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FERRUGEM DO COLMO DA AVEIA: FATORES GENÉTICOS DA VIRULÊNCIA DO PATÓGENO E DA RESISTÊNCIA DO HOSPEDEIRO

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FERRUGEM DO COLMO DA AVEIA: FATORES GENÉTICOS DA VIRULÊNCIA DO PATÓGENO E DA RESISTÊNCIA DO HOSPEDEIRO[1]

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RESUMO

A aveia (*Avena sativa* L.) é um cereal de importância mundial utilizada para a produção de grãos e forragem. A ferrugem do colmo da aveia, causada por *Puccinia graminis* f. sp. *avenae* (Pga), é uma das mais devastadoras doenças da aveia em todo o mundo. Para melhor entender as epidemias de ferrugem do colmo no Sul do Brasil, foram realizados levantamentos da virulência do patógeno durante dois anos em um programa de melhoramento de aveia. As epidemias foram caracterizadas por uma mistura de raças similares e de amplo espectro de virulência. A raça de maior virulência (TST) foi identificada em 2014, para a qual apenas o gene *Pg-10* foi parcialmente efetivo. Marcadores moleculares para estudos de genética de populações para Pga são escassos. Utilizando a sequência genômica de um isolado de Pga, 19 marcadores de sequências simples repetidas (SSR) foram desenvolvidos. Estes marcadores foram utilizados para avaliar a diversidade genética de 66 isolados de Pga da Austrália, Brasil e Suécia. Os isolados do Brasil e da Austrália foram caracterizados por uma e duas linhagens clonais predominantes, respectivamente. Por outro lado, os isolados da Suécia foram caracterizados por uma população recombinante e alta diversidade genética (nove genótipos distintos entre dez isolados). No hospedeiro, um limitado número de genes de resistência à ferrugem do colmo está disponível para o melhoramento da aveia. Para identificar novos genes de resistência, 61 genótipos brasileiros do Programa Internacional de Aveia da Quaker foram avaliados para a resposta de plântula e de planta adulta à ferrugem do colmo na Austrália. Nos testes de plântula, o patótipo de maior virulência da Austrália conferiu tipo de infecção susceptível à todas as linhagens diferenciais e genótipos testados, sendo utilizado para investigar a presença de resistência de planta adulta (RPA). Em um ensaio de campo, os genótipos UFRGS 087105-1 e UFRGS 087129-1 foram resistentes a moderadamente resistentes e representam uma promissora fonte de RPA para a ferrugem do colmo na Austrália. No Brasil, o genótipo UFRGS 995088-3 é uma importante fonte de resistência à ferrugem do colmo. Este estudo reporta a análise genética e o mapeamento molecular da resistência em UFRGS 995088-3. A análise genética foi realizada em duas populações de linhagens endogâmicas recombinantes (LERs) $F_{5:7}$. A razão de segregação para a resistência está em conformidade com a presença de um e três genes independentes. Um arranjo de 6000 SNPs da aveia foi utilizado para genotipar uma população de 85 LERs. Os dados moleculares fornecem evidências de um único gene para a resistência com distorção de segregação. Este gene foi mapeado em um intervalo de 0,7 cM entre dois marcadores que explicam 95 % da resistência. Estes marcadores poderão servir de base para estudos futuros de validação em outras populações.

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OAT STEM RUST: GENETIC FACTORS OF THE PATHOGEN VIRULENCE AND OF THE HOST RESISTANCE[1]

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ABSTRACT

Oat (*Avena sativa* L.) is a major cereal crop of global importance used for grain and forage production. Oat stem rust, caused by *Puccinia graminis* f. sp. *avenae* (Pga), is one of the most severe diseases of oats worldwide. To better understand the epidemics of stem rust in South Brazil, virulence surveys were carried out during two epidemic years in an oat breeding program. The epidemics were characterized by a mixture of similar and highly virulent races. The most virulent race (TST) was identified in 2014, for which only *Pg-10* was partially effective. Molecular markers suitable for population genetics of Pga are scarce. Using the genomic sequence of a Pga isolate, 19 simple sequence repeat (SSR) markers were developed. These markers were used to assess the genetic diversity of 66 Pga isolates from Australia, Brazil and Sweden. Brazilian and Australian isolates were characterized by one and two predominant clonal lineages, respectively. In contrast, the Swedish isolates were characterized by a highly diverse recombinant population (nine distinct genotypes out of ten isolates). In the host, a limited number of resistance genes to stem rust are available for oat breeding. In order to identify novel resistance sources, 61 Brazilian genotypes from Quaker International Oat Nursery were assessed for seedling and adult plant response to stem rust in Australia. In the seedling tests, the most virulent pathotype of Australia conferred susceptible infection type to all differential set and genotypes tested, and thus it was used to investigate the presence of adult plant resistance (APR). In a field trial, the genotypes UFRGS 087105-1 and UFRGS 087129-1 were resistant to moderately resistant and represent a promising source of effective APR to oat stem rust in Australia. In Brazil, the genotype UFRGS 995088-3 is a useful stem rust resistance source. This study reports the genetic analysis and the molecular mapping of the resistance in UFRGS 995088-3. The genetic analysis was performed using two recombinant inbred line (RIL) populations $F_{5:7}$. The segregation ratio of RIL populations for the resistance conformed to the presence of one and three independent genes. A 6 K oat single nucleotide polymorphism (SNP) array was used to genotype one population comprising 85 RILs. The molecular marker data provided evidence of a single-gene for the resistance with distorted segregation. This gene was mapped in a 0.7 cM interval between two markers that explained 95 % of the resistance. These markers can be used as a basis for further validation studies using other populations.

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1 INTRODUÇÃO

O desafio da agricultura nos dias de hoje é atender de forma sustentável a demanda crescente por produtos agrícolas. As doenças reduzem a qualidade e a produtividade dos cultivos e são controladas preferencialmente pelo uso de resistência genética. Entretanto, a capacidade dos patógenos em superar as resistências tem determinado um curto período de controle da resistência genética, tornando as cultivares susceptíveis poucos anos após o seu lançamento comercial e a sua adoção pelos produtores. Sendo assim, o melhoramento genético para resistência é um processo contínuo de identificação e caracterização de novas e efetivas combinações de genes de resistência a serem incorporados nas cultivares.

A aveia destaca-se na agricultura mundial pela produção de grãos para alimentação animal e humana. No Sul do Brasil, a aveia se ajusta ao sistema de rotação de culturas sucedendo a cultura principal de verão. Nesta região, a aveia é majoritariamente utilizada como forrageira para o gado ou como planta de cobertura, sendo caracterizada pelo baixo nível tecnológico empregado. Na produção de grãos, a lavoura brasileira de aveia vem experimentando aumentos consistentes em produtividade, tornando o grão uma opção cada vez mais rentável dentro do sistema de produção.

As ferrugens da aveia, principais doenças da cultura, são causadas por duas espécies distintas do gênero *Puccinia*, sendo *P. coronata* o agente causal da ferrugem da folha e *P. graminis* o agente causal da ferrugem do colmo. Fontes de resistência para a

ferrugem do colmo são escassas, com apenas 18 genes de resistência descritos em aveia. Levantamentos feitos no Canadá e na Austrália, mostram que um número ainda mais reduzido de genes de resistência tem sido utilizados em cultivares comerciais, e que raças do patógeno com virulência a estes genes estão presentes. Sendo assim, identificar e caracterizar novos genes de resistência para a ferrugem do colmo da aveia é de extrema importância para garantir o controle da doença através do melhoramento genético.

Para melhorar a eficiência no melhoramento genético é fundamental entender a dinâmica e a estrutura populacional do patógeno. A caracterização do patógeno pode ser baseada no espectro de virulência ou em marcadores moleculares. O espectro de virulência, determinado sob uma série diferencial de genes de resistência do hospedeiro, pode auxiliar na escolha da combinação de genes de resistência a ser utilizada no melhoramento. Para estudos de genética de populações, o desenvolvimento de marcadores moleculares tem contribuído para caracterizar migrações intercontinentais, além da importância de fatores locais, como, por exemplo, a presença do hospedeiro alternativo, no estabelecimento das epidemias.

Os objetivos deste trabalho foram: (1) caracterizar o espectro de virulência de *Puccinia graminis* f. sp. *avenae* em epidemias de ferrugem do colmo do Sul do Brasil; (2) desenvolver e caracterizar marcadores microssatélites para *Puccinia graminis* f. sp. *avenae*; (3) identificar e caracterizar fontes de resistência à ferrugem do colmo da aveia em germoplasma brasileiro e (4) caracterizar a resistência à ferrugem do colmo no genótipo UFRGS 995088-3 e identificar marcadores moleculares associados ao(s) loco(s) da resistência.

2 REVISÃO BIBLIOGRÁFICA

2.1 A aveia

2.1.1 Importância econômica

A aveia pertence a um grupo de espécies gramíneas de origem mediterrânea e está adaptada ao cultivo sob clima ameno. Em nível mundial, a aveia se destaca na produção de grãos destinados principalmente para a alimentação animal (Murphy & Hoffman, 1992). Além disso, a aveia vem ocupando cada vez mais espaço na dieta humana devido ao seu valor nutricional. Grãos de aveia são constituídos majoritariamente por amido, que corresponde 44 a 61 % do grão descascado, e possuem o maior teor de proteína e de lipídios entre os cereais com teores variando de 12 a 24 % para proteína, e de 3 a 12 % para lipídios (Peterson, 1992). Outros benefícios do consumo de aveia incluem o menor índice glicêmico quando comparado ao trigo, e a presença de β-glicanas, fibras solúveis associadas a redução dos níveis de colesterol e da pressão arterial em humanos (Jenkins *et al.*, 2002; Maki *et al.*, 2007).

Na América do Sul, a aveia é cultivada em três condições climáticas distintas: (1) zona temperada do Chile, Argentina e Uruguai; (2) zona tropical e subtropical do Centro-Sul do Brasil; e (3) zona tropical de altitude na região dos Andes e áreas altas da Bolívia, Equador e Peru (Federizzi & Mundstock, 2004). No Sul do Brasil, estima-se que 5.000.000 ha sejam cultivados com aveia preta (*Avena strigosa*) para cobertura do solo, supressão de plantas daninhas e reciclagem de nutrientes, estando associada ao sucesso do sistema de

plantio direto, iniciado na década de 1970 (Carvalho & Strack, 2014). Para a produção de grãos, a maior área cultivada com aveia branca (*A. sativa*) ocorreu em 2005 com 367 mil ha e uma produção de 522 mil toneladas (Federizzi *et al.*, 2014). No período de 2010 a 2013, apesar da retração na área cultivada para menos de 200 mil ha, a produção se manteve próxima das 400 mil toneladas, determinada pelo aumento da produtividade média da lavoura brasileira (Federizzi *et al.*, 2014). Em 2016, segundos dados da Companhia Nacional de Abastecimento (Conab, 2017), houve uma expansão da área para 291.500 ha com a produção estimada de mais de 827 mil toneladas.

O melhoramento genético da aveia branca no Brasil teve início em 1974, fomentado pelo programa internacional *Breeding Oat Cultivars Suitable for Production in Developing Countries*, concedido pela Agência dos Estados Unidos para o Desenvolvimento Internacional (USAID) (Federizzi & Mundstock, 2004). Com o término do projeto da USAID, em 1977 a empresa Quaker, através do Quaker International Oat Nursery (QION) manteve a troca de germoplasma entre diferentes programas de melhoramento de aveia do mundo. O melhoramento genético da aveia no Brasil tem buscado principalmente aumentos no potencial de rendimento e na qualidade de grãos, ciclo precoce e resistência a doenças (Federizzi & Mundstock, 2004).

2.1.2 Taxonomia e domesticação

O gênero *Avena*, da família *Poaceae*, é formado por um grupo de espécies cultivadas e silvestres com diferentes níveis de ploidia dos genomas A, B, C e D, com número genômico básico (x) igual a sete cromossomos (Loskutov & Rines, 2011). Diversas espécies diploides dos genomas A e C ocorrem na natureza, enquanto não são conhecidas espécies diploides dos genomas B e D (Loskutov & Rines, 2011). A ausência de ancestrais diploides dos genomas B e D e a similaridade de sequências nucleotídicas

entre os genomas A, B e D sugerem que os genomas denominados B e D são, na verdade, derivações do genoma A (Jellen *et al.*, 1994; Nikoloudakis *et al.*, 2008; Loskutov & Rines, 2011; Chew *et al.*, 2016).

As espécies domesticadas da aveia se diferenciam dos seus ancestrais silvestres pela desarticulação das espiguetas e a não-debulha natural dos grãos da panícula, sendo a espécie *A. sativa* (2n= 6x= 42 cromossomos, genoma ACD) a de maior importância econômica mundial (Loskutov & Rines, 2011). Além desta, outras três espécies foram domesticadas, incluindo: *A. strigosa* (2n= 2x= 14 cromossomos, genoma A), *A. abyssinica* (2n= 4x= 28 cromossomos, genoma AB) e *A. byzantina* (2n= 6x= 42 cromossomos, genoma ACD) (Loskutov & Rines, 2011).

A origem hexaploide da espécie *A. sativa* é atribuída a dois eventos sucessivos de poliploidização e duplicação cromossômica (Thomas, 1992). No primeiro deles, a fusão dos genomas A e C de dois ancestrais diploides deram origem ao tetraploide AC (ou DC). Esta espécie tetraploide intercruzada com outra espécie diploide teria dado origem à espécie hexaploide (Thomas, 1992). Com base em caracteres morfológicos, citogenéticos e de similaridade de sequências genômicas, os possíveis ancestrais diploides do genoma A são as espécies *A. longiglumis*, *A. canariensis* e *A. wiestii*, enquanto do genoma C são as espécies *A. clauda* e *A. eriantha* (Loskutov, 2008; Chew *et al.*, 2016). Já entre os possíveis ancestrais tetraploides AC (ou DC conforme sugerido por Yan *et al*. (2016)) estão as espécies *A. insularis*, *A. magna* e *A. murphyi*, todas endêmicas da região Noroeste da África, onde supostamente teriam ocorridos os eventos de poliploidização (Chew *et al*., 2016). A domesticação da espécie *A. sativa* teria ocorrido a partir de *A. sterilis*, na região do Crescente Fértil entre os mares Mediterrâneo, Cáspio e Negro que compreende aos territórios do Iran, da Georgia e da Rússia (Loskutov, 2008).

As espécies de aveia podem ser agrupadas em três pools gênicos de acordo com a fertilidade dos cruzamentos. Pertencem ao pool gênico primário todas as espécies hexaploides cultivadas e silvestres, incluindo, *A. sterilis*, *A. fatua* e *A. bizantina*. Estas espécies hibridizam facilmente e geram descendentes férteis. No pool gênico secundário estão as espécies tetraploides *A. magna*, *A. murphyi* e *A. insularis*, com as quais a espécie *A. sativa* hibridiza diretamente, porém, gerando progênies parcialmente estéreis. O pool gênico terciário inclui as espécies diploides e tetraploides que requerem técnicas avançadas de cultivo *in vitro* para a hibridização com *A. sativa* (Loskutov & Rines, 2011).

2.2 Ferrugem do colmo em aveia

A ferrugem do colmo da aveia, causada pelo fungo *Puccinia graminis* Pers. f. sp. *avenae* Eriks. & Henn., está presente em todos os continentes onde a aveia é cultivada, sendo conhecida pelo seu potencial de destruição, que pode levar a perda total das lavouras (Martens, 1985). No Brasil, as epidemias são normalmente limitadas ao final do ciclo da cultura com maior potencial de dano econômico em cultivares de ciclo tardio (Martinelli, 2004).

2.2.1 Descrição da doença

A doença ocorre preferencialmente nos colmos, produzindo pústulas alongadas que rompem a epiderme dos tecidos infectados liberando os esporos de coloração castanho escura. A dispersão dos esporos ocorre pelo vento, podendo percorrer centenas de quilômetros. O doença é policíclica com ciclos de 8 a 10 dias, sendo uma doença notoriamente de climas mais quentes (Agrios, 2005). Sob alta severidade da doença, a infecção causa a interrupção no fluxo de nutrientes na planta, além do enfraquecimento dos colmos, o que torna as plantas mais vulneráveis ao acamamento e reduz a qualidade e a

produtividade de grãos (Martens, 1985). A doença é controlada pela aplicação de fungicidas ou através da resistência genética do hospedeiro (Agrios, 2005).

2.2.2 Caracterização do patógeno

Puccinia é o gênero com maior número de espécies da família Pucciniaceae, incluindo diversos patógenos causadores das ferrugens nos cereais (Hiratsuka & Sato, 1982). *Puccinia graminis* é um patógeno biotrófico obrigatório causador da ferrugem do colmo em cereais. A fase gametofítica do patógeno ocorre apenas em espécies dos gêneros *Berberis* e *Mahonia* (Wahl *et al*., 1984), enquanto a fase esporofítica foi identificada em 72 gêneros na classe liliopsida, incluindo os cereais de importância agrícola (Cummins, 1971). Esta capacidade do fungo em infectar um grande número de espécies de gramíneas é caracterizada pela subdivisão em formas especializadas, as quais parasitam hospedeiros diferentes. Estas formas especializadas, indistinguíveis morfologicamente, são denominadas *formae speciales* (no singular *forma specialis*, abreviado por f. sp.) (Anikster, 1984). É possível ainda subdividir as *formae speciales* em raças fisiológicas, utilizando a formula de virulência/avirulência sobre uma série diferencial de genes de resistência do hospedeiro, ou seja, a habilidade específica de biótipos do patógeno em infectar diferentes genótipos de uma mesma espécie hospedeira (Stakman *et al*., 1962).

A diferenciação de raças em *P. graminis* f. sp. *avenae* foi primeiramente reportada por Stakman (1923), que utilizou três genótipos de aveia (*Victory*, *White Tartar* e *Monarch Selection*) para caracterizar quatro raças (I, II, III e IV) de acordo com o tipo de infecção na interação específica entre planta e patógeno. Posteriormente foram identificados novos genótipos de aveia diferenciais que passaram a ser denominados pela série numérica *Pg* (*Pg-1*, *Pg-2*, etc.), aumentando a capacidade de discriminação das raças do patógeno (Martens, 1985). Atualmente, 12 linhagens diferenciais contendo os genes *Pg* isolados (*Pg-*

1, Pg-2, Pg-3, Pg-4, Pg-6, Pg-8, Pg-9, Pg-10, Pg-12, Pg-13, Pg-15 e *Pg-16*) são utilizados internacionalmente para a caracterização de raças da ferrugem do colmo da aveia (Fetch Jr. & Jin, 2007).

2.2.3 Estudos genéticos em *Puccinia graminis* f. sp. *avenae*

Marcadores moleculares tem sido utilizados em estudos de genética de populações. Entre os marcadores de maior sucesso destacam-se as sequências simples repetidas (SSR), também conhecidos por microssatélites. SSR são segmentos do DNA genômico formadas por sequências de 1 a 6 pares de bases repetidas de maneira adjacente (tandem) (Bhargava & Fuentes, 2010). Originalmente desenvolvidos para a pesquisa em humanos, os microssatélites, por serem altamente polimórficos, multialélicos e de fácil detecção, foram rapidamente adotados para análise genética em outros organismos (Beckmann & Weber, 1992; Tóth *et al*., 2000; Karaoglu *et al*., 2005; Bhargava & Fuentes, 2010). Nos fungos, o desenvolvimento de marcadores moleculares tem possibilitado inferir sobre a contribuição da recombinação sexual, da migração em longas distâncias e da sobrevivência em plantas voluntárias para diversos patógenos (Kolmer, 2005; Ordoñez *et al*., 2010; Ali *et al*., 2014). Até o momento, marcadores microssatélites específicos para *P. graminis* f. sp. *avenae* não foram identificados.

Em alguns países, a diversidade genética e a estrutura populacional de *P. graminis* f. sp. *avenae* tem sido determinada utilizando marcadores moleculares. Berlin *et al*. (2013) e Berlin *et al*. (2014) utilizaram marcadores SSR desenvolvidos para *P. graminis* f. sp. *tritici* polimórficos em uma população de *P. graminis* f. sp. *avenae* da Suécia e do Tajiquistão. A diversidade genética revelada pelos marcadores permitiu inferir sobre a importância da presença do hospedeiro alternativo (*Berberis* sp.) na sobrevivência e recombinação sexual em epidemias de ferrugem do colmo da aveia nestes países (Berlin *et*

al., 2013, Berlin *et al*., 2014). Na Austrália, marcadores AFLP (*Amplified Fragment Lenght Polimorphism*) foram desenvolvidos para acessar a diversidade em uma coleção de isolados do patógeno representando 25 anos de coletas nas diferentes regiões do país (Haque *et al*., 2008). Neste caso, os marcadores revelaram a estrutura clonal na população e a divisão em três grupos distintos que representam introduções independentes do patógeno no continente (Haque *et al*., 2008).

2.3 Resistência genética

2.3.1 Resistência genética à ferrugem dos cereais

Nos cereais de inverno, a resistência às ferrugens pode ser caracterizada pela resposta de hipersensibilidade específica a raças do patógeno (resistência qualitativa), ou por seu efeito sobre a redução na taxa de progresso da doença (resistência quantitativa). A natureza qualitativa da resistência associada a resposta de hipersensibilidade permite sua caracterização em estádios iniciais do desenvolvimento da planta, sendo frequentemente denominada de resistência de plântula. Já o efeito quantitativo na redução da doença é melhor caracterizado em estádios avançados do desenvolvimento da planta e, por isso, este tipo de resistência é também denominada resistência de planta adulta.

A resistência de plântula tem sido amplamente caracterizada e utilizada no melhoramento dos cereais. Em alguns casos, como em trigo para o gene *Sr31* e cevada para o gene *Rpg1*, a resistência genética garantiu o controle da doença por mais de meio século na América do Norte (Steffenson, 1992; Kolmer, 2005). Entretanto, resistências duráveis são exceção, não a regra. Isso porque cultivares contendo resistência raça-específica quando cultivadas em larga escala ficam expostas à grande diversidade do patógeno. A pressão de seleção sobre o patógeno determina, neste caso, modificações nas

frequências das raças ou o surgimento de novas raças na população do patógeno levando à susceptibilidade da cultivar poucos anos após o seu lançamento comercial e a sua adoção pelos produtores.

A resistência de planta adulta normalmente atua sobre todas as raças do patógeno, e portanto, tende a ser mais durável. Os exemplos mais bem conhecidos são os genes *Sr2* e *Lr34*, de resistência a ferrugem do colmo e da folha do trigo, respectivamente (McIntosh *et al*., 1995; Lagudah, 2011). Estes genes tem conferindo resistência parcial em cultivares de trigo por mais de 100 anos cobrindo extensas áreas de cultivo sobre pressão de doença (Ellis *et al*., 2014).

Apesar de isoladamente genes de resistência de planta adulta não proporcionarem níveis adequados no controle da doença, a combinação de vários genes de efeito parcial conferem a quase completa imunidade (Singh *et al*., 2014). Neste caso, o efeito aditivo é determinado pela combinação de genes de diferentes mecanismos de resistência (Rubiales & Niks, 2000). Entretanto, a impossibilidade de realizar testes em plântula e a presença de genes de resistência completa que mascaram o efeito parcial são apontadas como limitações para a identificação e seleção da resistência de planta adulta no melhoramento (St. Clair, 2010; Singh *et al*., 2011; Ellis *et al*., 2014).

2.3.2 Caracterização da resistência

Os fatores genéticos envolvidos na interação planta x patógeno para a resposta de hipersensibilidade foi primeiramente descrita por Flor (1942). Flor estabeleceu que a resistência (incompatibilidade na interação) é determinada pela correspondência dos genes de resistência no hospedeiro e de avirulência no patógeno. Uma das aplicações dos princípios da teoria gene-a-gene de Flor é a postulação de genes de resistência em genótipos do hospedeiro. Desta forma, utilizando uma coleção de isolados diferenciais do

patógeno com virulência conhecida é possível inferir a presença de genes de resistência em um grande número de genótipos em um curto espaço de tempo (Loegering *et al*., 1971; Browder, 1973). Por outro lado, a postulação de genes não é eficaz para discriminar a presença de mais de um gene efetivo em um mesmo genótipo. Para determinar de forma definitiva o número e a identidade dos genes de resistência são realizados cruzamentos entre o genótipo resistente e genótipos susceptíveis (Kolmer, 1996). As plantas F_1 obtidas do cruzamento são autofecundadas e o número de genes segregando para a resistência pode ser determinado avaliando-se a população F_2 ou gerações avançadas (Kolmer, 1996).

A resistência pode ser descrita qualitativamente, analisando-se o tipo de infecção, ou quantitativamente, através da percentagem da área da planta ocupada pela doença (severidade). Testes em casa-de-vegetação utilizando inoculação artificial em plântula permitem acessar o tipo de infecção. Para descrever o tipo de infecção da ferrugem do colmo normalmente utiliza-se a escala de Stakman *et al*. (1962) (McIntosh *et al.*, 1995). O tipo de infecção varia desde a completa ausência de sintomas (hospedeiro imune) à máxima expressão da reprodução do patógeno (hospedeiro altamente susceptível). Para acessar a resposta de planta adulta diversas escalas tem sido propostas, sendo a mais utilizada a escala de Peterson *et al*. (1948), que descreve qualitativa e quantitativamente a doença (Stubbs *et al*., 1986). Além disso, escalas numéricas simplificadas, como a descrita por Bariana *et al*. (2007) tem grande aplicação no melhoramento pela possibilidade de avaliação de um grande número de genótipos de forma eficiente.

2.3.3 Resistência à ferrugem do colmo da aveia

Grandes esforços tem sido realizados na identificação e caracterização de genes de resistência não somente dentro da espécie cultivada, mas também em espécies silvestres de aveia, com o objetivo de ampliar as opções de resistência para o melhoramento. A busca

por novas fontes de resistência teve grande êxito para a ferrugem da folha da aveia, onde mais de 90 genes de resistência foram identificados (Chong *et al*., 2000). Entretanto, para a ferrugem do colmo apenas 18 genes de resistência são descritos na literatura (Fetch Jr. & Jin, 2007). Esse número é bastante inferior ao observado para a ferrugem do colmo do trigo onde são catalogados 58 genes de resistência (Yu *et al*., 2014).

Inicialmente, um conjunto de fontes de resistência à ferrugem do colmo foi caracterizado entre cultivares de aveia. Estes genes foram denominados *Pg-1* ao *Pg-12* de acordo com a ordem com que foram identificados. Com exceção do gene *Pg-6*, identificado na espécie diploide *A. strigosa*, os demais foram caracterizadas em cultivares de *A. sativa* (Martens *et al*., 1979). Na busca por novos genes de resistência, foram identificados em *A. sterilis*, os genes *Pg-13* (Mckenzie *et al*., 1970), *Pg-15* (Martens *et al*., 1980), *Pg-a* (Martens *et al*., 1981) e *Pg-17* (Harder *et al.*, 1990) e, em *A. barbata*, o gene *Pg-16* (Dinoor & Wahl, 1963). Todos estes genes foram transferidos para a espécie *A. sativa* (Martens *et al.*, 1979).

Visando identificar novas fontes de resistência à ferrugem do colmo da aveia, Gold Steinberg *et al.* (2005) avaliaram 9.978 acessos de aveia entre espécies cultivadas e silvestres, em condições de campo, utilizando inoculação artificial com a raça de maior frequência no Canadá. Destes, apenas 47 acessos (0,5% do total) apresentaram nível de resistência moderada à muito alta, sendo que, 36 deles pertenciam à espécie *A. strigosa*. A identidade da resistência destes acessos não foi determinada, entretanto, pela análise da genealogia, a maior parte dos acessos compartilham o mesmo gene, sendo esse possivelmente o já caracterizado *Pg-6* (Gold Steinberg *et al.*, 2005).

Dezesseis dos 18 genes Pg conferem resistência raça-especifica, podendo ser determinada sua presença em plântulas inoculando-se artificialmente a primeira folha completamente expandida (Fetch Jr. & Jin, 2007). Nestas condições, variações no tipo de

infecção em função da temperatura tem sido documentada para diversos genes Pgs (Martens, 1985). Sendo assim, os genes *Pg-3, Pg-9 e Pg-16* não são efetivos a 25 ºC, enquanto os genes *Pg-4, Pg-8, Pg-12, Pg-13, Pg-15* e *Pg-a* perdem sua efetividade sob temperatura acima de 27 ºC (Fetch Jr., 2006).

Alguns dos genes Pgs tem sido extensivamente utilizados no melhoramento da aveia. Os genes *Pg-2, Pg-4* e *Pg-a* foram detectados em grande parte das cultivares de aveia na Austrália (Adhikari *et al.*, 2000). Já no Canadá, os genes *Pg-2* e *Pg-13* estão presentes em maior número de cultivares (Harder, 1994; Mitchell Fetch *et al.*, 2007; Mitchell Fetch *et al.*, 2013). O uso da resistência tem contribuído para modificações na população do patógeno e atualmente todas as cultivares na Austrália e no Canadá são susceptíveis à ferrugem do colmo (Fetch Jr., 2009; Park, 2008).

A resistência parcial ou de planta adulta na aveia tem importância maior no controle da ferrugem da folha (*P. coronata* f. sp. *avenae*), sendo fundamental para garantir a durabilidade da resistência das cultivares (Leonard, 2002; Federizzi *et al.*, 2015). Para a ferrugem do colmo, a resistência em estádios avançados do desenvolvimento da planta foi caracterizada apenas para os genes *Pg-11* e *Pg-17* (Harder *et al.*, 1971; Harder *et al.*, 1990). A resistência do gene *Pg-11* está associada à deficiência de clorofila, o que tem limitado o seu uso no melhoramento (Harder *et al.*, 1971), enquanto o nível de resistência reportado em condições de campo para o gene *Pg-17* é baixo, com redução inferior a 30 % da quantidade da doença (Gold Steinberg *et al.*, 2005). Portanto, estes genes tem pouca aplicação no melhoramento.

2.4 Melhoramento molecular em aveia

A seleção assistida por marcadores moleculares no melhoramento genético depende da disponibilidade de marcadores associados às características de interesse e do custo da

genotipagem em larga escala. Apesar de disponíveis marcadores para genes de resistência em aveia, incluindo marcadores RAPD e RFLP para os genes *Pg-9* e *Pg-13* (O'donoughue *et al.*, 1996), AFLP para o gene *Pc68* (Kulcheski *et al.*, 2010) e SCARs para o gene *Pc91* (McCartney *et al.*, 2011), estes marcadores não foram incluídos em plataformas de genotipagem, o que tem inviabilizado o seu uso na rotina de laboratórios aplicados a programas de melhoramento.

SNPs (*Single nucleotide Polymorphism*) referem-se a polimorfismos de uma única base no DNA. Devido à sua abundância e compatibilidade com as tecnologias de genotipagem em larga escala (*high throughput*) disponíveis atualmente, os SNPs tem se tornado os marcadores de escolha para a seleção assistida por marcadores. Diferentes tecnologias para a genotipagem por SNPs estão disponíveis para uso na pesquisa e melhoramento genético. A tecnologia multiplex da Illumina (http://www.illumina.com) permite a genotipagem de um grande número de SNPs, sendo indicada, por exemplo, para a geração de mapas genéticos em populações de mapeamento (Semagn *et al.*, 2014). A tecnologia uniplex KASP (Kompetitive Allele Specific PCR), da LGC Genomics (http://www.lgcgenomics.com) tem aplicação para a genotipagem de pequeno a moderado número de SNPs em um grande número de indivíduos, como por exemplo, na seleção assistida por marcadores em programas de melhoramento (Semagn *et al.*, 2014).

Uma plataforma de SNPs para aveia tem sido recentemente desenvolvida e atualmente conta com 6.000 marcadores (Oliver *et al.*, 2013; Tinker *et al.*, 2014). Esta plataforma tem sido utilizada para estabelecer um mapa consenso em aveia (Oliver *et al.*, 2013; Chaffin *et al*., 2016) e aplicada no mapeamento de resistências à ferrugem da folha, incluindo os genes *Pc91* (Gnanesh *et al.*, 2013) e *PcKM* (Gnanesh *et al.*, 2015), e os QTLs de resistência parcial na linhagem MN841801 (Lin *et al.*, 2014) e em outras duas importantes fontes de resistência norte-americanas (Babiker *et al.*, 2015).

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3 ARTIGO 1

Pathogenic variation of the oat stem rust pathogen *Puccinia graminis* f. sp. *avenae* in two epidemic years in South Brazil[1]

Abstract

Stem rust is a major disease of oat worldwide and developing genetic resistant cultivars is the preferred method of control. Virulence survey provide useful information for rational deployment of resistance genes in cultivars. Such information is largely lacking for oat stem rust in growing conditions of South Brazil. The objective of this study was to characterize the virulence structure of the oat stem rust pathogen in two epidemics years in an oat breeding station in South Brazil. A total of 16 single-pustule isolates were obtained during 2013 and 2014 epidemic years. Based on the seedling response of the oat stem rust differential set, we detected three and six distinct physiologic races in 2013 and 2014 epidemics, respectively. The stem rust pathogen from the epidemics was characterized by a mixture of highly virulent races that showed slight differences in virulence from each other. TST was the most virulent race and were detected only in 2014. This race leaves only the *Pg-10* partially effective gene to the stem rust pathogen in South Brazil.

Key words: *Avena sativa,* virulence survey, physiologic race.

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Introduction

The cultivated oat belongs to a group of species with Mediterranean origin and well adapted to growing conditions of South Brazil. The hexaploide oat (*Avena sativa* L.) is grown for grain production while the black oat (*A. strigosa*) has been widely cultivated as forage for animal feed or as cover crop. The black oat occupies about 5 million ha every year in Brazil (Carvalho and Strack 2014). In 2016, the Brazilian oat production estimates was 827,800 tons of grain harvested in an area of 291,500 ha (Conab 2017). Both oat species are of major importance for crop rotation in the no-till crop system in South Brazil.

Stem rust, caused by the fungus *Puccinia graminis* Pers. f. sp. *avenae* Eriks. & Henn, is an important disease of oats around the world. The disease is characterized by pustules of several millimeters long formed by the rupture of the host epidermis from pressure of a mass of red-color urediniospores (Agrios 2005). Infections occur mainly on stems and on leaf sheaths, but occasionally they are found on the glumes and leaf blades as well (Agrios 2005). Fungicides application and genetic resistance are the main methods to control the disease. Genetic resistance is the preferred method, however usually the deployment of resistance genes into cultivars has no longer been effective due to the rapid pathogen evolution.

P. graminis f. sp. *avenae* is an heteroecious obligate biotrophic fungus that survive through asexual cycles in oats (the stem rust). The pathogen might complete the sexual cycle, infecting the alternate host barberry (*Berberis* spp.) where it is present. The sexual recombination is an important source of overwinter survival for the pathogen in extreme climates, contributing as a source of initial inoculum of stem rust in North Europe (Berlin et al. 2012), in Central Asia (Berlin et al. 2014) and North America (Peterson et al. 2005). Although sexual reproduction has not been reported for *P. graminis* in South America, the

pathogen can be maintained alive in volunteers and wild oats growing along roadsides and in non-cultivated field providing the initial inoculum for local epidemics (Martinelli 2004).

Rust virulence survey in cultivated grasses can be used to improve the efficiency of breeding rust resistant cultivars. Such surveys have been conducted regularly in North America (Fetch Jr. 2009) and in Australia (Park 2008) to early detect the novel races that could defeat the resistance genes in the growing cultivars, and are useful in the development of strategies based on informative anticipatory resistance breeding (McIntosh and Brown 1997; Kolmer 2005). A dynamic pathogenic variation was reported in Canada where the pathogen acquired virulence to most of the oat resistance genes (Martens and McKenzie 1979). Because there are a limited number of known resistance genes for stem rust, the rapidly evolving pathogen has led susceptible most of the cultivars in a wide growing area of Canada (Fetch Jr. 2009; Fetch Jr. et al. 2011). Similarly, in Australia no stem rust resistant cultivars have been released since the pathogen has acquired virulence to all known resistance genes (Park 2008). In South Brazil, virulence surveys were carried out during 1959-1972 (Coelho 1976). Such studies revealed a dynamic pathogenic variation along the years based on the infection type of six Pg-genes (*Pg-1*, *Pg-2*, *Pg-3*, *Pg-4*, *Pg-8*, *Pg-9*) and the cultivar Saia. The objective of this study was to characterize the virulence structure of the oat stem rust pathogen in two epidemics years in an oat breeding station in South Brazil.

Material and Methods

Sampling, isolation and multiplication of rust material

Rusted stems from different oat genotypes were collected in the Oat Breeding Program of the Universidade Federal do Rio Grande do Sul, located in Eldorado do Sul, RS, Brazil (30º05’27’’ S and 51º40’18’’ W) around 65 m above sea level. The samples

were collected during natural stem rust epidemics of 2013 and 2014. The samples were dried in a closed container with silica gel for at least 24 h and then stored in a refrigerator at 3-5 °C.

Methods used for single pustule isolation were as reported in Fetch Jr. (2009) with some modifications. Urediniospores from each sample were transferred to seedling leaves of a group of susceptible oat genotypes (UFRGS 035080, UFRGS 19 and UFRGS 04B7117-1). The inoculated seedlings were incubated in the dark for 16 h in a dew chamber. Plants were kept in the dew chamber under artificial light for at least 2 h to provide a period of slow drying and light. The plants were then transferred to a growth chamber at 20 ± 4 °C with a 14 h light/ 10 h dark photoperiod supplemented by artificial lighting. The pots containing inoculated seedling were individually covered with a plastic bottle to prevent cross-contamination of samples. At 10-15 days post inoculation, one single-pustule isolate was selected from each seedling pot, removing all the others pustules by cutting the leaf just above the selected pustule. The single pustule was incubated in a dew chamber, and the next day only spores from the selected pustule were transferred to a new group of seedling of the susceptible genotypes (UFRGS 035080, UFRGS 19 and UFRGS 04B7117-1). The process was repeated one time.

To increase the spores a new set of susceptible seedling plants were treated with the growth regulator maleic hydrazide following the protocol described by Liu et al. (2013). The plants at 0.5-1.5 cm tall were treated with 10 ml/ pot (5 cm diameter) of a maleic hydrazide solution at the concentration of 0.4 g/L. The spores were collected into gelatin capsules (00 size), and stored in -80 °C freezer.

Virulence phenotyping

For virulence phenotyping, 8-10 seeds of each single-gene differential line (Pg-1,

Pg-2, Pg-3, Pg-4; Pg-6, Pg-8, Pg-9, Pg-10; Pg-12, Pg-13, Pg-15, Pg-16) and the oat lines 'Pg-a', 'Omega', UFRGS 995088-3, UFRGS 03B7024-1, URS 21, and URS Tarimba were planted in 5 cm diameter pots filled with turf substrate (http://www.blumengarten.com.br). The inbred lines UFRGS 995088-3 and UFRGS 03B7024-1 are useful resistance source and a susceptible check of the breeding program, respectively. URS 21 and URS Tarimba represent commercial Brazilian oat cultivars. Mineral oil (Soltrol ® Isoparaffin, Chevron Phillips) was added to each gelatin capsule containing a single-pustule isolate. Seedlings at one leaf fully expanded stage were inoculated using a rust inoculator (G-R Manufacturing Company, Manhattan, Kansas) pressurized by an air pump to 20 kPa. Inoculated plants were incubated as described previously, then transferred to a growth chamber at 20 ± 4 °C with a 14 h light/ 10 h dark photoperiod supplemented by artificial lighting.

Seedling response assessments were made 12 days after the inoculation on the 0-4 infection type (IT) scale described by Stakman et al. (1962) where, IT=0 represents the lowest incompatible resistant reaction and IT=4 the fully compatible susceptible reaction. The physiological race of each isolate was determined by their reaction on the differential lines using the letter-code nomenclature for *P. graminis* f. sp. *avenae* (Fetch Jr. and Jin 2007).

Results

At the oat-breeding station of Eldorado do Sul, the stem rust was first detected on August 30th in 2013, and on September 23rd in 2014. The IT of each differential line, oat breeding line or oat cultivar to all isolates collected during 2013 and 2014 epidemic years are presented in Table 1. Susceptible IT to all isolates, including 2013 and 2014 collections, were identified among the differential lines *Pg-1*, *Pg-2*, *Pg-4*, *Pg-8* and *Pg-15* and for the line UFRGS 03B7024-1. A variation in the resistance IT (; to X-) was detected

for *Pg-3* to the isolates 1301, 1302, 1304 and 1401. *Pg-6* was effective to all isolates in 2013, but effective to only 33% of the 2014 isolates. The gene *Pg-10* conferred a unique and similar intermediate to high IT (IT = 2cn to 23+cn) to all isolates in both years. Resistance IT among the lines Pg-9, Pg-13, Pg-12, Pg-a, Omega and UFRGS 995088-3 was identified only in

Table 1 Infection type of the oat differential set lines and cultivars to 16 *Puccinia graminis* f. sp. *avenae* isolates obtained in Eldorado do Sul, RS, during 2013 and 2014 epidemics years.

Differential, inbred line or cultivar	Infection type[a] of single pustule isolates from 2013							Infection type of single pustule isolates from 2014								
	1301	1302	1303	1304	1305	1307	1308	1401	1402	1404	1405	1406	1407	1408	1409	1410
Pg-1	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Pg-2	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Pg-3	;1,X-	;1,X-	3+	;,X-	3+	3+	3+	;,X-	3+	3+	3+	3+	3+	3+	3+	3+
Pg-4	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Pg-6	;	;	;	;	;	;1+	;1-	3+	3+	;	3+	;	;	33+	3+	3+
Pg-8	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Pg-9	3+	3+	3+	3+	3+	3+	3+	;c	3+	3+	33+	3+	3+	3	3+	3+
Pg-10	2+cn	2+cn	2cn	2cn	2cn	23cn	23cn	23cn	23cn	2cn	23+cn	2cn	2cn	23cn	23+cn	23cn
Pg-12	3+	3+	3+	3+	3+	33+	3+	;	1+	3+	1c	3+	3+	3+	;1-c	3+
Pg-13	3+	3+	3+	3+	3+	3+	33+	;1	1+	33+	1c	3+	3+	3+	3+	3+
Pg-15	3	3+	3	3+	3	33+	33+	3+	3+	3-	3+	3	3	3+	3+	3+
Pg-16	1+	;1	1+c	;1	1++c	3+	3	1c	1++	1-c	3+	;1	;1	3	3	3+
Pg-a	3+	3+	3+	3+	3+	3+	3+	;n	;	3+	;n	3+	3+	3+	;cn	3+
omega	3+	3+	3+	3+	3+	3+	3+	;0	;c	3+	;n	3+	3+	3+	;cn	3+
UFRGS 995088-3	3-c	3-c	33-	3	3	3+	3+	;0	;c	3-	;n	3+	3+	3	;n	3+
UFRGS 03B7024-1	3+	3+	3+	3+	3+	3+	3+	3+	3+	3	33+	3+	3+	3+	3+	3+
URS 21	;1	;1-	3+	X-	3	3+	3+	;1-,X-	3+	3-	3+	3	3	3+	3+	3+
URS TARIMBA	;c	;1+	3+	;	3+	1+c	1+c	;1-	3+	3+	3+	3+	3+	3+	33+	3+

[a]Infection types were defined using a 0–4 scale (Stakman et al. 1923), where 0 = immune, no signs of disease; ; = hypersensitive flecks of dead host tissue present; 1 = tiny pustules with sharply defined hypersensitive flecking; 2 = small to medium-sized pustules with defined necrosis, sometimes showing green islands; 3 = medium-sized pustules, without hypersensitivity; and 4 = large pustules. The symbols + and – indicate slightly larger and smaller pustule sizes, respectively. The letters n and c indicate pronounced necrosis and chlorosis, respectively. The letter X indicates mesothetic reaction.

2014 to less than half of the isolates. The combination of IT found in UFRGS 995088-3 and URS 21 conformed to the presence of the genes *Pg-a* and *Pg-3*, respectively. URS Tarimba showed a unique pattern of IT combination, and probably carry an uncharacterized gene.

Based on seedling response, in 2013, three distinct physiologic races were found, including, RJS, TJS and TJT, with virulence to all Pg-genes except to *Pg-6* and *Pg-10* (Table 2). A wide pathogen variation was found among isolates from 2014, comprising six distinct races, including the race TST, which led only *Pg-10* partially effective. RJS was the most common race in 2013 with three out of seven isolates identified (Table 2). TJS with additional virulence to *Pg-3* was the most common race in 2014 comprising 33 % of the isolates found in this epidemic year.

Table 2 Races of *Puccinia graminis* f. sp. *avenae* obtained in Eldorado do Sul, RS, during 2013 and 2014 epidemic years.

Race[a]	Isolates (culture number)	Virulence/ avirulence to Pg-lines
2013		
RJS	1301, 1302, 1304	1, 2, 4, 8, 9, 12, 13, 15, a / 3, 6, 10, 16
TJS	1303, 1305	1, 2, 3, 4, 8, 9, 12, 13, 15, a / 6, 10, 16
TJT	1307, 1308	1, 2, 3, 4, 8, 9, 12, 13, 15, 16, a / 6, 10
2014		
RQD	1401	1, 2, 4, 6, 8, 15 / 3, 9, 10, 12, 13, 16, a
TSD	1402	1, 2, 3, 4, 6, 8, 9, 15 / 10, 12, 13, 16, a
TSF	1405	1, 2, 3, 4, 6, 8, 9, 15, 16 / 10, 12, 13, a
TJS	1404, 1406, 1407	1, 2, 3, 4, 8, 9, 12, 13, 15, a / 6, 10, 16
TSK	1409	1, 2, 3, 4, 6, 8, 9, 13, 15, 16 / 10, 12, a
TST	1408, 1410	1, 2, 3, 4, 6, 8, 9, 12, 13, 15, 16, a / 10

*Based on the letter-code nomenclature by Fetch and Jin (2007).

Discussion

All the isolates showed virulence to *Pg-1, Pg-2, Pg-4, Pg-8* and *Pg-15*. The absence of variation of virulence to these genes may be caused by the presence of these

genes in genotypes from the location of sampling (a breeding nursery), which led to a selection bias towards the virulence in the pathogen. It is also possible that the susceptible genotypes UFRGS 035080, UFRGS 19 and UFRGS 04B7117-1, used for single pustule isolation, carry some of these genes. They were chosen based on their susceptibility under field conditions, which does not mean they are universally susceptible genotypes. The frequency of virulence to *Pg-16* was low in both 2013 and 2014. In Canada, *Pg-16* has remained effective, but it has not been deployed into cultivars due to yield penalties (Gold Steinberg et al. 2005; Fetch Jr. 2009; Fetch Jr. et al. 2011). The low frequency of virulence to *Pg-16* among Brazilian isolates might be associated to the low frequency of this resistance gene in the host population. The frequency of virulence in *Pg-6*, *Pg-12*, *Pg-a* and *Pg-13* were quite different between 2013 and 2014, suggesting different sources for the first inoculum in each year. Both *Pg-a* (same IT of *Pg-12*) and *Pg-13* proved to be useful for oat breeding and have been deployed into cultivars of Australia and Canada (Adhikari et al. 2000; Fetch Jr. et al. 2011), and may be present in some of the Brazilian cultivars. The frequency of virulence for these resistance genes might be low in the original population, but dynamically changed in the pathogen population towards the virulence to the deployed resistance genes in the cultivars. The variation of *Pg-6* virulence could be explained by the presence of the diploid specie *A. strigosa*, the original and only known source of the gene *Pg-6* (Gold Steinberg et al. 2005; Fetch Jr. et al. 2011). This oat species has been widely grown in South Brazil for forage or as cover crop and it is usually not sprayed with fungicides (Federizzi and Mundstock 2004).

Based on the IT response of the international differential set three to six distinct races of *Puccinia graminis* f. sp. *avenae* were identified during the two epidemic years of stem rust at UFRGS oat breeding station. This race diversity is higher than the reported in a wide area of South Africa over two years, where there were present four distinct races

(Van Niekerk et al. 2001), and similar to the identified in three different regions of Canada in 2005, which comprised 3 to 8 races (Fetch Jr. 2009). The high virulence variation identified for only one location in the current study can be explained by a high genetic diversity on the host in respect to the resistance genes of the oat breeding germplasm, i.e. three Brazilian genotypes included in this study (UFRGS 995088-3, URS 21 and URS Tarimba) showed different IT combination suggestive of the presence of different resistance genes. The host genetic diversity may contribute to the presence of different adapted clonal races in the pathogen population.

Overall the two epidemic years were characterized by a mixture of races that showed slight differences in virulence spectrum from each other. The clonal-reproducing structure of the pathogen population with a single step mutation for virulence may explain this pathogenic variation, e.g., in 2013, the RJS race added virulence to *Pg-3* (TJS) and *Pg-16* (TJT). The clonal-reproducing structure is expected in South America where there is no reports of sexual reproduction of *P. graminis* (Chaves et al. 2008). The clonal-reproducing population structure occurs in Australia where a highly virulent pathotype, overcoming all Pg-genes was identified (Haque et al. 2008). In South Brazil, a wide spectrum of virulence and the occurrence of highly virulent races have been reported for others cereal rust pathogens, including the wheat leaf rust (*Puccinia triticina*) (Ordoñez et al. 2010) and the oat crown rust (*Puccinia coronata* f. sp. *avenae*) (Leonard and Martinelli 2005). Here we reported the occurrence of highly virulent races of *P. graminis* f. sp. *avenae*, including the TST with virulence to the majority of the known resistance Pg-genes. These results highlights the potential of the clonal-reproducing pathogen populations of Southern America in acquiring virulence to the host resistance genes.

The results found in the current study emphasize the need of search new and effective resistance genes to stem rust for the sustainability of oat production in South

Brazil. Breeding strategies must consider the combination of adult plant partial resistance genes as they are usually not associated to specific recognition of pathogen effectors and, thus, will not promote a rapid change in the pathogen population.

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4 ARTIGO 2

Development, characterization and application of genomic SSR markers for the oat stem rust pathogen *Puccinia graminis* f. sp. *avenae*[1]

Abstract

Oat stem rust, caused by the fungus *Puccinia graminis* f. sp. *avenae* (Pga), is one of the most severe diseases of oats worldwide. Population studies are scarce for this pathogen, mainly due to the lack of polymorphic molecular markers suitable for genetic analysis. We sequenced an Australian Pga isolate, determined the abundance of simple sequence repeats (SSRs), and developed PCR-based polymorphic markers suitable for genetic diversity analysis. The amplification of 194 primer pairs was initially assessed across a validation panel including 12 isolates of different cereal rust species and their *formae speciales*. A high cross-species amplification frequency was observed for most markers. A subset of 19 genomic-derived SSRs were deemed useful for genetic diversity analysis of Pga and were assessed on 66 Pga isolates from Australia, Brazil and Sweden. Brazilian and Australian isolates were characterized by one and two predominant clonal lineages, respectively. In contrast, the Swedish isolates previously shown to undergo sexual recombination were highly diverse (nine distinct genotypes out of 10 isolates) and divided into two sub-populations. The genomic-derived SSR markers developed in this study were well suited to the population studies undertaken, and have diagnostic capabilities that should aid in the identification of unknown rust pathogen species.

Key words: stem rust, microsatellite, population genetics, SSR transferability, *formae speciales*, *Avena sativa*

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Introduction

Puccinia graminis Pers. is an obligate biotrophic fungus that causes stem rust in grasses (Leonard & Szabo, 2005). *Puccinia graminis* is a complex species with distinct host-specialized *formae speciales* (f. sp.) that may undergo sexual reproduction on the alternate host barberry (*Berberis* spp.) in geographic regions where it is present. *P. graminis* f. sp. *avenae* (Pga), the causal pathogen of stem rust in oats, is one of the most destructive diseases of oat worldwide as it can cause total crop failure during severe epidemics. Although oat stem rust can be controlled using fungicide treatment, host genetic resistance is preferred. However, breeding oats with resistance to stem rust has been a challenge as few resistance genes are known and, in some regions (e.g. Australia; Park, 2008) virulence has been detected to almost all of them.

Understanding pathogen's population structure can help to predict and minimize the harmful impact of epidemics and assist breeding efforts in the quest for more durable resistance. Four main factors are known to influence and hence cause changes in cereal rust pathogen population structure, *viz*. sexual recombination (Peterson et al., 2005), somatic hybridization (Park et al., 1999), selection/mutation (Park et al., 2002) and genetic drift and exotic incursion (Zwer et al., 1992). The importance and effects of long-distance dispersal for rust species across continents are well known (Watson & De Sousa, 1983; Brown & Hovmøller, 2002). For example, in Australia, two independent exotic incursions of the wheat stripe rust pathogen (*P. striiformis* f. sp. *tritici*) have been recorded. The first was likely originated from Europe in 1979 and the second was likely from the USA in 2002, with the latter rapidly displacing the former to dominate the Australian *P. striiformis* f. sp. *tritici* population (Wellings, 2007).

Previous genetic diversity studies on Pga using sequence-tagged microsatellites (STMs) identified genetic variation among Australian Pga isolates was higher compared

with other cereal rust pathogens (Keiper et al., 2003; Keiper et al., 2006). Polymorphism among Australian isolates collected between 1974 and 1999 showed three and five distinct lineages or incursions based on AFLP and STM technology, respectively, with clear sub-population structure (Haque et al., 2008). It was however hypothesized that single step mutation was the most common mechanism by which Pga developed virulence to commonly deployed R genes in cultivated oats in conjunction with the build-up of inoculum in wild oat populations which commonly co-locate with cultivated oats (Keiper et al., 2006; Haque et al., 2008; Park, 2008). In contrast, the alternate host enables sexual recombination and contributes to overwintering in Pga populations in Asia, Europe and North America, giving rise to highly diverse Pga populations in these regions (McCallum et al., 2007; Berlin et al., 2012; Berlin et al., 2014).

The traditional method to monitor the genetic variation in the pathogenicity of cereal rust pathogens is to conduct virulence surveys using differential host genotypes, and defining the phenotypic pathotypes based on the virulence pattern (Fetch Jr. & Jin, 2007). Such surveys have proven valuable in both the detection of new pathotypes and in the development of informative anticipatory control strategies for breeding for rust resistance (McIntosh & Brown, 1997; Chen, 2005; Park, 2015). However, virulence markers are limited in number, are often under positive selection, and biases are introduced with the selective method of detection.

Molecular markers have been used for genetic differentiation, diagnostics and determination of population structure of numerous plant pathogens. Simple sequence repeats (SSRs) or microsatellites are tandem repeats and are hypervariable genome elements. They are highly reproducible co-dominant markers that are easy to use and have been extensively used in genetic studies of higher organisms (Putman & Carbone, 2014). A recent study by Ali et al. (2014) used SSR markers to genotype a worldwide collection of

P. striiformis f. sp. *tritici* (Pst) isolates and traced the center of origin for to the Himalayan region, where sexual recombination is known to occur. SSR markers have been used to confirm the likely mutational origin of several pathotypes of *P. graminis* f. sp. *tritici* (Pgt) in South Africa that are believed to be derived from Ug99, a highly virulent lineage of Pgt (Visser et al., 2011).

Although SSRs are presumed to be not associated with virulence, they have the ability to distinguish between pathotype groups and different *formae speciales* of cereal rust pathogens. Therefore, polymorphic SSR markers with cross-transferability both within and between rust taxa are valuable tools for identification and characterization of unknown disease samples and possible exotic pathogen incursions. In Sweden and Tajikistan, a selection of SSR markers developed for Pgt (Szabo, 2007; Zhong et al., 2009) was applied to successfully infer the importance of the alternate host barberry on the variability within Pga populations (Berlin et al., 2013, Berlin et al., 2014). Although these markers were highly informative using genetically diverse isolates from Asia and Sweden, they were largely monomorphic among isolates from geographic regions where there is no reported sexual recombination in which reproduction is considered to be primarily clonal, i.e. Australia and Brazil (H. Karaoglu and R.F. Park, unpublished results). We therefore developed and characterized SSR markers based on the genomic sequence of an Australian isolate of Pga. We present data demonstrating the utility and hence suitability of a collection of highly polymorphic Pga-derived SSR markers for both diagnostic and detailed pathogen population studies.

Material and Methods

SSR loci characterization

The genome size of the Pga isolate (PBIC culture no. 512, accession no. 962508) was estimated as 78.09 Mb based on a genome survey conducted by the Beijing Genomics Institute (BGI), China. A total of 4,900 Mb clean data, which have been filtered out the adapter and low quality sequences from the raw data, was accessed to detect different SSRs using a PYTHON-based program described by Karaoglu et al. (2005). The program uses a recursive algorithm to search for repeated nucleotides between 1 and 6 base pairs (bp) in length. SSR redundancy was excluded by counting only a single match when there was more than one record for the same SSR locus. SSRs were determined by the minimum length of 10 bp, which was equivalent to 10 repeats or more for mononucleotides and five or more repeats for di-, tri-, tetra-, penta- and hexanucleotides. For SSR abundance analysis, the motifs of different reading frames and complementary strands were grouped together, e.g. the motif class $(AG)_n$ includes $(AG)_n$, $(GA)_n$, $(TC)_n$ and $(CT)_n$. For simplicity, only the motif of one strand was shown.

The software Oligo7 (http://www.oligo.net/) was used to design the different combination of primers. The parameters of primer design included: between 18-21 bp in length, low probability of loop and hairpin formation, and the length of PCR product between 100-450 bp. To test the primers, a 10 µL PCR reaction was carried out using the final concentration of 1.4 ng/µL of genomic DNA, 0.7 ng/µL of each primer pair, 0.014 mM of dNTP, 3.5 mM of $MgCl_2$, 0.05 U/µL of BIOTAQ DNA Polymerase and 10× NH_4 Reaction Buffer (Bioline, Australia) according to the manufacturer's recommendation. The standard PCR procedure was as follows: initial denaturation at 95 °C for 4 min, then 35 cycles each of denaturation (30 s at 95 ºC), annealing (30 s at 54°C) and extension (30 s at

72°C), followed by a final extension (7 min at 72 °C). Two and a half microliters of 5x DNA loading buffer (Bioline) were added to each 10 µL PCR product, and 2 µL of the solution was loaded on 3 % agarose gels to visualize the amplicons.

Rust pathogen samples for SSR marker development

Five stem rust pathogen isolates that represent different *formae speciales*, including *P. graminis* f. sp. *avenae* (Pga), *P. graminis* f. sp. *tritici* (Pgt), *P. graminis* f. sp. *secalis* (Pgs), *P. graminis* f. sp. *tritici x secalis* (Pgts, the scabrum rust) and *P. graminis* f. sp. *phalaridis* (Pgp) and seven cereal rust pathogen isolates that represent different *Puccinia* species, including *P. striiformis* f. sp. *tritici* (Pst), *P. striiformis* f. sp. *pseudo-hordei* (Psph), *P. striiformoides* (Psd, the cocksfoot yellow rust), *P. striiformis* f. sp. *poeae* (Psp), *P. triticina* (Pt), *P. hordei* (Ph) and *P. coronata* f. sp. *avenae* (Pca) were used to assess the performance of the primer pairs for each SSR (Table 1). Genomic DNA from the 12 cereal rust isolates was extracted as described by Karaoglu and Park (2014). A further 66 Pga isolates from three geographic regions (Brazil, Australia and Sweden) were selected to assess the ability of a subset of the most polymorphic SSR markers to discriminate between diverse Pga isolates. For the Australian and Brazilian Pga isolates DNA was extracted from rust urediniospores increased from purified single pustules as described by Karaoglu and Park (2014). Field samples were collected in Sweden, inoculated on oats, and once disease symptoms were obvious after 12 days, multiple leaf segments with single pustules were sampled and DNA was extracted as described by Berlin et al. (2013).

Isolates from Australia represent all the distinct pathotypes identified between 1974 to 2000. The samples from Brazil were collected during two epidemic years from different oat lines of one research breeding station in Southern Brazil, and the samples from Sweden

were collected in one year from three different sites (Ingaberga, Pattala and Evertsholm) in the Southeastern Sweden (Table 2).

Table 1 *Puccinia* species used in the study.

Species	Host	Causal disease
Puccinia graminis f. sp. *avenae* (Pga)	*Avena sativa*	Oat stem rust
P. graminis f. sp. *tritici* (Pgt)	*Triticum aestivum*	Wheat stem rust
P. graminis f. sp. *secalis* (Pgs)	*Secale cereale*	Rye stem rust
P. graminis f. sp. *tritici x secalis* (Pgts)	*Agropyron scabrum*	Scabrum stem rust
P. graminis f. sp. *phalaridis* (Pgp)	*Phalaris* sp.	*Phalaris* stem rust
P. striiformis f. sp. *tritici* (Pst)	*Triticum aestivum*	Wheat yellow rust
P. striiformis f. sp. *pseudo-hordei* (Psph)	*Hordeum* sp.	Barley grass yellow rust
P. striiformoides (Psd)	*Dactylis glomerata*	Cocksfoot yellow rust
P. striiformis f. sp. *poeae* (Psp)	*Poa* sp.	*Poa* grass yellow rust
P. triticina (Pt)	*Triticum aestivum*	Wheat leaf rust
P. hordei (Ph)	*Hordeum vulgare*	Barley leaf rust
P. coronata f. sp. *avenae* (Pca)	*Avena sativa*	Oat crown rust

Identification of SSR markers for Pga population studies

The cross-amplification of 194 SSR loci was determined for each of the 12 isolates (species and *formae speciales*) on 3% agarose gels by considering the presence of bands with approximately the same size as the reference Pga isolate. Only the 171 primer pairs that showed amplification on the reference Pga isolate were selected to further assess polymorphism across a subset of 19 Pga isolates, comprising 13 from Australia, two from Brazil and four from Sweden. Based on the allelic diversity observed following gel electrophoresis, 19 selected markers were chosen to screen an expanded collection of 66 isolates of Pga from Australia, Brazil and Sweden to assess the suitability and utility of the SSR loci for genetic diversity analysis. Forward primers for each selected locus were 5′ labeled by incorporating the fluorophore dye FAM (Macrogen, Korea and Applied Biosystems, Australia).

Table 2 *Puccinia graminis* f. sp. *avenae* samples used in this study.

Culture no.	MLG	Virulence/ avirulence[a] to Pg-genes	Accession no.	Country/ region of origin	Year
228	1	2 / 1, 3, 4, 8, 9, Saia, 13, a	710110	Australia/ New South Wales	1971
229	1	2, 3 / 1, 4, 8, 9, Saia, 13, a	710112	Australia/ New South Wales	1971
238	1	2 / 1, 3, 4, 8, 9, Saia, 13, a	BC-3	Australia	?
241	1	1, 2 / 3, 4, 8, 9, Saia, 13, a	BC-3	Australia	?
245	1	1, 9, Saia / 2, 3, 4, 8, 13, a	710116	Australia/ New South Wales	1971
254	1	1, 2, 3, 9 / 4, 8, Saia, 13, a	770247	Australia/ New South Wales	1977
255	1	1, 3 / 2, 4, 8, 9, Saia, 13, a	770258	Australia/ New South Wales	1977
257	1	1, Saia / 2, 3, 4, 8, 9, 13, a	780034	Australia/ Victoria	1978
263	1	3 / 1, 2, 4, 8, 9, Saia, 13, a	780026	Australia/ South Australia	1978
276	1	3 / 1, 2, 4, 8, 9, Saia, 13, a	780313	Australia/ Victoria	1978
278	1	1, 3, 9, Saia / 2, 4, 8, 13, a	780144	Australia/ South Australia	1978
281	1	1, Saia / 2, 3, 4, 8, 9, 13, a	780553	Australia/ New South Wales	1978
286	1	1, 2, 4 / 3, 8, 9, Saia, 13, a	780890	Australia/ Queensland	1978
288	1	2, 3, 4 / 1, 8, 9, Saia, 13, a	781420	Australia/ New South Wales	1978
296	1	1, 3, 13 / 2, 4, 8, 9, Saia, a	781415	Australia/ New South Wales	1978
299	1	1, 2, 3, 4 / 8, 9, Saia, 13, a	780933	Australia/ Queensland	1978
310	1	1, 2 / 3, 4, 8, 9, Saia, 13, a	780256	Australia/ Queensland	1978
375	1	1, 2, 3, 4, 13 / 8, 9, Saia, a	810815	Australia/ Western Australia	1981
377	1	/ 1, 2, 3, 4, 8, 9, Saia, 13, a	810608	Australia/ New South Wales	1981
395	1	1, 3 / 2, 4, 8, 9, Saia, 13, a	BC-2W	Australia/ New South Wales	?
404	1	1, 2, 3, 4 / 8, 9, Saia, 13, a	820625	Australia/ Western Australia	1982
405	1	1, 2, 4 / 3, 8, 9, Saia, 13, a	820917	Australia/ Victoria	1982
409	1	/ 1, 2, 3, 4, 8, 9, Saia, 13, a	820593	Australia/ New South Wales	1982
463	1	4, 9, 13 / 1, 2, 3, 8, Saia, a	882945	New Zealand	1988
277	2	1 / 2, 3, 4, 8, 9, Saia, 13, a	780187	Australia/ New South Wales	1978
396	2	2, 4 / 1, 3, 8, 9, Saia, 13, a	BC59	Australia	?

Culture no.	MLG	Virulence/ avirulence[a] to Pg-genes	Accession no.	Country/ region of origin	Year
237	3	3 / 1, 2, 4, 8, 9, Saia, 13, a	BC-1	Australia	?
86	4	3 / 1, 2, 4, 8, 9, Saia, 13, a	730147	Australia/ New South Wales	1973
246	4	1, 2 / 3, 4, 8, 9, Saia, 13, a	770098	Australia/ Queensland	1977
432	4	3, 13 / 1, 2, 4, 8, 9, Saia, a	842589	Australia/ Queensland	1984
230	5	1, 3 / 2, 4, 8, 9, Saia, 13, a	BC-2	Australia	?
244	6	2, 3, 4 / 1, 8, 9, Saia, 13, a	720091	Australia/ New South Wales	1972
362	7	Saia / 1, 2, 3, 4, 8, 9, 13, a	800459	Australia/ Western Australia	1980
443	8	3, 4, 8 / 1, 2, 9, Saia, 13, a	853049	Australia	1985
453	8	4, 13 / 1, 2, 3, 8, 9, Saia, a	872731	Australia	1987
243	9	2, 4 / 1, 3, 8, 9, Saia, 13, a	R11AC22	Australia	?
462	10	2, 3, 4, 13 / 1, 8, 9, Saia, a	882966	New Zealand	1988
497	11	1, 2, 3, 4, 8, 9, 13 / Saia, a	923034	Australia/ New South Wales	1992
496	12	1, 2, 3, 4, 8, 9, Saia / 13, a	923117	Australia/ Victoria	1992
512	12	1, 2, 3, 4, 8, 9, Saia, 13, a /	962508	Australia/ Queensland	1996
546	13	1, 2, 4, 8, a / 3, 9, Saia, 13	992630	Australia/ Victoria	1999
1301	14	1, 2, 4, 8, 9, 12, 13, 15, a / 3, 6, 10, 16	NA	Brazil/ South	2013
1302	14	1, 2, 4, 8, 9, 12, 13, 15, a / 3, 6, 10, 16	NA	Brazil/ South	2013
1303	14	1, 2, 3, 4, 8, 9, 12, 13, 15, a / 6, 10, 16	NA	Brazil/ South	2013
1304	14	1, 2, 4, 8, 9, 12, 13, 15, a / 3, 6, 10, 16	NA	Brazil/ South	2013
1305	14	1, 2, 3, 4, 8, 9, 12, 13, 15, a / 6, 10, 16	NA	Brazil/ South	2013
1307	14	1, 2, 3, 4, 8, 9, 12, 13, 15, 16, a / 6, 10	NA	Brazil/ South	2013
1308	14	1, 2, 3, 4, 8, 9, 12, 13, 15, 16, a / 6, 10	NA	Brazil/ South	2013
1309	14	NA	NA	Brazil/ South	2013
1401	15	1, 2, 4, 6, 8, 15 / 3, 9, 10, 12, 13, 16, a	NA	Brazil/ South	2014
1404	16	1, 2, 3, 4, 8, 9, 12, 13, 15, a / 6, 10, 16	NA	Brazil/ South	2014
1405	17	1, 2, 3, 4, 6, 8, 9, 15, 16 / 10, 12, 13, a	NA	Brazil/ South	2014
1406	14	1, 2, 3, 4, 8, 9, 12, 13, 15, a / 6, 10, 16	NA	Brazil/ South	2014

Culture no.	MLG	Virulence/ avirulence[a] to Pg-genes	Accession no.	Country/ region of origin	Year
1407	14	1, 2, 3, 4, 8, 9, 12, 13, 15, a / 6, 10, 16	NA	Brazil/ South	2014
1408	14	1, 2, 3, 4, 6, 8, 9, 12, 13, 15, 16, a / 10	NA	Brazil/ South	2014
1410	14	1, 2, 3, 4, 6, 8, 9, 12, 13, 15, 16, a / 10	NA	Brazil/ South	2014
1.3	18	NA	NA	Sweden/ Southeastern	2014
1.7.2	19	NA	NA	Sweden/ Southeastern	2014
2.1.2	20	NA	NA	Sweden/ Southeastern	2014
2.10.2	21	NA	NA	Sweden/ Southeastern	2014
2.3.2	22	NA	NA	Sweden/ Southeastern	2014
2.4.2	23	NA	NA	Sweden/ Southeastern	2014
2.6.2	24	NA	NA	Sweden/ Southeastern	2014
2.7.2	24	NA	NA	Sweden/ Southeastern	2014
3.1.2	25	NA	NA	Sweden/ Southeastern	2014
3.9.2	26	NA	NA	Sweden/ Southeastern	2014

NA: not available, MLG: multilocus genotype

[a] Virulence/ avirulence formulae based on Australian differential set, including Pg-1, Pg-2, Pg-3, Pg-4, Saia, Pg-8, Pg-9, Pg-13 and Pg-a. For Brazilian isolates the virulence/ avirulence formulae was based on the lines Pg-1, Pg-2, Pg-3, Pg-4, Pg-6, Pg-8, Pg-9, Pg-10, Pg-12, Pg-13, Pg-15, Pg-16 and Pg-a.

For the genotyping of the selected 66 Pga samples, a 30 µL PCR reaction was carried out using the same protocol as above. The alleles for each SSR locus were scored either manually or correct allele sizes were determined automatically using the Peak Scanner Software v1.0 (Applied Biosystems). Distinct multilocus genotypes (MLG), i.e. an isolate or group of isolates with a unique combination of alleles, were detected using the software GENCLONE (Arnaud-Haond & Belkhir, 2007). The polymorphic information content (PIC) was calculated for each marker across distinct MLG (clone-corrected data set) using the formula: $PIC = 1 - \sum_{i=1}^{l} P_i^2 - \sum_{I=1}^{L-1} . \sum_{j=i+1}^{l} 2P_i^2 P_j^2$, where P_i and P_j are the population frequency of the *i*th and *j*th allele, respectively (Nagy et al., 2012). Estimates of heterozygosity for the 19 loci among 26 MLG were determined using the software GenAlEx6.4 (Peakall & Smouse, 2012).

Pga genetic diversity

Individual genotypes were identified by combining the data for co-dominant polymorphisms generated by each of the 19 genomic-derived SSR primer pairs. The number of different alleles and the percentage of polymorphic loci across the different countries were computed for the clone-corrected data set (distinct MLGs only) using GenAlEx6.4 (Peakall & Smouse, 2012). A Principal Coordinate Analysis (PCoA), which does not rely on the assumptions of random mating or linkage equilibrium, was performed using a genetic distance matrix in GenAlEx6.4 (Peakall & Smouse, 2012).

In order to infer the common ancestry, population structure was determined for the clone-corrected data set using the model-based Bayesian method implemented in Structure 2.3.4 software assuming that the population is in Hardy-Weinberg equilibrium (panmixia) (Pritchard et al., 2000). The parameters of the project were set as: (1) Admixture model (assuming that individuals may have part of the genome from each of the K populations)

and independent allele frequencies among populations; and (2) run length was given as 100,000 burning period length, followed by 200,000 Markov Chain Monte Carlo (MCMC) replications. Each K value was run 8 times with K value varying from 1 to 10. The true value of K was set according to the maximal value of log Pr(K)/K by plotting the log Pr(K) estimated against the given K value (Supplemental Figure S1). The Structure groups were visualized in a bar graph. Based on the Structure results, each isolate was assigned to one of the genotypic clusters. If a sample showed a hybrid of clusters, then the sample was assigned to the dominant cluster.

Results

Detection and classification of genomic SSR loci

Approximately 4,900 Mb of genomic DNA from Pga urediniospores was examined for the presence of SSR arrays. A total of 34,185 perfect SSRs (sequences with a minimum of 10 bp containing only the SSR repeat) were identified, with an average relative frequency of 1 SSR per 2.28 Kb within clean error free genomic sequence alignments (Table 3). Mononucleotides were the most frequent repeat in the genome, representing more than 66 % of all SSR motifs. Di- and trinucleotides were the second and third most abundant, comprising 10.9 % and 19.5 % of total SSR arrays, respectively. Others SSRs, including tetra-, penta- and hexanucleotides each comprised less than 1 % of total SSRs identified within the Pga genome. The A/T motif was the most frequent identified, representing approximately 80 % of all mononucleotide SSR loci (Table 3). The AG motif was the most frequent among dinucleotides (51.3 %), tetranucleotides (23.9 %) and pentanucleotides (26.5 %). Motifs containing only CG were the lowest class in mono-, di-, and trinucleotides and were absent in tetra-, penta- and hexanucleotides. The longest SSR was a mononucleotide A, which was 570 bp long (Table 3). Among mono-, di- and

Table 3 Abundance and characteristics of SSRs in the *Puccinia graminis* f. sp. *avenae*.

Type	Number	Frequency [a]	Motif class	Motif number	% of motif class	Longest [b]
Mono	22609 (66.14 %)	3.45	(T/A)n	18469	81.7	A (570)
			(C/G)n	4140	18.3	G (89)
Di	7200 (21.06 %)	10.85	(AG/TC)n	3694	51.3	GA (19)
			(AC/TG)n	1794	24.9	TG (18)
			(AT/TA)n	1682	23.4	AT (30)
			(CG/GC)n	30	0.4	CG (8)
Tri	4013 (11.74 %)	19.46	(AAC/TTG)n	909	22.7	TGT (52)
			(AAG/TTC)n	888	22.1	AGA (18)
			(ATC/TAG)n	525	13.1	TAG (13)
			(ATG/TAC)n	473	11.8	CTA (63)
			(CCT/GGA)n	382	9.5	GAG (13)
			(ACC/TGG)n	337	8.4	TGG (12)
			(ATT/TAA)n	188	4.7	ATT (53)
			(CTG/GAC)n	143	3.6	TGC (17)
			(AGC/TCG)n	134	3.3	TCG (11)
			(CGG/GCC)n	34	0.8	GCG (7)
Tetra	276 (0.81 %)	282.93	(AAAG)n	66	23.9	AAAG (15)
			(AAAT)n	37	13.4	AAAT (7)
			(AAAC)n	36	13.0	CAAA (7)
			(CCTT)n	21	7.6	GGAA (20)
			(AACC)n	17	6.2	ACCA (7)
			(AATC)n	15	5.4	ATCA (6)
			(ACAT)n	11	4.0	TATG (7)
			(ACTC)n	9	3.3	GAGT (7)
			(ATTG)n	8	2.9	TGAT (6)
			(ATAG)n	7	2.5	TAGA (59)
			Others	49	17.8	-
Penta	49 (0.14 %)	1593.67	(AAAAG)n	13	26.5	AAGAA (17)
			(AAAAC)n	9	18.4	TTGTT (6)
			(AAAAT)n	3	6.1	AAAAT (8)
			(AAAGG)n	3	6.1	AAAGG (5)
			Others	21	42.9	-
Hexa	38 (0.11 %)	2055	(TAGGGT)n	6	15.8	TAGGGT (5)
			(ACCCAA)n	3	7.9	ACCCAA (6)
			(CACCAA)n	3	7.9	CACCAA (7)
			Others	26	68.4	-
Total	34185 (100 %)	2.28				

[a] total genome size (78,090 Kb) divided by the number of SSR.
[b] number in the brackets indicate the number of repeats.

trinucleotides, the length of the SSR was associated with low frequency of C/G content, e.g. the CG motif was no longer than eight repeats and CGG no longer than seven repeats.

SSR marker cross-amplification in other *Puccinia* taxa

The extent of polymorphism amongst SSR loci was positively correlated with the number of repeats within a SSR. Consequently, the longest 194 potential SSR loci were selected with suitable flanking sequences for primer design, included 39 di-, 84 tri-, 11 tetra-, 32 penta- and 28 hexanucleotides. These 194 SSR loci were initially tested using the genome sequenced isolate Australian Pga isolate 512. Using standard PCR conditions, 171 (88 %) of the SSR markers amplified products with the expected size (Figure 1). To investigate the transferability of the SSR markers across different *formae speciales* and species, 12 distinct *Puccinia* rust isolates were assessed using the 194 SSRs (Table 1). Across the 194 primer pairs for each SSR marker, the percentage of cross-*formae speciales* amplification was higher than that of the cross-species amplification, indicating a clear correlation with inferred phylogenetic distance between the *Puccinia* taxa and Pga (Figure 1). For instance, as expected the highest cross transferability was to other *formae speciales* of *P. graminis* that were closely related to Pga. Based on the percentage of amplification, stem rust collected from *Phalaris* sp. (Pgp, 87.1 %) followed by the "scabrum" stem rust (Pgts, 74.2 %) were the most closely related to Pga. Among the different *Puccinia* species, nearly half of the SSR markers amplified DNA extracted from the barley leaf rust (*P. hordei*) and oat crown rust (*P. coronata*) pathogens. The examined isolates of *P. striiformis* were more distantly related to Pga based on the lower cross transferability frequency of SSR markers, with Pst (24.7 %), Psph (24.7 %) and Psp (24.2 %) being the least related (Figure 1). Interestingly, nearly twice the number of SSRs amplified fragments from the cocksfoot stripe rust (Psd) pathogen relative to other stripe rust

pathogens included in this study.

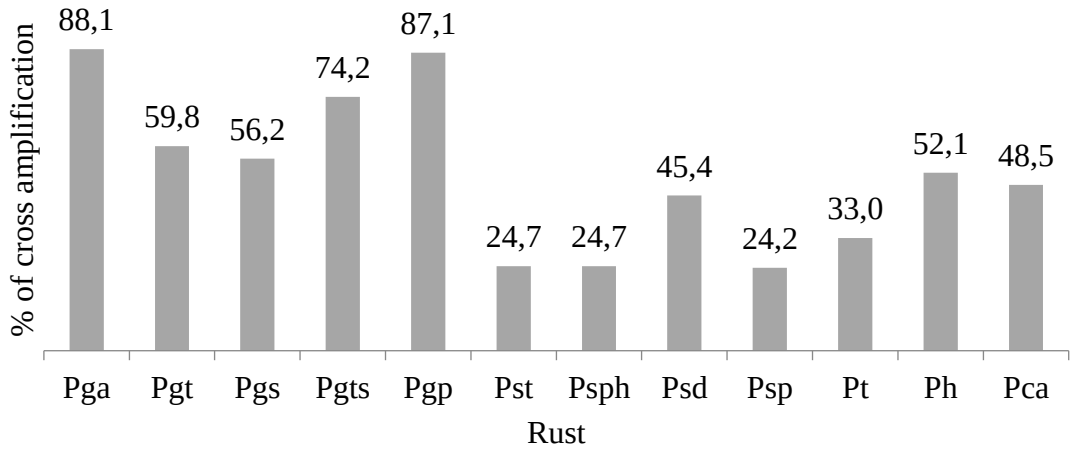

Figure 1 Percentage of loci amplified across different *formae speciales* and *Puccina* species using SSRs developed from *Puccinia graminis* f. sp. *avenae*. Pga: *P. graminis* f. sp. avenae, Pgt: *P. graminis* f. sp. *tritici*, Pgs: *P. graminis* f. sp. *secalis*, Pgts: *P. graminis* f. sp. *tritici x secalis*, Pgp: *P. graminis* f. sp. *phalaridis*, Pst: *P. striiformis* f. sp. *tritici*, Psph: *P. striiformis* f. sp. *pseudo-hordei*, Psd: *P. striiformoides*, Psp: *P. striiformis* f. sp. *poeae*, Pt: *P. triticina*, Ph: *P. hordei*, Pca: *P. coronata* f. sp. *avenae*.

Evaluation of SSR markers for Pga genetic diversity analysis

The further selection of SSR markers for detailed Pga population analysis was based on two amplification criteria: efficiency (expressed as the proportion of isolates showing clear amplification products), and polymorphism using a diverse panel of Pga isolates, which resulted in the 19 selected markers shown in Table 4. Using the clone-corrected data set (26 MLGs) the number of alleles per locus varied from three to nine with PIC values ranging from 0.40 to 0.79 (Table 4). Levels of observed heterozygosity (H_O) ranged from 0.218 to 1.000 and the expected heterozygosity (H_E) under random mating ranged from 0.288 to 0.628. A majority of the loci (63%) showed higher levels of observed heterozygosity (H_O) than the expected heterozygosity (H_E).

Table 4 Motif repeat, primer sequence, number of alleles (N), size range of amplicon (bp), polymorphic information content (PIC), observed (H_O) and expected (H_E) heterozygosity of 19 *Puccinia graminis* f. sp. *avenae* SSR markers.

Locus	Motif	Primer sequences	N	Size range	PIC[a]	H_O[a]	H_E[a]
F1-20	(AAC) 11	F:CCACCGCTGAAAAAAAAAAAA R:GATCAGTTCGGGAGTGTTAGG	11	154-189	0.82	0.792	0.670
F2-02	(TTC) 11	F:TTTGGGCTTTCTTGGTTTC R:CGAGGAACAACTACTAACA	9	269-308	0.78	0.974	0.645
F2-03	(TTC) 12	F:CGAGAAGTCCGAATGCCTCAG R:TCATCCCATCCGCTCATTTTT	6	247-276	0.64	0.285	0.316
F2-08	(GTT) 17	F:TCGGCTTCCTTCTCTTCAGCA R:GCTCCTCCGGCGTATCCAGTC	5	208-223	0.54	0.218	0.303
F2-12	(TTG) 11	F:CGAGGAGGATGATGTAGAGTT R:CGCTTCATCATCCTCATCATT	7	210-260	0.70	1.000	0.593
F2-17	(TGT) 23	F:AGTTTAGATCCAGTTCGTATT R:CTCGATCATCAAGTCTTTTCT	7	204-248	0.48	0.623	0.459
F2-42	(AAAAT) 10	F:GAGTGAGATGAAAATGTAGAA R:CTCACCTCCAAACTAACAAAA	9	238-273	0.72	0.507	0.616
F3-04	(TAATC) 6	F:GGAGGGAGGGTAAGGAGATGT R:AAAGCAAAAACATCAAAGAC	4	198-209	0.64	0.682	0.565
F3-09	(TTTGT) 14	F:AGGCTTGCATCCTCATAAGAC R:ACACATCATCACATCCCTAAC	10	147-231	0.72	0.489	0.499
F3-33	(ATTTTG) 5	F:TGGGCTTTTTTCTTAGGTCGT R:CAAGCAGAAGGAGACAGAGGA	3	205-217	0.39	0.322	0.287
F3-46	(GAACAA) 5 + (AACCAA) 6	F:ACCACCACATTACACAAAAAG R:CAGCCGTGAGTAGTTGATGGT	9	164-187	0.79	0.451	0.445
F4-22	(GA) 15	F:TTGGGTTTTTGGGTTCTTTTC R:CGTCTCCTTTTATACCTTATC	9	254-287	0.74	0.265	0.429
F4-26	(GA) 15	F:ACTCGTTGAATCGTTATTGC R:CTCGGGTTTGGTTTTTTCCT	6	257-304	0.72	0.424	0.449
F4-33	(GA) 19	F:ATCCCGTCCCAAAACAAGTAG R:GGGCCTTGGAGACGATGTTAG	7	182-207	0.69	0.432	0.474
F5-02	(TTG) 10	F:TGCCATTTCTTCCCTCTACCC R:AGCCCCAGCCTCAGTCGGTTC	5	240-261	0.42	0.444	0.357
F5-27	(TTCT) 10	F:AGGCTGATGACGATGAATGAA R:TGTGGATAGCGACTGTAAACT	5	272-310	0.43	0.484	0.378
F5-35	(TAAAA) 7	F:TTTGACTTTTTAGGGGATTTG R:CTCACCTCCAAACTAACAAAA	7	204-234	0.67	0.521	0.420
F5-40	(TTTGAA) 5	F:TGGTGGGTTTGTTGGTATGT R:AGTGGCAGATTTATGTTTTC	4	327-343	0.55	0.468	0.433
F5-46	(ACCCAA) 6	F:AACAGCACGGAAAGAAGAC R:GAGGCGTAGTTGTTTGGTT	8	242-262	0.80	0.872	0.610

[a] PIC, H_O and H_E were calculated using the 26 distinct MLG detected by the software GENCLONE.

Genetic diversity within Pga

A total of 19 SSR markers were used to assess genetic variation among a group of isolates representing three distinct countries: Australia, Brazil and Sweden. The SSR

markers were highly polymorphic in the Swedish isolates, and detected nine unique genotypes among the 10 isolates (Table 5). Within the presumed clonally reproducing populations of Australia and Brazil, a lower level of genetic differentiation was observed, with 13 MLGs from 41 isolates in Australia, and four MLGs from 15 isolates in South Brazil (Table 5). Allelic diversity varied from 4.05 in Swedish isolates to 1.79 in Brazilian isolates (Table 5). All 19 SSR markers were polymorphic for Australian and Swedish isolates, but only 63% were polymorphic among Brazilian isolates.

Table 5 Population statistics of 19 *Puccinia graminis* f. sp. *avenae* SSR markers across three different countries.

Origin	N	MLG	N_a	Polymorphic Loci (%)
Australia	41	13	3.32	100
Brazil	15	4	1.79	63.2
Sweden	10	9	4.05	100
Total	66	26	6.74	100

N: number of isolates, MLG: multi locus genotype, N_a: number of different alleles.

PCoA was used to characterize sub-groups among the tested isolates. A two-dimensional scatter plot involving all isolates showed that the first two PCoA axes accounted for 39 and 20 % of the genetic variation in the SSR data, respectively (Figure 2). The low diversity among Brazilian isolates and in the major group of Australian isolates was indicated by their tight clustering in the PCoA plot (Figure 2). A second group of isolates were identified among the Australian MLGs 12 and 13. The Australian MLG 11 was separated apart from both Australian sub-populations but in the third PCoA axis it remained closer to the MLGs 12 and 13 (Figure 2, lower panel). A clear separation between Brazilian and Australian sub-populations was identified in the third PCoA axis. The Swedish isolates appeared to be more diverse, with a diffuse clustering and a tendency toward two clusters.

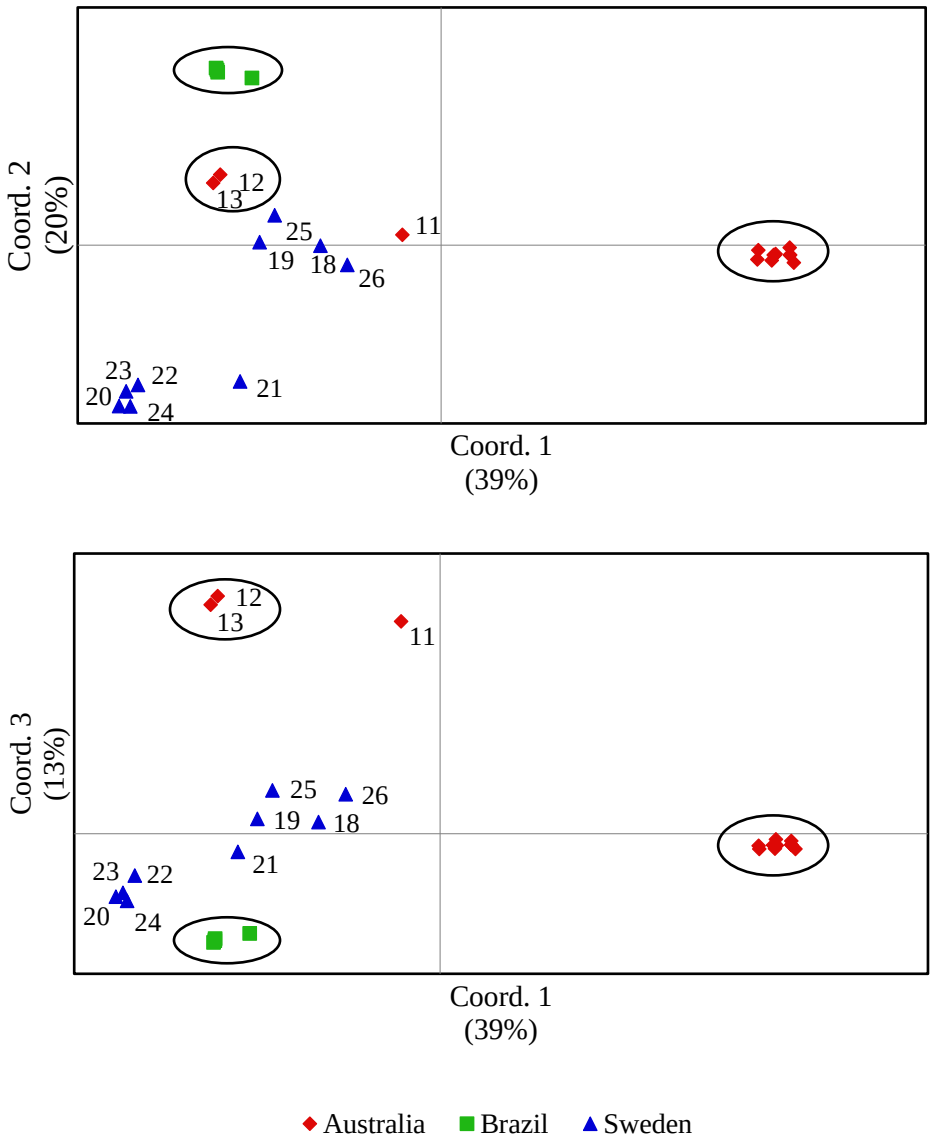

Figure 2 Principal coordinate analysis (PCoA) using 26 distinct multilocus genotypes (MLG 1 to 26, Table 2) of *Puccinia graminis* f. sp. *avenae* from Australia, Brazil and Sweden. Coordinate 1, 2 and 3 explain 39 %, 20 % and 13 % of the genetic variation between MLGs, respectively. Circles delimit the major clonal sub-populations in Australia and Brazil.

A clustering pattern was identified by the Structure analysis. Both Australian and Brazilian isolates showed a clear clonal population structure with discrete variation between isolates, except for the Australian MLG 11, which showed a pattern suggestive of a hybrid origin (Figure 3). Using ΔK, an ad hoc statistic based on the rate of change in the log probability of data between successive K values (Evanno et al. 2005), a K=2 was

identified, which suggested that the isolates were broadly divided into two sub-populations (Figure 3). As the maximum Log probability of data as a function of K/Log P(K) was identified at K=5 (Figure S1), we also investigated the population structure using K=3 to 5. Using K=3 and 4, the distinctness of Brazilian and Swedish populations from both Australian sub-populations became evident. A clear separation of Swedish samples into two sub-populations was seen only at K=5 (Figure 3).

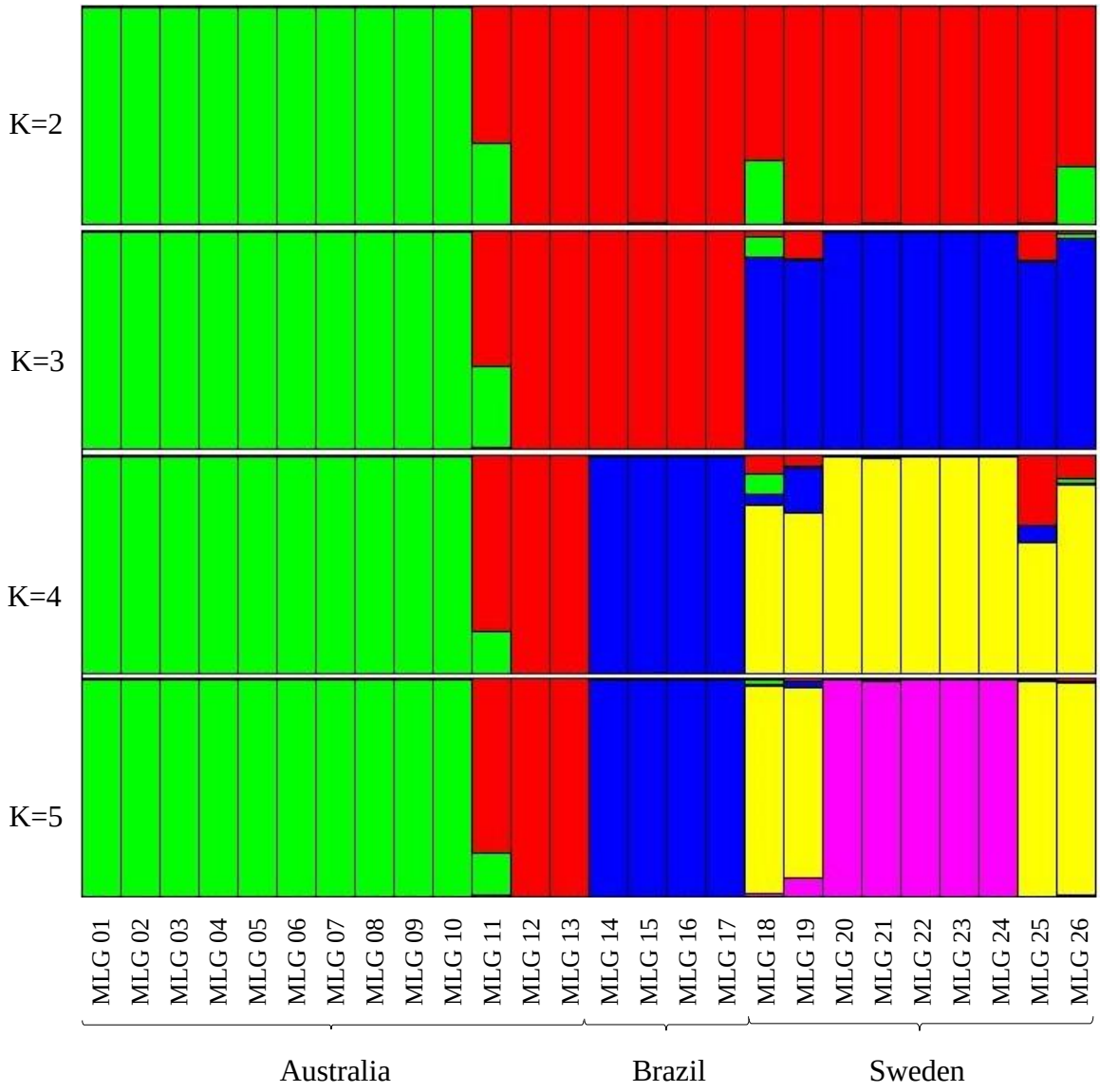

Figure 3 Population structure analysis of 26 distinct multilocus genotypes (MLG 1 to 26, Table 2) of *Puccinia graminis* f. sp. *avenae* isolates from Australia, Brazil and Sweden based on analysis performed in Structure software using (a) K=2, (b) K=3, (c) K=4 and (d) K=5 populations.

Discussion

Due to their abundance, ease of use, and high level of polymorphism, SSR markers are a valuable tool for genetic studies of plant pathogens. Using a genome sequence of an Australia isolate of Pga, we developed 19 SSR markers that were polymorphic, and demonstrated the utility for population studies by assessing Pga isolates from three diverse growing regions. We also demonstrated their diagnostic capabilities by comparing the specificity of the SSR markers to different cereal rust pathogen species and *formae speciales*.

We analyzed the abundance of 10 bp or longer perfect SSR motifs using a 78.09 Mb genome sequence of an Australian Pga isolate as a reference. The size and abundance of SSR loci within the Pga genome were similar to that reported previously for Pgt. The motif abundance was also similar to that reported by Karaoglu et al. (2013), with the exception of the lower frequency of tetra-, penta- and hexanucleotide repeats identified in the present study. In order to measure the polymorphism of the Pga SSR markers, the PIC value was calculated for each of the 19 markers. PIC takes into consideration both the number of alleles for a given locus and the relative frequency of these alleles. The SSR loci displayed a wide range of PIC values, with an average of 0.65 revealing a high level of polymorphism of the SSR loci among Pga isolates from populations regarded as being either clonal or sexually recombining. For the majority of the SSR loci, the observed heterozygosity (H_O) was higher than the expected (H_E) under random mating, which reflects the predominance of the asexual mode of reproduction.

The SSR markers developed and tested in this study were useful for diagnostic applications both between *formae speciales* of the same *Puccinia* species and differentiation between *Puccinia* taxa. A high cross-amplification frequency was observed, ranging from 24 % to 52 % within *Puccinia* species and from 56 % to 87 % within

different *formae speciales* of *P. graminis*. The variations in cross-amplification are explained by the high intra-species relatedness and the high sequence similarity, especially between *formae speciales* of the same species. Sequence similarity between different *formae speciales* of *P. graminis* previously contributed to the utility of Pgt markers in Pga population studies in both Sweden (Berlin et al., 2013) and Central Asia (Berlin et al., 2014). The extremely high (99 % of markers that amplified on Pga) cross-amplification observed for the Pga-derived SSR markers on the *P. graminis* isolate collected from *Phalaris* sp. suggested that it may be either Pga or a very closely related *forma specialis*. This isolate was previously named *P. graminis* f. sp. *phalaridis*, based on its host (*Phalaris canariensis*), and was able to infect some hexaploid and diploid oat genotypes but not wheat (Park, 1996). Although the extent of polymorphism was not determined in other rust pathogens, the markers revealed allelic diversity between different rust taxa and they are likely to be useful for diagnostic *Puccinia* spp. collected from wild grasses.

The Pga SSR markers developed in this study were evaluated across genetically diverse Pga populations from Australia, Brazil and Sweden to determine their suitability for genetic studies of populations undergoing both clonal and sexual Pga reproduction. Although the sampling selection was limited, the clustering of Swedish isolates using both PCoA and Structure showed a separation of the Swedish isolates into two clusters, confirming previous results that showed that the oat stem rust pathogen in Sweden was divided into two primary sub-populations (Berlin et al., 2012). In Australia and Brazil, sexual recombination is considered to be absent and clonal structures are therefore expected. However, we found one Australia isolate (MLG 11) that was not clustering with any clonal sub-population of Australia in PCoA and showed a hybrid pattern in Structure analysis. Based on both grouping analyzes it was evident some relatedness of this isolate to the MLGs 12 and 13. However, it is not possible to conclude about the origin of the

differentiation of this isolate apart from the sub-population and further studies are recommended to pursue this task. The concordance of grouping between PCoA and Structure analyses supported the distinctness of the clonal populations in Brazil and Australia.

Although different species of barberry are present South America (Landrum, 1999), there is no sign of sexual reproduction of *P. graminis* in the continent (Chaves et al., 2008). Instead, the low genetic variation observed among isolates collected in two years in South Brazil suggested that a single lineage was present and over-summer survival on wild species or volunteer oats must have provided the inoculum for annual epidemics.

The Australian isolates used in this study represented all pathotypes of Pga identified in annual pathogenicity surveys at the University of Sydney Plant Breeding Institute from 1974 to 2000, based on the responses of nine differential Pg-genes. The SSR marker analyses showed that only one genotype (MLG 1) was associated with many pathotypes. The lack of association between molecular markers and virulence patterns in phenotypes was evident in a previous study of Pga in Australia (Keiper et al., 2006), and is not surprising as different evolutionary forces drive changes in avirulence genes than in the genome as a whole. An exception was virulence to *Pg-a*, which was found only in one sub-population of Australia. Interestingly, the resistance gene *Pg-a* (Adhikari et al., 1999) remained effective until 1996 when the first *Pg-a* virulent pathotype was identified in Queensland, just three years after that sub-population was detected. The *Pg-a* virulence is attributed to selection in the pathogen after the deployment of cultivars with *Pg-a* resistance including, Culgoa II and Nobby in 1991 (Park, 2008). However, the significance of a new incursion on the loss of effectiveness of *Pg-a* in Australia needs to be considered. Because only two isolates used in this study are virulent on *Pg-a*, a further investigation using more isolates will show if all *Pg-a* virulent pathotypes belong to the same sub-

population in Australia and the contribution of each clonal population in recent epidemics in Australia.

The SSR markers developed in this study are suitable for population studies inferring genetic recombination and overseas migration for Pga. Moreover, the cross-amplification among different *formae speciales* makes them useful for rust identification in wild grasses or for related rust species.

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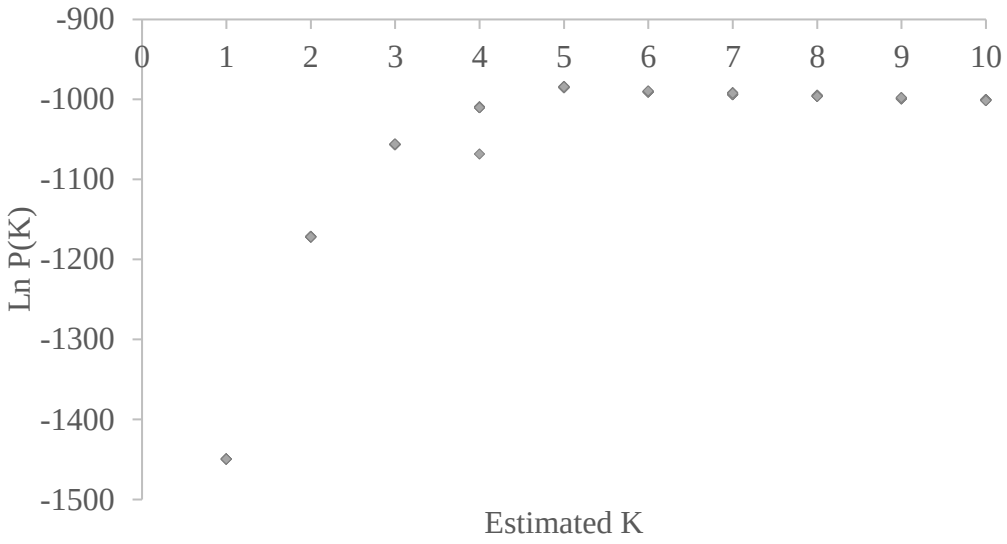

Figure S1 Log probability of K (Ln P(K)) as a function of K in the Structure software analysis of 26 distinct multilocus genotypes (MLG 1 to 26, Table 2) of *Puccinia graminis* f. sp. *avenae* from Australia, Brazil and Sweden based on admixture model and independent allele frequencies among populations.

5 ARTIGO 3

Seedling and adult plant resistance to stem rust in oat Brazilian genotypes[1]

Abstract

Oat stem rust, caused by the fungus *Puccinia graminis* f. sp. *avenae*, can cause significant yield losses wherever the conditions are favorable to the epidemics. There are a limited number of resistance genes available to breeding stem rust resistant oat cultivars. In order to identify novel resistance sources, 61 Brazilian genotypes derived from the Quaker International Oat Nursery were assessed for seedling and adult plant response to stem rust using an array of Australian isolates of *Puccinia graminis* f. sp. *avenae*. Seedling resistance was postulated for 52 genotypes, including six different resistance genes/alleles alone or in combination. The *Pg-a*, and the combination of *Pg-2+Pg-4+Pg-9* were the most common with 17 and 22 genotypes, respectively. The pathotype 41+Pg-9,-13,-a is virulent on all genotypes tested and was used to investigate the presence of adult plant resistance (APR) in a field experiment. Most of the genotypes showed either moderately resistant to moderately susceptible (8 genotypes = 13%) or susceptible (51 genotypes = 84%). Two genotypes were resistant to moderately resistant, which represent an effective stem rust resistance sources to the most virulent pathotype in Australia. These two promising sources of novel stem rust resistance belong to sister oat lines of the Universidade Federal do Rio Grande do Sul oat breeding program. A detailed genetic characterization to understand the mode of inheritance of the gene(s) of these sources of APR to oat stem rust is of major interest.

Key words: Gene postulation, *Avena sativa*, *Puccinia graminis* f. sp. *avenae*, seedling resistance

[1] Artigo formatado conforme as normas da revista Australasian Plant Pathology. http://link.springer.com/journal/13313

Introduction

Oat (*Avena sativa* L.) is a cereal crop of global importance used for grain, feed and forage production. Oats are infected by rust pathogens that cause yield and quality losses. The oat stem rust, caused by *Puccinia graminis* f. sp. *avenae* (Pga), is a highly destructive disease able to cause total crop failure (Martens 1985). Severe yield losses have been documented in epidemics in North America, Sweden and Australia (Martens 1985; Fetch Jr. 2009; Park 2008; Berlin et al. 2013). In South America, stem rust epidemics occur in the end of the crop season and represent a serious problem for later cultivars (Martinelli 2004). While fungicide application can be used to prevent disease losses, breeding rust resistant cultivars is the preferred method to control the stem rust in oats.

Rust pathogens are adapted to long distance migration through the wind and able to overcome resistance genes through single step mutation (Brown and Hovmøller 2002). Therefore, breeding for disease resistance is an ongoing process, which relies on the search of new effective resistance sources. The success of this process depends on selection of sources that provide a durable and satisfactory level of resistance.

Resistance genes to cereal rusts are broadly divided into two categories – seedling (R-genes) and adult plant resistance (APR-genes). Although R-genes are easy to identify and incorporate into elite germplasm, the major limitation of this type of resistance is its race-specificity and the rapidly evolving pathogen acquiring virulence to the resistance (McCallum et al. 2007). Therefore, there is a constant need to identify, characterize and deploy new effective R-genes. Contrasting to the complete absence of rust spore production in seedling genes, adult plant resistance (APR) provide partial resistance, reducing the spore production and extending the latent period. In wheat, the *Sr2* (stem rust resistance) and *Lr34* (leaf rust resistance) genes are race-non-specific APR-genes that have provide durable resistance, i.e. remained effective after a long period of deployment into

cultivars of wheat worldwide (Ellis et al. 2014). In oats, partial resistance, while not well characterized for stem rust, has being used to control oat crown rust, and similar to the observed for APR in wheat, has provide durable resistance (Leonard 2002; Graichen et al. 2011; Federizzi et al. 2015).

Based on gene-for-gene specificity, an array of pathotypes with known pathogenicity can be used to postulate which R-genes may be present in host genotypes (Loegering et al. 1971; Browder 1973). Multipathotype tests are a rapidly method to assess a large number of genotypes for the presence of R-genes and to increase the efficiency of advancing genetic analyses (Kolmer 1996). Furthermore, the presence of APR-genes can be assessed in field nurseries using a seedling virulent pathotype (Pathan and Park 2007).

For oat stem rust, 13 useful seedling Pg-genes are described, including *Pg-1*, *Pg-2*, *Pg-3, Pg-4, Pg-6* (*Pg-Saia*), *Pg-8, Pg-9, Pg-10, Pg-12, Pg-13, Pg-15, Pg-16* and *Pg-a* (Fetch Jr. and Jin 2007; Adhikari et al. 2000). Virulence has been detected to all Pg-genes in Australia (Park 2008), and in North America only *Pg-6*, *Pg-10,* and *Pg-16* has remained effective to all pathotypes (Fetch Jr. 2009). The only two known APR-genes, *Pg-11,* and *Pg-17* (Harder et al. 1990; McKenzie and Martens 1968) have not been widely used. The lack of stem rust resistance genes available to protect oats highlights the need of searching for new effective resistance sources.

The Quaker International Oat Nursery (QION) is a program of oat germplasm exchange worldwide (Harrison and Barnett 2008). It has given to oat breeders the access of a diverse germplasm with specific combinations of traits including milling quality and resistance to important diseases. Oats are grown in Southern Brazil as winter crop where environmental conditions are highly favorable to develop diseases, especially rusts. Therefore, rust resistance is of major importance for Brazilian oat germplasm (Federizzi and Mundstock 2004). In recent time, breeders have released resistant cultivars to both

crown and stem rust using classical breeding methods (Nava et al. 2016). These methods have permitted to accumulate both seedling and adult plant resistance genes in oat germplasm. The long-term breeding of resistant cultivars makes the Brazilian germplasm a potential source of valuable resistance genes to rust pathogens. The current study assessed the seedling and adult plant responses to oat stem rust in a set of 61 Brazilian genotypes available in the QION in Australia.

Material and Methods

Plant material

Sixty-one Brazilian oat genotypes from QION were tested for their response to different pathotypes of Pga (Table 1). Differential sets containing the genotypes with known single or combination genes used in Australia were included in order to confirm the virulence of the pathotypes (Table S1). The genotypes were grown through one generation in an Australian quarantine facility before being tested.

Seedling response in greenhouse tests

Seedling tests were carried out at Plant Breeding Institute, Cobbitty, Australia. Eight Pga pathotypes used to assess the seedling response were obtained from the Plant Breeding Institute Cereal Rust Collection (Table 2). Seedling tests were carried out in a greenhouse, where 10-15 seeds of each genotype were sown in 9 cm diameter pots filled with a mix of composted pine bark (8 parts) and coarse sand (2 parts). Pots were fertilized at sowing with a complete fertilizer (Aquasol®, 25 g/10 L water). At 1-2 leaf stage, the plants were sprayed with urediniospores suspended in a light mineral oil (Pegasol®). The inoculated plants were incubated for 48 h in a dew chamber at 18-20 °C using water-filled

steel trays covered with polythene hoods, under natural lighting. After the dew incubation, infected seedlings were transferred to a growth room at 22 ± 2 °C. The tests were repeated once to confirm the results.

Seedling response assessments were made on the 0-4 infection type (IT) scale described by Stakman et al. (1962) where IT=0 represents the lowest incompatible resistant reaction and IT=4 the fully compatible susceptible reaction. A mixture of infection types on the same leaf was referred as a mesothetic reaction (IT=X) and was included in the resistant category.

Adult plant response in field tests

Adult plant responses were assessed in 1m-row plots of each genotype during the crop season of 2015 at horse unit of Plant Breeding Institute (PBI), Cobbitty, Australia. The border row of each range of plots was sown with a mixture of susceptible lines. Supplying infected plants, using the pathotype 41+Pg-9,-13,-a (culture no 512), along the spreader row created an artificial epidemic. The rust response in the field was assessed two times from heading to grain maturity stage using the 1-9 scale described in Bariana et al. (2007).

Gene postulation

The principles of gene-for-gene specificity were applied to hypothesize which Pg genes may be present in the Brazilian oat genotypes. Eight different pathotypes, which represent a diverse collection of isolates of Pga that have been characterized for avirulence/ virulence in the oat stem rust differential set (Table S1) was used to postulate the genes in the Brazilian oat genotypes. Identity of Pg-genes was hypothesized by comparing the isolate/oat genotype combinations that result in low infection types with the isolate/ Pg-

line combinations that also result in low infection types.

Table 1 Pedigree and year of entry in the Quaker International Oat Nursery of 61 Brazilian oat genotypes.

Genotype	Pedigree	Quaker Nursery
UFRGS 015050-1	UPF 16 / UFRGS 950155	2006
UFRGS 017093-6	Cocker 492 / Starter-1 F_3 // UFRGS 10	2006
UFRGS 017147-2	UFRGS 881971 // Pc 68*5 / Starter F4	2006
UFRGS 01B7114-1-3	Pc 68 / 5* Starter F4 // UFRGS 10	2006
UFRGS 01B7121-2-4	Pc 68/ 5* Starter F4 // UFRGS 10	2006
UFRGS 028152-1	UFRGS 881971 // Pc 68*5 / Starter F4	2006
UFRGS 028153-2	UFRGS 881971 // Pc 68*5 / Starter F4	2006
UFRGS 036062-6	UFRGS 941699 / UFRGS 16	2006
UFRGS 036117-3	UFRGS 9252681 / UFRGS 911715	2006
UFRGS 037003-2	UFRGS 17 / UFRGS 950120	2006
UFRGS 037031-3	UPF 16 / UFRGS 950155	2006
UFRGS 038009-1	UFRGS 11 Sel. 1 / Belle	2006
UFRGS 038015-3	CTC-89B210 Sel. 4 / X6396-1	2006
UFRGS 046028-1	UFRGS 984081-2 / UFRGS 987015-2	2006
UFRGS 046048-1	UFRGS 970216-2 / UFRGS 970461	2006
UFRGS 046078-1	UFRGS 987016-1 / UFRGS 940548-5	2006
UFRGS 087045-3	UFRGS 995088-3 / UFRGS 960257-5	2012
UFRGS 087080-4	UFRGS 995078-2 / UFRGS 007003-1	2010
UFRGS 087105-1	UFRGS 995078-2 / URS 21	2010
UFRGS 087129-1	UFRGS 995078-2 / URS 21	2010
UFRGS 087157-3	UFRGS 995088-3 / UFRGS 006049	2010
UFRGS 087175-2	UFRGS 995088-3 / UFRGS 006049	2010
UFRGS 087212-1	UFRGS 995088-3 / UFRGS 006049	2010
UFRGS 097002-2	UFRGS 970488-1 / UFRGS 017149-2	2012
UFRGS 097018-3	UFRGS 970488-1 / UFRGS 017149-2	2012
UFRGS 097029-2	UFRGS 995088-3 / UFRGS 960257-5	2012
UFRGS 097032-1	UFRGS 995088-3 / UFRGS 960257-5	2012
UFRGS 098014-1	UFRGS 995088-3 / UFRGS 960257-5	2012
UFRGS 098036-1	UFRGS 995088-3 / UFRGS 960257-5	2012
UFRGS 098089-3	UFRGS 006198-7 / UFRGS 881920	2012
UFRGS 098167-1	UFRGS 995088-3 / UFRGS 960257-5	2012
UFRGS 098168-2	UFRGS 995088-3 / UFRGS 960257-5	2012
UFRGS 105025-1	UFRGS 036117-3 / UFRGS 017038-1	2012
UFRGS 22	UFRGS 841110 / UFRGS 884021-1	2006
UFRGS 9912002-2	UFRGS 86A1194-2 / UFRGS 8	2006
UFRGS 995080-1	UFRGS 10 / PAUL	2010
UFRGS Guapa	UPF 17 Resel. // Guaiba Sel. / CTC 84B993	2006
UPF 94174-1	NA	2006

Genotype	Pedigree	Quaker Nursery
UPF 95H1500-1	UPF 16 / UFRGS 7	2010
UPF 95H600-6	UPF 16 / UFRGS 16	2010
UPF 95H900-10	UPF 14 / UFRGS 16	2010
UPF 97H200-4	UFRGS 16 / UPF 16	2010
UPF 97H2200-7-2	UPF 16 / UPF 90H400	2010
UPF 97H300-14	UPF 90H400 / UFRGS 16	2010
UPF 97H300-2-1	UPF 90H400 / UFRGS 16	2010
UPF 97H300-2-11	UPF 90H400 / UFRGS 16	2010
UPF 97H300-2-12	UPF 90H400 / UFRGS 16	2010
UPF 97H300-6	UPF 90H400 / UFRGS 16	2010
UPF 97H400-2	UPF 90H400 / CTC5	2010
UPF 98H1600-2-1	UPF 7 / CTC5	2010
UPF 98H2000-4	UPF 16 / UPF 91AL100-1-4-3	2010
UPF 98H3100-2	UPF 90H400 / UPF 91AL100-1-4-3	2010
UPF 98H3200-2B	UPF 90H400 / UPF 89S080	2010
UPF 98H600-2	UPF 2 / UPF 89S080	2010
UPF 98H800	UPF 2 / UFRGS 14	2010
UPF 99H10-2	UPF 16 / UPF 18	2010
UPF 99H2-10-1	UPF 7 / UPF 18	2010
UPF 99H20-2-3	UPF 18 / UPF 92122-1	2010
UPF 99H43-23-1-1	OR2 / UPF 18	2010
UPF 99H43-5-5	OR2 / UPF 18	2010
UPF A20	UPF 80197 (X 2082-21 / CI 8428 // Steel)	2010

NA: not available.

Results

Over the period of 2006 to 2012, 61 Brazilian oat genotypes from QION were grown into quarantine and seeds were available for greenhouse and field tests in Australia. The oat genotypes evaluated in this study were developed by the UFRGS (Universidade Federal do Rio Grande do Sul) and UPF (Universidade de Passo Fundo) Oat Breeding Programs in Brazil. The pedigree of each genotype is shown in Table 1.

Seedling response

The Pga pathotypes selected for this work displayed a broad spectrum of virulence (Table 2). All the pathotypes were virulent on Swan, Pg-3, and Pg-17 differential lines.

The pathotypes 79, 4, 42 + Pg-9 and 28 showed the lower virulence with more than 85 % of the tested genotypes giving a resistant IT. The frequency of resistant lines dropped down to 48 % for pathotypes carrying combined virulence to *Pg-1,-2,-4,-9,-15,-16*. Using the pathotype 94+Pg-13, which has additional virulence to *Pg-13*, only 28 % of the genotypes remained resistant. The remaining resistant genotypes probably carry *Pg-a*. All 61 Brazilian genotypes tested were seedling susceptible to the pathotype 41+Pg-9,-13,-a.

Table 2 Accession number and susceptible differential lines of eight different pathotypes of oat stem rust.

Culture no.	Accession no.	Pathotype	Susceptible Pg-lines	Susceptible check lines	Resistance frequency[a]
254	770247	79	*1, 2, 3, 9, 17*	Swan, Mortlock	0.90
246	770098	4	*1, 2, 3, 8, 17*	Swan, Mortlock	0.88
245	710116	42+ *Pg-9*	*1, 3, 6, 9, 17*	Swan	0.88
443	853049	28	*3, 4, 17*	Swan	0.85
404	820625	20	*1, 2, 3, 4, 9, 15, 16, 17*	Swan, Mortlock	0.48
496	923117	41+ *Pg-9*	*1, 2, 3, 4, 6, 8, 9, 10, 15, 16, 17*	Swan, Mortlock	0.48
497	923034	94+ *Pg-13*	*1, 2, 3, 4, 8, 9, 13, 15, 16, 17*	Swan, Mortlock	0.28
512	962508	41+ *Pg-9,-13, -a*	*1, 2, 3, 4, 6, 8, 9, 10, 13, 15, 16, a, 17*	Swan, Mortlock, Barcoo	0.00

[a] frequency of Brazilian genotypes from the Quaker International Oat nursery with a low infection type.

Based on seedling tests, resistance genes/alleles could be postulated for 52 genotypes (Table 3). Six different resistance genes/alleles alone or in combination were postulated to be present in the Brazilian genotypes tested. Seventeen genotypes were resistant to the pathotype 94+Pg-13 and were postulated to carry *Pg-a*. The genotypes carrying *Pg-a* were resistant to all pathotypes except 41+Pg-9,-13,-a. The multipathotype test did not differentiate the IT combination given by *Pg-15*, *Pg-16*, *Pg-2+Pg-4* or *Pg-2+Pg-4+Pg-9*. For simplicity, only the combination *Pg-2+Pg-4+Pg-9* was shown (Table 3). This IT combination was identified in 22 genotypes. *Pg-13, Pg-4, Pg-8*, and *Pg-1* were

postulated alone in seven, four, one and one genotypes, respectively. Saia (*Pg-6*) and *Pg-10* gave a unique combination of IT and were not identified in any of the Brazilian genotypes tested. Four genotypes (UFRGS 046048-1, UFRGS 046078-1, UPF 97H300-6 and UPF 98H3100-2) were susceptible to all pathotypes tested. It was not possible to postulate which resistance genes were present in five genotypes (UFRGS 028152-1, UFRGS 22, UPF 98H2000-4, UPF 99H43-23-1-1 and UPF A20).

Adult plant response

All 61 Brazilian genotypes were screened in a field experiment over the sowing season of 2015, using the pathotype 41+Pg-9,-13,-a. None of the 61 genotypes displayed seedling resistance to this pathotype. Therefore, the potential presence of APR genes could be assessed in the field trial. The disease severity was high, with the susceptible check showing 100 % of infected stems. Fifty-one genotypes (84 % of total) were scored 6 or higher and were considered either susceptible or moderately susceptible (Table 3). Eight genotypes: UFRGS 087157-3, UFRGS 087212-1, UFRGS 097018-3, UFRGS 097029-2, UPF 98H600-2, UPF 98H800, UPF 99H10-2 and UPF 98H2000-4 showed intermediate response (score 5) being moderately resistance to moderately susceptible. Two genotypes, UFRGS 087105-1 and UFRGS 087129-1 sharing the same pedigree, showed stem rust response of 3, which were considered resistant to moderately resistant (Table 3, Figure 1).

Table 3 Field response and seedling infection type to eight pathotypes of *Puccinia graminis* f. sp. *avenae* on 61 Brazilian oat genotypes derived from the Quaker International Oat Nursery.

Line	Field response[a]	Seedling infection type[b]								Gene Postulation
		Pathotype[c]								
		79	4	42+Pg-9	28	20	41+Pg-9	94+Pg-13	41+Pg-9, 13, a	
UFRGS 028153-2	8	0;	1c	;cn	1-c	;1c	2-c	;1	3+	*Pg-a*
UFRGS 087045-3	8	;c	;	;c	;	1+c	2c	;1cn	3+	*Pg-a*
UFRGS 087080-4	6	;1-c	;1-	;c	;1-	;1+c	2-c	;1	3	*Pg-a*
UFRGS 087157-3	5	0;	;1-	0	0	;1cn	;1c	;1-c	3	*Pg-a* *
UFRGS 087175-2	8	;1	;1-c	;1c	1+	2+	;2c	;1-	3	*Pg-a*
UFRGS 087212-1	5	0;	;	0;	0;	;12c	;2c	;1-	3	*Pg-a* *
UFRGS 097002-2	8	;1-c	;c	;c	;	;1c	2-c	;1	3+	*Pg-a*
UFRGS 097018-3	5	;1-c	;1-c	;c	;	;1+c	2-c	11+	3+	*Pg-a*
UFRGS 097029-2	5	;1-c	;1-	;c	;	;1c	2-c	11+	3+	*Pg-a*
UFRGS 097032-1	6	;1-c	;1-c	;c	;	;1c	2-c	11+	3+	*Pg-a*
UFRGS 098014-1	8	;1c	;1	;c	;1	;1	2+	11+	3+	*Pg-a*
UFRGS 098036-1	8	0;	;1-c	;c	;1c	;1c	2c	11+	3+	*Pg-a*
UFRGS 098167-1	8	;1-	1	;1-c	;1	2c	2c	11+	3+	*Pg-a*
UFRGS 098168-2	8	;	;	;c	;	;1+c	2-c	;1	3+	*Pg-a*
UFRGS 995080-1	8	0;	;c	0;	0;	;1-cn	;1+c	;1-	3	*Pg-a* *
UPF 95H1500-1	6	0;	;cn	0;	;1-	;1-c	;1-	;	3	*Pg-a* *
UPF 99H43-5-5	8	11+	;1c	;1-c	;1	2+c	2+c	11+	3+	*Pg-a*
UFRGS 017147-2	8	2+	1c	;1c	;1c	2+	2+	3-	3+	*Pg-13* *
UFRGS 037003-2	7	;1	1+c	;11+c	;1+	1+c	22+c	3	3	*Pg-13* *

Line	Field response[a]	Seedling infection type[b]								Gene Postulation
		Pathotype[c]								
		79	4	42+Pg-9	28	20	41+Pg-9	94+Pg-13	41+Pg-9, 13, a	
UFRGS 098089-3	8	;1	;1c	1c	2-c	;c	2-c	3+	3+	*Pg-13* *
UPF 95H900-10	6	;1	;1-	;1-c	;11+	;1-c	2-c	3+	3	*Pg-13* *
UPF 97H300-2-1	8	12	11+	1+	2	2+	2+	3+	3+	*Pg-13*
UPF 97H300-2-12	8	;1	1c	1+	1+	1+	2+	3+	3+	*Pg-13* *
UPF 98H1600-2-1	8	;1	1+c	;1	;1	2+	2+	3+	3+	*Pg-13* *
UFRGS 015050-1	8	;1	;1-c	;1-c	;1+	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 01B7114-1-3	8	11+	1c	;1-c	;1+	3	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 01B7121-2-4	8	;1	1c	;1-c	;1-	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 036062-6	8	1-	1+c	;1-	;1	3+	3	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 046028-1	6	11+c	1c	;1c	;11+c	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 087105-1	3	;1	;1	;1	1-c	3+	3	3+	3	*Pg-2, Pg-4, Pg-9*
UFRGS 087129-1	3	1	;1	;1c	;1	3+	3	3+	3	*Pg-2, Pg-4, Pg-9*
UFRGS 105025-1	6	11+	;1-c	;1-c	;1c	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 9912002-2	8	;1	1+c	;1c	;1-	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UPF 97H200-4	8	;1	;1c	;1-c	;11+	3+	3+	3	3	*Pg-2, Pg-4, Pg-9*
UPF 97H2200-7-2	8	11+	1+	;1-c	1c	3	3+	3+	3	*Pg-2, Pg-4, Pg-9*
UPF 97H400-2	8	;1	;1c	;1c	1+	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UPF 98H3200-2B	6	11+	1c	;1	1-	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UPF 98H600-2	5	;1+	1+c	;1-	;11+	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UPF 98H800	5	11+	1	;1	;1-	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*

Line	Field response[a]	Seedling infection type[b]								Gene Postulation
		Pathotype[c]								
		79	4	42+Pg-9	28	20	41+Pg-9	94+Pg-13	41+Pg-9, 13, a	
UPF 99H10-2	5	;1-	;1-c	;1c	;1-	3+	3	3+	3+	*Pg-2, Pg-4, Pg-9*
UPF 99H2-10-1	8	;1-	;1-c	;1-c	;1-	3+	3	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 017093-6	8	2+	1+c	;1c	;1+	3+	3-	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 037031-3	8	11+c	1c	;1c	2c	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UPF 95H600-6	8	1+	1-	;1c	2	3+	3+	3+	3	*Pg-2, Pg-4, Pg-9*
UPF 99H20-2-3	8	;1-	;1-c	;1c	2-	3+	3	3+	3+	*Pg-2, Pg-4, Pg-9*
UPF 97H300-2-11	8	1	;1-c	;1	2+	3+	3+	3+	3+	*Pg-2, Pg-4, Pg-9*
UFRGS 038009-1	8	11+c	1+c	;1c	3	3+	33+	3+	3+	*Pg-4*
UFRGS 038015-3	8	11+c	1+	;1c	3+	3+	3+	3+	3+	*Pg-4*
UFRGS Guapa	8	11+	1+	1+c	3+	3+	3+	3+	3+	*Pg-4*
UPF 94174-1	8	;1	;1-c	;1c	3	3+	3	3+	3+	*Pg-4*
UPF 97H300-14	8	;1	3	;1-c	;1	0	3+	3+	3+	*Pg-8* *
UFRGS 036117-3	8	3	3-	3	12c	3+	3+	3	3	*Pg-1*
UFRGS 028152-1	8	2-	1+c	X-	1+	2+	3+	3+	3+	UR
UFRGS 22	8	;1-	3+	;c	3+	;1c	2-	3+	3+	UR
UPF 98H2000-4	5	2-	1+c	3+	2c	2+	2+	3+	3+	UR
UPF 99H43-23-1-1	8	3	;1c	X	2-	2+	2+c	3+	3+	UR
UPF A20	8	2	;12c	3	;12	33+	2+c	3+	3	UR
UFRGS 046048-1	8	3+	3+	3	3+	3+	3+	3+	3	None
UFRGS 046078-1	8	3+	3+	3	3+	3+	3+	3+	3	None

Line	Field response[a]	Seedling infection type[b]								Gene Postulation
		Pathotype[c]								
		79	4	42+Pg-9	28	20	41+Pg-9	94+Pg-13	41+Pg-9, 13, a	
UPF 97H300-6	8	3	3	3	3	3	3+	3+	3+	None
UPF 98H3100-2	8	3	3	3+	3+	2+	3+	3+	3+	None

[a] field response using the pathotype 41+Pg-9, 13, a. Data were scored on the 1-9 scale described in Bariana et al. (2007), where 1 is very resistant and 9 is very susceptible.

[b] Infection types were defined using a 0–4 scale (Stakman et al. 1923), where 0 = immune, no signs of disease; ; = hypersensitive flecks of dead host tissue present; 1 = tiny pustules with sharply defined hypersensitive flecking; 2 = small to medium-sized pustules with defined necrosis, sometimes showing green islands; 3 = medium-sized pustules, without hypersensitivity; and 4 = large pustules. The symbols + and – indicate slightly larger and smaller pustule sizes, respectively. The letters n and c indicate pronounced necrosis and chlorosis, respectively. The letter X indicates mesothetic reaction. UR: unknown resistance gene.

[c] Australian pathotype nomenclature (infection types of Australia differential set available on Table S1).

* slightly lower IT combination than the postulated Pg-line.

Discussion

The seedling tests confirmed the presence of major resistance genes for stem rust in most of the Brazilian oat genotypes. Based on the IT combination using eight pathotypes it was possible to identify 11 different resistance sources including five genotypes with unknown resistance genes. The diversity of seedling resistance genes suggests that the host has been selected over a diverse pathogen population. Previous studies showed that the pathogen population found in two epidemic years in an oat breeding station of South Brazil were characterized by a mixture of highly virulent pathotypes (Artigo 1). Although virulent pathotypes that overcome all the known resistance genes was reported, the deployment of different gene combination in different cultivars might preserve the pathogenic diversity in the pathogen population, delaying the predominance of the most virulent pathotypes.

Figure 1 Two oat genotypes showing APR to the most virulent stem rust pathotype in Australia. (A) The image shows rows of the two resistant oat genotypes UFRGS 087105-1 (right line) and UFRGS 087129-1 (left line) in a field nursery at 122 days after sowing. (B) Infected stems of UFRGS 087105-1 (left), UFRGS 087129-1 (middle), and the susceptible control Barcoo (right).

The *Pg-a* was the most common resistance gene and also the most effective against Australian pathotypes, conferring resistance to seven out of eight pathotypes used. The Pg-a resistance was characterized by the action of two complementary genes and was identified in several lines from the QION (Adhikari et al. 1999). The *Pg-a* gene has been deployed in Australian cultivars since 1991, but the emergence of a pathotype with virulence to this gene in Queensland in 1996 (Park 2008) might restrict its protection to oat stem rust in Australia. In Canada, virulence to *Pg-a* has remained low even after the deployment of cultivars with this gene (Fetch Jr. 2009; Fetch Jr. et al. 2011). The prevalence of this resistance gene among Brazilian genotypes might be related to the protection against a wide range of pathotypes in Brazil. A fitness cost of the *Pg-a* virulence for the pathogen may explain the worldwide use of *Pg-a*.

Given the existence of a pathotype that overcomes all known seedling Pg-genes, it is imperative to search for new oat stem rust resistance genes. The adult plant resistant genotypes UFRGS 087105-1 and UFRGS 087129-1 (Figure 1) to the most virulent pathotype of Australia (41+Pg-9,-13,-a) will contribute to breeding genetic resistant oats. Based on the differential rust response in the field, these genotypes probably carry different genes/alleles from the others previous described resistance sources. Additionally, oat crown rust seedling tests showed that these two genotypes carry effective resistance to a wide spectrum of virulence of *P. coronata*, the oat crown rust pathogen, found in Australia (Table S2). A detailed genetic characterization to determine the mode of inheritance of these valuable stem rust resistance sources are underway.

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Table S1 Infection type of eight *Puccinia graminis* f sp. *avenae* pathotypes on the Australia oat stem rust differential set.

Differential line	Seedling infection type							
	Pathotype							
	79	4	42 + Pg-9	28	20	41 + Pg-9	94 + Pg-13	41 + Pg-9,-13,-a
Pg-1	3	3+	3+	12+	3+	3	3	3+
Pg-2	3	3+	11+c	1+	3+	3+	3+	3+
Pg-3	3	3+	3+	3+	3+	3	3+	3+
Pg-4	11+	1+	;1+c	3	3+	3+	3+	3+
Pg-8	2	3-	11+	2+	2+	3+	3+	3+
Pg-9	3	2+	3+	12+c	3+	33+	3+	3+
Pg-13	2-	1+	2+	2	2+	2+	3+	3+
Saia (Pg-6)	;1	;1-	3+	;c	;	3+	;1	3+
Pg-4, -8	1+	;1-	;1-c	2	1+c	3	3+	3+
Pg-2, -4, -9	11+	;1-	;1-c	1+	3+	3+	3+	3+
Pg-2, -4	11+	;	;1-c	12-	3+	3+	3+	3+
Barcoo (Pg-a)	;	;	;cn	;1-	;1-	;1-c	;1-	3+
Mortlock (Pg-1, -2)	3+	3+	1+n	;1+c	3+	3+	3+	3+
Pg-a	;1	;1-	;1+c	;1	;1+c	2+	;1	3+
Swan	3+	3+	3+	3+	3+	3+	3+	3+
Pg-10	0	0	0cn	2-cn	2-cn	3+	2cn	3-
Pg-15	;1-	;1-	1c	1+c	3+	3+	3+	3+
Pg-16	1	11-	1c	1+c	3+	3+	3+	3+
Pg-17	3	3	3+	3+	3+	3+	3+	3+

[b] Infection types were defined using a 0-4 scale (Stakman et al. 1923), where 0 = immune, no signs of disease; ; = hypersensitive flecks of dead host tissue present; 1 = tiny pustules with sharply defined hypersensitive flecking; 2 = small to medium-sized pustules with defined necrosis, sometimes showing green islands; 3 = medium-sized pustules, without hypersensitivity; and 4 = large pustules. The symbols + and – indicate slightly larger and smaller pustule sizes, respectively. The letters n and c indicate pronounced necrosis and chlorosis, respectively.

Table S2 Seedling infection type to 12 pathotypes of *Puccinia coronata* f. sp. *avenae* on 61 Brazilian oat genotypes from Quaker International Oat Nursery and the Australia differential set.

Brazilian QION line or Australian differential line	Seedling infection type[a]											
	Puccinia coronata f. sp. *avenae* isolate from Plant Breeding Institute (culture number)											
	164	298	536	526	605	567	595	613	549	618	624	636
UFRGS 036062-6	;	0	;c	;1c	1	1-c	;1-cn	1	;1	;1-	1++	;
UFRGS 037003-2	0;	0	0	0	;1-	0	1cn	1	;1	1-c	1++	;1c
UFRGS 038015-3	0	0	;	;c	0	;	;cn	0	0	;cn	;n	;cn
UFRGS 046048-1	;	1cn	0	;c	0	0	0	0;	0	0;	;	0
UFRGS 046078-1	1c	;cn	0	0	0	0	0	0	0	0;	;	0
UFRGS 087045-3	0	0	0	0	1c	0	;1cn	1+c	0	;1c	2c	;1-c
UFRGS 087080-4	0	0	1-c	0	1-c	0	;1-c	1c	0	;1-c	;1+c	;1-c
UFRGS 087105-1	;n	;cn	;n	;c	1	;	;1	1	1-	;1-c	;1c	;1-c
UFRGS 087129-1	;n	;cn	;n	;c	1	;	;1-c	;1-	1-	;1-c	;1c	;1-c
UFRGS 087157-3	0	0	0	0	;cn	0	0	0	0	0	0	0
UFRGS 087212-1	0	0	0	0	0	0	0	0	0	0	0	0
UFRGS 097002-2	0	0	0;	0	1-	0	;c	;c	0	;c	;c	;cn
UFRGS 097018-3	0	0	0;	0	;cn	0	;cn	;c	0	;c	;cn	;cn
UFRGS 097032-1	;	0	0;	0	;cn	0	1-cn	1c	0	;cn	;c	;cn
UFRGS 098036-1	0	0	0;c	0	;cn	0	2-c	1c	0	1++c	1+c	1++c
UFRGS 098167-1	0	0	;c	0	;1-cn	0	2-c	1c	0	1++c	2++	;1-c
UFRGS 098168-2	0	0	0	0	;1-cn	0	1cn	1c	0	1c	1+c	;1-c
UFRGS 9912002-2	1-	1c	0	0	1-	0	1++	1+	1+c	1++	2-	1++
UPF 97H300-14	0	0	0	0;	1	0	;1c	1	1-	;1-	1+	;
UPF 97H300-2-1	0	;	0	0	2++	0	;	0	;	;cn	1c	;n
UPF 98H1600-2-1	0	0	;	;	;c	;	;n	;1-	1-	;cn	;n	;
UPF 97H300-2-12	0	0	0	0	3+	0	;n	;	3+	;cn	;n	;cn
UPF 95H900-10	0	;cn	0	0	2-cn	0	;cn	3+	3+	;cn	;n	;cn
UPF 97H300-2-11	0	;	0	2++	2cn	3+	;1+cn	3+	3	;cn	;n	;n
UPF 97H400-2	0,;n	;cn	2+cn	1cn	3+	0	;cn	3+	3+	;cn	;n	1-c;
UFRGS 028153-2	0	0	;cn	3+	3-	3-	;1-cn	;n	;cn	1c	2-	2++

Brazilian QION line or Australian differential line	Seedling infection type[a]											
	Puccinia coronata f. sp. *avenae* isolate from Plant Breeding Institute (culture number)											
	164	298	536	526	605	567	595	613	549	618	624	636
UFRGS 087175-2	;	1-cn	0	0	3	0	;cn	3+	3+	;cn	;n	;n
UPF 98H3100-2	;	;n	0	0	3	3+	3-	;	;	0	3+	33+
UFRGS 036117-3	0	0	0	1cn	;	1-c	NA	3+	2+c	;1-cn	3	2++
UPF 95H600-6	0	;	;n	;cn	0	3+	0	0	0	0	0	0
UPF 99H43-5-5	0	0	;	;cn	;cn	3+	;	1++cn	1-cn	;	;c	;c
UFRGS 098089-3	0	0	0	0	3-	0	3+	0	0	3+	3+	3+
UFRGS 037031-3	0	0	0	;c	0	;	11+cn	3+	0	2cn	3+	3+
UPF 95H1500-1	0	0	0,;	;cn	0	1+	;c	3+	0	;1	3+	3+
UFRGS 046028-1	;	;c	;	;c	3+	;	3+	3+	3+	3+	3+	3+
UFRGS 015050-1	0	0;	0	0	3-	0	3+	3+	0	3+	3+	3
UFRGS 017093-6	0	0	0	0	3+	0	3+	3+	0	3+	3+	3+
UFRGS 01B7121-2-4	0	;c	0	;	3+	0	0	3+	0	3+	3+	3+
UFRGS 105025-1	0	0	0	0	3-	0	33+	3+	0	3+	3+	2+c
UFRGS 01B7114-1-3	0	;c	;	;c	1c	X	;1	;c	3+	3+	3+	3-
UFRGS 995080-1	;c	0	;1-c	1-c	1c	1-	1-	;	3+	3+	3+	3+
UPF 97H2200-7-2	0	0	0	0	1	0;	1+c	1++	3+	3+	3+	3+
UPF 98H2000-4	;	;	2+cn	;c	1	;	;c	;	3+	3+	3+	3+
UPF 99H43-23-1-1	0	;	0	;c	;c	;c	1-c	;cn	3-	3+	3+	2+c
UFRGS 028152-1	0	;	;	;c	0	;	;11+	2	0	3+	3+	3+
UFRGS 038009-1	0	0	;	;c	0	1-c	;1c	0	0	3+	3+	3+
UFRGS Guapa	0	0	0	;c	0	;n	1-c	1+c	0	3+	3+	3+
UPF 97H200-4	0	0	0	;cn	;	0	;c	0;	0	3+	3+	2+c
UPF A20	0;	0	;	;cn	1+	;n	1-	;	;	3+	3+	3+
UFRGS 017147-2	0	;cn	;cn	;c	0	1cn	1cn	2-cn	;	3+	3	2cn
UFRGS 097029-2	0	0	1+c	0	1cn	0	2cn	2-cn	0	3+	2+cn	2cn
UFRGS 098014-1	0	0	0	0	1c	0	2	1c	0	3	3	1++c
UPF 99H2-10-1	0	;	;cn	;cn	;	;cn	;cn	;cn	2cn	3-	3	1cn
UFRGS 22	0	;n	3+	3+	0	3+	NA	2+	3+	3+	NA	3

Brazilian QION line or Australian differential line	Seedling infection type[a]											
	Puccinia coronata f. sp. *avenae* isolate from Plant Breeding Institute (culture number)											
	164	298	536	526	605	567	595	613	549	618	624	636
UPF 94174-1	0	0;	3+	1+c	0	1+	3+	3+	0	3+	3+	3+
UPF 98H800	0	;cn	;cn	2-cn	1+n	3+	3+	2+cn	3+	3+	3+	3+
UPF 99H10-2	1cn	;cn	1cn	2-cn	2+cn	3+	3+	2+cn	3+	3+	3	3+
UPF 99H20-2-3	1cn	;cn	1cn	2-cn	2+cn	3+	3+	2+cn	3+	3+	3	3+
UPF 97H300-6	0;	;cn	2+cn	2++	3+	3+	3+	3+	3+	3+	3+	3+
UPF 98H3200-2B	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
UPF 98H600-2	3+	3+	2-cn	3+	3+	3+	3+	3+	3+	3+	3+	3+
H458	0	;c	1-c	3-	;	3-	;c	;c	;cn	;c	;n	;
WIX4361-9	;n	;cn	;	;	;cn	;n	;1c	;c	;cn	;c	;c	;
Amagalon	0	0	0	0	0	;n	0	1+c	0	0	3+	3+
Culgoa	0	0	;c	3+	2++	3-	;c	2+c	2-c	2-c	;cn	1cn
Cleanleaf	n	;	;	0;	3+	0;	;1c	;c	3+	3+	;cn	;c
Pc68	0	0	0	0	3+	0	3+	3+	0	0	3+	3+
TAM.0301	1-	1c	3+	3+	2	3-	;c	2-cn	1-cn	1-c	;c	;c
TAM.0312	1	1c	3	3+	1+c	3-	;c	1cn	;cn	;c	;c	;c
Pc61	0	;cn	3	3	1c	3	;c	0	0;	0;	;c	;c
Pc38	1-	3+	;	;	3+	0	3	3+	3+	3+	3+	3+
Pc39	1-	;	1++	3+	3+	3+	3	3+	3+	3+	3+	3+
Swan	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Pc36	1c	;cn	;c	;c	1-cn	;cn	;cn	1cn	3+	3+	3+	3+
Pc46	3+	3+	0	0	;	;cn	;	;c	3+	3+	;c	;
Pc50	0	0	0	0	3+	0	0	3+	;n	;n	;c	0
Pc51	1-c	2c	33+	3+	3	3+	3+	3+	3+	3+	3+	3+
Pc52	0	;	0	;	3+	0	;c	;c	3+	3+	;c	;1c
Pc55	2	;	;	3+	3	3+	3+	3+	3+	3+	3-	3+
Pc56	;	1-c	;	;c	;c	;cn	;c	3+	;	;	3+	3+
Pc63	;	2	0	;	;c	0	;c	;c	0	0	3+	;
Pc64	;	;cn	;c	;c	;n	0;	;c	3+	;cn	;cn	;c	;c

Brazilian QION line or Australian differential line	Seedling infection type[a]											
	Puccinia coronata f. sp. *avenae* isolate from Plant Breeding Institute (culture number)											
	164	298	536	526	605	567	595	613	549	618	624	636
Pc71	0	;n	1++	3+	3+	3+	3+	1c	3+	3+	3+	3+
X716	;	1cn	;1c	;c	3+	1cn	1-c	;c	3+	3+	3+	3+
Warrego	0	;	0	;c	3-	0	3+	;	0	3	3+	3+
Bettong	0	;c	;cn	;cn	0	3-	0	0	0;	0	;c	0
Barcoo	0	0	;	;n	;c	3-	;c	0;	;	;c	;c	1-c
Landhafer	3+	3+	3+	3+	3-	3+	3+	3+	3+	3+	3+	3+
Santa fé	2+	2+	2+	3-	2cn	3-	3-	33-	3+	3	3+	3
Ukraine	3	0	1	3+	3+	3	1++	1++	1++	1++	3+	1++
Trispern	2+	3-	3+	3+	2-cn	3-	2-c	2+c	1+c	1c	2	2cn
Bondvic	2+	3-	3-	3	2cn	3+	1+c	2+	2-c	1-c	3	2+c
Pc45	;	;c	3+	3+	;	3+	;1-c	;c	;1	;1-	3	1-c
Pc48	0	;	3-	2-cn	0	1-c	2-c	3+	0	3+	3+	3-
Gwydir	0	;cn	0;c	;c	0;c	;cn	;	0;	3+	3	3+	3+
Volta	0	0	0	0	3-	0	0	3+	0	0	;c	0
Pc92	0	0	3+	;1-c	0	1cn	0	;	0	0	0	;
Pc94	0;	;	;c	;c	0	;	;c	;	0;	0	;c	;
Saia	0	0	0	0	;	0	;c	0	;cn	;c	;c	;c
Ensiler	0	;	0	;c	;	;	;c	;c	;	0	;c	;
Drover	0	0	0;	;c	1-c	;c	;c	1c	1	1c	2	3+
Genie	0	;n	0	0	0	0	;n	;n	0;	;n	3+	3+
Aladdin	0	0	0;	;c	;	;1n	0	0	0	0	0	3+
PCB 60-2	1-cn	1-cn	3+	3+	;c	3-	;c	;n	;c	;cn	;1c	;1-cn
PCB 62	;	;	;	;c	;	;	;	0;	;c	;	;c	;

[b] Infection types were defined using a 0–4 scale (Stakman et al. 1923), where 0 = immune, no signs of disease; ; = hypersensitive flecks of dead host tissue present; 1 = tiny pustules with sharply defined hypersensitive flecking; 2 = small to medium-sized pustules with defined necrosis, sometimes showing green islands; 3 = medium-sized pustules, without hypersensitivity; and 4 = large pustules. The symbols + and – indicate slightly larger and smaller pustule sizes, respectively. The letters n and c indicate pronounced necrosis and chlorosis, respectively. The letter X indicates mesothetic reaction.

6 ARTIGO 4

Genetic analysis and molecular mapping of stem rust resistance in hexaploide oat[1]

Abstract

Oat stem rust can cause severe economic losses worldwide and breeding genetic resistant oat cultivars is the preferred method to control the disease. The Brazilian oat line UFRGS 995088-3 have been used as a stem rust resistance source in South Brazil. The aims of this study were to investigate the identity of the seedling stem rust resistance genes in UFRGS 995088-3, to understand the genetics of the resistance and to map the resistance using a population of recombinant inbred line (RIL). The multipathotype tests suggests the presence of the *Pg-a* complex in UFRGS 995088-3. Genetic analysis was performed in two $F_{5:7}$ populations derived from the crosses 'UFRGS 03B7024-1 x UFRGS 995088-3' and 'UFRGS 995088-3 x UFRGS 035080'. The infection type in seedling tests conformed to the presence of three independent genes with two of them in a complementary mode of action in the UFRGS 03B7024-1 x UFRGS 995088-3 population and, a single gene in the UFRGS 995088-3 x UFRGS 035080 population. The 6 K oat Illumina Infinium single nucleotide polymorphism (SNP) array was used for genotyping 85 RILs derived from the UFRGS 03B7024-1 x UFRGS 995088-3 mapping population. The molecular marker data provided evidence of a single-gene locus with segregation distortion rather than the complementary gene action identified in the genetic analysis. The resistance gene was mapped in a 0.7 cM interval between two markers that explained 95 % of the observed resistance. These markers will provide the basis for further validation studies in others genetic populations.

Key words: *Avena sativa*, *Puccinia graminis* f. sp. *avenae*, segregation distortion, *Pg-a* complex

[1] Artigo formatado conforme as normas da revista Euphytica.
http://www.springer.com/life+sciences/plant+sciences/journal/10681

Introduction

Cultivated oat (*Avena sativa* L.) is a cereal of major importance worldwide, used for animal and human feed. Oats are infected by rust pathogens that cause losses in yield and quality of grain. Oat stem rust, caused by the fungus *Puccinia graminis* f. sp. *avenae*, is one of the most devastating disease of oats worldwide. Although not frequent as crown rust, the emergence of highly virulent pathotypes and the wind-born migration of the spores have threat the oat production. Oat stem rust epidemics have been reported causing economic losses in North America, Australia and North Europe (Fetch Jr. 2005; Park 2008; Berlin et al. 2013). The most economical method to prevent rust losses is breeding genetically resistant cultivars.

Most of the resistance genes in plants follow the Flor's gene-for-gene model, where the resistance is based on the race-specific recognition of pathogen avirulence gene products (Flor 1971). Once an effective resistance gene has been identified, the breeders might transfer it to the elite germplasm. Searching for a high level and long-lasting effective resistance has encouraged breeders to pyramid several resistance genes, including seedling-genes and adult plant resistance genes into the cultivars (Ayliffe et al. 2008). However, the phenotypic selection for such breeding strategy is complicated due to the pleiotropic effect of seedling genes that masks the effect of the gene combinations. Furthermore, the variation in the extent of the epidemics along the years and the absence of the disease in some environments may limit the efficiency of the selection for rust resistance because of the poor phenotypic information. The development of tightly linked molecular markers to support marker-assisted selection (MAS) facilitates the pyramiding approach, which may improve the efficiency of breeding rust resistance cultivars.

Among the molecular markers that have been associated to rust resistance in oats are random amplified polymorphic DNA (RAPD) (Penner et al. 1993), restriction fragment

length polymorphism (RFLP) (O'Donoughue et al. 1996) and amplified fragment length polymorphism (AFLP) (Kulcheski et al. 2010). These markers are either dominant, not suitable to discriminate heterozygotes in early generations, or labor/time consuming and have not been incorporated into high-throughput genotyping platforms. Hence, they have not been widely used, i.e., for MAS in oat breeding.

The abundance in the genomes and the compatibility with high-throughput automation, it makes single nucleotide polymorphism (SNP) the marker of choice for genetic analysis in plants (Mammadov et al. 2012). In oats, a SNP array has been developed and anchored in a consensus map (Oliver et al. 2013; Tinker et al. 2014; Chaffin et al., 2016). The oat SNP array was applied to successfully map crown rust resistance-associated loci (Gnanesh et al. 2013; Lin et al. 2014; Gnanesh et al. 2015; Babiker et al. 2015). Finally, KBioscience competitive allele-specific PCR (KASP) SNP genotyping system (http://www.lgcgenomics.com) has been used to develop PCR-compatible and cost-effective markers for crown rust resistance (Gnanesh et al. 2013; Lin et al. 2014; Gnanesh et al. 2015).

A promising oat stem rust resistance source was identified in South Brazil (Arruda 2011). In this region, under natural epidemics of stem rust, the oat line UFRGS 995088-3 has showed higher resistance levels than previous known resistance sources (L.C. Federizzi, Personal communication). Genetic studies, using five F_2 segregating populations were used to characterize the inheritance of this resistance (Arruda 2011). The genetic model of the inheritance varied from one to three genes controlling the resistance, two of them showing complementary action (Arruda 2011). This investigation aimed to investigate the identity of the seedling stem rust resistance genes in UFRGS 995088-3, to understand the genetics of the resistance in UFRGS 995088-3 using two recombinant

inbred line (RIL) populations, and to map the resistance in UFRGS 03B7024-1 x UFRGS 995088-3 mapping population.

Material and Methods

Plant material

The UFRGS 995088-3 is an oat line selected at the Universidade Federal do Rio Grande do Sul in 1999 derived from a cross between the lines UFRGS 881971 and Pc68/5*STARTER (F_4). UFRGS 995088-3 has been characterized as resistant to stem rust in the field under natural epidemics of oat stem rust pathogen at the Agronomy Station of Eldorado do Sul, RS, Brazil. Traces of stem rust have been detected in UFRGS 995088-3, only in the late growing seasons, probably associated with thermo-sensitivity of the resistance. UFRGS 0370B24-1 and UFRGS 035080 are oat lines well adapted to Brazilian conditions and susceptible to stem rust. Two genetic populations were used in this study: 'UFRGS 03B7024-1 x UFRGS 995088-3' and 'UFRGS 995088-3 x UFRGS 035080' consisting of 98 and 116 $F_{5:7}$ recombinant inbred lines (RILs), respectively. Both populations were developed at UFRGS Agronomy Experimental Station by single seed descent.

Seedling tests

Seedling tests were carried out in greenhouse facilities at the University of Sydney Plant Breeding Institute, Cobbitty, Australia. The lines were grown through one generation in an Australian quarantine facility before being tested. Seven *Puccinia graminis* f. sp. *avenae* pathotypes used for seedling tests were obtained from the Plant Breeding Institute Cereal Rust Collection. Ten to fifteen seeds of each RIL and parental line were sown in 9

cm diameter pots filled with a mix of composted pine bark (8 parts) and coarse sand (2 parts). Pots were fertilized at sowing with a complete fertilizer (Aquasol®, 25 g/10 L water). Seedlings at 1-2 leaf stage were sprayed with urediniospores of *P. graminis* f. sp. *avenae* suspended in a light mineral oil (Pegasol®). The inoculated plants were incubated for 48 h in a dew chamber at 18-20 °C using water-filled steel trays covered with polythene hoods, under natural lighting. After the dew incubation treatment, the infected seedlings were transferred to a greenhouse growth room at 20-24 °C. Australian oat stem rust differential lines were included to confirm the virulence spectrum of the pathotypes used.

Stem rust infection types (IT) were scored using seven different pathotypes (Table 1) 10 days after inoculation on a 0-4 scale described by Stakman et al. (1962): 0 (immune), ; (fleck), 1 and 2, resistant; 3 and 4, susceptible. Mixture of IT on the same leaf was referred as mesothetic reaction (IT=X) and was included in the resistant category. The experiment was repeated once to confirm the results. Chi-square (χ^2) tests were performed to test the goodness of fit of observed ratios to theoretically expected ratios.

DNA extraction

Genomic DNA was extracted from the fourth leaf of 5-10 plants of each parental line and RIL using the cetyltrimethyl ammonium bromide (CTAB) method described in (Saghai-Maroof *et al.*, 1984). Four pieces of 2 cm long leaves were collected and dried for 2-3 days in silica gel. The Qiagen™ Tissue Lyser with 2-mm-diameter tungsten beads was used at 25 Hz for 2 × 1 min to disrupt the leaf samples. The fine grounded powder was dispersed in 800 uL of CTAB extraction buffer (100 mM Tris, pH 8.0/ 1.4 M NaCl/ 20 mM EDTA/ 2 % β-mercaptoethanol), and incubated at 65 ºC for 30-40 min with occasional gentle swirling. To each sample, 600 uL of chloroform/phenol 24:1 (v/v) was added, and the solution was mixed by inversion to form an emulsion that was centrifuged

at 13,200 rpm for 20 min. The aqueous phase was removed, 1 volume of cold isopropanol was added and gently mixed by inversions to precipitate the nucleic acids, and stored in -20 ºC for 15 min. The samples were then centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The precipitated DNA was washed by gently inversion using a washing buffer solution (76 % ethanol/ 10mM NH_4OAc), and then dissolved in 100 uL of ddH_2O with 1 uL of RNAase A and incubated at 37 ºC for 2 h.

Genotyping

The parental lines and 85 RILs of the 'UFRGS 995088-3 x UFRGS 03B7024-1' were genotyped with the 6,000 oat Illumina Infinium SNP array (Illumina, San Diego, CA). The raw data were analyzed and the genotype calls were converted into allele scores for linkage mapping (Illumina, San Diego, CA).

Linkage map

The linkage map was developed with the software JoinMap 4 (Van Ooijen 2006). Markers with greater than 10% missing data or strong segregation distortion ($p < 0.001$, χ^2 test) were excluded from mapping. The logarithm of odds (LOD) threshold was set from three to 15 and adopted as indicator to cluster analysis with a maximum recombination frequency of 40 % using maximum likelihood mapping algorithm. Map distances were calculated using the Kosambi mapping function in centiMorgans (cM).

Mapping of seedling resistance gene

Phenotypic variation resulting from the seedling resistance loci was calculated by the software MapQTL®6 (Van Ooijen 2009). Regions with potential locus effects were identified using simple interval mapping. The loci were declared associated to the

resistance if the maximum LOD exceeded the genome wide results LOD threshold (calculated with 1000 permutations, mean error rate of 0.05). Loci associated to the resistance were characterized by the maximum LOD score and percentage of phenotypic variation explained.

Sequence similarity

The oat SNP sequences were reported in Tinker et al. (2014). To identify sequence similarity within the seedling resistance region, the sequences of the linked markers were compared with the rice (*Oryza sativa* L.) genome (http://rapdb.dna.affrc.go.jp/) and with the *Brachypodium distachyon* genome (https://phytozome.jgi.doe.gov/) using Blastn. Matches were filtered to retain those with an e-value less than or equal to 10^{-10} for each query sequence.

Results

Multipathotype tests

The seedling ITs of the parental lines UFRGS 995088-3, UFRGS 035080 and UFRGS 03B7024-1 were assessed across seven distinct pathotypes of *P. graminis* f.sp. *avenae* with broad spectrum of virulence (Table 1). The seven pathotypes can be divided into three groups based on their virulence pattern on the differential set. The first group that includes 42+Pg-9, 79 and 28, is avirulent to most of the differential lines (Table 1). The second group includes the pathotypes 94+Pg-13, 20 and 41+Pg-9 and have virulence to most of the differential lines, but are avirulent to *Pg-a*. Finally the isolate 41+Pg-9,-13,-a is the most virulent pathotype determining a susceptible IT to all differential lines tested. All three parental lines showed a resistant IT to the first group, which were IT= 0 in

UFRGS 995088-3, and IT= ;1 in both UFRGS 035080 and UFRGS 03B7024-1 (Table 1). Using pathotypes of the second group (the *Pg-a* avirulent pathotypes) a resistant IT (;1- to 2c) was identified in UFRGS 995088-3 contrasting with the susceptible IT (3 and 3+) of the others parental lines. The most virulent pathotype (41+Pg-9,-13,-a) conferred susceptible IT to all three parental lines (Table 1).

Inheritance of seedling resistance to stem rust

The resistance to the pathotypes 94+Pg-13 was assessed in seedling tests of two oat genetic populations. For the UFRGS 03B7024-1 x UFRGS 995088-3 population, the observed segregation of 3 resistant: 5 susceptible ratio (χ^2=0.133, p = 0.715) conformed with the hypothesis of three independent genes controlling the trait (Table 2). Based on the segregation ratio, two complementary genes are present in the resistant line, and a third gene is present in the susceptible parent. For the UFRGS 995088-3 x UFRGS 035080 population, χ^2 analysis of the segregation data indicated that the population does not significantly deviate from a 1 resistant: 1 susceptible ratio expected for a single gene (χ^2=0.138, p = 0.71, Table 2).

Genetic mapping of a single locus for the seedling resistance

Based on the resistant:susceptible ratio of the UFRGS 03B7024-1 x UFRGS 995088-3 genetic population a complex of three genes is associated to the resistance. Molecular markers were used to further characterize the genetic system involved in the resistance. The oat genotyping platform provided 1,192 polymorphic SNP markers. A linkage map constructed for the RIL population comprised 818 SNP markers arranged in 46 linkage groups spanning 1313.42 cM (Figure S1).

Table 1 Seedling infection type of parental lines to distinct pathotypes of *Puccinia graminis* f. sp. *avenae*.

Parental line or differential line	Seedling infection type[a]						
	Pathotype[b]						
	42+*Pg-9*	79	28	94+*Pg-13*	20	41+*Pg-9*	41+*Pg-9,-13,-a*
UFRGS 995088-3	0	0;	0;	;1-	;1c	2c	33+
UFRGS 035080	;1-c	;1-c	;1-c	3+	3+	3	3+
UFRGS 03B7024-3	;1-c	;1-c	;12-c	3+	3+	33+	3+
Pg-1	3+	3	12+	3	3+	3	3+
Pg-2	11+c	3	1+	3+	3+	3+	3+
Pg-3	3+	3	3+	3+	3+	3	3+
Pg-4	;1+c	11+	3+	3+	3+	3+	3+
Saia (Pg-6)	3+	;1	;c	;1	;	3+	3+
Pg-8	11+	2	2+	3+	2+	3+	3+
Pg-9	3+	3	12+c	3+	3+	33+	3+
Pg-10	0	0	2cn	2cn	2cn	3	3
Pg-13	2+	2-	2	3+	2+	2+	3+
Pg-15	1c	;1-	1+c	3+	3+	3+	3+
Pg-16	1c	1	1+c	3+	3+	3+	3+
Pg-4, -8	;1-c	1+	2	3+	1+c	3	3+
Pg-2, -4, -9	;1-c	11+	1+	3+	3+	3+	3+
Pg-2, -4	;1-c	11+	12-	3+	3+	3+	3+
Barcoo (Pg-a)	;cn	;	;1-	;1-	;1-	;1-c	3+
Mortlock (Pg-1, -2)	1+n	3+	;1+c	3+	3+	3+	3+
Pg-a	;1c	;1	;1	;1	;1c	2+	3+
Swan (none)	3+	3+	3+	3+	3+	3+	3+
Pg-17	3+	3	3+	3+	3+	3+	3+

[a] Infection types were defined using a 0-4 scale (Stakman et al. 1923), where 0 = immune, no signs of disease; ; = hypersensitive flecks of dead host tissue present; 1 = tiny pustules with sharply defined hypersensitive flecking; 2 = small to medium-sized pustules with defined necrosis, sometimes showing green islands; 3 = medium-sized pustules, without hypersensitivity; and 4 = large pustules. The symbols + and – indicate slightly larger and smaller pustule sizes, respectively. The letters n and c indicate pronounced necrosis and chlorosis, respectively.
[b] Australian pathotype nomenclature.

A total of 16 linked markers in the LG 24 with significant LOD score were associated with the resistance (Table 3, Figure 1). These markers showed segregation distortion ($p < 0.05$) with respect to the expected Mendelian inheritance in F_7 plants. A peak of LOD score was identified within 0.7 cM interval between the markers

GMI_ES05_c1719_239 and GMI_ES02_c7694_423 (Table 3, Figure 1). These markers explained more than 95 % of the resistance (Table 3, Figure 1).

Table 2 Segregation ratio of resistant:susceptible (R:S) and Chi-squared (χ^2) analysis of UFRGS 03B7024-1 x UFRGS 995088-3 and UFRGS 995088-3 x UFRGS 035080 populations when tested against *Puccinia graminis* f. sp. *avenae* (pathotype 94+Pg-13) at the seedling stage.

Genetic population	Segregation ratio (R:S)		Resistant genotypes	Susceptible genotypes	Total	χ^2	*p*
	Expected	Observed					
UFRGS 03B7024-1 x UFRGS 995088-3	37:61 (3:5)	35:63	$A_1B_1C_1$ [RP] $A_1B_1C_2$ $A_1B_2C_2$	$A_2B_2C_2$ [SP] $A_1B_2C_1$ $A_2B_1C_1$ $A_2B_1C_2$ $A_2B_2C_1$	98	0.133	0.715
UFRGS 995088-3 x UFRGS 035080	58:58 (1:1)	56:60	A_1 [RP]	A_2 [SP]	116	0.138	0.710

[RP] resistant parental line, [SP] susceptible parental line.

Table 3 Summary of significant markers for stem rust resistance detected in the oat UFRGS995088-3/ UFRGS03B7024-1 RIL population when tested against *Puccinia graminis* f. sp. *avenae* (pathotype 94+Pg-13) at the seedling stage.

Locus	Position[a]	χ^{2b}	% expl.[c]	LOD[d]
GMI_GBS_96371	15.3	3.57*	29.3	6.4
GMI_ES03_c2772_448	17.3	2.78*	38.6	9.0
GMI_ES_LB_11075	26.8	3.86**	85.7	35.9
GMI_ES03_c2880_225	27.4	2.71*	81.4	31.0
GMI_ES22_c3771_291	28.0	4.25**	85.7	35.9
GMI_ES02_c7694_423	29.3	4.25**	95.1	55.8
GMI_ES05_c1719_239	30.0	4.76**	95.1	55.8
GMI_ES01_c7989_189	31.0	3.86**	91.0	44.5
GMI_DS_LB_7921	31.1	3.86**	90.6	43.6
GMI_ES05_c16490_161	31.1	3.48*	90.6	43.7
GMI_ES02_c13827_987	32.4	4.76**	90.2	42.9
GMI_ES03_c13944_493	32.4	5.19**	90.2	42.9
GMI_DS_LB_1307	36.3	5.19**	63.9	18.8
GMI_ES01_c8445_691	36.3	5.76**	63.9	18.8
GMI_GBS_101905	36.4	3.57*	63.8	18.8
GMI_ES03_c21101_337	36.6	4.05**	62.9	18.3

[a] Position in cM on the linkage group 24 (Figure S1). [b] chi-square test of locus segregation ratio against the expected for Mendelian inheritance in F_7 generation. * and ** indicates the distorted segregation ratio at 0.05 and 0.01 levels of probability, respectively. [c] percentage of the phenotypic variance explained by the locus. [d] LOD significance threshold based on 1,000 permutations ($\alpha = 0.05$).

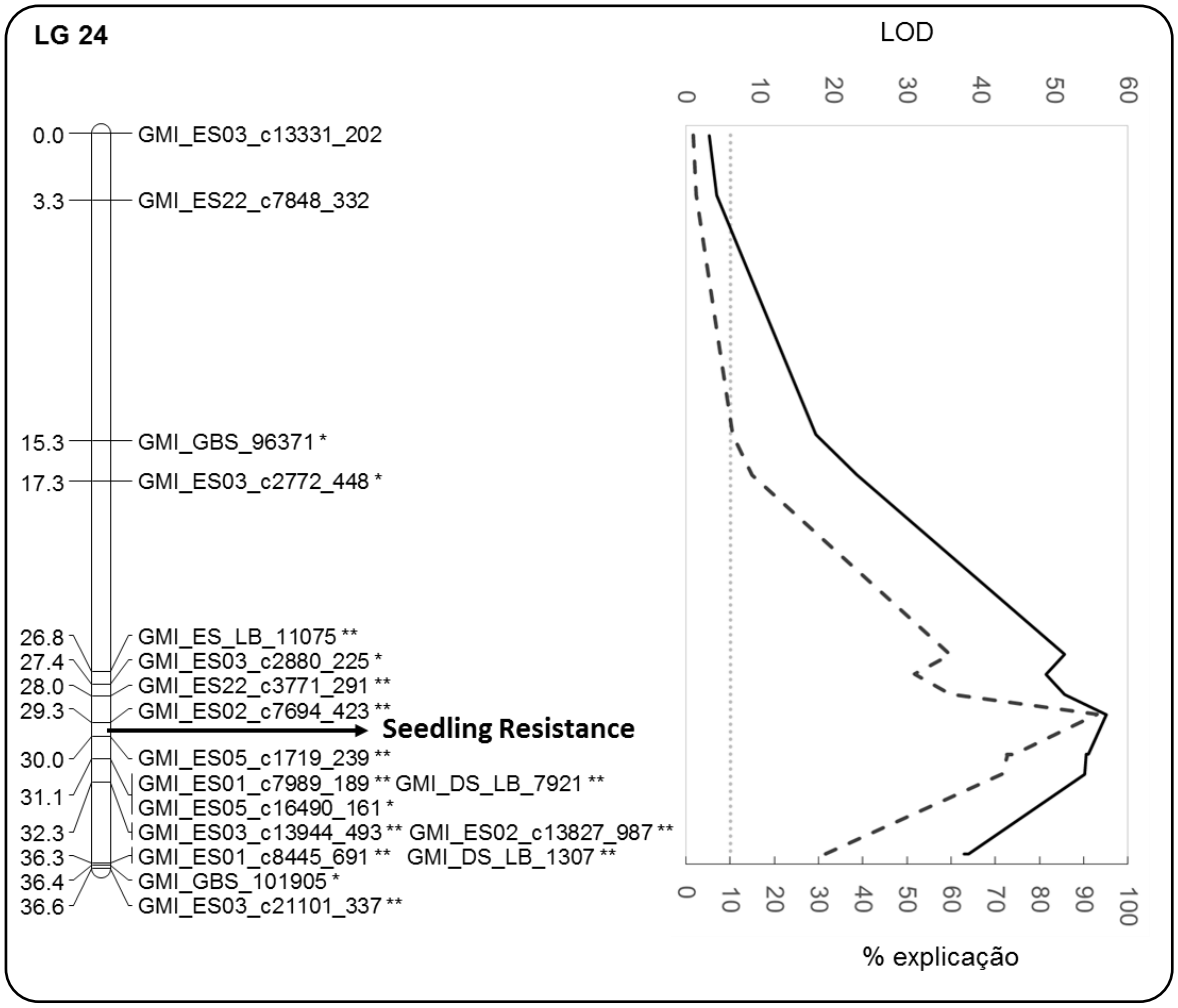

Figure 1 Stem rust resistance locus based on simple interval mapping identified in the genetic map of the UFRGS03B7024-1 x UFRGS995088-3 population. Seedling resistance locus was placed on the LG 24. Markers that showed segregation distortion are indicated with asterisks. LOD score and the significance threshold for the locus are presented as dashed and dotted lines, respectively. The percentage of the variance explained by the locus is presented as the solid line.

The LG 24 comprise 18 SNP markers spanning 36.6 cM. Based on the oat consensus map generated by Chaffin et al. (2016), 11 markers (GMI_GBS_96371, GMI_ES03_c2772_448, GMI_ES_LB_11075, GMI_ES03_c2880_225, GMI_ES22_c3771_291, GMI_ES01_c7989_189, GMI_ES05_c16490_161, GMI_DS_LB_1307, GMI_ES01_c8445_691, GMI_GBS_101905 and GMI_ES03_c21101_337) were mapped to the Mrg33 (Figure 2, Figure 3). The remaining

markers were assigned to different groups including: GMI_ES03_c13331_202, GMI_ES22_c7848_332 and GMI_ES03_13944_493 to Mrg15, GMI_ES05_c1719_239 and GMI_ES02_c13827_987 to Mrg13 and GMI_ES02_c7694_423 to Mrg08. The marker GMI_DS_LB_7921 was not anchored in the current oat consensus map.

Sequence similarity between cereal genomes and candidate genes for the resistance

The sequence of the oat SNP markers in the LG 24 were used as query Blastn to *Brachypodium distachyon* genome to identify the sequence similarity between the species. Among the markers associated to the resistance, GMI_ES_LB_11075, GMI_ES03_c2880_225, GMI_ES22_c3771_291, GMI_ES02_c7694_423, GMI_ES05_c1719_239, GMI_ES01_c7989_189, GMI_DS_LB_7921, GMI_ES03_c13944_493, GMI_DS_LB_1307 and GMI_ES03_c21101_337 showed significant sequence similarity with the *B. distachyon* sequences Bradi5g24220, Bradi5g24850, Bradi5g24220, Bradi5g20330, Bradi5g23570, Bradi5g22510, Bradi5g19367, Bradi5g16557, Bradi5g13920 and Bradi5g13727, respectively (Figure 2). These sequences cover a 9.18 Mbp region on chromosome 5 of *B. distachyon*. The markers GMI_ES03_c13331_202, GMI_ES22_c7848_332, GMI_GBS_96371 and GMI_ES03_c2772_448 showed sequence similarity to Bradi1g32410, Bradi1g32590, Bradi1g34572 and Bradi1g35490, respectively and spanned 3,31 Mbp region on chromosome 1 of *B. distachyon*.

The sequences of the oat SNP markers (LG 24) with significant similarity to the *Oryza sativa* genome were presented in the Figure 3. Among the 14 SNP markers closely associated with seedling resistance, GMI_ES_LB_11075, GMI_ES22_c3771_291, GMI_ES02_c7694_423, GMI_ES05_c1719_239, GMI_ES01_c7989_189,

GMI_ES03_c13944_493, GMI_DS_LB_1307 and GMI_ES03_c21101_337 showed significant sequence similarity with the *O. sativa* sequences Os04g0650800, Os04g0650800, Os04g0598200, Os04g0641450, Os04g0626300, Os04g0530700, Os04g0530700 and Os04g0483200, respectively. These sequences cover a 9.01 Mbp region on chromosome 4 of *O. sativa*. The markers GMI_ES03_c13331_202, GMI_ES22_c7848_332 and GMI_ES03_c2772_448 on LG 24 showed sequence similarity to Os06g0671700, Os06g0677400 and Os06g0634300 and spanned 2,42 Mbp region on chromosome 6 of *O. sativa*.

The sequences of the two closely linked markers flanking the resistance (GMI_ES02_c7694_423 and GMI_ES05_c1719_239) spanned a 2.45 Mbp sequence in the *O. sativa* chromosome 4 (Figure 3). To identify candidate genes for the resistance, we further investigated the genes within this region in the *O. sativa* genome using the genome browser in http://rapdb.dna.affrc.go.jp/. Using the Batch Retrieval we investigated the region between the positions 30,000,000 and 33,000,000 bp. A total of 477 genes were identified within the region including 10 genes related to biotic stress and defense response - two START-like domains (Os04t0593400 and Os04t0593500), five leucine-rich repeat domains (Os04t0605300, Os04t0616400, Os04t0647900, Os04t0648200 and Os04t0648400), and three NB-ARC domains (Os04t0620950, Os04t0621500 and Os04t0626450).

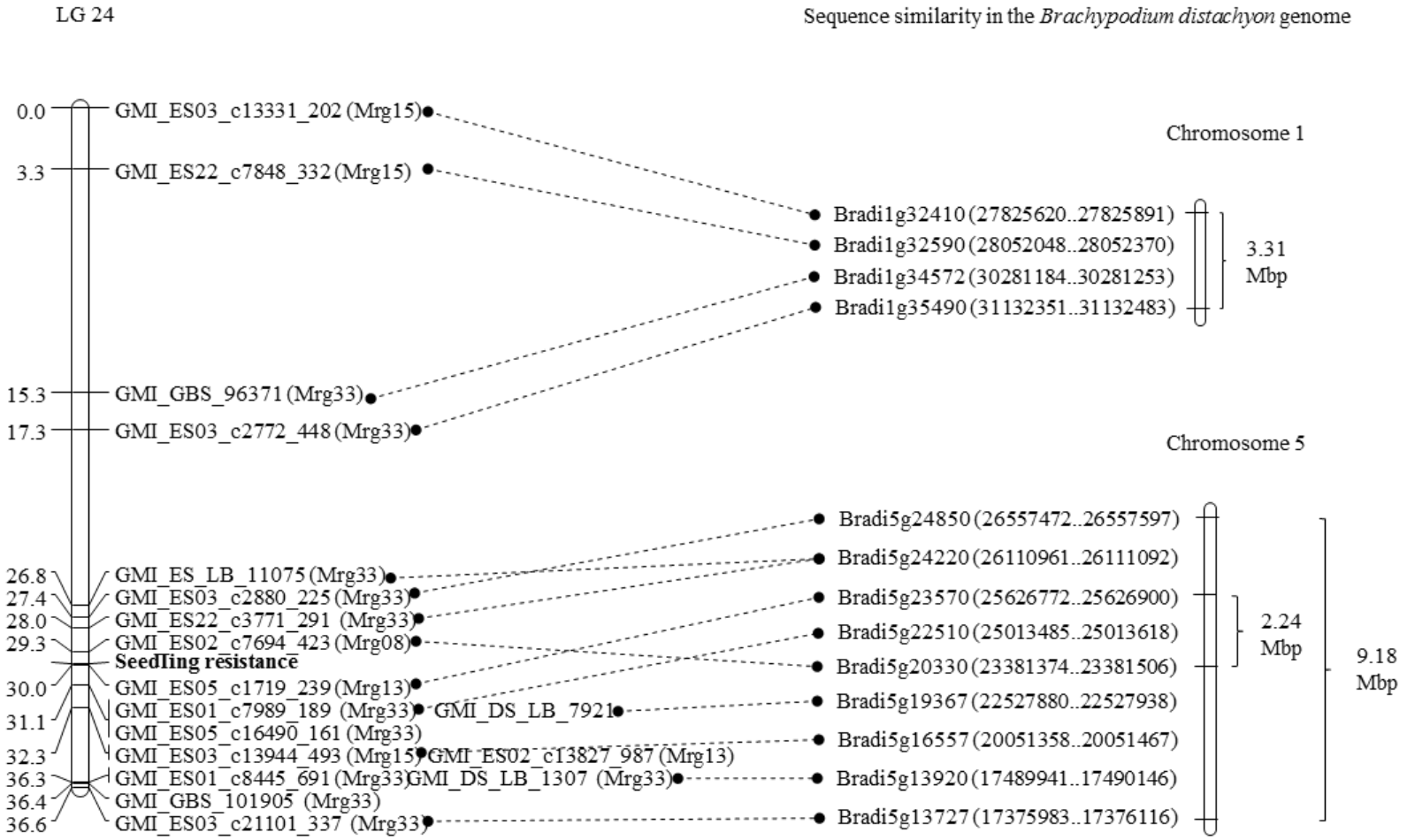

Figure 2 Markers in the linkage group 24 (LG 24) of the UFRGS03B7024-1 x UFRGS995088-3 RIL population map with significant similarity to the *Brachypodium distachyon* sequences. The position of the markers in the oat consensus map (Chaffin et al. 2016), when available is indicated in the parenthesis. The position of the loci in the *B. distachyon* genome is indicated in the parenthesis. The physical spanned region in the *B. distachyon* genome is on the right side.

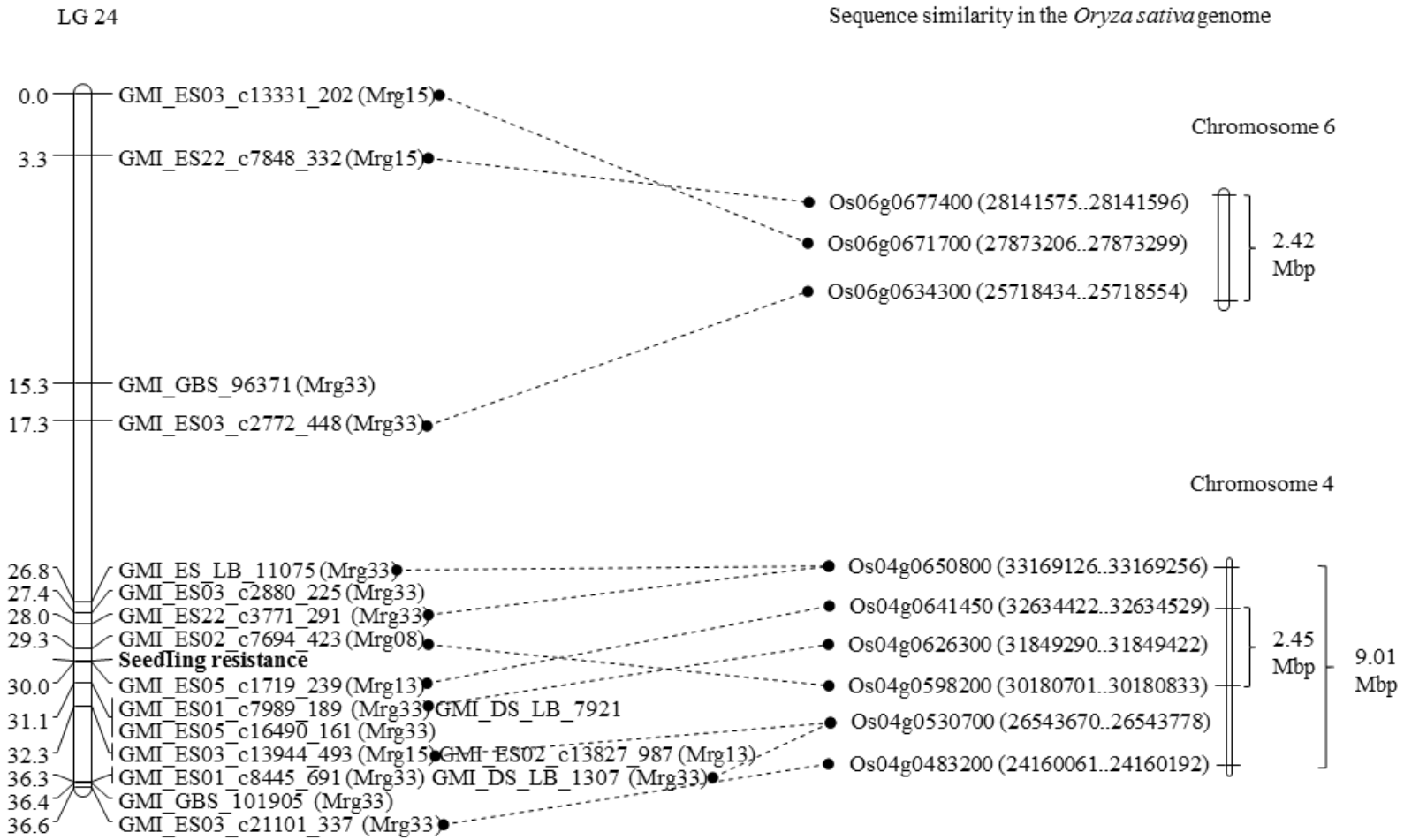

Figure 3 Markers in the linkage group 24 (LG 24) of the UFRGS03B7024-1 x UFRGS995088-3 RIL population map with significant similarity to the *Oryza sativa* sequences. The position of the markers in the oat consensus map (Chaffin et al. 2016), when available is indicated in the parenthesis. The position of the loci in the *O. sativa* genome is indicated in the parenthesis. The physical spanned region in the *O. sativa* genome is on the right side.

Discussion

The gene postulation using a multipathotype test indicates that all the three parental lines carry seedling resistance genes, as resistance IT were identified across the seven pathotypes tested. Based on known virulence to the resistance genes, the parental lines UFRGS 03B7024-1 and UFRGS 035080 do not carry *Pg-a* (Table 1). On the other hand, several evidences support the presence of *Pg-a* in the UFRGS 995088-3 inbred line. The multipathotype test showed similar IT combination between UFRGS 995088-3 and the differential lines known to carry *Pg-a* (Table 1). A genetic variability with respect to the number of genes, which varied from one to three genes, controlling the resistance to the stem rust in UFRGS 995088-3 was reported among five F_2 segregating populations (Arruda 2011). Similar segregation variability was observed for the *Pg-a* resistance (Adhikari et al. 1999; Adhikari and McIntosh 2001). Furthermore, the segregation ratio observed for the UFRGS 03B7024-1 x UFRGS 995088-3 population confirms previous studies which support the presence of complementary gene action resembling the *Pg-a* mode of inheritance (Martens et al. 1981; Adhikari et al. 1999). This genetic system has been unique among oat stem rust resistance as shown by the failed segregation among several crosses involving different sources of *Pg-a* resistance, including the non-related oat lines Omega and Amagalon (Adhikari et al. 1999; Adhikari and McIntosh 2001).

All the markers associated with seedling resistance were mapped in the LG 24 and explain more than 90 % of the phenotypic variation. These markers exhibited distorted segregation ratio from the expected Mendelian inheritance in F_7 generation. The segregation distortion represents the selection bias towards the resistant:susceptible ratio observed in the population. Altogether these results suggest that the genetic of the resistance in this population is controlled by a single locus located in a region with segregation distortion rather than the previously hypothesized genetic system of three

genes. Segregation distortion has been documented in crosses involving different stem rust resistance genes in oats, including *Pg-15* (Martens et al. 1980), *Pg-a* (Adhikari et al. 1999), and *Pg-13* (McKenzie et al. 1970). All these Pg-genes were introgressions from the wild relative *Avena sterilis* which might be involved in some level of hybrid incompatibility due to cytogenetic instability. In wheat, a chromosomal region showing segregation distortion has been reported surround the *Sr36* gene, a stem rust resistance locus introgressed from the wild relative *Triticum timopheevii* (Tsilo et al. 2008, Cavanagh et al. 2013). To our knowledge the current study is the first to show the association of an oat resistance gene to a genomic region with segregation distortion using molecular markers.

Most of the markers in the present study mapped to the same group as assigned in the current oat consensus map (Chaffin et al. 2016). However, several markers, including the ones from the LG 24, which was associated to the seedling resistance gene, mapped to different groups. In attempt to develop a consensus map for the hexaploid oat, Chaffin et al. (2016) identified several sub-genome rearrangement relative to the ancestral diploids and among oat populations due to chromosome translocation. The inconsistence of anchoring markers were reported in other mapping studies of oat (Gnanesh et al. 2013; Babiker et al. 2015). Interesting, in the current study, six of the resistance-linked markers that mapped to four different Mrg-groups on the oat consensus map, have similar sequences within a 9 Mbp interval of chromosome 4 in the *O. sativa* genome. We also investigated the similarities of the oat markers sequences in the genome of *B. distachyon*, and again, it was identified a consistence in grouping the similar sequences of the oat resistance-linked markers to the chromosome 5 of the *B. distachyon*. A recent rearrangement in the oat genomic region surrounding the resistance locus may explain the inconsistence of grouping the markers in the LG 24 to the oat consensus map.

Using a SNP array we identified two SNP markers (GMI_ES05_c1719_239 and GMI_ES02_c7694_423) linked to the resistance in the UFRGS 03B7024-1 x UFRGS 995088-3 mapping population. However, further validation studies are required to confirm the markers-resistance association in others populations. Developing KASP markers for the associated SNPs will facilitated the genotyping in the validation process and for future use in MAS. Using the homologous sequences in the rice genome we identified ten candidate genes within the closely linked markers in the oat map. These sequences can be used for cloning the genes from the parental lines, in an effort to identify the resistance gene.

In conclusion, multipathotype tests and genetic analysis support that the seedling resistance gene in UFRGS 995088-3 is the same known *Pg-a*. Genetic analysis using molecular markers data of the UFRGS 03B7024-1 x UFRGS 995088-3 population showed that the resistance was conferred by a single gene, in a genomic region characterized by a segregation distortion. We identified SNP markers linked to the stem rust resistance that will provide a basis for further validation studies and for the identification of the genes underlying the resistance.

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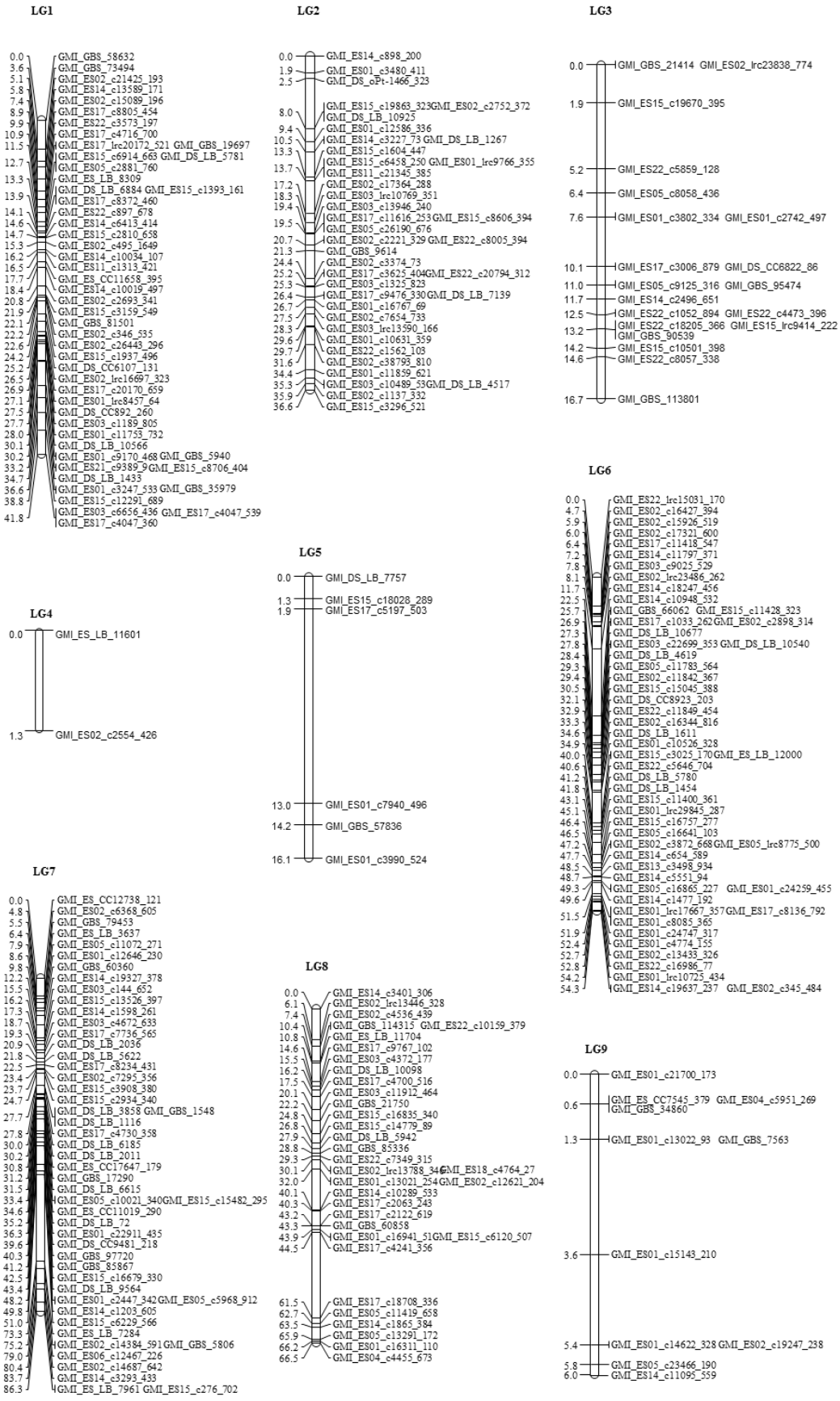


Figure S1 Framework map based on SNP markers in the UFRGS 03B7024-1 x UFRGS 995088-3 mapping population.

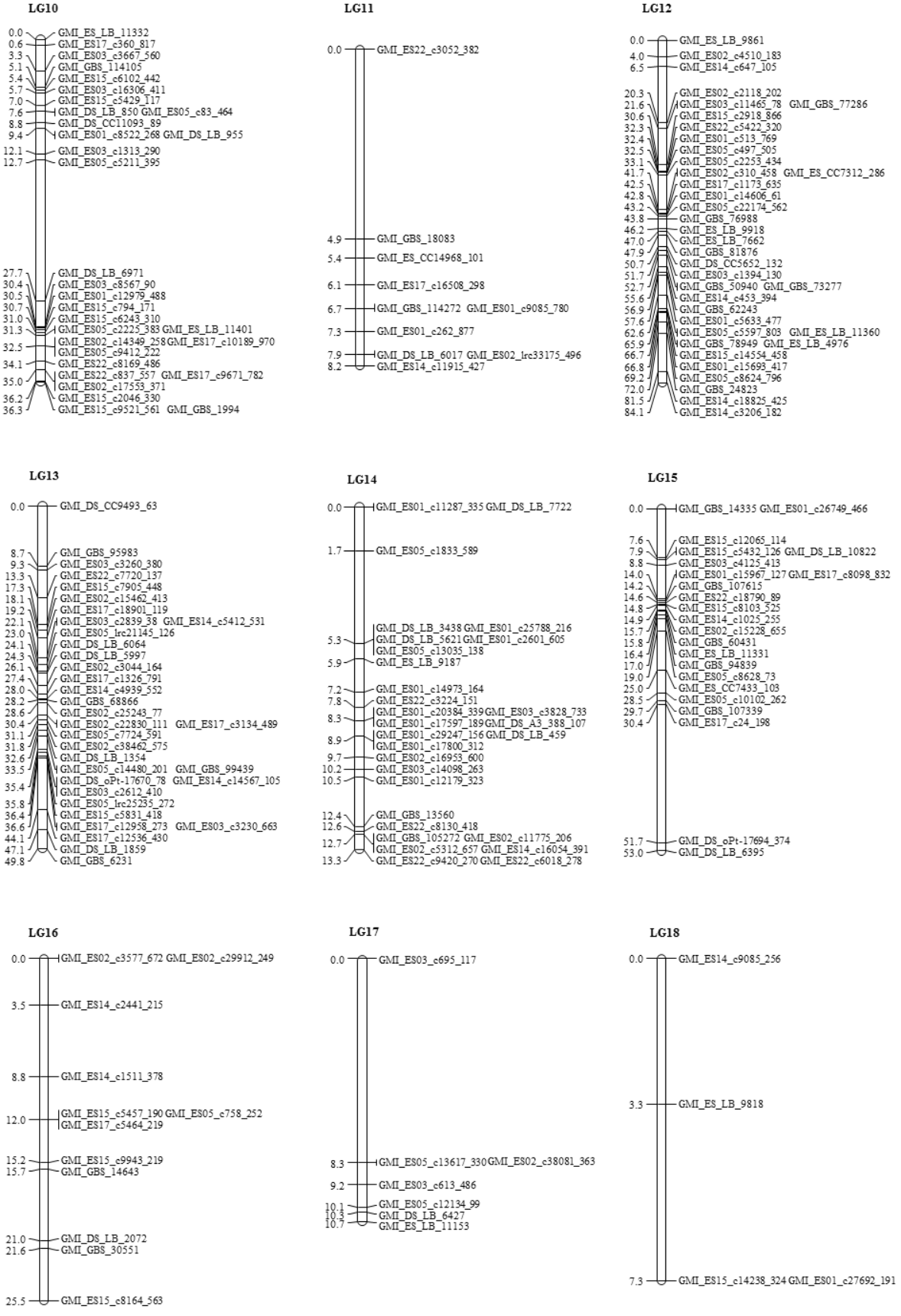
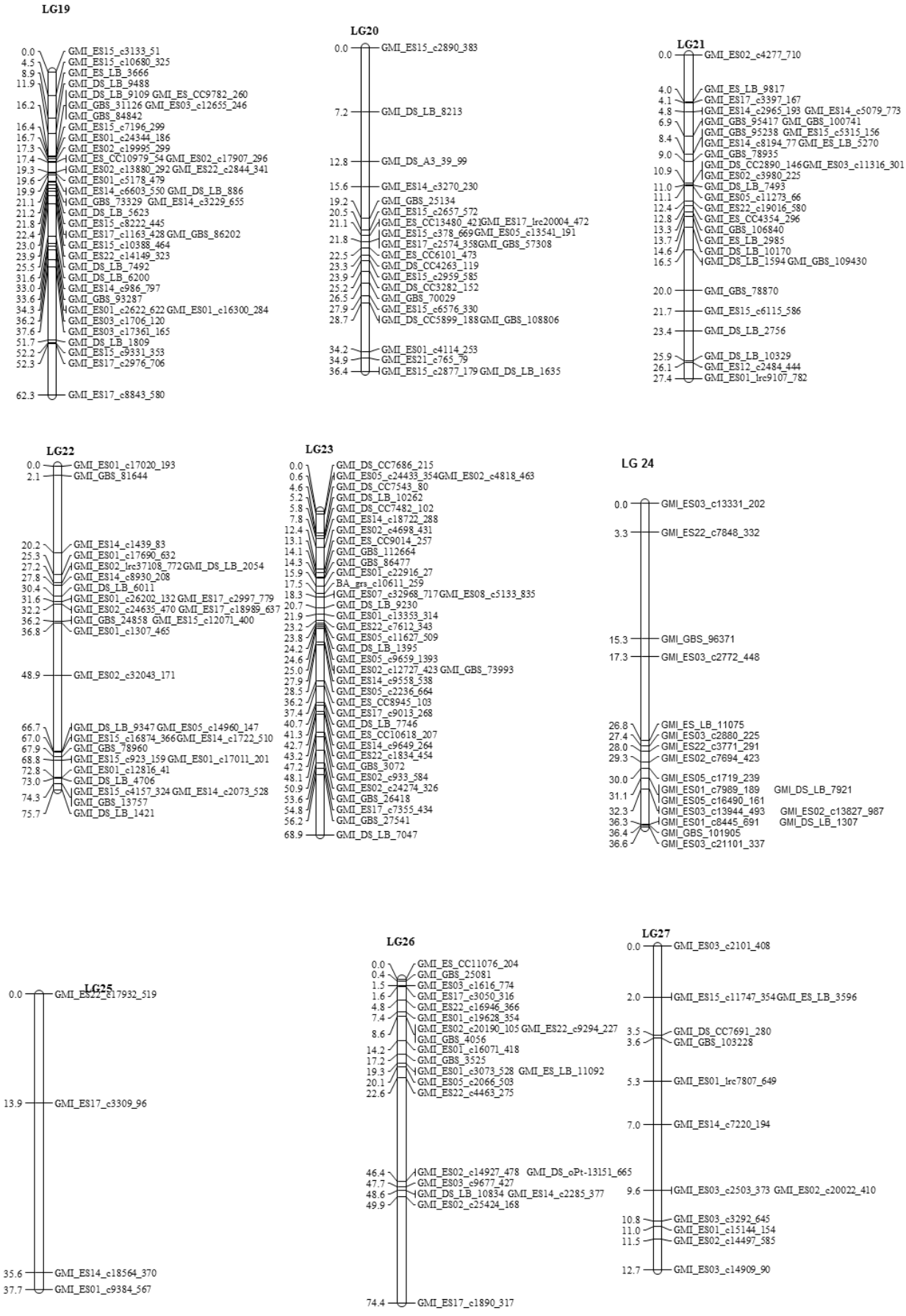


Figure S1 Continuation…



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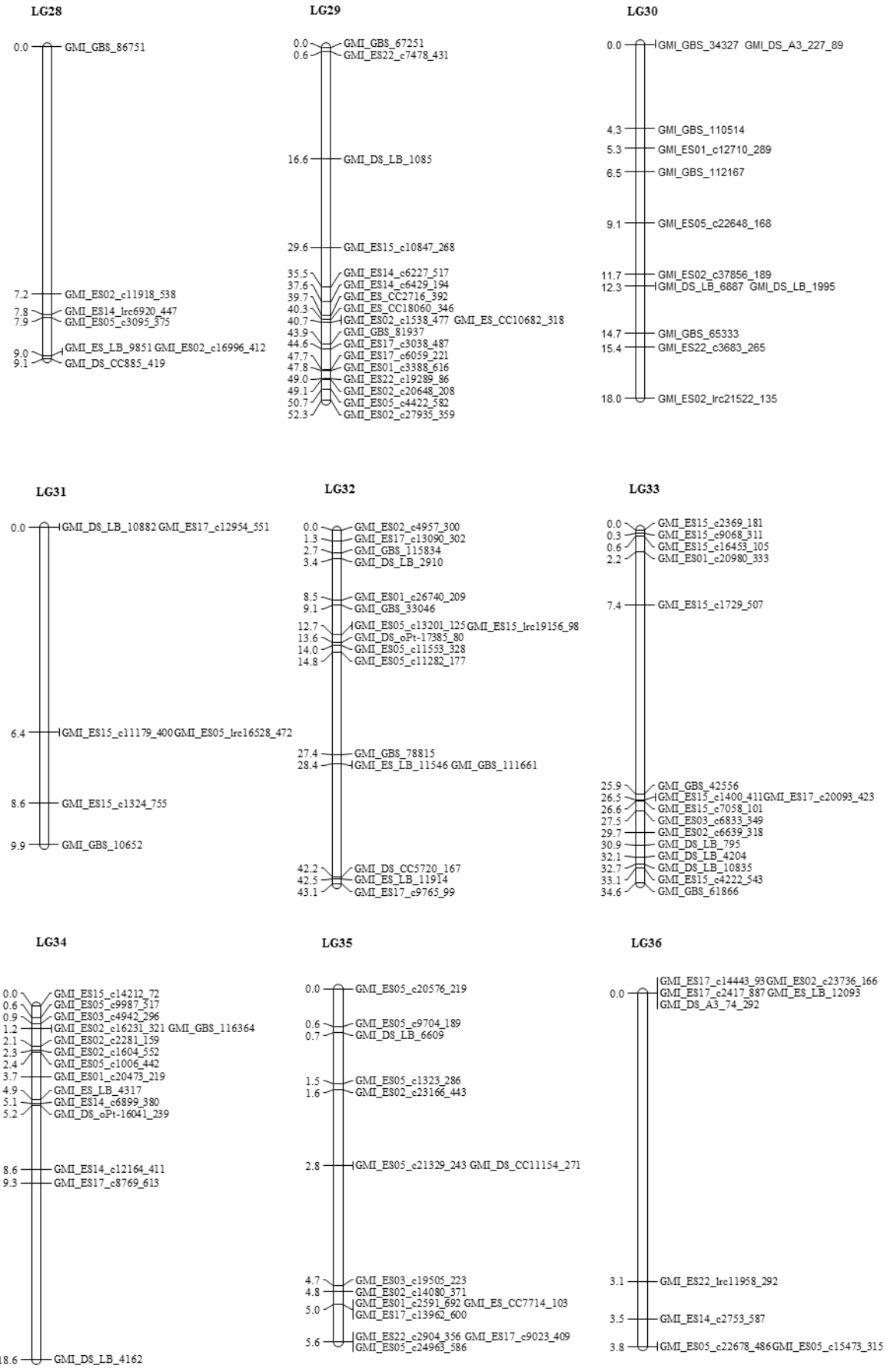

Figure S1 Continuation…

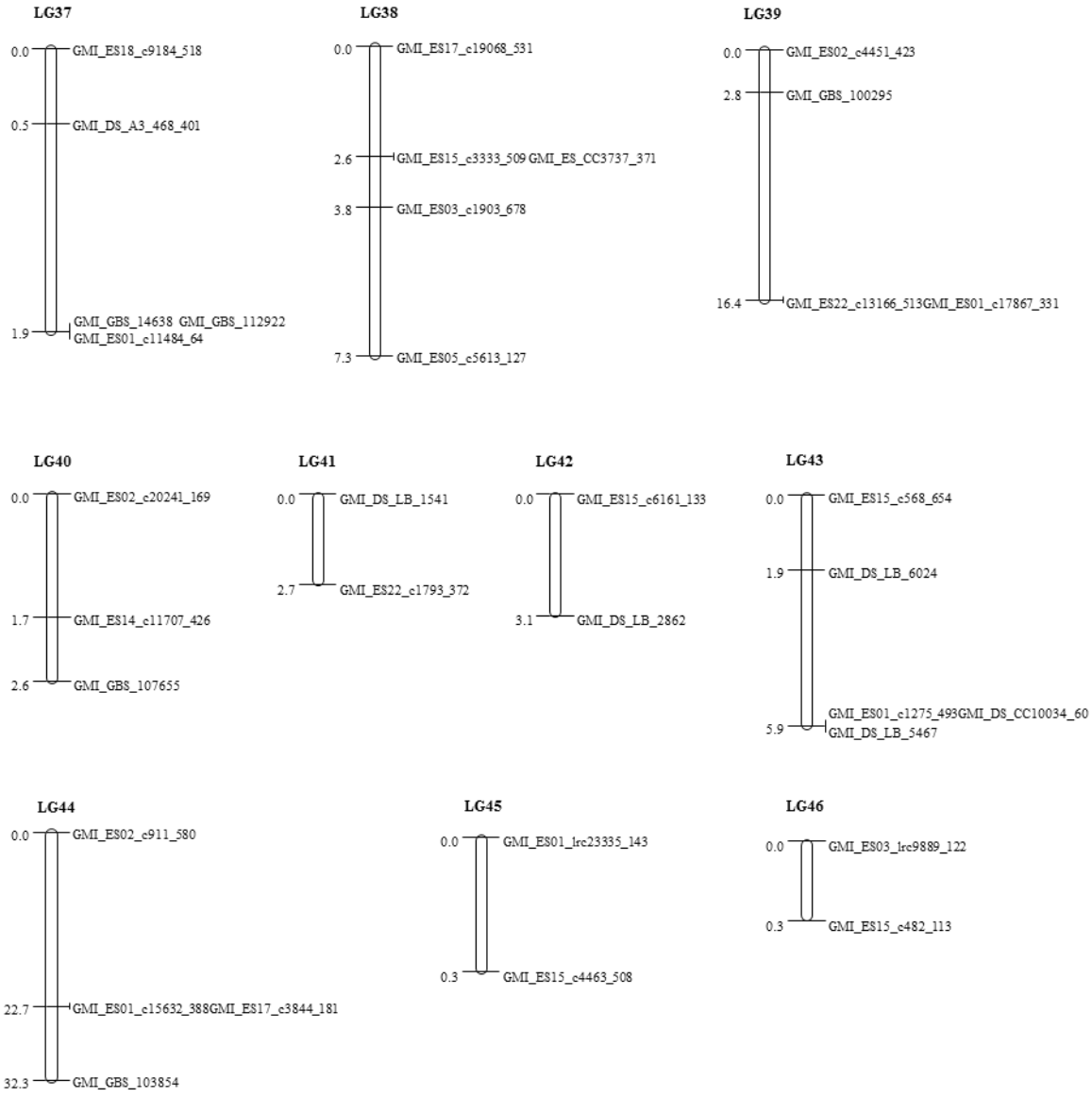

Figure S1 Continuation…

7 CONSIDERAÇÕES FINAIS

No Sul do Brasil, as epidemias de ferrugem do colmo foram caracterizadas pela presença de raças com amplo espectro de virulência, incluindo a raça TST em 2014, com virulência a todos os Pg-genes de resistência conhecidos. Entretanto, ao invés do predomínio da raça de maior espectro de virulência, as epidemias foram caracterizadas por uma mistura de raças. A diversidade de raças encontradas pode ser explicada pela diversidade de genes de resistência do hospedeiro no local da coleta (representado pelo germoplasma de um programa de melhoramento genético de aveia). Um estudo mais abrangente, com a amostragem em maior número de locais em lavouras comerciais e em plantas espontâneas de diferentes espécies hospedeiras, incluindo *A. strigosa*, permitirá caracterizar de forma mais precisa a dinâmica e a importância de cada raça para as epidemias no Sul do Brasil.

A diversidade revelada pelos 19 marcadores microssatélites desenvolvidos no presente estudo evidencia a existência de uma única população clonal entre os isolados de *Puccinia graminis* f. sp. *avenae* obtidos em dois anos de epidemias no Sul do Brasil. Na Austrália, a coleção de isolados obtida ao longo de 25 anos foi caracterizada por duas populações clonais, enquanto a maior diversidade genética foi observada entre as amostras da Suécia, onde as epidemias da doença e a sobrevivência do patógeno estão associadas ao ciclo sexual do patógeno utilizando *Berberis* spp. como hospedeiro alternativo. Apesar da reduzida variabilidade genética observada no Brasil e na Austrália, devido à ausência de recombinação sexual, as populações do patógeno foram caracterizadas pela existência de

patótipos (raças) com combinação de virulência a todos os Pg-genes de resistência. Estes resultados sugerem que as mutações para perda de genes de avirulência do patógeno são recorrentes. Sendo assim, no melhoramento para a resistência à ferrugem do colmo da aveia é fundamental adotar estratégias que permitam combinar genes de resistência parcial que garantam um bom nível de controle da doença e que não estejam associados à rápida evolução do patógeno.

Genótipos brasileiros do programa internacional da Quaker disponíveis na Austrália foram caracterizados para a resposta de resistência em plântula e em planta adulta. Todos os genótipos foram susceptíveis em plântula quando inoculados com o patótipo de maior virulência da Austrália. Utilizando o mesmo patótipo foi criada uma epidemia artificial em um ensaio de campo, onde foi identificado um elevado nível de resistência em duas linhagens irmãs - UFRGS 087105-1 e UFRGS 087129-1. Somente estes genótipos apresentaram níveis adequados de resistência em condições de campo, possivelmente associados à presença de genes de resistência de planta adulta. Este tipo de resistência é raro para a ferrugem do colmo da aveia sendo caracterizado em apenas dois genes (*Pg-11* e *Pg-17*). Estudos futuros deverão ser realizados para caracterizar as bases genéticas desta resistência e a sua efetividade em planta adulta contra diferentes patótipos.

A ação de um a três genes envolvidos na resistência da linhagem UFRGS 995088-3 previamente caracterizada em ensaios de campo na geração F_2, foi confirmada no presente estudo em duas populações utilizando a resposta de plântula e a análise genética na geração F_7. Utilizando dados moleculares foi possível identificar em uma das populações que a resistência previamente caracterizada pela ação de três genes é determinada por um único loco gênico em uma região com distorção de segregação. Foram identificados marcadores altamente associados a resistência que poderão ser utilizados em estudos futuros de validação em outras populações.

O controle genético da ferrugem do colmo da aveia é caracterizado por um número reduzido de genes de resistência no hospedeiro e pela habilidade do patógeno em superar as resistências já conhecidas. No Sul do Brasil, a população do patógeno foi caracterizada pela presença de um conjunto de raças com virulência a todos os genes de resistência o que determina que nenhuma cultivar seja resistente a todas as raças. Entretanto, a ferrugem do colmo não tem ocasionado grandes perdas para a produção de aveia no Brasil, com a epidemia da doença restrita ao final do ciclo da cultura. O sucesso do controle da doença é decorrente principalmente ao uso de fungicidas para controlar a ferrugem da folha (*P. coronata* f. sp. *avenae*) e a precocidade das cultivares que reduzem o período de exposição das lavouras ao patógeno. Neste contexto, a presença de genes de resistência raça-específica nas cultivares associada à diversidade de raças na população do patógeno é fundamental para reduzir o inóculo inicial da doença. Além disso, genes de resistência parcial ainda não caracterizados e expressos somente em planta adulta também podem estar contribuindo para a redução dos níveis da severidade da doença reduzindo a necessidade de aplicação de fungicidas para o controle da ferrugem do colmo nas áreas de produção comercial da aveia.