MANUELA ROCHA DE BRITO

CICLO DE VIDA ANFITÁLICO DE *Agaricus subrufescens*: ANÁLISE GENÉTICA E BIOLÓGICA

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutora.

Orientador

Dr. Eustáquio Souza Dias

Coorientador

Dr. Philippe Callac

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A Deus, por me abençoar e capacitar para tudo aquilo que Ele me destina.
*Aos meus pais Valmir (**in memoriam**) e Irení, por sempre me apresentarem a importância da honestidade e persistência.*
Ao amor do meu irmão Ícaro e da minha tia Lurdes.
Ao meu noivo, Rafael, que está presente em todos os momentos da minha vida, me dando forças, sempre com muita sabedoria.

DEDICO

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RESUMO

Agaricus subrufescens é uma espécie de cogumelo comestível com importantes propriedades medicinais. Esta espécie tem ciclo de vida anfitálico com produção de basidióporos homocarióticos e heterocarióticos no mesmo basidioma. Os esporos heterocarióticos podem resultar do emparelhamento de núcleos "irmãos" (= *Sister Nuclei Pair Progeny – SNPP*) ou núcleos não "irmãos" (=*Non Sister Nuclei Pair Progeny – NSNPP*), resultantes da segunda divisão da meiose. Este trabalho foi realizado com o objetivo de caracterizar geneticamente as três categorias de esporos (homocarióticos, *SNPPs* e *NSNPPs*), aliado a um estudo comportamental dos mesmos. A classificação foi baseada em homo-heteroalelismo, utilizando oito marcadores moleculares codominantes CAPS - sequencia polimorfica amplificada e clivada - sob análise de 225 esporos descendentes de uma estirpe brasileira WC837. A análise comportamental foi realizada pela (i) habilidade em testes de cruzamento entre os diferentes tipos de esporos descendentes, (ii) pela análise da taxa de crescimento micelial e por (iii) testes de frutificação. Testes de genótipos multilocos revelaram que 50% dos esporos foram heterocarióticos e, dentre estes, 24% foram considerados SNPP. A taxa de homoalelismo encontrada em marcadores CAPS não ligados ao centrômero foi de, aproximadamente, 50% para SNPP e NSNPP. Nos testes de frutificação, apenas NSNPP frutificaram. A taxa de crescimento micelial entre homocárions e SNPP não diferiu significativamente. Heterocárions SNPP e NSNPP podem cruzar-se com homocárions. Cruzamentos entre SNPP e SNPP também foram possíveis, desde que ambos compartilhem diferentes *mating type*. A partir destes resultados, foi possível propor uma estratégia simplificada do cruzamento de estirpes que têm esse tipo de anfitalismo e selecionar os esporos NSNPPs por meio de um único marcador ligado ao centrômero, enquanto os SNPPs seriam tratados como homocárions.

Palavras-chave: Cogumelo. Pseudo-homotalismo. Fenômeno Buller. Ciclo de vida.

ABSTRACT

Agaricus subrufescens is an edible mushroom with important medicinal properties. This species has amphithallic lifecycle producing homokaryotic and heterokaryotic spores in the same basidiomata. The heterocaryotic spores can result from the pairing of "sister" nuclei (= Nuclei Sister Pair Progeny - SNPP) or non "sister" nuclei (= Non Sister Nuclei Pair Progeny - NSNPP), resulting from the second division of meiosis. This study aimed to characterize genetically the three spores categories (homokaryons, SNPPs and NSNPPs), combined with a behavioral study of them. The classification was based on homo-heteroallelism through eight codominant molecular markers CAPS - Cleaved Amplified Polymorphic Sequence - under analysis of 225 offspring spores of a Brazilian strain WC837. The behavioral analysis was performed by (i) ability to cross tests between differents types of spores offspring (ii) analysis of mycelial growth rate and (iii) fruiting tests. Multilocus genotype test revealed that 50% spores were heterokaryotic and among them, 24% were considered to be SNPP. The homoallelism rate found by CAPS markers at centromere-unlinked was approximately 50% for SNPP and NSNPP. In fruiting tests, only NSNPP fruited. The mycelial growth rate between homokaryons and SNPP did not differ significantly. SNPP and NSNPP heterokaryons can cross with homokaryons. Crosses between SNPP and SNPP were also possible since both of them share different mating type alleles. From these data, it was possible to propose a simplified strategy to cross strains which have this type of amphithallism and select the NSNPPs spores through a single marker centromere-linked while SNPPs would be treated as homokaryons.

Keywords: Mushroom. Pseudohomothallism. Buller phenomenon. Life cycle.

SUMÁRIO

1 INTRODUCÃO

Os cogumelos são conhecidos, desde a Antiguidade, por sua utilização como alimento de elevado valor nutritivo e medicinal. Nos últimos anos, graças ao número crescente de estudos realizados com a finalidade de identificar suas propriedades nutricionais e terapêuticas, o cultivo de cogumelo vem se expandindo pelo mundo, tornando-se uma atividade agrícola de considerável importância econômica (DIAS; ABE; SCHWAN, 2004). Dentre os cogumelos comestíveis, o *Agaricus bisporus* (Lange) Imbach é uma das espécies mais consumidas do mundo. Entretanto, uma outra espécie de *Agaricus*, o *A. subrufescens*, vem ganhando destaque no mercado mundial, devido ao seu elevado valor nutricional, medicinal e agronômico (LARGETEAU et al., 2011).

O cogumelo *Agaricus subrufescens* Peck (= *A. blazei* Murrill *sensu* Heinemann; *A. refotegulis* Nauta ou *A. brasiliensis* Wasser) é conhecido por diferentes nomes populares, como ABM (por *A. blazei* Murrill [sensu Heinemenn]); royal sun *Agaricus*; cogumelo do sol, no Brasil; himematsutake, no Japão e jisongrong ou baximogu, na China (WISITRASSAMEEWONG et al., 2012b). O cogumelo *A. subrufescens* foi descoberto nos Estados Unidos, em 1893 e, na década de 1970, foi encontrado pela primeira vez no Brasil. Também foi formalmente reportado na Europa, na Oceania (KERRIGAN, 2005; PETERSON; DESJARDIN; HEMMES, 2000) e na Ásia (WISITRASSAMEEWONG et al., 2012a; ZHAO et al., 2011). Interfertilidade entre isolados da América do Sul, Europa e Ásia foi recentemente demonstrada por Thongklang et al. (2014), indicando que uma ampla base genética está agora disponível para o melhoramento genético da espécie.

Os estudos de Thongklang et al. (2014) estavam especialmente focados na análise do ciclo de vida de *A. subrufescens*. Estes autores mostraram que, assim como em *A. bisporus* var. *bisporus*, o *A. subrufescens* tem ciclo de vida

anfitálico e sistema de incompatibilidade sexual unifatorial e multialélico, com o loco *mating type* (MAT) ligado ao centrômero. Anfitalismo refere-se à produção de esporos homocarióticos e heterocarióticos no mesmo basidioma e, assim, combinam o ciclo heterotálico e pseudo-homotálico, respectivamente (KUHNER, 1977; LANGE, 1952). O anfitalismo em *A. bisporus* var. *bisporus* difere do anfitalismo em *A. subrufescens*, pelo fato de este último apresentar predominantemente basídios tetraspóricos, maior taxa de *crossing over* e pela presença de núcleos pós-meióticos pareados no mesmo esporo compartilharem o mesmo alelo *mating type* (THONGKLANG et al., 2014). Como resultado, esses autores classificaram três principais categorias de esporos descendentes: esporos heterocarióticos *Non Sister Nuclei Pair Progeny* (NSNPP), esporos heterocarióticos *Sister Nuclei Pair Progeny* (SNPP) e esporos homocarióticos.

NSNPP são esporos resultantes do emparelhamento de núcleos “não irmãos” provenientes da segunda divisão da meiose, mecanismo este que conserva o heteroalelismo no loco MAT e, por isso, são bem conhecidos por serem reprodutivamente competentes. Os SNPPs são tipos de esporos resultantes do pareamento de núcleos “irmãos” formados durante a segunda divisão da meiose, compartilham o mesmo loco *mating type* e, assim, não são reprodutivamente competentes. Finalmente, os homocárions são culturas haploides que podem ser oriundos de esporos que receberem apenas um núcleo pós-meiótico ou dois núcleos idênticos a partir de uma mitose pós-meiótica.

Este trabalho foi realizado com o objetivo de caracterizar geneticamente esses três tipos de esporos, aliado a um estudo comportamental dos mesmos, especialmente dos esporos heterocarióticos SNPP, nunca anteriormente estudados. Foi utilizado o isolado WC837, de origem brasileira, o mesmo usado por Thongkland et al. (2014), entretanto, foram obtidos um maior número de esporos descendentes e uma caracterização genética com maior número de marcadores codominantes CAPS (do inglês *cleaved amplified polymorphic*

sequence). Em um primeiro momento, (i) verificou-se o nível de ploidia de 225 descendentes de uma estirpe brasileira WC837, utilizando marcadores CAPS. Dentre os heterocárions, (ii) prováveis candidatos para serem SNPP e NSNPP foram selecionados. O estudo comportamental foi realizado por meio de (iii) *mating test* entre homocárions x homocárions, homocários x NSNPP, homocárions x SNPP e SNPP x SNPP. Foram analisadas (iv) a taxa de crescimento micelial entre os diferentes tipos de esporos e (v) a seleção de esporos homocarióticos e heterocarióticos (SNPP e NSNPP) para a avaliação da capacidade de frutificação.

2 REFERENCIAL TEÓRICO

2.1 *Agaricus subrufescens* Peck

O cogumelo *A. subrufescens* Peck é um fungo filamentoso pertencente à divisão Basidiomycota, classe Agaricomycetes e ordem Agaricales (NATIONAL CENTER OF BIOTECHNOLOGY INFORMATION - NCBI, 2014). Esse fungo foi descoberto no final do século XIX, na costa leste dos Estados Unidos e na década de 1970 foi encontrado em São Paulo. Além das Américas, *A. subrufescens* também foi encontrado na Europa, na Oceania e na Ásia (PETERSON; DESJARDIN; HEMMES, 2000; WISITRASSAMEEWONG et al., 2012a; ZHAO et al., 2011).

O *A. subrufescens* foi descrito pelo botânico americano Peck (1983), a partir de amostras coletadas dos Estados Unidos. As amostras brasileiras foram classificadas como *A. blazei* Murril, pelo botânico belga P. Heinemann, em 1967 (MIZUNO, 1995). Do ponto de vista taxonômico, a espécie *A. blazei* Murril *sensu* Heinemann tem sido referida erroneamente, uma vez que Wasser et al. (2002) constataram que a espécie cultivada no Brasil era diferente do *A. blazei* identificado por Murril em 1945. Assim, foi proposto como uma nova espécie, *A. brasiliensis* Wasser. Por sua vez, o nome *A. brasiliensis* já estava ocupado por uma espécie descrita por Elias Magnus Fries, em 1830 (CALLAC, 2007). Estudos do sequenciamento da região ITS1+2 e testes de fertilidade entre várias espécies de *Agaricus* realizados por Kerrigan (2005) indicaram que *A. brasiliensis* Wasser é biológica e filogeneticamente relacionada a *A. subrufescens* Peck.

O cogumelo *A. subrufescens*, também conhecido por cogumelo do sol, himematsutake e cogumelo da vida, entre outros, é uma espécie encontrada principalmente em ambientes de clima subtropical úmido, com temperaturas

oscilando entre 25 e 30 °C (ZHAO et al., 2011). Como decompositor secundário, é cultivado em compostos preparados a partir de diversos resíduos agrícolas, seguindo os processos gerais de cultivo desenvolvidos para *A. bisporus* (LLARENA-HERNÁNDEZ et al., 2013; ZIED et al., 2010). Entretanto, *A. subrufescens* requer temperaturas de, aproximadamente, 25 °C para frutificação, enquanto a temperatura ótima de *A. bisporus* é entre 16 e 19 °C (LARGETEAU et al., 2011). Devido à maior temperatura necessária para a sua produção, o *A. subrufescens* representa uma opção agrícola interessante para os países emergentes tropicais e poderia ser uma boa alternativa sazonal na região, durante o verão.

A morfologia dos basidiomas de *A. subrufescens* é variável, podendo ser robusta ou grácil, devido ao genótipo e a influências ambientais (KERRIGAN, 2005). O píleo varia de 20 a 70 mm, em fase de botão e de 60 a 150 mm, quando maduros, de forma convexa para plano convexa. A superfície do píleo é seca e, quando maduro, desenvolve pequenas escamas; sua cor varia de roxo, marrom, marrom alaranjada a completamente branco. O estipe varia de 3 a 6 cm de comprimento e 1 a 2 cm de diâmetro. Os basidiósporos são castanhos (5 x 4 µm) (FIRENZUOLI; GORI; LOMBARDO, 2008; WISITRASSAMEEWONG et al., 2012b). As amostras provenientes da Tailândia diferem dos padrões anteriormente relatados, em termos de tamanho e comprimento do estipe e a cor do píleo é mais avermelhada (WISITRASSAMEEWONG et al., 2012a).

Os cogumelos comestíveis são, em geral, alimentos de alto valor nutricional, com baixo teor de carboidratos e de gordura, além de possuírem significativas quantidades de minerais, proteínas e fibras (FIRENZUOLI; GORI; LOMBARDO, 2008). Suas propriedades bioquímicas atraem atenção na saúde alimentar, pois são ricos em vitaminas, tiamina, riboflavina, niacina, biotina, ácido ascórbico e outras relacionadas ao complexo B. Sua composição química

varia de acordo com a espécie e a linhagem avaliada (DAI et al., 2009; RAMBERG; NELSON; SINNOTT, 2010).

O basidiocarpo de *A. subrufescens* contém de 89% a 91% de água, geralmente menos que a quantidade encontrada em *A. bisporus*. Aproximadamente 48% da matéria seca consistem de proteína bruta, 18% de carboidratos e apenas 0,5 % de lipídeos. Seus corpos de frutificação contêm altos níveis de minerais, como, por exemplo, potássio, fósforo, cálcio, magnésio e zinco (GYÖRFI; GEÖSEL; VETTER, 2010).

Metabólitos bioativos de cogumelos podem ser isolados a partir do corpo de frutificação e da cultura pura de micélios (CHANG; MILES, 2004). Há relatos, na literatura, de que a espécie *A. subrufescens* produz vários compostos bioativos com atividade antitumoral, imunorregulatória e antimutagênica (FIRENZUOLI; GORI; LOMBARDO, 2008; WISITRASSAMEEWONG et al., 2012b). Esses compostos isolados de *A. subrufescens* são, principalmente, polissacarídeos, tais como riboglicanas (CHO et al., 1999), β-glicanas (GONZAGA et al., 2005) e glicomananas (HIKICHI; HIROE; OKUBO, 1999).

Além disso, outros compostos, como proteínas, ergosterol e lipídeos, podem atuar de forma isolada ou sinérgica com as glicanas. As proteínas demonstram atividade regulatória sobre células e ativação de macrófagos e linfócitos (HO et al., 2004). O ergosterol faz parte da fração lipídica e está presente na membrana celular de diversos fungos com ação antiangiogênica, além de precursor de ergocalciferol (Vitamina D), sendo possível que estes compostos atuem também como agentes antioxidantes (TAKAKU; KIMURA; OKUDA, 2001). Apesar do avanço nas pesquisas científicas a respeito das propriedades medicinais do *A. subrufescens*, seus mecanismos de atuação ainda não são completamente esclarecidos (DIAS; ABE; SCHWAN, 2004; FORTES; NOVAES, 2006; WASSER et al., 2002).

Além da questão medicinal, valores agronômicos do cogumelo *A. subrufescens* têm sido propostos. Esse cogumelo é capaz de converter resíduos lignocelulósicos em alimentos altamente nutritivos e a produção industrial dessas enzimas parece promissora. Além disso, sua aplicação na agricultura orgânica é utilizada para o controle de patógenos de plantas e para a obtenção de resistência, consistindo em uma alternativa para o uso de aditivos químicos (LARGETEAU et al., 2011). Dessa forma, o cultivo desta espécie contribui para uma agricultura mais sustentável e oferece um novo nicho de mercado e uma fonte potencial de diversificação para os produtores de *Agaricus*.

2.2 O ciclo de reprodução dos basidiomicetos

A reprodução nos basidiomicetos pode ocorrer de forma assexuada, em que se reproduzem, por fragmentação do micélio ou de forma sexuada, por meio dos basidiósporos, nos quais os núcleos são produto da meiose (CALLAC, 2007). O cogumelo corresponde à fase reprodutiva, sendo macroscopicamente constituído pelo estipe e píleo. No píleo situam-se as lamelas, em cujas superfícies localizam-se os basídios e os basidiósporos (CHANG; MILES, 2004).

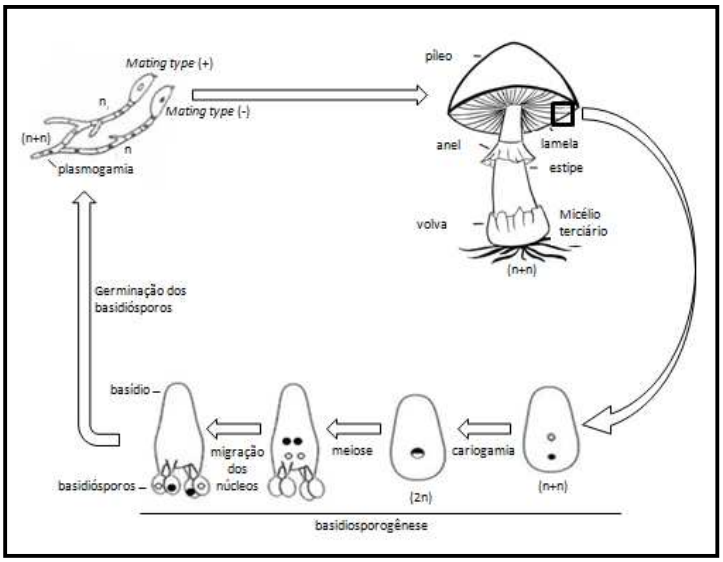

Figura 1 Ciclo de vida de um basidiomiceto típico

Na Figura 1 está esquematizado o ciclo de vida de um basidiomiceto típico. De modo geral, a reprodução sexual inicia-se com a germinação de basidiósporos e a posterior fusão do micélio monocariótico (n) contendo material genético compatível, resultando em um dicarioto (n+n). O dicarioto é caracterizado pela presença de dois núcleos por compartimento celular da hifa e por grampos de conexão entre as células adjacentes. Os grampos de conexão são importantes marcadores morfológicos para permitir a distinção entre monocariotos e dicariotos, como é o caso de *Lentinula edodes* (CHANG; MILES, 2004). Entretanto, nem todos os basidiomicetos formam um dicarioto típico. Espécies do gênero *Agaricus*, por exemplo, raramente apresentam grampos de conexão e as células que o constituem são multinucleadas, à exceção de *A. bitorquis*, cujos compartimentos são heterocariontes binucleados (DIAS et al., 2008; HOU; ELLIOTT, 1979).

O micélio dicarioto pode permanecer no estado vegetativo por um período ilimitado de tempo, dependendo das condições ambientais. Por outro

lado, o dicarioto é uma condição necessária para a consolidação do ciclo sexual. Uma vez estimulados com condições ambientais favoráveis, iniciam-se a fusão nuclear (cariogamia) que resulta na formação de núcleos diploides (2n). Normalmente, esta fase é extremamente curta porque é rapidamente sucedida pela meiose, a qual restaura o estado haploide do organismo, completando, assim, o ciclo sexual com a produção dos basidiósporos (STAMBERG; KOLTIN, 1972).

2.3 Modelos sexuais de reprodução e o sistema *mating type* em basidiomicetos

As espécies fúngicas têm três modelos básicos de reprodução sexual: o **homotalismo**, no qual todas as estirpes haploides são autoférteis e o acasalamento entre linhagens geneticamente diferentes não é requerido para completar o ciclo de vida; o **heterotalismo**, em que todas as estirpes haploides são estéreis e o acasalamento entre linhagens geneticamente compatíveis é necessário para completar o ciclo sexual e o **pseudo-homotalismo**, no qual dois núcleos sexualmente compatíveis são incorporados em um mesmo esporo, permitindo que a maioria destes seja autofértil (Figura 2) (NI et al., 2011; XU; HORGEN; ANDERSON, 1996). Segundo Duncan e Galbraith (1972), dois padrões de comportamento podem ser ocasionalmente encontrados dentro de uma mesma espécie, e aqueles que operam simultaneamente mais de um ciclo de vida são considerados anfitálicos. O termo **anfitalismo**, originalmente definido por Lange (1952), refere-se à espécie que produz esporos homocarióticos e heterocarióticos em um mesmo basidioma e assim combinam, respectivamente, os ciclos reprodutivos heterotálico e pseudohomotálico.

O anfitalismo não é um evento raro entre os basidiomicetos. Estima-se que, de 500 espécies de Agaricales, aproximadamente 8% sejam considerados

anfitálicos (LAMOURE, 1989). Este levantamento é, provavelmente, subestimado, pois, além de ser um levantamento desatualizado, este percentual foi baseado principalmente em observações morfológicas, como, por exemplo, alta proporção de basídios bispóricos.

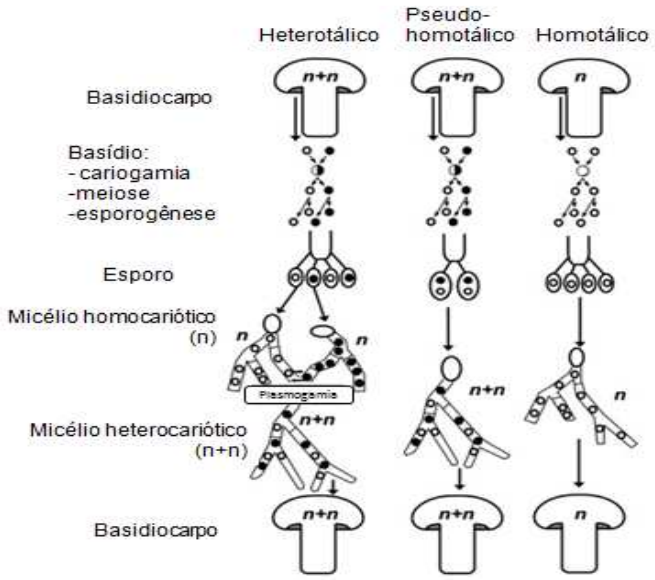

Figura 2 Representação esquemática mostrando os detalhes dos diferentes modos de reprodução
Fonte: Savoie et al. (2013)

Embora os modos de reprodução tenham sido estudados em poucas espécies de *Agaricus*, todos os tipos de modelos já foram encontrados, como demonstrado na Tabela 1.

Tabela 1 Comparação do sistema sexual no gênero *Agaricus*

Espécie	**N° de núcleos por célula/ heterocárions**	**Tipo de sexualidade**	**Referência**
A. bitorquis	Dicárion	Heterotálico	Anderson et al. (1984)
A. devoniensis	Multicárion	Heterotálico (?)	Callac et al. (2005)
A. arvensis	Multicárion	Heterotálico (?)	Calvo-Bado et al. (2000)
A.bisporus			
var. *burnettii*	Multicárion	Anfitálico/ heterotálico*	Kerrigan et al. (1994)
var. *bisporus*	Multicárion	Anfitálico/ pseudo-homotálico*	Lange (1952)
var. *eurotetrasporus*	Multicárion	Homotálico	Callac et al. (2003)
A. subrufescens	Multicárion	Anfitálico/ pseudo-homotálico* Heterotálico	Kerrigan (2005) Dias et al. (2008)

*com predominância

Os fungos, em sua maioria, não são diferencialmente separados em "machos" e "fêmeas", sendo indivíduos morfologicamente idênticos. A distinção como forma de evitar o autocruzamento é feita mediante mecanismos genéticos, por meio de um sistema de reconhecimento molecular de incompatibilidade conhecido como Sistema *Mating Type* (RAPER, 1966). Existem dois tipos de sistema de incompatibilidade: unifatorial (=bipolar), no qual a sexualidade é determinada por um único loco *mating type – MAT* (A), tendo diferentes alelos para serem sexualmente compatíveis (A1 + A2) e o bifatorial (= tetrapolar), no qual a sexualidade é controlada por dois locos *MAT* (A e B) e cada estirpe deve ter diferentes alelos em ambos os locos (A1B1 + A2B2) (Figura 3). O número de alelos no loco *MAT* é variável, podendo ser bialélico ou multialélico, a depender da espécie (NIEUWENHUIS; NIEUWHOF; AANEN, 2013). A fertilidade resulta quando dois indivíduos com núcleos carregando diferentes alelos se cruzam, originando um dicarioto.

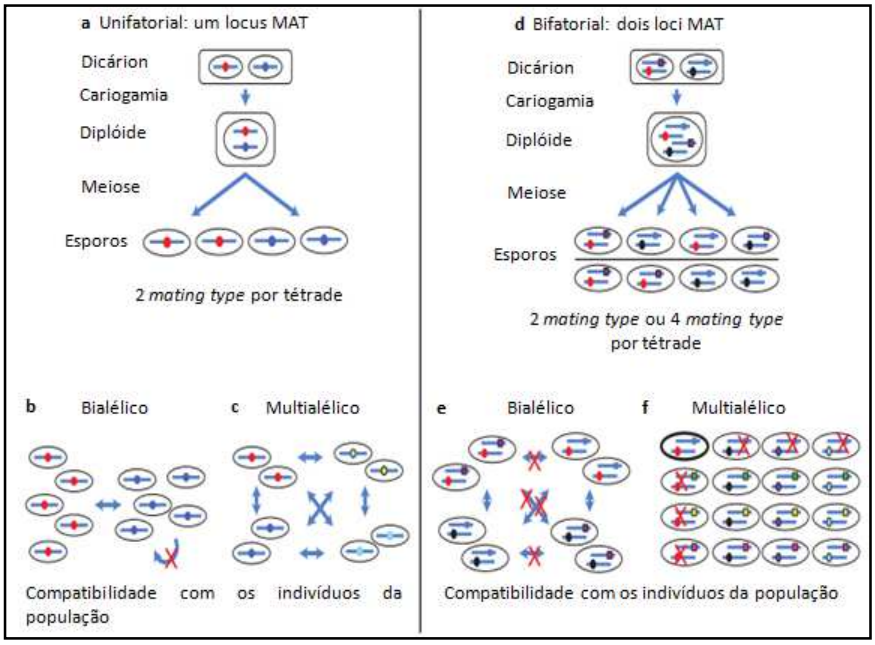

Figura 3 Visão geral de compatibilidade entre indivíduos haploides para sistemas unifatorial e bifatorial. (a) Um dicárion unifatorial produz gametas de dois diferentes *mating type* ; (b) e (c) compatibilidade entre populações de indivíduos bialélicos e multialélicos, respectivamente, em um sistema unifatorial ; (d) o dicárion bifatorial irá produzir gametas de quatro tipos diferentes. Em cada tétrade formada, serão produzidos dois (1/3 das tétrades) ou quatro (2/3 da tétrade) com diferentes *mating type* ; (e) e (f) compatibilidade entre populações de indivíduos bialélicos e multialélicos, respectivamente, em um sistema bifatorial. O "X" em vermelho indica a incompatibilidade entre os indivíduos
Fonte: Nieuwenhuis, Nieuwhof e Aanen (2013), com modificações

Aproximadamente 40% das espécies conhecidas de basidiomicetos apresentam o sistema de acasalamento unifatorial (NI et al., 2011). Em todas as espécies de *Agaricus* conhecidas, o sistema de incompatibilidade sexual é unifatorial e multialélico (CALLAC, 2007).

Seguindo a regra, *A. subrufescens* apresenta um sistema de incompatibilidade sexual unifatorial e multialélico (THONGKLANG et al.,

2014). Em relação ao tipo de modelo sexual de reprodução, há controvérsia na literatura, pois há estudos que propõem um modelo primariamente heterotálico (DIAS et al., 2008) e estudos que propõem um modelo anfitálico (HERRERA et al., 2012; KERRIGAN et al., 2005; THONGKLANG et al., 2014). Essa discussão será detalhada no tópico subsequente.

2.4 Variação do número de basidiósporos, comportamento nuclear e suas relações com o ciclo de vida

Importantes eventos nucleares, como cariogamia, meiose, mitose pós-meiótica e migração nuclear, são reportados por ocorrerem durante o ciclo de vida de basidiomicetos e são essenciais para a basidiosporogênese (HERRERA et al., 2012). Consequentemente, a correlação entre o nível de ploidia dos esporos (homocariótico n *vs* heterocariórico n+n) e o número de esporos por basídio interfere diretamente no tipo de ciclo de reprodução da espécie (SHIMOMURA et al., 2012).

Resumidamente, em determinada fase do ciclo de vida de um basidiomiceto típico, a célula terminal de certas hifas adquire a forma de uma clava e passa a ser denominado basídio. No basídio, dois núcleos fundem-se (cariogamia), originando um núcleo diploide que, imediatamente, divide-se por meiose e produz quatro núcleos haploides. Enquanto a meiose ocorre, formam-se, na superfície do basídio, quatro protuberâncias denominadas esterigmas. Cada um dos núcleos haploides gerados na meiose migra para o interior dos basidiósporos por meio dos esterigmas (Figura 4A) (WEBSTER; WEBER, 2007).

Embora a maioria dos basidiomicetos apresente quatro basidiósporos por basídio, esse número aparece de forma heterogênea no gênero *Agaricus*. Em *A. bisporus*, mais de 90% dos basídios têm dois esporos, cada um recebendo

dois núcleos, sendo a maioria dos esporos heterocariótica e autofértil (CALLAC et al., 2003; XU; DESMERGER; CALLAC, 2002).

A. subrufescens é uma espécie tipicamente tetraspórica, entretanto, a frequência de basidiósporos por basídios pode variar em função da linhagem e ambiente. Kerrigan e Ross (1987), por meio de estudos realizados com *A. subrufescens* cultivados em condições padrões, observaram que a maioria dos basídios era tetraspórica, porém, quando colocados em temperaturas mais baixas, a média de esporos por basídio declinava. Herrera et al. (2012) também observaram, em condições padrões de cultivo, que a maioria dos basídios era tetraspórica, entretanto, grandes variações do número de badisiósporos foram observadas de acordo a linhagem estudada. De acordo com os autores, duas linhagens apresentaram frequência de basídios tetraspóricos de apenas 54 e 30%, respectivamente. Neste caso, portanto, a variação do número de basidiósporos por basídio foi determinada pelas diferenças genéticas entre as linhagens e não por variações ambientais.

O efeito dos fatores genéticos sobre o número de basidiósporos por basídio já foi evidenciado anteriormente em *A. bisporus*. Para essa espécie, a frequência de basidiósporos por basídio é primariamente determinada pelo loco BSN, ligado ao loco *MAT* no cromossomo I (XU; HORGEN; ANDERSON, 1993). Os alelos *Bsn-t* e *Bsn-b* conferem o fenótipo com predominância de basídios tetraspóricos e bispóricos, respectivamente. O alelo *Bsn-t* é dominante em relação ao *Bsn-b* (CALLAC et al., 1998).

Em muitos basidiomicetos, a meiose é seguida por uma mitose, que pode ocorrer em diferentes locais. Quando ocorre no interior do basídio e os oito núcleos resultantes migram para os esporos, são gerados esporos maduros binucleados (Figura 4B). Quando a mitose ocorre depois que os núcelos migram para os basidiósporos, são gerados esporos binucleados, porém, homocarióticos (Figura 4C). E quando a mitose ocorre ainda no interior do basídio, porém

apenas quatro dos oito núcleos migram para os esporos e os remanescentes são degradados, são gerados esporos uninucleados (Figura 4D). Há, ainda, relato na literatura, de mitose pós-meiótica na base ou no interior dos esterigmas (não esquematizado na Figura) (WEBSTER; WEBER, 2007).

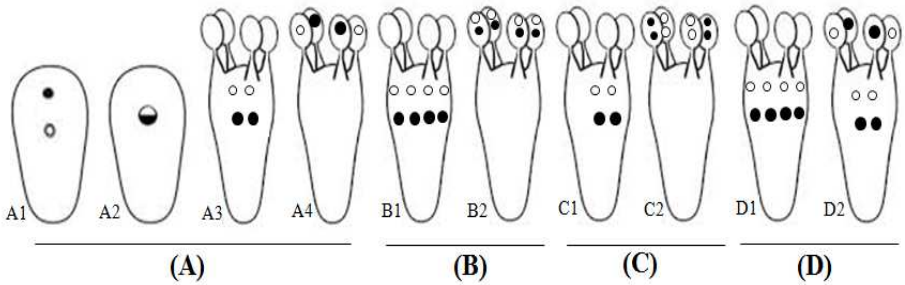

Figura 4 Desenho esquemático de basidiosporogênese, fundamentado por Dias et al. (2008), Thongklang et al. (2014) e Webster e Weber (2007). (A) A1: estagio dicariótico, A2: cariogamia, A3: meiose, A4: migração dos núcleos para os basidiósporos; (B) B1: mitose pós-meiótica ocorrendo no interior do basídio, B2: migração dos oito núcleos para o interior dos basidiósporos; (C) mitose pós-meiótica ocorrendo no interior dos basidiósporos e (D) D1: mitose pós-meiótica ocorrendo no interior do basídio, D2: Migração de apenas quatro dos oito núcleos e degeneração dos remanescentes

Em *A. subrufescens* já foram reportadas mitoses pós-meióticas. Dias et al. (2008) sugerem a ocorrência de mitose pós-meiótica no interior dos basidiósporos e um modelo reprodutivo primariamente heterotálico. Herrera et al. (2012), após observarem a ocorrência de basídios bispóricos e trispóricos em todas as linhagens estudadas, sugeriram que esta espécie seja anfitálica, uma vez poderia resultar na formação de esporos heterocarióticos autoférteis.

Vários mecanismos de empacotamento de núcleos para o interior dos esporos têm sido propostos, quando a migração é realizada em pares. No modelo de empacotamento nuclear proposto para *A. bisporus*, quatro células filhas resultantes da meiose são empacotadas em pares, com a predominância de emparelhamento de núcleos não "irmãos” no mesmo esporo (KERRIGAN et al.,

1994; RAPER; RAPER; MILLER, 1972; SUMMERBELL et al., 1989). Nesse modelo, a migração do núcleo pós-meiótico para dentro do esporo ocorre de forma não aleatória e a expectativa para esse modelo é a de que a maioria dos basidiósporos apresente dois núcleos e seus genótipos sejam altamente heterozigóticos. O modelo de migração para *A. subrufescens* difere do modelo não aleatório de *A. bisporus*, uma vez que, por meio de análises genéticas, Thongklang et al. (2014) caracterizaram três principais categorias de esporos (Figura 5), que são: os heterocarióticos, que recebem núcleos "irmãos", denominados *Sister Nuclei Pair Progeny* (SNPP); os que recebem núcleos não "irmãos", denominados *Non Sister Nuclei Pair Progeny* (NSNPP) e os homocarióticos, definidos resumidamente a seguir.

a) NSNPP: heterocárions que são reprodutivamente competentes, conhecidos por terem ciclo de vida pseudo-homotálico;
b) SNPPs: heterocárions que apresentam genótipos homoalélicos em regiões ligadas ao *Mating type* loco, embora sejam heteroalélicos em regiões mais distantes. Com isso, teoricamente, não são reprodutivamente competentes;
c) Homocárions: são esporos originados através de migração envolvendo dois núcleos geneticamente iguais.

A presença de esporos homocarióticos e heterocarióticos (SNPP e NSNPP) encontrados por Thongklang et al. (2014), confirma, mais uma vez, que o ciclo de vida de *A. subrufescens* é anfitálico. Estudos comportamentais desses diferentes tipos de esporos podem proporcionar avanços em programas de melhoramento genético, ao proporem que os mesmos sejam utilizados em testes de cruzamento, especialmente em relação aos esporos heterocarióticos SNPP, nunca anteriormente estudados.

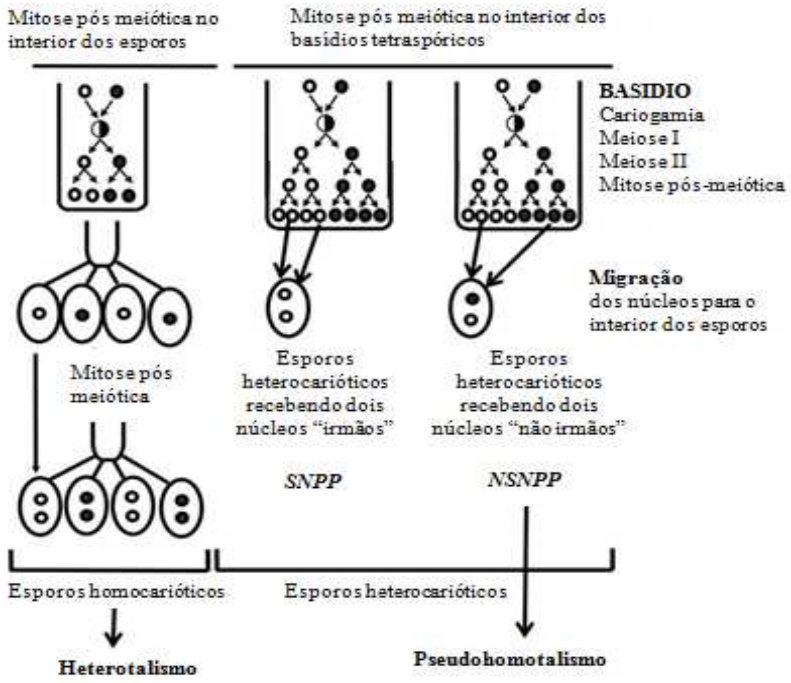

Figura 5 Categorias de esporos. Homocaritótico; SNPP = heterocariótico *Sister Nuclei Pair Progeny*; NSNPP = heterocariótico *Non-Sister Nuclei Pair Progeny*
Fonte: Thongklang et al. (2014)

2.5 Fenômeno Buller (= *Di-Mon mating*)

O cruzamento entre micélios compatíveis monocárion x monocárion é bem conhecido por sua capacidade de originar heterocárions férteis. Contudo, um famoso micologista canadense, Buller (1931), contribuiu significativamente para um entendimento ainda mais aprofundado da sexualidade em basidiomicetos. Ele encontrou dicárions com capacidade de fertilização de monocárions em *Corpeinus lagopus*, resultando em um novo dicárion. Este processo foi cunhado como fenômeno Buller, por Quintanilha (1937). Posteriormente, Papazian (1950) referiu-se a esse tipo de cruzamento como *di-*

mon mating. O cruzamento *di-mon* já foi observado em muitas espécies de fungos, em condições laboratoriais e é, geralmente, acompanhado por migração nuclear do heterocariótico para o homocariótico, que se torna heterocariótico (CALLAC et al., 2006).

O tipo de sistema *mating type* é um fator importante nas interações genéticas entre monocários e dicários. Quando um ou dois núcleos de um dicárion é compatível com o núcleo do monocárion, a dicariotização do monocárion ocorre prontamente (HEITEMAN et al., 2007). Dois tipos legítimos de combinações *di-mon mating* são possíveis. São eles: (i) o monocárion é compatível com todos os núcleos do dicárion (ex.: bipolar (A1+A2) x A3) ou tetrapolar (A1B1+A2B2) x A3B3) e (ii) em combinações de semicompatibilidade, em que o monocárion é compatível somente com um dos núcleos do dicárion (ex.: bipolar (A1+A2) x A1) ou tetrapolar (A1B1+A2B2) x A2B2). Combinações incompatíveis são encontradas em espécies tetrapolares, em que o monocárion não é compatível com nenhum dos núcleos do dicárion (ex.: (A1B1 + A2B2) x A1B2 ou A2B1) (WEBSTER; WEBER, 2007).

Como, no gênero *Agaricus*, as espécies até então estudadas são bipolares e multialélicas, o fenômeno Buller é legítimo em todos os tipos de combinação. Em *A. bisporus* esta via de cruzamento já foi reportada em experimentos laboratoriais *in vitro* e em substratos simultaneamente inoculados com esporos e micélio (CALLAC et al., 2006). Esse fenômeno ainda não foi reproduzido em *A. subrufescens*, porém, o desenvolvimento desse método é uma estratégia promissora que pode ser utilizada em estudos genéticos e programas de melhoramento (CALLAC, 2008).

2.6 Marcador molecular para distinção entre esporos homocários e heterocários

A distinção entre os diferentes tipos de esporos (homocários e heterocários) pode ser realizada a partir de heteromorfismos no DNA da estirpe parental, particularmente comum em certas espécies de *Agaricus* (FOULONGNE-ORIOL, 2012).

O sequenciamento de segmentos do DNA é analisado por meio de eletroferogramas, nos quais picos duplos correspondem a heteromorfismos (Figura 5). Um baixo nível de heteromorfismo é claramente um fator limitante para o desenvolvimento de marcadores moleculares operacionais (FOULONGNE-ORIOL, 2012).

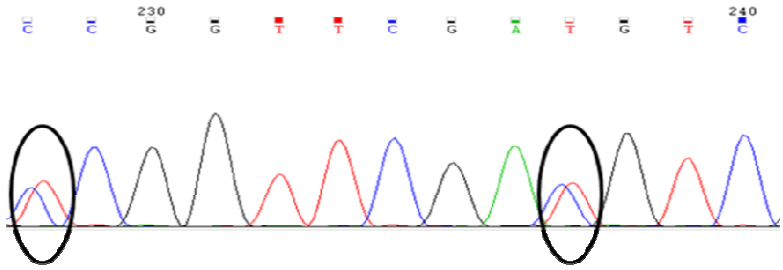

Figura 5 Heteromorfismo em duas regiões no eletroferograma resultante de um sequenciamento

O marcador CAPS, do inglês *cleaved amplified polymorphic sequences* ou sequência polimórfica amplificada e clivada, pode ser utilizado para acessar a diferença entre esporos homocarióticos e heterocarióticos, e já foi empregado especialmente para algumas espécies de *Agaricus* (THONGKLANG et al., 2014). CAPS são fragmentos de DNA amplificados via PCR utilizando *primers* específicos (20 a 30 pb), seguido da digestão com endonucleases de restrição. São marcadores codominantes que exploram posições polimórficas detectadas

nas sequências de DNA, e a base genética do polimorfismo revelado pelo marcador CAPS é a presença/ausência de sítios de restrição da sequência amplificada. Portanto, trata-se de uma técnica de alta reprodutibilidade, com a desvantagem da necessidade de conhecimento prévio da sequência de DNA para a construção de *primers* (WILLIAMS et al., 1990). Além de acessar o nível de ploidia, os marcadores CAPS foram bastante empregados na construção de mapas genéticos de *A. bisporus* (FOULONGNE-ORIOL, 2012; FOULONGNE-ORIOL et al., 2010, 2011).

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

SPORE BEHAVIORS REVEAL A CATEGORY OF MATING-COMPETENT HETEROKARYONS IN THE AMPHITHALLIC MEDICINAL FUNGUS *Agaricus subrufescens*

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Abstract: *Agaricus subrufescens* is an edible and medicinal mushroom with a unifactorial system of incompatibility and an amphithallic life cycle in which meiospores give rise two three types of mycelia: homokaryons and heterokaryons which are either non-sister nuclear pair progenies (NSNPPs) or sister nuclear pair progenies (SNPPs). NSNPPs are classical fertile heterokaryons; SNPPs are unconventional heterokaryons which are homoallelic at the mating type locus which is tightly linked to a centromere. The objective was to characterize these types of spores, particularly their mycelial growth rate and their ability to cross and to fruit. A large group of offspring (225 single spore isolates) of the Brazilian strain WC837 was analyzed with eight codominant molecular markers. Fifty percent of the spores were heterokaryotic and among them about one quarter were SNPPs. The rate of homoallelism (i.e. the loss of parental heterozygozity) at centromere-unlinked markers was consistently 50% in both SNPPs and NSNPPs. The mycelial growth rate of the NSNPPs was significantly higher than for the SNPPs and the homokaryons. Only NSNPPs inconsistently fruited. Both NSNPPs and SNPPs crossed with homokaryons and interestingly SNPPs bearing different mating type alleles crossed between each other and restored fertile heterokaryons. The interest of these genetically inbred heterokaryons lies in their ability to cross; however through this process, gametic selection is bypassed. We propose a simplified strategy of crossbreeding for strains having this type of amphithallism in which a single centromere-linked marker could be used to discard the NSNPPs while the mating-competent SNPPs would be treated as homokaryons.

Keywords: Mushroom Pseudohomothallism Buller phenomenon Life cycle Breeding program

Introduction

Outcrossing and recombination generate variability which is required for adaptation of fungi to their environment in natural populations and contributes to their evolution. Knowing how outcrossing occurs in a given species contributes not only to a better understanding of how reproductive strategies can impact the natural population structures but also of how to control hybridization between strains, for example in selective breeding programs for agronomic or technological purposes. Most fungal species of the phylum Basidiomycota have sexual reproduction with various reproductive strategies and life cycles (heterothallism, homothallism, and pseudohomothallism, also called secondary homothallism). Outcrossing or selfing may occur via the Buller phenomenon i.e. between a homokaryon [*n*] and a heterokaryon [*n*+*n*] (Buller 1931) or more classically between homokaryons via heterothallism: meiospores give rise to infertile homokaryotic mycelia (*n*) then plasmogamy between sexually compatible homokaryons restores a fertile heterokaryon [*n*+*n*]. In contrast, in pseudohomothallism two postmeiotic nuclei are paired in each spore. Such spores generally give rise to fertile heterokaryons. Amphithallism refers to the production of both homokaryotic and heterokaryotic spores in the same sporocarp, thus encompassing both heterothallism and pseudohomothallism (Lange 1952; Kuhner 1977). Amphithallism is not rare. For instance, of approximately 500 species of Agaricales about 8% were considered amphithallic (Lamoure 1989). This percentage is certainly an underestimate because it was mainly based on morphological observations of a high percentage of bisporic basidia.

Amphithallism is known in bisporic species such as *Agaricus bisporus* var. *bisporus* (Lange) Imbach, the button mushroom (Raper et al. 1972), but can also occur when basidia are tetrasporic. For example, in *Mycocalia denudata*

(Burnett and Boulter 1963), post-meiosis mitotic division (a 'third division') occurs in the basidia. *Agaricus subrufescens* Peck, the almond mushroom, was also found to be tetrasporic and amphithallic in the three strains recently studied by Thongklang et al. (2014).

The almond mushroom is cultivated in the Americas (mainly in Brazil) and in Asia (mainly China, Japan and Taiwan). It is popularly known as ABM (for *A. blazei* Murrill [sensu Heinemann]), Royal Sun *Agaricus*, Cogumelo do Sol or Sun Mushroom in Brazil, Himematsutake in Japan, and Jisongrong or Baximogu in China (Parra 2013; Wisitrassameewong et al. 2012b). Even though *Agaricus subrufescens* and *Agaricus bisporus* belong to the distinctly different *Agaricus* sections *Arvenses* and *Bivelares* respectively, both are secondary decomposers and can be cultivated on similar substrates (Largeteau et al. 2011; Llarena-Hernández et al. 2013). *Agaricus subrufescens* is worthy of study not only because it is a species of medicinal and nutritional interest but also because it is the *Agaricus* species having the broadest climatic and geographical range. Indeed, it was initially described from the USA in 1893 by Peck (Peck 1893), then under different names in Brazil and in Europe. Its main synonyms are *A. blazei* Murrill sensu Heinemann (misapplied), *A. brasiliensis* Wasser**,** M. Didukh, Amazonas & Stamets (illegitimate) and *A. rufotegulis* Nauta (Parra 2013; Wisitrassameewong et al. 2012b). Moreover, it has also been formally reported from Oceania (Peterson et al. 2000; Kerrigan 2005) and Asia (Zhao et al. 2011; Wisitrassameewong 2012a; Gui et al. 2014). Interfertility between isolates from South America, Europe and Asia has been recently demonstrated (Thongklang et al 2014), indicating that a broad genetic base is available for the genetic improvement of this species.

The reproductive strategies and life cycle of this species of interest are distinctive and qualify it as a model for studies on how outcrossing may occur in Basidiomycota. Thongklang et al. (2014) showed that *A. subrufescens*, like *A.*

bisporus var. *bisporus*, has a unifactorial system of sexual incompatibility with a mating type locus (*MAT*) tightly linked to a centromere. However, amphithallism in *A. subrufescens* differs from *A. bisporus* var. *bisporus* not only by its tetrasporic basidia but also by a higher rate of crossovers and, in certain strains such as WC867, by the fact that postmeiotic nuclei paired in a spore may carry the same mating type allele. As a result three primary categories of single spore isolates (SSIs) were considered by Thongklang et al. (2014):

- Non-sister nuclear pair progeny (NSNPP) heterokaryons: in *Agaricus*, the mating type locus *MAT* is linked to a centromere, therefore virtually all NSNPPs are reproductively competent 'self-fertile' sexual heterokaryons. These are the familiar heterokaryons of the pseudohomothallic life cycle allowing uniparental reproduction.

- Sister nuclear pair progeny (SNPP) heterokaryons: These are 'unconventional' heterokaryons having a pair of 'sister' nuclei arising from the same second meiotic division, and therefore having highly homoallelic genotypes in tightly centromeric-linked regions. They are theoretically not reproductively competent although they can be heteroallelic in regions distal to the centromeres. SNPPs had not been previously considered in classical concepts of amphithallism.

- Homokaryons are haploid cultures that can arise from spores in two different ways. In the traditional scenario, homokaryotic spores receive only one postmeiotic nucleus. However, in amphithallism involving nuclear pairs, some spores may receive two identical nuclei from one postmeiotic division, and these are also homokaryons.

The objective of the present work is to characterize the behavior of these three types of spores, particularly their mycelial growth rate and their ability to cross and to fruit, and finally to propose a simplified strategy to cross strains having this type of amphithallism. A large group of offspring (225 SSIs) was

analyzed with eight single-locus co-dominant cleaved amplified polymorphic sequence (CAPS) markers for characterizing NSNPPs, SNPPs and homokaryons. Then we compared the three types of SSIs in mating tests, mycelial growth rate tests and fruiting tests. To our knowledge SNPPs have never been studied previously. The functional role of each type of spore in the wild is discussed and consequences for breeding strategies in this cultivated species are examined.

Materials and methods

Parental strain and single spore isolates

We used the same strain, WC837, as Thongklang et al. (2014). This strain, provided by PSUMCC (USA), originated in Brazil and a subculture (CA454) is available in the INRA collection (CGAB). The strain WC837 was cultivated under standard conditions for sporocarp production as described by Llarena-Hernández et al. (2014). Scoring of basidia of a fresh sporocarp in one- to four-spored classes was performed using light microscopy as described by Callac et al. (1993) and a sporeprint was obtained from the same sporocarp. For germination, spore suspensions were prepared in a saline solution (NaCl 0.85 %) with trace of Tween80. Spore counts were estimated using a Malassez cell. One hundred microliters of the spore suspension were spread over 90-mm-diameter Petri dishes on complete yeast medium (Raper et al. 1972). Plates were placed upside down with the addition of grains colonized by *A. bisporus* mycelium in the lid (a germination stimulus; Rast and Stauble 1970) for one week. After germination, SSIs were subcultured on compost extract medium (aqueous extract of pasteurized commercial mushroom compost plus 1% glucose and 2% agar).

Choice of informative markers

EST sequences of *A. subrufescens* identified by Foulongne-Oriol et al (2014) with putative homologs in the genome of *A. bisporus* were selected. Information about their physical position on the *A. bisporus* genome was used to develop informative molecular markers for the distinction between the three expected types of spore among the WC837 SSIs (Thongklang et al. 2014). First, we selected *A. subrufescens*-identified sequences for which *A. bisporus* homologs were close to *MAT* and the centromere (RPB2 and PRS088 markers). Secondly, we chose two other *A. subrufescens* sequences with *A. bisporus* homologs located on chromosome I in pericentromeric (PRS113 marker) and distal positions (PRS095 marker). Thirdly, four other *A. subrufescens* sequences (PRS003, PRS016, PRS160 and ITS markers), for which *A. bisporus* homologs were found in distal positions on chromosomes IV, X, VII and IX were also used (Table 1).

Table 1 Characteristics of *A. subrufescens* markers used for WC837 SSIs type identification

marker	Seq ID *A. subrufescens*[(a)]	Homologs in *A. bisporus* (GM) V2.0 [(a,b)]	Genome position *A. bisporus* V2.0 (chromosome/scaffold - coordinates)[a]
PRS95	sp_isotig01625[b]	189140	I/1 - 12142-14030
PRS113	cl_GSIH7AY04H0M2U[b]	147275	I/1 - 373143-373641
PRS88	cl_isotig00274[b]	62238	I/1 - 850846-853302
Rpb2	KJ541801/KJ541802[c]	113824	I/1 - 869574-873431
PRS003	cl_isotig01560[b]	195831	IV/14 - 493735-495750
PRS016	cl_isotig01118[b]	194648	X/9 - 1326408-1327402
PRS160	cl_GSIH7AY04I5DEO[b]	193525	VII/7 - 232331-232917
ITS	KJ541796/KJ541796[c]	#	IX/29 – rDNA

[a]Morin et al., 2012; [b]Foulongne-Oriol et al., 2014; [c]Thongklang et al., 2014

Method based on homo/heteroallelism to classify SSIs in three categories

We used codominant single-locus markers that were heteroallelic in the parental strain. Heterokaryotic and homokaryotic SSIs were first distinguished as follows: SSIs heteroallelic at least at one of the loci were unambiguously heterokaryons (either NSNPPs or SNPPs). SSIs homoallelic at all loci were regarded as putative homokaryons. This classification was performed with several genetically independent markers (genetically unlinked loci), to avoid misinterpreting a heterokaryon as a homokaryon when it was homoallellic at any of these loci. For each of the genetically independent markers, the frequency of homoallelic SSIs was estimated from the experimental data. The probability of misinterpretation of a heterokaryon as a homokaryon was the product of these frequencies for all the markers used. Such a multilocus genotype test has been used in *A. bisporus* by Kerrigan et al. (1992, 1993, 1994) and in *A. subrufescens* by Thongklang et al. (2014).

Among the heterokarotic SSIs, NSNPPs are expected to be heteroallelic at loci tightly linked to centromeres while SNPPs are homoallelic. We used markers linked to the *MAT* locus which is tightly linked to the centromere (Xu et al. 1993). Homoallelic and heteroallelic SSIs at such loci were considered as putative SNPPs and NSNPPs respectively. The reliability of this method depends on the rates of crossovers between these markers and *MAT* and thus the centromere. Such rates can be estimated through the analysis of the homokaryotic offspring.

Molecular markers genotyping

CAPS markers were previously used for mapping in *Agaricus bisporus* (Callac et al. 1997; Foulongne-Oriol et al. 2010) and to study the life cycle of *A. subrufescens* (Thongklang et al. 2014). CAPS markers exploit heteromorphic positions detected in the sequences; informative loci are heteromorphisms which are included in the restriction sites of restriction endonucleases (Table 2). When the restriction site differed from the sequence, derived CAPS (dCAPS) markers were developed (Neff et al. 1998).

DNA extraction was done as described by Zhao et al. (2011). PCR was performed in a 30 µL reaction mixture containing 50 ng genomic DNA, 0.5 µM of each primer, 200 µM dNTPs, 2 µg BSA, 1 U Taq DNA Polymerase and 1x incubation buffer. Amplifications were carried out as follows: an initial denaturing step at 94 °C for 5 min; 35 cycles of 94°C for 1 min, 55°C for 90s, 72°C for 1 min; and a final extension at 72 °C for 5 min. The amplified region was sequenced by Beckman Coulter Genomics Inc. (England). Restriction enzymes recognizing a polymorphic position of the sequence were selected. For the cleavage reaction, 5 µL of the PCR product were digested with 2 U of the appropriate restriction enzyme for 120 min at the optimal incubation temperature recommended by the manufacturer. CAPS products were visualized in 2% agarose gels, running at 90 V for 90 min. Selected primer sequences and matching restriction enzymes for each marker are listed on Table 2. Loci were named as the sequenced DNA segment followed by the heteromorphic position in the amplified sequence. All the markers are heteroallelic in the parental strain W837. We analyzed all 225 SSIs in the same conditions as described above.

Table 2 CAPS markers used to study allelic segregation of the WC837

Sequenced DNA fragment and primers (5'-3')	Locus[a] Endonuclease Restriction site	Genotypes	Phenotypes[b] (fragment size in pb)
ITS1+5.8S+ITS2	*ITS:200*	*1* or *1/1*	**773**
ITS5:GGAAGTAAAAGTCGTAACAAGG	*Hind*III	*2* or *2/2*	**554+219**
ITS4: TCCTCCGCTTATTGATATGC	gtgaAA**R**CTTgct	*1/2*	**773+554+219**
Rpb2:	*Rpb2:715*	*1* or *1/1*	**617**+274+142+119
5F: GATGATCGTGATCATTTCGG	*Tsp*509I	*2* or *2/2*	**403**+274+**198**+142+119
7R: ACYTGRTTRTGRTCRGGGAAV	aaggA**R**TTgcga	*1/2*	**617+403**+274+**198**+142+119
PRS3:	*PRS3:212*	*1* or *1/1*	**678**
3F: CCCAAAGATTTCTCCAACCA	*Dde*I	*2* or *2/2*	**446+232**
3R: AAATCCCAACTTTGCGTCAC	aataC**Y**CAGcatc	*1/2*	**678+446+232**
PRS16:	*PRS16:1*	*1* or *1/1*	**464**
16F:CAGCAGTCTTGACAATGCTGTG	*Sac*I	*2* or *2/2*	**424**+40
GCTTCCGTGAGTCGAGCT(dCAPS[c])	agtcGAGCT**Y**tagt		
16R:CCGTCAAGGTCCTCAGTGAT	(dCAPS[c])	*1/2*	**464+424**+40
PRS88:	*PRS88:248*	*1* or *1/1*	**764**
88F :CTCGCAATTAGCTTCCAAGG	*Aat*II	*2* or *2/2*	**491+273**
88R :CGGTTGTCCAAGATCAAGGT	gttcGA**Y**GTcgac	*1/2*	**764+491+273**
PRS95:	*PRS95:266*	*1* or *1/1*	**380**+61
95F :CGCAACTTGAATAACGCTCA	*Hinf*I	*2* or *2/2*	**287+154**+61
95R :TATGCGCGAGATTACGACTG	agggg**R**AATCctgt	*1/2*	**380+287+154**+61
PRS113:	*PRS113:158*	*1* or *1/1*	**426**
113F :TAGTTTAGGGCGCATCAACC	*Bts*I	*2* or *2/2*	**238+188**
113R :CCTCCAACCAACACTCATCC	gaagGC**R**GTGAGttgg	*1/2*	**426+238+188**
PRS160:	*PRS160:134*	*1* or *1/1*	**524**
160F: CACTGAACGTGACCTGGAGA	*Ear*I	*2* or *2/2*	**365+159**
160R: AGGGTTTTCGGATGACATTG	tcgcCTCT**Y**CCctta	*1/2*	**524+365+159**

[a] Each locus is designed by name of the sequenced DNA fragment followed by its position on the amplified sequence (number of nucleotides after the forward primer); sequences including the recognition site (in capital) and heteromorphisms (in bold type) are indicated

[b] Fragments giving major characteristic bands in electrophoresis gel are in bold type

[c] derived CAPS primer: the underlined nucleotide C is in fact T in the sequence of WC837

Linkage map and Mendelian segregation analyses

To test genetic independence, contingency chi-square tests were performed for all pairwise combinations between the loci used. For the linked loci, their order and genetic distances were computed using MAPMAKER/EXP V3.0b software (Lander et al. 1987). The recombination rate was transformed into map distance (centimorgan cM) using the Kosambi function. For each marker, the hypothesis of the Mendelian allelic segregation ratio of 1:1 was tested among the homokaryotic offspring using chi-square tests.

Mating test and hybridizations

Mating tests were performed according to Kerrigan et al. (1994). *A. subrufescens* has a unifactorial system of incompatibility: two homokaryons give a positive reaction only when they bear different alleles (*Mat-1* and *Mat-2*) at the mating type locus *MAT*. The presence of fluffy, vigorous mycelium at the junction zone between the two mycelia characterizes a positive mating reaction. The test was intended to determine the mating type alleles of the SSIs and ultimately to estimate the linkage relationships between *MAT* and other markers used. A preliminary mating test was performed to identify homokaryotic strains with different mating type alleles. We paired all the homokaryotic SSIs and most of the heterokaryons SSIs (NSNPPs or SNPPs) with four testers previously selected (two *Mat-1* and two *Mat-2*), in duplicate.

Furthermore, mating tests between SNPPs bearing different mating type genotypes (*Mat-1/1* or *Mat-2/2*) were performed, in triplicate. When required, the hybrid mycelium was isolated on a compost-agar medium. Confirmation of hybridization was achieved using a marker tightly linked to *MAT* for evidence of heteroallelism and consequently the restoration of fertility (*Mat-1/2*). However if

two SNPP heterokaryons crossed, we assumed that the resulting 'hybrid' heterokaryon would receive one nucleus of each parental heterokaryon and therefore, four different hybrids were possible. To reveal whether the nuclei randomly assembled in the hybrid, we tested pairs of SNPPs having the genotypes *a1/a2 b1/b1* and *a1/a1 b1/b2* at two loci A and B, and then compared the genotypes of several hybrids isolated from matings between the same SNPPs. In this condition, one SNPP parent contained the nuclei *a1b1* and *a2b1* and the other contained the nuclei *a1b1* and *a1b2*. Consequently the four possible hybrids had four different genotypes: they were homoallelic respectively either at A, at B, at both loci, or at neither.

Mycelial growth rate test

Agar plugs of 4 mm diameter were transferred to Petri dishes (90 mm diameter) containing 1 % malt agar medium and incubated at 28 °C for 15 d. Colony diameters were measured on two perpendicular axes. These data were used to score the rate of the mycelial growth with two replicate plates for each SSI in a completely randomized experimental design. Mycelial growth rate was compared between each type of SSI using variance analysis and post-hoc Duncan's test applied for multiple means comparison.

Fruiting test

Strains were cultivated in plastic trays filled with 8 kg of conventional compost under standard conditions of cultivation as described by Llarena-Hernández et al. (2014). Forty five plastic trays were inoculated with the parental strain WC837, homokaryons (four strains, two replicates), NSNPPs (nine strains, two replicates), and SNPPs (nine strains, two replicates).

Results

Classification of the SSIs: homokaryons, NSNPPs, and SNPPs

Bisporic basidia were not detected and trisporic basidia were rare (less than 1 %) among basidia of the parental sporocarp. After incubation for 14 d, the rate of germination was estimated as 10% for a density of approximatively 1,000 spores spread on agar medium in a 90 mm diameter Petri dish. Three hundred and forty germinating spores were isolated from the same sporeprint, however many of them stopped growing and only 225 SSIs exhibited sufficient mycelial growth to be used.

SSIs were classified based on homo-/hetero-allelism at eight CAPS markers (Tables 3 and S1). Among 225 SSIs, 49.78% (112/225) were heteroallelic at least at one of the eight CAPS markers used and were therefore confirmed heterokaryons. The remaining SSIs were putative homokaryons with a high level of confidence. The rates of homoallelism among the heterokaryotic offspring, which are indicated for each locus in Table 3, represent the loss of the parental heterozygosity at these loci. For the five loci *PRS088:248*, *PRS095:266*, *PRS003:212*, *PRS016:1* and *PRS160:134* which are genetically independent from each other (= unlinked loci with alleles segregating independently), the probability that a heterokaryon should be homoallelic at all five loci and therefore misinterpreted as a homokaryon is the product of the five rates of homoallelism: $p = 31/112 \times 57/111 \times 55/111 \times 58/110 \times 58/111 = 0.019$. However it can be noticed that in fact, using only these five markers, only 111 heterokaryotic SSIs were detected since a single SSI found to be homoallelic at these five markers was heteroallelic at one of the three remaining loci used (SSI WC837-290 was heteroallelic only at locus *PRS113:158*). Finally by estimation we would expect $111/ (1-p) = 113$ heterokaryons. This is only one more than the

112 confirmed heterokaryons that we found. The final percentage of heterokaryons should be 50.22 % (113/125) i.e. close to 50%.

Among the heterokaryons, 21% (24/112) were likely SNPPs because they were homoallelic at both loci *RPB2:715* and *PRS088:248* tightly linked to the centromere and the *MAT* locus, while 67% (75/112) were likely NSNPPs because they were heteroallelic at these two loci. Twelve percent (13/112) of the heterokaryons that were homoallelic at only one of these two loci remained unclassified. Possibly, following a crossover between *RPB2:715* and *PRS088:248,* they received both one recombinant and one non-recombinant nucleus; theoretically they could be either SNPPs or NSNPPs with equal probability. Without these 13 unclassified SSIs, the rate of SNPPs was estimated as 24 % (24/99) among the heterokaryons. Taking them into account with the half considered as SNPPs this rate should be 27 % (30.5/112).

Table 3 Rate of homoallellism and allelic distribution at nine loci for 225 SSIs of the strain WC837 of *Agaricus subrufescens*

Loci	Complete offspring (225 SSIs)			Heterokaryotic offspring (99 SSIs)[a]								
	Homokaryons (113 SSIs)		112 SSIs)	Heterokaryons (75 SSIs) Homoallelism:			NSNPPs (24 SSIs) Homoallelism:			SNPPs Homoallelism:		
	Genotypes		Chisquare									
	1	*2*		Rate	*1/1*	*2/2*	Rate	*1/1*	*2/2*	Rate	*1/1*	*2/2*
MAT[b]	59	26	12.81*	Non-testable			Non-testable			16/16	11	5
RPB2:715	73	37	11.78*	29/109	16	13	0/73	0	0	23/23	15	8
PRS88:248	75	38	12.11*	31/112	23	8	0/75	0	0	24/24	16	8
PRS113:158	77	34	16.51*	35/112	28	7	17/73	16	1	13/24	10	3
PRS95:266	83	30	24.86*	57/111	47	10	36/74	31	5	12/24	10	2
PRS3:212	45	66	3.97*	55/111	21	34	36/74	12	24	12/24	5	7
PRS16:1	64	45	3.31	58/110	27	31	37/74	16	21	12/23	7	5
PRS160:134	54	59	0.22	58/111	30	28	38/74	18	20	11/24	6	5
ITS:200	50	55	0.24	62/104	25	37	38/72	16	22	13/20	6	7

* Mendelian segregation rejected (df = 1, $p < 0.05$)

[a] Only 99 of the 112 SSIs are considered. The 13 remaining heterokaryotic SSIs remain unclassified because they are homoallelic at only one of the two centromere-linked loci *RPB2:715* and *PRS88:248*.

[b] Deduced from mating tests

Mating tests between SSIs and tester homokaryons

In all the types of mating tests (homokaryon × homokaryon, homokaryon × SNPP, and homokaryon × NSNPP), positive and negative mating reactions were observed (Fig. 1). In positive reactions, more or less fluffy mycelium emerged from one or several points on the junction line between the two mycelia. The new putative hybrid mycelium often formed a new circular and vigorous colony overlapping its parent mycelia. Negative reactions were characterized by the absence of fluffy mycelium in the contact zone. Moreover the contact zone remained often poorly invaded, sometimes exhibiting a solid white line (Fig. 1d). With two replicates for each mating test, it was not rare to observe a positive reaction in only one of the two replicates; false negative reactions are common in *A. bisporus* (Kerrigan et al. 1994) as in some species of *Agaricus* section *Arvenses* to which *A. subrufescens* belongs (Calvo-Bado et al. 2000).

In mating tests between four tester homokaryons and the 113 remaining putative homokaryons identified with the CAPS markers, positive mating reactions were obtained for 75 % of them (Table 4) giving an unbalanced segregation ratio of 59 *Mat-1* : 26 *Mat-2*. These two alleles co-segregated completely with those of *PRS088:271* (0 recombinants) and almost completely with those of *RPB2:715* (2 recombinants).

In mating tests between four tester homokaryons and 66 putative NSNPP heterokaryons heteroallelic at the *MAT* locus, positive reactions were observed for 20 of them (30%): 13 had a positive reaction with tester(s) *Mat-1*, six with tester(s) *Mat-2*, and only one with two testers bearing either *Mat-1* or *Mat-2* allele. Such positive mating reactions between homokaryons and heterokaryons (the Buller phenomenon) are not rare in Basidiomycota (Buller 1931; Quintanilha 1937; Raper et al 1972; Callac et al 2006).

In mating tests between four tester homokaryons and 20 putative SNPP heterokaryons (homoallelic at the *MAT* locus), positive reactions were observed for 16 of them (80%). The data completely agreed with the expected homoallelic genotypes *Mat-1/1* and *Mat-2/2* of these SNPPs which gave positive reactions with *Mat-2* and *Mat-1* tester strains respectively. Finally in this limited sample, there was no evidence of recombination between the tightly linked loci *MAT*, *RPB:175* and *PRS088:248*: 11 had the genotype *Mat-1/1 Rpb2:175-1/1 PRS088:248-1/1* and five had the genotype *Mat-2/2 Rpb2:715-2/2 PRS088:248-2/2*. It is noteworthy that this unbalanced ratio 11 (*Mat-1/1*) / 5 (*Mat-2/2*) = 2.20 is similar to the ratio 59 (*Mat-1*) / 26 (*Mat-2*) = 2.27 observed among the homokaryons. It was expected that SNPPs would behave like homokaryons in mating tests but this had previously never been formally shown.

Table 4 Success in mating tests

Category of SSIs	Tested SSIs	Positive reactions[a]
Homokaryons	113	85 (75.2 %)
SNPP heterokaryons	20	16 (80 %)
NSNPP heterokaryons	66	20 (30.3 %)

[a] Mating tests with four testers (two *Mat-1* and two *Mat-2*) and two replicates. A reaction was positive when it was observed in at least one of the eight plates

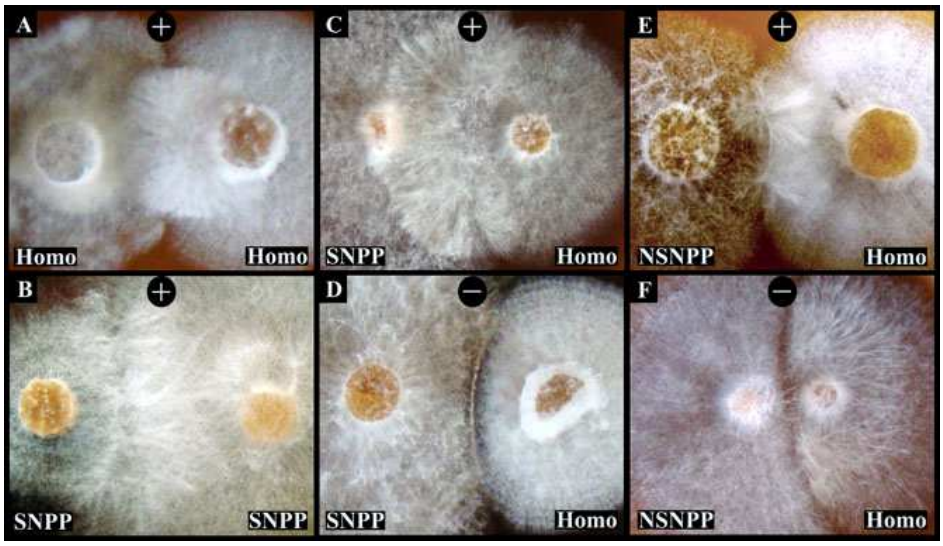

Figure 1 Positive and negative reactions in mating tests. A, positive between homokaryons (WC837-309, WC837-145). B, positive between SNPPs (WC837-142, WC837-18), C and D, between SNPP and homokaryon positive (WC837-142, WC837-20) and negative (WC837-142, WC837-145) respectively. E and F, between NSNPP and homokarons positive (WC837-97, WC837-145) and negative (WC837-121, WC837-20) respectively

Single locus segregation ratios

Chi-square value for Mendelian segregation (df = 1, p = 0.05) tests among the homokaryotic offspring are reported in Table 3. The 1:1 segregation was rejected for all the loci of the large linkage group (see next paragraph: *MAT, RPB2:715, PRS088:248, PRS113:158, PRS095:266*) and for *PRS003:212.* For the large linkage group we note that it was always allele *2* of each locus that belonged to the same parental chromosome and that was always the under-represented allele.

Although the number of identified SNPPs was low it must be noted that similar unbalanced segregations among the postmeiotic nuclei having migrated

in these heterokaryotic spores were observed since the genotype *1/1* was much more frequent than the genotype *2/2* at all five loci of the large linkage group listed above (11:5, 15:8, 16:8, 10:3, and 10:2 respectively).

Pairwise segregation ratios, linkage map, and synteny with *A. bisporus*

Using chi square contingency tests for nine loci, genetic independence was rejected ($p<0.001$, 1 *df*) for the following eight pairs of loci among the 36 tested pairs (Tables S2 and S3): *ITS* and *PRS160:134*, *PRS113:158* and *PRS095:266*, and all the pairwise combinations between the four loci *PRS113:158*, *PRS088:248*, *RPB2:715* and MAT. Therefore, there were two linkage groups: a large one including five loci (*MAT, RPB2:715, PRS088:248, PRS113:158, PRS095:266*) and a small one (*ITS:200*, *PRS160:134*).

The linkage map obtained by the maximum-likelihood method implemented in Mapmaker (Fig. 2) was consistent with the loci independence tests. By projection on the *A. bisporus* genome, the genetic order of markers in the large linkage group of *A. subrufescens* respects the physical order of their respective homologs observed in *A. bisporus*. Since no recombinant was found between *MAT* and *PRS088:248*, these two loci were found collocated. Two recombinants were observed between *MAT* and *RPB2:175*. Conversely, the small linkage group did not agree with *A. bisporus* data. *PRS160:134* and *ITS:200* loci are linked in *A. subrufescens* but are unlinked in *A. bisporus*, since the *ITS* locus belongs to chromosome IX and *PRS160* to chromosome VII.

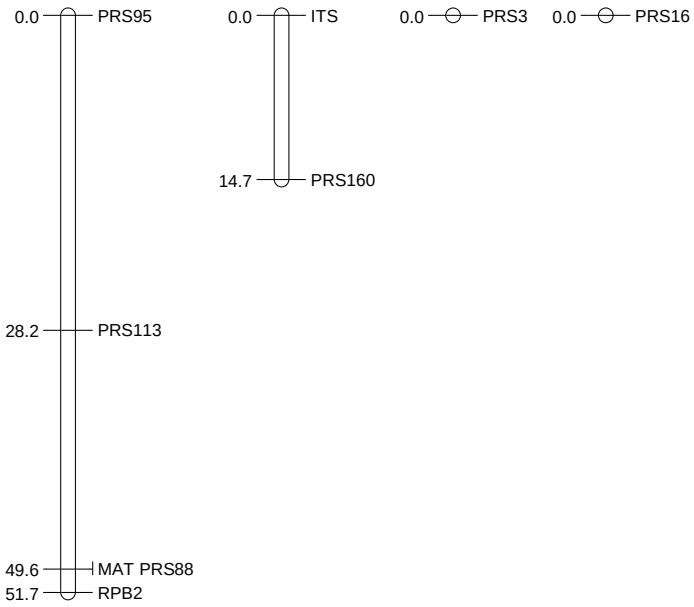

Figure 2 Linkage map obtained from the segregation analysis at 9 loci in 113 homokaryons progeny. Values indicated on the left side are the distance intervals in centimorgans (cM) using Kosambi function. Markers including molecular CAPS markers and the phenotypic MAT loci are written on the right side

Relationships between homoallelism and linkage to centromere

The mean rate of homoallelism among the heterokaryons (Table 3) varied from 27 % to 31 % for the three centromere-linked loci of the large linkage group (*RPB2:*715, *PRS088:248*, and *PRS113:158*) and varied from 50 % to 53 % for the centromere-unlinked markers except *ITS:200* that reached 60 %. Lethal or deleterious recessive alleles at centromere-linked loci might explain not only the lower rate of homoallelism observed at these loci among the heterokaryotic offspring but also the deviation from Mendelian segregation ratio observed among the homokaryotic offspring.

The mean rate of homoallelism among SNPPs and among NSNPPs depends on the linkage to the centromere since these heterokaryons inherit of homologous or heterologous centromeres respectively. This is illustrated in Fig. 3 where the mean rate of homoallelism has been calculated among 75 NSNPPs and 24 SNPPs from data of Table 3 for centromere-unlinked loci, centromere-linked loci and for the locus *PRS113:158* which is intermediary. However, the 13 heterokaryons remaining unclassified are not taken into account in Fig. 3; some of them are probably heteroallelic SNPPs or homoallelic NSNPPs at one of these two markers. If all SNPPs and SNPPs would have been classified, the true rates of homoallellism would not be exactly 0 for NSNPPs or 1 for SNPPs but there would be little variation from these values at the loci tightly linked to *MAT*. Finally, when the linkage to the centromere or the *MAT* locus increases, the rate of homoallelism increased from 50 % to 100 % for the SNPPs, whereas it decreased from 50 % to 0 % for the NSNPPs.

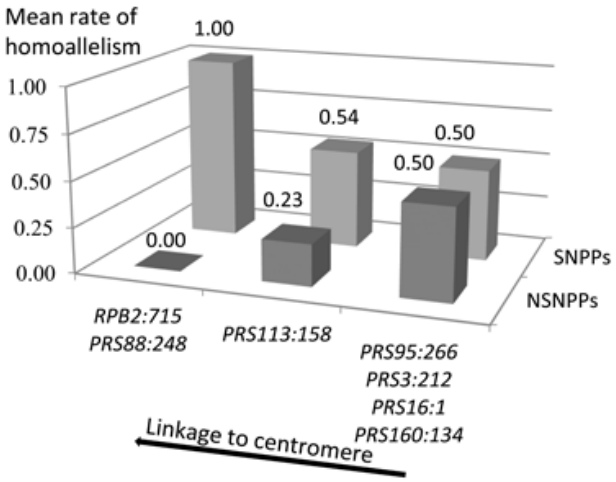

Figure 3 Relationships between linkage to centromere and homoallelism among 75 NSNPPs and 24 SNPPs. Mean rates of homoallelism was calculated for two loci tightly linked to the locus *MAT* and the centromere, for four centromere-unlinked loci which are genetically independent from each other, and for the locus *PRS113:158* which is intermediary Matings between SNPP heterokaryons and confirmation of the crosses

Since SNPPs behaved as homokaryons in mating tests when confronted to tester homokaryons we chose some of them to test whether they could cross among themselves. Seven confrontations with three replicates were performed between SNPPs having not only different mating type genotypes (*Mat-1/1* vs. *Mat-2/2*) but also appropriate genotypes at other loci in order to test whether the nuclei of two mated SNPPs are randomly paired in the resulting 'hybrid' heterokaryon (see materials and methods). In five of the seven confrontations, positive mating reactions were observed (Fig. 1b) and two to four putative hybrids were isolated from different replicate plates or from distinct reactions appearing in the same confrontation plate.

Sixteen putative hybrids isolated from positive mating reactions between SNPPs were all heterozygous at the *PRS088:248* locus (Table 5). This not only confirmed their hybrid status but also indicated that these unconventional crosses between infertile heterokaryons led to fertile heterokaryons since *PRS088:248* is tightly linked to the *MAT* locus. Genotypes at two other loci (only one in one case) were the same for all the hybrids isolated from matings between the same couple of SNPPs (Table 5). Moreover, except in the case where a single locus was used, these data showed that only one of the four possible pairings between the two nuclei of each 'parental' heterokaryon was found. In two cases there were four identical hybrid genotypes from the same type of mating. If the pairing was at random, the probability of finding the same pairing four times would be $4/4^4 = 0.016$. These data demonstrated that the pairings were not random and even suggested that only a single pairing was possible. However the process of pairing of nuclei remains unknown.

Table 5 Genotypes of confronted SNPPs and the isolated hybrids

Pairs of confronted SNPPs and isolated hybrids	Loci						N[a]
	PRS88: 248	*PRS113: 158*	*PRS95: 266*	*PRS3: 212*	*PRS16: 1*	*PRS160: 134*	
WC837-142 (*MAT 2/2*)	*2/2*	*2/2*	*1/1*	*2/2*	*1/2*	*2/2*	
WC837-006 (*MAT 1/1*)	*1/1*	*1/1*	*1/1*	*½*	*1/1*	*2/2*	
Hybrid (WC837-142 × WC837-006)	*1/2*			*½*	*1/2*		2
WC837-142 (*MAT 2/2*)	*2/2*	*2/2*	*1/1*	*2/2*	*1/2*	*2/2*	
WC837-018 (*MAT 1/1*)	*1/1*	*1/1*	*1/2*	*1/1*	*2/2*	*1/1*	
Hybrid (WC837-142 × WC837-018)	*1/2*		*1/2*		*2/2*		4
WC837-142 (*MAT 2/2*)	*2/2*	*2/2*	*1/1*	*2/2*	*1/2*	*2/2*	
WC837-232 (*MAT 1/1*)	*1/1*	*1/1*	*1/1*	*2/2*	*2/2*	*1/2*	
Hybrid (WC837-142 × WC837-232)	*1/2*				*2/2*	*2/2*	3
WC837-175 (*MAT 2/2*)	*2/2*	*1/2*	*2/2*	*½*	*2/2*	*1/1*	
WC837-120 (*MAT 1/1*)	*1/1*	*1/1*	*1/1*	*½*	*1/2*	*1/2*	
Hybrid (WC837-175 × WC837-120)	*1/2*				*1/2*		3
WC837-175 (*MAT 2/2*)	*2/2*	*1/2*	*2/2*	*½*	*2/2*	*1/1*	
WC837-251 (*MAT 1/1*)	*1/1*	*1/1*	*1/1*	*½*	*1/2*	*2/2*	
Hybrid (WC837-175 × WC837-251)	*1/2*	*1/2*			*2/2*		4

[a] Number of replicates: for each type of confrontation N hybrids were isolated and all had the same genotype

Mycelial growth rate test

The distribution of frequency of 91 SSI colony diameters (39 homokaryons, 37 NSNPPs and 15 SNPPs) measured after 15 days of growth is shown in Fig. 4. The mean diameter measured for homokaryons was 34.9 mm and did not differ significantly from the mean diameter for SNPPs (34.1 mm). The heterokaryons differed significantly ($p<0.05$) from both SNPPs and homokaryons with a mean diameter of 47.2 mm.

The homokaryotic SSIs presented a peak of growth rate in the range of 31-40 mm where 56.4% (22/39) are grouped. Heterokaryon SNPPs showed a peak in the range 31-40 mm with 33.3% of the individuals (5/16) in that range. The NSNPPs were present in all ranges represented in the graph, with a peak in the range 51-60 mm containing 37.8% (9/41) of them.

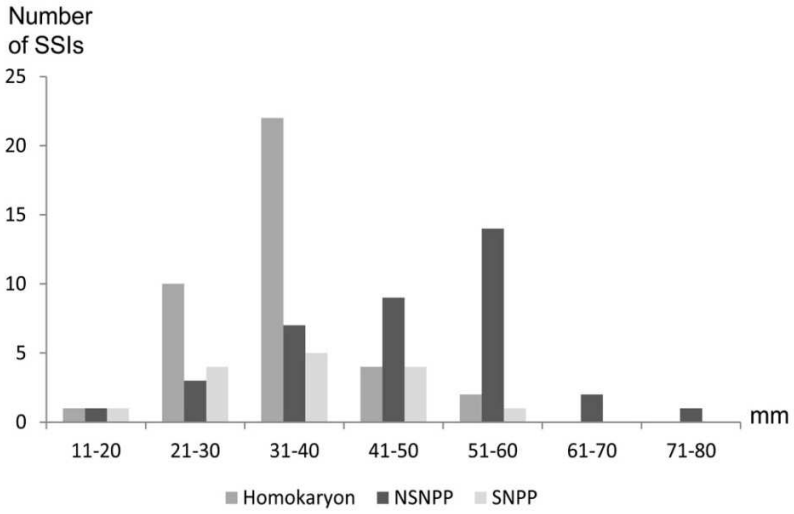

Figure 4 Frequency distribution of colony diameter of 91 SSI of WC837 (39 homokaryon, 37 NSNPPs and 15 SNPPs) after 15 days on malt agar medium

Behavior of single spore homokaryotic and heterokaryotic isolates (NSNPPs and SNPPs) in fruiting tests

The parental strain fruited normally. The homokaryons were unable to invade the culture substrate and were replaced by competitors or contaminants. Among the NSNPPs four failed to adequately invade the substrate; two invaded the substrate but did not fruited; and three fruited but only two of them produced mature sporocarps. Among the SNPPs, three did not adequately invade the substrate and among the six remaining, two fruited locally as a result of hybridization with uncontrolled inoculum (confirmed by sequencing, data not shown) but only one reached maturity; two produced balls of mycelium (popcorn-like); and two formed both immature fruiting bodies and balls of mycelium which also resulted from uncontrolled hybridizations. Finally, as expected, only the parental strain and NSNPPs were fertile but the latter poorly fructified. The SNPPs were more vigorous than the homokaryons in invading the substrate and appeared to easily cross with uncontrolled inoculum from the environment.

Discussion

The present data confirm the distinctiveness of the reproductive strategies and the amphithallic life cycle of *A. subrufescens* with tetrasporic basidia leading to three primary categories of SSIs. Thongklang et al. (2014) used a relatively small group of offspring (94 SSIs) of the strain WC 837 and three molecular markers. We used a larger group of progeny consisting of 225 SSIs, analyzed using eight markers, to perform more accurate assessments and to get enough SSIs of each category to study them. We found that 50% of the SSIs were heterokaryotic and among them the percentage of SNPPs was

estimated as 24-27 %. The rate of homoallelism among the heterokaryotic offspring varied from 27 % to 60 % depending of the loci. The rate of homoallelism at centromere-unlinked loci was consistently close to 50% (Table 2) among each category of heterokaryons (SNPPs and NSNPPs). These values are of the same order as the values estimated in the previous offspring of the same parent: 40 % of SSIs were heterokaryotic and 23 % of them were SNPPs; the rates of homoallelism among the heterokaryotic SSIs varied from 26 % for *RPB2:736* ([66-56]/38) to 50 % at each of the two centromere-unlinked loci *ITS:200* and *CEL4:236* ([75-56]/38 in both cases; calculated from Table 2 in Thongklang et al. 2014). In both experiments, the percentages of SNPPs among the heterokaryotic SSIs and the rate of homoallelism at centromere-unlinked markers appeared respectively smaller and higher than the 33% (1/3) expected if the postmeiotic nuclei migrated randomly in the spores. This 1/3 ratio of homoallelic SSIs from a heteroallelic parent was established by Langton and Elliott (1980) for the pseudohomothallism model with bisporic basidia (i.e. four nuclei migrating at random in two spores). For the model with tetrasporic basidia and postmeiotic mitosis occurring within basidia (i.e. eight nuclei migrating in four spores) a 3/7 ratio had been proposed (Langton and Elliott 1980). However this ratio of homoallelism is correct if all the spores (homokaryotic and heterokaryotic) are considered. In fact when the eight nuclei are paired at random in four spores, 1/7 of the spores receive two nuclei from the same postmeiotic mitosis and are true homokaryons. Thus, we calculated the ratio of homoallelism among the heterokaryons exclusively and found 1/3 as for the model with bisporic basidia. Following a similar reasoning a 1/3 ratio of SNPPs receiving homologous centromeres is also expected among the heterokaryotic SSIs under this model (Fig. S1).

As did Thongklang et al. (2014), we conclude that the process of migration of nuclei in the spores clearly differs from the non-random process

known in *A. bisporus* and that it remains to be clarified. Moreover we showed that for strain WC837 the observed frequency of of homoallelism among the homokaryotic SSIs at centromere-unlinked markers was higher than the 33% expected in a random model and consistently close to 50%. Thongklang et al. (2014) found similar frequencies but did not highlight these results which did not significantly differed from 33 % due to their smaller numbers of centromere-unlinked markers (two vs. four) and heterokaryons (39 vs. 113 in the present study). Finally, it remains also to clarify either how the frequency of homoallelism among the heterokaryotic SSIs might overestimate the frequency of homoallelism among the heterokaryotic spores under the random model, or how the process of migration of postmeiotic nuclei in the spores might really differ from the random model.

In *A. bisporus* var. *bisporus* the parental heterozygosity is highly maintained in the NSNPPs due to both a low rate of crossovers and the fact that non-sister nuclei preferentially migrate in the same spore (Kerrigan et al. 1993). In contrast, in the heterokaryotic offspring of strain WC837 the rate of homoallelism was consistently close to 50% at all five centromere-unlinked loci. This indicated that recombination was not suppressed and that the pairing of non-sister nuclei was not favored. This high rate of crossover has major consequences for the potential role of the heterokaryotic spores in nature as well as for developing breeding programs. Heterokaryotic SSIs of strain WC837 of *A. subrufescens* lost half of the parental heterozygosity exactly as this is classically expected in a selfing (a cross between homokaryotic SSIs from the same parent but not from spores of the same basidium) but with the exception of the centromeric regions which remained highly heterozygous in the NSNPPs or became highly homozygous in the SNPPs. A simple calculation using the data of Table 3 can roughly illustrate how the homoallelism varies: the mean rate of homoallelism at the eight markers used, which is 0% in the parental strain,

increased to 34% in the NSNPPs and to 65% in the SNPPs, and of course to 100% in the homokaryons which are haploid. As in selfing, inbreeding depression is expected even more so in SNPPs but less pronounced in NSNPPs. The lower mycelial growth rate of the SNPPs compared to the NSNPPs and the poor fruiting ability of the NSNPPs compared to the parental strain may reflect inbreeding depression that is known to impact these two characters when selfing is performed in *A. bisporus* (Xu 1995).

As Billiard et al. (2011, 2012) conjectured, we can wonder whether pseudohomothallism evolved to favor intramixis or to achieve universal mating compatibility (i.e. for example NSNPPs can potentially cross via the Buller phenomenon with homokaryons bearing any mating types). One of the potential points of interest in amphithallism in *A. bisporus* var. *bisporus* might be the maintenance or the selection of heterozygous genotypes adapted to a given environment through multiple intramictic generations; however in certain experimental conditions universal mating compatibility may be favored since heterokaryotic spores did not self but fertilized the homokaryons (Callac et al. 2006). In *A. subrufescens*, the inbred heterokaryotic descendants do not favor intramixis and thus their interest probably better lies in their ability to cross. However if the NSNPPs can achieve universal mating compatibility the role of the SNPPs remains unclear.

SNPP heterokaryons have been identified in only a few species such as *Coprinus bilanatus* Challen and Elliott (1989) and, to our knowledge, their behavior had not been studied previously. In our offspring these unconventional heterokaryons lost 50% percent of the parental heterozygozity at the centromere-unlinked loci and up to 100% at markers tightly linked to the centromere. They are homoallelic at the *MAT* locus. They represented 24-27 %, i.e. about the quarter of the heterokaryotic SSIs and one eighth of the total analyzed offspring. Their mycelial growth rate was on average lower than that of the NSNPPs and

similar to that of the homokaryons on malt agar medium; however, they better invaded the culture substrate. They were infertile in fruiting tests. In mating tests, 80% gave positive reactions with tester homokaryons and thus interacted as well as or possibly better than the homokaryons. The SNPPs crossed also among themselves but always the same pairs of nuclei were found in the resulting fertile heterokaryotic hybrids among the four possible parings. In conclusion, SNPPs were mating-competent heterokaryons behaving like homokaryons. They are possibly even more competent to cross and to survive in the wild due to their heterozygozity. However they must not be considered homokaryons because of a major difference: they can transmit not only recessive deleterious or lethal alleles but also advantageous alleles that could be on linked loci. Such a linkage has been showed through the Buller phenomenon in *A. bisporus*, for a locus involved in disease resistance (Callac et al. 2008). As in the Buller phenomenon, the gametic selection is bypassed when SNPPs cross together or with homokaryons. These types of crosses might be frequent in this species; as a result certain deleterious or lethal alleles could be maintained through multiple generations or could be accumulated in certain nuclei. The latter hypothesis could explain why the less frequent alleles of the unbalanced segregations at each of the five loci of the large linkage group were all from the same parental nucleus. The presence of deleterious or lethal alleles could also explain the relative lack of success in mating tests and the inbreeding depression.

For strains having such a type of amphithallism, our results highlight certain consequences for breeding strategies. For example, it is unrealistic to expect to improve such strains by simply selecting among a first generation of NSNPPs as can be done in *A. bisporus* (monospore selection; Kerrigan 1993; Moquet et al. 1998) because of inbreeding depression. Outcrossing between strains is therefore necessary and for this the large geographical range of this

species described in the introduction is useful. Mating tests are not reliable to discard NSNPPs because of false negative and false positive reactions. A better solution is to use single-locus codominant markers such as CAPS markers. Depending on the objective and the context, a simple strategy of treating mating-competent SNPPs as homokaryons may be appropriate for the two following reasons: first this can avoid selection against alleles of interest that could be present in SNPPs but absent in homokaryons; secondly this is faster and cheaper because five centromere-unlinked markers are necessary to identify the SNPPs, while a single marker tightly linked to the centromere is sufficient to identify and discard most of the NSNPPs. Such markers as *RPB2* and *PRS088* used in the present study or even better *MIP* (Thongklang et al., 2014) might be used. Although reproductive strategy varies among the different strains of *A. subrufescens* studied by Thongklang et al. (2014) this method should be efficient in all cases.

A first conclusion is that, for this type of amphithallic life cycle including mating-competent SNPPs, traditional methods cannot reliably correctly identify the categories of spores. Possibly this type of life cycle might be not infrequent but it has rarely been observed. A second conclusion is that both SNPP and NSNPP heterokaryons are able to cross. It would be interesting to study in which conditions they could be eventually more efficient than homokaryons for this function. On the other hand, the process of migration of the nuclei in the spores during sporogenesis and the process favoring certain pairings of nuclei when heterokaryons are crossed remain to be clarified. Finally we propose a simple method to cross amphithallic strains which does not require identifying all the types of spores. Our study is complementary to the recent work of Thongklang et al (2014) on the interfertility between geographically distant specimens of *A. subrufescens*, and together they respond to the questions:

what to cross and how to cross? This contributes to facilitate the genetic improvement of strains of *A. subrufescens* and other amphithallic species.

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Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary data

Supplemental Table S1 Genotypes of 225 SSIs at nine loci

SSI	*RPB2: 715*	*PRS088: 248*	*PRS113:1 57*	*PRS09: 266*	*PRS00: 212*	*PRS016: 1*	*PRS160: 134*	*ITS: 200*	*MAT*[a]
Homokaryons									
WC837-014	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*1*	-
WC837-027	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*1*	*1*
WC837-028	*1*	*1*	*1*	*1*	*2*	*1*	*1*	*2*	*1*
WC837-032	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*2*	*1*
WC837-042	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*
WC837-055	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*2*	*1*
WC837-060	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-095	*1*	*1*	*1*	*1*	*2*	*2*	*2*	-	*1*
WC837-101	*1*	*1*	*1*	*1*	*1*	*1*	*2*	-	-
WC837-106	*1*	*1*	*1*	*1*	*2*	*1*	*2*	-	*1*
WC837-109	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-110	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-125	*1*	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*1*
WC837-126	*1*	*1*	*1*	*1*	*2*	*1*	*1*	*2*	*1*
WC837-133	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*1*	*1*
WC837-136	*1*	*1*	*1*	*1*	*2*	*1*	*2*	-	*1*
WC837-137	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*2*	*1*
WC837-138	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*1*	*1*
WC837-140	*1*	*1*	*1*	*1*	*2*	-	*2*	*2*	*1*
WC837-150	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*	*1*
WC837-152	*1*	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*
WC837-169	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
WC837-177	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*2*	-

Supplemental Table S1, continuation

WC837-179	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*2*	*1*
WC837-186	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*1*	-
WC837-193	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*2*	*1*
WC837-198	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*
WC837-199	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-203	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-211	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	-
WC837-219	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	-
WC837-220	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*	-
WC837-222	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-229	*1*	*1*	*1*	*1*	*2*	*1*	*1*	*2*	*1*
WC837-230	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*
WC837-235	*1*	*1*	*1*	*1*	*2*	*1*	*1*	*2*	*1*
WC837-238	*1*	*1*	*1*	*1*	*2*	*1*	*1*	*2*	*1*
WC837-244	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*1*	*1*
WC837-252	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*	-
WC837-254	*1*	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*1*
WC837-255	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*2*	-
WC837-259	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*
WC837-264	*1*	*1*	*1*	*1*	*2*	*1*	*1*	*2*	-
WC837-266	*1*	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*1*
WC837-267	*1*	*1*	*1*	*1*	-	*2*	*1*	*2*	*1*
WC837-279	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
WC837-281	*1*	*1*	*1*	*1*	*2*	*1*	*1*	*2*	-
WC837-305	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*1*
WC837-309	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*2*	*1*
WC837-316	*1*	*1*	*1*	*1*	*2*	*2*	*1*	*2*	*1*

Supplemental Table S1, continuation

WC837-318	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-328	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-020	*1*	*1*	*1*	*2*	*2*	*1*	*1*	*2*	*1*
WC837-048	*1*	*1*	*1*	*2*	*2*	-	*2*	*1*	*1*
WC837-068	*1*	*1*	*1*	*2*	*2*	-	*2*	*1*	-
WC837-127	*1*	*1*	*1*	*2*	*1*	*1*	*1*	*2*	*1*
WC837-213	*1*	*1*	*1*	*2*	*2*	*2*	*1*	*2*	*1*
WC837-214	*1*	*1*	*1*	*2*	*1*	*1*	*2*	*1*	*1*
WC837-216	*1*	*1*	*1*	*2*	*1*	*1*	*1*	*2*	-
WC837-242	*1*	*1*	*1*	*2*	*1*	*1*	*2*	*1*	*1*
WC837-257	*1*	*1*	*1*	*2*	*2*	*1*	*1*	*2*	*1*
WC837-301	*1*	*1*	*1*	*2*	*1*	*1*	*1*	*2*	-
WC837-156	*1*	*1*	*2*	*1*	*1*	*2*	*2*	-	*1*
WC837-304	*1*	*1*	*2*	*1*	*1*	*1*	*1*	*1*	*1*
WC837-100	*1*	*1*	*2*	*2*	*2*	*1*	*2*	*1*	-
WC837-102	*1*	*1*	*2*	*2*	*2*	*2*	*2*	*1*	*1*
WC837-162	*1*	*1*	*2*	*2*	*2*	*2*	*2*	*1*	*1*
WC837-164	*1*	*1*	*2*	*2*	*1*	*2*	*1*	*2*	*1*
WC837-204	*1*	*1*	*2*	*2*	*1*	*1*	*2*	*2*	*1*
WC837-228	*1*	*1*	*2*	*2*	-	*2*	*1*	*2*	*1*
WC837-274	*1*	*1*	*2*	*2*	*2*	*1*	*1*	*2*	*1*
WC837-287	*1*	*1*	*2*	*2*	*1*	*1*	*1*	*1*	*1*
WC837-155	*1*	*2*	*1*	*1*	*2*	*1*	*2*	-	*2*
WC837-078	*2*	*1*	*1*	*1*	*2*	*2*	*2*	-	*1*
WC837-145	*2*	*2*	*1*	*1*	*2*	-	*2*	*1*	*2*
WC837-176	*2*	*2*	*1*	*1*	*1*	*1*	*1*	*1*	*2*
WC837-189	*2*	*2*	*1*	*1*	*2*	*2*	*2*	*1*	-

Supplemental Table S1, continuation

WC837-191	*2*	*2*	*1*	*1*	*2*	*2*	*2*	*1*	*2*
WC837-194	*2*	*2*	*1*	*1*	*1*	*2*	*2*	*2*	*2*
WC837-201	*2*	*2*	*1*	*1*	*2*	*2*	*2*	*1*	*2*
WC837-245	*2*	*2*	*1*	*1*	*2*	*2*	*1*	*2*	*2*
WC837-262	*2*	*2*	*1*	*1*	*1*	*1*	*2*	*1*	*2*
WC837-319	*2*	*2*	*1*	*1*	*1*	*1*	*1*	*2*	*2*
WC837-243	*2*	*2*	*1*	*2*	*2*	*2*	*1*	*2*	-
WC837-275	*2*	*2*	*1*	*2*	*1*	*1*	*2*	*1*	*2*
WC837-054	*2*	*2*	*2*	*1*	*2*	*2*	*2*	*1*	*2*
WC837-063	*2*	*2*	*2*	*1*	*2*	*1*	*1*	*2*	*2*
WC837-158	*2*	*2*	*2*	*1*	*1*	*1*	*2*	-	*2*
WC837-182	*2*	*2*	*2*	*1*	*2*	*2*	*1*	*2*	-
WC837-197	*2*	*2*	*2*	*1*	*2*	*1*	*1*	*2*	*2*
WC837-227	*2*	*2*	*2*	*1*	*1*	*1*	*1*	*2*	-
WC837-231	*2*	*2*	*2*	*1*	*1*	*1*	*2*	*1*	-
WC837-250	*2*	*2*	*2*	*1*	*2*	*1*	*1*	*2*	*2*
WC837-258	*2*	*2*	*2*	*1*	*1*	*1*	*2*	*1*	*2*
WC837-271	*2*	*2*	*2*	*1*	*2*	*1*	*2*	*1*	*2*
WC837-283	*2*	*2*	*2*	*1*	*2*	*2*	*1*	*2*	-
WC837-323	*2*	*2*	*2*	*1*	*2*	*1*	*2*	*1*	*2*
WC837-325	*2*	*2*	*2*	*1*	*1*	*2*	*1*	*2*	*2*
WC837-335	*2*	*2*	*2*	*1*	*1*	*2*	*1*	*1*	-
WC837-039	*2*	*2*	*2*	*2*	*2*	*2*	*1*	*2*	-
WC837-046	*2*	*2*	*2*	*2*	*2*	*1*	*1*	*1*	*2*
WC837-123	*2*	*2*	*2*	*2*	*2*	*1*	*2*	*2*	*2*
WC837-225	*2*	*2*	*2*	*2*	*1*	*2*	*1*	*2*	*2*
WC837-233	*2*	*2*	*2*	*2*	*2*	*1*	*1*	*2*	*2*

Supplemental Table S1, continuation

WC837-240	*2*	*2*	*2*	*2*	*1*	*2*	*1*	*2*	-
WC837-241	*2*	*2*	*2*	*2*	*2*	*1*	*1*	*2*	*2*
WC837-246	*2*	*2*	*2*	*2*	*1*	*1*	*1*	*2*	*2*
WC837-293	*2*	*2*	*2*	*2*	*1*	*2*	*2*	*1*	-
WC837-336	*2*	*2*	*2*	*2*	*1*	*2*	*2*	*1*	-
WC837-172	*2*	*2*	-	*1*	*2*	*2*	*2*	*2*	*2*
WC837-011	-	*1*	*1*	*1*	*2*	*1*	*2*	*1*	*1*
WC837-159	-	*1*	*1*	*1*	*1*	*2*	*2*	*1*	-
WC837-294	-	*2*	-	*1*	*1*	*2*	*1*	*2*	-
Putative NSNPP heterokaryons									
WC837-297	-	*1/2*	-	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	
WC837-089	-	*1/2*	*1/2*	*1/2*	*2/2*	*2/2*	*1/2*	*1/2*	
WC837-045	*1/2*	*1/2*	*1/1*	*1/1*	*1/2*	*1/1*	*1/2*	*2/2*	
WC837-069	*1/2*	*1/2*	*1/1*	*1/1*	*1/2*	*1/1*	*2/2*	*1/1*	
WC837-081	*1/2*	*1/2*	*1/1*	*1/1*	*1/2*	*1/2*	*2/2*	-	
WC837-083	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*	*1/2*	*1/1*	*2/2*	
WC837-099	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*	*1/2*	*1/1*	*2/2*	
WC837-119	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*	*2/2*	*1/2*	*1/2*	
WC837-130	*1/2*	*1/2*	*1/1*	*1/1*	-	-	*1/2*	*1/2*	
WC837-135	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*	*1/1*	*2/2*	*2/2*	
WC837-188	*1/2*	*1/2*	*1/1*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	
WC837-196	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*	*1/2*	*1/2*	*1/2*	
WC837-215	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*	*1/2*	*1/1*	*2/2*	
WC837-253	*1/2*	*1/2*	*1/1*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	
WC837-132	*1/2*	*1/2*	*1/1*	*2/2*	*1/1*	*1/1*	*1/2*	*1/2*	
WC837-134	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*2/2*	*2/2*	*1/2*	
WC837-157	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	

Supplemental Table S1, continuation

WC837-321	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*
WC837-124	*1/2*	*1/2*	*2/2*	*2/2*	*1/2*	*1/1*	*2/2*	*1/1*
WC837-295	*1/2*	*1/2*	-	-	*2/2*	*2/2*	*2/2*	*1/1*
WC837-091	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/1*	*2/2*
WC837-092	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/2*	*1/2*	*1/2*
WC837-097	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*2/2*	*1/2*	*1/2*
WC837-105	*1/2*	*1/2*	*1/2*	*1/1*	*1/1*	*1/2*	*2/2*	*1/1*
WC837-111	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/2*	*1/2*	*1/2*
WC837-114	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*
WC837-115	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/2*	*2/2*	*1/1*
WC837-118	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*2/2*	*1/1*
WC837-131	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*2/2*	*1/2*	*1/2*
WC837-195	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/2*	*2/2*	*1/2*
WC837-202	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/1*	-	*1/1*
WC837-210	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*
WC837-217	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/1*	*1/1*	*1/2*
WC837-226	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/2*	*1/2*	*1/2*
WC837-261	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	*2/2*
WC837-268	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/1*	*1/1*	*2/2*
WC837-307	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/1*	*1/2*	*1/2*
WC837-310	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*2/2*	*1/2*	*1/2*
WC837-322	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*2/2*	*2/2*	*1/2*
WC837-184	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*	*1/2*	*1/2*
WC837-326	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*	*2/2*	*1/1*
WC837-329	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/1*	*1/1*	*1/2*
WC837-005	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*
WC837-016	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/1*	*2/2*

Supplemental Table S1, continuation

WC837-019	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*
WC837-023	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*
WC837-025	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*2/2*
WC837-033	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	-
WC837-037	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*
WC837-043	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*	*1/2*
WC837-084	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/2*	*1/1*
WC837-086	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*2/2*	*1/1*	*2/2*
WC837-088	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*2/2*	*1/2*	*1/1*
WC837-103	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	-
WC837-112	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*
WC837-116	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/2*	*1/2*
WC837-121	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*
WC837-122	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*1/1*	*2/2*	*1/2*
WC837-128	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*	*1/1*
WC837-129	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/1*	*2/2*
WC837-160	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*	*2/2*
WC837-181	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*2/2*	*1/1*
WC837-183	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*
WC837-185	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/1*
WC837-206	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*
WC837-207	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/1*	*2/2*
WC837-209	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*
WC837-218	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/1*
WC837-248	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/1*	*2/2*	*1/2*
WC837-272	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*1/1*	*2/2*
WC837-280	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*

Supplemental Table S1, continuation

WC837-282	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/1*	*2/2*	
WC837-289	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*2/2*	*1/1*	
WC837-306	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	*2/2*	*2/2*	*1/1*	
WC837-333	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*1/1*	*1/1*	*2/2*	
Putative SNPP heterokaryons									
WC837-002	-	*1/1*	*1/1*	*1/1*	*2/2*	*2/2*	*1/2*	-	-
WC837-041	*2/2*	*2/2*	*1/2*	*1/2*	*1/1*	*1/1*	*1/2*	-	*2/2*
WC837-234	*2/2*	*2/2*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*	*1/1*	-
WC837-296	*2/2*	*2/2*	*1/2*	*1/2*	*2/2*	*1/1*	*1/2*	*1/2*	-
WC837-175	*2/2*	*2/2*	*1/2*	*2/2*	*1/2*	*2/2*	*1/1*	*1/1*	*2/2*
WC837-044	*2/2*	*2/2*	*1/2*	*1/1*	*1/2*	*1/2*	*1/1*	*2/2*	*2/2*
WC837-315	*2/2*	*2/2*	*1/2*	*1/1*	*1/2*	*1/1*	*1/2*	*1/2*	-
WC837-142	*2/2*	*2/2*	*2/2*	*1/1*	*2/2*	*1/2*	*2/2*	*1/1*	*2/2*
WC837-273	*2/2*	*2/2*	*2/2*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*
WC837-003	*1/1*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*	*2/2*	*1/1*
WC837-317	*1/1*	*1/1*	*1/2*	*1/2*	*1/2*	-	*2/2*	*1/1*	*1/1*
WC837-327	*1/1*	*1/1*	*1/2*	*1/2*	*1/2*	*2/2*	*1/1*	*2/2*	*1/1*
WC837-290	*1/1*	*1/1*	*1/2*	*2/2*	*1/1*	*1/1*	*1/1*	*2/2*	-
WC837-117	*1/1*	*1/1*	*1/2*	*1/1*	*1/2*	*1/1*	*1/2*	*2/2*	-
WC837-113	*1/1*	*1/1*	*2/2*	*1/2*	*2/2*	*1/1*	*1/2*	*1/1*	-
WC837-004	*1/1*	*1/1*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*
WC837-018	*1/1*	*1/1*	*1/1*	*1/2*	*1/1*	*2/2*	*1/1*	-	*1/1*
WC837-171	*1/1*	*1/1*	*1/1*	*1/2*	*1/1*	*1/2*	*2/2*	*2/2*	*1/1*
WC837-299	*1/1*	*1/1*	*1/1*	*1/2*	*2/2*	*1/2*	*1/2*	*1/2*	*1/1*
WC837-320	*1/1*	*1/1*	*1/1*	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	-
WC837-006	*1/1*	*1/1*	*1/1*	*1/1*	*1/2*	*1/1*	*2/2*	-	*1/1*
WC837-120	*1/1*	*1/1*	*1/1*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	*1/1*

Supplemental Table S1, conclusion

WC837-232	*1/1*	*1/1*	*1/1*	*1/1*	*2/2*	*2/2*	*1/2*	*2/2*	*1/1*
WC837-251	*1/1*	*1/1*	*1/1*	*1/1*	*1/2*	*1/2*	*2/2*	*1/1*	*1/1*
Unclassified heterokaryons									
WC837-270	*1/1*	*1/2*	*1/2*	*1/2*	*2/2*	*2/2*	*1/1*	*2/2*	
WC837-285	*2/2*	*1/2*	*1/1*	*1/1*	*1/1*	*1/1*	*2/2*	*1/1*	
WC837-300	*2/2*	*1/2*	*2/2*	*1/1*	*2/2*	*1/1*	*1/1*	*2/2*	
WC837-302	*2/2*	*1/2*	*2/2*	*1/1*	*1/1*	*2/2*	*1/1*	*2/2*	
WC837-334	*2/2*	*1/2*	*2/2*	*2/2*	*2/2*	*2/2*	*1/1*	*2/2*	
WC837-221	*2/2*	*1/2*	*1/2*	*1/1*	*1/2*	*2/2*	*1/1*	*2/2*	
WC837-263	*1/2*	*1/1*	*1/1*	*1/2*	*1/2*	*2/2*	*1/2*	*1/2*	
WC837-144	*1/2*	*1/1*	*1/2*	*1/1*	*1/2*	*1/1*	*1/2*	*2/2*	
WC837-205	*1/2*	*1/1*	*1/2*	*1/1*	*1/2*	*1/2*	*2/2*	*1/1*	
WC837-208	*1/2*	*1/1*	*1/2*	*2/2*	*1/2*	*1/2*	*1/2*	*1/1*	
WC837-331	*1/2*	*1/1*	*1/2*	*2/2*	*1/1*	*1/1*	*1/1*	*2/2*	
WC837-139	*1/2*	*1/1*	*1/2*	*1/2*	*1/2*	*1/2*	*2/2*	-	
WC837-149	*1/2*	*1/1*	*1/2*	*1/2*	*1/1*	*1/2*	*1/2*	*2/2*	

a Deduced from mating tests

Supplemental Table S2 Chi-square contingency tests for linkage between nine loci calculated from allelic segregations in the homokaryotic offspring of WC837

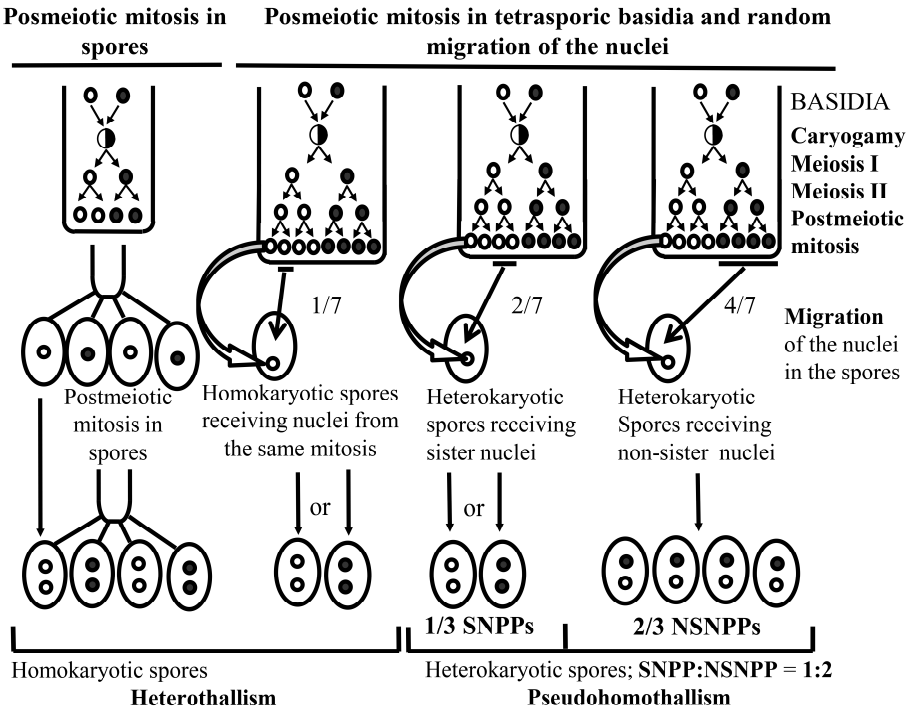
Pair of loci	P:R[a] ratio	χ^2
ITS200 and RPB2:715	51:51	0.03
ITS200 and PRS003:212	49:45	0.17
ITS200 and PRS016:1	38:44	0.24
ITS200 and PRS088:248	54:51	0.2
ITS200 and PRS095:266	56:49	0.8
ITS200 and PRS113:158	54:49	0.37
ITS200 and PRS160:134	90:15	53.36*
ITS200 and MAT	39:39	0.03
RPB2:715 and PRS003:212	48:60	0.24
RPB2:715 and PRS016:1	63:43	1.75
RPB2:715 and PRS088:248	108:2	81.64*
RPB2:715 and PRS095:266	67:43	0.54
RPB2:715 and PRS113:158	87:22	24.34*
RPB2:715 and PRS160:134	52:58	0.2
RPB2:715 and MAT	82:2	55.91*
PRS003:212 and PRS016:1	54:44	1.78
PRS003:212 and PRS088:248	49:62	0.37
PRS003:212 and PRS095:266	46:65	0.73
PRS003:212 and PRS113:158	45:64	1.06
PRS003:212 and PRS160:134	59:43	2.15
PRS003:212 and MAT	36:47	0.07
PRS016:1 and PRS088:248	63:46	1.09
PRS016:1 and PRS095:266	58:51	0.05
PRS016:1 and PRS113:158	60:47	0.3
PRS016:1 and PRS160:134	57:52	0.25
PRS016:1 and MAT	42:40	0.41
PRS088:248 and PRS095:266	69:44	0.53
PRS088:248 and PRS113:158	89:22	24.72*
PRS088:248 and PRS160:134	52:61	0.48
PRS088:248 and MAT	85:0	62.73*
PRS095:266 and PRS113:158	83:28	11.55*
PRS095:266 and PRS160:134	48:65	1.9
PRS095:266 and MAT	52:33	0.06
PRS113:158 and PRS160:134	47:64	2.04
PRS113:158 and MAT	65:19	12.12*
PRS160:134 and MAT	42:42	0.07

a Ratio parental: recombinant

*Rejection of genetic independency (P<0.001, 1 *df*)

Supplemental Table S3 Percentage of recombinant genotypes for each pair of linked loci

SSI		P: R[a] ratio; percentage of recombinants
Parental genotype	Recombinant genotype	
43 [ITS200-1/PRS160:134-1]	8 [ITS200-1/PRS160:134-2]	
47 [ITS200-2/PRS160:134-2]	7 [ITS200-2/PRS160:134-1]	90:15; 15/105 = 14 %
72 [RPB2:715-1/ PRS088:248-1]	1 [RPB2:715-1/ PRS088:248-2]	
36 [RPB2:715-2/ PRS088:248-2]	1 [RPB2:715-2/ PRS088:248-1]	108:2; 2/110 = 2 %
63 [RPB2:715-1/ PRS113:158-1]	10 [RPB2:715-1/ PRS113:158-2]	
24 [RPB2:715-2/ PRS113:158-2]	12 [RPB2:715-2/ PRS113:158-1]	87:22; 22/109 = 20 %
57 [RPB2:715-1/ Mat-1]	1 [RPB2:715-1/ Mat-2]	
25 [RPB2:715-2/ Mat-2]	1 [RPB2:715-2/ Mat-1]	82:2; 2/84 = 2 %
65 [PRS088:248-1/ PRS113-1]	10 [PRS088:248-1/ PRS113:158-2]	
24 [PRS088:248-2/ PRS113-2]	12 [PRS088:248-2/ PRS113:158-1]	89:22; 22/111 = 20 %
59 [PRS088:248-1/ Mat-1]	0 [PRS088:248-1/ Mat-2]	
26 [PRS088:248-2/ Mat-2]	0 [PRS088:248-2/ Mat-1]	85:0; 0/85 = 0 %
65 [PRS095:266-1/ PRS113:158-1]	12 [PRS095:266-1/ PRS113:158-2]	
18 [PRS095:266-2/ PRS113:158-2]	16 [PRS095:266-2/ PRS113:158-1]	83:28; 28/111 = 25 %
50 [PRS113:158-1/ Mat-1]	10 [PRS113:158-1/ Mat-2]	
15 [PRS113:158-2/Mat-2]	9 [PRS113:158-2/ Mat-1]	65:19; 19/84 = 23 %



Supplemental Figure S1 Amphithallic tetrasporic random model with precocious postmeiotic mitosis in basidia: expected ratio SNPP:NSNPP SNPP = heterokaryotic sister nuclei pair progenies; NSNPP = heterokaryotic non-sister nuclei pair progenies; black and white circles represent homologous centromeres or two alleles at a locus tightly linked to a centromere. The proportion of each type of spores is calculated by considering that a nucleus can be paired equally with any of the seven remaining nuclei of the basidium. In one-seventh of cases the resulting spore is homokaryotic since both nuclei come from the same mitosis. The final ratio SNPP:NSNPP is 1:2