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Genetic structure of natural populations of the grass endophyte *Epichloë festucae* in semiarid grasslands

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Abstract

Plants of red fescue (*Festuca rubra*), a commercially important turf grass, are infected by the fungal endophyte *Epichloë festucae* in semiarid natural grasslands, known as dehesas, in western Spain. We used amplified fragment length polymorphism (AFLP) markers to analyse the genetic polymorphism existing in two natural populations of *Epichloë festucae*. Linkage disequilibrium and the presence of clonal lineages indicated that nonrecombinant asexual reproduction predominates in both populations. However, most genetic variation detected was found to occur within populations, with only a moderate amount of genetic differentiation between populations (F_{ST}: 0.197). Overall, the study suggests that dehesa grasslands are useful reservoirs of *Epichloë festucae* endophytes, and provides information on population structure which is relevant to design sampling strategies.

Keywords: AFLP, clonal lineages, endophytes, *Epichloë festucae*, *Festuca rubra*, grasslands

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Introduction

Festuca rubra L. is an economically important turf grass which is used in ornamental lawns, sports fields and erosion control lawns. Characteristics such as good tolerance to drought, shadow and soils with low fertility make *F. rubra* a good species for use in low maintenance turfs.

Mutualistic fungal endophytes belonging to the *Epichloë* and *Neotyphodium* genera are known to infect several *Festuca* species. In general, these endophytes do not induce symptoms in infected plants, but produce several types of toxic alkaloids, and as a result, the host plants are more resistant to herbivores than uninfected plants (Siegel *et al*. 1990; Breen 1994). In addition, increased tolerance to drought and other abiotic stress factors has also been observed in endophyte-infected grasses (Malinowski & Belesky 2000). Specifically, *F. rubra* plants infected by the endophyte *Epichloë festucae* (Leuchtmann *et al*. 1994), are more resistant to several species of insect herbivores, and have a better appearance and survival rates under stressful conditions than uninfected plants (Saha *et al*. 1987; Bazely *et al*. 1997; Funk & White 1997).

Because of the benefits conferred to infected plants, there is a great interest in the use of endophytes for the improvement of commercial forage and turf grass cultivars, and endophytic cultivars have been obtained with increased resistance to insects and no toxicity to mammals (Fletcher & Easton 1997). Because endophytes are efficiently transmitted by seed and permanently infect the host plant, their incorporation into commercial cultivars is a simple process. Therefore, selection of fungal strains with useful alkaloid profiles, or improving the general performance of commercial cultivars is an objective of interest for the turf and forage grass seed industry.

Currently, there is a limited number of endophytic *F. rubra* turf grass cultivars. Furthermore, because of limited fungal genetic resources, many of these cultivars have been developed from a few strains of endophytes (Ruemmele *et al*. 1995). In order to select useful strains of endophytes for turf grass improvement, it is desirable to have wide fungal germplasm collections. Centres of diversity of the host and related species may be good choices for the collection of endophytes (Funk & White 1997). Indeed, semiarid natural grasslands of western Spain known as

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'*dehesas*' could be good areas of collection for *E. festucae* endophytes. The reasons are: the Iberian Peninsula is considered a centre of origin for *F. rubra* and related fine fescues (Saint Yves 1930), *F. rubra* is a very abundant species in dehesas, and their endophyte infection rates are very high (up to 70% of the plants) in these natural populations (Zabalgogeazcoa *et al*. 1999).

The abundance of endophytes does not necessarily imply that there is a great variety of endophyte genotypes in the ecosystems. *E. festucae* may propagate itself by means of sexual or asexual mechanisms; because of this, it is difficult to predict the genetic structure of natural populations of this endophyte. When reproduction is asexual, the host plant does not show any symptom of infection during its entire life cycle. However, at anthesis the ovules are infected by the endophyte, which is later transmitted through the seeds. As a result, the seeds give rise to plants infected by the same fungus genotype as that infecting the mother plant. Thus, in the asymptomatic type of grass–endophyte interaction, the transmission of the fungus is vertical and its reproduction is clonal (Leuchtmann & Clay 1997).

Alternative sexual reproduction of *Epichloë* species is timed with flower production in the host plant. The fungus forms an external stroma which wraps the flag leaf sheath and the enclosed developing inflorescence. The result is that the inflorescence cannot emerge and the stem is sterilized. This symptom is known as choke disease of grasses. If the fungal stroma is fertilized by conidia of a compatible mating type, then perithecia containing ascospores develop on the surface of the stroma. Ejected ascospores may penetrate and infect new hosts via florets or wounds. In this type of interaction reproduction of the fungus is sexual and transmission is horizontal (Leuchtmann *et al*. 1994; Schardl 1996; Leuchtmann & Clay 1997).

In natural populations of *F. rubra*, only a small proportion of the infected plants develop stromata. In addition, the occurrence of fertilized stromata bearing perithecia seems to be extremely rare (Sampson 1933; Leuchtmann *et al*. 1994; Zabalgogeazcoa *et al*. 1999). Therefore, in natural populations of *F. rubra* the main mechanism of propagation of the fungus is vertical transmission to seeds through fungal asexual reproduction. Because in a given plant all seeds are infected by the same fungal genotype, there should be little genetic variation in populations in which the fungus is propagated exclusively in this fashion. In the absence of mechanisms which generate genetic variability, only migration of infected seeds from different populations would incorporate substantial genetic variation in populations of *E. festucae*.

As mentioned above, dehesa ecosystems are potentially important reservoirs for endophyte germplasm useful for turf grass improvement. This potential is related to the genetic diversity encountered in populations, which is an unknown parameter. The objective of this work was to determine the extent of genetic variation and genetic structure of natural populations of *E. festucae,* using amplified fragment length polymorphisms (AFLP), as a way to improve the efficiency of sampling strategies to identify new genetic variants of *Epichloë* endophytes.

Materials and methods

Plants and fungi

The two natural populations of *Festuca rubra* studied (Palancar and Servandez) are located in dehesa-type natural grasslands in the province of Salamanca, in western Spain. Dehesa ecosystems occupy > 400 000 ha in this province. The straight line distance between populations is 41 km. At Palancar, 33 plants of *F. rubra* were dug and carried to the laboratory, and 40 plants were sampled at Servandez. To minimize the probability of sampling ramets belonging to the same genet, a distance of at least 10 m was left between each pair of plants. Samples were numbered in the order in which they were collected. Therefore, sample number is, to some extent, related to distance among samples. A tiller from each plant was separated, transplanted to a pot and maintained outdoors in a wirehouse. To isolate endophytic fungi from the plants, stem pieces or leaf sheaths from each selected tiller were cut, surface sterilized, and plated in potato dextrose agar (Bacon & White 1994). The fungal cultures used in the study were derived from the mycelium which emerged from a single piece of stem or leaf sheath. This precaution was taken to avoid sampling more than one fungal genotype per plant in case there were mixed infections in a single plant. In any case, this situation has been described in other *Epichloë* species, but not in *Epichloë festucae* (Meijer & Leuchtmann 1999). To avoid false-negative diagnostics, plants in which no endophytes were isolated the first time were plated again.

No *Festuca* plants bearing stromata were observed in the locations at which plants were collected. In the Palancar population, *E. festucae* was isolated from 26 of the 33 plants analysed. In the Servandez population, 31 of 40 plants were endophyte infected. Eighteen isolates from each population were used for the AFLP analysis. Three additional isolates of *E. festucae* (H18, H24, V23), were obtained from infected plants of *F. rubra* ssp. *pruinosa* growing in sea cliffs in the North Atlantic coast of Galicia (Spain). The places where these plants were found are ≈ 350 km away in a straight line from the area where dehesa endophytes were collected. The distance between H and V isolates is 42 km.

Four samples were used as outgroups in the analyses. Two of them, Z12 and Z21, corresponded to *E. typhina* and were obtained from stromata-bearing plants of *Lolium perenne* collected at the same location in a dehesa grassland.

Table 1 Sequences of primers and adaptors used for amplified fragment length polymorphism analysis

*Eco*RI adaptor	5′-CTCGTAGACTGCGTACC-3′
	3′-CTGACGCATGGTTAA-5′
*Mse*I adaptor	5′-GACGATGAGTCCTGAG-3′
	3′-TACTCAGGACTCAT-5′
*Eco*RI + 1 primer	5′-GACTGCGTACCAATTCA-3′
*Mse*I + 1 primer	5′-GATGAGTCCTGAGTAAC-3′
*Mse*I + 1 primer	5′-GATGAGTCCTGAGTAAT-3′
*Eco*RI + 2 primer	5′-GACTGCGTACCAATTCAC-3
*Mse*I + 2 primer	5′-GATGAGTCCTGAGTAACA-3
*Mse*I + 2 primer	5′-GATGAGTCCTGAGTAATA-3

Isolate Z21 was collected in 1997 and Z12 in 1999. The other two samples, T6 and T8, corresponded to *Neotyphodium lolii*, and were isolated from asymptomatic plants of *L. perenne*. Both isolates were obtained from plants growing at a distance > 20 m from each other. *E. typhina* and *E. festucae* are haploid organisms, like *N. lolii*, which is the closest known asexual relative of *E. festucae* (Schardl *et al*. 1994).

DNA purification and AFLP markers

To produce enough mycelium for DNA extraction, isolates were grown on top of cellophane disks in potato dextrose agar (PDA) plates. DNA was extracted from lyophilized 2-week-old cultures using the method described by Moon *et al*. (1999). AFLP analysis was performed according to Vos *et al*. (1995) with the modifications described by Cervera *et al*. (1998). DNA was digested with restriction endonucleases *Mse*I and *Eco*RI. After ligation of adaptors (Table 1), pre-amplification of DNA templates was performed with primers complementary to the adaptor sequences with an additional selective 3′-nucleotide (Table 1). Polymerase chain reaction (PCR) reactions were performed in a 20-μL volume containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 30 ng of each primer *Eco*RI + A, *Mse*I + C and *Mse*I + T, 0.4 U of *Taq* DNA polymerase and 5 μL of adaptor-ligated DNA fragments. Selective amplifications were performed using two combinations of primers (*Eco*RI + AC/*Mse*I + CA) and (*Eco*RI + AC/*Mse*I + TA) (Table 1) with ^{33}P-labelled *Eco*RI primers. We used 5 μL of the pre-amplification template for each PCR. Two replicates of the PCRs from independent DNA extractions were performed to determine the accuracy of the analysis. Samples amplified with different primer combinations were loaded onto 4.5% denaturing polyacrylamide gels and electrophoresed for 2 h. Gels were later dried onto chromatography paper, and exposed to autoradiographic film. Each amplified band was scored as the presence/absence of data. Only bands shown to be reproducible were scored. *E. festucae* is a haploid ascomycete. Therefore, AFLP data in this material should be equivalent to restriction fragment length polymorphism (RFLP) data. From these data a binary presence/absence matrix was constructed and imported to other software packages for data manipulation and analysis.

Data analyses

Genome-wide multilocus analysis of linkage disequilibrium was performed in the Palancar and Servandez populations using the multilocus association test described by Brown *et al*. (1980). In this test, the value $X(2)$ = [observed variance of k/expected variance] − 1, is an indicator of multilocus structure. The parameter k is the average number of heterozygous loci observed for all possible pairwise combinations of haplotypes in each population. $X(2)$ is equal to 0 when there is no linkage association between loci, and values > 1 imply that the variance observed in k because of multilocus structure is more than double that due to independent polymorphism. The value $X(2)$ is also known as the index of association (I_A) (Maynard Smith *et al*. 1993). Alternatively, linkage disequilibrium was also tested for all possible pairs of loci within each population (Weir 1979). POPGENE Version 3.2 software was used to perform both types of linkage disequilibrium analyses (Yeh *et al*. 1997).

The binary data matrix was converted to a genetic distance matrix by computing, for every pair of isolates, the Dice coefficient (Sneath & Sokal 1973). This coefficient was calculated as

$$GS_{ij} = 2a/(2a + b + c),$$

where GS_{ij} is the genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i.

A dendrogram was constructed by the unweighted pair group method with arithmetic averages (UPGMA) from the genetic distance matrix as a graphic representation of the relationships among samples. The goodness-of-fit of the tree to the distance matrix data was tested by cophenetic correlation. These analyses were performed using the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) software package Version 2 (Rohlf 1993).

Principal components analysis is a method of data reduction and structure detection (Manly 1994). When the data are highly correlated, a plot of the taxa against the first few principal components will account for a large proportion of the total variance. Such a plot would effectively summarize the structure contained in the full data set. Principal component analysis was applied to the AFLP data using STATISTICA Version 5.0. In the graphical representation

of the analysis, the variance was maximized with varimax normalized rotation.

The extent of genetic variation within each population was estimated by calculating the average gene diversity over all AFLP loci (H), and the difference in H between both populations was tested with a t-test (Nei & Kumar 2000). This index of gene diversity is equivalent to the probability that two isolates share the same haplotype at a randomly chosen AFLP locus, and estimates the expected heterozygosity if the population were going to mate randomly.

The genetic structure was examined by analysis of molecular variance (AMOVA) of haplotypic data using the ARLEQUIN software package (Schneider *et al*. 2000). For this analysis the haplotypic data were considered to be derived from RFLP analysis. The F_{ST} coefficient, obtained from the AMOVA was used as a measure of genetic differentiation among populations. The significance of F_{ST} and the variance components among populations were tested with 1023 random permutations of the distance matrix used to generate a null distribution of correlation coefficients.

Gene flow, calculated as the average number of individuals that migrate to each population per generation (Nm) was estimated with the formula $Nm = 1/2[(1/F_{ST}) - 1]$, where N is the population size and m the fraction of individuals that are immigrants in an haploid population (Hartl & Clark 1997).

Results

To obtain an optimum number of scorable polymorphic bands, different combinations of primers were tested on a few samples. The best results were obtained using the primers shown in Table 1. Combination of primers *Eco*RI + AC/*Mse*I + CA and *Eco*RI + AC/*Mse*I + TA yielded a total of 84 scorable polymorphic AFLP for all the samples analysed. The proportion and number of polymorphic markers detected at Palancar and Servandez populations are shown in Table 2.

The average gene diversity estimated from marker frequencies at each population was quite considerable, at least from the point of view of an organism whose reproduction seems to be predominantly clonal. Average gene diversity estimates were greater for the Servandez than for the Palancar population (Table 2). However, this difference was not statistically significant ($t_{0.95,62} = -1.9441$, $P = 0.0564$).

Linkage disequilibrium

Significant linkage disequilibrium was detected for both populations when Brown's multilocus association test was used (Table 3). When AFLP loci showing a very low frequency of polymorphism (< 0.1) were excluded from the analysis, linkage disequilibrium was still maintained at a significant level. In addition, tests of linkage disequilibrium performed in all pairwise combinations of loci detected significant linkage disequilibrium ($P < 0.05$) in 748 of the 1225 (61.06%) possible pairwise combinations of loci in the Palancar population and in 784 of the 1540 (50.90%) pairs of loci from the Servandez haplotype data. The number of pairs of loci showing linkage disequilibrium increased to 75.6 and 74.4% of the total when only loci with markers showing frequencies of < 0.9 where considered.

Genetic relationships

Genetic similarities among different isolates of grass endophytes were represented with a dendrogram derived from the genetic similarity matrix (Table 4) using the

Table 2 Percentage (L) and number (N) of amplified fragment length polymorphism loci which are polymorphic, and average gene diversity (H) observed in two natural populations of *Epichloë festucae*. The percentage and number of polymorphic loci in which marker frequencies are ≤ 0.90 are indicated with the .90 subscript

Population	L	N	$L_{.90}$	$N_{.90}$	$H \pm SD$
Palancar	79.4	50	61.9	39	0.247 ± 0.184
Servandez	88.9	56	69.8	44	0.299 ± 0.178

Table 3 Test of multilocus association in *Epichloë festucae* haplotypes from the Servandez and Palancar populations. K is the number of different marker loci observed when two haplotypes are compared. The null hypothesis of independence of loci is rejected when the observed variance of K (s^2_K) is out of the 95% confidence limits. The index of association (I_A) equals 0 when loci are independent. For each population, multilocus structure was tested using the total number of polimorphic loci, and a subset of loci at which marker frequencies were ≤ than 0.90

Popn (no. loci)	Mean K	Observed s^2_K	Expected s^2_K	95% confidence limits of s^2_K	I_A
Palancar	15.54	58.37 ± 3.18	9.62	3.38–15.86	5.07
$Palancar_{0.90}$	14.39	50.66 ± 2.83	8.59	3.03–14.14	4.90
Servandez	18.85	57.83 ± 3.72	11.24	3.95–18.54	4.14
$Servandez_{0.90}$	17.60	54.49 ± 3.34	10.12	3.57–16.66	4.38

Table 4 Matrix of Dice coefficients of genetic similarity between pairs of isolates of *Epichloë festucae* from the Palancar (p) and Servandez (s) populations, and from plants of *Festuca rubra* ssp. *pruinosa* (h,v). Outgroup species are represented by isolates of *Neotyphodium lolii* (t) and *Epichloë typhina* (z)

	p1	p4	p6	p9	p11	p12	p13	p14	p15	p17	p23	p25	p26	p27	p29	p31	p32	p33	s1	s2	s3	s4	s5	s6	s9	s10	s24	s26	s27	s28	s29	s30	s31	s32	s33	s40	h18	h24	v23	z12	z21	t6
p1	1.0																																										
p4	.89	1.0																																										
p6	.82	.85	1.0																																									
p9	.66	.66	.63	1.0																																								
p11	.79	.84	.83	.68	1.0																																							
p12	.70	.70	.71	.67	.83	1.0																																						
p13	.78	.83	.81	.69	.99	.84	1.0																																					
p14	.75	.81	.90	.56	.84	.62	.83	1.0																																				
p15	.58	.64	.69	.51	.65	.55	.63	.68	1.0																																			
p17	.75	.81	.90	.56	.84	.62	.83	1.0	.68	1.0																																		
p23	.70	.76	.80	.59	.77	.55	.76	.88	.64	.88	1.0																																	
p25	.73	.73	.77	.73	.92	.85	.90	.73	.62	.73	.65	1.0																																
p26	.74	.79	.81	.74	.90	.92	.89	.71	.63	.71	.66	.93	1.0																															
p27	.74	.79	.89	.58	.85	.64	.84	.99	.70	.99	.87	.75	.73	1.0																														
p29	.73	.79	.89	.57	.83	.63	.84	.99	.66	.99	.86	.71	.69	.97	1.0																													
p31	.75	.80	.78	.69	.97	.81	.98	.83	.60	.83	.76	.88	.86	.84	.84	1.0																												
p32	.58	.61	.66	.63	.62	.55	.63	.71	.70	.71	.75	.56	.60	.70	.72	.63	1.0																											
p33	.74	.74	.84	.56	.78	.63	.79	.89	.65	.89	.81	.72	.70	.88	.91	.79	.65	1.0																										
s1	.77	.71	.78	.56	.68	.66	.68	.74	.58	.74	.71	.60	.67	.73	.75	.66	.62	.83	1.0																									
s2	.59	.53	.51	.67	.57	.63	.55	.47	.51	.47	.48	.57	.64	.49	.45	.53	.55	.49	.66	1.0																								
s3	.56	.62	.63	.67	.73	.84	.74	.53	.58	.53	.52	.74	.81	.55	.54	.71	.58	.59	.56	.69	1.0																							
s4	.56	.56	.54	.69	.67	.73	.65	.50	.54	.50	.52	.68	.74	.52	.48	.63	.58	.52	.62	.85	.76	1.0																						
s5	.75	.83	.81	.75	.93	.86	.94	.77	.65	.77	.72	.88	.92	.79	.78	.92	.68	.74	.68	.59	.79	.67	1.0																					
s6	.71	.79	.83	.65	.90	.78	.91	.85	.70	.85	.81	.80	.84	.86	.86	.89	.70	.82	.76	.55	.70	.63	.92	1.0																		
s9	.81	.84	.79	.61	.77	.60	.76	.86	.62	.86	.82	.66	.69	.85	.85	.76	.72	.81	.84	.57	.51	.59	.73	.80	1.0																	
s10	.79	.82	.74	.60	.73	.57	.73	.79	.59	.79	.74	.60	.64	.78	.80	.71	.69	.75	.81	.54	.51	.56	.70	.78	.93	1.0																
s24	.62	.65	.69	.67	.78	.66	.78	.70	.62	.70	.74	.74	.72	.69	.71	.76	.72	.66	.72	.60	.60	.70	.76	.83	.77	.74	1.0															
s26	.71	.68	.78	.65	.80	.75	.81	.74	.63	.74	.72	.77	.81	.73	.75	.79	.70	.76	.82	.64	.64	.74	.79	.86	.80	.78	.92	1.0														
s27	.66	.63	.64	.71	.73	.78	.74	.57	.56	.57	.56	.75	.79	.59	.58	.72	.63	.63	.67	.76	.76	.86	.77	.70	.61	.61	.72	.79	1.0													
s28	.59	.59	.58	.73	.70	.74	.71	.54	.49	.54	.56	.68	.75	.56	.55	.68	.56	.60	.69	.80	.74	.92	.73	.69	.63	.63	.74	.77	.86	1.0												
s29	.55	.60	.58	.65	.71	.64	.72	.63	.50	.63	.66	.64	.70	.65	.64	.72	.63	.58	.64	.64	.55	.71	.74	.76	.72	.72	.75	.78	.70	.80	1.0											
s30	.54	.60	.67	.64	.71	.76	.72	.63	.59	.63	.62	.70	.76	.65	.64	.69	.59	.70	.67	.67	.86	.75	.77	.79	.61	.58	.70	.74	.74	.81	.65	1.0										
s31	.66	.71	.64	.76	.79	.79	.80	.61	.52	.61	.58	.74	.80	.63	.62	.78	.61	.56	.64	.74	.72	.85	.82	.75	.69	.69	.74	.77	.80	.88	.84	.70	1.0									
s32	.66	.68	.67	.77	.76	.72	.74	.58	.53	.58	.60	.75	.81	.59	.56	.72	.53	.61	.61	.75	.81	.77	.79	.70	.59	.56	.64	.68	.76	.80	.65	.82	.75	1.0								
s33	.62	.65	.63	.72	.77	.76	.75	.57	.50	.57	.59	.76	.79	.59	.55	.73	.50	.60	.63	.77	.77	.88	.75	.67	.61	.58	.68	.72	.83	.94	.74	.78	.87	.85	1.0							
s40	.55	.63	.64	.65	.68	.61	.67	.66	.63	.66	.66	.61	.68	.68	.64	.64	.70	.58	.64	.75	.67	.68	.71	.73	.72	.67	.72	.70	.65	.69	.70	.74	.72	.68	.67	1.0						
h18	.74	.68	.78	.43	.65	.57	.66	.79	.51	.79	.65	.57	.58	.78	.81	.66	.51	.82	.75	.44	.47	.41	.62	.70	.74	.69	.54	.64	.52	.49	.46	.57	.46	.49	.47	.46	1.0					
h24	.77	.71	.75	.46	.68	.59	.68	.77	.54	.77	.68	.60	.61	.76	.78	.68	.54	.86	.79	.46	.49	.43	.65	.73	.78	.72	.56	.67	.54	.51	.48	.60	.48	.52	.49	.48	.95	1.0				
v23	.64	.69	.70	.42	.62	.59	.63	.75	.48	.75	.64	.49	.55	.74	.76	.63	.52	.71	.71	.42	.51	.45	.62	.68	.73	.68	.54	.58	.49	.52	.50	.60	.54	.47	.50	.50	.85	.79	1.0			
z12	.42	.39	.36	.22	.34	.43	.34	.32	.20	.32	.32	.28	.35	.32	.33	.31	.29	.36	.44	.28	.34	.35	.34	.35	.41	.46	.23	.35	.37	.38	.38	.35	.39	.29	.36	.25	.38	.40	.43	1.0		
z21	.45	.42	.40	.29	.38	.43	.38	.37	.24	.37	.37	.33	.39	.36	.37	.35	.31	.41	.47	.30	.36	.37	.38	.39	.44	.49	.29	.39	.38	.41	.42	.39	.42	.33	.39	.28	.48	.50	.62	.85	1.0	
t6	.38	.50	.43	.32	.43	.39	.43	.46	.62	.46	.43	.41	.45	.45	.47	.43	.56	.44	.40	.29	.42	.38	.47	.49	.56	.51	.47	.49	.40	.37	.45	.43	.42	.34	.35	.45	.42	.44	.40	.19	.24	1.0
t8	.39	.47	.44	.33	.40	.44	.41	.43	.53	.43	.36	.42	.46	.42	.44	.41	.53	.50	.41	.30	.51	.43	.44	.46	.49	.44	.40	.46	.49	.42	.35	.52	.39	.38	.39	.38	.47	.50	.44	.24	.28	.77

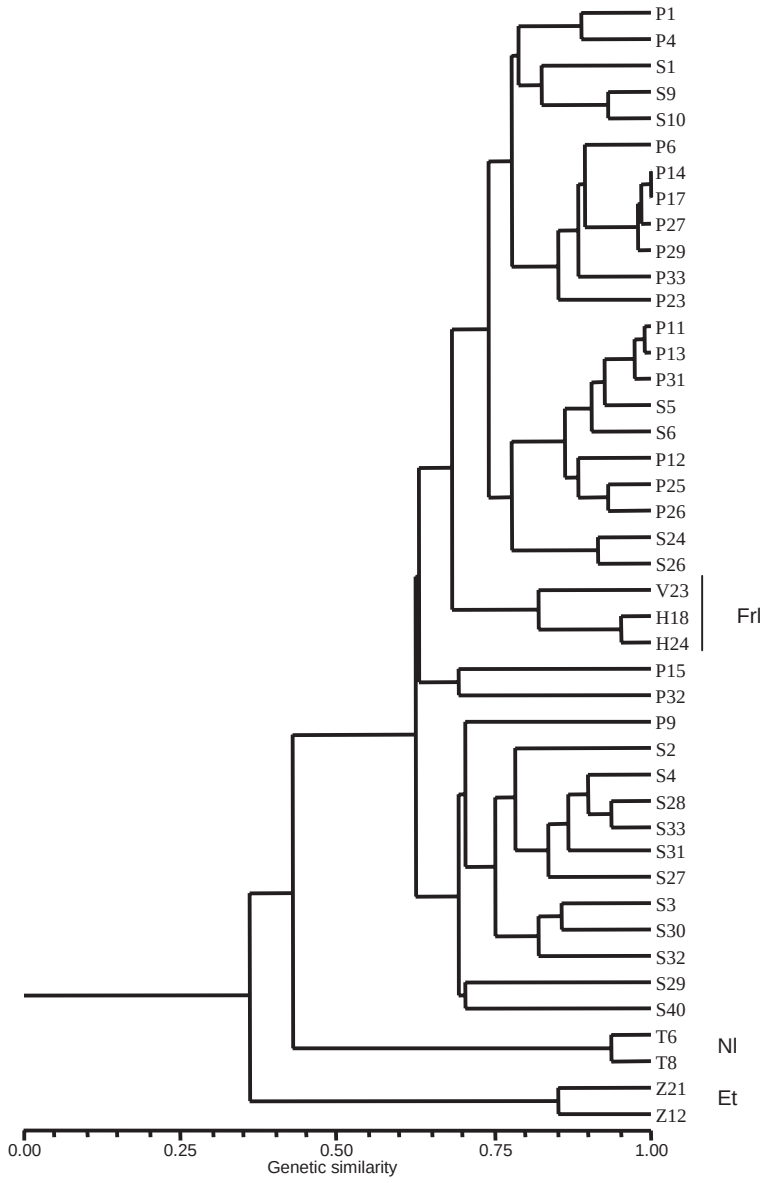

Fig. 1 Dendrogram generated by UPGMA clustering of Dice genetic similarity coefficients of isolates of endophytic fungi. *Epichloë festucae* isolates are from Palancar (P) and Servandez (S) grassland populations and from coastal populations of *Festuca rubra* (Frl group). Outgroups are *Neotyphodium lolii* (Nl group) and *Epichloë typhina* (Et group) isolates from *Lolium perenne*.

UPGMA method (Fig. 1). This dendrogram showed a good fit to the genetic similarity data, as reflected by a cophenetic correlation coefficient of 0.95. In this dendrogram, individuals from the same outgroup species (*Epichloë typhina* and *Neotyphodium lolii*) were clustered together, and separated from the *E. festucae* isolates with genetic similarity values < 0.5. Regarding *E. festucae*, most Palancar isolates were clustered in one group and most Servandez isolates in another. Within each dehesa population some clusters of isolates are very likely to represent clones, the most clear case being the groups formed by isolates P14, P17, P27 and P29, and isolates P11, P13 and P31 (Fig. 1) with similarities > 0.95. The maximum difference between any pair of isolates in each of these groups is one marker. In other clusters such as (S9, S10), (S28, S33) and (P25, P26), with similarities > 0.9, isolates within each group differ by 5 or fewer markers. All these clusters grouped individuals always coming from the same population, and in most cases corresponded to spatially contiguous samples.

Some isolates from the Servandez population were more similar to Palancar clusters than to clusters from their own population. This is the case of isolates S5 and S6 and S1, S9 and S10. This could be an indication of the existence of migrants from Palancar into the Servandez population.

Clusters of highly related isolates were related to other clusters with genetic similarities ranging between 0.6 and 0.75. Genetic similarities among *E. festucae* isolates from *Festuca rubra* ssp. *pruinosa*, and those of the clusters formed by the Palancar and Servandez dehesa populations were within this range.

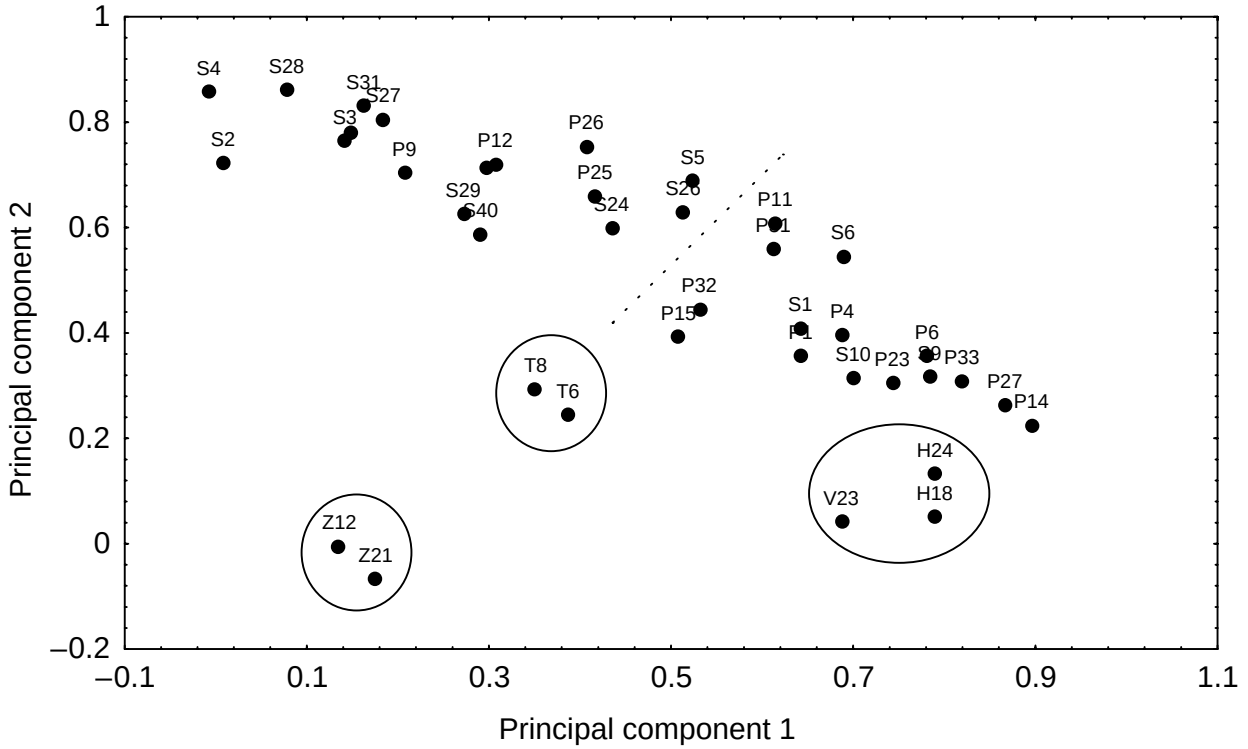

Fig. 2 Distribution of isolates of fungal endophytes on the two first components obtained from a principal component analysis. *Epichloë festucae* isolates belong to two populations: Servandez (S) and Palancar (P). The dotted line separates two groups predominantly formed by isolates from one population. Circles enclose isolates of *Neotyphodium lolii* (T6–T8), *Epichloë typhina* (Z12–Z21) and *Epichloë festucae* isolates from coastal populations of *Festuca rubra* ssp. *pruinosa* (H18, H24, V23). Principal components 1 and 2 describe 60.4% of the AFLP variation.

In order to further investigate the genetic structure of *Epichloë* populations, we subjected our data set to principal component analyses (Manly 1994). In this analysis, the first two principal components accounted for 60.4% of the existing variation (49.2% corresponding to component 1 and 11.2% to component 2), with the third component accounting only for 5.5% of the total variation. These results indicated that data were highly correlated and, based on these major effects of the first two components, a two-dimensional plot of the sample scores was made to represent the intersample similarities (Fig. 2). In this plot, the two principal factorial axes separate the two outgroup species, *E. typhina* and *N. lolii*, as well as the *F. rubra* ssp. *pruinosa* isolates, from the dehesa isolates of *E. festucae*. The first factor has higher loadings on Palancar isolates, whereas the second factor has higher loadings on Servandez isolates. This result shows that *E. festucae* isolates within populations are more similar than isolates from different populations.

The level of genetic differentiation between the Palancar and Servandez populations was estimated by AMOVA (Table 5). This analysis detected significant ($P > 0.001$) variation between both populations, and produced an F_{ST} value of 0.197, indicating a moderate level of differentiation among populations. However, this analysis also suggests that most of the variance detected corresponds to within population variation.

Table 5 Analysis of molecular variance from Palancar and Servandez populations of *Epichloë festucae*

Source	d.f.	Variance components	Percentage of variation	F_{ST}
Among populations	1	2.23475**	19.70	0.197**
Within populations	34	9.10784	80.30	
Total	35	11.342		

**significant with $P > 0.001$.

Discussion

Within each endophyte population studied, several clusters of highly similar isolates were detected. This is most evident in the Palancar population, in which two groups of isolates seem to be clearly clonal (14, 17, 27 and 29, and 11, 13 and 31) (Fig. 1). The isolates within these groups are identical at > 90% of the AFLP loci polymorphic

at this population. The genetic similarity observed among some isolates within clusters, together with the linkage disequilibrium detected in each population (Table 3), suggest that nonrecombining asexual or clonal reproduction predominates over sexual or parasexual mechanisms, which involve recombination. This point is in agreement with the absence of fungal sexual structures (stromata) in the host plant populations, as well as with the fact that in many instances consecutively numbered samples are very similar genetically. Therefore, clonal spread of the endophytic fungi may occur by means of infected seeds or vegetative growth of the host plants. Concerning the last mechanism, the age of some genets of *Festuca rubra* has been estimated to be of the order of several hundred years, and their spread at > 200 m in diameter (Harberd 1961).

In contrast to the presence of clonal lineages and the linkage disequilibrium observed in *Epichloë* populations, in a similar plant–fungus system formed by *Atkinsonella hypoxylon* and the grass *Danthonia spicata*, most fungal isolates within populations were derived from sexual reproduction (Kover *et al*. 1997). A crucial fact to relate the results of that study to the ones observed in *Epichloë festucae* is that in every plant infected by *A. hypoxylon*, the fungus can be propagated asexually by means of seed transmission, as well as sexually by means of ascospores. In the *E. festucae*/*F. rubra* interaction, the propagation of the fungus is mainly asexual, whereas sexual ascospores develop only in a few infected plants.

In clonal lineages of fungi, genetic variation caused by mutation is reflected in the accumulation of genetic differences among members of the same mitotic lineage. For example, in the ascomycete *Magnaporthe grisea*, variation generated by neutral chromosomal rearrangements has been shown to exist within clonal lineages. In this fungus, such variation appeared even after few serial transfers of cultures (Talbot *et al*. 1993; Anderson & Kohn 1995). The genetic similarity observed within some clusters of isolates supports the hypothesis of clonal lineages being important in *E. festucae* populations.

Because of intraclonal variation, it is not straightforward to determine the limits of some of the clonal lineages present in the dendrogram shown in Fig. 1. An approximation to these limits can be derived from the genetic similarity shown by the *F. rubra* ssp. *pruinosa* (FRL) isolates to the rest of the samples analysed. These isolates are not likely to belong to any of the clonal lineages present in dehesa grasslands. The plant hosts of these endophytes grow only in cliffs along the coastline, ≈ 350 km from our dehesa endophytes. The FRL cluster is separated from dehesa isolates by a genetic similarity coefficient of ≈ 0.70 (Fig. 1). Within this group, the H isolate was collected 40 km from the V isolates, and the similarity between the H and the V isolates is ≈ 0.80. If this range of genetic similarity of 0.7–0.8 is taken as an estimate of a distance between two different genotypes or clones, then a few different clonal lineages can be distinguished within each dehesa population.

The populations sampled in this study are natural grasslands used for livestock grazing, otherwise undisturbed by human activity. Dehesa grasslands are rich in plant species and because of plant competition it is likely that there are not many opportunities for the recruitment of new infected *F. rubra* individuals. Clonal lineages may be represented by grass/endophyte associations established long time ago in these populations. The presence of some isolates from Servandez such as S5, S6; within a Palancar cluster suggests that some clonal lineages could be broadly spread, > 40 km apart, likely because of seed dispersion.

Within each subpopulation, some isolates are very different from all others (i.e. P15, P32, P9, S29, S40). The genetic similarity among these isolates is at most 0.75. The contribution of genetically distant isolates to the genetic diversity of each population could be important. In this study 80% of the genetic variance of the data occurs within populations. In addition, the estimated average gene diversity is substantial at both populations (Table 2). These data suggest that in endophyte populations from dehesas there is a significant genetic variation.

E. festucae only occasionally exhibits sexual structures in certain natural populations of *F. rubra* (Sampson 1933; Leuchtmann *et al*. 1994; Zabalgogeazcoa *et al*. 1999). Therefore if sex is excluded, the genetic variation observed within populations has three possible sources: variation generated within clonal lineages by mutation, inmigration, and parasexual recombination. Genetic recombination by means of parasexual cycles is a phenomenon common in fungi, but not yet described in *Epichloë* species. However, vegetative compatibility allowing heterokaryon formation has been shown to occur in this genus (Chung & Schardl 1997), and this is a prerequisite for the existence of parasexual recombination. Further support for the hypothesis of parasexuality in *Epichloë* comes from studies that have identified hybrids of *Epichloë* with other species of this genus, and with related asexual endophytes (Schardl *et al*. 1994; Tsai *et al*. 1994). For parasexuality to occur, two different genotypes of fungi would need to be in contact. This could occur by means of the horizontal infection of a floret of an already infected plant by a sexual ascospore from another plant. In addition, White *et al*. (1996) have shown the presence of mycelium and conidia in the surface of two species of grasses infected by *Neotyphodium* endophytes. It is not known whether such a thing happens in infected plants of *F. rubra*, but the presence of endophytes in the phylloplane could make possible for two different strains to come in contact without the need of sexual ascospores.

Alternatively, although sexual structures seem to be extremely rare in natural populations, perhaps some environmental conditions affecting the transition to a sexual mode of reproduction may occur sporadically. For example,

Sun *et al*. (1990) reported that the addition of nitrogen fertilizer had a negative effect in the production of stromata in infected plants of *F. rubra*.

The results of UPGMA clustering, principal components analysis and AMOVA, all show that there is some genetic differentiation among the Palancar and Servandez endophyte populations. In the context of analyses of fungal populations, an F_{ST} value of 0.197 indicates a moderate level of genetic differentiation (Pimentel *et al*. 2000). The tendency to clonal reproduction in *E. festucae*, observed here, would favour strong genetic differentiation between populations by means of genetic drift. The moderate F_{ST} value observed may be attributed to the presence of migrants in the populations. The data presented show evidence of the presence of such individuals in the populations: the Palancar-like isolates within the Servandez population, and perhaps the genetically distant isolates observed in each population (Fig. 1). The level of genetic differentiation observed among populations could be maintained with only 2.03 migrants per generation in each population, the value obtained for *Nm*.

In conclusion, the analysis carried out in this study brings some insights about the structure of natural populations of *E. festucae* in dehesa grasslands. Within populations substantial genetic variation is found. However, clonal lineages are present. These lineages are produced by the clonal propagation of a fungal genotype by means of seed transmission and/or vegetative growth of the host plant. Clones of a given lineage may then differentiate by mechanisms other than sexual recombination (i.e. mutation and perhaps parasexuality). Populations are connected by migration, this probably happens when infected seeds are transported from one population to another. This gene flow works against the tendency towards strong differentiation which could be expected from the local dominance of clonal lineages. Sex could perhaps occur sporadically within populations and work as a recombinant of lineages.

Dehesas are natural semiarid grasslands in which *F. rubra* plants are very abundant, and in natural populations of this species, a high proportion of the plants are infected by *E. festucae*. If sampling for diverse genotypes of endophytic fungi is desired, few samples should be picked at each location, short distances between sampled plants should be avoided, and sampling in multiple locations will increase the chance of isolating genetically different samples.

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References

Anderson JB, Kohn LM (1995) Clonality in soilborne, plant pathogenic fungi. *Annual Review of Phytopathology*, **33**, 369–391.

Bacon CW, White JF (1994) Stains, media and procedures for analyzing endophytes. In: *Biotechnology of Endophytic Fungi of Grasses* (eds Bacon CW, White JF), pp. 47–56. CRC Press, Boca Raton, FL.

Bazely DR, Vicari M, Emmerich S, Filip L, Lin D, Inman A (1997) Interactions between herbivores and endophyte-infected *Festuca rubra* from the Scottish islands of St. Kilda, Benbecula and Rum. *Journal of Applied Ecology*, **34**, 847–860.

Breen JP (1994) Acremonium endophyte interactions with enhanced plant resistance to insects. *Annual Review of Entomology*, **39**, 401–423.

Brown AHD, Feldman MW, Nevo E (1980) Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics*, **96**, 523–536.

Cervera MT, Cabezas JA, Sancha JC, Martínez de Toda F, Martínez Zapater JM (1998) Application of AFLPs to the characterization of grapevine *Vitis vinifera* L. Genetic resources. A case study with accessions from Rioja (Spain). *Theoretical and Applied Genetics*, **97**, 51–59.

Chung KR, Schardl CL (1997) Vegetative compatibility between and within *Epichloë* species. *Mycologia*, **89**, 558–565.

Fletcher LR, Easton SH (1997) The evaluation and use of endophytes for pasture improvement. In: *Neotyphodium/Grass Interactions* (eds Bacon CW, Hill NS), pp. 209–227. Plenum Press, New York.

Funk CR, White JF (1997) Use of natural and transformed endophytes for turf improvement. In: *Neotyphodium/Grass Interactions* (eds Bacon CW, Hill NS), pp. 229–239. Plenum Press, New York.

Harberd DJ (1961) Observations on population structure and longevity of *Festuca rubra*. *New Phytologist*, **60**, 184–192.

Hartl DL, Clark AG (1997) *Principles of Population Genetics*, 3rd edn. Sinauer Associates, Sunderland, MA.

Kover PX, Dolan TE, Clay K (1997) Potential versus actual contribution of vertical transmission to pathogen fitness. *Proceedings of the Royal Society of London, Series B*, **264**, 903–909.

Leuchtmann A, Clay K (1997) The population biology of grass endophytes. In: *The Mycota, Vol. 5, Part A: Plant Relationships* (eds Caroll GC, Tudzynsky P), pp. 185–202. Springer Verlag, Berlin.

Leuchtmann A, Schardl CL, Siegel MR (1994) Sexual compatibility and taxonomy of a new species of *Epichloë* symbiotic with fine fescue grasses. *Mycologia*, **86**, 802–812.

Malinowski DP, Belesky DP (2000) Adaptations of endophyte-infected cool season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Science*, **40**, 923–940.

Manly FJ (1994) *Multivariate Statistical Methods, A Primer*, 2nd edn. Chapman & Hall, London.

Maynard Smith J, Smith NH, O'Rourke M, Spratt BG (1993) How clonal are bacteria? *Proceedings of the National Academy of Sciences of the USA*, **90**, 4384–4388.

Meijer G, Leuchtmann A (1999) Multistrain infections of the grass *Brachypodium sylvaticum* by its fungal endophyte *Epichloë sylvatica*. *New Phytologist*, **141**, 355–368.

Moon CD, Tapper BA, Scott B (1999) Identification of *Epichloë* endophytes in plants by a microsatellite-based PCR fingerprinting assay with automated analyisis. *Applied and Environmental Microbiology*, **65**, 1268–1279.

Nei M, Kumar S (2000) *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.

Pimentel G, Peever TL, Carris LM (2000) Genetic variation among natural populations of *Tilletia controversa* and *T.bromi*. *Phytopathology*, **90**, 376–383.

Rohlf FJ (1993) *NTSYS-PC Numerical Taxonomy and Multivariable Analysis System, Version 1.8*. Exeter Publications, Setauket, NY.

Ruemmele BA, Brilman LA, Huff DR (1995) Fine fescue germplasm diversity and vulnerability. *Crop Science*, **35**, 313–316.

Saha DC, Johnson-Cicalese JM, Halisky PM, van Heemstra MI, Funk CR (1987) Occurrence and significance of endophytic fungi in fine fescues. *Plant Disease*, **71**, 1021–1024.

Saint Yves A (1930) Aperçu sur la distribution géographique des *Festuca* (Subgen. Eu-Festuca). *Candollea*, **4**, 146–165.

Sampson K (1933) The systemic infection of grasses by *Epichloë typhina*. *Transactions of the British Mycological Society*, **18**, 30–47.

Schardl CL (1996) *Epichloë* species: fungal symbionts of grasses. *Annual Review of Phytopathology*, **34**, 109–130.

Schardl CL, Leuchtmann A, Tsai HF, Collett MA, Watt DM, Scott DB (1994) Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. *Genetics*, **136**, 1307–1317.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN, Version 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Siegel MR, Latch GCM, Bush LP *et al.* (1990) Fungal endophyte infected grasses: alkaloid accumulation and aphid response. *Journal of Chemical Ecology*, **16**, 3301–3315.

Sneath PHA, Sokal RR (1973) *The Principles and Practice of Numerical Classification*. W.H. Freeman, San Francisco.

Sun S, Clarke BB, Funk CR (1990) Effect of fertilizer and fungicide applications on choke expression and endophyte transmission in chewings fescue. In: *Proceedings of the International Symposium on Acremonium/Grass Interactions* (eds Quisemberry SS, Joost RE), pp. 62–66. Louisiana Agricultural Experiment Station, Baton Rouge.

Talbot NJ, Salch YP, Ma M, Hamer JE (1993) Karyotypic variation within clonal lineages of the rice blast fungus, *Magnaporthe grisea*. *Applied and Environmental Microbiology*, **59**, 585–593.

Tsai HF, Liu JS, Staben C *et al.* (1994) Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. *Proceedings of the National Academy of Sciences of the USA*, **91**, 2542–2546.

Vos P, Hogers R, Bleeker M *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Weir BS (1979) Inferences about linkage disequilibrium. *Biometrics*, **35**, 235–254.

White JF, Martin TI, Cabral D (1996) Endophyte–host associations in grasses. XXII. Conidia formation by *Acremonium* endophytes on the phylloplanes of *Agrostis hiemalis* and *Poa rigidifolia*. *Mycologia*, **88**, 174–178.

Yeh FC, Yang RC, Boyle T, Ye ZH, Mao JX (1997) *POPGENE, the User-Friendly Shareware for Population Genetic Analysis*. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.

Zabalgogeazcoa I, Vázquez de Aldana BR, García Criado B, García Ciudad A (1999) The infection of *Festuca rubra* by the fungal endophyte *Epichloë festucae* in Mediterranean permanent grasslands. *Grass and Forage Science*, **54**, 91–95.

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