High natural prevalence of a fungal prion

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Prions are infectious proteins that cause fatal diseases in mammals. Prions have also been found in fungi, but studies on their role in nature are scarce. The proposed biological function of fungal prions is debated and varies from detrimental to benign or even beneficial. [Het-s] is a prion of the fungus *Podospora anserina*. The *het-s* locus exists as two antagonistic alleles that constitute an allorecognition system: the *het-s* allele encoding the protein variant capable of prion formation and the *het-S* allele encoding a protein variant that cannot form a prion. We document here that *het-s* alleles, capable of prion formation, are nearly twice as frequent as *het-S* alleles in a natural population of 112 individuals. Then, we report a 92% prevalence of [Het-s] prion infection among the *het-s* isolates and find evidence of the role of the [Het-s]/*het-S* allorecognition system on the incidence of infection by a deleterious senescence plasmid. We explain the *het-s*/*het-S* allele ratios by the existence of two selective forces operating at different levels. We propose that during the somatic stage, the role of [Het-s]/*HET-S* in allorecognition leads to frequency-dependent selection for which an equilibrated frequency would be optimal. However, in the sexual cycle, the [Het-s] prion causes meiotic drive favoring the *het-s* allele. Our findings indicate that [Het-s] is a selected and, therefore, widespread prion whose activity as selfish genetic element is counteracted by balancing selection for allorecognition polymorphism.

heterokaryon incompatibility | self/nonself recognition | spore killing

Prions are infectious proteinaceous particles. Prions were first identified in mammals as the causal agents of a group of fatal neurodegenerative diseases termed spongiform encephalopathies including Scrapie, BSE, and Creutzfeldt-Jakob disease (1, 2). Many prion proteins were also identified in fungi, especially in the yeast *S. cerevisiae* (3). Currently, ~10 fungal prion proteins have been identified and 19 additional candidates await further characterization (4–6).

The proposed biological function of fungal prions is debated. Fungal prions could be proteinopathies analogous to the protein deposition diseases found in humans, but it was also proposed that these prions might be adaptive and confer a benefit to the host (7–14). Ongoing controversy in the field may be attributed, at least in part, to the scarcity of experimental studies on the evolutionary dynamics and natural occurrence of fungal prions. A first survey among wild yeast strains from various culture collections failed to find any proof of presence of the [URE3] or [PSI⁺] prion, but [RNQ⁺] was found in a fraction of the isolates (9). It was argued that absence of [URE3] and [PSI⁺] prions in wild isolates is caused by negative effects on their host and that these prions therefore should be considered as diseases (9). However, others have argued that the scarcity of fungal prions is consistent with a possible functionality of prions as a mechanism for generating heritable phenotypic diversity (7). Fungal prions should then be considered as epigenetic determinants that can infrequently lead to prion-associated phenotypes that, although benign or even detrimental under most conditions, may be beneficial occasionally. Recently [PSI⁺], [MOT3⁺], and [RNQ⁺] were found in an extensive study in several of 690 wild yeast strains, and data suggest that one-third of these isolates harbored unknown prions (12). In that study, [PSI⁺] was identified in 1.5% of the tested isolates.

A prion termed [Het-s] has been described in *Podospora anserina*, a coprophilic filamentous fungus whose natural habitat is herbivore dung. Fungal spores ingested with the plant material end up in the dung, germinate, and form mycelia that compete for the limited and short-lived resources. Sexual spores are forcibly ejected from the dung and may attach to surrounding vegetation with their appendages. The [Het-s] prion functions in a somatic allorecognition process called heterokaryon incompatibility, present in most if not all fungi (15). Heterokaryon incompatibility occurs when mycelia that differ genetically at specific recognition loci (termed *het* loci) fuse, leading to death of the fusion cells. Incompatibility acts as a barrier against transfer of infectious genetic elements such as mycoviruses and mitochondrial plasmids (16–18). Incompatibility could also prevent various forms of conspecific parasitism, a mechanism by which selfish nuclei act as parasites of individuals belonging to the same species. As a general rule, it is proposed that balancing selection is maintaining *het* alleles close to equal frequency in populations (19). This frequency-dependent selection favors rare alleles because their bearers are less likely to suffer infection upon contact with infected colonies than those carrying more common alleles. The *het-s*/*S* locus displays two different incompatible alleles, *het-s* and *het-S*, which code for the HET-s and HET-S proteins, respectively (20). The *het-s*-encoded protein can adopt a prion form de novo at low frequency. *Het-s* mycelium that is infected with HET-s in its prion form is referred to as [Het-s] mycelium; the nonprion state is termed [Het-s*]. The HET-S protein encoded by the antagonistic allele cannot form a prion. [Het-s] prion infection is rapidly transmitted between *het-s* colonies. The incompatibility reaction occurs when a [Het-s] mycelium fuses with a *het-S* mycelium, whereas [Het-s*] nonprion strains are compatible with *het-S* mycelium (Fig. 1). Thus, the incompatibility function is associated with the prion form of HET-s, which led to the suggestion that [Het-s] might be a beneficial prion (15, 21). HET-s and HET-S are two-domain proteins with an N-terminal globular domain termed HeLo domain and a C-terminal prion forming domain (PFD) (22), which adopts a highly ordered β -solenoid amyloid conformation (23). During the [Het-s]/HET-S encounter, it is proposed that [Het-s] induces amyloid folding of the HET-S C-terminal domain, which, in turn, triggers refolding of the HET-S HeLo domain leading to toxicity (22, 24). Thus, in the incompatibility reaction, HET-s and HET-S do not have equivalent roles, the HET-S HeLo domain represents the cell death execution entity, and the prion form of [Het-s] acts as a trigger for activation of this toxicity domain. The HeLo domain in HET-s is inactive and does not exert any toxicity. A number of HET-s orthologs have been identified in other peizizomycotina fungi. Key residues, critical for formation of the β -solenoid fold, are conserved, arguing for the existence of

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show presence of *repa* by PCR genotyping. In other words, *nwd2* is in an inactive pseudogene form in all analyzed *het-s* isolates.

All eight *het-s* alleles contained the same 13 amino acid substitutions compared with *het-S*. Also, the five sequenced *het-S* alleles proved identical. No new polymorphism between *het-s* and *het-S* was found. To further establish the nature of the *het-s/S* allele in the tested strains, they were tested for their sensitivity to *het-S* spore killing (29). When a paternal *het-S* strain is crossed with the *pGPD-het-s* strain (= Δ *het-s* strain overexpressing HET-s from an ectopically integrated plasmid copy) as a maternal counterpart, nearly 100% of the resulting asci show *het-S* spore killing (Fig. 2). This spore killing is a clear phenotype that allows for straightforward testing for whether a strain is *het-S* or *het-s*. All wild-type strains were therefore crossed with a maternal *pGPD-het-s* strain. Strains that showed sensitivity to *het-S* spore killing lacked the *repa* element and were therefore genotyped as *het-S* with the PCR test. All strains that did not show sensitivity to *het-S* spore killing contained the *repa* element in the *het-s* promoter region and were genotyped *het-s* (Table S1). Overall we found 72 *het-s* strains and 40 *het-S*, a significant deviation from equal distribution of the two alleles ($\chi^2 = 10.6$; $P < 0.005$), (Fig. 3A). No resistance to meiotic drive by [Het-s] was observed among the *het-S* isolates; all showed *het-S* spore killing.

The near 2:1 ratio of *het-s*:*het-S* found in the Wageningen population is comparable to the allele distribution found in a small collection of strains originally isolated in the 1940s from various locations in France. Among 14 strains, 9 *het-s* and 5 *het-S* strains were found (33).

Prevalence of [Het-s] Prion Infection. To find out whether isolation and storage of *P. anserina* strains asserts any influence on the [Het-s] prion infection state, we repeated the standard isolation procedure (34) on infected and noninfected *het-s* strains. Both [Het-s*] and [Het-s] isolates were allowed to self and shoot spores. The harvested spores were stored in glycerol-peptone at -80°C . After storage for 2 wk, mycelium was grown from the spores and was tested for the prion phenotype. All 10 tested [Het-s] ascospore isolates proved to be [Het-s] after storage. Similarly, all seven [Het-s*] isolates remained unchanged [Het-s*]. This result demonstrates that the procedure used for isolation and storage of

P. anserina isolates does not alter the state of [Het-s] prion infection and that the metastable [Het-s*] is not transformed into the prion state. Therefore, screening of our wild-type collection from the -80°C can provide information about the presence of the [Het-s] prion in the original strains as collected from nature.

The 112 *P. anserina* wild-type strains from our collection were tested for their somatic [Het-s]/[Het-s*] or [Het-S] phenotype (Table S1). These tests were performed by sequential cell fusion and prion transmission assays (Fig. S2). Of the 112 tested strains, 66 were capable of directly infecting a [Het-s*] tester strain with the [Het-s] prion and were therefore of the [Het-s] somatic phenotype. All of these [Het-s] strains proved to be of the *het-s* genotype after the *het-s/S* genotyping tests described above. Therefore, these 66 wild-type strains originally must have been [Het-s] prion infected. Six strains could only infect a [Het-s*] tester strain after initially being infected themselves by a [Het-s] strain. In other words, these strains were capable of adopting and passing on a [Het-s] infection, which makes them of the nonprion [Het-s*] somatic phenotype. All [Het-s*] strains were also genotyped as *het-s*. The remaining strains were not capable of infecting a [Het-s*] tester strain nor could they adopt or pass on a [Het-s] prion infection. These strains were of the [Het-S] somatic phenotype and were also genotyped as *het-S*.

Summarizing the above, it can be concluded that the results from the heterokaryon incompatibility prion assay were in complete concordance with the genotype of the natural strains based on *het-S* spore killing, presence of the *repa* element, and sequence data (Table S1). Of the 72 *het-s* strains, 66 were originally [Het-s] prion infected and 6 were [Het-s*] nonprion infected. The remaining 40 *het-S* strains showed the somatic [Het-S] phenotype and sensitivity to *het-S* spore killing. Clearly, the majority of strains (>91%) with the *het-s* genotype was [Het-s] prion infected (Fig. 3B).

Direct Isolation of [Het-s] from the Wild. To further ascertain presence of [Het-s] in strains growing on dung in the wild, we performed an infection experiment similar to the one we used to test the *Podospora* collection strains from the freezer and proceeded to a fresh isolation of unique *P. anserina* isolates. Twelve Petri dishes were inoculated with [Het-s*] and [Het-S], 2 cm apart. On six of these plates, close to the [Het-s*] inoculum, a sterilized piece of rabbit dung collected from the wild was placed. This sterilized dung yielded no barrage/incompatibility reaction between the [Het-s*] tester and [Het-S]. On the six other plates, unsterilized rabbit dung freshly collected from the wild was placed close to the [Het-s*] inoculum. Here, on four of six plates, a barrage was formed between the [Het-S] strain and the strain that was initially uninfected [Het-s*]. From the unsterilized dung fragment, all kinds of fungi and other microorganisms spawned after inoculation. Contact between these organisms and the [Het-s*] tester strain resulted in infection of this strain with the [Het-s] prion in four of six cases here. Direct recognition of *P. anserina* on these plates was not possible because for the isolation of *P. anserina* from dung, different media conditions are to be used. On specific ad hoc isolation medium, we were, however, able to isolate nine unique *P. anserina* strains (Wa121–Wa129), one from a rabbit dropping and eight from one fresh horse dung sample that were directly tested for presence of [Het-s], sequenced at the *het-s/S* locus, and PCR tested for presence of the *repa* transposon. Again, all *het-s* alleles were identical in sequence and contained the *repa* element; also, the *het-S* alleles were identical in sequence. Of five *het-s* strains, four had the [Het-s] prion and one was [Het-s*]. The other four isolates were *het-S*. The same horse dung sample yielded three [Het-s], one [Het-s*], and four *het-S* cultures.

Role of *het-s*/*het-S* on Transmission of a Deleterious Senescence Plasmid. A suggested biological role for the [Het-s] prion is heterokaryon incompatibility, which has been hypothesized to serve as a cellular defense mechanism against genetic infection with viruses, plasmids, or “selfish” nuclei from strain to strain in nature

Fig. 3. *het-s*/*het-S* allele distribution, [Het-s] prevalence, and infection by the pAL2-1 senescence plasmid in a natural population of *P. anserina*. (A) In total, 112 wild isolates of *P. anserina* were tested for *het-s*/*het-S* genotype, 72 were of the *het-S* and 40 were of the *het-s* genotype. (B) Sixty-six of 72 *het-s* isolates were prion infected (i.e., had the [Het-s] phenotype), whereas six were [Het-s*]. (C) Prevalence of infection by the pAL2-1 senescence plasmid is higher in *het-s* than in *het-S* isolates.

(16–18, 35). In support of this view are the common occurrence of heterokaryon incompatibility in fungi and the well-documented inhibition of transmission of deleterious genetic elements by polymorphism for heterokaryon incompatibility genes in fungi (36).

Forty percent of our *P. anserina* isolates were found to be infected with pAL2-1, a mitochondrial senescence plasmid that reduces the lifespan of *P. anserina* under all conditions tested (37). We combined these data with our *het-s* genotyping data and found 37 of the 72 *het-s* isolates and 8 of the 40 *het-S* isolates carried the plasmid, (Fig. 3C). This distribution of the plasmid infection was strongly biased toward *het-s* isolates ($\chi^2 = 10.540$, $df = 1$, $P = 0.001$). We also directly tested the effect of [Het-s]-based heterokaryon incompatibility on the spread of the pAL2-1 plasmid. We confronted two natural plasmid-infected strains (Wa32 and Wa118, [Het-s] and *het-S*, respectively), to [Het-s] and *het-S* laboratory strains. The two laboratory strains are isogenic, differing only at the *het-s* locus. Wa32 and Wa118 differ from these strains in one or more additional unknown heterokaryon incompatibility loci. Recipient colonies were tested for the presence of the plasmid after 7 d of growth. When using Wa32 ([Het-s]) as a donor, 21 of 39 *het-s* cultures and 8 of 40 *het-S* cultures were plasmid infected. When Wa118 (*het-S*) was used as a donor, none of 20 [Het-s] and 6 of 20 *het-S* were infected. Overall infection was higher when donor and recipient strain were either both [Het-s] or both *het-S* than when different (Table 1; $\chi^2 = 15.069$, $df = 1$, $P = 0.0001$). This result demonstrates that [Het-s]/*het-S* polymorphism reduces the spread of the senescence plasmid.

Discussion

The [Het-s] prion of the filamentous fungus *P. anserina* was early on suggested to be beneficial and to have a function in self/nonself recognition between strains, a system called heterokaryon incompatibility in fungi (38, 39). However, the existence of [Het-s] in nature or its effect on horizontal transfer of parasitic elements has never been formally demonstrated. Here, we have analyzed a natural population of *P. anserina* for the presence of [Het-s] and polymorphism at the *het-s* locus and compare this frequency distribution with the distribution of a senescence plasmid. We found a near 2:1 ratio of *het-s*:*het-S* alleles and a high prevalence of the [Het-s] prion infection among the *het-s* isolates. We also found that the senescence plasmid pAL2-1 is overrepresented in *het-s* strains and that its spread is reduced by [Het-s]-based heterokaryon incompatibility. The high frequency of [Het-s] in natural isolates indicates that both heterokaryon incompatibility and meiotic drive are biologically relevant and not phenomena restricted to laboratory conditions. The fact that we found [Het-s], [Het-s*], and [Het-S] strains in the same dung sample also indicates that somatic and sexual *het-s*/*het-S* interactions are likely to occur in nature.

[Het-s]-Based Incompatibility Limits Propagation of a Deleterious Plasmid. Classically, it is proposed that fungal incompatibility systems serve to limit propagation of deleterious cytoplasmic elements

such as mycoviruses and senescence plasmids (16–18). In a previous study, it was found that 40 percent of the *P. anserina* isolates from the population were infected with the cytoplasmic element pAL2-1, a well-studied mitochondrial senescence plasmid that reduces lifespan (37). If heterokaryon incompatibility restricts plasmid transmission in the wild, it is expected that strains carrying the rarer *het* allele are less likely to become infected with deleterious cytoplasmic elements. We thus tested the presumption that *het-S* isolates suffer less from the spread of parasites than *het-s* isolates. We found that the distribution of the plasmid infection was biased toward *het-s* isolates, which could be explained by a frequency-dependent disadvantage for the common *het-s* relative to the rare *het-S* allele. We also directly tested the effect of the [Het-s]-based heterokaryon incompatibility on the horizontal spread of the senescence plasmid and found that this incompatibility system has an additive effect on the infection efficiency between otherwise incompatible colonies. These results indicate that [Het-s]/*het-S* incompatibility impacts plasmid transmission and imply that [Het-s]/*het-S*-based allorecognition is of benefit for the fitness of the species. [Het-s*] strains, in turn, are devoid of this allorecognition function and can be viewed as a weak point in this allorecognition system. Existence of the inactive [Het-s*] state associated to the *het-s* genotype might contribute to the higher infection rate in *het-s* isolates. Globally, if the [Het-s]/*HET-S* system is to limit plasmid propagation efficiently, it appears critical that abundance of [Het-s*] is kept at a minimum in the population.

***het-s* and *het-S* Alleles Are Subject to Counteracting Selection During Different Life Stages.** *Het*-allorecognition alleles are predicted to be subjected to frequency-dependent balancing selection because rarer *het* genotypes will have a fitness advantage over common genotypes. This selective pressure will allow *het*-allele frequency to reach an equilibrated distribution and will then maintain this optimal distribution. For a different heterokaryon incompatibility locus, the *het-c* locus of *Neurospora crassa*, which reduces the spread of senescence plasmids among strains in the laboratory (17), balancing selection maintaining polymorphic alleles was evidenced (19). Equilibrated allele frequencies and balancing selection were also reported for the *het-6* locus in the same species (40). In our collection, the ratio of *het-s* to *het-S* isolates deviates from equal frequencies ($\chi^2 = 5.95$, $df = 1$, $P = 0.0146$), which would be predicted for optimal self/nonself recognition (19). We found that the distribution of the pAL2-1 plasmid infection was biased toward *het-s* isolates, consistent with its overrepresentation in the population. If balancing selection is operating at this incompatibility locus as occurs for other *het*-loci, one needs to explain why *het-s*:*het-S* allele ratios deviate from equal frequency. Overrepresentation of the *het-s* allele can readily be explained by its meiotic drive behavior. However, meiotic drive has not led to fixation of *het-s* and loss of *het-S* polymorphism. We propose that balancing selection for heterokaryon incompatibility alleles is responsible for the maintenance of the *het-s*/*S* polymorphism and provides a counteracting force to meiotic drive of *het-s* in favor of the rare *het-S* allele. The reciprocal formulation of the same idea is that balancing selection operates on [Het-s]/*HET-S* but that meiotic drive deviates allele frequency from equilibrated distribution by bringing the *het-s* allele to a suboptimal frequency, which leads to increased plasmid infection. Not surprisingly for a meiotic drive element found in natural populations, [Het-s] comes at a cost at another stage of the life cycle, which prevents it from going to fixation and losing its function.

The *het-S nwd2* gene pair forms a putative functional unit conserved in a range of fungal species, arguing for a fitness advantage associated with the maintenance of *het-S nwd2* activity. It has been proposed that *nwd2* and other *nwd* gene family members may have a function in host defense and be analogous to pathogen recognition receptors described in plants and animals (27, 28). In this model, NWD2 recognized a nonself cue and activates toxicity

Table 1. Effect of [Het-s]/[Het-S] polymorphism on the infection with the senescence plasmid pAL2-1

Plasmid containing donor	Recipient strain*	
	Strain s [Het-s]	Strain S [Het-S]
Wa32 ([Het-s], pAL2-1)	21/39	8/40
Wa118 ([Het-S], pAL2-1)	0/20	6/20

*Colonies of laboratory strains s ([Het-s]) and S ([Het-S]) were inoculated at 1 cm distance from plasmid containing strain Wa32 ([Het-s]) or Wa118 ([Het-S]). After 1 d, colonies touched; after 7 d, samples were taken from the laboratory strains, 2 mm from the barrage-zone and tested for pAL2-1 by PCR. The number of recipient cultures infected with pAL2-1 after confrontation to the naturally infected donor as fraction of the number was tested.

of HET-S as a defense reaction. This gene pair is inactive in *het-s* strains because *nwd2* is in a pseudogene form (resulting from insertion of the *repa* transposon) and because the HET-s HeLo domain is unable to induce toxicity in response to β -solenoid folding of the PFD region. The *het-s nwd2* haplotype could thus be envisioned as an inactive mutant derivation of the *het-S nwd2* haplotype. Thus, maintenance of the *het-S nwd2* function could also participate in counteracting the meiotic drive imposed by the [Het-s] element. This perspective of viewing *het-s* as a mutant form of *het-S* implies that the [Het-s]/*het-S* system evolved from an ancestral *het-S* gene by exaptation (an evolutionary phenomenon by which a character previously shaped by selection is coopted for a new use) (41). Inactivation of the HeLo domain turned *het-S* into the prion-forming *het-s* allele, which would have subsequently thrived in virtue of balancing selection for allorecognition function and by meiotic drive.

Effect of Sexual [Het-s]/*het-S* Interactions on [Het-s] Prevalence. The presence of the neutral [Het-s*] phenotype in the population, be it at a low frequency, indicates that [Het-s]-prion curing occurs in the wild. Because the only known natural mechanism of [Het-s] prion curing is outcrossing with a *het-S* strain, it can reasonably be hypothesized that these [Het-s*] strains result from such outcrossing events. When outcrossing occurs at elevated temperature (26 °C), the [Het-s] prion is completely cured from the *het-s* progeny and there is normal segregation of the *het-s* and *het-S* alleles. At low temperature (18 °C), in a fraction of the asci, *het-S* spore killing occurs and [Het-s] is maintained in the two *het-s* progeny (resistance to prion curing). There is drive of the *het-s* prion-forming allele and maintenance of the [Het-s]-prion form in the driving progeny. *het-s* $\times$ *het-S* outcrossing is expected to have a negative effect on [Het-s] prion prevalence in the population because it leads to total or partial prion curing. However, the *het-S* spore killing phenomenon (defined as the concomitant of abortion of the *het-S* progeny and maintenance of [Het-s]) favors [Het-s] because it represents an exception to the prion curing otherwise associated with outcrossing. Outcrossing allows expansion of the *het-s* allele at the expense of *het-S*. This segregation advantage can be expected to have an indirect positive effect on the spread of [Het-s], because abundance of the *het-s* genotype in the population will favor horizontal transmission of the prion between strains. A further possibility to consider is that there might exist specific fitness effects associated to the [Het-s] progeny originating from two-spored asci in which *het-S* spore killing occurred. Such [Het-s] ascospores could benefit from higher resource availability during maturation or suffer lower levels of competition during colonization of fresh substrate after ascospore discharge and dispersal.

Conclusions

We here show that the [Het-s] prion of *P. anserina* is highly prevalent in nature with both detrimental and beneficial effects on the host. [Het-s] is a selfish meiotic drive element that is kept in balance by frequency-dependent selection favoring the rarer *het-S* allele (Fig. 4). The distribution of the three phenotypes, [Het-s], [Het-s*], and [Het-S], indicates that the interactions between [Het-s] and [Het-S] occur in the wild and result in the sexual cycle, in *het-S* spore killing and curing of [Het-s], and at the somatic level, in heterokaryon incompatibility between [Het-s] and [Het-S]. Selection forces at both levels are conflicting: Segregation advantage of *het-s* in the sexual cycle is counteracted by its active role as a prion in somatic self/nonself recognition for which equal frequencies of the two alleles would be optimal. Although the *het-s* allele is selected by meiotic drive and increases in frequency, it is selected against by its diminishing effect in heterokaryon incompatibility. This frequency-dependent disadvantage of *het-s* (compared with *het-S* strains) is reflected in a high plasmid infection of *het-s* strains. These counteracting selective forces shape the *het-s*:*het-S* allele ratios in the population. The possible fitness advantage

Fig. 4. Counteracting forces maintain [Het-s] prevalence and *het-s/het-S* polymorphism. The figure graphically summarizes the evolutionary forces that shape the *het-s/het-S* allele distribution and lead to high prevalence of [Het-s] prion infection in the population. Each phenotype/genotype is represented by a circle whose size reflects the prevalence of the given phenotype/genotype in the population (the percentage of each phenotype/genotype in the population is given). For each phenotype/genotype, the evolutionary forces favoring it are listed. The mechanisms controlling [Het-s*] to [Het-s] interconversion are also given.

associated with the *het-S nwd2* function may also participate in preventing the *het-s* allele from going to fixation by meiotic drive. Balancing selection for the allorecognition function is operating both on [Het-s] and *het-S* but not on [Het-s*] because it is devoid of allorecognition capacity. The function of [Het-s] in heterokaryon incompatibility thus provides [Het-s] prion-infected strains with an advantage over [Het-s*] prion-free strains. The highly infectious nature of [Het-s], combined with a beneficial role for the prion form, can explain the extreme prevalence of this prion in the analyzed natural fungal population.

Methods

Strains and Culture Conditions. The *P. anserina* laboratory strains were all derived from strain S and have been described (15, 42). The natural *P. anserina* isolates were collected by growing mycelium from fragments of herbivore feces collected on fields around Wageningen, The Netherlands, since 1991 and were stored at -80°C (37, 43). Spores were collected from emerging *P. anserina*-like fruiting bodies. After germination of the spores, the fungus was tested for sexual compatibility with *P. anserina* tester strains (44). Culture conditions and genetic analysis methods were as described by Esser (45, 46). Standard growth medium was cornmeal agar. All crosses were performed on moistened copromes (47) placed on sterile filter paper on top of cornmeal agar by spermatization of monokaryotic tester strains or either monokaryotic or dikaryotic wild-type strains from our collection with microconidia. Microconidia were isolated from strains growing up to 4 d on cornmeal agar at 27°C . This suspension was achieved by adding 2–4 mL of saline to this fungal colony. After some gentle shaking, the saline with the conidia was removed and poured over monokaryotic strains (spermatization).

Analysis of [Het-s/S/s*] Phenotype and *het-s/S* Genotype of a Natural Isolate of *P. anserina* at Somatic Level. *P. anserina* has, next to the *het-s/S* locus, some other heterokaryon incompatibility loci (36) that all need to have compatible alleles if two different mycelia are to be somatically compatible. Therefore, any direct encounter between a wild-type strain and a tester strain grown on cornmeal agar may result in an incompatibility

reaction or barrage. The short-lived fusion of hyphae preceding the incompatibility reaction, however, is long enough to infect a [Het-s*] mycelium with the [Het-s] prion of an already-infected mycelium (33). Applying this principle, it is possible to test wild-type strains of *P. anserina* for their *het-s/s* genotype and [Het-s/S/s*] phenotype, as is depicted in Fig. S1.

Genotype Analysis Based on Sexual *het-S* Spore Killing Phenotype. All natural isolates were used as a paternal (spermatial) parent in a cross to pGPD-*het-s* as a maternal strain, and overexpression of *het-s* increases the appearance and abundance of [Het-s] (38). When the paternal strain involved is genotypically *het-S*, then this cross will phenotypically result in >90% *het-S* spore killing (29) as shown in Fig. 2. Non-*het-S* strains (*het-s*, *het-s**, or Δ *het-s*) show no spore killing, unless another *Psk* spore killer gene is active (43). *Psk* spore killer genes also disturb a potential *het-S* spore killing phenotype. Strains in

the collection that are *Psk* spore killers therefore are less suitable for straight forward genotype analysis based on *het-S* spore killing.

***het-s/S* Genotype by PCR and Sequencing.** The following forward (TGACG-GAGGAGCTCGGTTTCG) and reverse (CCTGGAAAGAAGCATGATGCCTTTC) primers were applied in a PCR with annealing at 63 °C and elongation at 72 °C in 35 cycles. After PCR, gel separation of the amplicon showed either a 1,500-bp product or a product that is ~350 bp longer. The longer product reflects the original prototypical *het-s* gene with a *repa* element in its promoter region that is absent in *het-S* (32, 48). For sequence analysis of the ORF of the *het-s/S* locus, the primers used were as follows: CTCAGTGCCTTCCTGGCAACATC (forward) and CCTGGAAAGAAGCATGATGCCTTTC (reverse).

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