Positively selected effector genes and their contribution to virulence in the smut fungus *Sporisorium reilianum*

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Abstract

Plants and fungi display a broad range of interactions within natural and agricultural ecosystems ranging from symbiosis to parasitism. Pathogenic interactions are governed by secreted fungal effector proteins, which are thought to coevolve with their host targets. Biotrophic smut fungi which belong to the division of Basidiomycota are well-suited to investigate the evolution of plant pathogens, because several quality draft genomes and genetic tools are available for these species. Here, we used the genomes of *Sporisorium reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum*, two closely related formae speciales infecting maize and sorghum, respectively, together with the genomes of *Ustilago hordei*, *U. maydis* and *S. scitamineum* to identify effector genes showing signs of positive selection. The largest numbers of such genes were identified in the two pathovariants of *S. reilianum* and between paralogues in *U. hordei*, where many belong to families showing species-specific expansions. Next, we assessed the contribution to virulence of candidate effector genes in *S. reilianum* f. sp. *zeae* by deletion of individual genes in a solopathogenic strain. While eight of nine deletions mutants were unaffected in virulence, one mutant had lost virulence. This shows that despite the relatively recent divergent time of the two formae speciales, a signature of positive selection in candidate effector genes in *S. reilianum* is a poor indicator for the identification of genes with virulence functions.

Key words

Positive selection; effector evolution; smut fungi; comparative genomics; virulence

Introduction

Plants and fungi have a long history of coevolution since the emergence of pioneering land plants approximately 400 million years ago. The development of early plants was likely supported by associations with symbiotic fungi, as suggested by analyses of ribosomal RNAs and fossil records (Gehrig et al. 1996; Martin et al. 2017; Remy et al. 1994). Different forms of plant-fungus interactions evolved, including mutualistic symbiosis where both plant and fungus benefit (Parniske 2008), and pathogenic interactions where fungal colonization greatly reduces plant fitness (Dean et al. 2012). Pathogenic interactions play critical roles in natural and agricultural ecosystems, and understanding the evolutionary mechanisms shaping such interactions is an active field of research (Bagchi et al. 2014; Fisher et al. 2012). Pathogenic interactions are governed by secreted fungal effector proteins, which can protect and shield growing hyphae, suppress plant defense responses and change plant physiology to support growth of the pathogen. Many effector proteins lack known functional domains, and their expression is linked to plant colonization (Franceschetti et al. 2017; Lanver et al. 2017; Lo Presti et al. 2015; Toruño et al. 2016). Effector proteins and their plant targets are thought to evolve either in an arms race or a trench-warfare scenario. In the former, effectors manipulating the host for the benefit of the pathogen are selected, whereas plant targets evolve to detect effectors or inactivate their function (Rovenich et al. 2014). In the latter scenario, sets of alleles are maintained by balancing selection on both host and pathogen sides (Brown and Tellier 2011; Tellier et al. 2014). Several methods are in place for identifying genomic regions under selection (Aguileta et al. 2010; Aguileta et al. 2009; Nielsen 2005). These approaches detect genes subject to positive selection in host and pathogen genomes, and it is thought that genes with signs of positive selection have important functions during host pathogen interaction or have contributed to host specialization (Aguileta et al. 2009). Depending on the aim of an investigation, studies identifying genes with signatures of positive selection are carried out

within or between species. Whereas studies on the population level focus on recent and ongoing selective processes, and are therefore instrumental in understanding the process of adaptation, comparative genomic studies employing different species encompass a broader time span and can for instance help to unravel the genetic basis of host specialization (Plissonneau et al. 2017). Indeed, positive selection studies in a number of plant pathogen systems revealed that genes encoding secreted effector proteins are enriched in signatures of positive selection. Such studies include investigations in diverse plant pathogens like *Microbotryum* species causing anther-smut disease of Caryophyllaceae species (Aguileta et al. 2010), the wheat pathogen *Zymoseptoria graminicola* (Stukenbrock et al. 2011), the rust fungus *Melampsora larici-populina* (Hacquard et al. 2012), the rice blast fungus *Magnaporthe oryzae* (Huang et al. 2014), the Irish potato famine pathogen *Phytophthora infestans* (Dong et al. 2014), and a group of smut fungi parasitizing different grasses and a dicot host (Sharma et al. 2014). Links between genes showing signatures of positive selection and the actual function being selected are only beginning to be understood. For example, the population genomics study in the wheat pathogen *Z. tritici* which identified candidate effector genes under positive selection (Stukenbrock et al. 2011) was followed up experimentally, and in this case it was shown that the deletion of three of four candidate genes reduced virulence (Poppe et al. 2015). In the grey mold fungus *Botrytis cinerea* four positively selected genes were deleted without affecting virulence and this finding was attributed to functional redundancy, the limited number of tested host plants or experimental conditions which are different from natural infections (Aguileta et al. 2012). An elegant study with the oomycete effector protein EpiC1 showed that a single amino acid substitution under positive selection leads to targeting different host proteases determining host specificity (Dong et al. 2014). Many studies stop at the level of scanning for positive selection or conduct only initial functional experiments like gene expression profiling (Cantu et al. 2013; Guyon et al. 2014; Hacquard et al. 2012; Joly et al. 2010; Sharma et al. 2014; Sharma et al.

2015; Silva et al. 2015; Sperschneider et al. 2015; Sperschneider et al. 2014). Therefore, it remains largely open whether genes with signatures of positive selection in general play a role in virulence and/or host specificity.

Smut fungi belonging to the division of Basidiomycota are an important group of about 2,500 species parasitizing mostly grasses, including important crops like maize, sorghum, oat, barley and sugarcane (Vánky 2012). In smut fungi, sexual reproduction is linked to pathogenic development and smut fungi therefore depend on successful plant colonization to complete their life cycle. As biotrophic pathogens, they require living plant tissue for establishing a successful interaction (Martinez-Espinoza et al. 2002). With few exceptions like *Ustilago maydis*, smut fungi usually develop symptoms only in the female or male inflorescence of their respective host plants. During the last ten years, quality draft genome sequences of prominent species were obtained, among which are *U. maydis*, the causative agent of smut disease on maize and teosinte (Kämper et al. 2006), *Ustilago hordei* infecting barley (Laurie et al. 2012), *Sporisorium scitamineum* parasitizing sugarcane (Dutheil et al. 2016; Que et al. 2014; Taniguti et al. 2015) and *Sporisorium reilianum* causing head smut (Schirawski et al. 2010). *S. reilianum* occurs in two formae speciales that infect maize (*S. reilianum* f. sp. *zeae*) and sorghum *(S. reilianum* f. sp. *reilianum*), respectively (Zuther et al. 2012). The speciation of *U. hordei*, *U. maydis*, *S. scitamineum* and *S. reilianum* is hypothesized to have occurred between seven and 50 million years ago (Munkacsi et al. 2007). The availability of genome sequences of several species with different host ranges, together with established tools for genetic manipulations (Brachmann et al. 2004; Kämper 2004; Khrunyk et al. 2010; Schuster et al. 2016) make this group of smut fungi particularly interesting for studying the evolution of effector genes as well as their contributions to virulence, speciation and host specificity. Here, we employed the genome of the recently sequenced strain *S. reilianum* f. sp. *reilianum* SRS1_H2-8 (http://www.ebi.ac.uk/ena/data/view/LT795054-LT795076) (Zuther et al. 2012) together with

the genomes of *U. maydis*, *U. hordei*, *S. scitamineum* and *S. reilianum* f. sp. *zeae* to identify potential effector genes with signatures of positive selection. To determine whether such candidate effectors play a role in virulence, they were individually deleted in *S. reilianum f. sp. zeae* and the phenotype of the deletion strain assessed after infection of maize. Only one of nine tested candidate effectors showed a strong contribution to virulence, suggesting that genes with signatures of positive selection are rarely selected for traits related to virulence in *S. reilianum*.

Materials and Methods

Construction of protein families

Fungal species used in this study, their number of gene models, number of predicted secreted proteins and sources of genome data are listed in supplementary table S1. The predicted proteome of the five smut *Ustilago hordei*, *U. maydis*, *Sporisorium scitamineum*, *S. reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum* were used to perform an all-against-all blastp search (Altschul et al. 1990). To identify settings for coverage and identity resulting in the maximum number of families comprised of 1:1 orthologues (*i.e.* families that have an equal number of members in each species), SiLiX (Miele et al. 2011) was run with a range for coverage and identity between 5 % and 95 % in 5 % steps. An identity of 40 % and coverage between 5 % and 45 % lead to the maximum number of families with 1:1 orthologues (5,394; supplementary fig. S1) while settings with 40 % identity and 80 % coverage lead to 5,326 families with 1:1 orthologues (supplementary fig. S1). Since using a higher coverage had only a cost of 68 core families, the stricter criteria were applied for family clustering. Families with at least two members were aligned on the codon level using MACSE 1.01b (Ranwez et al. 2011) and on the protein level using PRANK v.100802 (Löytynoja and Goldman 2008). Only consistently found alignment sites with a maximum of 30 % gaps were used for further analysis.

Estimation of genome-wide divergence values and divergence times

The five genomes of *U. hordei*, *U. maydis*, *S. scitamineum*, *S. reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum* were aligned using the multiz genome aligner from the TBA package (Blanchette et al. 2004). The resulting multiple genome alignment was further processed to remove coding and ambiguously aligned regions. More specifically, the following filters were applied:

- Alignments blocks with paralogous sequences were discarded.
- Aligned regions containing sequences annotated as part of a protein coding gene in any of the five species were discarded.

This led to an alignment of 2.1 million nucleotides.

- A window of 5 nucleotide long was slid along the alignment by 1 nucleotide. Windows containing at least 2 indels were discarded.
- A window of 50 nucleotide long was slid along the alignment by 1 nucleotide. Windows containing more than 150 gaps were discarded.
- A window of 300 nucleotide long was slid along the alignment by 1 nucleotide. Windows containing more than 500 gaps were discarded.
- Finally only alignment blocks of at least 300 nucleotides were kept.

The final alignment had a total length of 936,984 nucleotides. Given the strict criteria used to filter the alignment, the divergence estimates obtained for intergenic sequences might represent an underestimate of the true divergence times.

Gene families with exactly one member in each species were concatenated and pairwise protein sequence similarities computed using the seqinr package (Charif and Lobry 2007) for R. Protein alignments were also used to infer dates of divergence, using a relaxed clock model. The PhyloBayes version 4.1 software (Lartillot et al. 2009) was used with the auto-correlated model of Thorne et al. (Thorne et al. 1998) under a GTR + CAT model. Two calibration points were

used, based on the speciation times of the host lineages: the split between *U. maydis* and the *Sporisorium* species was assumed to have occurred between 11 and 24 million years (Myr) ago, corresponding to the estimated speciation time of the maize lineage with the sugarcane lineage, and the split between *U. hordei* and the other species was constrained to have occurred between 24 and 70 Myr ago (Munkacsi et al. 2007). Uniform priors were used on these intervals for the Monte-Carlo Markov Chain. As convergence issues arise when large alignments (more than 20k positions) are used, we followed the PhyloBayes authors' recommendation and performed a jackknife procedure and generated three datasets of ca 20,000 amino acids by randomly sampling families and concatenating the corresponding alignments. Two chains were run in each case and convergence assessed. Sampling was performed after a burning of 10,000 iterations, and every 10 subsequent iterations. Chains were run to ensure that the minimum effective sample size was greater than 30 and maximum relative difference lower than 0.3 in at least one sample. Results are summarized in supplementary table S2 and supplementary fig. S2 shows the six chains for the three samples. In addition to the convergence of the two chains for each sample, our results reveal extremely consistent results between samples. Figure 1A shows estimates from one chain of the first data set.

Detection of positive selection

For gene families with at least three members, translated sequences were employed to create maximum likelihood phylogenetic trees using PhyML 3.0 (Guindon et al. 2010) with a minimum parsimony starting tree and the LG amino acid substitution model with a four-classes gamma distribution of site-specific substitution rate (Le and Gascuel 2008). The best tree topology obtained from nearest neighbor interchange (NNI) and subtree pruning recrafting (SPR) searches was kept (Guindon et al. 2010). BppML (Dutheil and Boussau 2008) was then used to re-estimate branch lengths from the codon alignment using the YN98 substitution model

(Nielsen and Yang 1998). Next, MapNH (Romiguier et al. 2012) was used for mapping substitutions on the resulting tree. PartNH (Dutheil et al. 2012) was subsequently employed to fit time non-homogeneous models of codon substitutions. PartNH uses the previously inferred substitution maps in order to perform model comparisons and select a non-homogeneous model with minimal number of parameters. Both methods 'free' (differing parameters between neighboring branches of a tree) and 'join' (shared parameters between neighboring branches) were applied to scan for positive selection. Finally, putative secreted effector proteins were identified by predicting secretion using SignalP 4.0 (Petersen et al. 2011) and proteins were considered as secreted if the program indicated the presence of a signal peptide but no transmembrane domain.

To detect residues under positive selection in homologues of *pit2*, the branch-site model with Bayes Empirical Bayes (BEB) analysis as implemented in PAML4 (Yang 2007) was applied. We employed information about family composition, alignment and phylogeny as outlined above and defined *sr10529* and *srs_10529* as foreground branches. A posterior probability threshold of > 95 % was used for the BEB analysis.

Association of positively selected genes with repeats in U. hordei

Since *U. hordei* shows the highest content of repetitive elements in the group of smut fungi investigated here, it was tested whether genes under positive selection are located significantly closer to repetitive elements than average genes in the genome. For this analysis, only a group of "uncharacterized interspersed repeats" was investigated, because it was shown previously that this is the only category showing a strong association with candidate effector genes (Dutheil et al. 2016). Binary logistic regressions were conducted in R using the rms package. The 'robcov' function of the rms package was used in order to get robust estimates of each effect.

The variable “distance to the closest interspersed repeat” was transformed by log(x+1) because of its extreme distribution.

Comparing d_N/d_S ratios of genes residing in virulence clusters

Previous work has identified several virulence gene clusters in *U. maydis,* some of them playing important roles during pathogenic development (Kämper et al. 2006; Schirawski et al. 2010). In total, these clusters contain 163 genes, where 100 reside in clusters without virulence phenotype and 63 reside in clusters with virulence phenotype upon deletion. Both types of clusters contain each 32 genes for which a d_N/d_S ratio could be determined (the missing genes are part of families that do not have at least three members and were therefore not analyzed). The d_N/d_S ratios of all genes in clusters were compared between clusters with and without virulence phenotype (Wilcoxon Rank-Sum Test).

Gene Ontology terms enrichment analysis

All proteins in *S. reilianum* f. sp. *zeae* and in *S. reilianum* f. sp. *reilianum* were considered for Gene Ontology (GO) term enrichment analysis. GO terms were assigned using iprscan 1.1.0 (http://fgblab.org/runiprscan; developed by Michael R. Thon) which links GO information provided by Interpro to each protein. In this way, 1,759 unique GO terms could be assigned to 4,130 proteins in *S. reilianum* f. sp. *zeae* and 1,744 unique GO terms could be assigned to 4,124 proteins in *S. reilianum* f. sp. *reilianum* (supplementary table S3). The Bioconductor package topGO (Alexa et al. 2006) was then used in R to link each GO term to the three major categories “Cellular Component”, “Biological Process” or “Molecular Function”. Enrichment analysis was performed by computing *P* values for each GO term using Fisher’s classic test with parent-child correction (Grossmann et al. 2007). Cytoplasmic proteins with and without signatures of

positive selection were compared for the two species separately and differences were considered to be significant at the 5 % level.

Strains and growth conditions

The *Escherichia coli* derivative Top10 (Invitrogen, Karlsruhe, Germany) and the *Saccharomyces cerevisiae* strain BY4741 (*MAT*a *his*3Δ1 *leu*2Δ *met*15Δ *ura*3Δ; Euroscarf, Frankfurt, Germany; kindly provided by M. Bölker, Marburg) were used for cloning purposes. *Sporisorium reilianum* strains used in this study are listed in supplementary table 4. They are derivatives of the haploid solopathogenic strain JS161 (Schirawski et al. 2010). *E. coli* was grown in dYT liquid medium (1.6% (w/v) Trypton, 1.0% (w/v) Yeast Extract (Difco), 0.5% (w/v) NaCl) or YT solid medium (0.8 % (w/v) Trypton, 0.5% (w/v) Yeast-Extract, 0.5% (w/v) NaCl, 1.3% (w/v) agar) supplemented with 100 mg/mL Ampicillin when needed. *S. cerevisiae* was maintained in YPD solid medium (1% (w/v) yeast extract, 2% (w/v) Bacto-Pepton, 2% (w/v) Bacto-Agar, 2% (w/v) glucose) and grown on SC URA^- medium (1.7% (w/v) Yeast Nitrogen Base without ammonium sulfate, 0.147% (w/v) dropout-mix without Uracil, 2% (w/v) glucose) for selecting transformants containing the plasmid pRS426 (Sikorski and Hieter 1989) (kindly provided by M. Bölker, Marburg) or derivatives of pRS426. *S. reilianum* strains were grown in liquid $YEPS_{light}$ medium (1.0 % (w/v) yeast extract, 0.4% (w/v) peptone, 0.4% (w/v) sucrose) at 28°C on a rotary shaker at 200 rpm.

Construction of S. reilianum strains

Polymerase chain reactions were performed using the Phusion High-Fidelity DNA Polymerase (New England Biolabs). Templates were either JS161 genomic DNA or indicated plasmid DNAs. Restriction enzymes were obtained from New England Biolabs. *S. reilianum* was transformed by protoplast-mediated transformation following a method established for *U.*

maydis (Schulz et al. 1990). Transformants were selected on RegAgar plates (1.0% (w/v) yeast extract, 0.4% (w/v) Bacto-Pepton, 0.4% (w/v) Sucrose, 1 M Sorbitol, 1.5% (w/v) Bactoagar) supplemented with 200 µg/mL Geneticin and true resistance was tested by growing single colonies on PD plates (3.9% (w/v) Potato-Dextrose Agar, 1% (v/v) Tris-HCl (1M, pH 8.0)) supplemented with 50 µg/mL Geneticin. Gene replacements with resistance markers were generated with a PCR-based method employing the previously described *Sfi*I insertion cassette system (Brachmann et al. 2004; Kämper 2004) and were confirmed by Southern blot analysis. Genomic regions residing about 1 kb upstream (left border) or downstream (right border) adjacent to open reading frames of candidate genes were PCR-amplified using the listed primer pairs (supplementary table S5) and genomic DNA of JS161 as template. The resulting fragments were used for cloning plasmids containing the respective deletion constructs.

To obtain deletion constructs for the genes *sr10529* and *sr14347*, PCR fragments containing the left and right borders of each gene were ligated to the hygromycin resistance cassette of pBS-hhn (Kämper 2004) via *Sfi*I restriction sites and cloned into pCRII-TOPO (Life Technologies) to generate pTOPO Δsr10529 #1 and pTOPO Δsr14347 #1, respectively. Since the use of Geneticin as selection marker resulted in much less false positive transformants compared to the use of Hygromycin B, the hygromycin resistance cassettes in these plasmids were replaced by the Geneticin resistance cassette of pUMA 1057 (Brachmann et al. 2004) by ligation via *Sfi*I restriction sites, yielding plasmids pTOPO Δsr10529 G418 and pTOPO Dsr14347 Gen #1, respectively. Deletion constructs were PCR-amplified from plasmids pTOPO Δsr10529 G418 and pTOPO Dsr14347 Gen #1 using the listed primers (supplementary table S5) and used to transform the *S. reilianum* strain JS161 to generate the gene deletion strains JS161Δsr10529 and JS161Δsr14347, respectively.

The drag and drop cloning method in yeast (Jansen et al. 2005) was used to generate plasmids pRS426 Dsr12968 Hyg #1, pRS426 Dsr14944 Hyg #2, pRS426 Dsr10059 Hyg #1, pRS426

Dsr10182 Hyg #1, pRS426 Dsr14558 Hyg #1 and pRS426 Dsr12897 Hyg #1 which contain deletion constructs for deleting the candidate genes *sr12968*, *sr14944*, *sr10059*, *sr10182*, *sr14558* or *sr12897*. These plasmids are a derivate of plasmid pRS426, which can be maintained in *E. coli* and *S. cerevisiae* (Sikorski and Hieter 1989). PCR-amplified left and right borders of each candidate gene and the hygromycin resistance cassette were integrated in pRS426 by homologous recombination in *S. cerevisiae*. Subsequently, the hygromycin resistance cassette was replaced with the Geneticin resistance cassette by ligation via *Sfi*I restriction sites, yielding plasmids pRS426 Dsr12968 Gen #1, pRS426 Dsr14944 Gen #3, pRS426 Dsr10059 Gen #1, pRS426 Dsr10182 Gen #1, pRS426 Dsr14558 Gen #1 and pRS426 Dsr12897 Gen #5, respectively. Gene deletion constructs were PCR-amplified from the respective plasmid using listed primers (supplementary table S5). The obtained deletion constructs were transformed into the *S. reilianum* strain JS161 to generate the gene deletion strains JS161Δsr12968, JS161Δsr14944, JS161Δsr10059, JS161Δsr10182, JS161Δsr14558 and JS161Δsr12897, respectively.

The drag and drop cloning method was also used to generate plasmid pRS426 Dsr12084 Gen #1. PCR-amplified left and right borders of *sr12084* and the Geniticin resistance cassette were integrated in pRS426 by homologous recombination in *S. cerevisiae*. The gene deletion construct for deleting the candidate gene *sr12084* was PCR-amplified from plasmid pRS426 Dsr12084 Gen #1 using primers sr12084_lb_fw/sr12084_rb_rv and transformed into the *S. reilianum* strain JS161 to generate the gene deletion strain JS161Δsr12084.

Virulence assays

The solopathogenic strain JS161 and derivatives thereof were grown in $YEPS_{light}$ liquid medium to an optical density at 600 nm (OD_{600}) of 0.8 - 1.0 and cell cultures were adjusted to an OD_{600} of 1.0 with sterile water prior to injection into one week old maize (*Zea mays*) seedlings of the

dwarf cultivar ‘Gaspe Flint’ (originally kindly provided by B. Burr, Brookhaven National Laboratories and maintained by self-pollination). Plants were sowed in T-type soil of ‘Fruhstorfer Pikiererde’ (HAWITA, Vechta, Germany) and grown in a temperature-controlled greenhouse (14h-/10h- light/dark cycle, with 28/20°C and 25,000 – 90,000 lux during the light period). Virulence symptoms were scored nine to ten weeks post infection according to previously described symptoms (Ghareeb et al. 2011) and the following categories were distinguished: the plant did not develop ears, the plant developed healthy ears shorter or equal to 1 cm or the plant developed healthy ears longer than 1 cm, the plant developed spiky ears, phyllody in ears or phyllody in tassels. Spore formation was only observed occasionally and rarely, the plant died due to the infection. Three independent infections were carried out per strain, mock treated plants were infected with water as control and at least three independent deletion strains were tested for virulence.

Results

S. reilianum f. sp. zeae and S. reilianum f. sp. reilianum diverged around one million years ago

To establish a frame for our comparative analysis we first estimated the divergence of the five smut fungi *U. hordei*, *U. maydis*, *S. scitamineum*, *S. reilianum* f. sp. *zeae* and *S. reilianum* f. sp. *reilianum*, both at the non-coding and protein coding levels. We used the information that the speciation of *U. hordei*, *U. maydis*, *S. scitamineum* and *S. reilianum* likely followed the divergence pattern of the respective crop ancestors (Munkacsi et al. 2007) and performed a molecular dating analysis based on the complete proteomes of the five pathogens. We supposed that the speciation time of the maize and sorghum / sugarcane lineages occurred between 11 and 24 million years (Myr) ago while constraining the speciation of the barley lineage to have occurred between 24 and 70 Myr ago to calibrate the molecular dating analysis. Based on these

assumptions, we estimated the oldest split between *U. hordei* and the other four species to have occurred ca 30 Myr ago with a 95% posterior interval ranging from 24 to 47 Myr ago, (fig. 1A and supplementary table S2). *U. hordei* shares 58 % identity in alignable intergenic regions with *S. reilianum f. sp. reilianum* and 77 % identity at the protein level (fig. 1B). The split between *U. maydis* and the *Sporisorium* species was estimated to have occurred around 20 Myr ago (95% posterior interval 13 to 24 Myr; fig 1A and supplementary table S2). *S. reilianum* f. sp. *reilianum* shares 61% nucleotide identity in alignable intergenic regions with *U. maydis*, and 79% sequence identity at the protein level (fig. 1B). *S. scitamineum* and the two formae speciales of *S. reilianum* were found to have diverged 12 Myr ago (95% posterior interval 7.15 to 16.61 Myr; fig. 1A and supplementary table S2), which is consistent with the mean divergence estimated from eight studies between the hosts sorghum and sugarcane (10 Myr with a posterior interval of eight to 13 Myr, source: timetree.org (Kumar et al. 2017)). *S. reilianum* f. sp. *reilianum* and *S. scitamineum* share 75% non-coding nucleotide identity and 88% identity at the protein level (fig. 1B). Finally, the two *S. reilianum* strains diverged 1.4 Myr ago (95% posterior interval 0.65 to 3.63 Myr; fig. 1A and supplementary table S2) and share 98% noncoding nucleotide identity and 99% protein identity (fig. 1B). Some studies suggested that the speciation of sugarcane and sorghum might have been as recent as 5 Myr ago. In order to test how this assumption impacts the estimation of the divergence between the two formae speciales of *S. reilianum*, we constrained the split of *S. scitamineum* and *S. reilianum* to have occurred in the last 5 Myr. This did not significantly impact the split date of *U. hordei* (30 Myr ago with a 95% posterior interval of 24 to 55 Myr; supplementary table S2), but lead to a more recent split time for *U. maydis*, around 12 Myr ago (with a 95% posterior interval of 11 to 15 Myr ago), and as expected for *S. scitamineum*, around 4.75 Myr ago (with a 95% posterior interval of 4 to 5 Myr ago, supplementary table S2). Under this assumption, the split between the two *S. reilianum* species is predicted to be more recent, ca 0.33 Myr ago

(95% posterior interval 0.17 to 0.55 Myr; supplementary table S2), but still much before the beginning of agriculture. This suggests that the two variants did not diverge by a host jump between the domesticated species *Sorghum* and maize, but rather between some of the wild ancestors of these two crop plants. The comparison of the five smut genomes therefore encompasses a broad evolutionary time. The speciation times involved all predate 10,000 yrs, which implies that several "domestication" events of these agricultural pathogens occurred independently. A consequence is (with the putative exception of the two *S. reilianum* formae speciales), that adaptation to the agricultural host will be negligible when interpreting the inter-specific patterns of sequence divergence, as it represents a marginal proportion of the time since the divergence from the ancestral species.

S. reilianum contains the largest number of positively selected genes

To identify homologous genes with signs of positive selection in the five smut fungi analyzed here, we first built gene families using the SiLiX clustering algorithm (Miele et al. 2011). To maximize the occurrence of orthologues and minimize the number of paralogues within each gene family we optimized the clustering parameters for our data set. A total of 8,761 families was inferred, among which 5,326 families consisted of 1:1 orthologues with up to six members in each species (supplementary table S6). In this set of families, 245 families contained predicted secreted proteins only. Given that each species encodes on average 588 secreted proteins predicted with SignalP (supplementary table S1), about 40 % of all putatively secreted proteins were present in all five species. Interestingly, we observed several species-specific family expansions in *U. hordei*. There were 17 families which encompassed five to 25 members, but no members in other species (supplementary table S6). Moreover, we identified three families with up to 62 members in *U. hordei*, but only one member in up to three of the other species (supplementary table S6).

To detect positive selection, families with at least three members (supplementary table S6) were, regardless of their species composition, aligned on the codon and amino acid level and a phylogenetic tree was inferred. Obtaining accurate alignments is critical for detecting positive selection since alignment errors frequently inflate the false discovery rate (Jordan and Goldman 2012; Schneider et al. 2009). We therefore developed a stringent bioinformatics pipeline for the filtering of sequence alignments by masking ambiguous alignment positions for further analysis (see Methods). A non-homogeneous model of sequence evolution allowing d_N/d_S ratios to vary along the phylogeny was employed to scan for positive selection (Dutheil et al. 2012; Nielsen and Yang 1998). The largest number of genes with signs of positive selection was found in *S. reilianum* f. sp. *zeae* (84 genes, of which 25 encode predicted secreted proteins) and *S. reilianum* f. sp. *reilianum* (111 genes of which 27 encode predicted secreted proteins) (fig. 1C). In addition, a substantial number of positively selected candidate genes was also found in *U. hordei* (49, and of these 22 genes are predicted to code for secreted proteins), but only very few in *U. maydis* (2 genes) and *S. scitamineum* (7 genes) (fig. 1C). A list of all proteins with their associated d_N/d_S ratios in each species is provided in supplementary table S3. Predicted secreted proteins were significantly enriched in the group of proteins under positive selection in *U. hordei* and in the two investigated pathovariants of *S. reilianum* (*P* values $< 10^{-5}$; Fisher's exact test). This corroborates results of earlier studies that showed that predicted secreted proteins are often under positive selection, which can be attributed to their direct interaction with host proteins. Notably, all genes found under positive selection in the two strains of *S. reilianum*, in *S. scitamineum* and in *U. maydis* share orthologous genes in the other species (supplementary tables S2 and S5). In contrast, genes with signs of positive selection in *U. hordei* belong largely (36 out of 49 genes) to families showing species-specific expansions (supplementary tables S3 and S6).

Genes under positive selection in U. hordei are associated with uncharacterized interspersed repeats

Among the species compared here, the genome of *U. hordei* shows the highest fraction of repetitive elements (Dutheil et al. 2016). Such elements are known to contribute to gene family expansions (Kazazian 2004), and have been suggested to contribute to adaptation by providing advantageous mutations, for instance by RIP leakage (Dong et al. 2015; Rouxel et al. 2011). Therefore, we tested whether genes under positive selection in *U. hordei* are physically associated with repetitive elements. We performed a binary logistic regression with the prediction of positive selection as a response variable (that is, whether the underlying branch has a dN/dS ratio higher than one) and we considered three putative explanatory variables: (1) whether the gene was predicted to encode a secreted protein, (2) whether the gene is duplicated and (3) the distance of the gene to the closest interspersed repeat. The complete linear model explains of 50% of the observed variance, and the three explanatory variables are all significant at the 0.1% level (supplementary table S7). These results suggest that positively selected genes in *U. hordei* are associated with duplication events, and positive selection is more likely to occur at genes encoding putative effectors. In addition, the proximity of interspersed repeats increases the odds of positive selection, independently of the two other effects, and is confirmed by a stratification approach: the effect still holds when only duplicated genes are considered, or only genes encoding a secreted protein, or the combination of the two (supplementary table S7). This finding corroborates previous results obtained in other microbial plant pathogens where it was described that effector genes tend to localize in repeat rich regions and where it was suggested that such regions contribute to the rapid evolution of effector genes (Dong et al. 2015).

Positively selected genes encoding cytoplasmic proteins in S. reilianum

While we expect effector genes to be under positive selection, we find that the majority of positively selected genes in *S. reilianum* encodes cytoplasmic proteins (fig. 1C). To assess the putative functional role of these genes, we performed a Gene Ontology term enrichment analysis, comparing cytoplasmic proteins under positive selection to cytoplasmic proteins not under positive selection (table 1). This analysis revealed that genes with a potential role in metabolic processes, like sulfur compound metabolism, molybdopterin cofactor metabolic process, RNA metabolic process, organic cyclic compound metabolic process and oxidoreductase activity, as well as responses to starvation and extracellular stimuli are significantly overrepresented (table 1). This could indicate that cytoplasmic proteins under positive selection contribute to metabolic changes which might be needed to survive with the limited nutrients available on the surface or in the biotrophic interface of different host plants.

Virulence contribution of effector genes showing signs of positive selection in S. reilianum

Candidate effector genes inferred to be under positive selection in a particular species could play a critical role in pathogenicity. Therefore, we sought to assess the contribution to virulence of such candidate genes by creating individual deletion mutants. In total nine candidate genes with high d_N/d_S ratios were chosen which were all predicted to encode secreted proteins: three with signatures of positive selection only in *S. reilianum* f. sp. *zeae*, three with signatures of positive selection in *S. reilianum* f. sp. *zeae* as well as in *S. reilianum* f. sp. *reilianum* and three with signatures of positive selection only in *S. reilianum* f. sp. *reilianum*. All nine chosen candidate genes together with their selection criteria are summarized in table 2. Deletion mutants were generated in the haploid solopathogenic strain JS161 of *S. reilianum* f. sp. *zeae*. This strain is capable to colonize maize plants and cause disease without a compatible mating partner (Schirawski et al. 2010). Deletion mutants were also generated in strain JS161 in cases

where positive selection was only detected in *S. reilianum* f. sp. *reilianum* (table 2), because no solopathogenic strain is presently available for *S. reilianum* f. sp. *reilianum*. For each gene at least three independent deletion mutants were generated and tested for virulence. To determine virulence, Gaspe Flint, a dwarf variety of corn, was infected and symptoms were scored in male and female flowers (fig. 2). Only the deletion of *sr10529*, a gene showing positive selection in both formae speciales of *S. reilianum*, showed a strong reduction in virulence (table 2 and fig. 2). The gene *sr10529* in *S. reilianum* f. sp. *zeae* is orthologous to the previously identified and characterized gene *pit2* (*UMAG_01375*) in *U. maydis*.

Pit2 plays an essential role in virulence as inhibitor of a group of maize papain-like cysteine proteases that are secreted to the apoplast (Doehlemann et al. 2011; Mueller et al. 2013). Previous work identified a conserved domain of 14 amino acids (PID14) in Pit2 as required and sufficient for the inhibition of maize cysteine proteases (Mueller et al. 2013). When the branch-site model of PAML 4 (Yang 2007) was used to identify amino acid residues under positive selection in the Pit2 orthologues of the two *S. reilianum* species, only two residues residing in the PID14 domain were found under positive selection. However, 24 positively selected residues were detected outside this domain in the 57 amino acids long C-terminus (fig. 3).

Discussion

We used evolutionary comparative genomics of five related smut fungi infecting four different host plants to identify positively selected genes encoding predicted secreted proteins, as such genes were suggested to contribute to virulence in various plant pathogenic microbes (Aguileta et al. 2010; Dong et al. 2014; Hacquard et al. 2012; Huang et al. 2014; Sharma et al. 2014; Stukenbrock et al. 2011). Our analysis revealed that positive selection is found between paralogous genes in *U. hordei*, where they belong to families with species-specific expansions.

In contrast, genes under positive selection in the other four species belong to families of orthologous sequences. While we find evidence for a large set of genes under positive selection in the *S. reilianum* species, signatures for positive selection are hardly detectable in the more distant relatives *U. hordei*, *U. maydis* and *S. scitamineum* that diverged earlier (fig. 1A). Finding evidence for positive selection over time spans of several millions of years is notoriously difficult (Gillespie 1994) because of two main reasons: (1) periods where genes are evolving under positive selection occur episodically and may be followed by long episodes of purifying selection, leading to an average d_N/d_S below 1 on long periods of time and (2) fast evolving genes may diverge to an extent where their homology is difficult to infer and where they can no longer be aligned reliably. To overcome this problem, more genome information of species with intermediate branching points is needed (Gillespie 1994).

Predicted secreted proteins were about three times overrepresented in the set of positively selected genes and this illustrates the importance of secreted proteins in adaptation processes of smut fungi. This corroborates results in other plant pathogenic microbes like *Melampsora sp.*, *Z. tritici* and the wheat powdery mildew *Blumeria graminis* (Joly et al. 2010; Poppe et al. 2015; Wicker et al. 2013). However, the majority of positively selected genes encodes cytoplasmic proteins (fig. 1C), suggesting that important targets of adaptation like plant colonization are not only governed by secreted proteins. A Gene Ontology analysis in *S. reilianum* showed that mainly processes related to metabolism and its regulation as well as responses to starvation and external stimuli are enriched in cytoplasmic proteins under positive selection (table 1). This points at a role of these proteins in adaptation to differences in nutrient availability in the respective host plants maize and sorghum as well as responses to cues originating from the respective host (Haueisen and Stukenbrock 2016). A study conducted in *U. maydis* has shown that the fungus induces major metabolic changes in the host plant upon infection during establishment of biotrophy and undergoes a series of developmental transitions

during host colonization which are likely influenced by the host environment (Doehlemann et al. 2008). It is thus conceivable that the two *S. reilianum* accessions have adapted to their different hosts that differ significantly for example in their amino acid and vitamin composition (Etuk et al. 2012).

Out of nine deletions of positively selected genes, only one mutant, lacking *sr10529*, was affected in virulence. While six genes deleted are single genes in *S. reilianum* f. sp. *zeae* for which we failed to identify paralogs, *sr12084* has two paralogs, *sr14347* has five paralogs and *sr10182* has ten paralogs (table 2). In our analysis we have restricted ourselves to generating deletion mutants in the genes under positive selection only. This leaves open the possibility that the paralogous genes have redundant functions in virulence. Adapting the CRISPR-Cas9 technology allowing multiplexing (Schuster et al. 2017) to *S. reilianum* will help to address this in future studies. Alternatively, the candidate effectors we investigated may be needed under conditions which differ from those tested here. For example, *S. reilianum* f. sp. *zeae* can systemically colonize maize plants via root infection (Mazaheri-Naeini et al. 2015), a colonization route we have not assessed in our experimental setup. Knowing the expression profile of effector genes might help to look for characteristic differences in development of the mutants compared to wild type strains. Since we lack this information, we scored disease symptoms only in the inflorescences about nine weeks after infection. In three cases positive selection was detected in orthologous genes in *S. reilianum* f. sp. *reilianum* (table 2) while candidate effector genes were for experimental reasons deleted in *S. reilianum* f. sp. *zeae*. Therefore, it cannot be excluded that these effectors might have a virulence function in *S. reilianum* f. sp. *reilianum.* In this case, the positively selected effector genes might have evolved during adaptation to the sorghum host and present host specificity genes. Candidate effector proteins might also be positively selected for traits which are not directly linked to pathogenicity. Such traits could for instance involve competition with large numbers of other

plant colonizing microbes (Rovenich et al. 2014; Zhan and McDonald 2013). Secreted proteins of *S. reilianum* could act for example as toxin or could efficiently utilize resources from the environment and thereby limit the growth of other microbes. In these cases, a contribution to virulence is not expected to be observed in effector gene mutants. Moreover, our molecular dating analysis showed that the common ancestors of the investigated smut species originated before the beginning of crop domestication. Therefore, positive selection, whose signs we detect by our approach, has most likely occurred on ancestral host plants and not on the domesticated host maize. Consequently, some of the candidate effector genes under positive selection might not be important for the colonization of crop plants, but for infection of related wild species.

In *U. maydis*, we note that effector genes residing in clusters whose deletion affected virulence (Kämper et al. 2006; Schirawski et al. 2010) have similar d_N/d_S ratios as effector genes in clusters where the deletion had no effect on virulence (median d_N/d_S ratio 0.0619 vs.0.1094; Wilcoxon rank test with P value = 0.1848). Furthermore, orthologues of the effectors Pep1, Stp1 and Cmu1, which were shown to have important roles in pathogenicity of *U. maydis* (Djamei et al. 2011; Doehlemann et al. 2009; Schipper 2009) showed no signatures of positive selection. These observations could suggest that certain fungal effector proteins are for unknown reasons under evolutionary constraint and are therefore not free to accumulate non-synonymous mutations. Such effectors are conserved over long time spans (Hemetsberger et al. 2015; Schirawski et al. 2010; Sharma et al. 2015) and this illustrates that they are instrumental for successful infections in a large group of smut fungi. They probably target molecules shared by several host plants, for example housekeeping functions that cannot easily evolve in response to the binding of an effector.

One candidate gene (*sr10529*) under positive selection in both formae speciales of *S. reilianum* showed a strong contribution to virulence upon deletion. It is orthologous to the previously

described protease inhibitor Pit2 in *U. maydis*, where the deletion also abolished virulence (Doehlemann et al. 2011; Mueller et al. 2013). Positively selected residues in the PID14 domain of Pit2 might reflect that different proteases need to be inhibited in maize and sorghum. Pit2 might thus contribute to determining the host range of the respective species. A role of cysteine protease inhibitors in host specificity was demonstrated in the pathosystem *Phytophthora infestans*, a pathogen of potato and its sister species *Phytophthora mirabilis*, which infects the ornamental plant *Mirabilis jalapa*. Positively selected orthologous protease inhibitors where shown to inhibit proteases specific to the respective host plants and this specificity could be traced back to a single amino acid substitution (Dong et al. 2014). Surprisingly, 24 positively selected sites in Pit2 were detected outside the PID14 domain in the 57 amino acid long C-terminus in both *S. reilianum* f. sp. *zeae* and f. sp. *reilianum*. This finding raises the intriguing possibility that the C-terminus of Pit2 might possess a second function, which is independent of protease inhibition. Earlier work has shown that the *pit1* gene encoding a transmembrane protein is located next to the *pit2* effector gene and both genes contribute similarly to virulence (Doehlemann et al. 2011). Furthermore, *pit1* and *pit2* are divergently transcribed which makes it likely that the expression of *pit1* and *pit2* is co-regulated. In addition, this gene arrangement of *pit1* and *pit2* is conserved in *U. hordei*, *U. maydis*, *S. scitamineum* and *S. reilianum* (Sharma et al. 2015). This finding has led to the speculation that Pit1 and Pit2 somehow act together to govern virulence of *U. maydis* and related smut fungi. It was hypothesized that that Pit2 shuttles apoplastic maize proteins towards Pit1, thereby scavenging damage-associated molecules (Doehlemann et al. 2011). In this scenario, the positively selected amino acids in the C-terminus of Pit2 could have been selected for scavenging such molecules as adaptation to the two hosts. In future studies it will be highly interesting to complement the *pit2* mutant of *S. reilianum* f. sp. *zeae* with the *pit2* orthologue of *S. reilianum* f. sp. *reilianum* to see if this promotes virulence on sorghum.

Conclusions

Screens for genes with signs of positive selection are commonly used to identify candidate effector genes in various plant pathogenic microbes. However, it is currently largely open whether positively selected effector genes play indeed a role in virulence. Here, we used comparative genomics of five smut fungi and showed that only one out of nine genes under positive selection contribute to virulence of *S. reilianum*. Moreover, the majority of positively selected genes did not encode predicted secreted proteins. Our results therefore suggest that many genes with signatures of positive selection are involved in traits that are not directly related to pathogenicity and that positive selection alone is not a good indicator for a virulence function of a certain gene.

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Figure legends

FIG. 1. Phylogeny, divergence estimates and number of genes under positive selection in five related smut fungi species parasitizing different host plants. A) Chronogram of the five fungal pathogens as estimated under a relaxed molecular clock. Boxes represent 95% posterior intervals, with corresponding values indicated below. B) Pairwise sequence differences, for both the non-coding genome and the proteome (non-synonymous differences). C) Number of positively selected genes on each terminal branch (total number of genes and genes predicted to encode a secreted protein).

FIG. 2. Virulence phenotypes of single deletion mutants of positively selected genes in *S. reilianum* f. sp. *zeae*. Plants of the maize variety 'Gaspe Flint' were infected with water, the solopathogenic strain JS161 or independent deletion mutants of candidate genes as indicated below each bar. Deletion of *sr10529* lead to a strong reduction in virulence (left panel in first row). In contrast, deletion of the candidate genes *sr12968* (left panel in second row), *sr14558* (right panel in second row), *sr14944* (left panel in third row), *sr14347* (right panel in third row), *sr10059* (left panel in forth row), *sr12897* (right panel in forth row), *sr10182* (left panel in fifth row) and *sr12084* (right panel in fifth row) did not alter virulence. Symptoms were scored about 9 weeks post infection and categorized according to severeness as illustrated in the legend below the bar plots. Results are shown as mean of three independent experiments in relation to the total number of infected plants, which is indicated above each bar (n). Note that strains JS161ΔSr10529 #G4 and #G5 (left panel in first row) were only infected in one replicate.

FIG. 3. Distribution of positively selected amino acids in the cysteine protease inhibitor Pit2. The alignment shows the protein sequences of orthologues in *U. hordei* (UHOR_02064), *U. maydis* (UMAG_01375), *S. scitamineum* (SPSC_03677), *S. reilianum* f. sp. *zeae* (sr10529) and *S. reilianum* f. sp. *reilianum* (srs_10529). Sites under positive selection detected by a branch-site model are indicated by colored bold letters. Residues colored in red indicate positive selection detected in the respective species and purple residues indicate sites found under positive selection in both species. The yellow shaded area is orthologous to the previously identified conserved PID14 domain, which is required and sufficient for inhibition of a group of papain-like cysteine proteases. Green sequences indicate secretion signal peptides and bold numbers above the alignment indicate positions in UHOR_02064.

Supplementary figure 1. Number of families consisting of 1:1 orthologues in relation to varying settings for coverage and identity in the clustering program SiLiX. The maximum number of families containing 1:1 orthologues can be obtained with a coverage between 5 % and 45 % and an identity of 40 %.

Supplementary figure 2. Trace of the Monte-Carlo Markov chains for 3 gene samples (see Methods). Vertical lines show the burning phase (10,000 iterations). A) With 2 calibration points B) With 3 calibration points.

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Table 1: Gene Ontology terms significantly overrepresented in positively selected genes encoding cytoplasmic proteins in *S. reilianum*

Gene Ontology Id	Gene Ontology Description	Category	*P* values[a)]	Species with significantly enriched category
GO:0030532	small nuclear ribonucleoprotein complex	Cellular Component	0.023	*S. reilianum* f. sp. *reilianum*
GO:0045263	proton-transporting ATP synthase complex, coupling factor F(o)	Cellular Component	0.038 and 0.036	*S. reilianum* f. sp. *reilianum* and f. sp. *zeae*
GO:0044425	membrane part	Cellular Component	0.047	*S. reilianum* f. sp. *reilianum*
GO:0031668	cellular response to extracellular stimulus	Biological Process	0.015 and 0.013	*S. reilianum* f. sp. *reilianum* and f. sp. *zeae*
GO:0051186	cofactor metabolic process	Biological Process	0.015	*S. reilianum* f. sp. *reilianum*
GO:0042594	response to starvation	Biological Process	0.017 and 0.012	*S. reilianum* f. sp. *reilianum* and f. sp. *zeae*
GO:0009267	cellular response to starvation	Biological Process	0.020 and 0.014	*S. reilianum* f. sp. *reilianum* and f. sp. *zeae*
GO:0006790	sulfur compound metabolic process	Biological Process	0.022	*S. reilianum* f. sp. *reilianum*
GO:0051301	cell division	Biological Process	0.023	*S. reilianum* f. sp. *reilianum*
GO:0006777	Mo-molybdopterin cofactor biosynthetic process	Biological Process	0.027	*S. reilianum* f. sp. *reilianum*
GO:0009605	response to external stimulus	Biological Process	0.027 and 0.012	*S. reilianum* f. sp. *reilianum* and f. sp. *zeae*
GO:0043545	molybdopterin cofactor metabolic process	Biological Process	0.034	*S. reilianum* f. sp. *reilianum*
GO:0043413	macromolecule glycosylation	Biological Process	0.036	*S. reilianum* f. sp. *reilianum*
GO:0022402	cell cycle process	Biological Process	0.037	*S. reilianum* f. sp. *reilianum*
GO:0051189	prosthetic group metabolic process	Biological Process	0.039	*S. reilianum* f. sp. *reilianum*
GO:0006139	nucleobase-containing compound metabolic process	Biological Process	0.012	*S. reilianum* f. sp. *zeae*
GO:0016070	RNA metabolic process	Biological Process	0.023	*S. reilianum* f. sp. *zeae*
GO:1901360	organic cyclic compound metabolic process	Biological Process	0.016	*S. reilianum* f. sp. *zeae*
GO:0006725	cellular aromatic compound metabolic process	Biological Process	0.024	*S. reilianum* f. sp. *zeae*
GO:0046483	heterocycle metabolic process	Biological Process	0.023	*S. reilianum* f. sp. *zeae*
GO:0035383	thioester metabolic process	Biological Process	0.041	*S. reilianum* f. sp. *reilianum*
GO:1902589	single-organism organelle organization	Biological Process	0.048	*S. reilianum* f. sp. *reilianum*
GO:0010181	FMN binding	Molecular Function	0.047 and 0.022	*S. reilianum* f. sp. *reilianum* and f. sp. *zeae*
GO:0030515	snoRNA binding	Molecular Function	0.021	*S. reilianum* f. sp. *zeae*
GO:0016491	oxidoreductase activity	Molecular Function	0.028	*S. reilianum* f. sp. *zeae*

[a)]calculated by Fisher's classic test with Parent-Child correction; only entries with P value ≤ 0.05 are shown

Table 2: Positively selected genes that were deleted in *S. reilianum* f. sp. *zeae* and their selection criteria

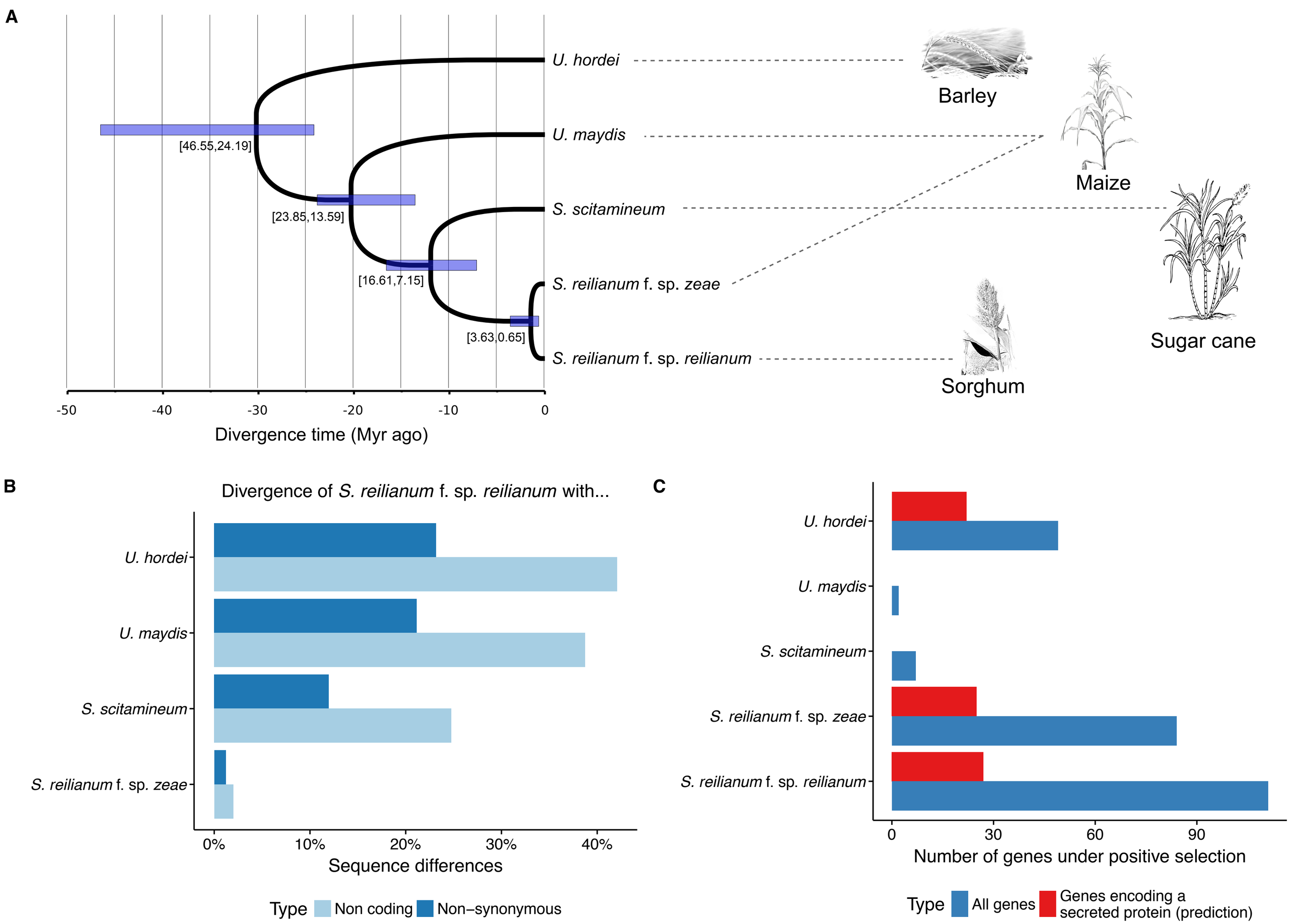
Family	Gene	Description	d_N/d_S (model free)	d_N/d_S (model join)
FAM001428	*sr10529*	conserved hypothetical protein	31.147	31.147
FAM005472	*sr10059*	conserved hypothetical *Ustilaginaceae* -specific protein	6.539	6.539
FAM000532	*sr10182*	conserved hypothetical protein	1.575	1.575
FAM002067	*sr12968*	conserved hypothetical protein	37.901	37.901
FAM003728	*sr14558*	conserved hypothetical protein	24.355	24.355
FAM004113	*sr14944*	conserved hypothetical *Ustilaginaceae* -specific protein	4.305	4.305
FAM003465	*sr14347*	conserved hypothetical protein	544.340	544.340
FAM001868	*sr12897*	conserved hypothetical protein	infinite[a]	infinite[a]
FAM000842	*sr12084*	conserved hypothetical protein	infinite[b]	infinite[b]

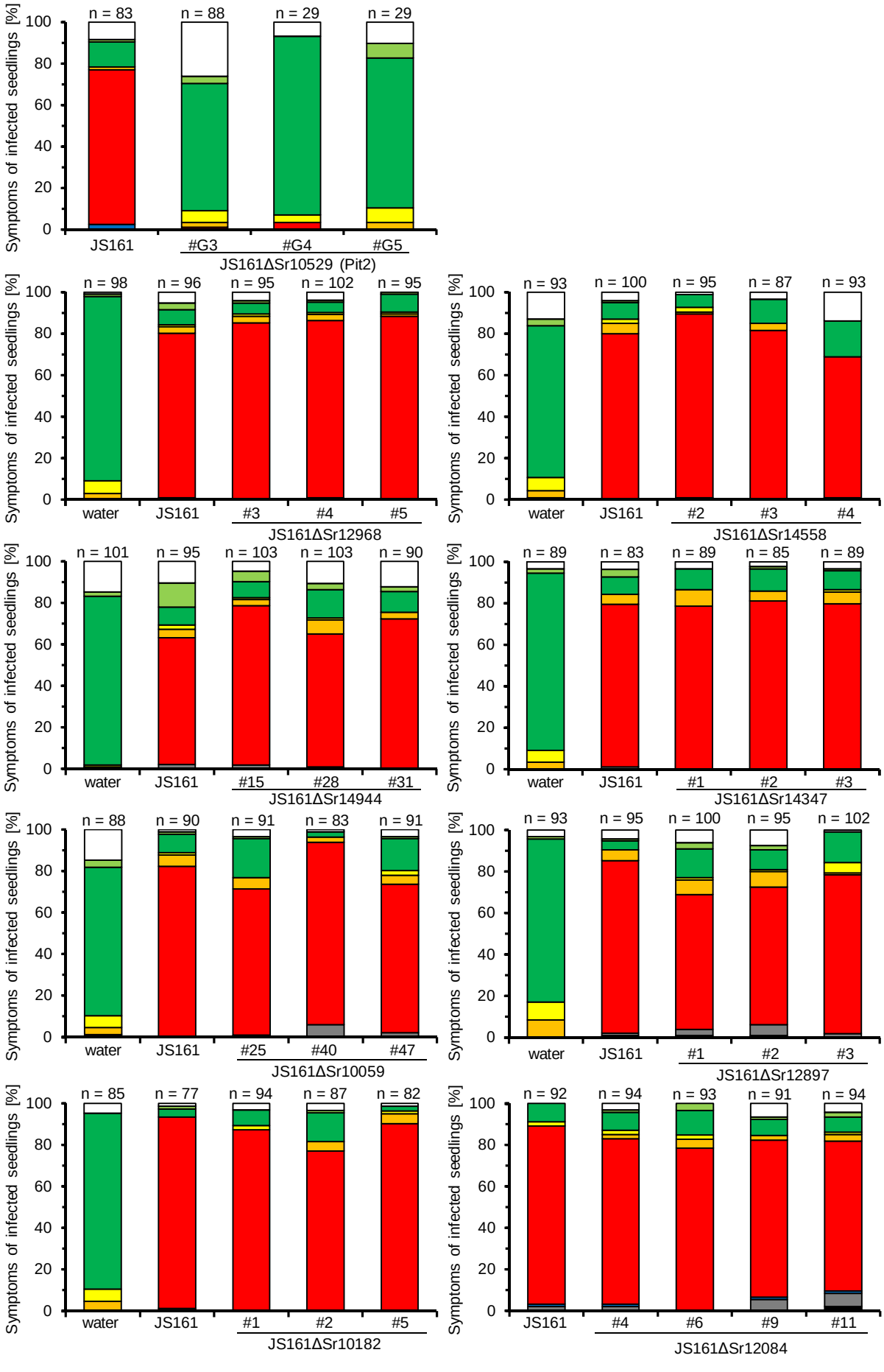
Table 2: Positively selected genes that were deleted in *S. reilianum* f. sp. *zeae* and their selection criteria (continued)

Gene	Species with detected positive selection	Number of paralogs[c]	Virulence phenotype of candidate gene deletion	Closest ortholog in *U. maydis* [d]
sr10529	*S. reilianum* f. sp. *zeae* and f. sp. *reilianum*	0	virulence abolished	*UMAG_01375* (*pit2*)[e]
sr10059	*S. reilianum* f. sp. *zeae* and f. sp. *reilianum*	0	virulence unaffected	*UMAG_05306* (cluster 19A)[f]
sr10182	*S. reilianum* f. sp. *reilianum*	10[1)]	virulence unaffected	*UMAG_00492*
sr12968	*S. reilianum* f. sp. *reilianum*	0	virulence unaffected	*UMAG_02006*
sr14558	*S. reilianum* f. sp. *zeae*	0	virulence unaffected	*UMAG_03564*
sr14944	*S. reilianum* f. sp. *zeae and* f. sp. *reilianum*	0	virulence unaffected	*UMAG_04034* (cluster 11-16)[g]
sr14347	*S. reilianum* f. sp. *zeae*	5[2)]	virulence unaffected	*UMAG_03349*
sr12897	*S. reilianum* f. sp. *reilianum*	0	virulence unaffected	*UMAG_01820*
sr12084	*S. reilianum* f. sp. *zeae*	2[3)]	virulence unaffected	UMAG_00792 (cluster 1-32)[g]

a) infinity due to low value of d_S
b) infinity due to long branch for *sr12085* , a species-specific duplicate
c) based on blastp search with an e-Value cutoff of 0.001
d) based on blastp search
e) as described in Doehlemann *et al* ., 2011 and Mueller *et al., 2013*
f) as described in Kämper *et al* ., 2006
g) as described in Schirawski *et al* ., 2010

[1)] The ten paralogs include: *sr13431* , *sr11876* , *sr16607* , *sr11405* , *sr10621* , *sr16723* , *sr16877* , *13293* , *sr11163.2* and *sr15970*
[2)] The five paralogs include: *sr12257* , *sr11661* , *sr13976* , *sr14607* and *sr11273*
[3)] The two paralogs include: *sr12085* and *sr12086*


A
U. hordei
U. maydis
S. scitamineum
S. reilianum f. sp. zeae
S. reilianum f. sp. reilianum
[46.55,24.19]
[23.85,13.59]
[16.61,7.15]
[3.63,0.65]
-50
-40
-30
-20
-10
0
Divergence time (Myr ago)
Barley
Maize
Sugar cane
Sorghum
B
Divergence of S. reilianum f. sp. reilianum with...
U. hordei
U. maydis
S. scitamineum
S. reilianum f. sp. zeae
0%
10%
20%
30%
40%
Sequence differences
Type
Non coding
Non−synonymous
C
U. hordei
U. maydis
S. scitamineum
S. reilianum f. sp. zeae
S. reilianum f. sp. reilianum
0
30
60
90
Number of genes under positive selection
Type
All genes
Genes encoding a secreted protein (prediction)


Symptoms of infected seedlings [%]
n = 83
n = 88
n = 29
n = 29
JS161
#G3
#G4
#G5
JS161ΔSr10529 (Pit2)
n = 98
n = 96
n = 95
n = 102
n = 95
water
JS161
#3
#4
#5
JS161ΔSr12968
n = 93
n = 100
n = 95
n = 87
n = 93
water
JS161
#2
#3
#4
JS161ΔSr14558
n = 101
n = 95
n = 103
n = 103
n = 90
water
JS161
#15
#28
#31
JS161ΔSr14944
n = 89
n = 83
n = 89
n = 85
n = 89
water
JS161
#1
#2
#3
JS161ΔSr14347
n = 88
n = 90
n = 91
n = 83
n = 91
water
JS161
#25
#40
#47
JS161ΔSr10059
n = 93
n = 95
n = 100
n = 95
n = 102
water
JS161
#1
#2
#3
JS161ΔSr12897
n = 85
n = 77
n = 94
n = 87
n = 82
water
JS161
#1
#2
#5
JS161ΔSr10182
n = 92
n = 94
n = 93
n = 91
n = 94
JS161
#4
#6
#9
#11
JS161ΔSr12084
plant without ears
healthy ears ≤ 1 cm
healthy ears > 1 cm
spiky ears
phyllody in ears ≤ 1 cm
phyllody in ears > 1 cm
phyllody in tassels
spore formation
dead plants

```
                                  20                                  40
UHOR_02064   MLHYLGRLFLVAALAVACLR PATQ---NRPLRRAIV---- -GDNNDNYITKLHRRWYFLW
UMAG_01375   MLFRSAFVLLIVAFASACLV QHVQAI---PVRRSLSTDAS M----SSAAGKLNRRWWFGF
SPSC_03677   MLVHSAP-AFIATLVALCLA QHVQAIQLPAIRRSLTHNDD A--------ANLERRWFWNF
srs_10529    MLVHSAR-AFVAALL-LGLV LHVHAIQMPAMRRSLSSHAD AGAAGGSTLGKLARRWFFNF
sr10529      MLVHSAR-AFVAALL-LGLV LHVHA--MPAMRRSLSSHAD AGAAGGSTLGKLARRWFFDF

                   60                  80                  100
UHOR_02064   PGSLAPKPPREGEEHKIIYA DWIVHHDPAYNSNVQKEIEL ARLQNPTFIQVSVGESSSSS
UMAG_01375   TGSLGKEPDNGQVQIKIIPD ALIIKNPPANKDDLNKLIEN LKRKHPRFKTVVMPTDPNGD
SPSC_03677   GSSLGRSPDNN---ALIVPE DMIKKHTAALVTEWQTYLNE MHRQHPNWKRIDWRDDGPAG
srs_10529    GGSLAPLDAVP---IFEIPK SLIKTHKPAEVTKWEVFLQR VHRKHPDWTHVHWTTDGPVG
sr10529      GGALSRWDVAP---ILRIPE DVAKAHSRAEVARWEVYLER VHREHPDWQYVHWTDNGPIG

                 120
UHOR_02064   SSSSSKKS----- 120
UMAG_01375   VVIWE-------- 118
SPSC_03677   FARWESEKQGRSH 121
srs_10529    YKGH--------- 119
sr10529      YKSH--------- 117
```