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Molecular identification of *Tilletia controversa* and *T. caries*, the causal agent of wheat dwarf and common bunt

B. Sharifnabi ✉

Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan, Iran

F. Ghaderi

Department of Plant Protection, College of Agriculture, Yasouj University, Yasouj, Iran

F. Haghighi

Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan, Iran

Abstract: Common and dwarf bunt of wheat are recognized as being caused by three closely related species, *Tilletia caries* and *T. laevis* as common bunt, and *T. controversa* as dwarf bunt. The morphological characteristics of two species including *T. controversa and T. caries* were studied from wheat grown in two provinces of Iran, Lorestan and Chaharmahal and Bakhtiari during 2014—2015. In this study, morphological characters could not completely distinguish these pathogens, as in some properties, they showed similarity to *T. laevis.* So, a collection of twenty wheat bunt isolates was used to compare species in morphological characteristics and phylogenetic relationships. Phylogenetic analyses were presented based on three PCR amplified nuclear DNA fragments including elongation factor 1 alpha (*EF1α*), the second largest subunit of RNA polymerase II (*RPB2*) genes and ITS–rDNA region. Maximum likelihood (ML) method was used to determine the phylogenetic relationship among isolates using MEGA v.6 and BEAUti and BEAST v1.6.1 software. Maximum likelihood bootstrap (BS) values and Bayesian posterior probabilities (PP) values were applied as criteria for strongly supported clades. Two species including *T. controversa* and *T. caries* were distinguished as different species with DNA sequence information.

Key words: Morphology, multilocus phylogeny, *Tilletia*, *EF1α*, *RPB2*, ITS–rDNA

INTRODUCTION

Smut fungi belong to the Ustilaginomycetes class and Basidiomycota phylum (Stamatakis et al. 2007). Among smut and bunt fungal diseases causal agents, *Tilletia* species which are the causal agent of bunt and smut of Poaceae family, contain about 140 recognized species (Vánky 1994). *Tilletia* species produce sori which contain teliospores and sterile cells replaced completely or partially the developing ovary and on the other hand, produce their sori in vegetative organs of grass host. *Tilletia caries* (DC.) Tul. [=*T. tritici* (Bjerk.) Wint.], the type species of the genus causes common bunt of wheat, which is one of the destructive diseases of wheat in human history (Durán & Fischer 1961, Goates 1996).

Two concepts of morphological and phylogenetic analyses have provided sufficient evidence that most species of smut fungi are host genus or species specific (Carris et al. 2007, Bao 2010). Fifty–five percent of 600 smut species which are reported from Europe occurred on a single host, 86% on five or fewer hosts and 93% on ten or fewer hosts (Vánky 1994, Begerow et al 2004). Multilocus phylogenetic analysis based on the sequence data from ITS–rDNA, elongation factor 1 alpha (*EF1α*) and a portion of the second largest subunit of RNA polymerase II (*RPB2*) supported *Tilletia* host association between smut species and host genus/species (Carris et al. 2007, Bao 2010).

Common bunt caused by *T. laevis* and *T. caries* and dwarf bunt caused by *T. controversa*, play a considerable role as the important cereal diseases. In Iran, the common bunt is commonly caused by *T. laevis* as it has been occurred by *T. caries* in other regions. Morphologically *T. caries* and *T. controversa* are morphologically similar and differentiation based on solely spore morphology is quite difficult and somehow impossible, although there are some differences in teliospore gelatinoids sheath thickness and spore reticulate ornamentation, they could be biologically separable. Moreover, genetic structures of these two species are so similar that lots of studies recognized them as a single species and consider *T. controversa* as a mutant of *T caries*. Numerous

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✉ Corresponding Author E-mail: sharifnabib@gmail.com
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attempts such as application of precise teliospore morphology, fluorescence microscopy, serology and total proteins methods could not differentiate these species and none of them could show trusty differences between two species (Bao 2010). A PCR–RFLP method was developed using *RPB2* gene to discriminate *Tilletia caries* from *T. controversa* (Zgraja et al. 2016). Monoclonal antibody D–1 against teliospores of *T. controversa* was also applied (Gao et al. 2014). In this study, we conducted multilocus phylogenetic analysis based on sequence data from ITS rDNA, elongation factor 1 alpha (*EF1α*) and a portion of the second largest subunit of RNA polymerase II (*RPB2*) to examine the hypothesis that multilocus phylogenetic trees can provide evidence to support the morphological species concept of the wheat bunt pathogens.

MATERIALS AND METHODS

Sampling and morphological characterization

Symptomatic ears were collected from two provinces of Iran viz., Lorestan and Chaharmahal and Bakhtiari during 2014–2015, and taken to the laboratory. Sori were detached from infected wheat ears and placed on a smooth surface and were measured (Bao 2010.). Length and width of 3–5 sori were recorded per specimen where sufficient material was available. Teliospores from bunted florets were soaked in sterile distilled water for 30 min at room temperature and mounted on a microscope slide in Shear's mounting medium. Spore morphology including teliospore diameter with gelatinous sheath (Durán & Fischer 1961), the thickness of exospore, number of meshes (areolae) per spore diameter and sterile cell diameter were recorded for 20 spores per sample using differential interference contrast microscopy at ×1000. For germination of teliospores, one part of teliospore suspension was plated on 2% water agar with antibiotics and incubated at 16°C from 10 to 15 days, while the other part of suspension, which was also plated on 2% antibiotic water agar, was incubated at 5°C in the presence of light from 3 to 6 weeks. These are environmental conditions required for teliospore germination and production of primary sporidia and small colonies with secondary sporidia (Goates 1996). For Autofluorescence, teliospore suspended in water were placed on a microscope slide and allowed to air dry. The spores were then covered with a small amount of nonfluorescing immersion oil and a coverslip (Stockwell & Trione 1986) for visualizing the hyaline sheaths and teliospore were mounted in water and photographed with background lighting.

Genomic DNA extraction

Total genomic DNA was isolated using a modified CTAB (cetyltrimethyl ammonium bromide) technique. Approximately 100 mg teliospore powder was suspended in 900 µL of extraction buffer (100 mMTris–HCl, 100 mM EDTA, 250 mM NaCl) then 100 µL of 10% *N*–Lauroylsarcosine (Sigma– Aldrich, Germany) was added. The suspension was incubated at 60° C for 60 min and centrifuged for 10 min at 13000 ×g in a Beckman microfuge (Beckman, USA). Upper phase suspension was transferred to a new microtube. One hundred milliliter of NaCl 5 mM and 200 mL CTAB 5% were added to each tube respectively and the tubes were placed at 65° C for 10 minutes. According to the volume of material contained in each tube, chloroform/isoamyl alcohol (24:1) was added and after mixing, the tubes were centrifuged for 10 minutes at 13000 × g. Upper phase of suspension was transferred to a new 1.5 ml tube and the equal volume of isopropanol was added where the DNA was precipitated at –20 °C. To precipitate DNA, tubes were centrifuged for 10 minutes at 13000 ×g then the supernatant was discarded gently. The sediment was washed with about 100 ml of 75% ethanol and once the DNA was precipitated, the pellet was diluted in 100 ml of deionized double–distilled sterile water (Murray & Thompson 1980).

Phylogenetic analysis

Three PCR amplified nuclear DNA fragments including elongation factor 1 alpha (*EF1α*), the second largest subunit of RNA polymerase II (*RPB2*) genes and ITS–rDNA region were used in this study (Table 1). PCR amplifications were performed in 20 µL reactions containing 0.05 µM of each primer, 1 × Dream *Taq* Buffer (MBI Fermentas), 0.4 µM dNTPs (MBI Fermentas) and 0.5 units of Dream *Taq* DNA polymerase (MBI Fermentas). The PCR cycle parameters were as follow: 2 min initial denaturation at 96 °C followed by 35 cycles of 96 °C for 30 s, annealing for 45 s, and extension at 72 °C for 1 min. A final extension for 10 min was applied at 72°C. Finally, the quantity and quality of PCR products were evaluated and visualized on 1.5% agarose gel. The gels were stained with ethidium bromide and visually analysed under UV light (GelDoc, Bio–Rad Laboratories). The PCR products were purified with QIA quick columns according to the manufacturer's instructions and amplified products were sequenced in Macrogen, South Korea.

The consensus regions of *EF1α*, *RPB2* and ITS–rDNA were blasted against the NCBI's GenBank sequence database using Megablast to identify their closest neighbors. The obtained sequences from GenBank together with the novel generated sequences during this study (Table 2), were aligned with MAFFT v.7 online interface using default settings (http:// mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013). Maximum likelihood (ML) methods were used to determine the phylogenetic relationship among isolates using MEGA (Molecular Evolutionary Genetics Analysis) v. 6 software (Tamura et al. 2007) and BEAUti and BEAST v1.6.1 (Drummond & Rambaut 2007). Maximum likelihood bootstrap (BS) values (Reeb et al. 2004) and Bayesian posterior probabilities (PP) values (Drummond & Rambaut 2007) were used as criteria for strongly supported

clades. An isolate of *Ustilago hordei* was used as an outgroup in the combined anonymous loci dataset according to results from multilocus phylogenetic analyses of *Tilletia* spp.

Table 1. Primers characteristics used in this study.

Locus	Sequence (5'–3')	Annealing Temp. (°C)	Reference
RPB2_F	GATGGACGCGGTTTGTAATG	58	Carris, *et al.* 2007
RPB2_R	TCGAAGAGCCAACACTGAGACG		
EF1α –F	TCAACGTCGTCGTCATCGG	55	Carris*et al.* 2007
EF1α –R	CCGTGCCGATACCACCGATCTT		
ITS –F	GGAAGTAAAAGTCGTAACAAGG	59	White *et al*. 1990
ITS –R	TCCTCCGCTTATTGATATGC		

Table 2. Characteristics of *Tilletia* species isolates used in phylogenetic analysis.

Host	Location	Host Species	GenBank accession numbers		
			EF1–α	***RPB2***	***ITS***
Tilletia vankyi	Australia	*Lolium perenne*	EU257587 [a]	EU257620 [a]	EU257554 [a]
T. vankyi	US	*Festuca rubra*	EU257585[a]	EU257618[a]	EU257552[a]
T. fusca	US	*Vulpia microstachys*	EU257567 [a]	EU257601 [a]	EU257537 [a]
T. brevifaciens	Poland	*Thinopyrum intermedium*	EU257565 [a]	EU257599 [a]	EU257535 [a]
T. brevifaciens	Austria	*T. intermedium*	EU257566 [a]	EU257600 [a]	EU257536 [a]
T. bromi	US	*Bromus tectorum*	EU257555 [a]	EU257592 [a]	EU257528 [a]
T. bromi	US	*B. hordeaceus*	EU257557 [a]	EU257594 [a]	EU257530 [a]
T. caries	Australia	*Triticum aestivum*	EU257559 [a]	EU257596 [a]	EU257532 [a]
T. caries	Sweden	*T, aestivum*	EU257560 [a]	EU257597 [a]	EU257533 [a]
T. controversa	US	*T. aestivum*	EU257561 [a]	EU257598 [a]	EU257534 [a]
T. controversa	Germany	*T. aestivum*	EU257562 [a]	EU257588 [a]	EU257526a
T. elymi	US	*Elymus glaucus*	EU257564 [a]	EU257591 [a]	EU257527 [a]
T. laevis	US	*Triticum aestivum*	EU257571 [a]	EU257605 [a]	EU257541 [a]
T. laevis	Iran	*T. aestivum*	EU257573 [a]	EU257607 [a]	EU257543 [a]
T. goloskokovii	US	*Apera interrupta*	EU257569 [a]	EU257603 [a]	EU257539 [a]
T. goloskokovii	US	*Apera interrupta*	EU257568 [a]	EU257602 [a]	EU257538 [a]
T. trabutii	Iran	*Hordeum murinum*	EU257581 [a]	EU257614 [a]	EU257548 [a]
T. trabutii	Australia	*H. murinum*	EU257582 [a]	EU257615 [a]	EU257549 [a]
T. puccinelliae	US	*Puccinellia distans*	EU910060 [a]	EU910066 [a]	EU910054 [a]
T. puccinelliae	US	*P. distans*	EU910061 [a]	EU910067 [a]	EU910055 [a]
T. lolii	Iran	*Lolium rigidum*	EU257575[a]	EU257609[a]	EU257544[a]
T. lolii	Iran	*Loliolum subulatum*	EU257576[a]	EU257610[a]	EU257545[a]
T. caries	Iran (Lorestan)	*Triticum aestivum*	MH781452 [b]	MH781428 [b]	MH781414 [b]
T. caries	Iran (Lorestan)	*T. aestivum*	MH781449 [b]	MH781430 [b]	MH781415 [b]
T. caries	Iran (Lorestan)	*T. aestivum*	MH781460 [b]	MH781429	MH781416 [b]
T. caries	Iran (Lorestan)	*T. aestivum*	MH781462 [b]	MH781431 [b]	MH781417 [b]
T. caries	Iran (Lorestan)	*T. aestivum*	MH781453 [b]	MH781441 [b]	MH781418 [b]
T. caries	Iran (Chaharmahal)	*T. aestivum*	MH781450 [b]	MH781433 [b]	MH781419 [b]
T. caries	Iran (Chaharmahal)	*T. aestivum*	MH781461 [b]	MH781438 [b]	MH781420 [b]
T. caries	Iran (Chaharmahal)	*T. aestivum*	MH781463 [b]	MH781434 [b]	MH781421 [b]
T. caries	Iran (Chaharmahal)	*T. aestivum*	MH781454 [b]	MH781443 [b]	MH781422 [b]
T. caries	Iran (Chaharmahal)	*T. aestivum*	MH781451 [b]	MH781442 [b]	MH781423 [b]
T. controversa	Iran (Chaharmahal)	*T. aestivum*	MH781444 [b]	MH781424 [b]	MH781404 [b]
T. controversa	Iran (Chaharmahal)	*T. aestivum*	MH781445 [b]	MH781425 [b]	MH781405 [b]
T. controversa	Iran (Chaharmahal)	*T. aestivum*	MH781455 [b]	MH781426 [b]	MH781406 [b]
T. controversa	Iran (Chaharmahal)	*T. aestivum*	MH781457 [b]	MH781427 [b]	MH781407 [b]
T. controversa	Iran (Lorestan)	*T. aestivum*	MH781446 [b]	MH781432 [b]	MH781408 [b]
T. controversa	Iran (Lorestan)	*T. aestivum*	MH781447 [b]	MH781440 [b]	MH781409 [b]
T. controversa	Iran (Lorestan)	*T. aestivum*	MH781456 [b]	MH781435 [b]	MH781410 [b]
T. controversa	Iran (Lorestan)	*T. aestivum*	MH781458 [b]	MH781436 [b]	MH781411 [b]
T. controversa	Iran (Lorestan)	*T. aestivum*	MH781459 [b]	MH781439 [b]	MH781412 [b]
T. controversa	Iran (Lorestan)	*T. aestivum*	MH781448 [b]	MH781437 [b]	MH781413 [b]
Ustilago hordei	Germany	*Hordeum vulgare*	AY740068 [a]	KF706521 [a]	JN367380 [a]

a. Cited from Carris et al. 2007

b. From this study;

RESULTS

Morphology analysis

The beginning of teliospores germination for collections Tcar1–10 occurred between 2–3 days at 16°C. Whereas, none of the teliospores from collections Tcon1–10 germinated at 16°C over a period of 6 weeks. At 5 °C, teliospores from all the collections germinated, but the onset of germination varied greatly. Teliospore from collections Tcar1–10 germinated within 7–12 days. In contrast, the germination of teliospores from collections Tcon1–10 began between 28–30 days at 25 °C. Based on the germination properties of teliospores, collections Tcon1–10 would have been assigned to *T. controversa* because they did not germinate at 16°C and collections Tcar1–10 would have been designated *T. caries.* Teliospores from collections Tcon1–10 appeared frequently spherical and reticulated and can be visualized reticulations and hyaline sheaths on spores. The teliospores from collections Tcar1–10 were frequently aspherical and slightly reticulated and appeared devoid of sheaths, but had small reticulations. If the presence of reticulations and hyaline sheaths is used for taxonomic characteristic, the teliospores from collections Tcon1–10 are species *T. controversa* and those from Tcon 1–10 are from either *T. caries* or *T. laevis.* Teliospores mounted in immersion oil and viewed by epifluorescence microscopy can be grouped on the basis of autofluorescence as described by Stockwell and Trionc (1986). Teliospores from collections Tcon1–10 had reticulated wall layers and a netlike appearance on the surfaces. These were characteristics that described for teliospores of *T. controversa* (Stockwell & Trionc 1986). However, teliospores from the Tcar1–10 collections were typically devoid of autofluorescing reticulations, but occasionally nonfluorescent reticulations.

Phylogenetic analysis

The ITS region (800bp), partial nucleotide sequences of the second largest subunit of RNA polymerase II (*RPB2)* (600 bp) and sequences of elongation factor 1 alpha (*EF1α*) (800 bp) with a total of 1957 characters, were concatenated and applied for phylogenetic analysis of 40 *Tilletia* isolates. We used the known pathogenic relatives of *Tilletia* spp., *Ustilago hordei* species as outgroups. New Sequences described in this manuscript are deposited in GenBank. A maximum likelihood tree was inferred by combined analysis of all three loci (Fig. 1). Isolates of *T. controversa* grouped with isolates of *T. controversa* (Carris et al. 2007) in a well–supported clade (ML–BS: 100%, PP: 98%). Isolates of *T. caries* grouped with isolates of *T. caries* (ML–BS: 100%, PP: 97%) (Carris et al. 2007). Other well–supported clades include those containing isolates of *T. vankyi* (ML–BS: 99%, PP: 94%), *T. brevifaciens* (ML–BS: 100%, PP: 99%), *T. goloskokovii* (ML–BS: 99%, PP: 100%), *T. puccinellia* (ML–BS: 96%, PP: 95%), *T. trabutii* (ML–BS: 97%, PP: 96%), *T. fusca* (ML–BS: 100%, PP: 99%), *T. elymi* (ML–BS: 100%, PP: 100%), *T. bromi* (ML–BS: 97%, PP: 100%), *T. laevis* (ML–BS: 98%, PP: 100%) and *T. lolii* (ML–BS: 97%, PP: 97%)

DISCUSSION

Two teliospore collections obtained from bunted wheat grown in two provinces of Iran viz., Lorestan and Chaharmahal and Bakhtiari were examined for the following characteristics currently used in differentiating *T. caries* from *T. controversa*, including presence or absence of a prominent hyaline sheath (Duran & Fischer 1961), the temperature at which the teliospores germinated (Hoffmann 1982) and the autofluorescence properties of the teliospores (Stockwell&Trione 1986). Our results showed that in some cases, these traits could not successfully distinguish *T.controversa* from *T. caries* and sometimes failed to distinguish both species from *T. laevis.*

Criteria used in separation of *T. caries* from *T. controversa* is one of degree only (Holton & Kenddrick 1956), although these characters such as host stunting could be considered as identifier of dwarf bunt, but some races of *T. caries* or *T. laevis* could do it on certain varieties of wheat (Holton & Rodenhiser 1942).

Teliospore germination requirements are more difficult to ascertain, requiring six weeks or longer for *T. controversa*, and cannot be determined for old or otherwise nonviable collections. Moreover, the range of germination conditions between a common bunt and dwarf bunt were shown to be overlapped (Russell & Mills 1994). For example, light is not necessary for the germination of some isolates of dwarf bunt (Meiners&Waldher 1959). Teliospore size and sorus shape have not been emphasized as characters in wheat bunt identification in previous studies. Kühn (1874) observed that spores of *T. controversa* were on average 1 μm smaller than those of *T. caries* and Fischer (1952) noted the sori of *T. brevifaciens* were “hard and compact” in comparison to those of *T. caries*.

Teliospore ornamentation and thickness of gelatinous sheaths were also highly variable between isolates and among teliospores within isolates (Holton & Kendrick 1956). Moreover, Hoffmann (1982) acknowledged that extreme variation in the depth of the reticulations and the presence or absence of a prominent hyaline sheath are also characteristics of the teliospores of other species that infect grasses, which makes it difficult, if not impossible, to use these morphologic criteria for species identification.

Russell & Mills (1994) suggested that the genes conferred different teliospore morphologies and germination phenotypes are likely to be alternate alleles that can arise through mutation and recombination in natural populations. Although dwarf and common bunt are acknowledged to be epidemiologically distinct diseases, the etiologic agents appear to be essentially indistinct. Genetic and

biochemical analyses provide strong evidence that *T. caries*, *T. controversa*, and *T. laevis* are variant forms of one species, which occur because of genetic variation present in natural populations. The classification schemes presently used to differentiate *T. caries* and *T. controversa* rely on phenotypes that are common to both pathogens.

Although some previous studies have provided evidence supporting the recognition of three morphological species of wheat bunt pathogens, their conclusions must be assessed in the context of number and diversity of the isolates that were analyzed (Stockwell & Trione 1986, Liu et al. 2009). Moreover, an anonymous molecular marker was developed to distinguish *T. controversa* from common bunt species and tested on 15 common bunt isolates and *T. controversa* races D1–D17 isolates (Liu et al 2009).

Number of isolates and the selection of the loci are two common problems in molecular phylogenetic studies that may produce bias in the results (Leigh et al. 2003).

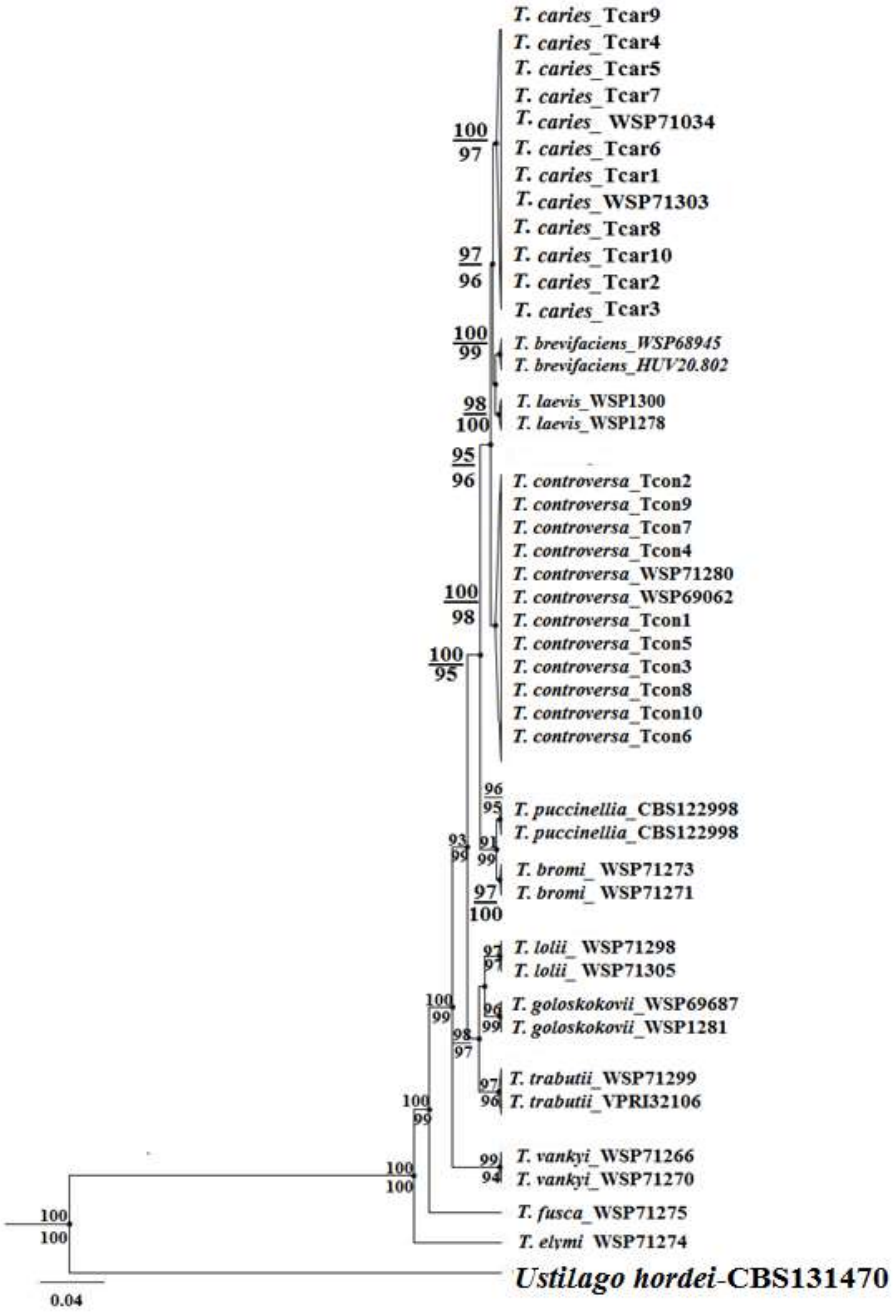

Fig 1. Maximum likelihood tree of *T. controversa and T. caries* isolates and related species with the combination of EF1A, ITS1 and RPB2 genes. Branch length indicates the substitution rate. Maximum likelihood bootstrap (BS) values are indicated on branches, and Bayesian posterior probabilities (PP) values are indicated under branches.

Utilization of monoclonal antibody D–1 revealed that the orange cycle fluorescent signal was stronger in *T. controversa* teliospores, whereas only the green signal was observed in of *T. caries* teliospores (Gao et al 2014). In addition, a PCR–RFLP method which was developed for species discrimination using *Hin*fI in *RPB2* gene showed that *T. controversa* produced two fragments, whereas *T. caries* was digested into three fragments (Zgraja et al 2016), but still with no clear distinguishing species.

In the present study, ML consensus tree was supported by Bayesian inference and bootstrap values. We could separate the morphological species for the common and dwarf bunt pathogens of wheat using multi–gene phylogenies. The lack of correlation between phylogenetic species and morphological species is supported by previous studies examining the relationship among the wheat bunt pathogens and related species. Russell and Mills (1994) compared wheat bunt isolates from Oregon, Pakistan and Turkey and observed that two Pakistan collections were intermediate between common bunt and dwarf bunt in taxonomic criteria used for species determination. Shi et al (1996) used RAPD loci to analyze the genetic relationships among 66 isolates of wheat bunt fungi and their results showed that most of the dwarf bunt isolates fell into one group and a mixture of dwarf and common bunt isolates in the second group; however, neither group was supported by bootstrap analysis. Shi et al (1996) also found two distinct restriction digest patterns in 5.8s and ITS rDNA among wheat bunt isolates. One haplotype was associated with 85% of the dwarf bunt isolates and the second haplotype was associated with all of the common bunt and four dwarf bunt isolates.

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شناسایی مولکولی گونه‌های *Tilletia controversa* و *T. caries*، عوامل سیاهک پاکوتاه و معمولی گندم

بهرام شریف‌نبی[1]، فریبا قادری[2] و فضیلت حقیقی[1]

۱– گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه صنعتی اصفهان، اصفهان، ایران

۲– گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه یاسوج، یاسوج، ایران

چکیده: عوامل مولد بیماری سیاهکهای پنهان معمولی (*T. caries* و *T. laevis*) و پاکوتاه (*T. controversa*) گندم به عنوان سه گونه نزدیک به هم تشخیص داده شده اند که تمایز آنها از یکدیگر به سادگی امکان پذیر نمی باشد. در این پژوهش، خصوصیات مورفولوژیکی دوگونه *T.controversa* و *T. caries* در مناطق گندم خیز استان های لرستان و چهارمحال بختیاری در سالهای 1394–1393 مورد بررسی قرار گرفت. تلاش برای تمایز دقیق گونه ها با ویژگی های مورفولوژیکی تقریبا مبهم بود، زیرا در برخی از ویژگی ها مشابه و یا شبیه گونه *T. laevis* بودند. بنابراین، به منظور بررسی روابط فیلوژنتیکی و امکان تمایز گونه ها، تعداد ۲۰ جدایه به طور تصادفی انتخاب گردید و ژن های *RPB2*، *EF1α* و ناحیه ITS–rDNA با استفاده از آغازگرهای اختصاصی تکثیر و توالی یابی شدند. درخت فیلوژنتیکی با روش Maximum likelihood با نرم افزارهای MEGA v.6 و BEAUti and BEAST v1.6.1 ترسیم و با توجه به نتایج درخت فیلوژنی بدست آمده، همه جدایه های توالی یابی شده در این مطالعه در دو کلاد *T. controversa* و *T. caries* قرار گرفتند.

واژگان کلیدی: ویژگی های مورفولوژیکی، فیلوژنی چند ژنی، *Tilletia*، *EF1–α*، *RPB2*، ITS–rDNA

مکاتبه کننده: بهرام شریف نبی Email: sharifnabib@gmail.com

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