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Genetic diversity and specialisation of *Eudarluca caricis* on some graminaceous *Puccinia* species.

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Eudarluca caricis is a common hyperparasite of rusts. A total of 100 cultures were isolated from six *Puccinia* species or forms growing on 10 species of British grasses at two sites approximately 3 km apart. 82 isolates collected in 2005 were partially sequenced at the ITS locus, and amplified fragment length polymorphism profiles generated for 86 isolates from 2005 and 12 from 2007. Partial ITS sequences of most isolates grouped closely, in a clade with previously reported graminaceous *Puccinia* isolates and a number of *Melampsora* isolates. A second clade was very distinct and contained mostly isolates from *P. poarum* on *Poa trivialis*. All isolates had distinct AFLP haplotypes. The *P. poarum* isolates were very distinct from isolates collected from other rusts at the same site. Isolates from *P. brachypodii* f. sp. *arrehenatheri* growing on *Arrhenatherum elatius* in 2005 and 2007 at the same location were distinct (P < 0.001). Isolates from each rust or grass in one year and site were more similar than expected from overall variation between isolates (P<0.001). Isolates from *P. coronata* on different grasses clustered together (with isolates from *P. brachypodii* f. sp. *poae-nemoralis),* suggesting partial host rust specialisation in *E. caricis*.

Introduction

Rusts are ubiquitous pathogens of grasses, and are among the most serious problems in agricultural cereal production. Conversely, rusts may play a part in reducing the competitiveness of otherwise invasive plants and increasing biodiversity (Peters & Shaw, 1996). The enemy release hypothesis suggests that organisms which become invasive may do so because they have moved to a new geographic area without the natural enemies – pathogens and pests – which regulate them in their range of origin (Evans, 2008). Natural enemies of rusts are therefore of interest from two points of view: they may help regulate agricultural and horticultural pests (Fleming, 1980; Vandermeer *et al.*, 2009; Gordon & Pfender, 2012); and they may reduce the effectiveness of rusts as biocontrol agents.

Natural enemies of rusts include a variety of fungi, for example *Lecanicillium* spp.*,* and animals, for example *Mycodiplosis sp.* flies, of varying degrees of specialisation. The ascomycete *Eudarluca caricis* has attracted considerable interest because the asexual form (*Sphaerellopsis filum)* is very common and has a wide host range among the rusts (Kranz & Brandenburger, 1981). It can easily be cultured in artificial media, although it is not found sporulating in nature except in association with rusts. In favourable systems, it can sometimes usefully reduce losses due to certain rusts (Morris *et al.*, 1994; Gordon & Pfender, 2012), although this requires the population of *E. caricis* to be substantial at the start of the season and therefore able to increase sufficiently rapidly to slow down rust development before the rust becomes damaging. This is unlikely in most agricultural settings, but can occur if, for example, a population of rust on a weed pre-exists the development of severe epidemics on the crop, providing a reservoir from which *E. caricis* can spread to the rust on the crop. The question of the host range of individual hyperparasite genotypes is therefore of practical interest, as well as having theoretical relevance to testing ideas about the functioning of tri-trophic and other co-evolutionary systems (Thompson, 1999).

Host specificity of *E. caricis* was shown by Yuan *et al.* (1999) who inoculated isolates onto the rust *Melampsora larici-epitea* on willow. Isolates from willow and one from a grass were infectious, but isolates from rusts on *Larix* (Gymnosperm) and *Rubus fruticosa* agg. (Roseacae) were not. Within isolates from *Melampsora* species infecting *Salix* there were very substantial quantitative differences both in effect on rust isolates and on the spore production of *E. caricis*, and significant quantitative hyperparasite-pathogen interactions (Pei *et al.*, 2010). Similarly, Nischwitz *et al.* (2005) found quantitative interactions between *E. caricis* isolates and *Melampsora* species (or isolates: multiple isolates were not tested) on poplar. Pei *et al.* (2010) found quantitative interactions between 12 *E. caricis* isolates and 5 *Melampsora larici-epitea* isolates. Two isolates from *Puccinia* rusts on grass did not infect *Melampsora.* Previously, Keener (1934) had shown that single isolates from 11 diverse rusts had clear, individually distinct, patterns of host specificity on a test range of 19 angiosperm rusts. However, there is no evidence for specialisation of isolates of *E. caricis* to rusts on an individual host plant species.

Several recent publications have surveyed genetic variation in *E. caricis*, concentrating on isolates from *Melampsora* rusts because of the problems they cause in willow and poplar plantations. Bayon *et al.* (2006) found little variability among isolates from *Melampsora* rusts in willow and poplar plantations in England with the population dominated by a few clones, but populations changed greatly between years (Bayon *et al.*, 2008). ITS sequences indicate a number of distinct clades within the taxon, and Liesebach and Zaspel (2004) suggested that there were actually two species present. They hypothesised a degree of host separation between these, noting that all their isolates from *Puccinia* lay within one subgroup of the group they denoted "I".

Despite the importance of rusts as pathogens of cereals and wild grasses, there has been little recent study of *E. caricis* on rusts of grass hosts, and none concerning the genetic structure on

different hosts. The aim of the present work was to test three hypotheses: (1) All the isolates from *Puccinia* would lie in the same clade as Liesebach and Zaspel's isolates from *Puccinia*; (2) the population of *E. caricis* on grass-infecting rusts would be largely clonal; (3) the population structure of *E. caricis* on grass rusts would be consistent with there being no specialisation of populations on individual species of rust.

Materials and methods

Sampling

Samples were collected from two locations (Table 1). The first was a teaching collection of grasses maintained since the mid 1980s at the University of Reading as parallel strips 2 m long, about 50 cm wide, and separated by approximately 1 m of bare ground (51.436852N,-0.941505E). The order of strips was *Bromus erectus* - 4 unsampled strips – *Holcus mollis* – unsampled – *H. lanatus* – *Dactylis glomerata* – *Anthoxanthum odoratum* – *Cynosurus cristatus* – 2 unsampled strips – *Festuca pratensis* – *Agrostis gigantea*. Samples from the second site, Shinfield, about 3 km south (51.411437N,-0.937411E), were from an ungrazed mixed grassland maintained by mowing twice annually. One isolate per leaf was collected. Leaves were collected from distinct stems across the sampling area, but it is not possible to say whether these were always distinct genets. Identification of rust was by host, uredium morphology and uredospore morphology, following Wilson and Henderson (1966) and Ellis and Ellis (1997), noting Dennis's (1989) verification that most herbarium records on B. erectus were *P. recondita* f.sp. *bromina*. Most isolates were collected in 2005 from both sites, It was not possible to produce a balanced design, both because the range of hosts differed across sites and because the degree of rust infestation differed between grass hosts. In 2007, a second collection from *Arrenatherium elatius* at Shinfield was made for comparison across years

Isolation and culturing of *E. caricis*.

Rust infected leaves were examined under a stereo microscope. Portions bearing rust sori with visible black pycnidia of the asexual phase of *E. caricis, Sphaerellopsis filum,* were placed on tap water agar containing 15 mg/L of both penicillin and streptomycin, in a box covered with absorbent paper. After 48 h at 20 °C emerging cirrhi were picked with a fine

sterile needle and suspended in 0.5 mL sterile distilled water. This suspension was spread on potato dextrose agar (PDA). After a further 48 h, single germinating spores were picked and transferred to PDA with antibiotics as before. Long-term storage was as mycelial slants on PDA at 4 °C. For DNA extraction, mycelial plugs were inoculated into potato dextrose broth and grown in shake culture (110 rpm) for 14 d at 20 °C.

DNA extraction and characterisation.

Mycelium was filtered from the culture medium using muslin. Approximately 100 mg of the mycelial mat was used for extraction with a DNEasy Plant Mini Kit (Qiagen, Crawley , UK) following the manufacturer's instructions. The concentration was determined using a PicoGreen quantitation kit (Invitrogen, Paisley, UK) and adjusted to 10 ng/µL.

ITS sequences

The primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC) and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G) of White *et al.* (1990) were used to amplify an approximately 700 bp fragment of the internal transcribed spacer region of the rDNA of the isolates collected in 2005, using 40 cycles of 94 °C 30 s, 52 °C 45 s, 72 °C 90 s. Amplification products were purified with QIAquick PCR purification kits (Qiagen, Crawley, UK), according to the manufacturer's specifications. Sequencing was done by Macrogen DNA sequencing services, Korea.

AFLP profiles

The method of Bayon *et al.* (2006), which purifies the restriction digested, ligated DNA before selective amplification, was followed. Genomic DNA (200 ng) was restricted with 12.5 units *PstI* and *MseI* (both New England Biolabs, Ipswich, Mass.) in One Phor All buffer (Pharmacia, Milton Keynes, UK) for 1 h at 37 °C. The digested DNA was ligated to *MseI* and biotinylated *PstI* adaptor sequences (5'-GACGATGAGTCCTGAG and 5'-

biotinCTCGTAGACTGCGTACATGCA respectively; Sigma, Poole UK) using T4 DNA ligase (New England Biolabs) in One Phor All buffer with 1 mM ATP (Sigma, Poole, UK). Biotinylated fragments were selected by binding to streptavidin coated magnetic beads (Dynal Biotech, Oslo, Norway) and then resuspended in TE buffer. Selective PCR then used primer combinations 6-FAM *MseI* CA + *PstI* AA; NED *MseI* CA + *PstI* CA; or HEX *MseI*CA + *PstI* CC. Products labelled with 6-FAM, NED and HEX were mixed before fragment analysis on an ABI 3130xl capillary electrophoresis instrument, with a length standard extending to 500 bp. Bands with length in the range 50 – 580 bp and an intensity greater than 50 (against a background of about 10) were scored as present; bands just above the end of the length ladder were included, because the relationship between retention time and length was closely linear up to 500 bp.

Alignment and Phylogenetic Analysis

ITS trace files were assembled and edited with SeqMan and EditSeq in DNASTAR (Madison, Wisconsin). Sequences were aligned in BioEdit using CLUSTAL W (Thompson *et al.*, 1994) and optimised manually. Publicly available sequences of *E. caricis* from previous studies were incorporated into our matrix, in order to achieve greater geographic coverage, as well as taxonomic coverage from rust and plant host species and to allow direct comparability with previous results. Sequences from *Alternaria* and *Ulocladium* were used as an outgroup to root the tree. Our matrix included 110 sequences, 83 of which were generated in this study from samples collected in 2005 and 27 were available from earlier submissions to Genbank . Sequence numbers in Genbank of the isolates sequenced here are: from rust at Shinfield on *Arrenatherum elatius*: **KM285288** – **KM285302**; on *H. lanatus*: **KM285345** - **KM285351**; on *P. trivialis*: **KM285360** - **KM285369**; from rust at Reading on *Agrostis gigantea*: **KM285303**, **KM285304**; on *Anthoxanthum odoratum*: **KM285305** - **KM285311**; on *B. erectus*: **KM285312** - **KM285319**; on *C. cristatus*: **KM285320** - **KM285327**; on *D.

glomerata: **KM285328, KM285329**; on *F. pratensis*: **KM285330** - **KM285336**; on *H. lanatus*: **KM285337** - **KM285345**; and on *H. mollis*: **KM285352** - **KM285359**.

Phylogenetic analyses were conducted under Maximum Likelihood (ML) criteria with RAxML (Stamatakis *et al.*, 2008), which selected the most appropriate model of sequence evolution for our data (GTR+G) and assessed clade support with 100 bootstrap (BP) replicates (Felsenstein, 1985). Pairwise genetic divergence was calculated between all pairs of sequences in the alignment with DIVEIN (Deng *et al.*, 2010). Sequence divergence within our data was within the range of that observed in data from similar studies (e.g. Liesebach & Zaspel, 2004). Parts of the alignment where unambiguous alignment was not possible at the ends of the sequences were removed from the analysis.

AFLP analysis

AMOVA (Excoffier *et al.*, 1992) based on Euclidean distance measures between individuals was used to characterise and test for significance of differentiation between groups. The analysis was made on the 100 isolates for which *PstI* CA patterns were available. The results were very similar using the combined *PstI*CA and *PstI*AA band patterns and for separate analyses using the isolates for which *PstI*AA and *PstI*CC band patterns were available. Three distinct analyses were made. (1) Using the data from Reading in 2005, an heirarchical AMOVA was conducted, calculating the sum of squares between rusts, between grass hosts of the same rust, and between isolates. A randomisation test of the rust variance was conducted by randomly re-assigning groups of isolates from each grass hosts to rusts in such a way as to preserve the number of grasses infected by each rust.   (2) The differentiation of isolates from each host of *P. coronata* in the Reading 2005 data was tested against the variation between isolates. (3) Differentiation of isolates between hosts at Shinfield was tested against the variation between isolates.   Canonical variate analysis (CVA) of

polymorphic PstICA bands with a randomisation test on the trace of the matrix of eigenvectors was used to visualise and test for differentiation between groups as in Rajaguru and Shaw (2010). All calculations were done with Mathematica v8 (Wolfram Research, Champaign, IL); code is available on request.

Results

ITS sequence comparisons

Successful sequences were obtained from 82 isolates (Table 1). Sequence divergence in our data is within the range of that observed in data from similar studies (e.g. Liesebach and Zaspel). The sequences generated in this study fall into two well supported clades (Fig 1). The first clade (bootstrap probability 83%) includes 20 out of 22 isolates from *Puccinia brachypodii* (both formae speciales), all isolates from *P. coronata, P. graminis, P. recondita* and sequences from *Puccinia* species on grasses from other studies. It corresponds to group I of Liesebach and Zaspel (2004) on the basis of isolates included in both analyses. It includes several sequences from *Melampsora* isolates on *Salix* and *Euphorbia* and is sister to a very well supported clade (bootstrap probability 99%) comprising more sequences from *Melampsora* and corresponding to Liesebach and Zaspel's group II. The second clade containing sequences from this study was not closely related to the first one (Fig 1) and did not appear in Liesebach and Zaspel's grouping. It has bootstrap probability 100% and includes all isolates from *P. poarum* (collected only at Shinfield) with two isolates from *P. brachypodii* f. sp. *arrhenatheri*, also from the Shinfield site in 2005.

AFLP comparisons

Useful profiles were obtained for 100 isolates using PstICA, 95 using PstIAA and 78 with PstICC. Over a hundred different fragment lengths were obtained from each primer.

Variation between isolates with identical ITS sequences was substantial, with differences between AFLP profiles in up to 43% of bands. Identical haplotypes were rare: isolates AE9 and AE13 from *Arrhenatherum elatius* at Shinfield differed only in a few short bands and two bands adjacent to others. They were considered possible clones: the analysis was repeated with and without removal of one of these isolates, with no substantive effect. Similarly, analyses were repeated with and without isolate AE12 from *Arrhenatherum elatius* at Shinfield in 2005 which grouped with the *P. poarum* isolates in the ITS phylogeny, and analyses were repeated ignoring bands which appeared only once in the dataset . (No AFLP fragment pattern was obtained from the other isolate from *A. elatius* that grouped with the isolates from *Poa trivialis).* The results were essentially identical; to maximise sample sizes in rusts and grasses only the PstICA results are presented, using all bands.

In the data from isolates collected at Reading in 2005, isolates from the same grass or rust were substantially more similar than isolates from different grasses ($P \ll 0.001$ by AMOVA on 6, 47 df; Table 2a; Fig 2a). At Shinfield in 2005 isolates from *P. poarum* on *Poa trivialis* were clearly separated from the isolates from *P. brachypodii* f. sp. *arrhenatheri* on *Arrhenatherum elatius* and the proportion of variation associated with differences between rusts was correspondingly large (Table 2c; $P < 0.001$ by AMOVA between isolates from all three grass/rust combinations 2, 31df; Table 3; Fig 2b).

P. coronata and *P. graminis* were present on more than one host. Isolates of *E. caricis* from different rusts were not significantly more different than isolates from different host grasses within a single rust ($P=0.08$ using a randomisation test re-assigning groups of isolates from a grass to rusts at random; Fig 2a; Table 2b; Table 4). This result must be viewed cautiously because the sampling only two rusts occurred on multiple hosts, and one host of *P. graminis, Dactylis glomerata,* had a sample size of 2. If *D. glomerata* is removed, the heirarchical AMOVA has a marginally more significant variance between rusts ($P=0.05$). This is

potentially misleading, because the "host within rust" stratum of variation refers only to *P. coronata.* We therefore analysed isolates from *P. coronata* separately. This showed that isolates from *P. coronata* on different host grasses were more distinct than expected from variation between isolates (Table 2b). At Reading, all isolates from hosts of *P. coronata* were clustered in CVA but clearly separate from isolates taken from *P. recondita* f. sp. *bromina* on *Bromus erectus* and *P. graminis* on *Cynosurus cristatus* (Fig 2a; Table 3). The isolates from *P. coronata* on *H. lanatus* at Shinfield, plotted on canonical axes separating isolates from distinct rust hosts calculated from the Reading data only, clustered with the isolates from Reading (Fig. 3) and were close in Euclidean distance (Table 4). By contrast, the isolates from *P. brachypodii* f.sp. *arrhenatheri* at Shinfield in 2005, plotted in the same way, were dispersed over the PCA space and not associated with existing clusters.

The groups of isolates from *P. brachypodii* on *Arrhenatherum elatius* at Shinfield in 2005 and 2007 were more different than expected from the differences within the groups (AMOVA, 1, 22 df, $F_{ST} = 0.25$, P<0.001). The genetic distance between the two groups was 23.0, larger than the average distance between groups of isolates taken from single grasses at Reading in 2005 (Table 4).

Discussion

We put forward and tested three hypotheses with regards to the genetic diversity of *E. caricis* relative to its host rust and plant species. First, Liesebach and Zaspel (2004) noted that all their isolates from graminaceous *Puccinia* species lay in a single subclade of their group I, and suggested this might be a consistent grouping. The majority of isolates indeed fall in Liesebach and Zaspel's group I but all the isolates from *Poa* fall into a clade separate from their clades I-IV (Fig 1). In view of the variability shown, it seems premature to start

assigning particular ITS sequence clades to distinct taxa before much wider study has been undertaken.

The second hypothesis was that the population of *E. caricis* on grass-infecting rusts was largely clonal. In *Melampsora* on *Salix* populations were dominated by a few very successful clones, certainly by the end of the season (Pei *et al.*, 1996; Bayon *et al.*, 2008). Using the same AFLP technique, we found that the population of *E. caricis* on grass-infecting rusts under study here was very variable, with few isolates which could have been clones. This difference may not be because the host rusts, on willow and grasses, are intrinsically different, but instead be because of differences in population dynamics and diversity of the host rusts. The populations examined here came from a stable multi-species community with a small proportion of the area occupied by any one grass host, and therefore a rather varied rust population; the rapid turnover of rust-infected grass leaves also means that the annual range of variation in rust population is probably less than in a willow plantation. This means there is less scope for single *E. caricis* clones to expand and dominate the population.

The third hypothesis tested was that patterns of genetic variation of *E. caricis* would be independent of their host rust. This was refuted. There was good evidence of a degree of host specificity in *E. caricis*. At both Reading and Shinfield, the similarity between isolates recovered from a single rust species was greater than expected by chance, either assuming a well-mixed population, or assuming specific associations with the grasses attacked by that rust (Fig 2a). At Reading, the hosts were organised as distinct strips, so simple spatial separation could cause separation of the groups of isolates. However, plots of *Anthoxanthum odoratum* and *Cynosurus cristatus*, hosting *Puccinia brachypodii* and *P. graminis* respectively, were adjacent, whereas the grass plots hosting *P. coronata* were up to 7 m apart. Also, isolates from *P. graminis* on *Cynosurus cristatus* were clearly differentiated from those from *P. coronata* hosts on either side. There was strong evidence from both ITS and AFLP

data that the isolates recovered from *Puccinia poae* on *Poa trivialis* at Shinfield in 2005 were distinct from those from *P. brachypodii* on *Arrhenatherum elatius* intermixed with the *P. trivialis* (Figs. 1, 2b). However, since two isolates from *P. brachypodii* also lay in the same clade as the *P. poarum* isolates, this is unlikely to represent a fixed host association. The isolates from *P. coronata* on *Holcus lanatus, H. mollis* and *Festuca pratensis* group together, as do the isolates from *P. coronata* at Shinfield (Fig 3, Table 4). (These isolates were not used in calculating the projection of the data, so the association is unlikely to be due to chance). Isolates from *P. brachypodii* f. sp. *poae-nemoralis* at Reading were similar to the isolates from *P. coronata* (Fig 2a, Table 3). Thus, a possible interpretation of the data is that there were separate populations of *E. caricis* virulent on *P. recondita* f. sp. *bromina*; on *P. coronata and P. brachypodii* f. sp. *poae-nemoralis;* and on *P. graminis*. This could be tested by quantitative cross-inoculation studies; unfortunately these are very labour-intensive and were beyond the scope of the present study.

The difference, established by a randomisation test, between the populations of *E. caricis* on *P. brachypodii* on *A. elatius* at Shinfield in 2005 and 2007 is not surprising, since both rust and (therefore) hyperparasite have a wide annual abundance range (Kajamuhan, 2008), and stochastic demographic variation in which genotypes are successful in a given year would be expected to be considerable.

The population genetic evidence for host specificity agrees with the results of inoculation experiments (Keener, 1934; Yuan *et al.*, 1999; Pei *et al.*, 2010) which have shown quantitative specificity of particular isolates of *E. caricis* to particular rusts. Such specificity does not need to be absolute for the hyperparasite to be unable to maintain itself in some rust populations or even species because the basic reproduction rate in a natural setting drops below zero. Such partial specificity can therefore cause a strong association with particular rusts in natural settings. There seems no reason to suppose that specificity of *E. caricis*

towards particular genotypes of rust will correspond to general taxonomic groupings in the rust hosts, since it must depend on particular aspects of both host rust defence systems and hyperparasite virulence mechanisms and these are unlikely to correspond to the virulence-host plant groupings which constrain the evolution of the rust groups.

In summary, our results show substantial and wide variation in naturally occurring populations of *E. caricis,* with some evidence of specialisation of sympatric populations to particular graminaceous rusts. There are two practical implications. First, the ecosystem service provided by *E. caricis* is not likely to be general suppression of rust, but instead will act on particular rust populations or sub-populations for genetical as well as population dynamic reasons. This could either stabilise or destabilise the population dynamics of host plant species and their rusts in unmanaged vegetation (Shaw, 2014). Second, it seems possible that a population of the hyperparasite maintained at moderate levels on a rust species common on one host would be able to attack the same species of rust on an economically important host which had a different annual cycle of abundance. For example, *P. coronata* is abundant for much of the year on weedy populations of *H. lanatus* which is itself abundant in many areas of Europe. *P. coronata* can be a serious problem in growing seed crops of *Lolium perenne* and may be partly controlled by *E. caricis* (Gordon & Pfender, 2012). The present results suggest that *E. caricis* on *P. coronata* and perhaps other rusts infecting wild or weedy grasses such as *H. lanatus* might be virulent on strains of *P. coronata* infecting *L. perenne* and assist in their management. Understanding patterns in such systems could be useful in devising strategies for biological control. Under some circumstances hyperparasites such as *E. caricis* could affect the effectiveness of rusts as biological control agents or regulate a rust in the home range of an invasive weed. Under other circumstances, hyperparasites could be exploited to reduce the intensity of attack of rusts on crop plants and

increase crop productivity. The results here suggest that this could be quite targeted, which might be advantageous, but would also make a detailed understanding of the system crucial.

Acknowledgements

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Table 1. Numbers of isolates of *Eudarluca caricis* characterised by AFLP and ITS from each host.

		Number of isolates					
		Reading		Shinfield			
		2005		2005		2007	Total
Rust species	Host grass	AFLP	ITS	AFLP	ITS	AFLP	
Puccinia brachypodii f. sp. *poae-nemoralis*	*Anthoxanthum odoratum*	7	7				7
P. brachypodii f. sp. *arrhenatheri*	*Arrenatherum elatius*			13	15	12	29
P. recondita f. sp. *bromina*	*Bromus erectus*	8	8				8
P. coronata	*Agrostis gigantea*	4	2				4
	Festuca pratensis	8	7				8
	Holcus lanatus	8	8	6	7		15
	H. mollis	7	8				8
P. graminis	*Cynosurus cristatus*	9	8				9
	Dactylis glomerata	2	2				2
P. poarum	*Poa trivialis*			16	10		16
	Total ITS		50		32		82
	Total AFLP	53		35		12	100

Table 2 AMOVA and F_{ST} estimates (a) between samples of *Eudarluca caricis* taken from *P. coronata* (infecting *Agrostis gigantea, Holcus lanatus, H. mollis, Festuca pratensis*), *P. brachypodii* f. sp. *poae-nemoralis (*infecting *Anthoxanthum odoratum), P. graminis* (infecting *Cynosurus cristatus, Dactylis glomerata*), *P. recondita* f. sp. *bromina* (infecting *Bromus erectus*) at Reading in 2005; (b) between samples taken from *P. coronata* on its four grass hosts; and (c) between samples taken from *P. brachypodii* f. sp. *arrhenatheri* (infecting *Arrhenatherum elatius), P. coronata* (infecting *H. lanatus)* and *P. poarum (*infecting *Poa trivialis*) at Shinfield in 2005, based on all bands in AFLP length profiles generated with the primers *PstI*CA + *MseI* CA.

Data group	Source of Variation	F_{ST} [a]	n	P [b]	Mean band diversity[c]
(a) Reading 2005	Between groups of isolates from different rusts	0.16	4	0.10[e]	0.20
	Between grasses within a rust[d]	0.11	4	<0.001	0.15
	Between isolates within single grasses	0.73	45		0.11
	Total	1	53		
(b) Reading 2005, *P. coronata* only	Between groups of isolates from different grasses	0.17	4	0.004	0.15
	Between isolates from a single grass	0.83	27		0.11
	Total	1	31		
(c) Shinfield 2005	Between groups of isolates from different hosts[f]	0.40	3	<0.001	0.25
	Between isolates from a single host	0.60	34		0.14
	Total	1	37		

[a]Proportion of the total variability between individuals associated with grouping factor.

[b]Proportion of randomisations yielding a ratio larger than that in the data, between the variance in successive levels of the heirarchy.   For variation among rusts or among grasses judged against variation among isolates within rusts, $P < 0.001$.

[c]Probability that any two bands from distinct groups differ, averaged over band positions present in the sample = proportion of bands differing between any two haplotypes from distinct groups.

[d] *P. brachypodii* and *P. recondita* were present on single grass hosts; *P. graminis* was present on both *Cynosurus cristatus* and *Dactylis glomerata* but there were only two isolates from *D. glomerata.*

[e] For the randomisation test of the rust-(grass within rust) comparison, all isolates from four grass hosts were randomly assigned to one "rust", and two to another, so as to match the actual data structure.

[f] Only one host rust was present on each grass species so classifications by grass or rust are equivalent.

Table 3. Euclidean distances between centroids of groups of isolates of *Eudarluca caricis* taken from the specified rusts in 2005, calculated from AFLP amplicon presence-absence data using PstICA.

			Reading				Shinfield
		Sample size	*P. brachypodii* f. sp. *poae-nemoralis*	*P. coronata*	*P. recondita*	*P. graminis*	*P. brachypodii* f. sp. *arrhenatheri*
Reading	*P.brachypodii* f.sp. *poae-nemoralis*	7	0	4.3	8.3	16.1	10.5
	P. coronata	23		0	5.5	12.2	7.3
	P. recondita	8			0	14.0	9.2
	P. graminis	9				0	5.5
Shinfield	*P. brachypodii* f. sp. *arrhenatheri*	12	10.5	7.3	9.2	5.5	0
	P. poarum	16	48.8	42.4	41.6	33.4	37.3

Table 4. Average euclidean distances between centroids of groups of *Eudarluca caricis* taken from *P. coronata* growing on distinct grasses in 2005, calculated from AFLP amplicon presence-absence data using PstICA.

		Sample size	Reading			
			Agrostis gigantea	*Holcus lanatus*	*H. mollis*	*F. pratensis*
Shinfield	*Holcus lanatus*	6	8.3	3.1	4.3	5.7
Reading	*Agrostis gigantea*	4	0	9.0	9.2	8.5
	H. lanatus	8		0	5.9	7.9
	H. mollis	7			0	6.9
	Festuca pratensis	8				0

Fig. 1. Unrooted maximum likelihood cladogram from ITS sequences of *Eudarluca caricis* from different rust (name on left) and plant (name on right) host species. Localities (GER=Germany) and accession numbers from GenBank sequences from other studies are also provided. All sequences generated in this study are from samples in or near Reading, UK. Numbers above branches are bootstrap (BP) support values indicating the percentage of resampled replicates in which the subsequent branches form a monophyletic group. Branch lengths are proportional to the change between isolates or groupings; numbers on the nodes are the percentage of bootstrap replicates in which the division appeared. Latin numbers shown on the right represent Liesebach and Zaspel (2004)'s groups. Liesebach and Zaspel (2004)'s group I isolates belong in the large clade shown at the top of the cladogram. *Puccinia brachypodii* f.sp. *arrenatheri* in clade 1: **KM285288** –**KM285298**, **KM285300**, **KM285301** and in unnamed clade **KM285299**, **KM285302**; *Puccinia brachypodii* f. sp. *poae-nemoralis* **KM285305** - **KM285311** ; *P. recondita* f. sp. *bromina* **KM285312** - **KM285319** *P. coronata* **KM285303**, **KM285304**, **KM285330** - **KM285359**; *P. graminis* **KM285320** - **KM285329**; *P. poarum* **KM285360** - **KM285369.**

Figure 2. Separation of *Eudarluca caricis* isolates from rusts on different host grasses by Canonical variate analysis of AFLP (PstICA) patterns of isolates collected from (a) the Reading site in 2005 (b) the Shinfield site in 2005. (a) Symbols represent: Ao, isolates from *Puccinia brachypodii* var *poae-nemoralis* growing on *Anthoxanthum odoratum*; isolates from *P. coronata* growing on Ag, *Agrostis gigantea,* Hl, *Holcus lanatus*, Hm, *H. mollis,* Fp, *Festuca pratensis*;. Be, isolates from *P. recondita* growing on *Bromus erectus;* Cc, *P. graminis* growing on *Cynosurus cristatus.* (b) Symbols represent: Ae, isolates from *Puccinia brachypodii* var *arrhenatheri* growing on *Arrhenatherum elatius*; Hl, *P. coronata* growing on *Holcus lanatus*, Pt *P. poarum* growing on *Poa trivialis.* Variation between groups of isolates

from the same grass host is maximised relative to variation within groups. Axis length is

proportional to the proportion of variation explained by the axis (i.e. to the eigenvalue

associated with the axis); axes scaling is arbitrary. At both sites separation between groups is

greater than if isolates are assigned to hosts at random, P < 0.001.

Figure 3. Separation of *Eudarluca caricis* isolates from various graminaceous rusts by

Canonical variates analysis of AFLP (PstICA) patterns of isolates collected at Reading in

2005. Projection maximises variation between groups of isolates from the same rust host at

Reading. Axis length is proportional to the proportion of variation related to the axis.

Symbols: br, *P. brachypodii* f. sp. *poae-nemoralis*; co, *P. coronata;* gr, *P. graminis* ; re, *P.*

recondita f. sp. *bromina;* ○ , *P. coronata* isolates from Shinfield projected on the axes

calculated from the Reading data. Separation between groups is greater than if isolates are

assigned to hosts at random, P << 0.001.

Puccinia brachypodii on *Anthoxanthum odoratum* (7 isolates)
Puccinia brachypodii on *Arrhenatherum elatius* (13 isolates)
Puccinia coronata on *Agropyron* sp. UK (AY836372)
Puccinia coronata on *Agrostis gigantea* (2 isolates)
Puccinia recondita f.sp. *bromina* on *Bromus erectus* (8 isolates)
Puccinia coronata on *Festuca pratensis* (7 isolates)
Puccinia sp. on *Holcus lanatus* USA (AY587135)
Puccinia coronata on *H. lanatus* (15 isolates)
Puccinia coronata on *H. mollis* (8 isolates)
Puccinia graminis on *Cynosurus cristatus* (8 isolates)
Puccinia graminis on *Dactylis glomerata* (2 isolates)
Puccinia sp. on *Phalaris* sp. USA (AY587136)
Melampsora sp. on *Salix* sp. GER (AY607011)
Melampsora sp. on *Salix* sp. GER (AY607012)
Melampsora sp. on *Salix* sp. GER (AY607013)
Melampsora sp. on *Salix* sp. UK (AY836374)
Melampsora sp. on *Salix* sp. UK (AY836370)

I

Melampsora sp. on *Salix* sp. GER (AY607014)
Melampsora sp. on *Salix* sp. GER (AY607015)
Melampsora sp. on *Salix* sp. S. Korea (EF600974)
Melampsora sp. on *Salix* sp. S. Korea (EF600978)
Melampsora medusae USA (AY587134)

II

Triphragmiopsis on *Larix kaempferi* China (AY836371)
Puccinia boroniae Australia (AY572490)
Melampsora sp. on *Salix* sp. GER (AY607023)
Melampsora sp. on *Salix* sp. Ethiopia (AY607022)
Phragmidium violeaceum on *Rubus fruticosus* UK (AY836373)

Melampsora sp. on *Salix* sp. Belgium (AY607021)
Melampsora sp. on *Salix* sp. GER (AY607019)
Melampsora sp. on *Salix* sp. GER (AY607020)
Melampsora sp. on *Salix* sp. GER (AY607016)
Melampsora sp. on *Salix* sp. GER (AY607017)
Melampsora sp. on *Salix* sp. GER (AY607018)

IV+V

Puccinia brachypodii on *A. elatius* (2 isolates)
Puccinia poarum on *Poa trivialis* (10 isolates)

Alternaria sp. on *Acer microphyllum* USA (AY587132)
Alternaria sp. on *Populus trichocarpa* USA (AY587133)
Ulocladium sp. on *Populus trichocarpa* USA (AY587137)

0.2

A

B