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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.

Key words: Hyperparasite, host specialisation, Puccinia graminis, biological control, Sphaerellopsis filum, Puccinia recondita, Holcus, Bromus, Anthoxanthum, Cynosurus
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 fruticosus agg. (Roseaceae) 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. cariciis on grass-infecting rusts would be largely clonal; (3) the population structure of E. cariciis 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
MseICA + 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.

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.

\textbf{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 PstICA patterns were available. The results were very similar using the combined PstICA and PstIAA band patterns and for separate analyses using the isolates for which PstIAA and PstICC 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 << 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 hierarchical 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-infesting 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-infesting 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. poae 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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References


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

<table>
<thead>
<tr>
<th>Rust species</th>
<th>Host grass</th>
<th>Reading</th>
<th>Shinfield</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2005</td>
<td>2005</td>
<td>2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFLP</td>
<td>ITS</td>
<td>AFLP</td>
</tr>
<tr>
<td>Puccinia brachypodii f. sp. poae-nemoralis</td>
<td>Anthoxanthum odoratum</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>P. brachypodii f. sp. arrhenatheri</td>
<td>Arrenatherum elatius</td>
<td>13</td>
<td>15</td>
<td>12</td>
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<tr>
<td>P. recondita f. sp. bromina</td>
<td>Bromus erectus</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
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<td>P. coronata</td>
<td>Agrostis gigantea</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Festuca pratensis</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Holcus lanatus</td>
<td>8</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>H. mollis</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>P. graminis</td>
<td>Cynosurus cristatus</td>
<td>9</td>
<td>8</td>
<td></td>
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<td></td>
<td>Dactylis glomerata</td>
<td>2</td>
<td>2</td>
<td></td>
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<tr>
<td>P. poarum</td>
<td>Poa trivialis</td>
<td>16</td>
<td>10</td>
<td></td>
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<tr>
<td>Total ITS</td>
<td></td>
<td>50</td>
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<tr>
<td>Total AFLP</td>
<td></td>
<td>53</td>
<td>35</td>
<td>12</td>
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</table>
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 PstICA + MseI CA.
<table>
<thead>
<tr>
<th>Data group</th>
<th>Source of Variation</th>
<th>$F_{ST}^a$</th>
<th>n</th>
<th>$P^b$</th>
<th>Mean band diversity $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Reading 2005</td>
<td>Between groups of isolates from different rusts</td>
<td>0.16</td>
<td>4</td>
<td>0.10$^e$</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Between grasses within a rust $^a$</td>
<td>0.11</td>
<td>4</td>
<td>&lt;0.001</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Between isolates within single grasses</td>
<td>0.73</td>
<td>45</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1</td>
<td>53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Reading 2005, P. coronata only</td>
<td>Between groups of isolates from different grasses</td>
<td>0.17</td>
<td>4</td>
<td>0.004</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Between isolates from a single grass</td>
<td>0.83</td>
<td>27</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) Shinfield 2005</td>
<td>Between groups of isolates from different hosts $^f$</td>
<td>0.40</td>
<td>3</td>
<td>&lt;0.001</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Between isolates from a single host</td>
<td>0.60</td>
<td>34</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1</td>
<td>37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$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 hierarchy. For variation among rusts or among grasses judged against variation among isolates within rusts, $P < 0.001$.  

For variation among hosts judged against variation among isolates within hosts, $P < 0.01$.  

$^c$Proportion of the total variability between individuals.
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.

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.

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.

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.

<table>
<thead>
<tr>
<th>Reading</th>
<th>P. brachypodii f. sp. poae-nemoralis</th>
<th>P. coronata</th>
<th>P. recondita</th>
<th>P. graminis</th>
<th>Shinfield P. brachypodii f. sp. arrhenatheri</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading P. brachypodii f. sp. poae-nemoralis</td>
<td>7</td>
<td>0</td>
<td>4.3</td>
<td>8.3</td>
<td>16.1</td>
</tr>
<tr>
<td>P. coronata</td>
<td>23</td>
<td>0</td>
<td>5.5</td>
<td>12.2</td>
<td>7.3</td>
</tr>
<tr>
<td>P. recondita</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>14.0</td>
<td>9.2</td>
</tr>
<tr>
<td>P. graminis</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.5</td>
</tr>
<tr>
<td>Shinfield P. brachypodii f. sp. arrhenatheri</td>
<td>12</td>
<td>10.5</td>
<td>7.3</td>
<td>9.2</td>
<td>5.5</td>
</tr>
<tr>
<td>P. poarum</td>
<td>16</td>
<td>48.8</td>
<td>42.4</td>
<td>41.6</td>
<td>33.4</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Holcus lanatus</th>
<th>Agrostis gigantea</th>
<th>Reading</th>
<th>H. mollis</th>
<th>F. pratensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shinfield</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Holcus lanatus</td>
<td>6</td>
<td>8.3</td>
<td>3.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Reading</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agrostis</td>
<td>4</td>
<td>0</td>
<td>9.0</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>gigantea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Holcus lanatus</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>H. mollis</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>Festuca</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
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** - **KM285339**; 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, $P << 0.001$. 