Plant Pathology (2005) **54**, 161–168 Doi: 10.1111/j.1365-3059.2005.01145.x

Ustilospores of *Tilletia ehrhartae*, a smut of *Ehrharta calycina*, are common contaminants of Australian wheat grain, and a potential source of confusion with *Tilletia indica*, the cause of Karnal bunt of wheat

I. G. Pascoe[a]*†, M. J. Priest[b], R. G. Shivas[c] and J. H. Cunnington[a]

[a]*Primary Industries Research Victoria, Department of Primary Industries, Knoxfield, Private Bag 15, Ferntree Gully Delivery Centre, Victoria 3156;* [b]*Orange Agricultural Institute, NSW Agriculture, Forest Road, Orange, NSW 2800; and* [c]*Plant Science, Queensland Department of Primary Industries, 80 Meiers Road, Indooroopilly, Queensland 4068, Australia*

Australian wheat consigned for export from Australian ports was surveyed in March 2004 using a national diagnostic protocol for detection and identification of *Tilletia indica*. No ustilospores of *T. indica* were detected, confirming previous surveys which have failed to detect *T. indica* in Australia. However, the survey detected moderate levels of the common smuts *Tilletia caries* (syn. *Tilletia tritici*), *Tilletia laevis* and *Urocystis agropyri*, and very low levels (average fewer than six ustilospores per 150 g sample) of an unidentified dark, tuberculate-spored *Tilletia* in ≈ 60% of samples tested. Comparison with herbarium specimens enabled identification of the majority of the tuberculate ustilospores as *Tilletia ehrhartae*, a smut fungus known to infect only *Ehrharta calycina* (perennial veldt grass) and which is common in southern Australia. A smaller number of tuberculate smut ustilospores were identified as *Tilletia walkeri*, a smut of *Lolium* spp. recorded in Australia but apparently uncommon. Both *T. ehrhartae* and *T. walkeri* bear sufficient resemblance to *T. indica* for misidentifications to be possible where only a very few ustilospores are seen, although *T. ehrhartae* ustilospores are always <25 μm in diameter. The frequent presence of ustilospores of both *T. ehrhartae* and *T. walkeri* as contaminants of Australian wheat grain exports has significance for diagnosticians testing Australian export wheat, as it demonstrates the potential for tuberculate ustilospores of species other than those covered in existing diagnostic protocols to be misidentified as *T. indica*. This paper describes *T. ehrhartae* in detail, and provides criteria for its differentiation from *T. indica*, *T. walkeri* and some other species.

Keywords: diagnostic protocols, quarantine pathogens, taxonomy

Introduction

Tilletia indica Mitra, the cause of Karnal bunt of wheat, has never been detected in Australia, but is regarded as a high-risk exotic pathogen with potential to disrupt market access for Australian wheat (Murray & Brennan, 1998). Karnal bunt was first recognized as a new disease in India and *T. indica* was described in 1931 (Mitra, 1931); it is present in several other Asian countries and has more recently become established in Mexico and parts of the southern USA (Ykema *et al*., 1996). Surveys to determine its distribution in the USA resulted in the development of diagnostic protocols including a size-selective sieving technique (Peterson *et al*., 2000), molecular diagnostics (Bonde *et al*., 1997; Frederick *et al*., 2000), and identification of a similar tuberculate-spored *Tilletia*, *Tilletia walkeri* Castlebury and Carris from *Lolium perenne* and *Lolium multiflorum* (Castlebury & Carris, 1999). Subsequently, the European Union developed a diagnostic protocol in 2003 (Inman *et al*., 2003; Anonymous, 2004), based largely on USDA protocols. These protocols rely on extraction and morphological identification of tuberculate *Tilletia* ustilospores followed by molecular confirmation. The EU protocol (Inman *et al*., 2003) provides a useful pictorial guide to distinguishing three tuberculate *Tilletia* species: *T. indica*, *T. walkeri* and *T. horrida* Takah, which are known contaminants of wheat seed.

To ensure an adequate state of preparedness for a potential incursion of *T. indica* to Australia, a national diagnostic protocol was prepared in 2003 (Wright *et al*., 2003), based on consultation with EU and USA experts.

*To whom correspondence should be addressed.

†E-mail: ian.pascoe@dpi.vic.gov.au

Accepted 28 October 2004

A workshop to train plant pathologists from around Australia in the use of the protocol was conducted at Menangle, NSW, in November 2003.

In February 2004 a shipment of Australian wheat was rejected by an importing country on the grounds that it allegedly contained ustilospores of *T. indica*. Australian authorities, although confident that *T. indica* was not present in Australia, were anxious to reassure trading partners of the absence of *T. indica* from Australian shipments and to determine which ustilospores, if any, had been misidentified as *T. indica*. In March 2004 a combined task force of quarantine authorities and agriculture departments from all Australian states carried out a national survey of grain samples collected by the Australian Wheat Board from all wheat-exporting ports and from all ships carrying export Australian grain. Although not a survey of wheat crops, this survey sampled all wheat harvested in the 2003/04 season and either still in storage awaiting export, or in the process of being exported. Retained samples were tested from 34 ships already at sea.

This paper reports on the identities of the tuberculate *Tilletia* ustilospores extracted from samples, and provides a guide to their differentiation from *T. indica*.

Materials and methods

Extraction and identification

The survey utilized Australia's *National diagnostic protocol for the identification of* Tilletia indica*, the cause of Karnal bunt* (Wright *et al*., 2003). This protocol is closely modelled on the EU protocol (http://www.csl.gov.uk/science/organ/ph/diagpro/tipro.pdf and Anonymous, 2004) and the USDA protocol (http://www.aphis.usda.gov/ppq/manuals/pdf_files/KB_new.pdf).

Samples taken from all Australian wheat-exporting ports were obtained from the Australian Wheat Board store in Werribee, Victoria. Each sample consisted of about 200 g grain sourced either from ships during loading, or from zone samples obtained from grain stores at each of the ports. The procedure consisted of the following steps.

Visual examination
Visual examination of grain samples for presence of bunted grains and weed seeds.

Subdivision
Subdivision of each grain sample into three 50 g subsamples for ustilospore extraction and identification.

Size-selective sieving of samples followed by centrifugation
Each 50 g sample was placed in a flask with 100 mL 0·066% Tween 20 solution and placed on a rotary shaker at 200 r.p.m. for 3 min. The contents of the flask were then poured through a 53 μm sieve above a 15 μm sieve. The flask was then rinsed twice with 100 mL water, and the water poured through the sieves. The 15 μm sieve was then rinsed gently to concentrate the ustilospores at one edge, and the remaining small quantity of water containing ustilospores was pipetted into a 15 mL centrifuge tube. Centrifuge tubes containing the grain washings were then centrifuged at 1000 ***g*** for 3 min. The majority of the supernatant was poured off and discarded.

Ustilospore detection and identification
Aliquots (20 μL) were pipetted from the centrifuge tube onto a microscope slide, covered with an 18×18 mm cover slip and examined microscopically using brightfield optics. Any dark, tuberculate ustilospores seen were examined at higher magnifications and measured. All tuberculate *Tilletia* ustilospores seen were compared with the illustrations published in the Australian national protocol and the EU protocol, with reference slides of *T. indica*, *T. walkeri* and *T. horrida* and with herbarium specimens of *Tilletia ehrhartae* Talbot and *Tilletia rugispora* Ellis.

Culturing
Ustilospores were washed off slides and surface-sterilized according to the national protocol before being plated on water agar containing streptomycin and penicillin. Plates were incubated under lights for 12 days prior to examination for germinated ustilospores. The protocol then required transfer of sporidia to potato dextrose broth and harvesting of the resulting mycelial mat for DNA extraction and subsequent PCR. However, because ustilospore germination was not successful, this part of the procedure was not carried out.

Morphological examination of herbarium specimens

Tuberculate *Tilletia* ustilospores found during the survey were compared with a set of reference slides of *T. indica*, *T. horrida* and *T. walkeri* and with herbarium specimens of candidate species from the Plant Disease Herbarium, Department of Primary Industries, Victoria (VPRI) and the Plant Pathology Herbarium, Department of Primary Industries, Queensland (BRIP). Ustilospores from mature sori on herbarium specimens were mounted both in water and in lactic acid. A compound microscope using both brightfield and interference-contrast microscopy was used for observations and photographs.

Morphology of bleached ustilospores

Chesmore *et al*. (2003) trialled an image-analysis system for identification of bleached ustilospores of *T. indica*. To provide a point of comparison with ustilospores found in this study, ustilospores from herbarium specimens of the identified species were subjected to bleaching and photographed. Ustilospores were removed from the sorus, placed on a microscope slide in a drop of water, and dried down over a small flame to cause the ustilospores to adhere to the slide. A drop of 0·5% sodium hypochlorite was placed on the ustilospore deposit, allowed to stand for 4 min, then washed off with several washes of water. The slide was then mounted in either water or lactic acid,

sealed with nail varnish, and examined using interference-contrast microscopy.

DNA sequencing and phylogenetic analysis

The ribosomal DNA internal transcribed spacer region from *T. ehrhartae* (BRIP 28392) was amplified and sequenced according to the methods of Cunnington & Shivas (2004). DNA was extracted by grinding a small amount of ustilospores (1 mm^3) in 50 μL 5% Chelex-100 (Biorad). The material was spun down briefly in a microcentrifuge. PCR was performed according to Cunnington & Shivas (2004). The 25 μL reactions contained 1 μL DNA extract, 200 μM of each dNTP, 1·5 mM $MgCl_2$, 2·5 μL 10× buffer, 4 ng each of primers ITSF1 (Gardes & Bruns, 1993) and ITSUR (5′-TGTTCGCTATCGGTCTCTCC-3′; Cunnington & Shivas, 2004) and 0·5 U *Taq* polymerase. Reaction cycles were 35 cycles of: 30 s at 94°C, 30 s at 50°C, 1 min at 72°C. As only a small amount of product was amplified, nested PCR was performed on this product using 1 μL of the first-round product with primers ITS5 (White *et al*., 1990) and ITSUR. Reaction conditions were otherwise the same as for the first PCR. The nested PCR products were then purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced directly using primers ITS5 and ITS4 (White *et al*., 1990) with an ABI PRISM BIGDYE Terminator Cycle Sequencing Kit (Perkin-Elmer), according to the manufacturer's instructions. The sequence was deposited on GenBank (accession AY770433).

The sequence was aligned with a wide range of ITS sequences for other *Tilletia* species (obtained from GenBank) using CLUSTALX (Thompson *et al*., 1997). The alignment was then adjusted manually. The ITS sequence for *Entyloma microsporum* was included as an outgroup. A neighbour-joining tree was created using the Kimura-2-parameter method with a complete deletion of gaps using MEGA (Kumar *et al*., 2001). One thousand bootstrap replicates were performed.

Results

No bunted grains of either wheat or weeds were found during examination of samples.

Examination of slides prepared from sample extractions showed the presence of tuberculate *Tilletia* ustilospores in about 60% of samples. These samples yielded an average of 5·8 tuberculate *Tilletia* ustilospores per 150 g sample. *Tilletia indica* was not detected in any samples. However, a tuberculate-spored *Tilletia* with comparatively small ustilospores (17–25 μm diameter) and tuberculate, irregularly polygonal, but not cerebriform ornamentation was detected in many samples from temperate Australia. Examination of herbarium specimens held in VPRI and BRIP resulted in identification of the fungus as *Tilletia ehrhartae* Talbot, a smut of perennial veldt grass, *Ehrharta calycina*. A smaller number of ustilospores with coarsely cerebriform ornamentation and measuring 27–40 μm were tentatively identified as *Tilletia walkeri* Castlebury & Carris. In total, 665 ustilospores of *T. ehrhartae* were identified from 81 of the 119 samples, and 22 ustilospores of *T. walkeri* were identified from eight samples (Table 1). The common smut fungi *T. laevis* Kuhn, *T. caries* (DC.) Tul. (= *T. tritici*) and *Urocystis agropyri* were also found very frequently.

Table 1 Samples examined and number of ustilospores of *Tilletia ehrhartae* and *Tilletia walkeri* detected for each port of consignment

Port	Samples tested	Ustilospores detected	
		T. ehrhartae	*T. walkeri*
Geraldton WA	7	9	16
Fremantle WA	17	36	6
Albany WA	6	29	0
Esperance WA	4	18	0
Thevenard SA	5	1	0
Port Lincoln SA	8	57	0
Port Pirie SA	3	9	0
Wallaroo SA	7	36	0
Port Giles SA	7	63	0
Port Adelaide SA	8	171	0
Portland Vic	11	183	0
Geelong Vic	6	34	0
Melbourne Vic	3	6	0
Port Kembla NSW	8	9	0
Newcastle NSW	6	3	0
Brisbane Qld	6	1	0
Gladstone Qld	5	0	0
Mackay Qld	2	0	0
Total	119	665	22

Ports ordered anticlockwise around the Australian coast from Geraldton in the west to Mackay in the east.
WA = Western Australia, SA = South Australia, Vic = Victoria, NSW = New South Wales, Qld = Queensland.

Tilletia ehrhartae was recorded only in samples consigned from ports in southern Australia, and was most prevalent in samples from ports in south-eastern South Australia and western Victoria. Table 1 shows the distribution of *T. ehrhartae* and *T. walkeri* relative to ports from which samples were shipped. No ustilospores of *T. ehrhartae* were detected in samples from the ports of Mackay and Gladstone (central Queensland coast), which ship wheat from inland Queensland. *Tilletia ehrhartae* was detected from only one sample obtained from the port of Brisbane, which ships wheat from southern Queensland and northern New South Wales. The 22 ustilospores identified as *T. walkeri* were recovered only from samples obtained from the ports of Fremantle and Geraldton in Western Australia, areas of Australia from which the fungus has not previously been recorded. A small number of other large (35–55 μm) tuberculate *Tilletia* ustilospores were found (four in total: two from Portland, one from Fremantle, one from Newcastle), but could not be identified; they were clearly not *T. indica*, *T. walkeri* or *T. ehrhartae*, but had coarser, columnar ornamentation. Candidate species were *Tilletia inolens* McAlpine and *Tilletia eragrostidis* Clinton & Ricker (both fungi occur in Australia), but the small number of ustilospores found did

not allow resolution of their identities, or any certainty that they all belonged to the same species.

Attempts to culture from ustilospores detected on slides were unsuccessful. Consequently, no molecular confirmation of the identification of *T. ehrhartae* could be carried out. (As there are no previously published sequences for this fungus, successful sequencing of the ustilospores detected may not have proven its identity.)

For future comparison, the rDNA ITS region was obtained from a good specimen of *T. ehrhartae* on *E. calycina* (BRIP 28392). Phylogenetic analysis revealed *T. ehrhartae* to lie within a basal assemblage of predominantly tuberculate-spored species of *Tilletia* (see Fig. 3). These included *T. nigrifaciens*, *T. horrida*, *T. barclayana*, *T. indica* and *T. walkeri*. However, *T. ehrhartae* was not very closely related to these species, being most similar to *T. nigrifaciens* (82% similarity).

Tilletia ehrhartae Talbot (1958), *Bothalia* 7, 109–111

Holotype: on *Ehrharta calycina*, Nature Reserve, Lambert's Bay, Clanwilliam district, South Africa, October 1956, leg. R. Storey, PREM 41604 (not seen).

Ustilospores (Fig. 1) globose, 17–25 μm in diameter (including ornamentation), very dark olivaceous brown when mature, ornamentation in surface view consisting of opaque, heavily melanized polygonal scales, approx. 6–10 per diameter, 1–3 μm across, separated by the dark olivaceous exospore wall, in profile as cylindrical or slightly tapered spines, broadly truncate to slightly rounded at the apex (appearing 'worn-down'), 1–2·5 μm high and 2–3 μm wide, with an apparently more darkly melanized apex in dark, mature ustilospores, sheath (when present) extending to the apex of the spines or slightly beyond. Sterile cells globose, generally larger than the ustilospores, hyaline, 19–28 μm in diameter, with a thick, multilayered wall up to 5 μm thick, the wall soft and easily deformed by pressure from surrounding ustilospores.

Specimens examined: on *Ehrharta calycina* Sm. (Poaceae); Kaniva, Vic., Australia, Earles J, 9 November 1987, VPRI 15641a; Kaniva, Vic., Australia, Joannides C, 14 November 1991, VPRI 17743a; Pinnaroo, SA, Australia, Shivas RG, Vánky K, 6 October 2001, BRIP 28392a; Naracoorte, SA, Australia, Shivas RG, Vánky K, 11 October 2001, BRIP 39762a. The Australian Plant Pest Database contains additional records of the fungus on *E. calycina* from Hindmarsh Island SA, Meningie SA, Tintinara SA, Victor Harbour SA, Esperance WA, Cranbrook WA, Albany WA, Cascade WA, Jerrangamup WA, Woogenellup WA and Perth WA.

The above description agrees well with Talbot's (1958) description of the holotype (PREM 41604), except that Talbot describes the ustilospore ornamentation as 'rounded warts up to 1·5 μm high'. In very mature ustilospores the warts do appear apically rounded in profile, but in young ustilospores they are cylindrical and

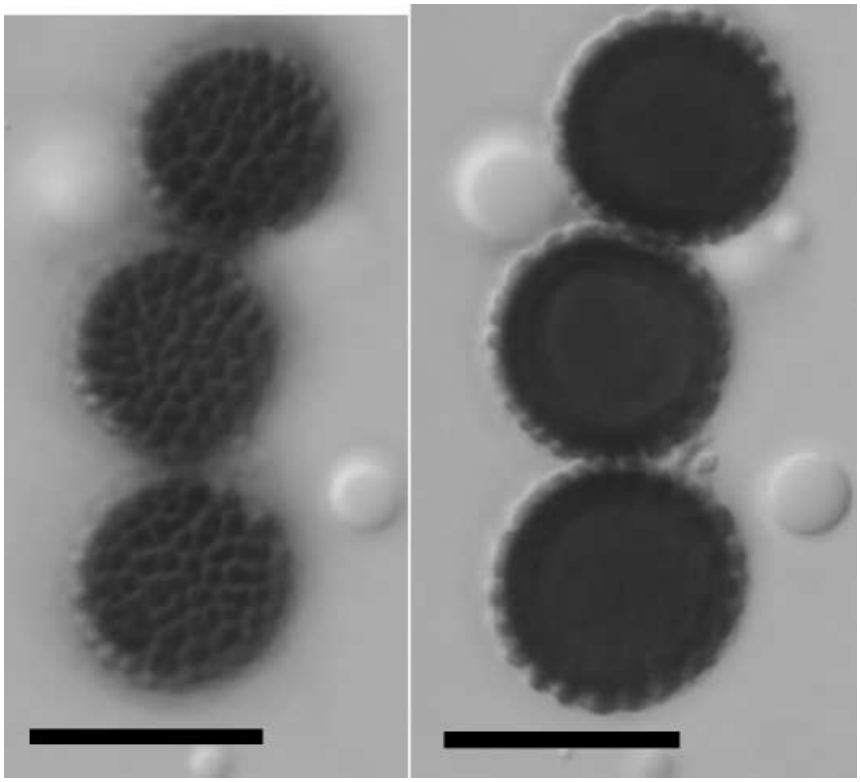

Figure 1 Ustilospores of *Tilletia ehrhartae* from sori on *Ehrharta calycina* (BRIP 28392) mounted in water. Left, surface view; right, profile view. Scale bar = 20 μm.

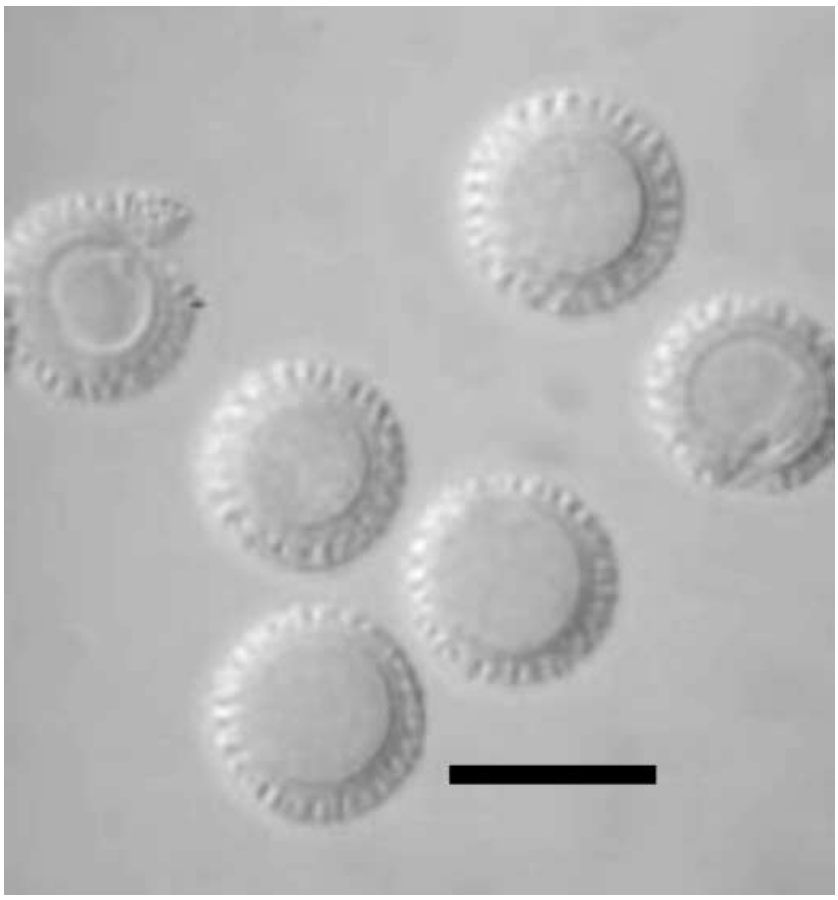

Figure 2 *Tilletia ehrhartae* ustilospores from sori on *Ehrharta calycina* (BRIP 28392) after bleaching in 0·5% sodium hypochlorite and remounting in lactic acid. Scale bar = 20 μm.

truncate. In surface view the ornamentations are irregularly polygonal with sharp corners, and resemble the pattern made in dried, cracked mud. K. Vánky (personal communication) examined the holotype and, in a draft description based on the type, described ustilospore ornamentation in profile as '1·5–2·5(–3) μm high, cylindrical or subpyramidal ornamentation with flattened tips' and in surface view as 'darker, irregularly polygonal areas, 6–11 per spore diameter'. Although the type was not examined in the present study, the information provided by Vánky confirmed the fungus as *T. ehrhartae*.

Examination of bleached ustilospores (Fig. 2) showed spines significantly longer (2·5–3·5 μm) and narrower (≈1 μm) than those of natural ustilospores. The spines were cylindrical or slightly tapered, truncate, often with 'growth rings' at uniform levels around the circumference of the ustilospore. After bleaching, spines were 0·5–1 μm longer when mounted in water than in lactic acid. Bleached ustilospores were seen to have a clearly defined two-layered wall, an outer layer 1–1·5 μm thick, in which the spines were seated, and an inner layer of about the same thickness. Although this technique differed in detail from that of Chesmore *et al*. (2003), the morphology of bleached ustilospores of *T. ehrhartae* appeared to differ significantly from that of *T. indica*, *T. walkeri* and *T. horrida*, especially in the erect, uniformly cylindrical, truncate spines.

In water mounts of ustilospores extracted from wheat grain samples, *T. ehrhartae* could be distinguished from *T. indica* by the smaller ustilospores (max. 25 μm diameter) with larger, more acutely polygonal scales in surface view, which were broadly truncate in profile. *Tilletia ehrhartae* could be distinguished from *T. walkeri* by its smaller ustilospores, and by its polygonal scales that very rarely formed cerebriform aggregations, which had sharply angled outlines compared to the rounded corners of scales in *T. walkeri* and were broadly truncate in profile.

Discussion

No *T. indica* was detected in a survey that tested three replicate subsamples from 119 samples obtained from stores at 18 ports around Australia. The survey demonstrated that other *Tilletia* taxa, in particular *T. ehrhartae*, can be present as contaminants in wheat grain, and therefore are at risk of being misidentified as *T. indica*.

Tilletia ehrhartae is the bunt of *E. calycina*, perennial veldt grass. *Erharta calycina* is a grass of dry, cool temperate climates, is native to southern Africa, and is a significant

environmental weed in California. The grass is present in all Australian states except Queensland and Northern Territory. Its distribution in Australia roughly corresponds with the wheat belt, although it does not extend into Queensland. Herbarium records of *T. ehrhartae* exist from western Victoria, southern South Australia and southern Western Australia. These areas correspond to the greatest frequency of survey samples containing *T. ehrhartae*. During a survey for smut fungi in South Australia in October 2001, heavily smut-infected plants of *E. calycina* were often found on roadsides adjacent to wheat crops and occasionally within the crop (R.G.S. and K. Vánky, unpublished), indicating that *T. ehrhartae* ustilospores can be expected to contaminate wheat during harvesting.

Tilletia ehrhartae was treated by Durán & Fischer (1961) as a synonym of *T. rugispora* Ellis. *Tilletia rugispora* was originally described from *Paspalum plicatulum*. Durán & Fischer (1961) listed as hosts *Paspalum paniculatum*, *Paspalum scrobiculatum* and *Paspalum virgatum* as well as *E. calycina*, and the US National Fungus Collection, Beltsville, MD, USA holds specimens from *Paspalum dicotulum* and *Paspalum nicorae*. However, while there are 14 records of *Tilletia* from *E. calycina* in Australian herbaria, there are no Australian records of any *Tilletia* from *Paspalum* spp. Examination of material of *T. rugispora* on *P. plicatulum* (BRIP 27375, Santo Tome, Argentina, coll. C. & K. Vánky, 26 June 1999) shows differences in ornamentation, with *T. rugispora* having more distinctly conical or pyramidal spines than *T. ehrhartae*, and in colour, with ustilospores of *T. rugispora* being mid-reddish brown compared with the very dark olivaceous ustilospores of *T. ehrhartae*. Durán (1987) described *T. rugispora* with conical ornamentations tapering to a sharp point and coloured reddish-brown to copper, clearly a different morphology from *T. ehrhartae* as described here. Pimentel *et al*. (1998) included *T. rugispora* in a study of relationships among tuberculate-spored species of *Tilletia*, and showed *T. rugispora* to be rather isolated from *T. indica*, *T. barclayana* (Bref.) Sacc. & P. Syd. and the other tuberculate species studied. Their molecular data (RAPD and PCR-RFLP) are not directly comparable with those in the present study, and no rDNA ITS sequence is available on GenBank. The present study was unsuccessful in extracting DNA from the specimen of *T. rugispora* examined (BRIP 27375). K. Vánky (personal communication) has examined holotypes of both *T. rugispora* and *T. ehrhartae* and considers them to be morphologically distinct. Consequently, *T. ehrhartae* has been retained here as distinct from *T. rugispora*.

The presence of very dark, tuberculate *Tilletia* ustilospores in shipments of Australian wheat raises the possibility of confusion with *T. indica*, which has not been recorded from Australia. *Tilletia ehrhartae* can be distinguished from *T. indica* and *T. walkeri* by its consistently smaller size (17–25 μm diameter in water), dark olivaceous colour and coarsely polygonal scales, which are rarely cerebriform in surface view and which appear broadly truncate (or 'worn down') in profile. However, some individual, isolated ustilospores of *T. ehrhartae* can appear similar to typical ustilospores of *T. indica* or *T. walkeri*, just as occasional ustilospores of those species can appear similar to typical ustilospores of *T. ehrhartae*. Table 2 compares the morphology of some taxa of *Tilletia* which can potentially be confused with *T. indica*, but a perusal of smut taxonomy literature will reveal many other species with tuberculate ornamentation which are just as liable to be confused with *T. indica*.

The ITS sequence for *T. ehrhartae* obtained in this study (Fig. 3) provides a further mechanism by which this species can be identified. The presence of *T. ehrhartae* in a shipment of Australian wheat in China was recently confirmed by comparison of ITS sequences of those ustilospores with the sequence obtained from the herbarium specimens used during the present work (Yi Jianping, Shanghai Inspection and Quarantine Bureau, personal communication, September 2004).

Table 2 Comparative morphology of mature ustilospores of tuberculate *Tilletia* species most likely to be confused with *T. indica*

Species	Teliospore size (μm)	Ornamentation		Colour
		Surface view	Profile	
*T. indica**	28–54	Densely tuberculate to narrowly cerebriform	Conical to truncate	Dark reddish brown to black, opaque
*T. walkeri**	23–45	Coarsely cerebriform to coralloid scales	Conical to truncate	Pale yellow to dark reddish-brown
T. horrida	17–36	Polygonal to cerebriform scales	Sharply pointed to truncate	Chestnut brown
T. barclayana	14–36	Fine, polygonal to cerebriform scales	Sharply pointed to truncate	Chestnut brown
*T. ehrhartae** Talbot	18–25	Coarse polygonal scales	Cylindrical, truncate	Olivaceous, dark
*T. rugispora**	17–28	Coarse polygonal scales	Conical, bluntly pointed	Mid-reddish brown
*T. inolens**	31–41	Coarse polygonal scales, made up of many fine spines	Coarse, cylindrical, flared and broken at tips	Dark golden yellow to dark chocolate brown
*T. eragrostidis**	28–37	Coarse polygonal scales	Coarse truncate projections	Light to dark reddish brown
T. cathcartae	30–40	Densely verruculose to tuberculate	Cylindrical, truncate	Golden brown

Sourced from a consensus of Inman *et al.* (2003); Durán (1987); Durán & Fischer (1961); Vánky & McKenzie (2002); USDA web page 'The Genus *Tilletia* in the United States' [http://nt.ars-grin.gov/taxadescriptions/Tilletia]; observations of the authors.

* = species seen by the authors. All other descriptions derived from the literature.

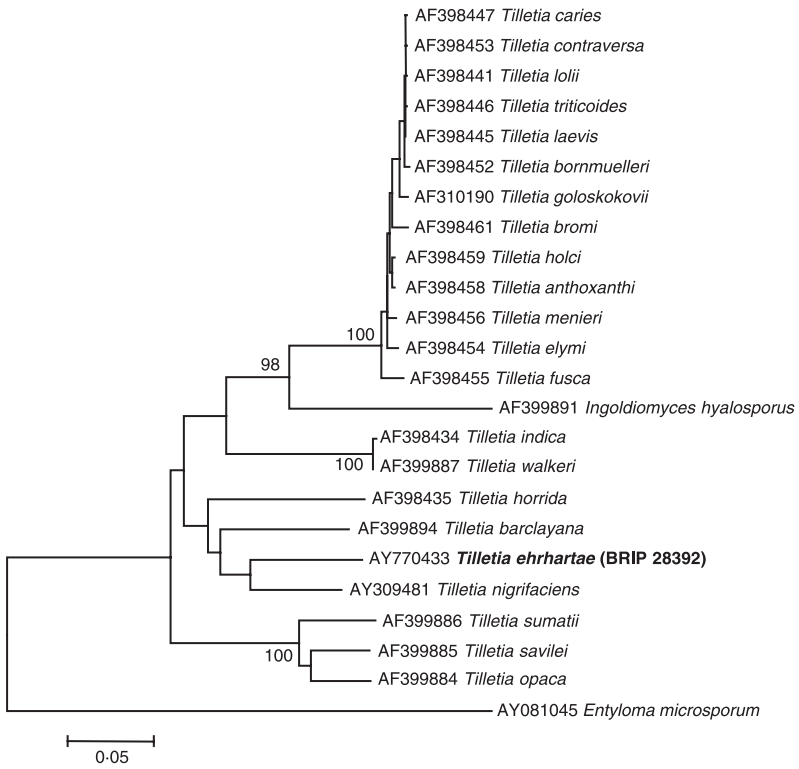

Figure 3 Neighbour-joining tree based on the entire ITS sequence, showing the relationship of *Tilletia ehrhartae* (BRIP 28392) to various other *Tilletia* species. Only bootstrap values over 90% (approximate) are shown. Scale bar = five changes per 100 bases.

It is clear that even with well developed protocols for detection of *T. indica* in grain shipments, it is possible for a number of tuberculate-spored species of *Tilletia* to be confused with *T. indica*, especially when very low numbers of tuberculate ustilospores are detected. As can be seen in the illustrations accompanying the EU protocol (Inman *et al*., 2003), *T. walkeri*, for example, can have some ustilospores which resemble typical *T. indica*, and some which resemble typical *T. ehrhartae*. This throws into doubt the veracity of any identification based on one or two ustilospores. However, once 10–20 ustilospores have been seen in a sample or series of samples, some consistency in ornamentation and size should have emerged, and a reasonably reliable identification can be reached. Importing countries and their quarantine authorities need to be very careful before identifying a few tuberculate ustilospores as *T. indica*, and need to consider carefully other candidate species that may be present in the country of origin of the grain. Of particular importance are *T. walkeri* (on ryegrass), *T. horrida* (on rice), *T. barclayana* (on *Panicum* spp. and other hosts), *T. ehrhartae* (on *E. calycina*), *T. rugispora* (on *Paspalum* spp.), *T. eragrostidis* (on *Eragrostis* spp.), *T. cathcartae* Durán & G.W. Fischer (on *Poa* spp.) and *T. inolens* (on *Agrostis* and *Deyeuxia* spp.) (Table 2). Of these, *T. cathcartae* appears most similar in its ornamentation to *T. indica*, although its ustilospores are never described as opaque.

In addition to *T. ehrhartae*, other species of *Tilletia* with tuberculate ornamentation which have not yet been reported as contaminants of wheat grain must also be considered as potential sources of confusion with *T. indica*. Other, as yet undescribed but similar, species of *Tilletia* may also exist. New techniques for identification of *T. indica*, such as that of Chesmore *et al*. (2003), need to be validated across a wider range of species than *T. indica*, *T. walkeri* and *T. horrida*. It is especially important that suspect detections be subjected, where possible, to examination of a suitable number of ustilospores and to molecular confirmation, before a positive identification of *T. indica* is made.

Acknowledgements

The following people contributed to the results reported in this paper. Dominie Wright and Gordon Murray, authors of the national protocol, provided ongoing technical advice on implementation of the protocol and participated significantly in the survey, in Dominie Wright's case leading the extraction laboratory for the duration of the survey. Fiona McBeth provided policy advice from a federal government perspective and liaised with the Australian Wheat Board. Officers of the Australian Wheat Board provided access to shipping and zone samples from their store at Werribee, and staff of the National Residue Service managed sampling protocols. The Plant Standards Branch, Department of Primary Industries oversaw quarantine aspects of the survey and vetted all procedures. Incident managers and team leaders, Jacky Edwards, Jo Luck, Gordon Berg, Sharon Morley, Martin Mebalds, Chris Wilmshurst, Krishan Sharma and Jane Moran, managed the laboratories and overall operation of the survey. A team of over 20 scientists from WA, SA, NSW,

Victoria, Tasmania and Queensland did all the hard work. Alan Inman, CSL, York, UK provided valuable comments on the identity of ustilospores sent to him as photos.

References

Anonymous, 2004. EPPO standards – diagnostic protocols for regulated pests – PM7/29 *Tilletia indica*. *Bulletin OEPP/EPPO Bulletin* **34**, 219–27.

Bonde MR, Peterson GL, Schaad NW, Smilanick JL, 1997. Karnal bunt of wheat. *Plant Disease* **81**, 1370–7.

Castlebury LA, Carris LM, 1999. *Tilletia walkeri*, a new species on *Lolium multiflorum* and *L. perenne*. *Mycologia* **91**, 121–31.

Chesmore D, Bernard T, Inman AJ, Bowyer RJ, 2003. Image analysis for identification of the quarantine pest *Tilletia indica*. *Bulletin OEPP/EPPO Bulletin* **33**, 495–9.

Cunnington JH, Shivas RG, 2004. The phylogenetic position of *Tilletia nigrifaciens*. *Australasian Mycologist* **22**, 53–6.

Durán R, 1987. *Ustilaginales of Mexico: Taxonomy, Symptomatology, Spore Germination and Basidial Cytology*. Pullman, WA, USA: Washington State University Press.

Durán R, Fischer GW, 1961. *The genus* Tilletia. Pullman, WA, USA: Washington State University Press.

Frederick RD, Snyder KE, Tooley PW, Berthier-Schaad Y, Peterson GL, Bonde MR, Schaad NW, Knorr DA, 2000. Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. *Phytopathology* **90**, 951–60.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes – applications to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**, 113–8.

Inman AJ, Hughes KJD, Bowyer RJ, 2003. EU recommended protocol for the diagnosis of a quarantine pathogen, *Tilletia indica*. http://www.csl.gov.uk/science/organ/ph/diagpro/tipro.pdf

Kumar S, Tamura K, Jakobsen IB, Nei M, 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–5.

Mitra M, 1931. A new bunt of wheat in India. *Annals of Applied Biology* **18**, 178–9.

Murray GM, Brennan JP, 1998. The risk to Australia from *Tilletia indica*, the cause of Karnal bunt of wheat. *Australasian Plant Pathology* **27**, 212–25.

Peterson GL, Bonde MR, Phillips JG, 2000. Size-selective sieving for detecting teliospores of *Tilletia indica* in wheat seed samples. *Plant Disease* **84**, 999–1007.

Pimentel G, Carris LM, Levy L, Meyer RJ, 1998. Genetic variability among isolates of *Tilletia barclayana*, *T. indica* and allied species. *Mycologia* **90**, 1017–27.

Talbot PHB, 1958. New and interesting records of South African fungi. Part 3. *Bothalia* **7**, 109–11.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**, 4876–82.

Vánky K, McKenzie EHC, 2002. Smut fungi of New Zealand. *Fungi of New Zealand, Vol. 2*. Hong Kong, China: Fungal Diversity Press.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York, NY, USA: Academic Press, 315–22.

Wright D, Murray G, Tan M-K, 2003. *National Diagnostic Protocol for the Identification of* Tilletia indica, *the Cause of Karnal Bunt*. South Perth, WA, Australia: Department of Agriculture, Government of Western Australia.

Ykema RE, Floyd JP, Palm ME, Peterson GL, 1996. First report of Karnal bunt of wheat in the United States. *Plant Disease* **80**, 1207.