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Abstract

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What causes flag smut of wheat?

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ABSTRACT

The causal agent of flag smut of wheat is currently subject to strict quarantine regulations in many countries and is believed to have a wide host range on wild and cultivated grasses. This fungus has been classified as both *Urocystis agropyri* and *Urocystis tritici*. *Urocystis agropyri* was first described from *Elymus repens* in Germany and *U. tritici* was first described from *Triticum vulgare* (= *T. aestivum*). In 1953, G.W. Fischer placed *U. tritici* and a large number of other *Urocystis* species in synonymy with *U. agropyri*. The present study is the first attempt to clarify the taxonomy and phylogeny of flag smut pathogens of grasses using molecular analyses. Three loci, the internal transcribed spacer (ITS) region of rDNA, the RNA polymerase II subunit 2 (RPB2), and translation elongation factor (TEF) protein-coding regions were used for phylogenetic

reconstruction to determine the species boundaries of 24 *Urocystis* specimens from triticoid hosts. Results indicate that there are several distinct lineages of flag smut pathogens, including the causal agent of flag smut of wheat, which is supported as a separate species, *U. tritici*. Sequences from specimens on *E. repens*, which are retained as *U. agropyri*, grouped in a clade distinct from those on wheat and rye. The closest relatives of *U. tritici* were found to be *U. hispanica* from *Aegilops* and *Urocystis* sp. from *Thinopyrum junceiforme* and *Elymus trachycaulis*. Recognition that *U. tritici* is genetically distinct from *U. agropyri* sensu stricto will impact regulatory policy and facilitate the development of diagnostic tests.

INTRODUCTION

The causal agents of flag smuts of grasses (*Urocystis* spp., Urocystidales, Basidiomycota) are widespread on wild and cultivated grasses throughout the temperate and subtropical regions of the world. The pathogens cause systemic infection on host plants, forming sori in vegetative parts, most commonly in leaves, as narrow stripes between the leaf veins (Mordue & Walker, 1981). The sori are filled with spore balls consisting of one to five brown, smooth-walled, permanently agglutinated teliospores, surrounded by complete (thorough) to incomplete (partial) layers of mostly hyaline sterile cells. The causal agents of flag smuts of grasses overwinter in soil; although, in autumn-sown cereals the systemic mycelium may overwinter in infected seedlings and become a source of infection next spring. Typically, infected plants fail to produce any seeds or have malformed inflorescences due to sorus formation (Purdy, 1965). As a result, flag smuts may cause yield losses of up to 100% under favorable environmental conditions (Purdy, 1965).

The causal agent of flag smut of wheat is a regulated pathogen in numerous countries around the world, including the United States, Canada, Brazil, Australia, and Kenya. Because of these regulations, significant economic losses often occur due to the quarantining of infested regions. For example, the US maintains a quarantine on imported wheat, including straw and seed, as it may carry "foreign strains" of flag smut that could be introduced (Cline & Farr, 2006).

The causal agent responsible for flag smut of wheat was first discovered in South Australia in 1868 (McAlpine, 1910). However, it is possible that the disease had been there long before the first official report, referred to by local farmers as "black rust" (McAlpine, 1910). The disease was subsequently reported in Australia from New South Wales, Victoria, and Queensland, and finally

Western Australia by 1921 (Purdy, 1965). The first record outside of Australia was in Italy in 1897 (Baldrati, 1928). Subsequently, it was reported from China (Miyake, 1912), Japan (Hori, 1907), India (Mundkur & Thirumalachar, 1952), South Africa (Putterill, 1920), and a number of Middle Eastern countries (Rieuf, 1954). The pathogen was reported from the North American continent in 1918, when it was observed in a small field in Missouri (Tisdale, 1926). Five years later, it was established in Missouri, Kansas and Illinois, and in 1940 it was reported in the Pacific Northwest (Fischer, 1942). In the US, flag smut of wheat was mainly a problem in the Midwest in the 1920s–1930s, and in the Pacific Northwest in the 1950s–1960s (Purdy, 1965).

In general, flag smut of wheat is more common in areas with warm, dry summers, where the soil temperature and moisture level are favorable for teliospore germination and subsequent infection of wheat seedlings (Purdy, 1965). Success in combating flag smut of wheat was due to the development of new wheat cultivars with flag smut resistance as well as fungicidal seed treatments. Sporadic resurgences of the disease have been reported in different parts of the world since 1970. A substantial outbreak occurred in New South Wales in the early 1990s, due to the release and use of a susceptible wheat cultivar (Ballantyne, 1993). In the United States, the most recent report of flag smut of wheat was in localized areas in south-central and southeastern Washington (Nelson & Durán, 1984). In 2015, it reemerged in seven western counties of Kansas for the first time in 70 years (https://agriculture.ks.gov/divisions-programs/plant-protect-weed-control/wheat-flag-smut).

The taxonomy of causal agents of flag smuts of grasses is controversial. It began with the description of *Uredo agropyri* as the causal agent of the flag smut of couch grass (*Elymus repens*) in Germany in 1848 (Preuss, 1848). Later, Fischer v. Waldheim transferred the species to *Urocystis agropyri* (Fischer v. Waldheim, 1867). Wolff (1873) identified the fungus causing flag smut of wheat in South Australia as *Urocystis occulta,* a species that had been described earlier as a pathogen of rye (Wallroth, 1833). After a detailed morphological examination, Körnicke (1877) determined that the pathogen of wheat from South Australia was distinct from that on rye and erected a new species, *Urocystis tritici.* Fischer (1943, 1953), on the basis of morphology, and Fischer and Holton (1943), on the basis of physiological specialization, proposed that *U. tritici,* as well as a number of species of *Urocystis* species on other grasses, were synonymous with *U. agropyri.* Since then, some authors (mostly North American mycologists and phytopathologists) followed the morphological concept of Fischer, while others (mostly European and Australian mycologists) treated *U. tritici* as a separate species, restricted to wheat. The latter approach was reflected in a world monograph of smut fungi (Vánky, 2012) where about 60 different species of

Urocystis were reported on grasses. Among them, ten (*U. agropyri* s.l., *U. agropyri-campestris, U. arxanensis, U. hispanica, U. hordeicola, U. occulta, U. secalis-silvestris, U. sichuanensis, U. tianschanica,* and *U. tritici*) were reported from triticoid hosts.

Studies on experimental host ranges of *Urocystis* species on grasses have produced conflicting results. Some researchers demonstrated that individual isolates of flag smuts from different grasses exhibited substantial host specialization (Mordue & Walker, 1981; Sampson & Watson, 1985). Conversely, other authors reported that at least some species of *Elymus* and *Aegilops* had been successfully infected by isolates from wheat in artificial inoculation tests (Purdy, 1965; Rees & Platz, 1973; Fischer & Holton, 1943).

Morphologically, the causal agent of flag smut of wheat is similar to *Urocystis agropyri* and some other species on triticoid grasses with dark-brown to brown teliospores agglutinated in spore balls and surrounded by hyaline to yellowish layers of sterile cells. In most cases, the identification of flag smut spores is very difficult if the host is not actually identified. Furthermore, the reported host ranges of different species causing flag smuts often overlap. Both *Urocystis hispanica* and *U. tritici* are reported to infect *Aegilops* (Purdy, 1965; Vánky, 2012). *Urocystis agropyri* and *U. tritici* are both capable of infecting species of *Elymus* (Fischer & Holton, 1943; Purdy, 1965), while three different species (*Urocystis hordeicola, U. agropyri, U. tianschanica*) have been reported from barley (Fischer, 1953; Vánky, 2012).

A molecular approach to resolve the species boundaries of flag smuts of grasses has been recommended, due to limited availability of taxonomic characters and contradictory reports on host-specificity (Vánky, 2012). In this study, the phylogenetic relationships of *Urocystis* species causing flag smuts on different triticoid hosts were investigated using recently collected field material and herbarium specimens. The internal transcribed spacer (ITS) rDNA region, the RNA polymerase II subunit 2 (RPB2), and the translation elongation factor (TEF) protein-coding regions were used for phylogenetic reconstruction to determine species boundaries. These data will help to understand the origin and genetic diversity of flag smuts of grasses and in the development of DNA-based methods of detection using pathogen-specific molecular markers.

MATERIALS AND METHODS

Taxon selection

Fresh specimens were collected in Washington, Idaho and Kansas (United States) in 2014-2015 and in Ukraine in 2009–2012, and used together with herbarium specimens from WSP, VPRI, KH, DAOM, BPI and BRIP (Table 1). A specimen of *Urocystis bolivari* from a non-triticoid host, *Lolium perenne,* was selected as an outgroup taxon for the phylogenetic analyses.

Morphology

Sorus and spore characteristics were studied from fresh and dried herbarium material. Specimens were examined by light microscopy (LM) and scanning electron microscopy (SEM). Images of sori were taken with a Canon Power Shot G10 camera. For LM, spore balls were mounted in 90% lactic acid on a microscope slide, covered with a cover glass, gently heated to boiling point and cooled, and then examined under a Carl Zeiss Axiostar microscope at 1000× magnification. LM photographs were taken with a Canon Power Shot G10 camera. For SEM studies the morphology of spores was observed at 15kV and photographed with a scanning electron microscope Hitachi TM3030. At least 30 teliospores were measured from each collection. Hierarchical clustering analysis (available at www.wessa.net) was used to assess the differences between *Urocystis agropyri* s. str. and *U. tritici* in the number of teliospores per spore ball and spore length.

DNA extraction, PCR and sequencing

The majority of sequences generated in this study were from specimens of *Urocystis* species causing flag smuts on grasses collected after 1990 (Table 1). Genomic DNA was isolated from spore balls using FastPrep 24 (MP Biomedicals, Irvine, California). Tubes were incubated in a water bath for 5 hours at 55° C, and DNA extracted using DNeasy Plant Mini Kit (Qiagen, Valencia, California) following the manufacturer's instructions. All amplifications were performed in 20 µl aliquots on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California). ITS1-F primer was used as the forward primer and ITS4-B was used as the reverse primer for the ITS region (Gardes & Bruns, 1993). In some cases the M-ITS was used as a forward primer (Stoll et al. 2003). The TEF region was amplified with Ef1-983F and EF1-2218R primers and nested with EF1-cf and EF1-1953R (Sascha Lotze-Engelhard, pers. comm.). RPB2 was amplified with fRPB2-

5f and bRPB2-7R and in some cases nested with bRPB2-6R2 and bRPB2-6F (Matheny et al., 2007). Primers are listed in Table 2. Standard cycling parameters with an annealing temperature of 47°C were used for the amplification of the ITS region. PCR cycle conditions for amplification of TEF were performed as follows: 95° C for 3 min, followed by 10 cycles of 95° C for 45 sec, 60° C (with 1° C decrease/ each cycle) for 1 min, and 72° C for 1 min 30 sec, followed by 34 cycles of 95° C for 45 sec, 50° C for 1 min, and 72° C for 1 min 30 sec, followed by a final extension at 72° C for 10 min. PCR cycle conditions for amplification of RPB2 were performed as follows: 95° C for 3 min, followed by 10 cycles of 95° C for 30 sec, 66° C (with 1° C decrease/ each cycle) for 30 sec, and 72° C for 1 min 30 sec, followed by 34 cycles of 95° C for 30 sec, 56° C for 45 sec, and 72° C for 1 min 30 sec, followed by a final extension at 72° C for 7 min. PCR products were purified with ExoSAP-IT (USB, Cleveland, Ohio) according to the manufacturer's instructions and amplified with respective forward and reverse PCR primers with the BigDye 3.1 terminator kit (Applied Biosystems, Foster City, California). PCR products were sent to Elim Biopharm for direct sequencing. Sequences were submitted to GenBank under the accession numbers listed in Table 1.

Phylogenetic analyses

Consensus sequences were assembled, aligned, and edited with Geneious 7.1.8 for Mac OS (Biomatters, Auckland, New Zealand) and with MAFFT 6.853 (Katoh & Toh, 2008) using the L-INS-i option. As suggested by Giribet & Wheeler (1999), manipulation of the alignment by hand was avoided, to obtain reproducible results. The sequences from each locus were concatenated in a supermatrix and run as partitioned datasets with Maximum Likelihood and Bayesian inference as phylogenetic criteria. The combined data set was tested for incongruence with the partition homogeneity test (PHT) as implemented in PAUP version 4.0b10 software. Maximum Likelihood was implemented as a search criterion in RAxML (Stamatakis, 2014). HKY+I+G was specified as the model of evolution for nucleotide sequence data in MrModeltest (Nylander et al., 2004). The RAxML analyses were run with a rapid Bootstrap analysis (command –f a) using a random starting tree and 1000 maximum likelihood bootstrap replicates. A Markov Chain Monte Carlo (MCMC) search in a Bayesian analysis was conducted with MrBayes (Ronquist & Huelsenbeck, 2003). Four runs were implemented for 5 million generations. The cold chain was heated at a temperature of 0.25. Substitution model parameters were sampled every 500 generations and trees were saved every 1000 generations. Convergence of the Bayesian analysis was confirmed using AWTY (Nylander et al., 2008) and a burn-in of 18000 generations was calculated. The ML and Bayesian analyses were run three times to test accuracy.

RESULTS

Phylogenetic analysis

The MAFFT analyses resulted in alignments of length 630 bp for ITS, 600 bp for TEF, and 570 bp for RPB2. The partition homogeneity test showed that no significant conflict existed between the phylogenies of the ITS, RPB2 and TEF regions. The combined data matrix contained 25 specimens including the outgroup and a total of 1797 characters (including gaps). The different runs of BA and the ML analyses yielded consistent topologies in respect to well-supported branches (*a posteriori* probability greater than 90 in most cases). The consensus tree of one run of Bayesian phylogenetic analyses is presented in Fig. 1.

Specimens identified as *Urocystis agropyri* s.l. were polyphyletic with at least four different lineages in our analysis. The first lineage consisted of a specimen from *Hystrix japonica,* a host native to Japan; the second lineage consisted of a specimen from *Elymus* sp. from the United States; the third lineage consisted of specimens from slender wheatgrass (*Elymus trachycaulis*) from the United States and sea wheatgrass (*Thinopyrum junceiforme*) from Greece; and the fourth lineage included all the American and European specimens of *U. agropyri* from *Elymus repens* and *Pseudoroegneria spicata.* As *Elymus repens* is the type host of *U. agropyri*, the latter clade was designated as *U. agropyri* s. str. Within *U. agropyri* s. str., two separate subclades were evident based mainly on the geography of collections.

All sequences of the six specimens on wheat from Australia and the United States, including one from the 2015 outbreak in Kansas, were identical. Specimens on wheat were distinct from *U. agropyri* s. str. The causal agent of flag smut of wheat was most closely related to *Urocystis hispanica* from *Aegilops* and *U. agropyri* s. l. from slender wheatgrass and sea wheatgrass.

An additional clade consisted of *Urocystis* species causing flag smut of rye and barley. A specimen morphologically identified as *Urocystis hordeicola* causing flag smut of barley was closely related to two specimens identified as *U. occulta* causing flag smut of cultivated rye, indicating that the two species may be conspecific, but type specimens would need to be examined to confirm this synonymy. A specimen of *U. secalis-silvestris* from the wild rye *Secale sylvestre* was basal to this clade.

Morphological analysis

Morphological characters for *Urocystis* spp. on different grass genera are summarized in Table 3. The specimens used in this study did not differ significantly in spore ball morphology, spore width, spore wall thickness, color of teliospores, morphology of sterile cells and color of spore mass. The morphological analysis focused on a comparison of the specimens representing causal agent of flag smut of wheat (*U. tritici*) with *U. agropyri* s.str. from *Elymus repens*. Mean spore ball length of *U. tritici* specimens from *Triticum* was larger than those of the specimens of *U. agropyri* s.str. from *Elymus repens*. In *U. tritici*, 42% of spore balls had one spore, 42% had two spores, 14% had three spores, and 4% had four or five spores, while in *U. agropyri* s.str., 1% had only sterile cells, 53% had one spore, 35% had two spores, 10% had three spores, and 1% had four spores. In many cases the spores of *U. tritici* were surrounded by complete layers of generally larger sterile cells, while in *U. agropyri* s.str. incomplete layers of generally smaller sterile cells were common.

DISCUSSION

Nucleotide sequence data from three genes were analyzed to infer the phylogeny and species limits of *Urocystis* species causing flag smuts on triticoid grasses. The most significant conclusion is that the analysis strongly supports the separation of the *Triticum*-infecting *Urocystis tritici* from the *Elymus*-infecting *U. agropyri* s.str. The results of the phylogenetic analysis further indicates that species of *Urocystis* on triticoid hosts have narrow host ranges. These results coincide with similar studies on other groups of smut fungi, supporting the hypothesis that most smut fungi are restricted to a single genus or, sometimes, to a single species of plant (Bauer et al., 2008; Kemler et al., 2009; Lutz et al., 2008, 2012a, 2012b; Piątek et al., 2011, 2012, 2013a, 2013b; Savchenko et al., 2013, 2014a, 2014b, 2015; Vánky & Lutz, 2007, 2010). These findings may affect regulatory policy, as they indicate that flag smuts on other hosts are unlikely to spread to wheat.

Because *Urocystis tritici* is a regulated pathogen, accurate identification is extremely important for biosecurity purposes. *Urocystis tritici* and *U. sichuanensis* appear to be the only flag smuts found on wheat, but the latter species is known from the type collection in China only and has not been recollected since 1952 (Vánky, 2012). However, spore balls from other hosts may contaminate grain without infection, for example from *Urocystis* species infecting *Elymus* species

that may grow alongside wheat fields. The presence of these spores could trigger regulatory action if they were misidentified as *U. tritici*. *Urocystis tritici* can to some extent be distinguished from *U. agropyri* s.str. using morphology when hosts are known. The spore balls of *U. tritici* tend to have more teliospores and generally darker sterile cells than *U. agropyri*, and the sterile cells of *U. tritici* were generally larger than those of all other examined flag smuts. Nevertheless, we do not think that spore ball morphology should be used for the delimitation of these pathogens due to the intergradation of morphological characters. *Urocystis tritici* can be distinguished from *U. agropyri* and other morphologically similar species of flag smut of grasses using the ITS region, a more reliable way of identification under these circumstances.

Four lineages were observed that had been classified as *Urocystis agropyri*. One lineage consisted of specimens from *Elymus repens* and *Pseudoroegneria spicata* from Europe and North America, which was identified as *U. agropyri* s. str. as *E. repens* is the type host for this species. The inclusion of the specimen on *P. spicata* is interesting, because *Pseudoroegneria* is one of the genome donors for the hexaploid *Elymus repens* (Mason-Gamer, 2008). The specimens of *U. agropyri* s. str. showed additional intraspecific variation. Two morphologically identical subclades were detected, one with specimens mostly from North America and the other with specimens from Europe. Whether these phylogenetic lineages should be recognized as species or not remains unclear, with additional work and more extensive sampling needed to clarify them. The other lineages previously classified as *U. agropyri* correspond to: (1) a specimen from *Hystrix japonica* from Japan, (2) a specimen from *Elymus* sp. from the US, and (3) specimens from wheatgrasses *Thinopyrum junceiforme* and *Elymus trachycaulus* from Greece and the US, respectively. These lineages appear to represent undescribed species of *Urocystis,* or they may represent one of the species placed in synonymy of *U. agropyri* by Vánky (2012), phylogenetically and ecologically different from *U. agropyri* s. str, but nearly indistinguishable morphologically (Table 3). The only morphological difference observed was that the sterile cells of the *Urocystis* specimens on wheatgrasses were hyaline while those of the two other lineages were slightly darker, hyaline-yellowish. However, there is currently insufficient information regarding the biology and ecology of these groups to resolve the specific status of these collections.

Another clade in the phylogenetic analysis consisted of the isolates of *Urocystis occulta* from cultivated rye (*Secale cereale*), *U. secalis-silvestris* from wild rye (*Secale sylvestre*), and *U. hordeicola* from cultivated barley (*Hordeum vulgare*). The analysis indicated a high level of genetic similarity among the barley and cultivated rye specimens of *U. hordeicola* and *U. occulta,*

respectively. The two smut species were identical in all three studied gene regions but have clear differences in spore morphology: *U. hordeicola* has usually two teliospores in larger spore balls (26 μm) surrounded by a complete layer of sterile cells, while *U. occulta* has often one teliospore in smaller spore balls (15.5 μm) surrounded by an incomplete layer of sterile cells. Additional studies are needed to address host specificity in the *U. occulta–U. hordeicola* complex, including the phylogenetic placement of *U. tianschanica,* another species reported on wild *Hordeum* species in Central Asia.

Host shifts probably played an important role in the species radiation of *Urocystis* species causing flag smuts. Both Bayesian and ML analyses clustered the specimens from wheat, wild wheat (*Aegilops*), rye, wild rye, barley, and the wheatgrass in one major clade. The close phylogenetic relationships among these smuts likely reflects the close relationships among the triticoid hosts, and may possibly reflect the origin of their wild and cultivated hosts in the Fertile Crescent of the Middle East (Salamini et al., 2002). For example, *Urocystis hispanica* on *Aegilops* is closely related to *U. tritici* on wheat. The hexaploid *Triticum aestivum* is thought to have arisen through natural hybridization between tetraploid wheat cultivars and diploid *Aegilops* and *Triticum* species (Kilian et al., 2007), such as the hybridization of a wild wheat species *Aegilops tauschii* and the tetraploid *T. turgidum* (McFadden & Sears, 1944), which may itself have an ancestor in the current wild wheat species, *Aegilops speltoides,* a host of *U. hispanica* (Chapman et al., 1976). This hybridization and diversification of the host species may have affected the speciation of the flag smut pathogens. It would not be surprising if the center of diversity of these pathogens coincides with the center of origin of their hosts in the Middle East, as this has been found in population genetic studies of different cereal pathogens such as *Blumeria graminis* and *Zymoseptoria tritici* (Stuckenbrock et al., 2007; Wyand & Brown, 2003). If true, this would explain the lack of morphological or phylogenetic differences between *U. tritici* from the United States and Australia, which might have originated from a single population in the Middle East and have been introduced to other regions of the world with its cultivated host. *Urocystis* species causing flag smuts may also have shifted to new hosts as they were moved around the world. An example of this type of host shift was provided by Munkacsi et al. (2007), who suggested that corn parasite *Sporisorium reillianum,* which originated approximately 3 million years ago on *Sorghum* in Africa, moved to maize only after the introduction of sorghum to the New World in 1700. However, these hypotheses about *Urocystis* species causing flag smuts cannot be proved or disproved without investigations of the specimens of flag smuts from the Middle East.

Until recently, molecular studies in plant pathogenic smut fungi have relied primarily on the LSU and internal transcribed spacer (ITS) regions of the nuclear rDNA to estimate evolutionary relationships (Bauer et al., 2008; Kemler et al., 2009; Lutz et al. 2008, 2012a, 2012b; Piątek et al., 2011, 2012, 2013a, 2013b, 2015a, 2015b; Savchenko et al., 2014a; Vánky & Lutz, 2007, 2010). Fewer studies have incorporated additional gene regions, such as the TEF (Bao et al., 2010; Carris et al., 2007; McTaggart et al., 2012; Munkacsi et al., 2007), GAPDH (McTaggart et al., 2012; Munkacsi et al., 2007), ATP6 (Munkacsi et al., 2007), COX3 (Munkacsi et al., 2007), and RPB1 (Munkacsi et al., 2007). Nuclear genes that encode the two largest subunits of RNA polymerase II (RPB2) are proving useful to infer the phylogenies of organisms across the fungal kingdom (Hibbett et al., 2007). However, thus far only a few studies have used RPB2 for species delimitation in plant pathogenic smut fungi (Bao et al., 2010; Carris et al., 2007; Piątek et al., 2016). In this study, LSU was found to be too conservative with almost no differences between taxa. Thus, we decided to use the internal transcribed spacer gene region (ITS), used in fungal barcoding, the protein-coding translation elongation factor 1 alpha region (TEF) that proved to be informative in another study on *Urocystis* phylogeny (Lotze-Engelhard, pers. comm.) and the protein coding RPB2 region, which we found to be informative after preliminary testing.

The tribe Triticeae includes both economically important grasses such as cereal and forage crops and troublesome weeds of worldwide distribution, such as couch grass *Elymus repens* (Palmer & Sagar, 1963). The results of this study underscore the need for more extensive study of *Urocystis* species causing flag smut of grasses. Problems in the identification of smut fungi become critical when the pathogens are the focus of international biosecurity regulations including quarantines. A better understanding of the species boundaries among flag smuts of grasses is important for selecting the most appropriate taxa to develop diagnostic tests. As additional isolates of flag smuts of different grasses from more diverse geographic areas become available, it will be possible to assess more fully the inter- and intra-specific variability in this important group of plant pathogens.

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Fig. 1 Bayesian inference of phylogenetic relationships resulting from the concatenated nucleotide sequence data. A 60% majority-rule consensus tree is shown. Bolded branches indicate posterior probabilities greater that 90%. Color boxes represent different lineages of flag smut (green for *Urocystis agropyri* s.l., purple for *U. agropyri* s.str., yellow for the flag smut of rye and barley and brown for the flag smut of wheat).

Fig. 2. Sori and spore balls of *Urocystis agropyri* s.str. from *Elymus repens.* **A.** Sori. **B, C, D.** Spore balls. Scale bars: **A** = 5 mm, **B** = 100 µm, **C, D** = 20 µm.

Fig. 3. Sori and spore balls of *Urocystis tritici* from *Triticum aestivum.* **A, B.** Sori. **C, D.** Spore balls. Scale bars: **A** = 5 mm, **B** = 1 mm, **C, D** = 20 µm.

Species of *Urocystis*	Host plant	Location	Date of collection / collector (s)	Voucher Number	Genbank Accession Number		
					ITS	TEF	RPB2
U. bolivari	*Lolium perenne*	Australia, South Australia, 9 km E of Pinnaroo	06.10.2001 / R.G. Shivas, K. Vánky	BRIP 47107	KX057771	KX057796	KX057748
U. agropyri s.l.	*Hystrix japonica*	Japan, Hiroshima, Sandan-Kyo	24.05.1959 / Y. Marimoto	DAOM 63674	KX057783	KX057809	KX057759
	Elymus sp.	United States, Washington, Chelan County, Lake Chelan State Park, 47.8709 N, 120.1966 W	04.07.2014 / K.G. Savchenko	WSP 72767	KX057784	KX057808	KX057770
	Elymus repens	United States, Washington, Whitman County, Pullman, 46.7356 N, 117.1595 W	17.06.2014 / K.G. Savchenko	WSP 72763	KX057787	KX057812	KX057762
		United States, Idaho, Lewis County, Kamiah, 46.2344 N, 116.0556 W	20.07.2014 / L.M. Carris, K.G. Savchenko	WSP 72768	KX057788	KX057813	KX057765
		United States, Washington, Whitman County, Pullman, 46.7390 N, 117.1554 W	06.06.2014 / K.G. Savchenko	WSP 72762	KX057789	KX057814	KX001804
		Ukraine, Kyiv, Holosiiv District, 50.3834 N, 30.4776 E	12.06.2009 / K.G. Savchenko	WSP 72765	KX057790	KX057815	KX057763

		United States, Washington, Pullman, 46.7378 N, 117.1632 W	08.06.2014 / K.G. Savchenko	WSP 72774	KX057792	KX057816	KX057766
		Ukraine, Cherkasy region, Trakhtemyriv Regional Landscape Park, 49.9687 N, 31.3416 E	22.05.2010 / K.G. Savchenko	WSP 72764	KX057794	KX057817	KX057767
		Ukraine, Donetsk region, Donetsk, Proletarskyi district, 47.9739 N, 37.9278 E	05.06.2011 / M.V. Savchenko	WSP 72769	KX057793	KX057818	KX057768
		Ukraine, Zaporizhia region, Berdyansk, 46.7651 N, 36.7745 E	18.06.2011 / M.V. Savchenko	WSP 72766	KX057795	KX057819	KX057769
	Elymus trachycaulus	Idaho, Shoshone County, Wallace, King Rd, 47.4480 N, 115.9357 W	18.08.2014 / K.G. Savchenko	WSP 72761	KX057785	KX057810	KX057761
	Pseudoroegneria spicata	Idaho, Latah Co., Silver Valley reservoir	28.06.2014 / K.G. Savchenko	WSP 72760	KX057791	KX057820	KX057764
	Thinopyrum junceiforme	Greece, Kos Island, Marmari	21.04.1990 / H. Scholz, I. Scholz	Vánky Ustil. Ex. 769, WSP 70519	KX057786	KX057811	KX057760
U. hispanica	*Aegilops lorentii*	Azerbaijan, 5 km SW of Baku	22.06.1986 / E. Tagi-Zade, K. Vánky	Vánky Ustil. Ex. 625, WSP 69160	KX057772	KX057801	KX057752

U. occulta	*Secale cereale*	Germany, Berlin, Spandau, ad Weinmeister hornweg pr. Karolinenho he	22.06.1986 / H. Scholz, I. Scholz	Vánky Ustil. Ex. 587, WSP 69121	KX057773	KX057797	KX057749
		Canada, Manitoba, 12 km SW of La Prairie	06.07.1987 / B.R. Nielsen, J.J. Nielsen	Vánky Ustil. Ex. 696, WSP 69497	KX057774	KX057798	KX057750
U. secalis-silvestris	*Secale sylvestre*	Ukraine, Dnipropetro vsk reg., Dniprovsko-Orilskij Reserve	15.06.2009 / K.G. Savchenko	WSP72 772	KX057775	KX057799	KX001805
U. hordeicola	*Hordeum vulgare*	Turkey, Aksehir	30.05.1992 / Mamluk, Saari	VPRI 20770	KX057776	KX057800	KX057751
U. tritici	*Triticum aestivum*	Australia, Victoria, Horsham	1992 / J. Brown	VPRI 19511	KX057777	KX057802	KX057753
		Australia, Victoria, Horsham	1992 / J. Brown	VPRI 18612	KX057778	KX057803	KX057754
		Idaho, Moscow	07.1992 / Anonymou s	WSP 72771	KX057779	KX057805	KX057755
		Idaho, Rockland	06.2010 / B. Goates	WSP 72770	KX057780	KX057806	KX057756
		Kansas, Rooks County, 39.4222 N, 99.2508 W	08.04.2015 / R.D. Buhler	BPI 910042	KX057781	KX057807	KX057757
		Idaho, Idaho Falls	Anonymou s	WSP 72773	KX057782	KX057804	KX057758

Table 1. Specimens used in this study, their geographic origin, collection number, herbarium, and GenBank accession numbers.

Primer name	Nucleotide sequence 5'–3'	Source
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns, 1993
ITS4-B	CAGGAGACTTGTACACGGTCCAG	Gardes and Bruns, 1993
M-ITS	GGTGAACCTGCAGATGGATC	Stoll et al. 2003
Ef1-983F	GCYCCYGGHCAYCGTGAYTTYAT	http://ocid.nacse.org/research/aftol/primers.php; Rehner and Buckley, 2005
EF1-2218R	ATGACACCRACRGCRACRGTYTG	http://ocid.nacse.org/research/aftol/primers.php; Rehner and Buckley, 2005
EF1-cf	ATYGCYGCNGGTACYGGYGARTTCGA	http://ocid.nacse.org/research/aftol/primers.php
EF1-1953R	CCRGCRACRGTRTGTCTCAT	http://ocid.nacse.org/research/aftol/primers.php
fRPB2-5f	GAYGAYMGWGATCAYTTYGG	Liu et al., 1999
bRPB2-7R	GAYTGRTTRTGRTCRGGGAAVGG	Matheny, 2005
bRPB2-6R2	GGRCANACCATNCCCCARTG	Matheny et al. 2007
bRPB2-6F	TGGGGYATGGTNTGYCCYGC	Matheny, 2005

Table 2. PCR primers used in this study.

Species of *Urocystis*	Host	Diameter of spore balls	Number of spores per spore ball	Diameter of spores	Character of sterile cells layer	Color of sterile cells	Length of sterile cells
Urocystis agropyri (Preuss) A.A. Fisch. Waldh.	*Elymus repens* (L.) Gould	20.3 µm	0 = 1% 1 = 53%, 2 = 35%, 3 = 10%, 4, 5 = 1%	14.4 µm	Complete to incomplete	Hyaline-yellowish	3–8 µm
	E. trachycaulus (Link) Gould ex Shinners	25 µm	1 = 45%, 2 = 50%, 3 = 5%	16.7 µm	Mostly complete	Hyaline	4–10 µm
	Hystrix japonica (Hack.) Ohwi	20.5 µm	1 = 60%, 2 = 35%, 3 = 5%	15.1 µm	Mostly complete	Hyaline-yellowish	3–8 µm
	Thinopyrum junceiforme (Á. Löve & D. Löve) Á. Löve	26.4 µm	1 = 32%, 2 = 53%, 3 = 10%, 4 = 5%	13.9 µm	Mostly complete	Hyaline	5–10 µm
U. hispanica (Syd.) Zundel	*Aegilops lorentii* Hochst.	29.2 µm	1 = 60%, 2 = 20%, 3 = 20%	15 µm	Complete to incomplete	Hyaline-yellowish	5–10 µm
U. hordeicola (Lavrov) Schwarzman	*Hordeum vulgare* L.	26 µm	1 = 30%, 2 = 60%, 3 = 10%	16.3 µm	Complete	Hyaline-brownish	4–7 µm

U. occulta (Wallr.) Rabenh.	*Secale cereale* L.	15.5 µm	1 = 55%, 2 = 43%, 3 = 2%	12.2 µm	Incomplete	Hyaline-yellowish	4–7 (8) µm
U. secalis-silvestris (Uljan.) Schwarzman	*Secale sylvestre* Host	22 µm	1 = 56%, 2 = 44%	13.5 µm	Mostly complete	Hyaline-yellowish	5–10 µm
U. tritici Körn.	*Triticum aestivum* L.	27.1 µm	1 = 41%, 2 = 41%, 3 = 14%, 4, 5 = 4%	15.5 µm	Mostly complete	Hyaline-yellowish	8–12 (15) µm

Table 3. Morphology of *Urocystis* from different triticoid hosts.