Genetic Variation in *Puccinia graminis* Collected from Oats, Rye, and Barberry

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ABSTRACT

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Puccinia graminis, the causal agent of stem rust, was collected from its alternate host barberry (*Berberis* spp.) and two different uredinial hosts, oats (*Avena sativa*) and rye (*Secale cereale*). The samples were analyzed using 11 polymorphic simple sequence repeat (SSR) markers. There were

large differences between fungal populations on oats (*P. graminis* f. sp. *avenae*) and rye (*P. graminis* f. sp. *secalis*), and the genetic variation within the different *formae speciales* was also high. It was possible to distinguish between the two *formae speciales* on barberry. Additional genotypic groups not present in the field samples from oats and rye were also identified on barberry. Our results confirm the importance of barberry in maintaining the populations of *P. graminis* in Sweden and the importance of the sexual stage for the survival of the pathogen.

Stem rust, caused by *Puccinia graminis* (Pers.), is a serious disease of cereal crops in grain producing areas of the world. *Puccinia graminis* is a biotrophic fungus and its urediniospores have been reported to infect 365 different grass species, including economically important crops such as wheat, rye and oats (24). Being a heteroecious rust, it needs an alternate host to complete its sexual life cycle. Gäumann (13) reported over 70 *Berberis* species and some species of *Mahonia* to be alternate hosts for *P. graminis*. The most frequently found alternate host in Sweden is common barberry (*Berberis vulgaris*), and the incidence of barberry has increased during the last decades (5,9,11,14, 22,32,34) due to the repeal of the Swedish law of barberry eradication, which was in force between 1918 and 1994. Other *Berberis* species and hybrids are also present in this country. The presence of both barberry and the uredinial hosts is needed by the pathogen to complete its life cycle, and allows it to permanently occupy a certain geographic area. Barberry also provides a source of initial inoculum for infection on the seasonal, uredinial, host (30). The sexual reproduction enables the fungus to recombine its genes and resulting in new virulence combinations (25), some of which may have serious consequences for cereal crop production (17).

Formae speciales and subspecies denomination. Eriksson and Henning (10) subdivided *P. graminis* into specialized forms or *formae speciales* (f. sp.), which reflect the fungus' parasitic adaptation to certain genera of grass hosts. The subdivisions have been supported by crossing studies and comparisons of DNA sequences (24). Johnson (20) found that the *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *secalis* infecting wheat and rye respectively, were closely related whereas the *P. graminis* f. sp. *avenae*, infecting oats, clearly differed from the other two. Urban (35) divided *P. graminis* into different subspecies and varieties based primarily on morphology, of which *P. graminis* subsp.

graminis var. *graminis* mainly occurred on *Triticum*, *Aegilops*, and *Elymus* while *P. graminis* subsp. *graminis* var. *stakmannii* mainly occurred on *Avena*, *Hordeum*, and *Elytrigia*. Mating between different *formae speciales* of *P. graminis* may occasionally occur on barberry, but signs of intervarietal sterility were shown between *formae speciales* (20). This agrees with Zambino and Szabo (38), who found that the sequences of the ITS (internal transcript spacer) region could be used to separate *P. graminis* f. sp. *tritici* and *secalis* from *P. graminis* f. sp. *avenae*, *dactylis*, *lolii*, and *poae*.

Berberis spp. is not the alternate host only for *P. graminis*. A number of *Puccinia* species form aecia on *Berberis* spp. including *P. brachypodii*, *P. pygmaea*, and *P. montanensis* (7). It was recently found that *P. striiformis*, causing yellow or stripe rust on wheat also undergoes sexual reproduction on barberry (18). The presence of the alternate host potentially advances disease onset, increases the initial inoculum levels, and increases the number of races of the pathogen. The races will be more diverse in the presence of the alternate host than if the pathogen only reproduces clonally (30). Johnson (20) concluded that the hybridization of two *formae speciales* may broaden the range of pathogenicity, and suggested that it was accompanied by a reduction in "pathogenic intensity" on the tested hosts. Previous studies of *P. graminis* f. sp. *avenae* have shown that if the alternate host is present, the genetic diversity is large (4).

In Sweden most wheat and rye is planted in the fall, and *P. graminis* can survive mild winters on these crops, but stem rust in wheat is only seen on older landraces and is not a problem in modern cultivars due to effective resistance genes. The first visible signs of stem rust in rye are usually reported in the end of June or beginning of July when the crop is in the heading or soft dough stage (DC 55 to 85 [37]). For oats, which is only grown as a spring crop in Sweden, the first disease reports come approximately 2 weeks later (also DC 55 to 85), in the middle or end of July (26).

The purpose of this study was to investigate the genetic differentiation between *formae speciales* of *P. graminis* on two different grass hosts and to determine whether *P. graminis* collected from barberry could be matched to these *formae speciales*. Aecia were collected from barberry and uredinia were collected

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains one supplementary table.

from oats (*Avena sativa*) and rye (*Secale cereale*). The genetic relationship was evaluated by using simple sequence repeat (SSR) markers, which previously have been used to study *P. graminis* (4,19,33,40).

MATERIALS AND METHODS

Sample collection. Samples were collected from both the uredinial hosts and the aecial hosts at different locations in southern Sweden (Fig. 1). Samples from the aecial host, *Berberis* spp. were collected in June each year (2008 to 2010). If more than one bush was present at a site, several collections could be made. The distance between bushes in a single collection was never greater than 20 m. At one site, when two different species of barberry were present, the samples were divided into two collections. From each collection, three to five clearly defined aecia were cut out and analyzed as separate samples.

Fig. 1. Location of collection sites, oat fields (+), rye fields (x), and barberry locations (Δ). Numbers correspond to the population numbers in Table 2. Bar = 100 km.

The collections from the grass host were done between the end of July and the end of August 2008 and 2009 (DC 83 to 92). In each field, five to eight stems were collected at 10 points, 10 m apart, along a straight line. In all but one field, this was repeated in three replications 10 m apart, creating a regular grid design of 30 × 100 m. In one field (Bränne Övregård) there was only one replication with 10 collections. From each collection point within a field, the stems with pustules were kept dry in a paper bag. Samples collected from oats were considered to be *P. graminis* f. sp. *avenae* and those from rye *P. graminis* f. sp. *secalis*. For DNA extraction, a single pustule from each collection was carefully cut out and used as a sample. When developing the method (4), a subset of single pustule isolates was made from the same source pustule and then genotyped. The genotypes from the different isolates from the same source pustules were identical or within the error margin of the allele determination procedure (data not shown). All samples from a field were treated as one population. At all field sites, the close surroundings were inspected for occurrence of *Berberis* spp., but no bushes were found in the field or its immediate surroundings. The collections from oats have previously been used and discussed in a study evaluating the oat stem rust population in Sweden (4).

SSR marker analysis and evaluation. The DNA extractions were carried out with OmniPrep kits (GenoTech, St. Louis) according to the manufacturer's instructions for fungal tissues with minor modifications as described by Berlin et al. (4). The samples were analyzed using 11 polymorphic SSR markers (Table 1). Amplification of the SSR markers was carried out as a multiplex reaction according to Berlin et al. (4). The samples were analyzed at the Uppsala Genome Center at Rudbeck laboratory, Uppsala University, by capillary electrophoresis analysis (ABI 3730XL DNA Analyzer). The lengths of the fragments were determined with GeneMarker (Softgenetics) to identify the different genotypes. If any sample showed more than two clear peaks at a locus, the alleles were coded as missing.

The internal transcribed spacer (ITS) region was sequenced for most of the samples collected from *Berberis* spp. to confirm the species identity. The rust-specific primers ITS1RustF10d and StdLSUR2a (3) were used both for polymerase chain reaction (PCR) amplification and sequencing. The same DNA samples as for the SSR marker analysis were used for the ITS region amplification. The ITS region was amplified in 50-μl reactions, including each of the following in the final concentrations: 2 ng/μl DNA template, 0.02 mM dNTP, 0.2 μM of each primer, 2.75 mM MgCl₂, 0.05 U/μl of DreamTaq DNA polymerase and DreamTaq buffer according to the manufacturer's recommendation. The PCR program included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C, annealing at 57°C and extension at 72°C, each for 30 s, and finally an extension step at 72°C for 7 min. The PCR products were purified with AMPure (Agencourt Bioscience Corporation, MA) according to the manu-

TABLE 1. Performance of the 11 simple sequence repeat (SSR) markers used in this study^a

SSR marker	N _a	H _o (s.e.)	H _e (s.e.)	F _{IS}	% missing values			Reference
					Barberry	Oats	Rye	
Pgestssr021	8	0.412 (0.045)	0.574 (0.040)	0.283	2.1	2.7	5.8	Berlin et al. (4)
PgtSSR21	7	0.457 (0.053)	0.470 (0.034)	0.027	0.7	6.3	10.1	Szabo (34)
Pgestssr024	15	0.610 (0.049)	0.647 (0.026)	0.057	1.4	0.5	2.9	Berlin et al. (4)
Pgestssr109	10	0.513 (0.046)	0.533 (0.035)	0.038	2.1	5.0	2.9	Zhong et al. (40)
Pgestssr171	22	0.432 (0.040)	0.743 (0.026)	0.419	21.4	19.5	23.2	Zhong et al. (40)
Pgestssr255	14	0.589 (0.069)	0.548 (0.055)	-0.074	2.8	5.0	5.8	Berlin et al. (4)
Pgestssr279	10	0.434 (0.047)	0.475 (0.040)	0.087	7.6	9.5	4.3	Zhong et al. (40)
Pgestssr280	15	0.172 (0.025)	0.635 (0.047)	0.729	17.9	28.5	21.7	Berlin et al. (4)
Pgestssr368	11	0.570 (0.047)	0.527 (0.034)	-0.081	5.5	6.8	2.9	Zhong et al. (40)
PgtCAA53	16	0.253 (0.104)	0.297 (0.091)	0.149	1.4	2.7	14.5	Jin et al. (19)
PgtCAA93	18	0.385 (0.097)	0.582 (0.088)	0.337	9.7	29.4	8.7	Jin et al. (19)

^a N_a, number of observed alleles; H_o, observed heterozygosity (s.e. in parenthesis); H_e, expected heterozygosity (s.e. in parenthesis); and F_{IS}, fixation index F_{IS} = (mean H_e - mean H_o)/mean H_e.

facturer's description and sequenced by capillary electrophoresis (3730 XL, Macrogen, South Korea). The forward and reverse ITS region sequences were aligned in SeqMan Pro (DNA Star). The species identity was controlled by comparing the acquired sequences to sequences deposited in GenBank through a BLASTN search (39) and only samples with a high similarity (at least 98% similarity and E-value of 0.0) were included in the study.

Genetic data analysis. Since different *Puccinia* species are present on barberry bushes in Sweden, the species identity of samples collected from barberry was investigated before applying the SSR markers to the samples (Supplementary Table 1). A combination of both ITS sequence identity and successful amplification of SSR markers was therefore obligatory for each aecial sample to be included in further analyses. Samples where no sequence of the ITS region was obtained were included if at least seven SSR loci gave amplification products. All samples with more than four missing values were removed from the dataset. In order to check for possible bias due to missing values, analyses were done with and without the loci with particularly high frequencies of missing values, but this did not affect the results.

To evaluate the usefulness of the primers (Table 1), the observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated for each SSR marker using GenAlEx6.4 (28). H_o is the proportion of samples that are heterozygous at a locus, and H_e is the proportion of samples expected to be heterozygous at the locus under random mating. The gene diversity is reported as the number of alleles at each locus and the gene allele frequencies were visualized in graphs.

Samples from each field site were grouped into one population, giving eight oat populations and three rye populations (Table 2). For each field population, the genotypic diversity is reported as the number of unique genotypes divided by the total number of samples within the population (G/N). H_o and H_e within field populations were calculated using GenAlEx6.4 (28), where H_o is the average observed heterozygosity and H_e is the expected heterozygosity within a population at random mating. The inbreeding coefficient (F_{IS}) was calculated using the computer program Fstat (15). To test for association among different loci, the index of association (I_A) was calculated for each population separately, using the software Multilocus 1.3b (2). Due to the high percentage of missing values within particular loci, the I_A test for significance was carried out with the option "missing data fixed" and replicated 1,000 times. Estimation of the selfing rate ($s(g2)$) from the distribution of multilocus heterozygosity in each field population was performed in the software REMS using 1,000 iterations (8). This method has been shown to be insensitive to null alleles (8).

Analyses of molecular variance (AMOVA) were carried out with Genalex6.4 with 9,999 permutations. Separate analyses were

done to estimate the difference between *P. graminis* f. sp. *avenae* and *P. graminis* f. sp. *secalis*, and the difference among field populations of *P. graminis* f. sp. *avenae*, and among field populations of *P. graminis* f. sp. *secalis*. Clone correction was done separately for each AMOVA.

The Bayesian cluster computer program Structure (29) was used to examine the population structure within the total set of samples in order to investigate the number of genotypic clusters and to determine the ancestry of the different samples. This program assumes panmixia, i.e., that each locus is at Hardy-Weinberg equilibrium and independent of other loci, and it has proved to be robust even when these assumptions are not met. The hypothesis in our case was that the *formae speciales* form different populations, and the assumptions made in the analysis were mixed populations and independent alleles. The range of presumed number of genotypic clusters (K) was set to 1 to 15 and the program was set to a burn-in period of 100,000 iterations followed by 100,000 iterations. The program calculates the Log likelihood for each value of K , and the highest likelihood value indicates the most likely number of genotypic clusters. Three independent simulations were performed to test the consistency of the result. The graphical display of the output was made in the software Distruct (31). In addition, a principal coordinate analysis (PCA) was performed with GenAlEx 6.4 to visualize the genetic pattern among all samples, based on the calculated genetic distances.

RESULTS

Eleven polymorphic SSR loci were used to genotype 290 uredinia collected from rye and oat fields, and 145 aecia collected from barberry in southern Sweden. All SSR loci were polymorphic and the number of alleles ranged between 7 and 22 for the different loci (Table 1). The H_o and H_e values were not statistically different from each other for seven of the 11 loci. For the remaining four loci (Pgtestssr021, Pgtestssr171, Pgtestssr280, and PgtCAA93) H_o was significantly lower than H_e , indicating an excess of homozygotes, which was also shown by the large F_{IS} values (Table 1). The allele frequencies for samples from the three hosts (barberry, oats, and rye) are presented in Figure 2. For the primers Pgtestssr021, PgtSSR21, Pgtestssr171, Pgtestssr255, Pgtestssr279, Pgtestssr 280, Pgtestssr368, and PgtCAA93, the allele frequencies clearly overlap among the different hosts. For three other primers, Pgtestssr024, Pgtestssr109, and PgtCAA53, the allele sizes are different between samples collected from the two grass hosts, oats and rye. A bimodal distribution of alleles from barberry was present for Pgtestssr024, where one peak corresponds to those from oats and the other to those from rye. A tendency for the same bimodal pattern is also seen for Pgtestssr109.

TABLE 2. Population genetic data for the oats and rye field populations^a

Pop	Crop	Site	Day of collection ^b	N	G/N	H_o (s.e.)	H_e (s.e.)	F_{IS}	I_A	P	$s(g2)$	P
Pop1	Oats	Ingvasta	06.08.08	28	1	0.365 (0.074)	0.520 (0.060)	0.322	0.398	0.027	0.017	0.477
Pop2	Oats	Å	11.08.08	27	1	0.388 (0.060)	0.504 (0.044)	0.251	0.067	0.333	0.000	0.678
Pop3	Oats	Bränne Övregård	13.08.08	30	0.9	0.440 (0.088)	0.454 (0.072)	0.054	0.065	0.353	0.032	0.389
Pop4	Oats	Götala	26.08.08	25	1	0.443 (0.087)	0.567 (0.054)	0.244	-0.215	0.998	0.000	0.522
Pop5	Oats	Skarpenberga	30.07.09	27	1	0.414 (0.066)	0.560 (0.060)	0.281	0.326	0.063	0.330	0.001
Pop6	Oats	Ingvasta	05.08.09	25	1	0.324 (0.052)	0.571 (0.045)	0.453	0.101	0.370	0.000	0.595
Pop7	Oats	Bränne Övregård	06.08.09	29	1	0.422 (0.047)	0.560 (0.047)	0.266	-0.096	0.585	0.000	0.778
Pop8	Oats	Klostergården-Dala	07.08.09	30	1	0.421 (0.067)	0.474 (0.066)	0.130	0.332	0.001	0.000	0.704
Pop9	Rye	Beteby	22.07.08	30	1	0.556 (0.077)	0.628 (0.068)	0.135	0.302	0.125	0.130	0.042
Pop10	Rye	Råby, Kölby	29.07.09	29	1	0.572 (0.066)	0.682 (0.051)	0.181	0.979	0.001	0.041	0.298
Pop11	Rye	Bränne Övregård	05.08.09	10	1	0.481 (0.073)	0.510 (0.070)	0.110	-0.183	0.799	0.067	0.271

^a N, number of successfully genotyped samples; G/N, number of genotypes divided by number of samples; H_o , observed heterozygosity (s.e. in parenthesis); H_e , expected heterozygosity (s.e. in parenthesis); F_{IS} , fixation index $F_{IS} = (\text{mean } H_e - \text{mean } H_o) / \text{mean } H_e$; I_A , index of association and its P value with the option missing value fixed; and $s(g2)$, estimated rate of selfing and its P value (H_o : $g2=s=0$).

^b Day.month.year.

All but one population had a G/N ratio of 1 (Table 2), indicating high genotypic diversity and thus little clonality within each field population. The H_o spans between 0.324 and 0.572 and the H_e spans between 0.454 and 0.682. The inbreeding coefficient (F_{IS}) ranged between 0.054 and 0.453. In one population, collected from an oat field (Bränne Övregård), the F_{IS} was close to zero which indicates random mating (Table 2). The estimation of selfing rates $s(g2)$ was significant for two field populations, one collected from oats (Skarpenberga) and one collected from rye (Beteby). This indicates a substructure within those two locations. The I_A ranged between -0.215 and 0.979 (Table 2). For eight of the 11 studied field populations the I_A was not significantly different from zero. Thus there is no evidence that these populations were not in linkage equilibrium.

Three AMOVAs were used to examine the variation between the two *formae speciales*, and the variation among populations within the two *formae speciales* separately (Table 3). The first AMOVA showed that the difference between *formae speciales* was 34% ($P < 0.001$), and the variation within *formae speciales* was 66%. The variation among field populations within *P. graminis* f. sp. *avenae* was 10% ($P < 0.001$). Within *P. graminis* f. sp. *secalis*, the variation among field populations was 12% ($P < 0.001$). This means that there was a clear differentiation between the two *formae speciales*. A large proportion of the variation was also present within the field populations of each *forma specialis*.

By using the program Structure, the number of inferred clusters (K) that had the highest Log likelihood was six (Fig. 3). Samples

Fig. 2. Allele frequencies of 11 simple sequence repeat markers used for genotyping *Puccinia graminis* collected from barberry (black bars), oats (white bars), and rye (striped bars).

from oats showed ancestry primarily from two of these inferred clusters (yellow and purple) either singly or in combination (Fig. 3). Those from rye showed ancestry primarily from a third cluster (light blue). Samples collected from barberry showed ancestry from all six inferred clusters. Some of the barberry samples shared ancestry with the samples from oats or rye, whereas other barberry samples did not (red, green, gray).

The PCA showed that the samples collected from oats and rye were different and that the samples collected from barberry formed a larger group in which samples from both *formae speciales* were represented (Fig. 4). The first axis of the PCA explained 41% and the second explained 19% of the differences between the individual samples.

DISCUSSION

P. graminis collected from the studied grass hosts, oats and rye, could be divided into two genetically separate groups, as indicated by the AMOVA analysis. This is also supported by the Structure analysis (Fig. 3) and the PCA (Fig. 4), where it is shown that these *formae speciales* are genetically different, and that they are genetically similar to some of the *P. graminis* samples found on the barberry bushes. The genetic differentiation was further proved by non-overlapping allele distributions for the two *formae speciales* at three SSR loci.

An alternate explanation of this differentiation is that it is due to geographical differences. The collections from Bränne Övregård (populations 7 and 11, from oats and rye, respectively) however, were collected in adjacent fields and show clear differentiation. Eriksson and Henning (10) described the phenotypic differences between samples of *P. graminis* infecting different grass hosts, both by inoculation and morphological studies. Our study confirms their results in that genetic differences were found between the two studied *formae speciales*.

In addition to *P. graminis* f. sp. *avenae* and *P. graminis* f. sp. *secalis* three additional groups, and possibly hybrids between the different groups, were found on barberry (Fig. 3). *P. graminis* can infect several hundred species (24) and the barberry samples (other than the ones that could be derived as *P. graminis* f. sp. *avenae* or *P. graminis* f. sp. *secalis*) may belong to any of the other *formae speciales* infecting wild grasses present in Sweden. Different *formae speciales* of *P. graminis* exist in parallel and seem to rarely exchange genetic material on barberry. Earlier studies (20,21) have shown that the fitness of hybrids is lower than for pure *formae speciales*. Hybrids will occur only in low frequencies, if present at all, since their ability to compete with pure *formae speciales* is poor. In this study, one sample collected from a rye field appears to be a hybrid between *P. graminis* f. sp. *secalis* and *P. graminis* f. sp. *avenae* (Fig. 3). The allelic composition of the sample supported this conclusion since it had one allele characteristic of each *forma specialis* at four loci (alleles 165 and 172 for Pgestssr21, 171 and 180 for Pgestssr279, 233 and 242 for Pgestssr255, and 234 and 237 for Pgestssr368; Fig. 2). As demonstrated by Johnson (20), hybridization is possible but it probably occurs rarely and the lack of fitness of the progeny will reduce their importance. The reproductive barrier appears to be in fertilization of the receptive hyphae by pycniospores since artificial crossings between *P. graminis* f. sp. *trititci/secalis* and *P. graminis* f. sp. *avenae* rarely are successful (20).

Samples genetically similar to one or the other of the two *formae speciales* were found on barberry. This, together with the high genetic diversity within both *formae speciales* studied, and no or low significance of the Index of Association is consistent with sexual reproduction, which also has been shown in a previous study (4). The presence of the alternate host and the possibility for sexual recombination will enable the fungus to become extremely diverse in Sweden compared with populations developing in the absence of barberry, where clonal reproduction

TABLE 3. Analyses of molecular variance between *Puccinia graminis* f. sp. *avenae* and *P. graminis* f. sp. *secalis*, among field populations of *P. graminis* f. sp. *avenae*, and among field populations of *P. graminis* f. sp. *secalis*^a

Source	df	Sum of squares	Mean of squares	Estimated variance	%	P value
Between <i>formae speciales</i>	1	536.1	536.1	5.04	34%	<0.001
Within <i>formae speciales</i>	282	2,751.7	9.8	9.76	66%	
Total	283	3,287.8		14.80	100%	
Among field populations of <i>P. graminis</i> f. sp. <i>avenae</i>	7	241.0	34.43	0.95	10%	<0.001
Within field populations of <i>P. graminis</i> f. sp. <i>avenae</i>	210	1,827.3	8.70	8.70	90%	
Total	217	2,068.3		9.65	100%	
Among field populations of <i>P. graminis</i> f. sp. <i>secalis</i>	2	73.3	36.66	1.28	12%	<0.001
Within field populations of <i>P. graminis</i> f. sp. <i>secalis</i>	66	632.5	9.58	9.58	88%	
Total	68	705.8		10.86	100%	

^a Clone correction was done separately for each analyzed population.

Fig. 3. Inferred ancestry of *Puccinia graminis* samples to six genotypic groups based on 11 simple sequence repeat markers using the program Structure. Each color represents one genotypic group.

dominates, such as those in Australia (23). Currently the race diversities of *P. graminis* f. sp. *avenae* and *P. graminis* f. sp. *secalis* in Sweden are not known, but we can only speculate that the large number of genotypes could lead to a large number of races (25).

Limited inbreeding has produced local populations and this can be seen since the F_{IS} value is larger than zero for most field populations. The analyses for two of the field populations (Skarpenberga and Beteby) also indicate significance for what is referred to as the rate of selfing ($s(g2)$), which in this case indicates inbreeding. For both of these fields, barberry bushes were found at a distance of 0.5 to 2.0 km from the field (Fig. 1), and such a situation may facilitate development of localized populations.

The importance of barberry as an alternate host may differ between the *formae speciales*. The host shift in *P. graminis* does not only enable genetic recombination but also enables the rust to infect annual crops, which are present during a relatively short growing season. A small amount of clonal survival may be one possible explanation why *P. graminis* f. sp. *secalis* is genetically more homogenous than *P. graminis* f. sp. *avenae* in Sweden (Fig. 3), since rye is a fall-sown crop where uredinia may overwinter. Oats is only sown in the spring in Sweden, and *P. graminis* f. sp. *avenae* cannot overwinter clonally on this host. Therefore a host shift to barberry, including obligate genetic recombination each year is the only way of survival. This hypothesis is supported by the observations that the first signs of disease are reported from rye approximately 2 to 3 weeks before the disease is found in oats. This also comports with both Eriksson's (10) and Hermansen's (16) findings that *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *secalis* were reported earlier in the field than *P. graminis* f. sp. *avenae* where pustules appeared approximately 2 weeks later. The genotypes infecting earlier will dominate the population at the end of the season (27,36).

Analyses did not uncover any signs of recent bottlenecks for field populations of either *P. graminis* f. sp. *avenae* or *P. graminis* f. sp. *secalis* (calculations of M value (12), data not shown),

though one may surmise that the populations experience them yearly or that there was one large bottleneck during the barberry eradication period.

The percentages of missing values of the SSR markers were high for some loci (Table 1). Pgestssr171 and Pgestssr280 have many missing values for all hosts, while PgtCAA53 and PgtCAA93 had many missing values for rye and oats respectively. One possible explanation is that null alleles are present. Species with large effective population sizes, like *P. graminis* in this case (data not shown), may be expected to have high null allele frequencies since the genetic variation within the species increases with the population size and mutations in the flanking regions and primer sites may be present (6). The primers used in this study were originally designed for *P. graminis* f. sp. *tritici*, and this may also have affected the null allele frequencies.

The many methods used to classify the divisions in *P. graminis*, of which *formae speciales*, subspecies, and varieties represent some of these attempts, indicate that it is not a simple species. We show that the genetic diversity is high within each *forma specialis* and that *P. graminis* f. sp. *avenae* and *P. graminis* f. sp. *secalis* are clearly different in Sweden. Abbasi (1) studied the sequences of the ITS region and found that the species is highly complex, and that neither morphological nor the genetic characters provide a full answer to the evolutionary relationship between samples from different parts of the world. The reproductive barrier reported here for *P. graminis* clearly separates *P. graminis* f. sp. *secalis* and *P. graminis* f. sp. *avenae* despite the fact that they meet on barberry. But, it may be premature to assume that this phenomenon applies on a worldwide basis.

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Fig. 4. Principal coordinate analysis (PCA) based on the calculated genetic differences between each individual sample. The first axis represents 41% and the second represents 19% of the total genetic differences between the individual samples.

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