Phylogenetic considerations for predicting the host range of *Ustilago sporoboli-indici*, a potential biological control agent for *Sporobolus* species in Australia

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Summary
The ribosomal DNA internal transcribed spacer region was amplified and sequenced from a selection of specimens of the *Sporobolus* smut *Ustilago sporoboli-indici*. Phylogenetic comparison with other *Ustilago* and *Sporisorium* species revealed strong support for an evolutionary radiation of *Ustilago* species infecting the Chloridoideae and Pooideae, of which *U. sporoboli-indici* forms a major lineage. Comparisons are made with other groups of plant pathogenic fungi, and it is concluded that phylogenetic analyses of potential biocontrol agents are useful for identifying pathogens that are derived from evolutionary lineages that parasitize a wide range of unrelated plants. Such pathogens are less desirable as biocontrol agents as they may have a greater likelihood of infecting plants outside their normal host ranges.

Keywords: co-evolution, Ustilaginomycetes, Chloridoideae

Introduction
Sporobolus grasses (*Sporobolus* spp.) are a major group of unpalatable weedy plants in Australia. The South African sporobolus grasses in the complex comprising *S. africanus* (Poir.) Robyns & Tournay, *S. fimbriatus* (Trin.) Nees, *S. natalensis* (Steud.) Dur. & Schinz and *S. pyramidalis* P.Beauv. are becoming increasingly important weeds that adversely affect agricultural and environmental areas in eastern Australia (Anon. 2001). There are no biological control agents currently available to manage these grasses (McFadyen 1999). In a preliminary attempt to identify potential biological control agents for these species the second author visited South Africa in December 2002 to survey for plant pathogenic fungi on *Sporobolus* spp. During this survey the smut fungus *Ustilago sporoboli-indici* Y.Ling was often collected, particularly on *S. pyramidalis*. These specimens have been deposited in herbaria BRIP (Queensland Department of Primary Industries and Fisheries, Plant Pathology Herbarium) and HUV (Herbarium Ustilaginales Vánky). *Ustilago sporoboli-indici* produces sori in the leaves, leaf sheaths and stems, rendering shoots sterile (Vánky 2003). The smut appears to be systemic and usually all shoots of an infected plant are sterile. The smut is characterized by sori that appear as bullate, lead-coloured striae and yellowish-brown spores 7–11.5 µm long. It is known on *S. africanus*, *S. elongatus* R.Br. and *S. pyrimadalis*, from Africa, China and the Philippines (Vánky 2003).

A phylogenetic analysis of the genus *Ustilago* (and the closely related genus *Sporisorium*) has recently been investigated by Stoll *et al*. (2003) using ribosomal DNA internal transcribed spacer sequences. Among other evolutionary trends, this study revealed that particular lineages of smut fungi were found to occur only on specific groups (often tribes) of grasses. This project aimed to determine the phylogenetic position of *U. sporoboli-indici* in relation to previously identified evolutionary relationships between smuts and grasses.

Materials and methods
Five specimens held in BRIP were used for the study (Table 1). DNA was extracted by grinding a small amount of spores (1 mm^3) in 50 µL of 5% Chelex-100 (Biorad). The material was spun down briefly in a microcentrifuge. The PCR was performed according to Cunnington and Shivas (2004). The 25 µL reactions contained 1 µL DNA extract, 200 µM of each dNTP, 1.5mM $MgCl_2$, 2.5 µL 10x buffer, 4 ng each of primers ITS1-F and ITSUR, and 0.5 units of Taq polymerase. Reaction cycles were 35 cycles of: 30 sec at 94°C, 30 sec at 50°C, 1 min at 72°C. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced directly using primers ITS5 and ITS4, with an ABI PRISM® BIGDYE™ Terminator Cycle Sequencing Kit (Perkin-Elmer) according to the manufacturers instructions. These sequences were aligned with a wide range of ITS sequences for other *Ustilago* and *Sporisorium* species (obtained from GenBank) using ClustalX (Thompson *et al*. 1997). 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.

Of the five specimens chosen for this work, the ITS region was successfully amplified and sequenced from four. These sequences were identical and have been deposited in GenBank (Table 1.). The reason for the failure to amplify the fifth specimen was probably due to a poor DNA extraction. A nested amplification may have overcome this (Cunnington and Shivas 2004), however as the sequences from the other four specimens were identical it was deemed that further work on the fifth, or indeed any further specimens, was unwarranted.

Results and discussion
Phylogenetic analysis revealed a tree (Figure 1) similar to that produced by Stoll *et al*. (2003). It consists of four main lineages, two of these comprise *Sporisorium* species, one contains only *Ustilago maydis*, while the final lineage comprises *Ustilago* species. *Ustilago sporoboli-indici* resides in this

Table 1. Specimens used and GenBank accessions for rDNA ITS sequences.

Specimen	Host	Locality	Collection Date	GenBank accession
BRIP 39706	*Sporobolus pyramidalis*	Nylstroom, Limpopo, South Africa	8 Dec. 2002	AY772736
BRIP 39709	"	Bergville, KwaZulu-Natal, South Africa	10 Dec. 2002	AY772737
BRIP 39711	"	Kokstad, Eastern Cape, South Africa	11 Dec. 2002	AY772738
BRIP 39712	"	Bizana, Eastern Cape, South Africa	12 Dec. 2002	AY772739
BRIP 44549	"	Nelspruit, Mpumalanga, South Africa	20 Dec. 2002	N/A

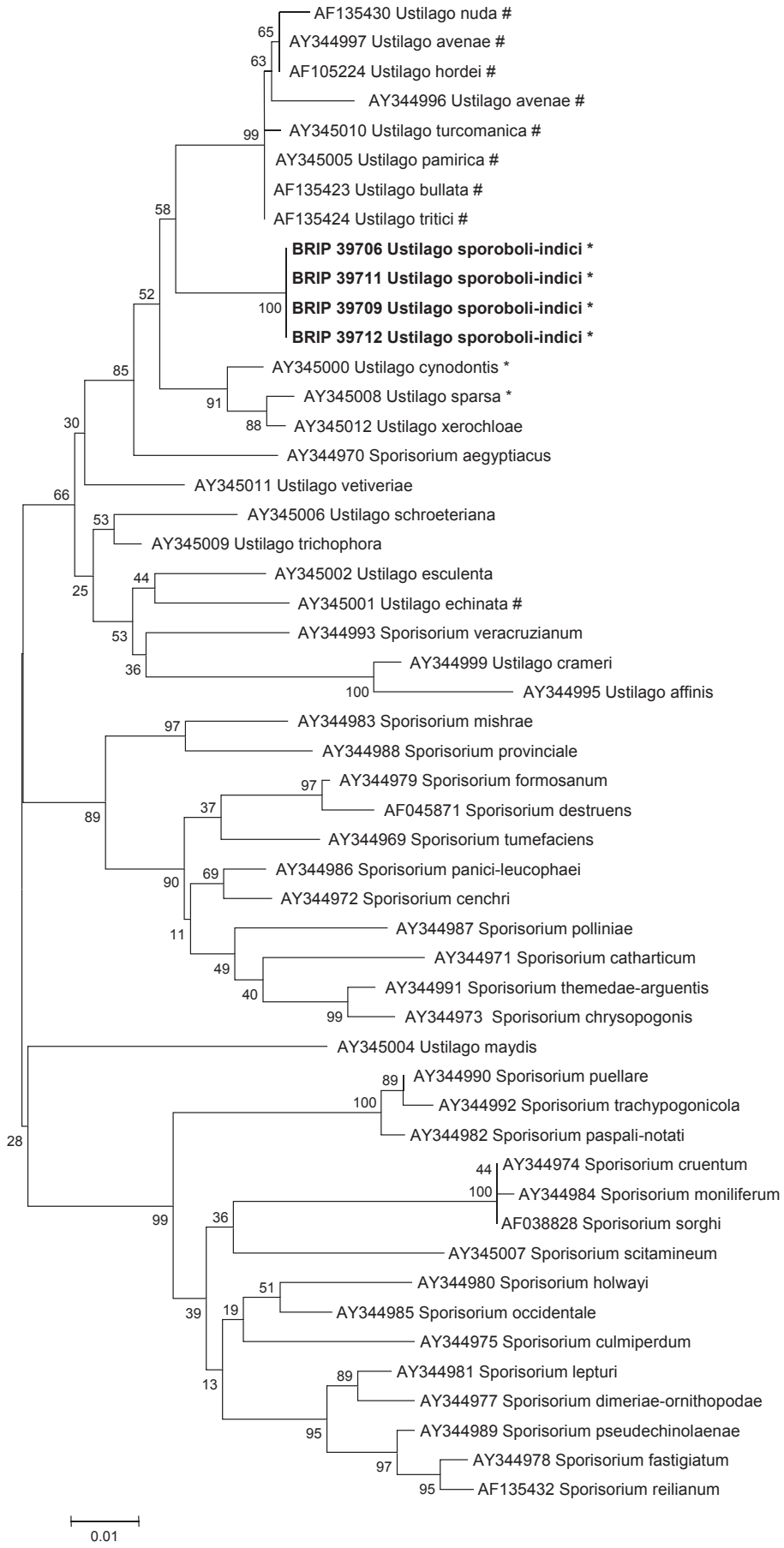

Figure 1. The phylogenetic position of *Ustilago sporoboli-indici*, in relation to the *Ustilago*/*Sporisorium* complex. #Pooideae, *Chloridoideae. Scale bar equals 1 change per 100 bases.

Ustilago lineage, which, like that found by Stoll *et al*. was found to have weak bootstrap support (66). Within *Ustilago*, Stoll *et al*. found two major lineages. *Ustilago sporoboli-indici* falls within the first of these (bootstrap support of 85), which contains a lineage of smut fungi predominantly infecting the Pooideae (temperate cereals) and a lineage predominantly infecting the Chloridoideae (Figure 1). *Ustilago sporoboli-indici* appears to form a third lineage within this group. As *Sporobolus* grasses are also members of the Chloridoideae, the results here agree with the findings of Stoll *et al*., by demonstrating that the *Ustilago* and *Sporisorium* species occurring on the Chloridoideae are from a single evolutionary lineage within *Ustilago*. The remainder of *Ustilago* and *Sporisorium* species in Figure 1 occur almost entirely on the Panicoideae.

Using these results to infer the potential host range for *U. sporoboli-indici* is more difficult. The broad implication is that this smut is much more likely to infect the Chloridoideae and Pooideae, than any other grass groups. However, *U. sporoboli-indici* is not particularly close to other groups of smut fungi infecting these tribes. This is in contrast to the group of *Ustilago* species on the Pooideae, which are closely related as shown by the short branch lengths between the species (Figure 1.). Sequences from other *Ustilago* and *Sporisorium* species that infect the Chloridoideae, and specifically *Sporobolus* species, would provide a clearer resolution of the relationships between *Ustilago* species infecting the Chloridoideae.

There have been few reports that used phylogenetic data for plant pathogenic fungi when looking for biocontrol agents. Berthier *et al*. (1996) used ITS region polymorphisms to infer two host-specialized taxa within *Puccinia carduorum* Jacky, but this was more a molecular taxonomic study, than a phylogenetic analysis of this group of rusts. The lack of use of phylogenetic analysis when looking for plant pathogenic fungi in weed control is probably due to an absence of broad phylogenetic data for these fungi. This has changed in recent years with many publications revealing the evolutionary relationships between plant pathogenic fungi (particularly obligate pathogens) and their hosts. The study by Stoll *et al*. (2003) is a good example, as it clearly demonstrates several lineages of smut fungi that predominantly occur on a single grass tribe. Another example is the phylogenetic analysis of powdery mildew fungi in the genus *Golovinomyces* (Matsuda and Takamatsu 2003) in which several evolutionary lineages were found to be specialized on tribes in the Asteraceae. But, perhaps more importantly, Matsuda and Takamatsu found a lineage of closely related *Golovinomyces* species that infect plants from a wide range of families.

When a group of plant pathogenic fungi is radiating onto unrelated host plants, the usefulness of the centrifugal phylogenetic method for selecting hosts for specificity testing must be questioned. Similarly, Voglmayr (2003) found some plant family delimited groups of *Peronospora* species (downy mildew fungi), but in addition, found a recent evolutionary lineage that was specialized to flower petals of a phylogenetically diverse range of plants.

The prospect of modernizing the centrifugal phylogenetic method has been discussed recently by Briese (2003), and although plant-pest coevolution was examined, emphasis was placed on insect-plant relationships. With more phylogenetic data appearing in the mycological literature, it is likely this information will be increasingly used to select potential biocontrol pathogens for host specificity testing. This information may be more useful for selecting species or species groups that could have previously unpredictable broad host ranges, thus eliminating them from further investigation.

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References

Anon. (2001). 'Weedy sporobolus grasses strategy'. (Natural Resources and Mines Coorparoo, Queensland)

Berthier, Y.T., Bruckart, W.L., Chaboudez, P. and Luster, D.G. (1996). Polymorphic restriction patterns of ribosomal internal transcribed spacers in the biocontrol fungus *Puccinia carduorum* correlate with weed host origin. *Applied and Environmental Microbiology* 62, 3037-41.

Briese, D.T. (2003). The centrifugal phylogenetic method used to select plants for host-specificity testing of weed biological control agents: Can and should it be modernised? *In* Improving the selection, testing and evaluation of weed biocontrol agents, eds J. Spafford and D.T. Briese, pp. 23-33. (CRC for Australian Weed Management, Technical Series 7).

Cunnington, J.H. and Shivas, R.G. (2004). The phylogenetic position of *Tilletia nigrifaciens*. *Australasian Mycologist* 22, 53-6.

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* 17, 1244-5.

Matsuda, S. and Takamatsu, S. (2003). Evolution of host-parasite relationships of *Golovinomyces* (Ascomycete: Erysiphaceae) inferred from nuclear rDNA sequences. *Molecular Phylogenetics and Evolution* 27, 314-27.

McFadyen, R. (1999). Prospects for biological control of weedy Sporobolus grasses. Proceedings of the Workshop, Weedy Sporobolus Grasses Research Update, Gympie, 19 Nov. 1999. pp. 51-3. (Queensland Department of Primary Industries).

Stoll, M., Piepenbring, M., Begerow, D. and Oberwinkler, F. (2003). Molecular phylogeny of *Ustilago* and *Sporisorium* species (Basidiomycota, Ustilaginales) based on internal transcribed spacer (ITS) sequences. *Canadian Journal of Botany* 81, 976-84.

Thompson, J.D., Gibson, T.J., Plenwniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24, 4876-82.

Vánky, K. (2003). The smut fungi (Ustilaginomycetes) of *Sporobolus* (Poaceae). *Fungal Diversity* 14, 205-41.

Voglmayr, H. (2003). Phylogenetic relationships of *Peronospora* and related genera based on nuclear ribosomal ITS sequences. *Mycological Research* 107, 1132-42.