate	Assembly accession number	Genome size (bp)	Reference
1	*T. caries*	AA11 (CBS 144825)	GCA_905072865	31,511,149	Sedaghatjoo et al
2	*T. caries*	AI (CBS 145171)	GCA_905068135	31,849,506	Sedaghatjoo et al
3	*T. caries*	AO (CBS 145172)	GCA_905071735	30,466,127	Sedaghatjoo et al
4	*T. caries*	AZH3 (CBS 145166)	GCA_905071745	31,386,298	Sedaghatjoo et al
5	*T. caries*	DAOMC 238032	GCA_001645005.2	28,142,201	(Nguyen et al., 2019)
6	*T. caries*	WSP 72095 (517)	GCA_004334575.1	35,802,276	-
7	*T. controversa*	DAOMC 236426	GCA_001645045.2	29,878,810	(Nguyen et al., 2019)
8	*T. controversa*	DAOMC 238052	GCA_009428265.1	28,565,061	(Nguyen et al., 2019)
9	*T. controversa*	OA2 (CBS 145169)	GCA_905071725	32,055,341	Sedaghatjoo et al
10	*T. controversa*	OL14 (CBS 145167)	GCA_905071785	30,830,153	Sedaghatjoo et al
11	*T. controversa*	OR (CBS 144827)	GCA_905071765	49,872,806	Sedaghatjoo et al
12	*T. controversa*	OV (CBS 145170)	GCA_905071775	29,542,762	Sedaghatjoo et al
13	*T. controversa*	OW (CBS 145168)	GCA_905071705	31,249,642	Sedaghatjoo et al
14	*T. horrida*	QB-1	GCA_001006505.1	20,105,270	(Wang et al., 2015)
15	*T. indica*	DAOMC 236408	GCA_009428345.1	29,678,000	(Nguyen et al., 2019)
16	*T. indica*	DAOMC 236414	GCA_009428365.1	28,967,515	(Nguyen et al., 2019)
17	*T. indica*	DAOMC 236416	GCA_001645015.2	30,384,772	(Nguyen et al., 2019)
18	*T. indica*	PSWKBGH_1	GCA_001689995.1	37,460,344	(Sharma et al., 2016)
19	*T. indica*	PSWKBGH_2	GCA_001689945.1	37,216,861	(Sharma et al., 2016)
20	*T. indica*	RAKB_UP_1	GCA_002220835.1	33,771,691	(Gurjar et al., 2019)
21	*T. indica*	Tik_1	GCA_002997305.1	31,836,179	(Kumar et al., 2018; Kumar et al., 2017)
22	*T. laevis*	ATCC 42080	GCA_009428275.1	28,777,633	(Nguyen et al., 2019)
23	*T. laevis*	DAOMC 238040	GCA_009428285.1	28,279,804	(Nguyen et al., 2019)
24	*T. laevis*	LLFL (CBS 144826)	GCA_905071755	30,985,200	Sedaghatjoo et al

Table 4-3 *(continued)*

No.	Species	Isolate	Assembly accession number	Genome size (bp)	Reference
25	*T. laevis*	L-19 (CBS 145173)	GCA_905071715	31,001,062	Sedaghatjoo et al
26	*T. walkeri*	DAOMC 236422	GCA_001645055.2	23,943,196	(Nguyen et al., 2019)
27	*T. walkeri*	DAOMC 238049	GCA_009428295.1	24,274,610	(Nguyen et al., 2019)

4. 4. Results

4. 4. 1. Sequence alignments and molecular phylogenetic reconstruction

In total the phylogenetic study comprises 70 taxa of which sequences for 24 taxa are newly generated for this study. The concatenated alignment consists of 716 characters for *EF1a*, 638 for ITS, and 578 for *RPB2* (including gaps). Of 1932 total number of characters, 1655 were constant, 186 were parsimony-informative, and 91 were variable but not parsimony-informative.

Topology of the three trees (BPP consensus, balanced ME, and ML tree) remained the same for majority of the well-supported clades and were recovered similarly in all three trees, however exceptions were shown for less supported clades. The consensus tree of one run of the Bayesian phylogenetic analyses is presented with the support values of all three methods given in the order (Bayesian *posterior probabilities* / minimum evolution bootstrapping / maximum likelihood bootstrapping) (Figure 4-1). The combined analyses of the three loci distinguished several species with maximum to high support values: *T. bromi* (1/99/100)*, T. goloskokowii* (1/100/100), *T. puccinelliae* (0.99/99/ 97), *T. sphaerococca* (1/ 80/97), *T. togwateei* (1/100/100), and *T. vankyi* (1/100/100*)*. Additionally, three highly supported groups were recovered in all three analyses. One of these group contained two *Tilletia* samples on *Aegilops cylindrica* (1/100/98). The other one accommodated two collections, which initially were identified as *T. controversa* on *Alopecurus myosuroides* (1/98/100) and the third group comprised three samples of *T. olida* (1/95/100) on *Brachypodium* species. Moreover, a well-supported lineage (1/86/100) was recovered that grouped samples of *T. brevifaciens*, *T. controversa* collected from *Elymus repens,* and *Thinopyrum intermedium* together with an undetermined *Tilletia* sample on *Agropyron* sp. Interestingly *Tilletia* samples on *E. repens* clustered within a clade together with *T. brevifaciens* only and did not appear in different clades. This was unlike *T. controversa* samples on *Agropyron* spp., *Secale cereale*, and *Th. intermedium*.

One of the high to moderately supported group (1/75/77) was comprised of one of the *T. secalis* and two *Tilletia* samples, one collected from *Agropyron* sp. (WSP 73158) and another from *Aegilops biuncialis* (WSP 73154). The latter was initially identified as *T. caries* by B. Metzger, which we could not confirm based on morphological features of teliospores using light microscopy. Position of European originated *T. secalis* (GD 1707) remained unclear. Three representatives of *T. trabutii* collected from *Hordeum* spp. were clustered together with high support in ML (93) and ME (99) analysis. Polytomous nodes (multifurcations rather than bifurcations) were mostly representing *T. caries*, *T. laevis*, and all the *T. controversa* collected from *Triticum* sp. Among them were also samples of *T. controversa* on different grasses such as;*Ag. cristatum*, *Bromus marginatus*, *Secale cereale*, and *Th. intermedium*.

The incongruence between the single gene trees suggesting that gene trees cannot be successfully presented in a single concatenated phylogenetic tree, therefore we applied a network phylogeny analysis. The phylogenetic reconstruction of 70 taxa depicted by Neighbor-Net showed tree-like relationships and was in agreement with well-supported groupings in the phylogenetic tree of combined loci (data not shown). However, substructures in two clades containing *T. brevifaciens* and *T. secalis* may exist based on the reticulation of Network (Additional Figure 4-1).

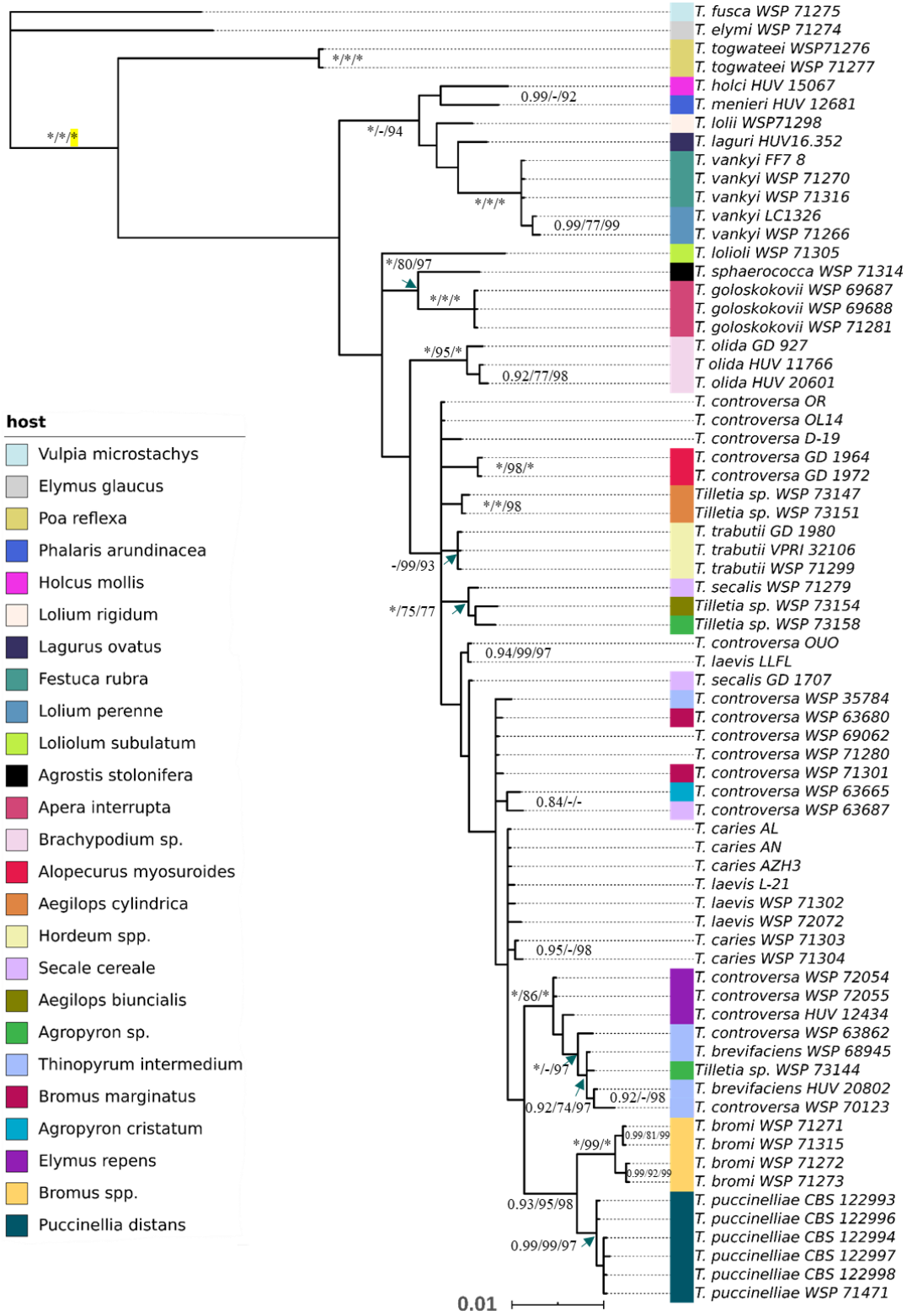


Figure 4-1 Unrooted phylogram obtained by Bayesian inference of phylogenetic relationship of *Tilletia* spp. based on a concatenated alignment of DNA sequences of *EF1α*, ITS1 and *RPB2* regions. Support values are given for branches in the following order Bayesian posterior probabilities (BPP) >0.9, and bootstap values >70% balanced minimum evolution and maximum likelihood are given at first, second, and third positions, respectively. A minus sign denotes lacking support for the present topology, 100% and 1 are shown with * and. Samples are color coded based on host. Samples without color code are collected from *Triticum* spp.

4. 4. 2. Phylogenomic inferences

From the total of 303 initial BUSCO genes, 241 were included for construction of individual gene trees. The multispecies coalescent analyses recognized five groupings corresponding. A clade containing common and dwarf bunt fungi from the rest of the species was supported with maximum support level (LPP 1). Seven samples of *T. controversa* were clustered together and separated from common bunt fungi, however between two species of *T. caries* and *T. laevis* no grouping corresponding to each species was achieved. Moreover, two distinct clades; one containing all *T. indica* isolates and the other both isolates of *T. walkeri* with maximum support (Figure 4-2). The concatenated analysis of 416,222 aligned nucleotides in the Neighbor-Net was in agreement with Astral II results (Additional Figure 4-2).

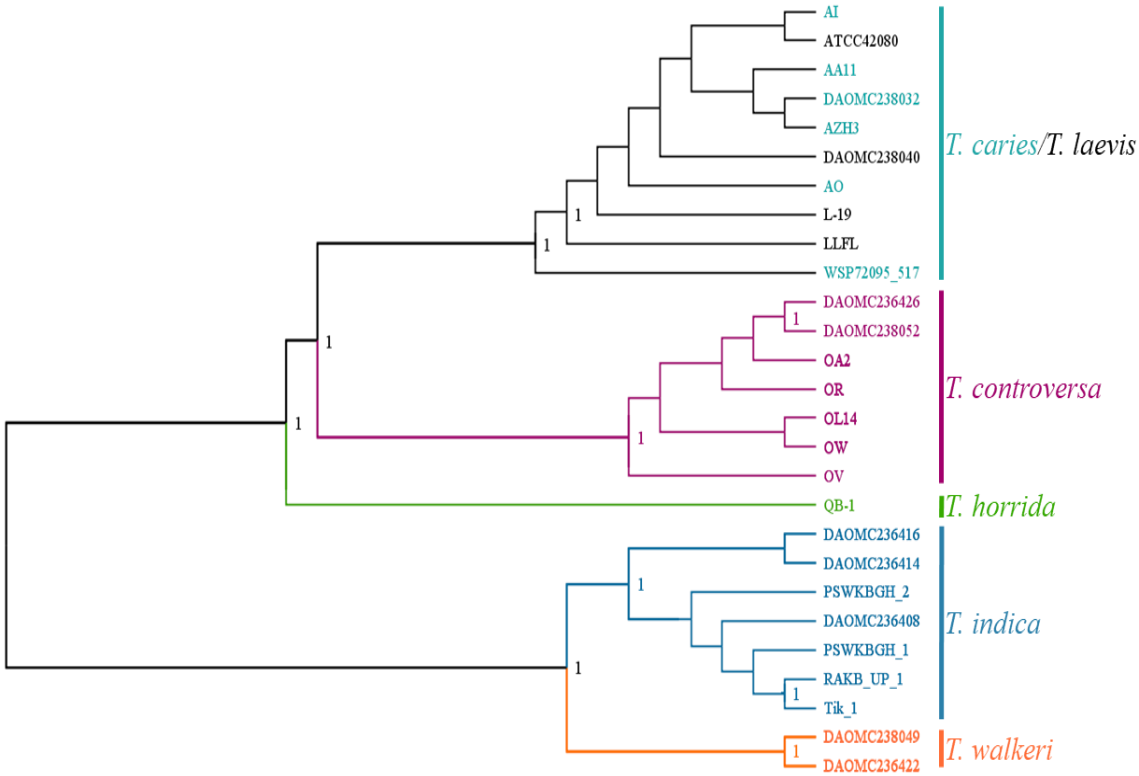


Figure 4-2 Proportional cladogram inferred on the set of 241 genes. Phylogeny inferred from input trees derived from single-partitioned ML per loci analyses (each gene tree reconstructed using a single partition) with ASTRAL II. Each isolate is color coded based on identified species. Node values indicate local posterior probability above 0.95 are shown.

4. 5. Discussion

4. 5. 1. Molecular phylogenetic reconstruction of *Tilletia* with a special focus on disentangling potentially host specific lineages within *T. controversa*

In this study, molecular phylogenetic analyses based on three genetic loci (*EF1a*, ITS, and *RPB2*) was performed to understand the delimitation of *T. controversa* species on species of Pooideae. To this end *T. controversa* samples were analyzed that had been obtained not only from *Triticum* species but different hosts from five additional genera. In general, we found a weak resolution in the backbone of the phylogenetic tree based on the three genes. Most terminal taxa could however be resolved as distinct taxa either due to their significant genetic distances discriminating them from other species or based on high support values in those species where several representatives had been included. The present study reproduced several phylogenetic groupings such as *T. bromi, T. goloskokowii*, *T. puccinelliae*, *T. sphaerococca*, *T. togwateei*, *T. trabutii,* and *T. vankyi* that have been observed previously (Carris et al., 2007). Yet, current study suggests that *T. controversa* is a multispecies parasite. Several sequences of *T. controversa* obtained from *Thinopyrum intermedium*, *Bromus marginatus*, *Agropyron cristatum* and one from *Secale cereale* clustered without any or significant support intermingled with sequences obtained from *T. caries*, *T. controversa* and *T. laevis* from wheat, suggesting that *T. controversa* can infect different hosts. This is rather contrary to previous studies showing that generally there is a strong host specificity in *Tilletia* species parasitizing wild grass species (Boyd and Carris, 1997; Boyd and Carris, 1998). The evidence that *T. controversa* can infect different grasses is important since in nature those grasses can serve as disease inoculum sources not only for these grass species but also wheat. On the other hand, artificial cross infection of *T. controversa* comprises of a broad number of hosts, yet the occurrence of dwarf bunt on wild grasses is limited (Purdy et al., 1963). Therefore, to what extent these sources of inoculum may play a role in the natural dispersal of dwarf bunt is unknown.

Despite a wider sampling both in number and geographic range, the here presented phylogenetic analyses based on three loci could not clearly distinguish the three bunt species of *T. caries*, *T. laevis* and *T. controversa* from one another. This is in line with previous reports (Bao, 2010; Bao et al., 2010; Carris et al., 2007). The sequences of the three species clustered polytomously without significant support for the whole group showing that sampling a larger number of representatives, the three species of *T. caries*, *T. controversa*, and *T. laevis* did not form a well-supported monophyletic group as suggested previously (Bao et al., 2010; Carris, 2008; Carris et al., 2007). Despite lacking resolution, several subgroups within this polyphyletic cluster emerged which partially

correlated with host species. Two specimens of *T. controversa* parasiting *Alopecurus myosuroides*, as well as two undetermined specimens on *Aegilops cylindrica,* formed highly supported subclusters. Both samples collected from *Al. myosuroides* were originally identified as *T. controversa* based on their teliospores morphological features. These finding might hint at cryptic species hidden within *T. controversa* and in the whole wheat bunt complex, because of insufficient morphological features to readily distinguish them. Classical taxonomy of *Tilletia* species is only based on teliospores morphology and morphometric, soral shape, and host. Because teliospore features can overlap among several species (Carris, 2008; Vánky, 2012), the identification of such species based on morphology only is difficult and consequently, their genetic diversity can remain unnoticed (Shi et al., 1996). Another example of such a morphologically difficult to impossible distingushable species from *T. controversa* is *T. secalis* (rye bunt). Two specimens of *T. secalis* from (*Secale cereale*), one collected from US (WSP 71279) and the other from Germany (GD 1707) did not cluster together and are thus unlikely conspecific. The analyzed sample of *T. secalis* from US was the first report of rye bunt in North America and was phylogenetically distinct from the common and dwarf bunt fungi according to (Carris, 2008) while the specimen of European origin clustered within common and dwarf bunt of wheat. Since *T. secalis* and *T. controversa* are both widespread in Central Europe (Fischer, 1956) and identification of these two species is problematic due to their undistinguishable morphological features (Niemann, 1954; Niemann, 1956), the suggestion of nonconspecificity of the European *T. secalis* to American one should be taken with caution because it may be only wrong identification. Moreover, the specimens of *T. secalis* from the US clustered with two other undetermined specimens of *Tilletia* obtained from *Aegilops biuncalis*, and *Agropyron* sp. which are morphologically distinct from *T. secalis* (data not shown). More representatives of those two samples are needed to resolve this clade, which may accommodate distinct species.

One group comprised of three *T. controversa* samples collected from *Elymus repens* (the type host), WSP 72054, WSP 72055, and UV 12434) two from *Thinoyprum intermedium* (WSP 63862 and WSP 70123), in addition to two *T. brevifaciens* (HUV 20802 and WSP 68945) specimens also from *Th. intermedium,* and one undetermined specimen on *Agropyron* sp. (WSP 73144) and separately from common and dwarf bunt of wheat. *Tilletia brevifaciens* originally was described on *Th. intermedium* (syn. *Agropyron intermedium*) by Fischer (1952) to distinguish dwarf bunt of wheat distinct from *T. caries*. It was later synonymized with *T. controversa* by Conners (1954) and confirmed by Durán and Fischer (1961) and treated accordingly by Vánky (1994) and Vánky (2012). However, *T. brevifaciens* on *Th. intermedium* was reclassified as a distinct species from *T. controversa* based on multilocus phylogenetic study and several lines of evidence (number of basidiospores per basidium and

temperature of teliospore germination) by Carris et al. (2007). Our multilocus phylogeny is in line with this reclassification since *T. brevifaciens* does not cluster with any of the phylogenetic groups comprising *T. controversa* obtained from *Triticum* spp. Due to the age of the samples, it remains unknown to us if these *T. controversa* samples within this lineage could be re-identified as *T. brevifaciens* by other lines of evidence. Additionally, both the internal support values within this clade in the phylogram and Neighbor-Net network suggest that further substructures may be present within this lineage which may be host associated. Additional variable genes and representatives are needed to obtain higher resolution and clearer picture within this lineage.

We also sequenced a few *Tilletia* species such as *T. holci*, *T. menieri*, and *T. olida* for the first time for the three loci phylogenic analysis. In general, host specificity of *Tilletia* species varies. Some *Tilletia* species are restricted to a genus such as *T. olida* on *Brachipodium* spp. and *T. trabutii* on *Hordeum* species. Some can parasitise more than one host species such as *T. controversa* and *T. vankyi*. However, such lineages that appear to infect more than one host species could actually be host-specific but too recently diverged for our markers to detect their differentiation. Presumably, higher mutational rate loci are better options for studying them as shown also by (McDonald et al., 2000; Zupunski et al., 2011) using inter simple sequence repeat regions.

One of the current limitations in phylogenetic studies of *Tilletia* is the lack of known phylogenetically informative loci. We tested the ribosomal protein L4 (rpl4A) gene with the suggested primers (rpl4_F1 and rpl4_R1) by Kruse et al. (2017) for a subset of samples including *T. caries* (n = 1), *T. controversa* collected from different hosts (n = 3), *T. holci* (n = 1), *T. lolioli* (n = 1), *T. olida* (n = 2), *T. secalis* (n = 2), and *T. trabutii* (n = 1). The 580 bp amplified protein coding region contained five substitutions for *T. olida* and four in *T. holci* only. Since the focus of this study was *T. controversa* and closely related species, we did sequence this region in our analysis.

We did not observe any correlation between obtained sequencing quality and age of herbarium specimens (data not shown). This observation is in agreement with the importance of teliospores storage rather than the age of teliospores collection material for the sequencing quality (Savchenko et al., 2014). The storage factor that plays the role remained unknown to us.

4. 5. 2. Phylogenomic inference of species boundaries of wheat bunt fungi

We additionally employed a phylogenomic approach to study the phylogenetic relation of *T. controversa* to the two genetically (chapter 2) and phylogenetically (Carris et al., 2007) closely related species of *T. caries* and *T. laevis,* with all currently available genomes (accessed Dec 2019). We used 241 single-copy genes to estimate the phylogenetic relation between 27 isolates of six *Tilletia* species.

Using a summary method of the multispecies coalescent approach, the causal agent of dwarf bunt, *T. controversa*, could be differentiated from the causal agents of common bunt; *T. caries* and *T. laevis*. However, despite the fact that Astral II takes into account the incomplete lineage sorting, the specimens of *T. caries* and *T. laevis* included in this study could not be resolved into individual lineages correlating with these two species. Nguyen et al. (2019) using a phylogenomic approach based on 4896 single copy orthologous genes suggested that species of *T. caries* and *T. laevis* are distinct. This is in contrast to our finding using a smaller number of genes but including genomes of four additional *T. caries* and two more *T. laevis* collected from Europe. This may suggest that the two common bunt species are only two morphotypes of one species. Vanky (2008) used the term pseudomorphospecies for smuts with morphological differences showing no genetic differences. The conspecificity of *T. caries* and *T. laevis* is also supported by a high degree of genomic identity (Nguyen et al., 2019), low number of single nucleotide polymorphism among the two species (chapter 2), indistinguishable protein profiles of *T. caries* and *T. laevis* using MALTI TOP-MS (Forster et al unpublished data), possibility of the hybridization of them (Flor, 1932), and similar electrophoretic karyotyping of them (Russell, 1994). *Tilletia indica* (wheat karnal bunt) and *T. walkeri* (ryegrass smut) which are only distantly related to common and dwarf bunt of wheat (Carris et al., 2006) were grouped together in a clade confirming their reported close relatedness (Castlebury and Carris, 1999).

Finally, as there are numerous additional hosts for *T. controversa* that could not be included in the current study, it seems likely that more detailed patterns regarding the reported natural and artificial hosts await detection. The result of this study once again highlights the importance of phylogenetically re-investigation of broad host range of *Tilletia* genus.

4. 6. Declaration

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4. 7. Acknowledgments

The Federal Ministry of Food and Agriculture of the Federal Republic of Germany funded this research project based on a decision of the German parliament (grant numbers 2812NA128 and 2812NA017). We especially thank Prof. Dr. Günter Deml for using his personal herbarium

specimens and those who freely shared their samples with us. Monique Slipher, Prof. Jack D. Rogers, and Prof. Lori M. Carris (Washington State Plant Pathology Herbarium (WSP) Pullman, WA, USA), and Dr. Roger Shivas (Queensland Plant Pathology Herbarium (BRIP) Brisbane, Queensland, Australia) are greatly acknowledged for providing specimens and specimens information confirmation. We appreciate the technical assistance of Anke Brißke-Rode and Katrin Balke.

4. 8. References

Bao, X. (2010). Host specificity and phylogenetic relationships among *Tilletia* species infecting wheat and other cool season grasses, Department of Plant Pathology, Washington State University.

Bao, X., Carris, L. M., Huang, G., Luo, J., Liu, Y., & Castlebury, L. A. (2010). *Tilletia puccinelliae*, a new species of reticulate-spored bunt fungus infecting *Puccinellia distans*. *Mycologia*, *102*(3), 613-623. https://doi.org/10.3852/09-135

Bauer, R., Begerow, D., Sampaio, J. P., Weiβ, M., & Oberwinkler, F. (2006). The simple-septate basidiomycetes: a synopsis. *Mycological Progress*, *5*(1), 41-66. https://doi.org/10.1007/s11557-006-0502-0

Bauer, R., Oberwinkler, F., & Vánky, K. (1997). Ultrastructural markers and systematics in smut fungi and allied taxa. *Canadian Journal of Botany*, *75*(8), 1273-1314. https://doi.org/10.1139/b97-842

Begerow, D., Bauer, R., & Oberwinkler, F. (1997). Phylogenetic studies on nuclear large subunit ribosomal DNA sequences of smut fungi and related taxa. *Canadian Journal of Botany*, *75*(12), 2045-2056. https://doi.org/10.1139/b97-916

Begerow, D., Göker, M., Lutz, M., & Stoll, M. (2004). On the evolution of smut fungi and their hosts (M. P. Reinhard Agerer , Paul Blanz, Ed.). IHW-Verlag & Verlagsbuchhandlung.

Begerow, D., Stoll, M., & Bauer, R. (2006). A phylogenetic hypothesis of Ustilaginomycotina based on multiple gene analyses and morphological data. *Mycologia*, *98*(6), 906-916. https://doi.org/10.3852/mycologia.98.6.906

Boyd, M. L., & Carris, L. M. (1997). Molecular relationships among varieties of the *Tilletia fusca* (*T. bromi*) complex and related species. *Mycological research*, *101*(3), 269-277. https://doi.org/Doi 10.1017/S0953756296002523

Boyd, M. L., & Carris, L. M. (1998). Evidence supporting the separation of the *Vulpia*- and *Bromus*-infecting isolates in the *Tilletia fusca* (*T. bromi*) complex. *Mycologia*, *90*(6), 1031-1039. https://doi.org/10.1080/00275514.1998.12027002

Bryant, D., & Moulton, V. (2004). Neighbor-Net: an agglomerative method for the construction of phylogenetic networks. *Molecular Biology and Evolution*, *21*(2), 255-265. https://doi.org/10.1093/molbev/msh018

Carris, L. (2008). Is rye bunt, *Tilletia secalis*, present in North America? *North American Fungi*, *3*, 147-159. https://doi.org/10.2509/naf2008.003.0078

Carris, L. M., Castlebury, L. A., & Goates, B. J. (2006). Nonsystemic bunt fungi *Tilletia indica* and *T. horrida:* a review of history, systematics, and biology. *Annu Rev Phytopathol*, *44*, 113-133. https://doi.org/10.1146/annurev.phyto.44.070505.143402

Carris, L. M., Castlebury, L. A., Huang, G., Alderman, S. C., Luo, J., & Bao, X. (2007). *Tilletia vankyi*, a new species of reticulate-spored bunt fungus with non-conjugating basidiospores infecting species of *Festuca* and *Lolium*. *Mycological research*, *111*(Pt 12), 1386-1398. https://doi.org/10.1016/j.mycres.2007.09.008

Castlebury, L. A., & Carris, L. M. (1999). *Tilletia walkeri*, a new species on *Lolium multiflorum* and *L. perenne*. *Mycologia*, *91*(1), 121-131. https://doi.org/10.2307/3761200

Castlebury, L. A., Carris, L. M., & Vinky, K. (2005). Phylogenetic analysis of *Tilletia* and allied genera in order Tilletiales (Ustilaginomycetes; Exobasidiomycetidae) based on large subunit nuclear rDNA sequences. *Mycologia*, *97*(4), 888-900. https://doi.org/10.3852/mycologia.97.4.888

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol*, *17*(4), 540-552. https://doi.org/10.1093/oxfordjournals.molbev.a026334

Chernomor, O., von Haeseler, A., & Minh, B. Q. (2016). Terrace aware data structure for phylogenomic inference from supermatrices. *Syst Biol*, *65*(6), 997-1008. https://doi.org/10.1093/sysbio/syw037

Conners, I. L. (1954). The organism causing dwarf bunt of wheat. *Canadian Journal of Botany*, *32*(3), 426-431. https://doi.org/10.1139/b54-038

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods*, *9*(8), 772. https://doi.org/10.1038/nmeth.2109

Degnan, J. H., & Rosenberg, N. A. (2006). Discordance of species trees with their most likely gene trees. *PLOS Genetics*, *2*(5), e68. https://doi.org/10.1371/journal.pgen.0020068

Denchev, C., & Denchev, T. (2013). Erratomycetaceae, fam. nov., and validation of some names of smut fungi recently described from India. *Mycobiota*, *1*, 63-70. https://doi.org/10.12664/mycobiota.2013.01.07

Denchev, T. T., & Denchev, C. M. (2018). Two new smut fungi on *Ventenata Poaceae*: *Tilletia elizabethae* from Slovakia and *T. ventenatae* from Turkey. *Willdenowia*, *48*(2), 177-183, 177. https://doi.org/10.3372/wi.48.48201

Denchev, T. T., Van Der Zon, A. P. M., & Denchev, C. M. (2018). *Tilletia triraphidis* (Tilletiaceae), a new smut fungus on *Triraphis purpurea* (Poaceae) from Namibia. *Phytotaxa*, *375*(2), 182-186. https://doi.org/10.11646/phytotaxa.375.2.5

Durán, R., & Fischer, G. W. (1961). The genus *Tilletia*. Pullman, WA,Washington State University.

Felsenstein, J. (1984). Distance methods for inferring phylogenies: a justification. *Evolution; international journal of organic evolution*, *38*(1), 16-24. https://doi.org/10.1111/j.1558-5646.1984.tb00255.x

Fischer, G. W. (1952). *Tilletia brevifaciens* sp. nov., causing dwarf bunt of wheat and certain grasses. *Research Studies. Washington State University*, *20*(1), 11-14 pp.

Fischer, G. W. (1956). The species of *Tilletia* on rye. *Research Studies of the State College of Washington*, *24*, 15.

Flor, H. H. (1932). Heterothallism and hybridization in *Tilletia tritici* and *T. levis*. *Journal Of Agricultural Research*, *44*, 9.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol Ecol*, *2*(2), 113-118. https://doi.org/10.1111/j.1365-294x.1993.tb00005.x

Goates, B. J. (1996). Common bunt and dwarf bunt. In R. D. Wilcoxson & E. E. Saari (Eds.), *Bunt and smut diseases of wheat; concepts and methods of disease management*. CIMMYT.

Gurjar, M. S., Aggarwal, R., Jogawat, A., Kulshreshtha, D., Sharma, S., Solanke, A. U., Dubey, H., & Jain, R. K. (2019). De novo genome sequencing and secretome analysis of *Tilletia indica* inciting Karnal bunt of wheat provides pathogenesis-related genes. *3 Biotech*, *9*(6), 219. https://doi.org/10.1007/s13205-019-1743-3

Hardison, J. R., Meiners, J. P., Hoffmann, J. A., & Waldher, J. T. (1959). Susceptibility of Gramineae to *Tilletia contraversa*. *Mycologia*, *51*(5), 656-664. https://doi.org/10.2307/3755895

Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q., & Vinh, L. S. (2017). UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, *35*(2), 518-522. https://doi.org/10.1093/molbev/msx281

Holton, C. S. (1954). Natural hybridization between common and dwarf bunt as related to the problem of delimitation of species of *tilletia* occurring on wheat. *Phytopathology*, *44*(9), 493-493.

Holton, C. S., & Kendrick, E. L. (1956). Problems in the delimitation of species of *Tilletia* occurring on wheat. *Research Studies of the State College of Washington*, *24*, 318 - 325.

Huson, D. H., & Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol*, *23*(2), 254-267. https://doi.org/10.1093/molbev/msj030

Jayawardena, R. S., Hyde, K. D., Jeewon, R., Ghobad-Nejhad, M., Wanasinghe, D. N., Liu, N., Phillips, A. J. L., Oliveira-Filho, J. R. C., da Silva, G. A., Gibertoni, T. B., Abeywikrama, P., Carris, L. M., Chethana, K. W. T., Dissanayake, A. J., Hongsanan, S., Jayasiri, S. C., McTaggart, A. R., Perera, R. H., Phutthacharoen, K., Savchenko, K. G., Shivas, R. G., Thongklang, N., Dong, W., Wei, D., Wijayawardena, N. N., & Kang, J.-C. (2019). One stop shop II: taxonomic update with molecular phylogeny for important phytopathogenic genera: 26–50 (2019). *Fungal Diversity*, *94*(1), 41-129. https://doi.org/10.1007/s13225-019-00418-5

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods*, *14*(6), 587-589. https://doi.org/10.1038/nmeth.4285

Katoh, K., Rozewicki, J., & Yamada, K. D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20*(4), 1160-1166. https://doi.org/10.1093/bib/bbx108

Kemler, M., Lutz, M., Göker, M., Oberwinkler, F., & Begerow, D. (2009). Hidden diversity in the non-caryophyllaceous plant-parasitic members of *Microbotryum* (Pucciniomycotina: Microbotryales). *Systematics and Biodiversity*, *7*(3), 297-306. https://doi.org/10.1017/s1477200009990028

Kruse, J., Mishra, B., Choi, Y. J., Sharma, R., & Thines, M. (2017). New smut-specific primers for multilocus genotyping and phylogenetics of Ustilaginaceae. *Mycological Progress*, *16*(9), 917-925. https://doi.org/10.1007/s11557-017-1328-7

Kruse, J., Pia Tek, M., Lutz, M., & Thines, M. (2018). Broad host range species in specialised pathogen groups should be treated with suspicion - a case study on *Entyloma* infecting *Ranunculus*. *Persoonia*, *41*, 175-201. https://doi.org/10.3767/persoonia.2018.41.09

Kühn, J. (1874). *Tilletia controversa*. In *Hedwigia* (Vol. 13). C. Heinrich.

Kumar, A., Mishra, P., Maurya, R., Mishra, A. K., Gupta, V. K., Ramteke, P. W., & Marla, S. S. (2018). Improved draft genome sequence of a monoteliosporic culture of the karnal bunt (*Tilletia indica*) pathogen of wheat. *Genome Announc*, *6*(20), e00015-00018. https://doi.org/10.1128/genomeA.00015-18

Kumar, A., Pandey, V., Singh, M., Pandey, D., Saharan, M. S., & Marla, S. S. (2017). Draft genome sequence of Karnal bunt pathogen (*Tilletia indica*) of wheat provides insights into the pathogenic mechanisms of quarantined fungus. *PLoS One*, *12*(2), e0171323. https://doi.org/10.1371/journal.pone.0171323

Larget, B., & Simon, D. (1999). Markov chasin monte carlo algorithms for the bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, *16*(6), 750-750. https://doi.org/10.1093/oxfordjournals.molbev.a026160

Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics*, *30*(22), 3276-3278. https://doi.org/10.1093/bioinformatics/btu531

Lefort, V., Desper, R., & Gascuel, O. (2015). FastME 2.0: a comprehensive, accurate, and fast distance-based phylogeny inference program. *Molecular Biology and Evolution*, *32*(10), 2798-2800. https://doi.org/10.1093/molbev/msv150

Letunic, I., & Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research*, *47*(W1), W256-W259. https://doi.org/10.1093/nar/gkz239

Li, Y.-M., Shivas, R. G., & Cai, L. (2014). Three new species of *Tilletia* on Eriachne from north-western Australia. *Mycoscience*, *55*(5), 361-366. https://doi.org/10.1016/j.myc.2013.12.003

McDonald, J. G., Wong, E., & White, G. P. (2000). Differentiation of *Tilletia* species by rep-PCR genomic fingerprinting. *Plant Dis*, *84*(10), 1121-1125. https://doi.org/10.1094/PDIS.2000.84.10.1121

Mirarab, S., & Warnow, T. (2015). ASTRAL-II: coalescent-based species tree estimation with many hundreds of taxa and thousands of genes. *Bioinformatics (Oxford, England)*, *31*(12), i44-i52. https://doi.org/10.1093/bioinformatics/btv234

Mishra, P., Maurya, R., Gupta, V. K., Ramteke, P. W., Marla, S. S., & Kumar, A. (2019). Comparative genomic analysis of monosporidial and monoteliosporic cultures for unraveling the complexity of molecular pathogenesis of *Tilletia indica* pathogen of wheat. *Sci Rep*, *9*(1), 8185. https://doi.org/10.1038/s41598-019-44464-0

Nguyen, H. D. T., Sultana, T., Kesanakurti, P., & Hambleton, S. (2019). Genome sequencing and comparison of five *Tilletia* species to identify candidate genes for the detection of regulated species infecting wheat. *IMA Fungus*, *10*(1), 11. https://doi.org/10.1186/s43008-019-0011-9

Nguyen, L. T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*, *32*(1), 268-274. https://doi.org/10.1093/molbev/msu300

Niemann, E. (1954). Zwergsteinbrand bei Roggen und Gerste nach kunstlicher Infektion mit verschiedenen *Tilletia*-Arten (Dwarf bunt of rye and barley with respect to artificial infection with various *Tilletia* species). *Zeitschrift fur Pflanzenbau und Pflanzenschutz 5*, 168-172.

Niemann, E. (1956). Taxonomie und Keimungsphysiologie der *Tilletia*-Arten von Getreide und Wildgräsern (Taxonomy and germination physiology of the *Tilletia* species of cereals and wild grasses). *Phytopathologische Zeitschrift*, *28*, 53.

Piatek, M., Lutz, M., & Chater, A. O. (2013). Cryptic diversity in the *Antherospora vaillantii* complex on *Muscari* species. *IMA Fungus*, *4*(1), 5-19. https://doi.org/10.5598/imafungus.2013.04.01.02

Pizarro, D., Divakar, P. K., Grewe, F., Leavitt, S. D., Huang, J. P., Dal Grande, F., Schmitt, I., Wedin, M., Crespo, A., & Lumbsch, H. T. (2018). Phylogenomic analysis of 2556 single-copy protein-coding genes resolves most evolutionary relationships for the major clades in the most diverse group of lichen-forming fungi. *Fungal Diversity*, *92*(1), 31-41. https://doi.org/10.1007/s13225-018-0407-7

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLOS ONE*, *5*(3), e9490. https://doi.org/10.1371/journal.pone.0009490

Purdy, L. H., Kendrick, E. L., Hoffmann, J. A., & Holton, C. S. (1963). Dwarf bunt of wheat. *Annu. Rev. Microbiol.*, *17*(1), 199-222. https://doi.org/10.1146/annurev.mi.17.100163.001215

Rannala, B., & Yang, Z. (2003). Bayes estimation of species divergence times and ancestral population sizes using dna sequences from multiple loci. *Genetics*, *164*(4), 1645-1656.

Roberson, R. W., & Luttrell, E. S. (2018). Dolipore septa in *Tilletia*. *Mycologia*, *81*(4), 650-652. https://doi.org/10.1080/00275514.1989.12025800

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol*, *61*(3), 539-542. https://doi.org/10.1093/sysbio/sys029

Russell, B. W. (1994). Morphological, physiological, and genetic evidence in support of a conspecific status for *Tilletia caries, T. controversa*, and *T. foetida*. *Phytopathology*, *84*(6), 576-582. https://doi.org/10.1094/Phyto-84-576

Russell, B. W., & Mills, D. (1993). Electrophoretic karyotypes of *Tilletia caries*, *T. controversa*, and their F1 progeny: further evidence for conspecific status. *Molecular plant-microbe interactions : MPMI*, *6*(1), 66-74. https://doi.org/10.1094/mpmi-6-066

Savchenko, K. G., Carris, L. M., Castlebury, L. A., Heluta, V. P., Wasser, S. P., & Nevo, E. (2014). Stripe smuts of grasses: one lineage or high levels of polyphyly? *Persoonia*, *33*, 169-181. https://doi.org/10.3767/003158514X685202

Sayyari, E., & Mirarab, S. (2016). Fast coalescent-based computation of local branch support from quartet frequencies. *Molecular Biology and Evolution*, *33*(7), 1654-1668. https://doi.org/10.1093/molbev/msw079

Schuhmann, G. (1960). Kunstliche Infektion von Roggensorten und Grasern mit Zwergsteinbrand (Artificial infection of rye varieties and grasses with dwarf bunt). *Bayerische Landwirt*, *38*, 3.

Sharma, P., Tiwari, R., Saharan, M. S., Sharma, I., Kumar, J., Mishra, S., Muthusamy, S. K., Gupta, R. K., Jaiswal, S., Iquebal, M. A., Angadi, U. B., Kumar, N., Fatma, S., Rai, A., & Kumar, D. (2016). Draft genome sequence of two monosporidial lines of the karnal bunt fungus *Tilletia indica* Mitra (PSWKBGH-1 and PSWKBGH-2). *Genome Announc*, *4*(5), e00928-00916. https://doi.org/10.1128/genomeA.00928-16

Shi, Y. L., Loomis, P., Christian, D., Carris, L. M., & Leung, H. (1996). Analysis of the genetic relationships among the wheat bunt fungi using RAPD and ribosomal DNA markers. *Phytopathology*, *86*(3), 311-318. https://doi.org/DOI 10.1094/Phyto-86-311

Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210-3212. https://doi.org/10.1093/bioinformatics/btv351

Soreng, R. J., Peterson, P. M., Romaschenko, K., Davidse, G., Teisher, J. K., Clark, L. G., Barberá, P., Gillespie, L. J., & Zuloaga, F. O. (2017). A worldwide phylogenetic classification of the Poaceae (Gramineae) II: An update and a comparison of two 2015 classifications. *Journal of Systematics and Evolution*, *55*(4), 259-290. https://doi.org/https://doi.org/10.1111/jse.12262

Stewart, J. E., Andrew, M., Bao, X., Chilvers, M. I., Carris, L. M., & Peever, T. L. (2013). Development of sequence characterized amplified genomic regions (SCAR) for fungal systematics: proof of principle using *Alternaria*, *Ascochyta* and *Tilletia*. *Mycologia*, *105*(4), 1077-1086. https://doi.org/10.3852/12-287

Vanky, K. (2008). Smut fungi (*Basidiomycota* p.p., *Ascomycota* p.p.) of the world. novelties, selected examples, trends. *Acta Microbiol Immunol Hung*, *55*(2), 91-109. https://doi.org/10.1556/AMicr.55.2008.2.2

Vánky, K. (1994). European smut fungi. Gustav Fischer. http://dx.doi.org/10.1002/fedr.19961070103

Vánky, K. (2012). Smut fungi of the world.American Phytopathological Society.

Wägele, J. W., & Mayer, C. (2007). Visualizing differences in phylogenetic information content of alignments and distinction of three classes of long-branch effects. *BMC Evolutionary Biology*, *7*(1), 147. https://doi.org/10.1186/1471-2148-7-147

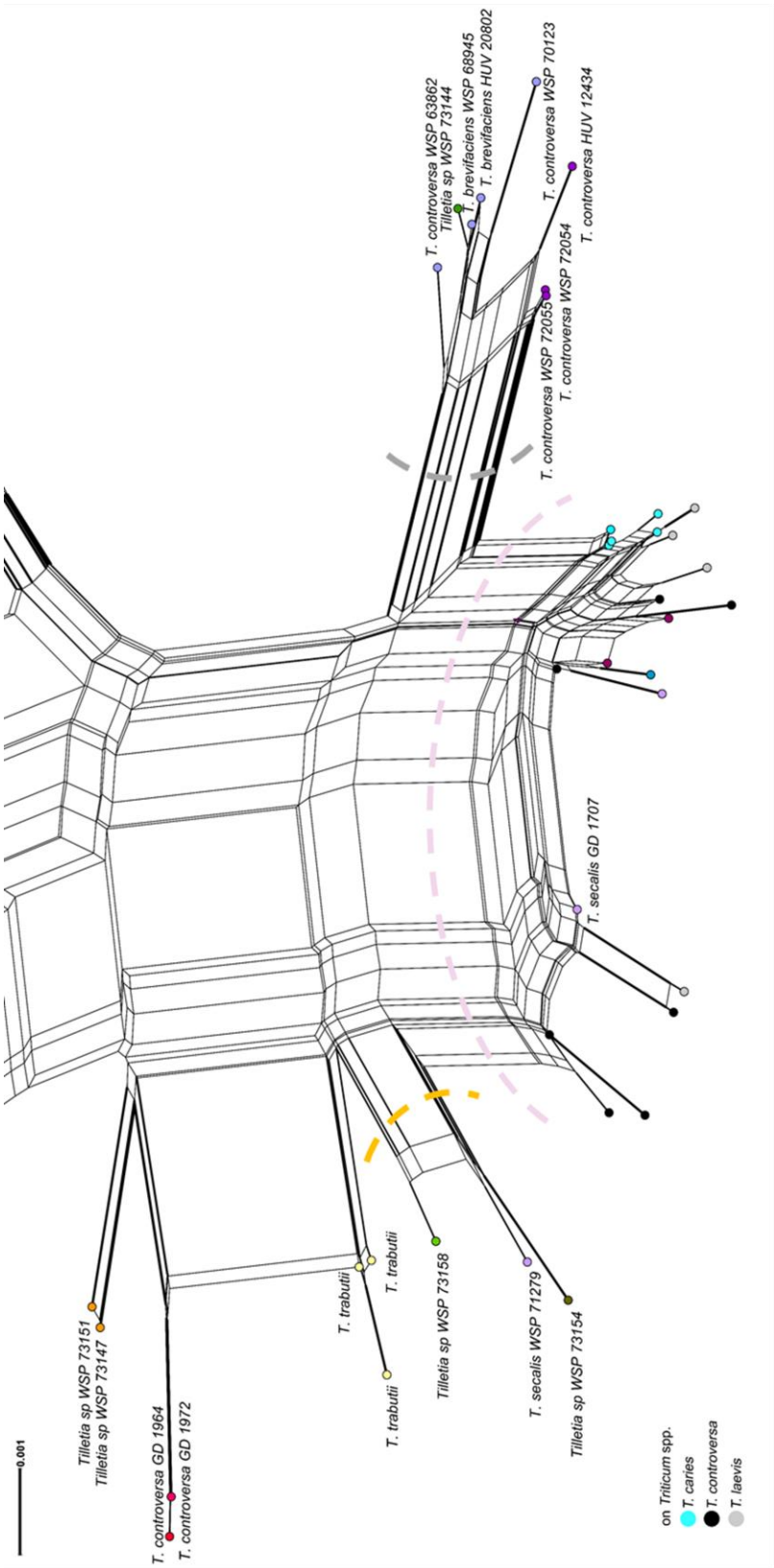
Wang, N., Ai, P., Tang, Y., Zhang, J., Dai, X., Li, P., & Zheng, A. (2015). Draft genome sequence of the rice kernel smut *Tilletia horrida* Strain QB-1. *Genome Announc*, *3*(3), e00621-00615. https://doi.org/10.1128/genomeA.00621-15

Waterhouse, R. M., Tegenfeldt, F., Li, J., Zdobnov, E. M., & Kriventseva, E. V. (2013). OrthoDB: a hierarchical catalog of animal, fungal and bacterial orthologs. *Nucleic acids research*, *41*(Database issue), D358-365. https://doi.org/10.1093/nar/gks1116

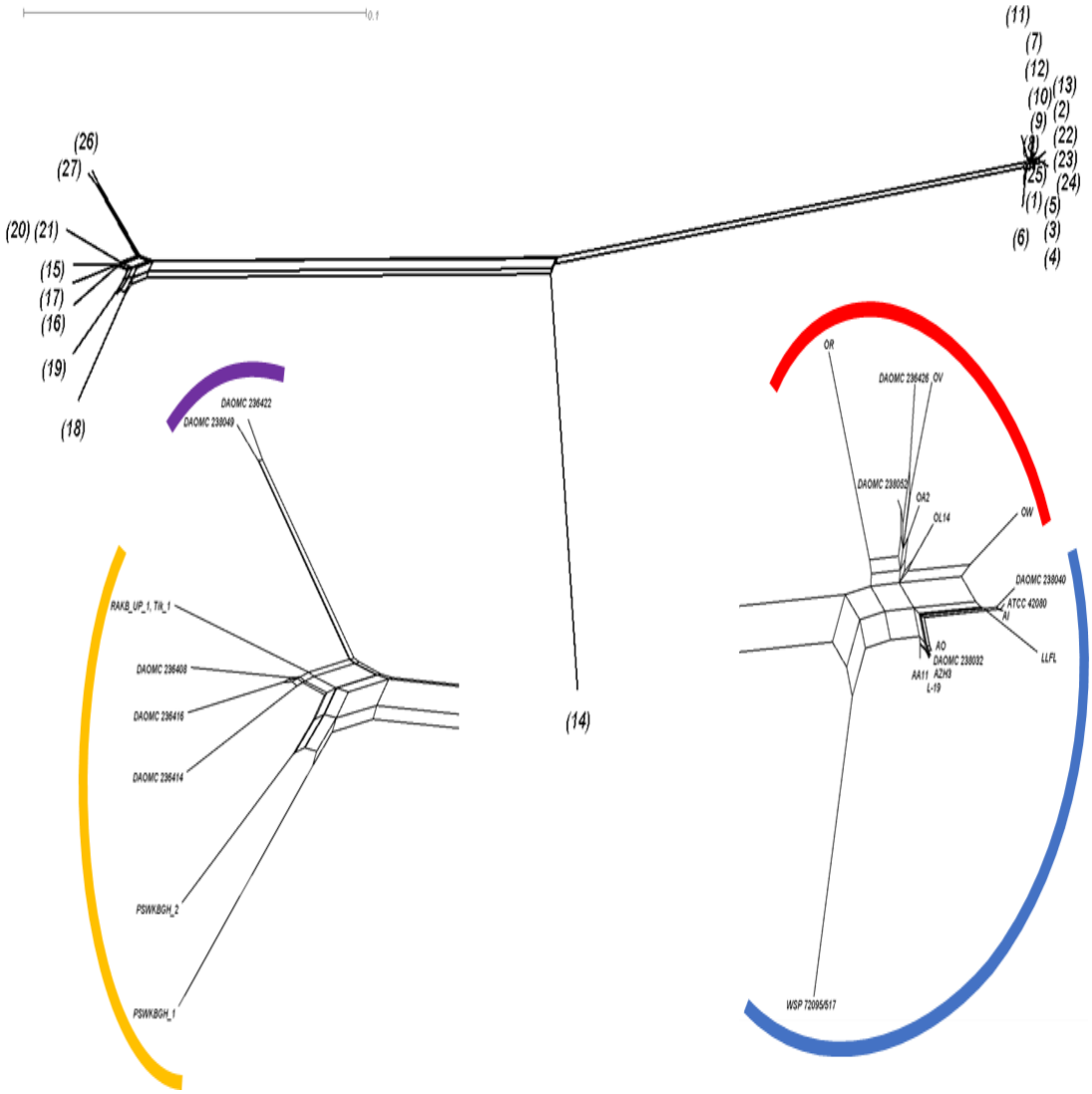
White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal rna genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR Protocols* (pp. 315-322). Academic Press. https://doi.org/10.1016/b978-0-12-372180-8.50042-1

Ziegler, R., Lutz, M., Piatek, J., & Piatek, M. (2018). Dismantling a complex of anther smuts (*Microbotryum*) on carnivorous plants in the genus *Pinguicula*. *Mycologia*, *110*(2), 361-374. https://doi.org/10.1080/00275514.2018.1451697

Zupunski, V., Ignjatovic-Micic, D., Nikolic, A., Stankovic, S., Jevtic, R., Levic, J., & Ivanovic, D. (2011). Identification of *Tilletia* species using REP-PCR fingerprinting technique. *Genetika-Belgrade*, *43*(1), 183-195. https://doi.org/10.2298/Gensr1101183z



Additional Figure 4-1 Neighbor-Net showing relation of *Tilletia* species using concatenated EF1α, ITS, and RPB2 sequences. More than 50% confidence on the edges are shown in thickened line. Scale bar indicates number of nucleotide substitutions per site. The groupings recognized by phylogeny is congruently recognized by Neighbor-Net network analysis. No resolution is observed between *T. caries*, *T. controversa*, and *T. laevis* (separated by dash lines in pink). The network suggests that there may be substructure present in *T. brevifaciens* lineage (separated by dash lines gray and *T. secalis* (separated by dash lines in yellow). Non wheat hosts are color coded identical to Figure 4-1.



Additional Figure 4-2 Visualization of incongruent splits among the used phylogenomic data by planar Neighbor-Net network (SplitsTree4). The concatenated analysis of 416,222 aligned nucleotides of 241 conserved loci from 27 isolates of six *Tilletia* species to highlight the character conflicts. The scale bar represents the number of character-state changes. The numbers correspond to the specimens in Table 3. Below a snapshot of the two wings of the network. Purple, yellow, red, and blue is representing isolates of *T. walkeri*, *T. indica*, *T. controversa*, and *T. caries* together with *T. laevis* (common bunt causal agents). Number (14) corresponds to the single representative of *T. horrida*. The Neighbor-Net is in agreement with Astrall II result in recognition of five groups and no structure is observed in the common bunt fungi network corresponding to each species.

Chapter 5. General discussion

After successful control of common and dwarf bunt by effective chemical seed treatments, the diseases were almost forgotten for decades, as the damage caused by these diseases in conventional farming was minor (Rudloff et al., 2020). This changed over the last few years after the European law for organic wheat production was amended in a way that conventionally produced (chemically treated) seeds were not any longer permitted to be used in organic farming. This shift resulted in the resurgence of bunt diseases, especially under favorable climate conditions in low-input and organic farming (Borgen and Davanlou, 2001; Matanguihan et al., 2011). In the meanwhile, however, the knowledge about the causal agents (*T. caries*, *T. controversa*, and *T. laevis*) remained limited and published literature was scarce. This thesis was designed to gain insights into inter- and intraspecies genomic variation of these three reemerging pathogens which may explain their partial ecological and physiological differences, and their phylogenetic and phylogenomic relationships through genome sequencing. Moreover, one specific aim was to develop a LAMP assay for the detection of *T. controversa* from teliospores and pure culture. I have highlighted the most important findings of this thesis below.

5. 1. Insight into genomic features of wheat common and dwarf bunts

5. 1. 1. Genomic features of the three species are very similar

In this study, the whole genomes of five isolates of *T. controversa*, four of *T. caries*, and two of *T. laevis* were sequenced, *de novo* assembled, and *in silico* functionally annotated. All isolates except one originated from recent European populations. Together with five recently published genomes (one *T. caries*, two *T. controversa*, and two *T. laevis* isolates) mostly collected from the Northern United states (Nguyen et al., 2019), they were compared for the variety of different genomic features at inter- and intraspecies level. Due to the relatively large genomic sample, the composition of genomes as well as genomic differences among these important wheat diseases could be examined on such a broad scale for the first time.

The results in chapter I suggests that the causal agents of common and dwarf bunt were very similar in many genomic features, such as the proportion of repetitive elements including the content of simple sequence repeats and transposable elements, the genome size, the number of protein-coding genes and tRNA-genes. Protein-coding genes also did not differ in their codon usage between the three species. The genomes displayed a generally high synteny and could be aligned by 82.7% to 94.3% of their size (excluding repetitive regions). In line with the observation of Nguyen et al. (2019),

the causal agents of common and dwarf bunt also shared high average nucleotide identity (>98.7%) within the aligned regions. The high genomic similarity among the species is also supported by several other lines of evidence such as electrophoretic karyotypes, electrophoretic patterns of phenole-soluble peptides, DNA fingerprinting, and MALDI TOF-MS analyses of teliospores proteins (Forster et al. under review; Kawchuk et al., 1988; Russell, 1994; Russell and Mills, 1993; Shi et al., 1996). The extensive genomic conservation and whole-genome macrosyntheny provide further evidence that the three bunt species have a common ancestor (Carris et al., 2007) and raise the question whether they can be regarded as three distinct species at all.

The prediction of genomic functions in the three species revealed that the identified biological pathways and functional categories were remarkably similar across the three species. For example, among the putative secondary metabolite gene clusters, which were predicted in *T. caries*, *T. controversa*, and *T. laevis* for the first time, nine gene clusters comprising 65 genes were highly conserved across the three species showing >95% identity and similar gene arrangements. Almost half of the total predicted secreted proteins (up to 519 proteins per species) were conserved and shared across all 16 studied isolates. The remaining predicted secreted proteins were also shared between the three species, but they were not present in all isolates. A small proportion of the *T. caries*, *T. controversa*, and *T. laevis* secreted proteins were made up of effector-like proteins (maximum detection of 144 proteins), of which 47% were shared and conserved across the 16 studied isolates. None of the effector-like proteins was species-specific. The three biotrophic fungi encoded for a limited number of enzymes for plant cell wall decomposition and starch catabolism (*T. caries* isolates with 189 to 212 proteins, *T. controversa* isolates with 188 to 213 proteins, *T. laevis* isolates with 191 to 209), of which 84% were shared and conserved among the 16 studied isolates showing carbohydrate-active enzyme content was very similar across the three species.

5. 1. 2. Variable content of repetitive elements within the three species isolates

In general, the proportion of repetitive elements within the isolates of *T. caries* varied from 7.8% to 13.7% of the total genome size, in *T. controversa* from 8.9% to 37.7% and *T. laevis* isolates from 9.1% to 11.8%. This diversity however did not result in differences between the three species genome size excluding the draft genome of one *T. controversa* isolate with 49 Mb genome size. Moreover, five already published *Tilletia* genomes collected from Northern United States had a significantly lower number of several TE superfamilies compared to the genomes sequenced in this work. This observation raised the question whether the difference in the abundance of TE was due to an unequal amount of sequencing data, differences in the read lengths, or was related to the

geographical origin of the isolates. Differences in the proportion of TE among isolates of fungal species had previously been shown previously (Badet et al., 2020; Le Cam et al., 2019; Lorrain et al., 2020). For example, in *Zymoseptoria tritici* the causal agent of Septoria leaf blotch, the TE amounted from 17 - 24% of the genome studying 19 isolates (Badet et al., 2020). Furthermore, Oggenfuss et al. (2020) showed a substantial genome-wide expansion of certain TE families from isolates in the pathogen's center of origin to more recently founded populations. Sequencing of more samples from different geographical origins is needed to reveal whether geographical origin plays a role in the diversity of TE in the causal agents of common and dwarf bunt.

5. 1. 3. *Tilletia controversa* has the highest intraspecies genomic variation and highest genetic diversity compared to *T. caries* and *T. laevis*

We showed that *T. controversa* had the highest genomic diversity compared to *T. caries* and *T. laevis*. These results were based on single nucleotide polymorphisms (SNP)s and small insertions or deletions (indel)s in aligned proportions of the genomes. Between the two species of *T. caries* and *T. laevis*, the genome-wide genetic identity was very high which resulted in equal genetic distances between *T. controversa* and *T. caries* and also *T. controversa* and *T. laevis*. In line with this observation, hierarchical clustering analysis based on *k*-mer comparisons of the whole genomes failed to separate isolates of *T. caries* from *T. laevis,* while isolates of *T. controversa* were clustered separately. Moreover, we identified only seven genes specific to *T. caries* and ten genes specific to *T. laevis*, while when both were taken together the number of genes specific for the two (*T. caries* and *T. laevis*) in comparison with *T. controversa* increased to 40. The limited number of species-specific genes and the highly identical gene content are in line with the fact that *T. caries* and *T. laevis* are the causal agents of the common bunt disease, causing identical disease symptoms and sharing identical requirements for teliospore germination. Separation of dwarf bunt from common bunt rather than from each bunt species was in agreement with the results obtained by randomly amplified polymorphic DNA (RAPD) analysis done by Shi et al., (1996). They, however, reported that some dwarf and common bunt individuals represent reciprocal characteristics of both. We did not find such a group in our limited number of compared genomes and suppose that discrepancy can be explained by the well-known low reliability of RAPD, which should not be used for serious work. All in all, due to the limited number of species-specific genes and overall high genomic synteny, SNPs and indels may be the key factors that are significant in the differences between these species.

5. 2. Insight into the genomic diversification of *Tilletia* species

The majority of functionally well-characterized genes in other members of *Ustilaginomycotina*, which play a role in pathogenicity, virulence, and life cycle (Benevenuto et al., 2018; Skibbe et al., 2010), either lacked homologs in the proteomes of common and dwarf bunt or the protein sequences were poorly conserved, indicating that *Tilletia* spp. has developed a unique mechanism for infection and pathogenicity. Compared to necrotrophic and saprotrophic fungi, genomes of the three biotrophic species encode relatively few secreted proteins, which is in line with the expectation that they underly selection limiting the damage of plant cells, which can trigger hypersensitive reactions and other defense responses (Girard et al., 2013). Moreover, common and dwarf bunt lacked many core enzymes for plant cell wall decomposition and starch catabolism. In CAZyme comparison of a broad set of Ustilagomycotina where members of *Tilletia* were missing, Kijpornyongpan et al. (2018) reported that Ustilagomycotina harbored enzymes GH5_16 (β-1,6-galactanase), GH8, GH42 (β -galactosidase), GT34 (α-galactosyltransferase), and AA10 (lytic polysaccharide monooxygenase) that are absent from other members of Basidiomycota. We reported the presence of the GH8 enzyme family (hydrolases with broad activities) in *Tilletia* spp. only and the others were absent in all 16 studied isolates. In addition, *Tilletia* spp. harbored gene families coding for PL14 and AA2 enzymes that are involved in lignin decomposition, which were completely absent in other studied Ustilagoinmycotina, but present in Agaricomycotina (Kijpornyongpan et al., 2018). Putative genes encoding for PL14 and AA2 were also reported from *T. indica* CAZyme analyses (Gurjar et al., 2019).

Common and dwarf bunt fungi are known to produce trimethylamine, which is responsible for the fishy odor of the teliospores (Hanna et al., 1932; Nielsen, 1963). In the present study, the genes involved in the trimethylamine production identified in bacteria (Craciun and Balskus, 2012) could not be identified in common and dwarf bunt. Therefore, we hypothesize that the trimethylamine synthesis pathway in *Tilletia* species is different from those known from bacteria. We also reported the lack of those genes in *Ustilago maydis,* the model organism with a nearly complete assembled genome up to the chromosome level (Kamper et al., 2006).

5. 3. Genomic comparison of six *Tilletia* species for finding DNA segments specific to *T. controversa* and the lack of DNA segments specific for *T. caries* or *T. laevis*

The whole-genome comparison approach generates a large number of species-specific candidate regions for the wet lab testing and validation and is quickly becoming the preferred option (Karim et al., 2019; Thomsen et al., 2017). It has especially become a method of choice for developing

markers between closely related species (Behr et al., 2016) or subspecies (Burbank and Ortega, 2018) where the genomic differences are minor. *K*-mer-based (based on a sequence's subsequences of length) whole-genome comparisons which include intergenic regions too, facilitate the identification of specific loci without prior knowledge of their function.

The lack of suitable species-specific genomic loci has hampered the development of *T. controversa* detection assay. The lack of suitable loci was mostly due to the very close phylogenetic relationship of *T. controversa* to *T. caries* and *T. laevis* (Bao et al., 2010; Carris et al., 2007; Castlebury et al., 2005; Jayawardena et al., 2019). Here, a whole-genome comparison approach based on *k*-mers of whole genomes was applied employing six *Tilletia* species (*T. caries*, *T. controversa*, *T. horrida*, *T. indica, T. laevis*, and *T. walkeri*) to find regions unique to and conserved in *T. controversa,* suitable for the development of a LAMP assay. Using this approach, 22 DNA segments longer than 1500 bp were selected *in silico* and screened for their specificity in the lab.

Employing the same approach, it was found that DNA regions suitable for specie-specific LAMP assays were lacking from both *T. caries* and *T. laevis*. The number of extracted DNA segments was very low (*T. caries* 16 segments, and *T. laevis* 43 segments), and they were too short (N50 = 39 bp for both species) for the development of the LAMP assays (optimum of >200 bp). Therefore, the development of LAMP assays for species-specific detection of *T. caries* and *T. laevis* appeared difficult. However, when common bunt causal agents were taken together, DNA segments suitable for a LAMP assay to specifically detect common bunt fungi were found. Especially the top 11 candidate regions and their genes could be used for the development of any DNA-base assay including LAMP.

5. 4. DNA-based identification of *T. controversa* by loop-mediated isothermal amplification (LAMP) and assay validation in an interlaboratory performance study

We established a LAMP assay to detect *T. controversa* DNA using an anonymous locus with a visual readout using neutral red as an indicator dye. The differentiation of *T. controversa*, as a regulated pathogen, from several other *Tilletia* species such as *T. caries*, *T. trabutii* (on *Hordeum* sp.), *T. brevifaciens* (on *Thinopyrum intermideum*) and *T. secalis* (on *Secale cereale*) based solely on morphological features of the teliospores is difficult to impossible. This is due to overlaps in the teliospores morphology of these species with highly variable teliospores of *T. controversa* (Bao, 2010; Carris, 2008; Fischer, 1952; Holton, 1954; Holton and Kendrick, 1956). For instance, the differentiation of *T. secalis* from *T. controversa* based on solely morphological features of teliospores is not possible at all (Niemann, 1954; Niemann, 1956) while it has been suggested that the two species are phylogenetically distinct

(Carris, 2008; Carris et al., 2007). These studies, however, suffered from a limited number of isolates used.

LAMP technique has been successfully applied for the detection of a variety of important clinical organisms as well as quarantine ones such as bacteria (Aglietti et al., 2019; Boehme et al., 2007; Kuboki et al., 2003), fungi (Stehlíková et al., 2020; Tan et al., 2016), nematodes (Zhang and Gleason, 2019) and viruses (Lee et al., 2015; Yan et al., 2020). The simplicity and ease of use make LAMP the method of choice for high throughput applications. The developed LAMP assay was optimized for 45 min at 65 °C using betaine and four primers with a colorimetric end-point readout. Afterward, the LAMP assay was successfully validated against 223 fungal samples, of which 132 were common bunt fungi and 39 were *T. controversa* samples. To the best of my knowledge, this study used the broadest sample collections among all assays developed for *T. controversa* detection. Due to the high degree of genetic similarity of *T. caries* and *T. laevis* to *T. controversa* shown in this study, a broad collection of common bunt fungi was also used to evaluate the specificity of the assay. The LAMP assay developed in this study accurately identified all *T. controversa* samples (n=39) with no false positive of common bunt. However, we reported cross amplification of *T. secalis* (GD 1707) and *T. trabutii* (HUV 15036/ WSP 71299). The only representative of *T. secalis* used in this study should be taken cautiously. Our phylogenetic analysis showed that the sample was phylogenetically similar to *T. controversa* collected from wheat. Therefore, a well-characterized specimen of *T. secalis* is needed to confirm the cross amplification. *Tilletia bromi* is morphologically and biologically similar to *T. controversa* and is reproductively compatible under artificial conditions (Pimentel et al., 2000a; Pimentel et al., 2000b). We did not have access to any *T. bromi* sample; therefore, the specificity of the LAMP assay toward this species could not be estimated.

Finally, the developed LAMP assay was validated in an interlaboratory performance study involving five national plant protection agencies and seed testing laboratories. The results showed 100% sensitivity and 97.7% specificity of the test. The only false positive result in one of the labs, most likely was due to cross contamination. The values obtained suggesting that it has potential for application in seed testing, for example in wheat export and import control.

5. 5. *Tilletia controversa* representing a species complex with hidden diversity and a parasite with a broad host range

As quoted by Taylor et al., (2000) "understanding the nature of species' boundaries is a fundamental question in evolutionary biology". The majority of species within the genus *Tilletia* are described based on a combination of their host specificities, and by their distinguishable

morphological characters especially teliospore and sterile cell ornamentation and sizes (Vánky, 2012). *Tilletia controversa* unlike other *Tilletia* species, was recognized as a broad host range pathogen By Durán and Fischer (1956) and Hardison et al., (1959), However, the phylogenetic relationship between *T. controversa* isolates obtained from grasses and the isolates from *Triticum* spp. has not been addressed before. To clarify whether they are indeed generalist species with a broad host range or rather represent complexes of cryptic species with narrow host ranges phylogenetic relationships of those species and close relatives were inferred using sequences of three gene regions (ITS rDNA, *EF1a, RPB2*).

In general, the analysis resolved various species with narrow host ranges parasitizing wild grasses as distinct lineages (i.e. the basal lineages from *T. fusca* up to *T. olida* representing 12 species and the crown lineage with *T. bromi* and *T. puccinelliae*, respectively). The analysis however failed to separate *T. controversa* collected from wheat from the samples obtained from other hosts such as *Agropyrum cristatum*, *Bromus marginarus*, *Secale creale*, and *Th. intermedium,* suggesting the ability of *T. controversa* to infect different grasses genera is conserved. The artificial cross-infection of *T. controversa* comprises a broad number of hosts, yet the occurrence of dwarf bunt on wild grasses is limited (Purdy et al., 1963). Whether these grasses play a role as an inoculum source for wheat infection in nature is a phytopathologically important question, which remains to be answered.

The current phylogenetic analysis reconfirmed that *T. brevifaciens* on *Th. intermedium* is distinct from *T. controversa* collected from wheat since *T. brevifaciens* did not cluster with any of the phylogenetic groups comprising *T. controversa* obtained from *Triticum* spp. *Tilletia brevifaciens* was reclassified as a species different from *T. controversa* based on multilocus phylogenetic study and several lines of evidence (number of basidiospores per basidium and temperature requirements for teliospore germination) by Carris et al. (2007). Our multilocus phylogeny was in line with this reclassification. Within this group samples of *T. controversa* from *Elymus* spp. also clustered; however, our Neighbor-Net analysis suggested that this cluster may comprise several host-specific lineages. More variable loci and additional samples are needed to gain a better understanding of the host spectrum of *T. brevifaciense.*

We showed that the lack of distinguishable morphological characters lumped genetically isolated groups into morphological species such as in the two samples collected from *Alopecurus myosuroides*. Both samples were originally identified as *T. controversa.* While the differences in morphological characters of teliospores to *T. controversa* were hard to define, the two samples were resolved as phylogenetically distinct from *T. controversa* from wheat. Additional features such as the number and nuclear condition of primary basidiospores and the ability of primary basidiospores to

conjugate and form infective dikaryon (Carris et al., 2007; Castlebury et al., 2005) would be needed to physiologically differentiate these two samples from *T. controversa* while the teliospores morphological features alone failed. This was not possible due to the age of the specimens.

We should be aware that the species concept and boundaries shown here may change after additional samples are examined. Moreover, isolates from several of the proposed *T. controversa* host species have not been studied yet using DNA-based approaches. Our study provides molecular evidence that morphologically dissimilar smut species (e.g. *T. caries* and *T. laevis*) may be genetically closer then species which are morphologically similar but obtained from different hosts such as *T. controversa* collected from *Triticum* spp. and the *Tilletia* samples obtained from *Alopecurus myosuroides* or *T. secalis* from *S. cereale)*. The assumption has been proposed by Huang and Nielsen, 1984 and Nielsen, 1968.

5. 6. Broader genomic sampling suggests conspecificity of *T. caries* and *T. laevis*

Genome data provide utmost genetic information for the estimation of evolutionary relationships among organisms (Misof et al., 2013; Spatafora et al., 2017). Conserved single-copy genes are of high importance for inferring the phylogeny of eukaryota (Ren et al., 2016). We used OrthoDB v.9.1 (Zdobnov et al., 2016) database of 303 orthologs (www.orthodb.org) as reference for the identification of homologous single-copy genes in *Tilletia* spp. genome sequences. We inferred the phylogenomic relation of 27 *Tilletia* isolates (six *T. caries*, seven *T. controversa*, one *T. horrida*, seven *T. indica*, four *T. laevis*, and two *T. walkeri*) based on 241 out of the total 303 single-copy orthologs. Using a summary of multispecies coalescent approach (Rannala and Yang, 2003), five lineages were recognized. In one lineage, we obtained a well-supported phylogeny for the separation of dwarf bunt (*T. controversa*) isolates from common bunt (*T. caries* and *T. laevis*). It is unlike the previous studies that suggested the conspecificity of the two bunts by assessing electrophoretic karyotypes and morphological characters of the three species (Russel, 1993; Russell, 1994; Russell and Mills, 1993). In our phylogenomic analysis, five *T. caries* and four *T. laevis,* which were collected from four continents, could not be separated from each other. We therefore suggested that the isolates, which exhibited two morphotypes, were either conspecific or have just recently diverged. This finding was in an agreement with other results obtained in our studies, such as very few species-specific genes to each of common bunt agents, the lack of DNA segments (including intergenic regions) specific to each species, the high genomic identity of *T. caries* and *T. laevis*, and hierarchical clustering of the genomes based on *k*-mer comparisons. Nguyen et al. (2019) analyzing 4,896 single-copy orthologous

genes and testing a limited number of isolates (one *T. caries* and two *T. laevis* isolates) suggested that *T. caries* and *T. laevis* are two distinct species. We could not confirm this finding using broader genomic sampling while analyzing fewer loci.

Finally, *T. indica* (causal agent of Karnal bunt) and *T. walkeri* (causal agent of ryegrass smut) grouped together, confirming their close relationship among them (Castlebury and Carris, 1999; Nguyen et al., 2019; Tan et al., 2016). The position of the single isolate of *T. horrida* used in this study remained unclear. Additional samples of this species will be required to investigate relationship of *T. horrida* to other studied *Tilletia* species.

5. 7. References

Aglietti C., Luchi N., Pepori A.L., Bartolini P., Pecori F., Raio A., Capretti P., Santini A. (2019) Real-time loop-mediated isothermal amplification: an early-warning tool for quarantine plant pathogen detection. *AMB Express* 9:50. DOI: 10.1186/s13568-019-0774-9.

Badet T., Oggenfuss U., Abraham L., McDonald B.A., Croll D. (2020) A 19-isolate reference-quality global pangenome for the fungal wheat pathogen *Zymoseptoria tritici*. *BMC Biology* 18:12. DOI: 10.1186/s12915-020-0744-3.

Bao X. (2010) Host specificity and phylogenetic relationships among *Tilletia* species infecting wheat and other cool season grasses, PhD thesis, Department of Plant Pathology, Washington State University.

Bao X., Carris L.M., Huang G., Luo J., Liu Y., Castlebury L.A. (2010) *Tilletia puccinelliae*, a new species of reticulate-spored bunt fungus infecting *Puccinellia distans*. *Mycologia* 102:613-23. DOI: 10.3852/09-135.

Behr J., Geissler A.J., Schmid J., Zehe A., Vogel R.F. (2016) The identification of novel diagnostic marker genes for the detection of beer spoiling *Pediococcus damnosus* strains using the BlAst diagnostic gene findEr. *Plos One* 11:e0152747. DOI: ARTN e015274710.1371/journal.pone.0152747.

Benevenuto J., Teixeira-Silva N.S., Kuramae E.E., Croll D., Monteiro-Vitorello C.B. (2018) Comparative genomics of smut pathogens: insights from orphans and positively selected genes into host specialization. *Front Microbiol* 9:660. DOI: 10.3389/fmicb.2018.00660.

Boehme C.C., Nabeta P., Henostroza G., Raqib R., Rahim Z., Gerhardt M., Sanga E., Hoelscher M., Notomi T., Hase T., Perkins M.D. (2007) Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *J Clin Microbiol* 45:1936-40. DOI: 10.1128/jcm.02352-06.

Borgen A., Davanlou M. (2001) Biological control of common bunt (*Tilletia tritici*). *Journal of Crop Production* 3:157-171. DOI: 10.1300/J144v03n01_14.

Burbank L.P., Ortega B.C. (2018) Novel amplification targets for rapid detection and differentiation of *Xylella fastidiosa* subspecies *fastidiosa* and multiplex in plant and insect tissues. *J Microbiol Methods* 155:8-18. DOI: 10.1016/j.mimet.2018.11.002.

Carris L. (2008) Is rye bunt, *Tilletia secalis*, present in North America? *North American Fungi* 3:147-159. DOI: 10.2509/naf2008.003.0078.

Carris L.M., Castlebury L.A., Huang G., Alderman S.C., Luo J., Bao X. (2007) *Tilletia vankyi*, a new species of reticulate-spored bunt fungus with non-conjugating basidiospores infecting species of *Festuca* and *Lolium*. *Mycol Res* 111:1386-98. DOI: 10.1016/j.mycres.2007.09.008.

Castlebury L.A., Carris L.M. (1999) *Tilletia walkeri*, a new species on *Lolium multiflorum* and *L. perenne*. *Mycologia* 91:121-131. DOI: 10.2307/3761200.

Castlebury L.A., Carris L.M., Vinky K. (2005) Phylogenetic analysis of *Tilletia* and allied genera in order Tilletiales (Ustilaginomycetes; Exobasidiomycetidae) based on large subunit nuclear rDNA sequences. *Mycologia* 97:888-900. DOI: 10.3852/mycologia.97.4.888.

Craciun S., Balskus E.P. (2012) Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. *Proc Natl Acad Sci U S A* 109:21307-12. DOI: 10.1073/pnas.1215689109.

Durán R., Fischer G.W. (1956) Further studies on the synonymy and host range of the dwarf bunt fungus. Research Studies of State Collge Washington XXIV:8.

Fischer G.W. (1952) *Tilletia brevifaciens* sp. nov., causing dwarf bunt of wheat and certain grasses. *Research Studies*. Washington State University 20:11-14.

Girard V., Dieryckx C., Job C., Job D. (2013) Secretomes: the fungal strike force. *Proteomics* 13:597-608. DOI: 10.1002/pmic.201200282.

Gurjar M.S., Aggarwal R., Jogawat A., Kulshreshtha D., Sharma S., Solanke A.U., Dubey H., Jain R.K. (2019) De novo genome sequencing and secretome analysis of *Tilletia indica* inciting Karnal bunt of wheat provides pathogenesis-related genes. *3 Biotech* 9:219. DOI: 10.1007/s13205-019-1743-3.

Hanna W.F., Vickery H.B., Pucher G.W. (1932) The isolation of trimethylamine from spores of *Tilletia levis*, the stinking smut of wheat. *Journal of Biological Chemistry* 97:351-358.

Hardison J.R., Meiners J.P., Hoffmann J.A., Waldher J.T. (1959) Susceptibility of Gramineae to *Tilletia contraversa*. *Mycologia* 51:656-664. DOI: Doi 10.2307/3755895.

Holton C.S. (1954) Natural hybridization between common and dwarf bunt as related to the problem of delimitation of species of *Tilletia* occurring on wheat. *Phytopathology* 44:493-493.

Holton C.S., Kendrick E.L. (1956) Problems in the delimitation of species of *Tilletia* occurring on wheat. *Research Studies of the State College of Washington* 24:318 - 325.

Huang H.Q., Nielsen J. (1984) Hybridization of the seedling-infecting *Ustilago* spp. pathogenic on barley and oats, and a study of the genotypes conditioning the morphology of their spore walls. *Canadian Journal of Botany* 62:603-608. DOI: 10.1139/b84-091.

Jayawardena R.S., Hyde K.D., Jeewon R., Ghobad-Nejhad M., Wanasinghe D.N., Liu N., Phillips A.J.L., Oliveira-Filho J.R.C., da Silva G.A., Gibertoni T.B., Abeywikrama P., Carris L.M., Chethana K.W.T., Dissanayake A.J., Hongsanan S., Jayasiri S.C., McTaggart A.R., Perera R.H., Phutthacharoen K., Savchenko K.G., Shivas R.G., Thongklang N., Dong W., Wei D., Wijayawardena N.N., Kang J.-C. (2019) One stop shop II: taxonomic update with molecular phylogeny for important phytopathogenic genera: *Fungal Diversity* 94:41-129. DOI: 10.1007/s13225-019-00418-5.

Kamper J., Kahmann R., Bolker M., Ma L.J., Brefort T., Saville B.J., Banuett F., Kronstad J.W., Gold S.E., Muller O., Perlin M.H., Wosten H.A., de Vries R., Ruiz-Herrera J., Reynaga-Pena C.G., Snetselaar K., McCann M., Perez-Martin J., Feldbrugge M., Basse C.W., Steinberg G., Ibeas J.I., Holloman W., Guzman P., Farman M., Stajich J.E., Sentandreu R., Gonzalez-Prieto J.M., Kennell J.C., Molina L., Schirawski J., Mendoza-Mendoza A., Greilinger D., Munch K., Rossel N., Scherer M., Vranes M., Ladendorf O., Vincon V., Fuchs U., Sandrock B., Meng S., Ho E.C., Cahill M.J., Boyce K.J., Klose J., Klosterman S.J., Deelstra H.J., Ortiz-Castellanos L., Li W., Sanchez-Alonso P., Schreier P.H., Hauser-Hahn I., Vaupel M., Koopmann E., Friedrich G., Voss H., Schluter T., Margolis J., Platt D., Swimmer C., Gnirke A., Chen F., Vysotskaia V., Mannhaupt G., Guldener U., Munsterkotter M., Haase D., Oesterheld M., Mewes H.W., Mauceli E.W., DeCaprio D., Wade C.M., Butler J., Young S., Jaffe D.B., Calvo S., Nusbaum C., Galagan J., Birren B.W. (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* 444:97-101. DOI: 10.1038/nature05248.

Karim S., McNally R.R., Nasaruddin A.S., DeReeper A., Mauleon R.P., Charkowski A.O., Leach J.E., Ben-Hur A., Triplett L.R. (2019) Development of the automated primer design workflow uniqprimer and diagnostic primers for the broad-host-range plant pathogen *Dickeya dianthicola*. *Plant Disease* 103:2893-2902. DOI: 10.1094/pdis-10-18-1819-re.

Kawchuk L.M., Kim W.K., Nielsen J. (1988) A comparison of polypeptides from the wheat bunt fungi *Tilletia laevis*, *T. tritici*, and *T. controversa*. *Canadian Journal of Botany* 66:2367-2376. DOI: 10.1139/b88-321.

Kijpornyongpan T., Mondo S.J., Barry K., Sandor L., Lee J., Lipzen A., Pangilinan J., LaButti K., Hainaut M., Henrissat B., Grigoriev I.V., Spatafora J.W., Aime M.C. (2018) Broad Genomic Sampling Reveals a Smut Pathogenic Ancestry of the Fungal Clade Ustilaginomycotina. *Mol Biol Evol* 35:1840-1854. DOI: 10.1093/molbev/msy072.

Kuboki N., Inoue N., Sakurai T., Di Cello F., Grab D.J., Suzuki H., Sugimoto C., Igarashi I. (2003) Loop-mediated isothermal amplification for detection of african trypanosomes. *Journal of Clinical Microbiology* 41:5517-5524. DOI: 10.1128/jcm.41.12.5517-5524.2003.

Le Cam B., Sargent D., Gouzy J., Amselem J., Bellanger M.-N., Bouchez O., Brown S., Caffier V., De Gracia M., Debuchy R., Duvaux L., Payen T., Sannier M., Shiller J., Collemare J., Lemaire C. (2019) Population Genome Sequencing of the Scab Fungal Species *Venturia inaequalis*, *Venturia pirina*, *Venturia aucupariae* and *Venturia asperata*. *G3* (Bethesda, Md.) 9:2405-2414. DOI: 10.1534/g3.119.400047.

Lee S., Kim J.-H., Choi J.-Y., Jang W.-C. (2015) Loop-mediated isothermal amplification assay to rapidly detect wheat streak mosaic virus in quarantined plants. *The plant pathology journal* 31:438-440. DOI: 10.5423/PPJ.NT.06.2015.0110.

Lorrain C., Feurtey A., Möller M., Haueisen J., Stukenbrock E. (2020) Dynamics of transposable elements in recently diverged fungal pathogens: lineage-specific transposable element content and efficiency of genome defences. *bioRxiv*:2020.05.13.092635. DOI: 10.1101/2020.05.13.092635.

Matanguihan J.B., Murphy K.M., Jones S.S. (2011) Control of common bunt in organic wheat. *Plant Dis* 95:92-103. DOI: 10.1094/PDIS-09-10-0620.

Misof B., Meyer B., von Reumont B.M., Kück P., Misof K., Meusemann K. (2013) Selecting informative subsets of sparse supermatrices increases the chance to find correct trees. *BMC Bioinformatics* 14:348. DOI: 10.1186/1471-2105-14-348.

Nguyen H.D.T., Sultana T., Kesanakurti P., Hambleton S. (2019) Genome sequencing and comparison of five *Tilletia* species to identify candidate genes for the detection of regulated species infecting wheat. *IMA Fungus* 10. DOI: 10.1186/s43008-019-0011-9.

Nielsen J. (1963) Trimethylammonium compounds in *Tilletia* Spp. *Canadian Journal of Botany* 41:335-339. DOI: 10.1139/b63-032.

Nielsen J. (1968) The inheritance of spore wall characteristics in *Ustilago avenae* and *U. kolleri*. *Canadian Journal of Botany* 46:497-500. DOI: 10.1139/b68-074.

Niemann E. (1954) Zwergsteinbrand bei Roggen und Gerste nach kunstlicher Infektion mit verschiedenen *Tilletia*-Arten (Dwarf bunt of rye and barley with respect to artificial infection with various *Tilletia* species). *Zeitschrift fur Pflanzenbau und Pflanzenschutz* 5:168-172.

Niemann E. (1956) Taxonomie und Keimungsphysiologie der *Tilletia*-Arten von Getreide und Wildgräsern (Taxonomy and germination physiology of the *Tilletia* species of cereals and wild grasses). *Phytopathologische Zeitschrift* 28:53.

Oggenfuss U., Badet T., Wicker T., Hartmann F.E., Singh N.K., Abraham L.N., Karisto P., Vonlanthen T., Mundt C.C., McDonald B.A., Croll D. (2020) A population-level invasion by transposable elements in a fungal pathogen. *bioRxiv*:2020.02.11.944652. DOI: 10.1101/2020.02.11.944652.

Pimentel G., Carris L.M., Peever T.L. (2000a) Characterization of interspecific hybrids between *Tilletia controversa* and *T. bromi*. *Mycologia* 92:411-420. DOI: Doi 10.2307/3761499.

Pimentel G., Peever T.L., Carris L.M. (2000b) Genetic Variation Among Natural Populations of *Tilletia controversa* and *T. bromi*. *Phytopathology* 90:376-83. DOI: 10.1094/PHYTO.2000.90.4.376.

Purdy L.H., Kendrick E.L., Hoffmann J.A., Holton C.S. (1963) Dwarf bunt of wheat. *Annual Review of Microbiology* 17:199-222. DOI: 10.1146/annurev.mi.17.100163.001215.

Rannala B., Yang Z. (2003) Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. *Genetics* 164:1645-1656.

Ren R., Sun Y., Zhao Y., Geiser D., Ma H., Zhou X. (2016) Phylogenetic resolution of deep eukaryotic and fungal relationships using highly conserved low-copy nuclear genes. *Genome Biology and Evolution* 8:2683-2701. DOI: 10.1093/gbe/evw196.

Rudloff J.E., Bauer R., Büttner P., Sedaghtjoo S., Kirsch N., Maier W. (2020) Monitoring zum Vorkommen von *Tilletia controversa* (Zwergsteinbrand) an konventionell erzeugtem Winterweizen in den Bundesländern Brandenburg, Mecklenburg-Vorpommern, Niedersachsen, Nordrhein-Westfalen, Sachsen, Sachsen-Anhalt und Schleswig-Holstein. *Kulturpflanzen* 72:7. DOI: 10.5073/JfK.2020.08.16.

Russel B.W. (1993) Electrophoretic Karyotypes and Molecular Genetic Analysis of *Tilletia caries* and *T. controversa*, Oregon State University.

Russell B.W. (1994) Morphological, physiological, and genetic evidence in support of a conspecific status for *Tilletia caries*, *T. controversa*, and *T. foetida*. *Phytopathology* 84:576-582. DOI: 10.1094/Phyto-84-576.

Russell B.W., Mills D. (1993) Electrophoretic karyotypes of *Tilletia caries*, *T. controversa*, and their F1 progeny: further evidence for conspecific status. *Mol Plant Microbe Interact* 6:66-74. DOI: 10.1094/mpmi-6-066.

Shi Y.L., Loomis P., Christian D., Carris L.M., Leung H. (1996) Analysis of the genetic relationships among the wheat bunt fungi using RAPD and ribosomal DNA markers. *Phytopathology* 86:311-318. DOI: DOI 10.1094/Phyto-86-311.

Skibbe D.S., Doehlemann G., Fernandes J., Walbot V. (2010) Maize tumors caused by *Ustilago maydis*; require organ-specific genes in host and pathogen. *Science* 328:89. DOI: 10.1126/science.1185775.

Spatafora J.W., Aime M.C., Grigoriev I.V., Martin F., Stajich J.E., Blackwell M. (2017) The fungal tree of life: from molecular systematics to genome-scale phylogenies, the fungal kingdom, *American Society of Microbiology*.

Stehlíková D., Luchi N., Aglietti C., Pepori A.L., Diez J.J., Santini A. (2020) Real-time loop-mediated isothermal amplification assay for rapid detection of *Fusarium circinatum*. *BioTechniques* 69:11-17. DOI: 10.2144/btn-2019-0168.

Tan M.K., Raman H., Chambers G., Sharma I., Chen Z., Deshpande N., Wilkins M.R. (2016) Characterization of SNP and structural variations in the mitochondrial genomes of *Tilletia indica* and its closely related species formed basis for a simple diagnostic assay. *PLoS One* 11:e0166086. DOI: 10.1371/journal.pone.0166086.

Taylor J.W., Jacobson D.J., Kroken S., Kasuga T., Geiser D.M., Hibbett D.S., Fisher M.C. (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics And Biology* 31:21-32. DOI: https://doi.org/10.1006/fgbi.2000.1228.

Thomsen M.C.F., Hasman H., Westh H., Kaya H., Lund O. (2017) RUCS: rapid identification of PCR primers for unique core sequences. *Bioinformatics* 33:3917-3921. DOI: 10.1093/bioinformatics/btx526.

Vánky K. (2012) *Smut fungi of the world* American Phytopathological Society, Minnesota, USA.

Yan C., Cui J., Huang L., Du B., Chen L., Xue G., Li S., Zhang W., Zhao L., Sun Y., Yao H., Li N., Zhao H., Feng Y., Liu S., Zhang Q., Liu D., Yuan J. (2020) Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. *Clinical Microbiology and Infection 26*:773-779. DOI: https://doi.org/10.1016/j.cmi.2020.04.001.

Zdobnov E.M., Tegenfeldt F., Kuznetsov D., Waterhouse R.M., Simão F.A., Ioannidis P., Seppey M., Loetscher A., Kriventseva E.V. (2016) OrthoDB v9.1: cataloging evolutionary and functional annotations for animal, fungal, plant, archaeal, bacterial and viral orthologs. *Nucleic Acids Research* 45:D744-D749. DOI: 10.1093/nar/gkw1119.

Zhang L., Gleason C. (2019) Loop-mediated isothermal amplification for the diagnostic detection of *Meloidogyne chitwoodi* and *M. fallax*. *Plant Disease* 103:12-18. DOI: 10.1094/pdis-01-18-0093-re.

Summary

The fungal genus *Tilletia* is generally recognized by the production of darkly pigmented teliospores, which replace mostly the host ovary. Currently, nearly 200 species of *Tilletia* species infecting Poaceae are included in this smut genus. Three species, namely *T. caries*, *T. controversa*, and *T. laevis*, cause economically important diseases of wheat in central Europe. Common bunt is caused by *T. caries* and *T. laevis*, whereas dwarf bunt is caused by *T. controversa*. The three species are described based on the morphology of their teliospores. However, they could not be reliably distinguished by using molecular phylogenetic analyses.

To obtain deeper insights into the inter- and intraspecies genetic variation and to compare gene contents of these three species we sequenced and functionally and structurally annotated the whole-genomes of four strains of *T. caries*, five of *T. controversa*, and two of *T. laevis*. The obtained data was analysed together with five publicly available genomes (one *T. caries*, two *T. controversa*, and two *T. laevis* strains). In general, our findings demonstrated that the three species were highly similar with regards to genome size and predicted gene content. There was no evidence for expansion or decrease of transposable elements in any of the species. The nine predicted secondary metabolites gene clusters, 84% of the total carbohydrate active enzymes, 72% of secreted proteins, and 50% effector-like proteins were conserved across 16 studied strains. The species-specific proteins made only 0.1% of all predicted proteins, and their function were mainly unknown. In non-repetitive regions, the number of single nucleotide polymorphisms (SNPs) and small insertions or deletions (indels) were lowest within *T. laevis* (max. 0.52 SNPs/kb and 1.09 bp indels/kb), while they were highest within *T controversa* (max. 1.47 SNPs/kb and 2.48 indels bp/kb). We also observed extensive sequence identity between the two species of *T. caries* and *T. laevis* (0.51 SNPs/kb and 1.04 indels bp/kb on average). At the same time both species showed comparable distances to *T. controversa*. Accordingly, phylogenomic analysis of 241 protein coding genes revealed two groupings where isolates of *T. caries* and *T. laevis* were intermingled in a monophyletic group together, but separated from those of *T. controversa*, which formed another monophyletic group. Taken together these results suggest that *T. caries* and *T. laevis* have either diverged very recently or could be conspecific. These observations also correlate well with the fact that the two species are causing identical disease symptoms, need the same germination conditions, and have similar infection biology. These characteristics are different from those of *T. controversa*.

Dwarf bunt is a quarantine pathogen in several countries. Consequently, its accurate identification is of high priority for plant health as well as to wheat exporters. The current international diagnostic protocol for detection of dwarf bunt in wheat seeds is based on a filtration

method and the teliospores morphology. The method is however laborious and requires expert knowledge. To facilitate identification of *T. controversa*, a loop-mediated isothermal amplification (LAMP) assay was developed. To do this, the generated genomic data were extended further with publicly available genomic data from ten *Tilletia* isolates in order to identify DNA segments that were conserved in and unique to *T. controversa.* The developed assay was based on one of these genome regions. The assay specificity was validated against 223 fungal phytopathogens including 11 *Tilletia* species. The end-point colorimetric based detection LAMP assay had 5 pg limit of detection and showed 100% sensitivity and 97.7% specificity in an interlaboratory test performance study.

The majority of smuts are reported to have relatively narrow host ranges including *Tilletia* species. *Tilletia caries* and *T. controversa* however are reported to infect different host species representing several grass genera. To clarify whether they are indeed generalist species with a broad host range or rather represent complexes of cryptic species with narrow host ranges phylogenetic relationships of those species and close relatives were inferred using sequences of three gene regions (ITS rDNA, *EF1a*, and *RPBII).* In total 70 specimens were analysed of which 20 specimens were newly produced for this study. In general, the multi locus phylogenetic analysis resolved various species with narrow host ranges parasitizing wild grasses as distinct lineages (*i.e.,* the basal lineages from *T. fusca* up to *T. olida* representing 12 species and the crown lineage with *T. bromi* and *T. puccinelliae*, respectively). Several small clusters of *T. controversa* from wild grasses (*Thinopyrum intermedium*, *Bromus marginatus*, *Agropyron cristatum*) and rye (*Secale cereale*), respectively, clustered as subgroups in a polytomous manner between different clusters of *T. caries, T. controversa* and *T. laevis* on wheat. Interestingly, one group of *T. controversa* sequences obtained from *Elymus repens*, *Th. intermedium,* and *Agropyron* sp. clustered with high support values clearly separate from this polytomous group and together with *T. brevifaciens* isolates also obtained from *Th. intermedium*. These representatives of *T. controversa* with high likelihood represent at least one cryptic species restricted to these wild grasses as hosts and might potentially be conspecific with *T. brevifaciens*. In the latter case, *T. brevifaciens* would represent a species with board host range.

Acknowledgments

I would like to express my sincere gratitude to my doctoral supervisor, Prof. Dr. Petr Karlovsky, who accepted me as his student and provided critical comments and insightful suggestions during my work. His enthusiasm for research is always inspiring me.

My gratitude to my direct supervisor, Dr. Wolfgang Maier, for his guidance and the chance he gave me to work in his group.

I would like to give the sincerest thanks to the members of my dissertation committee, Prof. Dr. Andreas von Tiedemann for participating in my examination committee and reviewing my dissertation.

My collaborating partners, Monika K. Forster and Dr. Berta Killermann for their great collaboration are highly appreciated. I heartily thank them for their punctuality, responsiveness, and great support along with their great role in the administration of the project, acquisition of findings, and effort for gathering our sample collection. I greatly appreciate more than 60 collectors and distributors of the fungal specimens who shared their samples freely with us. A special thanks to Prof. Dr. Günter Deml for using his treasure collection of bunts which he affectionately has gathered during years. The curators of WSU Herbarium of the USA and Queensland Plant Pathology Herbarium of Australia (Herbarium Ustilaginales Vánky) are gratefully acknowledged for providing specimens. Special thanks to Ms. Monique Slipher for her kind assistance in the reconfirmation of WSU samples. The four national seed testing laboratories and plant health agencies that performed the test performance study are highly appreciated.

I thank Prof. Dr. Marco Thines and Mishra Bagdevi for their support on genome assemblies, Dr. Holger Budahn, Prof. Eva Zyprian for providing laboratory facilities of the work which is not included in this thesis, and Dr. Yvonne Becker for critically reviewing the first chapter. The professional recommendations of Prof. Dr. Niessen, Dr. Anne Fiebig, and Dr. Jens Keilwagen, Dr. Anna Schwandner are greatly acknowledged. I would like to thank Anke Brißke-Rode, Carina Moock, Thomas Berner, Matthias Voigtländer, and Ruth Pilot for their great technical support.

A special thanks to my parents and my siblings (Mina, Farideh, Mohammad) for keeping me inspired. Dr. Stephan Fuchs, to whom I am in debt for his support, critical discussion, and encouragement. I would like to thank Dr. Andreas Westphal, my former supervisor, who without his encouragement this dissertation could not have been completed. All my JKI colleagues of the mycology group especially Katrin Balke, and my friends Sonia Mayel, Mahboube Jarrah, Mascha Hoffmeister, Samad Ashrafi, and Yahya Ghaffar for their understandings, and motivations.

The federal ministry of food and agriculture of the federal republic of Germany is greatly acknowledged for funding this research project based on a decision of the German parliament (grant numbers 2812NA128).

Attribution

Chapter 2.

SS conceptualized the study, designed, and performed the experiments, analyzed the data; BM assembled the genomes; MKF acquired samples, assisted in the fungal identification, and sample preparation for PacBio sequencing; YB revised the manuscript; JK assisted in genomes annotations; BK administrated the project and acquired funding and samples; MT assembled the genomes; PK conceptualized the study and contributed to the data analysis; WM administrated the project, acquired funding, contributed to the methodology; SS, WM, and PK wrote the manuscript with the input of other coauthors; All co-authors approved the final version.

Chapter 3.

SS acquired samples, designed, and performed the experiments, analyzed the data, and drafted the manuscript; MKF acquired samples and assisted in the identification of fungal specimens and experiments; LN participated in study design and LAMP development; PK participated in study design and manuscript writing; BK acquired samples, financial support and administered the project; WM conceived and supervised the study, contributed to writing, acquired financial support, and administered the project. All authors reviewed and approved the manuscript.

Chapter 4.

SS conceptualized the study, acquired samples, designed, and performed the experiments, analyzed the data, and drafted the manuscript; MKF acquired samples and assisted in the identification of fungal specimens and experiments; PK revised the manuscript; BK acquired samples, financial support and administered the project; WM supervised the study, contributed to writing, acquired financial support, and administered the project.