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Electrophoretic karyotypes of *Rhynchosporium commune, R. secalis and R. agropyri*

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Abstract Pulsed-field gel electrophoresis was used to generate electrophoretic karyotypes for 15 strains representing the three closely related plant-pathogenic fungi *Rhynchosporium commune*, *R. secalis* and *R. agropyri*. Between 13 and 16 chromosomes ranging in size from 0.9 to 6.4 Mb were found among the strains, leading to estimated genome sizes ranging from 54 to 63 Mb. Southern hybridization was used to identify homologous chromosomes, allowing detection of chromosome-length polymorphisms. There were no obvious differences in genome sizes or structures among the three species. The avirulence gene *NIP1* is present on a large chromosome that is not likely to be dispensable. Two strains of *R. commune* that were proposed in earlier studies to be aneuploid as a result of a parasexual cycle did not possess a larger number of chromosomes. The reported information on genome size and chromosome number will be useful for genome sequencing projects that aim to identify genes involved in speciation and host specialization.

Keywords Barley scald · CHEF · Chromosome-length polymorphism · PFGE · Pulsed-field gel electrophoresis

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Introduction

The fungus *Rhynchosporium secalis* (Oudem) J.J. Davis is a globally distributed plant pathogen that causes the scald disease on barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), triticale (x *Triticosecale* Wittmack) and other grasses (Caldwell 1937; Welty and Metzger 1996). Although *R. secalis* was originally described on rye (Oudemans 1897), the fungus is better known as a pathogen of barley due to its importance in producing animal feed, beer and whiskey. No sexual stage has been found for *R. secalis*, but it is known that the pathogen is related to *Tapesia*, *Pyrenopeziza*, *Phialocephala* and other Helotiales (Goodwin 2002; Zaffarano et al. 2010) and it evolves quickly in response to deployment of resistance genes and fungicides (e.g. Brown 1990; Kendall et al. 1993; Salamati and Tronsmo 1997) and that populations possess a high degree of diversity for various genetic markers including isozymes (Goodwin et al. 1993), restriction fragment length polymorphisms (McDonald et al. 1999), and microsatellites (Linde et al. 2005). Parasexual recombination (Newman and Owen 1985; Newton 1989) and cryptic sexual reproduction (Linde et al. 2003; McDonald et al. 1999; Salamati et al. 2000) have been proposed as mechanisms to generate the observed genetic diversity, but Goodwin et al. (1994) presented compelling evidence against parasexual recombination, suggesting that the observed variation could also be due to mutation and migration.

The lack of a recognized sexual stage precludes analysis of the inheritance of genes involved in pathogenicity, including the *NIP1* avirulence gene (Rohe et al. 1995). A large fraction of the global *R. secalis* population does not possess *NIP1* (Schürch et al. 2004), but the nature of the deletion is unknown. It was recently shown that some plant-pathogenic fungi have dispensable chromosomes (e.g., Wittenberg et al. 2009; Ma et al. 2010) and these chromosomes may offer an important mechanism for pathogens to become virulent on plants carrying corresponding resistance genes (Hatta et al. 2002; van der Does and Rep 2007).

A comprehensive analysis of the global population genetics of *R. secalis* led to the discovery that *R. secalis* is composed of three host-specialized species (Zaffarano et al. 2008). These species have recently been named according to their primary host as *R. commune* (infecting barley and other *Hordeum* spp.), *R. secalis* (infecting rye and triticale) and *R. agropyri* (infecting *Agropyron repens* and other *Agropyron* spp.) (Zaffarano et al. 2011). It was postulated that these *Rhynchosporium* spp. most likely originated from a common ancestor in northern Europe between 1,200 and 3,600 years ago (Zaffarano et al. 2008). Following the emergence of these pathogens, they are thought to have been disseminated globally through the spread of agriculture and trade in agricultural products (Linde et al. 2009). Although all three species appear to have arisen very recently and appear to be reproductively isolated, it is not known which genes were affected by the speciation process or whether host specialization affected their overall genome organization. We anticipate that whole-genome sequence comparisons enabled by inexpensive next-generation sequencing technologies will make it possible to understand the genetic basis of host specialization and speciation underlying this closely related group of plant-pathogenic fungi. Before undertaking a genome sequencing project, it is useful to have some basic knowledge of genome structure, including the overall genome size and the number of chromosomes to be expected in the assembly.

Electrophoretic karyotyping based on pulsed-field gel electrophoresis (PFGE) has been used to separate the chromosomes of many filamentous fungi (Walz 2004). In this study we used contour-clamped homogeneous gel electrophoresis (CHEF) to generate electrophoretic karyotypes for 15 strains of *Rhynchosporium* representing all three species. These karyotypes were used to: 1) make estimates of genome size and chromosome numbers for all three species; 2) compare overall genome structures of the three species; 3) physically map some well-studied genes onto particular chromosomes; 4) test the hypothesis that the *NIP1* gene is located on a small chromosome that may be dispensable; and 5) determine if previously characterized strains that possessed duplications for neutral markers are aneuploid, consistent with parasexual recombination.

Materials and methods

Fungal isolates

The 15 *Rhynchosporium* isolates used in this study included three host-specialized species originating from different locations in Australia and Switzerland and different hosts including cultivated barley, rye and triticale, as well as the wild grasses *Hordeum murinum* and *Agropyron repens* (Table 1). Four isolates from Zürich, Switzerland, and two from Horsham, Australia, were collected from the same barley field. All isolates had different genotypes for 6–8 restriction fragment length polymorphism (RFLP) loci (McDonald et al. 1999; Zaffarano et al. 2006). Isolation and culturing of the fungal isolates were as described previously (McDonald et al. 1999; Zaffarano et al. 2006).

Preparation of protoplasts

Isolates were grown on lima bean agar (LBA) plates amended with 50 μg/l of kanamycin. The LBA growth medium was prepared by autoclaving 65 g of dried Lima beans in 1 l of water. The autoclaved mixture was blended at maximum speed in a mixer for 10 s and filtered through 2 layers of cheesecloth. The resulting slurry was autoclaved with 12 g of agar, cooled and poured into Petri plates. Isolates were transferred from silica gel storage at −80°C onto LBA plates and grown for 14 days at 18°C. Single colonies were then transferred to fresh LBA plates and kept at 18°C for 14 days. Spores and mycelial fragments were harvested by adding 5 ml of sterile water and gently scraping the surface of the mycelium with a sterile glass microscope slide. 200 μl of the resulting suspension of spores and mycelium were transferred

Table 1 *Rhynchosporium* isolates and species used for electrophoretic karyotype analysis

Fungal isolate	Previous publication or source and year of collection (in parentheses)	Location	Host	Species
00CH-A7a	Linde et al. 2003	Zürich, Switzerland	*Hordeum vulgare*	*R. commune*
00CH-F4b	Linde et al. 2003	Zürich, Switzerland	*Hordeum vulgare*	*R. commune*
00CH-D2b	Linde et al. 2003	Zürich, Switzerland	*Hordeum vulgare*	*R. commune*
00CH-B2a	Linde et al. 2003	Zürich, Switzerland	*Hordeum vulgare*	*R. commune*
99CH-6C6B	Linde et al. 2003	Cugy, Switzerland	*Hordeum vulgare*	*R. commune*
99CH-2A2B	Linde et al. 2003	Leuggern, Switzerland	*Hordeum vulgare*	*R. commune*
AUS-3E6a[a]	McDonald et al. 1999	Horsham, Australia	*Hordeum vulgare*	*R. commune*
AUS-3F4b[a]	McDonald et al. 1999	Horsham, Australia	*Hordeum vulgare*	*R. commune*
04CH-WPK-A11.1	P.L. Zaffarano, C.C. Linde (2004)	Zürich, Switzerland	*Hordeum murinum*	*R. commune*
04CH-WPK-1B6.1	P.L. Zaffarano, C.C. Linde (2004)	Zürich, Switzerland	*Hordeum murinum*	*R. commune*
02CH-4-14a.1	C.C. Linde (2002)	Maur, Switzerland	*Secale cereale*	*R. secalis*
99CH-1B8	Zaffarano et al. 2006	Zofingen, Switzerland	*Secale cereale*	*R. secalis*
FRI/D-Nr.2	L. Jestin (2002)	Theix, France	x *Triticosecale* Wittmack	*R. secalis*
04CH-KB-2B-5A1.2	P.L. Zaffarano, M. Zala, C.C. Linde (2004)	Zürich, Switzerland	*Agropyron repens*	*R. agropyri*
04CH-WPK-2-4A1.2	P.L. Zaffarano, C.C. Linde (2004)	Zürich, Switzerland	*Agropyron repens*	*R. agropyri*

[a] Putative partial diploid based on earlier RFLP analysis (Salamati et al. 2000)

to each of 50 LBA plates and incubated at 18°C for 10 to 14 days.

Protoplasts were prepared as described in Rohe et al. (1996) with some modifications. Spores and mycelium were harvested from each plate as described above. The resulting suspension of spores and mycelium was filtered through two layers of cheesecloth to remove the suspended agar and placed into 50-ml Falcon tubes. The suspension was incubated overnight at room temperature to allow the spores to germinate. The suspension was then centrifuged for 5 min at 3,400 rpm (2,600 × g). The supernatant was removed and the pellet was resuspended in 20 ml of protoplast buffer (10 mM Tris/HCl, pH 7.5, 0.7 M KCl, 50 mM $CaCl_2$). The spore suspension was again centrifuged for 5 min at 3,400 rpm (2,600 × g). The supernatant was removed and the pellet was re-suspended in 20 ml of enzyme mix buffer [20 ml of protoplast buffer with 200 mg of Driselase from *Basidiomycetes* sp. (Sigma D9515)] and incubated with occasional gentle agitation at room temperature until protoplasts were observed, between 6 and 24 h. The protoplast solution was filtered through four layers of cheesecloth to separate cell wall debris from protoplasts. The cheesecloth was rinsed with 20 ml of protoplast buffer to minimize the loss of protoplasts during the filtering process. The Driselase was removed by centrifuging the filtrate in Falcon tubes at 4°C for 5 min at 2,000 rpm (900 × g), the supernatant was removed and the pellet was washed with protoplast buffer. The protoplast-buffer suspension was centrifuged again at 4°C for 5 min at 2,000 rpm (900 × g) and the pellet was re-suspended in 1 ml of protoplast buffer. The concentration of protoplasts in the suspension was adjusted to 4×10^8 protoplasts/ml using a haemocytometer.

Preparation of intact chromosomal DNA for pulsed-field gel electrophoresis

The concentrated protoplast suspension was mixed carefully with an equal volume of 2% low-melting-point agarose (molecular biology grade, Biofinex) with low-melting buffer (1 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris/HCl, pH 7.5) at 40°C. This mixture was transferred into a plug-forming stand and hardened on ice for 15 min. The protoplast plugs were incubated in lysis buffer [0.25 M EDTA, 10 mM Tris/HCl, pH 7.5, 1% N-Lauroylsarcosyl, 1 mg/ml Proteinase K (Sigma)] for 48 h at 37°C in a 15-ml Falcon tube. The plugs were transferred to a 50-ml Falcon tube and washed three times for 30 min with 20 ml of washing buffer (0.25 M EDTA in TE-Buffer) and then stored at 4°C in washing buffer.

Contour-clamped homogenous electric field (CHEF) gel electrophoresis

Chromosomal DNA was resolved using a Bio-Rad CHEF-DRII apparatus. The electrophoresis was conducted in 1% TAE agarose gels using a running buffer of 1% TAE at 13°C. Three sets of running conditions were used to separate chromosomes in different size range: The conditions for separating small chromosomes (1–3 Mbp) used a ramped switch time of 400–900 s for 96 h at 100 V in 1.2% agarose gels. To resolve intermediate-sized chromosomes (3–5 Mbp) the ramped switch time was 1,600–1,900 s for 92 h at 70 V and 600–800 s for 48 h at 100 V using 0.925% agarose gels. Large chromosomes (>5 Mbp) were resolved by a ramped switch time of 3,000–4,000 s for 140 h at 50 V followed by 2,500–3,000 s for 96 h at 50 V using 0.8% agarose gels. Chromosomes of *Saccharomyces cerevisiae* (Biorad), *Hansenula wingei* (Biorad) and *Schizosaccharomyces pombe* (Biorad) were used as size standards. Gels were stained for 30 min in ethidium bromide (5 μg/ml), de-stained for 1 h in ddH_20 and then photographed in a UV transilluminator (BIORAD, GEL DOC 2000). The gel pictures were analyzed with the program Quantity One (Biorad) to estimate the band sizes and measure the relative intensity of each band. Band sizes were estimated using a linear model as recommended in the Quantity One instructions for CHEF gels.

Southern blotting and DNA hybridization

Chromosomal DNA separated by CHEF was depurinated in 0.25 M HCl for 40 min. The gel was then rinsed briefly with distilled water and vacuum-blotted onto a Hybond N^+ membrane (Amersham Biosciences UK Limited) in 0.4 M NaOH with a vacuum blotter (2016 Vacugene, LKB Bromma) for 90 min at 50 cm H_2O (49 mbar). The membranes were rinsed with distilled water to remove small pieces of agarose and washed for 5 min in 2X SSC. The membranes were air dried and stored in plastic wrap until used.

Eight DNA probes were hybridized to the Southern blots to identify homologous chromosomes and detect chromosome length polymorphisms. Anonymous DNA probes pRS2, pRS6, pRS47, and pRS52 were used previously for RFLP analysis (McDonald et al. 1999; Salamati et al. 2000; Zaffarano et al. 2006). PCR amplifications as described in Linde et al. (2003), Schürch et al. (2004) and Zaffarano et al. (2008) were used to generate the probes for α-tubulin, the mating-type idiomorphs *MAT1-1* and *MAT1-2* and the avirulence gene *NIP*1. Nick translation with ^{32}P-labeled dCTP (Amersham Bioscience UK Limited), hybridization and autoradiography were as described previously (Zaffarano et al. 2006).

Results

The electrophoretic karyotypes of 15 isolates of *Rhynchosporium* from five hosts and representing three closely related species were analyzed using CHEF gel electrophoresis. The number of chromosomal bands resolved in each isolate by CHEF ranged from 10 to 14 (Table 2). In all isolates some chromosomal bands showed a higher fluorescence than neighbouring bands on the ethidium bromide-stained gels. Densitometry analysis of the gel images revealed that these bands fluoresced approximately 2 or 3 times as much as the neighbouring bands in the same isolate (Fig. 1), indicating that these bands most likely contained two or three co-migrating chromosomes. After taking into account these putative double or triple chromosomal bands, 13 to 16 chromosomes could be resolved in each isolate (Table 2).

The size of each chromosome was estimated using *S. cerevisiae*, *H. wingei* and *S. pombe* chromosomes as size standards, assuming that chromosome sizes were a linear function of migration distance in a gel. Estimates of genome size for each isolate were based on summing the individual chromosome sizes, taking into account the cases where two or three chromosomes migrated to the same position on the gel. With these assumptions, the number of chromosomes ranged from 13 to 16 and the genome size ranged from approximately 54–63 Mb (Table 2). Among the 10 *R. commune* isolates, the chromosome number was either 15 or 16 and the genome size estimate ranged from approximately 54–63 Mb. Among the 3 *R. secalis* isolates, the chromosome number was 13 or 14 and the genome size ranged from approximately 55 to 58 Mb. For the two *R. agropyri* isolates, there were either 15 or 16 chromosomes and the genome size was approximately 54 Mb. For all isolates, the majority of chromosomes were between 2 to 6 Mb in size and only one isolate had a chromosome smaller than 1 Mb (Table 2, Fig. 1).

Table 2 Estimated sizes (Mb) of *Rhynchosporium* chromosomes

Fungal isolate	Chromosome														Total	No of chr.
	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14		
00CH-A7a	6.4	5.9	5.7	4.9	4.4	4.3	4.1	3.7	3.3	3.2	2.9	**2.8**[c]	2.6	2.4	59.6	15.0
00CH-F4b	6.4	6.0	5.8	4.9	4.7	4.4	4.2	3.9	3.7	3.4	3.1	**2.9**[c]	2.8	2.7	61.8	15.0
00CH-D2b	6.2	**5.8**[b,c]	5.1	4.8	4.3	4.2	**3.9**[c]	3.3	3.1	3.0	2.9	2.7	2.6	1.3	63.0	16.0
00CH-B2a	6.3	5.9	5.7	4.8	4.6	4.2	4.0	**3.6**[c]	**3.2**[c]	2.9	2.8	2.5	1.2	1.1	59.6	16.0
99CH-6C6B	6.4	**5.8**[c]	5.0	4.6	4.3	4.0	**3.9**[c]	3.2	3.1	2.9	**2.7**[c]	2.4			60.5	15.0
99CH-2A2B	6.4	5.9	5.6	5.0	4.5	4.4	4.1	3.8	3.5	3.1	2.9	**2.9**[c]	2.8	2.3	60.0	15.0
AUS-3E6a[e]	6.3	5.7	**5.4**[c]	4.3	4.1	3.9	3.6	3.4	3.1	2.9	**2.8**[c]	2.5	2.2	0.9	59.0	16.0
AUS-3F4b[e]	6.3	5.7	5.5	5.0	**4.1**[c]	3.7	3.5	**3.0**[c]	2.9	2.8	2.7	2.4	2.2	1.0	58.1	16.0
04CH-WPK-A11.1	6.2	**5.5**[c]	4.4	4.2	3.9	3.7	3.2	3.0	2.9	2.8	**2.4**[d]	2.2			54.4	15.0
04CH-WPK-1B6.1	6.4	**5.1**[c]	4.6	**3.9**[c]	3.6	3.2	2.9	2.9	2.8	**2.6**[c]	2.3	2.2	1.3		55.2	16.0
02CH-4-14a.1	6.3	6.0	5.7	5.1	4.6	**4.0**[c]	3.7	**3.1**[c]	3.0	**2.9**[c]	2.6				56.9	14.0
99CH-1B8	6.3	6.2	5.7	4.9	4.7	4.3	**3.9**[c]	3.7	3.1	**3.0**[c]	**2.7**[c]				58.0	14.0
FRI/D-Nr.2	6.3	6.0	5.7	4.9	4.3	4.1	**3.9**[c]	3.6	**3.1**[c]	2.9	2.8				54.6	13.0
04CH-KB-2B-5A1.2	6.4	5.5	**4.7**[c]	**3.8**[c]	**3.6**[c]	3.2	3.0	**2.7**[d]	2.3	1.5					54.0	15.0
04CH-WPK-2-4A1.2	6.3	**5.4**[c]	4.4	4.0	3.7	3.2	3.0	2.9	2.8	**2.7**[c]	2.5	2.2	1.5	1.5	54.0	16.0

[a] Chromosome numbers were assigned from largest to smallest

[b] Bands containing multiple chromosomes according to fluorescent densitometry analysis are indicated in bold

[c] Represents bands that likely contain two co-migrating chromosomes

[d] Represents bands that likely contain three co-migrating chromosomes

[e] Putative partial diploid based on earlier RFLP analysis (Salamati et al. 2000)

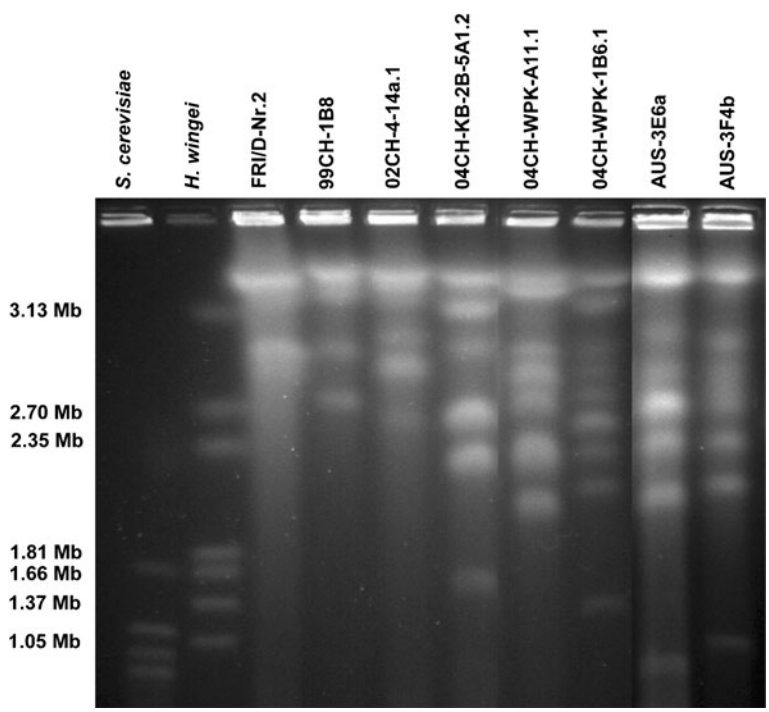

Fig. 1 A composite image showing the electrophoretic karyotypes of the smallest chromosomes for eight *Rhynchosporium* strains including all three species. The left-most lanes show chromosome size standards from *Saccharomyces cerevisiae* and *Hansenula wingei*. The next three lanes show *R. secalis* followed by one lane with *R. agropyri* and the final four lanes show *R. commune*

Southern hybridizations with different probes detected chromosome length polymorphisms between homologous chromosomes in different isolates (Fig. 2). Probe hybridization data are summarized in Table 3. Complete hybridization data were not available for all isolates for all probes due to membrane degradation, poor hybridization or high levels of non-specific background hybridization on some Southern blots. RFLP probes pRS47 and pRS2 hybridized to chromosomes 2 or 3 in all isolates while pRS6 hybridized to chromosomes 4–5 Mb in size. Probe pRS6 hybridized to two chromosomes in three of the isolates (Table 3). The *MAT1-1* and *MAT1-2* probes hybridized to chromosomes 3–4 Mb in size. The *NIP1* probe hybridized to a single chromosome in isolates 00CH-B2a, 99CH-6C6B and to two chromosomes in 04CH-WPK-A11.1.

Discussion

A recent series of studies showed that the fungal plant pathogen named as *Rhynchosporium secalis* is composed of three host-specialized species that most likely originated in Scandinavia approximately 1,200–3,600 years ago (Linde et al. 2009; Zaffarano et al. 2006, 2008). Here we refer to these three species using their new names (Zaffarano et al. 2011): *R. commune*, *R. secalis* and *R. agropyri*. Based on their karyotypes, we believe that these *Rhynchosporium* species contain 13 to 16 chromosomes ranging from approximately 0.9–6.4 Mb in size and have an overall genome size of approximately 54–63 Mb, with a mean value across all 15 isolates of approximately 58 Mb. This information will be useful for genome projects that seek to assemble the complete DNA sequence of *Rhynchosporium*. Our findings are similar to results from other plant-pathogenic fungi, which often contain between 10 and 20 chromosomes (Walz 2004). The precision of these genome size estimates is limited by many factors inherent to PFGE analysis, especially the fact that all of these strains had chromosomes larger than the largest chromosome of *S. pombe* (5.7 Mb) and thus the estimated sizes of the largest chromosomes were not

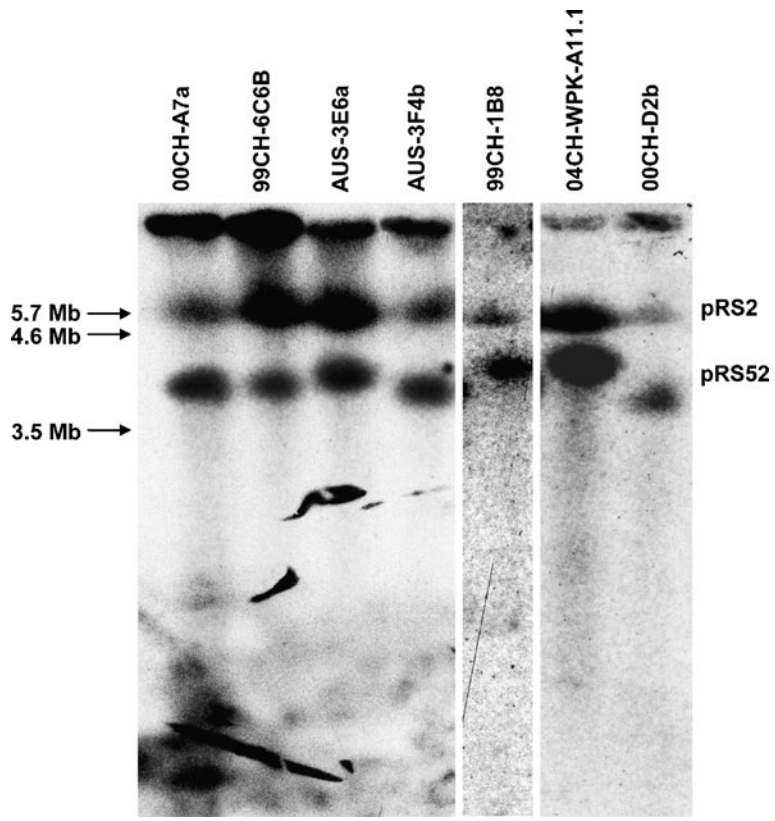

Fig. 2 Composite autoradiogram of Southern blots of *Rhynchosporium* electrophoretic karyotypes of medium-sized chromosomes. RFLP probe pRS2 hybridized to a chromosome ±5.7 Mb in size for each isolate while pRS52 hybridized to a smaller chromosome in each isolate. Chromosome length polymorphism can be seen with pRS52. The first four lanes are *R. commune* followed by one lane with *R. secalis,* one lane with *R. agropyri* and the final lane with *R. commune*

Table 3 Chromosomes that hybridized to probes on Southern blots of *Rhynchosporium* chromosomes indicated in Table 2

Fungal isolate	Probe							
	pRS6	pRS47	MAT1-1	MAT1-2	NIP1	pRS52	pRS2	alpha-tubulin
00CH-A7a	5	2	8		ND[a]	6	3	ND
00CH-F4b	5	2	8		ND	7	ND	ND
00CH-D2b	5	2		8	ND	6	2	2
00CH-B2a	5	2	7		4	6	3	ND
99CH-6C6B	4	2		8	10	5	2	ND
99CH-2A2B	5	2		7	null [b]	7	ND	ND
AUS-3E6a	4	2		7	ND	5	3	ND
AUS-3F4b	5	2		7	ND	5	3	ND
04CH-WPK-A11.1	3	2	4		3 and 4	3	2	2
04CH-WPK-1B6.1	4	2		6	null	5	2	2
02CH-4-14a.1	6	3		7	null	ND	ND	2
99CH-1B8	5 and 7	3	8		ND	6	3	2
FRI/D-Nr.2	5 and 7	3	7		ND	ND	ND	2
04CH-KB-2B-5A1.2	4	2	7		null	ND	ND	3
04CH-WPK-2-4A1.2	3 and 4	2		5	ND	ND	ND	3

[a] No data

[b] Null allele due to *NIP1* deletion

constrained by a chromosome known to have a larger size.

Though the three species appear to be reproductively isolated (Zaffarano et al. 2008), there does not appear to be a significant difference in their overall genome size or organization. But too few isolates of *R. agropyri* and *R. secalis* were included to make a robust statistical analysis. The lack of obvious differences in electrophoretic karyotypes likely reflects their close evolutionary relationship and extremely recent divergence (Zaffarano et al. 2008). The most notable pattern was that the three *R. secalis* isolates were the only ones where all chromosomes were larger than 2.5 Mb. If this difference is still present after examining the karyotypes of a larger number of *R. secalis* strains, this would suggest that the speciation event, probably driven by host specialization, led to a significant difference in chromosome structure in a relatively short period of time. Comparisons of the complete genome sequences of the different species will provide additional insight into the nature of any differences in genome structure. Since genetic mapping is not yet possible in *Rhynchosporium*, physical mapping using electrophoretic karyotypes may provide an important tool to assist with assembly and ultimately comparison of these genomes.

The hybridization data provided compelling evidence for chromosome length polymorphisms (CLPs, Fig. 2), suggesting that the genomes of these *Rhynchosporium* species are plastic, as found in many other phytopathogenic fungi (e.g., Wittenberg et al. 2009). CLPs in fungi have been detected in laboratory strains (Brody and Carbon 1989; Carle and Olsen 1985), natural isolates from widely separated geographical locations (Kinscherf and Leong 1988), and among isolates sampled from the same field (McDonald and Martinez 1991). In this case, CLPs were found among *R. commune* strains sampled from the same field, illustrating that CLPs can be distributed on very small spatial scales.

Though the avirulence gene *NIP1* was present in only three isolates (00CH-B2a, 99CH-6C6B and 04CH-WPK-A11.1), it was always found on a relatively large chromosome. This suggests that the *NIP1* deletions known to be frequent and widely distributed (Schürch et al. 2004) must be due to deletion of small regions within chromosomes, rather than being due to loss of dispensable chromosomes. Interestingly, *NIP1* was located on two chromosomes

in isolate 04CH-WPK-A11.1 from *H. murinum*. *NIP1* encodes a necrosis-inducing peptide that may act as an effector for *Rhynchosporium* and it also functions as an avirulence protein that prevents disease development in barley cultivars carrying the *Rrs1* resistance gene (Rohe et al. 1995). This is the first evidence for two copies of this gene in a strain of *Rhynchosporium*.

The mating type loci *MAT1-1* and *MAT1-2* were found on chromosomes with sizes of 3–4 Mb, while the RFLP loci pRS6 and pRS47 were located on chromosomes 4–6 Mb in size. It is clear that these loci are on different chromosomes so that any nonrandom associations found among these loci in population genetic analyses cannot be due to linkage. Interestingly, pRS6 hybridized to two chromosomes in two of the three *R. secalis* strains and in one of the two *R. agropyri* strains. In a recent study (Zaffarano et al. 2006), the pRS6 RFLP locus had the highest variability of all RFLP loci tested among 1343 *R. commune* isolates sampled from 30 barley populations around the world. In contrast, only one unique allele was found among 23 *R. secalis* isolates sampled from a Swiss rye population. RFLP variability at the pRS6 locus has not been studied in *R. agropyri*.

In an earlier study, two of the isolates included in this analysis (AUS-3E6a and AUS-3F4b) had been shown to possess more than one allele for at least one RFLP locus even after DNA was extracted from a colony that was started from a single spore (Salamati et al. 2000). Parasexual recombination has been proposed as a mechanism for creating the high level of variability found in *Rhynchosporium* populations because a teleomorph has not yet been discovered (Newman and Owen 1985; Newton 1989). In our earlier work, we hypothesized that the heterozygous isolates could be a by-product of parasexual recombination and we proposed to use PFGE to determine if these isolates were aneuploids, carrying extra chromosomes as a by-product of parasexual recombination. Our analysis of these karyotypes provided no evidence for extra chromosomes in these isolates compared to the other *R. commune* strains. Thus we could not support the hypothesis of parasexual recombination as a source of the genotype diversity that has been described in earlier studies. Instead we believe that the observed variation at these loci in these isolates were the result of duplications, as we observed earlier in *M. graminicola* (McDonald and Martinez 1991).

We anticipate that these findings will be very helpful for informing genome sequencing projects that aim to identify genes involved in host specialization and speciation in these closely related *Rhynchosporium* species.

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