Gypsy-like retrotransposons in *Pyrenophora*: an abundant and informative class of molecular markers

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Abstract: This paper describes the development of S-SAP (sequence-specific amplified polymorphism) using a primer derived from the LTR (long terminal repeat) of the *Pyggy* retrotransposon isolated from *Pyrenophora graminea*. Fragments were amplified by S-SAP from different *Pyrenophora* spp., indicating the presence of *Pyggy*-like sequences in these genomes. The bands were highly polymorphic between isolates and the number of bands differed by as much as 10-fold between species, demonstrating the potential of this method for genetic analysis in fungi. The phylogenetic relationship among the isolates as deduced using S-SAP data is presented, and shows evidence of genetic exchange between *P. graminea* and *P. teres*.

Key words: Pyrenophora, retrotransposon, molecular markers, S-SAP.

Résumé : Les auteurs décrivent le développement de marqueurs S-SAP (« sequence-specific amplified polymorphism ») à l'aide d'une amorce inspirée de la région LTR (« long terminal repeat ») du rétrotransposon *Pyggy* du *P. graminea*. Des produits d'amplification ont été obtenus par S-SAP chez différentes espèces du genre *Pyrenophora*, ce qui indique la présence de séquences de type *Pyggy* au sein de ces génomes. Les amplicons étaient très polymorphes entre isolats et le nombre de bandes pouvait différer par un facteur dix entre deux espèces. De telles caractéristiques démontrent le potentiel d'une telle méthode pour l'analyse génétique chez les champignons. Les relations phylogénétiques parmi les isolats, telles que déduites à l'aide des données S-SAP, sont présentées et montrent des évidences d'échanges génétiques entre le *P. graminea* et le *P. teres*.

Mots clés : Pyrenophora, retrotransposon, marqueurs moléculaires, S-SAP.

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Introduction

Pyrenophora graminea is a fungal seed-borne pathogen of barley (Cockerell and Rennie 1996; Paveley et al. 1996), which causes the economically important disease leaf stripe. *Pyrenophora graminea* is very closely related to other *Pyrenophora* spp. that infect barley, but little is known about the population structure of this pathogen.

Recently, a diagnostic test was developed that could detect *P. graminea* in infected barley seed and distinguish it from the very closely related *P. teres* (Taylor et al. 2001). Analysis of the DNA sequence has shown that it is derived from a retrotransposon belonging to the Ty3-*gypsy* class based upon its relationship (79% identity) to the corresponding region of the *REAL* retrotransposon of *Alternaria alternata* (Kaneko et al. 2000). This element has been designated *Pyggy* (*Py*renophora *g*raminea *gy*spy-like retrotransposon).

Retrotransposons are valuable genetic markers for studying the evolution of genomes (Flavell et al. 1992; Ellis et al. 1998). Their integration into new sites can be used as genetic markers (Lee et al. 1990) and, as they transpose via an RNA intermediate (Boeke et al. 1985), they do not hop about as DNA-mediated transposable elements but become fixed. Their location throughout the genome serves to chronicle the transpositional history of the retrotransposon family (Pearce et al. 2000), making them particularly suitable for phylogenetic studies. The solo long terminal repeats (LTRs) found both in fungi (Cameron et al. 1979) and plant genomes (Shirasu et al. 2000) suggest that excision of the element is possible by intraelement LTR to LTR recombination. These solo LTRs still serve as "footprints", indicating where the elements have been.

Retrotransposon markers can be exploited by the S-SAP technique (Waugh et al. 1997) that amplifies from a specific primer derived from a retroelement to a restriction site outside the element via an adapter. Since the markers generated by S-SAP are more polymorphic than bands amplified by AFLP (Waugh et al. 1997; Ellis et al. 1998), the discovery of *Pyggy* offered an opportunity to develop this sequence family as a multi-marker system for genetic analyses of *Pyrenophora* species.

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In *P. graminea*, little is known about the genetics or the amount of genetic variation within the species, though phenotypic variability has been confirmed by reports on its virulence, morphology, and karyotype (Gatti et al. 1992). Its sexual cycle is rare in the field and so the predominant mode of reproduction appears to be asexual. The discovery of *Pyggy* presented an opportunity to exploit the historic activity of this class of sequence to assess the genetic variation present in *Pyrenophora* spp., and to use these data to gain greater insight about the relationship between these species.

Materials and methods

Origin and maintenance of fungal isolates

The *Pyrenophora* spp. isolates used in this study were obtained from geographically diverse areas (Table 1). Fungal isolates derived from single conidia were identified, cultivated, and maintained as described by Stevens et al. (1998).

DNA extraction from fungal cultures

Genomic DNA was extracted from fungal cultures as described previously (Taylor et al. 2001).

S-SAP procedure

Genomic DNA (500 ng) was restricted as described by Waugh et al. (1997), with the following modification: *Eco*RI (5 U) (Hitchin, UK, NEB) was used in conjunction with *Mse*I (5 U) (NEB) to digest the genomic DNA for 1 h at 37 °C. Five microlitres of adapter mix (0.5 μL of 25 μM *Mse*I adapters, 0.5 μL of 2.5 μM *Eco*RI adapters, 1× RL buffer (10 mM Tris–acetate (pH 7.5), 10 mM magnesium acetate, 5 mM dithiothreitol (DTT), 5 ng BSA/μL), 0.2 μM ATP (Amersham Pharmacia Biotech, Bucks, UK), and 0.5 U T4 ligase (Amersham Pharmacia Biotech)) was then added to the restriction digest and incubated at 37 °C overnight. Five microlitres of the restriction–ligation reactions were removed and the restriction digest checked by agarose gel electrophoresis. A non-selective amplification was performed on 1 μL of the restriction digest with the following reaction components: 1× PCR buffer (750 mM Tris–HCl, 200 mM $(NH_4)_2SO_4$), 1% v/v Tween 20, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Abgene, Epsom, UK), 1 U *Taq* polymerase (Roche, East Sussex, UK), 30 ngμL^{-1} each of *Eco*RI and *Mse*I non-selective primers). The PCR conditions were as follows: 30 cycles of 94 °C for 1 min, followed by 60 °C for 1 min, and then 72 °C for 1 minute: there was a final extension step of 72 °C for 7 min. Five microlitres of this PCR was removed so that it could be checked by agarose gel electrophoresis. The remaining 20 μL in each reaction was diluted with 50 μL TE (10 μL Tris–Cl; 1 μM EDTA, pH 8). Selective amplification was then performed using 1 μL of the diluted non-selective amplification with similar reaction components except with 30 ngμL^{-1} *Mse*I selective primer and 31.5 ngμL^{-1} *Pyggy*_S-SAP primer designed from *Pyggy* sequence data (EMBL accession Nos. AF533703 and AF533704). This S-SAP primer, positioned along nucleotides 46 to 25 of the sequence, was labelled at the 5′ end with an ^{700}IRD label (MWG Biotech AG, Ebersberg, Germany) to allow fragment detection on the LI-COR® DNA analyser Gene ReadIR 4200 (LI-COR Inc, Lincoln, Nebr.). The PCR cycles were as described by Vos et al. (1995). The primer and adapter sequences are detailed in Table 2.

Electrophoresis / visualization of products

Amplified fragments were separated and analysed on a LI-COR® DNA analyser Gene ReadIR 4200 following the method of Jackson and Matthews (2000) with minor modifications: PCR products were diluted 1:1 with loading buffer and loaded on Sequagel XR® (National Diagnostics, Hull, UK) acrylamide gels. The labelled fragments were automatically converted to band images by the sequencer, then scored manually as present or absent. The intensity or contrast of the image was varied to ensure that all the band differences scored were true polymorphisms and not differences in the band intensity.

Phylogenetic analysis

Bands in the size range of 100–270 nucleotides were scored for presence or absence. These data were analysed using Treecon software (Van de Peer and De Wachter 1994). A phylogenetic tree was constructed using the neighbour-joining algorithm (Saitou and Nei 1987) by simple matching. Bootstrap analysis with 100 replicates was performed to estimate the robustness of the evolutionary tree.

Results and discussion

Pyggy LTR-derived S-SAPs using different selective bases

The results of the S-SAP analysis using a single selective base on the *Mse*I primer in combination with an LTR-specific primer designed from the *Pyggy* sequence data indicated that there was an abundance of polymorphisms within and between *Pyrenophora* spp. (Figs. 1 and 2). Each combination involving the *Pyggy*-derived primer in conjunction with one of four selective *Mse*I primers generated a different S-SAP fingerprint for the same set of six fungal isolates (Fig. 1). The *Mse*I primer combination with no selective bases on the 3′ end generated a weak S-SAP fingerprint compared with the *Mse*I primer combinations that did contain a selective base. This may be due to competition between the different copies of *Pyggy* in the PCR. Adding a selective base to the *Mse*I primer reduces this competition as only a subset of the *Mse*I fragments are amplified in the PCR and so produce a more intense and simpler fingerprint (Fig. 1). Addition of two or three selective bases to the *Mse*I primer significantly reduced the number of fragments amplified by approximately one quarter per selective nucleotide owing to the increased specificity in the PCR (results not shown). From these experiments, one selective base was considered optimal for *Pyggy*-based S-SAP in *Pyrenophora*. The primer *Mse*I+T was selected as the combination to screen further isolates of *Pyrenophora*, as it produced a DNA fingerprint with many well-separated intense bands that could be easily scored (Fig. 1).

An intense band of approximately 440 nucleotides in size is amplified in all six fungal isolates tested for both *Mse*I+0 and *Mse*I+G primer combination (band A in Fig. 1). This band corresponds to the expected internal fragment amplified by S-SAP both in size and selective base (EMBL accession Nos. AF533703 and AF533704). The amplification of

Table 1. Summary of *Pyrenophora* isolates used in this study.

Isolate identity	Host	Country of origin	Year of isolation	No. of observed bands for *Mse*I+T S-SAP primer combination
Pg 99-1	Barley	Norway	1999	40
Pg 98-2	Barley	UK	1998	41
Pg 97/BS 96-10	Barley	Denmark	1997	45
Pg 97/BS 4-7	Barley	Italy	1997	30
Pg 97 BS 5-6	Barley	Italy	1997	40
Pg 97 BS 6-1	Barley	Yugoslavia	1997	39
Pg 97-13	Barley	UK	1997	42
Pg 97-76a	Barley	UK	1997	42
Pg 97-78a	Barley	UK	1997	30
Pg 97-81a	Barley	Italy	1997	41
Pg 97/ BS 96-11	Barley	Denmark	1997	42
Pg 95-3	Barley	UK	1995	34
Pg 95-7	Barley	UK	1995	43
Pg 95-9	Barley	Sweden	1995	41
Pg 95-17	Barley	Norway	1995	35
Pg 93-3	Barley	UK	1993	13
Pg 92-7	Barley	UK	1992	18
Pg 91-19	Barley	UK	1991	41
Pg 90-38	Barley	UK	1990	39
Pt 99-47	Barley	Bulgaria	1999	7
Pt 99-48	Barley	Bulgaria	1999	3
Pt 99-49	Barley	Bulgaria	1999	11
Pt 99-51	Barley	Bulgaria	1999	8
Pt 98-76	Barley	Netherlands	1998	4
Pt 97/ BS 55-6	Barley	France	1997	14
Pt 97/BS 755-10	Barley	France	1997	12
Pt 96/BS 96-7	Barley	Russia	1996	9
Pt 95-49	Barley	Norway	1995	5
Ph 94-12	Barley	Australia	1994	4
Pa 98-2	Oats	UK	1998	15

Note: Pg, *P. graminea*; Pt, *P. teres*; Ph, *P. hordei*; Pa, *P. avenae*.

Table 2. Primer and adapter sequences used for S-SAP in this study.

Adapter/primer	Sequence (5′→3′)
Pyg_SSAP	CCGAGACCCATCGAGGATTTA
*Mse*I non selective primer	GATGAGTCCTGAGTAA
*EcoR*I non-selective primer	GACTGCGTACCAATTC
*Mse*I selective primer	GATGAGTCCTGAGTAA + selective base
*Mse*I adapter	GACGATGAGTCCGAG
	TACTCAGGAACTCAT
*EcoR*I adapter	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTCTAC

this band in both *P. graminea* and *P. teres* isolates shows that, not only is *Pyggy* present in *P. teres*, but the nearest *Mse*I site to the 3′ LTR is also conserved spatially. Together this strongly suggests a high degree of sequence conservation between elements found in the two species. A slightly smaller product (approx. 395 nucleotides in size) is amplified with the *Mse*I+C primer in the four *P. graminea* isolates but not in the two *P. teres* isolates (band B in Fig. 1). This product may be due to a subclass of *Pyggy* elements with an *Mse*I site closer to the primer (LTR) than the main 440-nucleotide product. This subclass of elements is not found in *P. teres* or any of the other isolates. Variation in size of the internal *Mse*I fragment has been demonstrated for *BARE*-1 retroelements in barley (Waugh et al. 1997).

The existence of subclasses of *Pyggy* elements that exists in *Pyrenophora*, as revealed by S-SAP, would explain previous findings. During the development of a specific PCR test for *P. graminea*, it was found that the first set of primers designed from the RAPD fragment was not specific and could amplify from other *Pyrenophora* spp. Specificity was attained only by sequencing these products and using the interspecific polymorphisms to design new primers, presum-

Fig. 1. S-SAP fingerprints of genomic DNA using different selective primer combinations. DNA samples labelled 1–6 are as follows: 1, PtBS 55-6; 2, PtBS 756/10; 3, Pg 95-9; 4, Pg 95-17; 5, Pg 99-1; and 6, Pg 95-7. Lanes marked with M represent DNA size standards in nucleotides. Arrows A and B denote amplified internal retrotransposon bands.

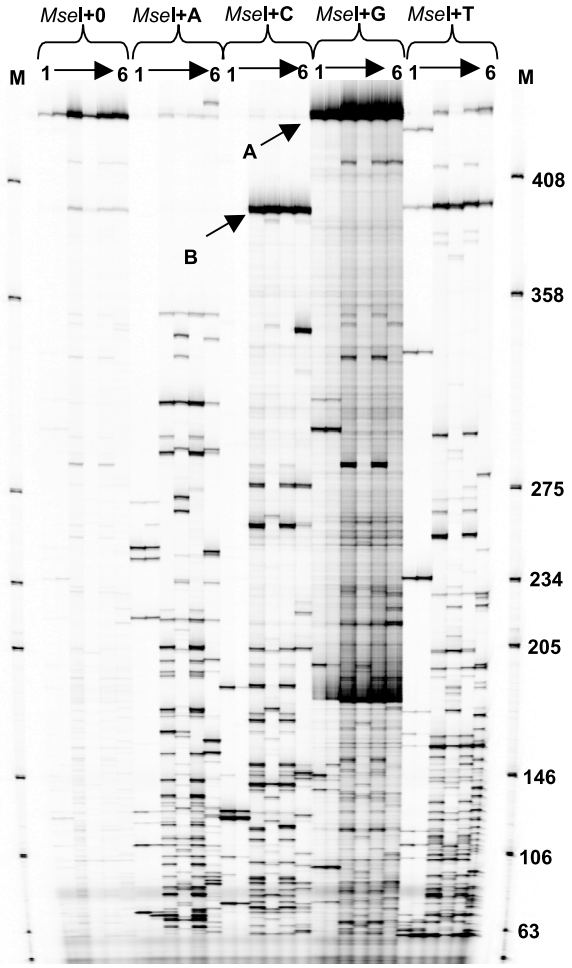
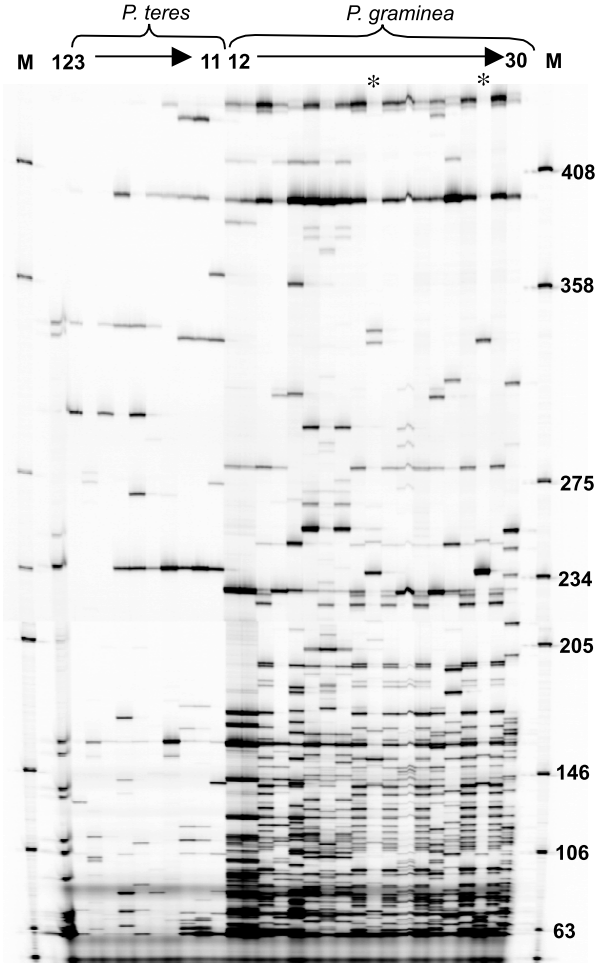


Fig. 2. S-SAP fingerprints of genomic DNA using the selective primer *Mse*I+C. DNA samples labelled 1–30 are as follows: 1, PA 98-2; 2, PH 94-12; 3, Pt 99-47; 4, Pt 99-48; 5, Pt-49; 6, Pt-99-51; 7, Pt 98-76; 8, Pt 95-49; 9, Pt 97/ BS 55-6; 10, Pt 97/BS 755-10; 11, Pt96/BS 96-7; 12, Pg 97/BS 6-1; 13, Pg 97–81a; 14, Pg 97-76a; 15, Pg 97-78a; 16, Pg 97/BS 5-6; 17, Pg 95-9, 18, Pg 95-17; 19, Pg 99-1; 20, Pg 95-7; 21, Pg 92-7; 22, Pg 97/BS 96-11; 23, Pg 95-3; 24, Pg 98-2; 25, Pg 91-79; 26, Pg 94-7; 27, Pg 90-38; 28, Pg 93-3; 29, Pg 97-13; and 30, Pg 97-10. Lanes marked with M represent DNA size standards in nucleotides.



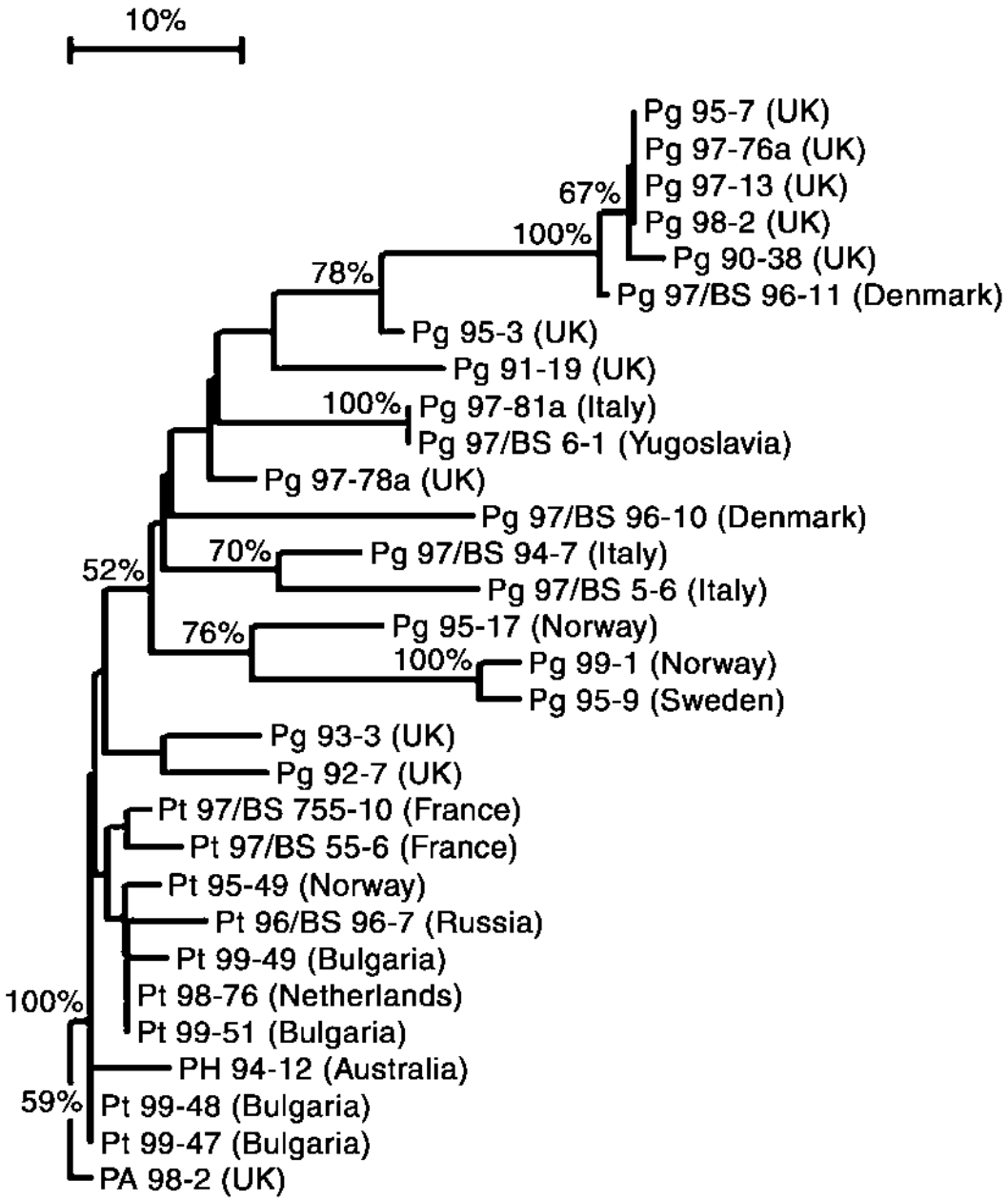
ably by amplifying them from a subclass of *Pyggy* elements specific to *P. graminea* (Taylor et al. 2001).

DNA polymorphisms in *Pyrenophora* spp. revealed by S-SAP

The sizes of the S-SAP products detected ranged from 50–500 nucleotides with more than 100 polymorphic bands amplified with a single selective primer. This indicates that there are many copies of the *Pyggy* retrotransposon distributed in *Pyrenophora* spp. just as other *gypsy*-like elements are widespread in the plant kingdom (Kumar et al. 1997; Suoniemi et al. 1998). It is estimated from the number of bands in this S-SAP analysis, with each of four selective primer combinations, that there are 120–180 copies of *Pyggy* in *P. graminea* and between 12–60 copies in other *Pyrenophora* spp. tested (Table 1). These data indicate a 10-fold higher copy number of *Pyggy* elements by S-SAP than estimates obtained from genomic southern blots of *Pyrenophora* spp. DNA probed with the *gag* and *pol* genes (P. Konstantinova, unpublished). This discrepancy can be explained by S-SAP amplification from solo LTRs or from the LTRs of other retroelements that do not share sequence homology with *Pyggy*, or amplification from unrelated sequences. Ellis et al. (1998) showed that 10% of S-SAP amplicons did not hybridize to their LTR sequence. Sequencing of the S-SAP fragments may explain the discrepancy and, if confirmed, LTR numbers that vastly exceed numbers of retrotransposons suggest a major role of the *Pyggy* family in genome evolution via intra- and interelement LTR-mediated recombination.

The S-SAP analysis (Fig. 2) shows there are many more copies of the *Pyggy* retrotransposon in the *P. graminea* isolates than in the other *Pyrenophora* spp. owing to the greater number of PCR products detected. This corroborates the data obtained from genomic southern blots of *Pyrenophora* spp. probed with the *Pyggy gag* and *pol* genes (P.

Fig. 3. Phylogenetic tree formed by neighbour-joining method (Saitou and Nei 1987) with simple matching that shows the relationship among *Pyrenophora* isolates based on S-SAP analysis. The distance scale is proportional to the evolutionary distance. One hundred samples were used for the bootstrap analysis and values above 50 are shown.



Konstantinova, unpublished). The difference in copy numbers between *P. teres* and *P. graminea* may be due to the fact that transposition has occurred at a higher frequency in *P. graminea* compared with *P. teres*. Alternatively, it may reflect a difference in the frequency of sexual recombination within the two species. It is known that the sexual stage of *P. graminea* is rare (Smedegard-Peterson 1972) compared with *P. teres* and so sexual recombination would be less in *P. graminea*. Retrotransposons may generate the diversity that is lacking for asexually reproducing species. This inabilty to generate diversity by sexual means may be the reason why the *P. graminea* genome is more tolerant of *Pyggy* elements.

Phylogeny of isolates as revealed by *Pyggy*

The S-SAP analysis was confined to bands in the 100- to 270-nucleotide size range. This was due to the difficulty in accurate band scoring because of the high numbers of fragments within a small size range and it was considered that the DNA polymorphisms in the selected size range would be sufficient to resolve the majority of the isolates. The bands within this range were scored from the gel (Fig. 2) and the data used to construct a phylogenetic tree (Fig. 3). Even using a subset of the data available, the results of this tree show that the majority of the fungal isolates may be uniquely identified based on their S-SAP fingerprint. The few isolates that were not separated could be resolved by us-

ing other polymorphisms from the gel image. The data also show a division of *P. graminea* and *P. teres* isolates into two clades. The isolates of *P. hordei* and *P. avenae* appear to be more closely related to *P. teres* with the former found within the *P. teres* clade (Fig. 3). Further screening of other isolates of *P. hordei* and *P. avenae* would produce a more detailed analysis of these pathogens' relationship within the genera of *Pyrenophora*.

Two *P. graminea* isolates (Pg 92-7 and Pg 93-3) appear from the S-SAP profiles to share many bands in common with the *P. teres* isolates (Fig. 2) and this is reflected in their grouping within the phylogenetic tree (Fig. 3). These two isolates also have a reduced number of S-SAP bands compared with other *P. graminea* isolates and their numbers are more akin to *P. teres* isolates (Table 1) suggesting they may be hybrids. There is evidence that *P. graminea* and *P. teres* can hybridize and produce fertile progeny under laboratory conditions and speculations have been made that this also occurs in the field (Smedegard-Peterson 1983). Ribosomal RNA internal transcribed spacer (ITS) sequence data demonstrated that there was only one consistent base pair difference between these two species (Stevens et al. 1998). There was also greater intraspecific variation compared with the interspecific variation in the ITS region for these two species. Additional sequence data, ITS, and glyceraldehyde-3-phosphate dehydogenase (*gpd*) (Zhang and Berbee 2001) from a wide range of *Pyrenophora s*pp. also indicated that, owing to their high sequence similarity, *P. graminea* and *P. teres* should be considered as synonymous rather than separate species. It is interesting to observe that the majority of the S-SAP profiles separate *P. graminea* and *P. teres* into separate clades even though the combination of data from the ITS, *gpd* sequence, biological crossing experiments, and morphology all indicate the close relationship of *P. graminea* and *P. teres*. If there is interbreeding between *P. graminea* and *P. teres* in the field this would have important consequences for disease resistance breeding programmes and disease control management strategies. It has been suggested by Smedegard-Peterson (1983) that "*P. teres* as well as *P. graminea* should be considered as forms, or physiological races, of one and the same biological, natural species."

S-SAP analysis also revealed a correlation with the geographic regions from which these *Pyrenophora* spp. were isolated. For example, two Italian *P. graminea* isolates (Pg 97/BS 94-7 and Pg 97/BS 5-6) are grouped closely together, two Norwegian (Pg 95-17 and Pg 99-1) and the Swedish *P. graminea* isolate (Pg 95-9) are grouped together, and two French *P. teres* isolates (Pt 97/BS 755-10 and Pt 97/BS 55-6) are grouped together. Agricultural practices are probably the main cause of this: the use of farm-saved seed, effective seed treatment, limited exchange of seed over long distances, and genotype–environment interactions (both for barley and pathogens) would all serve to limit the dissemination of the pathogens. However it does demonstrate the effectiveness of S-SAP to identify isolates which would be invaluable for population studies.

Further research using S-SAP should be used to elucidate more fully the population genetics of *Pyrenophora* spp. pathogenic on barley and determine the relative importance of sexual recombination compared with transposition of retroelements for genetic variation in *P. teres* and *P. graminea*.

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