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D. Rau · A. H. D. Brown · C. L. Brubaker · G. Attene · V. Balmas · E. Saba · R. Papa

Population genetic structure of *Pyrenophora teres* Drechs. the causal agent of net blotch in Sardinian landraces of barley (*Hordeum vulgare* L.)

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Abstract Monoconidial cultures of *Pyrenophora teres*, the causal agent of barley net blotch, were isolated from leaves collected from six populations of the barley landrace "S'orgiu sardu" growing in five agro-ecological areas of Sardinia, Italy, and genotyped using AFLPs. The 150 isolates were from lesions of either the "net form" (*P. teres* f. sp. *teres*) or the "spot form" (*P. teres* f. sp. *maculata*) of the disease. Of 121 AFLP markers, 42%, were polymorphic. Cluster analysis resolved the isolates into two strongly divergent groups (F_{ST} = 0.79), corresponding to the net (45% of the isolates) and the spot (55% of the isolates) forms (designated the *NFR* and *SFR* groups, respectively). The absence of intermediate genotypes and the low number of shared markers between the two groups indicated that hybridization between the two *formae* is rare or absent under the field condition of Sardinia. Five of the barley populations hosted both forms but in different proportions. The *SFR* populations were similar in overall polymorphism to the *NFR* populations. However, compared to the *SFR* form, the *NFR* occurred in all fields sampled and showed a higher population divergence (F_{ST} = 0.43 versus F_{ST} = 0.09 with all isolates; F_{ST} = 0.37 versus F_{ST} = 0.06 with clone corrected samples) probably due to a lower migration rate. AFLP fingerprints resolved 117 distinct genotypes among the 150 isolates sampled (78%), 87% in *SFR* and 68% in *NFR* isolates. Although the absolute numbers may be a function of the number of AFLP markers assayed, the relative difference suggests that clonality is more prevalent among the *NFR* isolates (with 11 of 46 haplotypes observed more than once), compared with *SFR* isolates (7 of 71 haplotypes). Both digenic and multilocus linkage disequilibrium analyses suggested that sexual reproduction occurs at significant levels within the *NFR* and *SFR* populations, and that the relative contribution of sexual and asexual reproduction varies among different environments.

Keywords Pathogen diversity · Migration · Linkage disequilibrium · Sexual reproduction · AFLP

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D. Rau (✉) · G. Attene · E. Saba
Dipartimento di Scienze Agronomiche e Genetica Vegetale Agraria, Università degli Studi di Sassari, Via E. de Nicola, Sassari, Italy
e-mail: attene@ssmain.uniss.it
Tel.: +39-079-229225
Fax: +39-079 229222

A. H. D. Brown · C. L. Brubaker
Centre for Plant Biodiversity Research, CSIRO Plant Industry, Canberra, ACT 2601, Australia

V. Balmas
Dipartimento di Protezione delle Piante, Università degli Studi di Sassari, Via E. de Nicola, Sassari, Italy

R. Papa
Dipartimento di Biotecnologie Agrarie ed Ambientali, Università degli Studi di Ancona, Via Brecce Bianche, Ancona, Italy

Introduction

The population genetic structure of each partner in a host plant-pathogen interaction affects the dynamics of their evolutionary relationship (Thrall and Burdon 1997). For crop plants, farmer decisions on varietal choice and crop husbandry act to determine the host genetic diversity on a vast geographic scale (McDonald et al. 1996). Thus, we usually know much about the genetic structure of the host crop. Whereas, far less is known about the genetic structure of any pathogen population.

DNA markers, such as RFLPs, AFLPs and SSRs, provide a means to redress this imbalance and to research pathogen genetic structure (Majer et al. 1996; Milgroom 1997; Taylor et al. 1999a), both in a natural system such as on *Microbotryum violaceum* (Delmotte et al. 1999; Bucheli et al. 2001) and in an agricultural system such as on *Botrytis cinerea* (Giraud et al. 1997), *Colletotrichum lindemunthianum* (Sicard et al. 1997a, b), *Magnaporthe grisea* (Kumar et al. 1999) and *Rhynchosporium secalis* (Burdon et al. 1994).

The utility of AFLP fingerprinting (Vos et al. 1995), in particular, has been demonstrated with several fungi (Rosendhal and Taylor 1997). AFLPs are reproducible and sample the genome widely, allowing discrimination among closely related individuals within a species (Majer et al. 1996; Mueller and Wolfenbarger 1999). Moreover, the high resolution of AFLP markers also enables testing for clonal identity among individuals and thus permits inferences about the contribution of sexual versus asexual modes of reproduction to the observed pattern of genetic variation (Rosendhal and Taylor 1997). This is an important factor in the coevolution of several host-pathogen relationships (Chen and McDonald 1996; Kumar et al. 1999). *Pyrenophora teres* Drechsler [anamorph: *Drechslera teres* (Sacc.) Shoemaker] is the causal agent of barley net blotch, a disease with a world-wide distribution that can cause substantial yield losses (Jordan et al. 1985; Steffenson et al. 1991). Several studies have documented high variability in the pathogenicity of the fungus (Harrabi and Kamel 1990; Tekauz 1990; Steffenson and Webster 1992). More recently, studies on molecular variation in *P. teres* have complemented the pathogenicity data. Peever and Milgroom (1994) used RAPD markers to assay the level and the distribution of genetic variation within and between populations from USA, Canada and Germany. On this scale, genetic diversity and divergence between populations (G_{ST} = 0.46) were high. Moreover, Peever and Milgroom (1994) deduced from the multilocus structure within populations of *P. teres* that sexual reproduction was frequent in four of the five populations analyzed. On the country scale, Peltonen et al. (1996) found a high level of RAPD variation within their collection of *P. teres* isolates from Finland. However, the sampling of isolates in different years and the small number of isolates from each location precluded inferences about the population structure of this variation.

Population genetic studies in *P. teres* must take into account that two morphologically similar intraspecific *formae* of the pathogen with different leaf symptoms may co-occur. The 'net' form (*P. teres* f. sp. *teres*) produces elongated, light brown lesions with dark brown necrotic reticulations, whereas the lesions of *P. teres* f. sp. *maculata* are ovoid, dark-brown and surrounded by a distinct chlorotic area (Smedegård-Petersen 1971). The two forms closely resemble each other morphologically. These closely related pathogens (http://www.bspp.org.uk/mppol/1998/1111stevens) can be hybridised to produce lesions that are morphologically intermediate between the "spot" and "net" forms, although it is not clear if this occurs naturally (Campbell et al. 1999). Even in the absence of intermediates, Williams et al. (2001) found that AFLP assays distinguished between isolates of *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* more reliably than leaf symptoms.

In this study, 150 isolates from infected leaves sampled from six different fields of a barley landrace growing in Sardinia island, Italy (Attene et al. 1996; Papa et al. 1998), were collected in 2000 and genotyped using AFLPs. The collection included isolates from lesions classified as either the spot form (*P. teres* f. sp. *maculata*) or the net form (*P. teres* f. sp. *teres*) of the fungus. The isolate genotypes were used to determine: (1) the genetic relationship between the two forms of the pathogen, (2) the comparative levels and patterns of genetic variation in both forms, and (3) the degree of multilocus structure and the contribution of sexual *versus* asexual reproduction in *P. teres* populations infecting heterogeneous barley landraces in Sardinia.

Materials and methods

Sampling and isolation of P. teres

P. teres infected-leaves (4th leaf to heading stage) were collected between January and April 2000 from six fields of the barley landrace "S'orgiu sardu" growing in five agro-ecological areas of Sardinia (Attene et al. 1996; Table 1). The fields were of similar area (2–3 hectares), and the sampling procedure was the same in each field. Single monoconidial isolates were obtained, each from a single leaf collected from randomly selected, non-contiguous plants (far apart about 10 m) along the diagonals of the field. Surface-sterilized leaf sections, each with a single lesion, were placed on 12 g/l of water agar (Technical No. 3, Oxoid) and incubated at 20–22 °C for 3–7 days (12 h light; 12 h dark). Single conidia were transferred to 39 g/l PDA plates (Potato Dextrose Agar, Oxoid) and incubated at 20 °C for 7 days before DNA extraction. For long-term storage, isolates were maintained at room temperature in 44-ml tubes on PDA (39 g/l) covered with mineral oil.

Each isolate is designated as *P. teres* f. sp. *teres* (net form) or *P. teres* f. sp. *maculata* (spot form) based on the morphology of the originating lesion following Smedegård-Petersen (1971). *P. teres* isolates from the United States, Canada, Germany, China and the Italian Peninsula, and isolates of three other barley pathogens, *Pyrenophora graminea* Ito e Kuribay. [anamorph: *Drechslera graminea* (Rabenh. ex Schlecht.) Shoemaker], *Cochliobolus sativus* Ito e Kuribay. [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoemaker] and *Rhynchosporium secalis* (Oud.) Davis, were included for comparison (Table 2).

Inoculation assays for seedlings and detached leaves

Inoculation tests were performed using 12 of the putatively misclassified isolates (see below) (SEC 3N, 8N, 11N, 26N, 34N, 51N, 56N, SIR 5N, 17bN, 16N, PIR 7S, TER 11S) and two American reference samples (PT-AB-91-23A, donor: Prof. M.G. Milgroom; ND89-12, donor: Prof. B.J. Steffenson) to confirm their phenotypes. Three different barley genotypes were used; a line extracted from a Sardinian barley landrace (Erdas), a commercial variety (Aliseo) and a universal susceptible genotype to *P. teres* f. sp. *teres* (Hector). Two different inoculation methods were adopted (1) on barley plant at the 3rd leaf stage (Tekauz 1990) and (2) on detached leaves in a Petri dish (Afanasenko et al. 1995). For plant inoculation, isolates were grown on 39 g/l of PDA at 20 °C for 5 days. Cultures were then flooded with sterile water, and conidia and mycelia were collected, gently scraping the colony surface with a metal spatula. Inoculum, adjusted to 1,000 infective unit/ml (Jonsson et al. 1997), was then sprayed until runoff on barley plants. After inoculation, each plant was put in a plastic bag (Jonsson et al. 1997). For inoculation in Petri dish, segments (4–6 cm) of third seedling leaves were placed on 12 g/l of water agar (Technical No. 3, Oxoid). Small plugs (approximately 1 mm) from the PDA culture of the fungus were transferred aseptically and separately onto the sterilized (95% ethanol for 10 s, 1% NaOCl for 90 s) leaf segments in each Petri dish (Scott 1991). Plants and Petri dishes were then incubated for 48 h at 100% relative humidity, at

Table 1 Location, climate and cropping system of sites of the barley host populations for the *Pyrenophora teres* samples in Sardinia

Site[a]	Number	Latitude	Longitude	Elevation (m)	Annual rainfall[b] (mm)
1. SEC (Nurra)	31	40°44'18"N	8°27'39"E	74	577 ± 108
2. PIR (Sinis)	24	39°59'49"N	8°32'28"E	9	645 ± 153
3. TER (Ogliastra)	26	39°41'54"N	9°34'34"E	139	822 ± 291
4. BAC (Trexenta)	36	39°36'07"N	9°07'47"E	374	560 ± 151
5. SIR (Trexenta)	21	39°31'55"N	9°08'06"E	186	533 ± 124
6. SES (Campidano)	12	39°17'51"N	9°04'02"E	48	426 ± 127

[a] Cropping system: sites 2 and 6 only grain; remainder green forage and grain production
[b]Average and standard deviation over a period of 30 years (1961–1990) according to the Mondial Organization of Meteorology

Table 2 Source of isolates of *Pyrenophora teres*, *Pyrenophora graminea*, *Cochliobolus sativus* and *Rynchosporium secalis*

Taxon and *formae speciales* (where known)	Number of isolates	Origin	Collector or donator
P. teres – net and spot	150	Six barley landrace populations Sardinia, Italy	V. Balmas and D. Rau
P. teres	4	Alberta, Canada	M.G. Milgroom
P. teres	3	Saskatchewan, Canada	M.G. Milgroom
P. teres	2	Germany	M.G. Milgroom
P. teres	6[a]	Italy	G. Vannacci and A. Porta-Puglia
P. teres – net	2	North Dakota	B.J. Steffenson
P. teres – net	2	California	B.J. Steffenson
P. teres – net	1	China	B.J. Steffenson
P. graminea	1	Italy	N. Pecchioni
C. sativus	3	Italy	G. Vannacci
R. secalis	3	New South Wales, Australia	J.J. Burdon

[a]Except for these six isolates, which were from seeds, all others were obtained from barley leaves

20 °C in the dark. After this period, plastic bags were removed and both plates and Petri dishes were incubated at 20 °C (Tekauz 1990) (14 h dark, 10 h light) for 6 more days.

DNA extraction

Four mycelial plugs from 7-day old cultures grown on PDA were transferred to Petri dishes (five plates/isolate) containing 10 ml of 24 g/l PDB (Potato Dextrose Broth, Oxoid) amended with yeast extract (5 g/l), kanamycin (50 μg/ml) and streptomycin (10 μg/ml), and incubated for 5–7 days at 20–22 °C in the dark. Mycelia were centrifuged in 50-ml Falcon tubes (5,000 rpm, 10 min at 4 °C). The supernatant was decanted and replaced with 20 ml of sterile water followed by vigorous shaking for 20 s. The samples were re-centrifuged (5,000 rpm, 10 min at 4 °C), and the supernatant discarded (Peever and Milgroom 1994). The mycelia were stored at –80 °C for at least 12 h before lyophilising for 48–72 h. The desiccated mycelia were stored at –20 °C until the DNA was extracted following Migheli et al. (1996).

AFLP analysis

Amplified fragment length polymorphisms (AFLPs) were obtained using the Gibco BRL Micro-organism AFLP kit (Gibco BRL, Grand Island, N.Y.) following the manufacturers instructions. Two selective amplifications were performed using two *Eco*RI (E) primers with two selective nucleotides (E-AC and E-GC) and one *Mse*I (M) primer with one selective nucleotide (M-C). The AFLPs were resolved on 6% denaturing polyacrylamide gels run at 50 W using 1 × Tris-Taurine-EDTA (TTE) buffer (10.8 g Trizma base; 3.6 g Taurine; 0.2 g $Na_2EDTA \cdot 2H_20$), fixed [10% glacial acetic acid and 20% methanol (v/v)], oven dried at 65 °C from 3 hours to overnight, and exposed to BiomaxMR (Kodak) film 1–4 days. Negative controls for each PCR reaction were run simultaneously. Polymorphic bands of consistent intensity were scored as a single biallelic (present or absent) locus; polymorphic bands with variable intensities were omitted to prevent confounding two co-migrating loci as a single locus. In each gel, a reference set of six isolates, including both forms of the pathogen, were used to check for the repeatability of AFLP fragments across and within gels.

Statistical analysis

Single locus analysis

(1) A UPGMA topology of individual isolates using the Nei and Li (1979) distances estimator was generated using NTSYS-pc ver. 2.02i (Rohlf 1992).

(2) Diversity statistics for each group of populations, or single population, included the percentage of polymorphic loci and the average diversity over loci both using the unbiased Nei's gene diversity (Nei 1978) and Shannon's information index (Shannon and Weaver 1949; Hutcheson 1970). All were calculated using Arlequin software ver. 2.0 (Schneider et al. 1997) and PopGene ver. 1.31 (Yeh and Yang 1999).

(3) Divergence statistics were estimated using the hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992). The total variance was partitioned into three levels, namely individuals, populations and the two *formae speciales* using Arlequin software (Schneider et al. 1997). Wright's *F*-statistics (Wright 1951) were calculated separately for the spot-form related group and the net-form related group, and confidence intervals (C.I.) were calculated from a bootstrap over loci using TFPGA (Miller 1997). The jack-knife C.I. over populations was calculated as the average $F_{ST} \pm 3\sigma$ where σ is the standard deviation between replicates from sequential elimination of populations (C.I. $\approx$ 99.73%). F_{ST} standard deviations were calculated by jack-knifing over loci using TFPGA (Miller 1997). The difference of the average F_{ST} values in the two groups was tested using the Wilcoxon non-parametric test for all possible pair-wise F_{ST} between populations within each group using JMP software ver. 3.1.5 (SAS Institute 1995).

(4) Genetic distances between populations were also calculated using Nei's unbiased genetic distance (Nei 1978) and used to infer a UPGMA topology using TFPGA (Miller 1997). A dendrogram was drawn from the pair-wise (11 × 11) Nei's unbiased distance matrix (Nei 1978) using the UPGMA clustering method implemented in TFPGA (Miller 1997). To infer the relative strength of the nodes produced by UPGMA analysis, we calculated: (1) the proportion of permuted data sets that resulted in the formation of each node seen in the original dataset when new data sets were constructed by resampling with replacement over loci, and (2) the number of loci that individually support the topology of the tree defined by the original combined data ("Consistency Indices").

(5) The correlation between the Nei's genetic distance matrix and a geographic distance matrix was tested (Mantel 1967; Sokal 1979) using TFPGA software with 10,000 permutations and log transformation of the geographic distances.

Multilocus analysis

Five of the *P. teres* populations were of sufficient size for this analysis.

(1) The exact test of digenic linkage disequilibrium for haploid data, which is an extension of Fisher's exact test for contingency tables (Slatkin 1994), was applied using Arlequin software ver. 2.0 (Schneider et al. 1997).

(2) The genotypic diversity within each *P. teres* populations was evaluated using the Shannon diversity index (Shannon and Weaver 1949; Hutcheson 1970). To correct for differences in sample size, I indices were normalized as $I_{nor} = I/\ln(n)$ (Liu et al. 1996; Kumar et al. 1999).

(3) The level of clonality versus sexual reproduction in different *P. teres* populations was measured using the index of association (I_A) test (Brown et al. 1980; Maynard Smith et al. 1993). The index I_A is a multilocus summary statistic of association between loci. The observed value of I_A, its simulated sampling distribution and the significance of its deviation from zero, was obtained using MultiLocus ver. 1.2 software (http://www.bio.ic.ac.uk/evolve/software/multilocus/), coded by P.M Agapov and A. Burt. In order to have a more useful statistic than I_A as a relative measure of panmixis, another measure of multilocus linkage disequilibrium (r_d) was calculated using MultiLocus ver. 1.2 software. This statistic increases monotonically with I_A but its expectation is largely independent of the number of loci analyzed (Burt et al. 1999).

To assess the effect of clonality on our estimates of population genetic variation, we compared the results of two analyses: one using all individuals and one in which each distinct multilocus genotype was represented only once (Maynard Smith et al. 1993; Chen and McDonald 1996; Xu et al. 1999).

Results

Of the 150 isolates 85 (57%) were obtained from "net"-form lesions (*P. teres* f. sp. *teres*) and 65 (43%) originated from "spot"-form lesions (*P. teres* f. sp. *maculata*). Five barley populations hosted both forms, whereas the BAC population hosted only isolates classified as *P. teres* f. sp. *teres*.

Single locus diversity

Polymorphisms were clear-cut and readily scored and the two primer combinations yielded 332 markers (154 from E-GC/M-C and 178 from E-AC/M-C). No bands were monomorphic across the four sampled species (*P. teres*, *P. graminea*, *C. sativus*, *R. secalis*). Omitting the *R. secalis* samples, only ten (4.2%) of the 236 remaining bands were monomorphic among the three other species. Between the two *Pyrenophora* spp., 52 (35%) of the 149 *Pyrenophora* bands were monomorphic. Within the total widespread sample of *P. teres* isolates, 64 of the 121 polymorphic markers were common to all isolates (26 of 55 from E-GC/M-C and 31 of 66 from E-AC/M-C or a total of 47% were polymorphic). Remarkably, the percentage of polymorphic bands among the Sardinian isolates (51 out of 121 bands; 42%) was only slightly lower than in the total sample with the 20 reference isolates.

Cluster analysis showed that all the isolates classified as *P. teres* are clearly separated from *P. graminea*, *C. sativus* and *R. secalis* (Fig. 1). The analysis also showed that the *P. teres* isolates were much closer to *P. graminea* (average genetic distance = 0.22) than to *C. sativus* (average genetic distance = 0.80). Moreover, *C. sativus* was closer to *P. teres* than either species was to *R. secalis* (average genetic distance = 0.91).

Within the *P. teres* species cluster, clear intra-specific variation is evident with two strongly defined clusters. The first cluster (Cl.1) comprises 56 Sardinian isolates classified by lesion morphology as the spot form (*P.t.* f. sp. *maculata*), (26 from Sardinia) classified as the net form (*P. t.* f. sp. *teres*), and three isolates one from Canada-Alberta and two from the Italian Peninsula of undetermined form (Fig. 1). Eighteen of the 27 (67%) net form isolates in this cluster were from the SEC site (Fig. 1). In contrast, the second cluster (Cl.2) comprises 59 Sardinian isolates and five reference samples (four from the USA and one from China) classified as the net form, nine Sardinian isolates classified as the spot form and twelve undetermined isolates (three from Alberta, Canada, three from Saskatchewan, Canada, two from Germany and four from the Italian Peninsula) (Fig. 1). The two clusters differ from each other in the proportion of spot-form and net-form Sardinian isolates (contingency $\chi^2 = 45.9$, $df = 1$, $P < 0.0001$). The controlled inoculation assays for seedlings and detached leaves of ten *SFR* isolates from Net-type lesions (SEC 3N, 8N, 11N, 26N, 34N, 51N, 56N, SIR 5N, 17bN, 16N) and with two *NFR* isolates from Spot-type lesions (PIR 7S, TER 11S), found that isolates produced symptoms in accord with their AFLP classification. Both American reference samples (PT-AB-91-23A and ND 89-12) produce net-type symptoms.

Therefore, we conclude that the Cl.1 cluster is a spot-form related cluster (*SFR*), whereas Cl.2, with the five known net isolates, is a net-form related cluster (*NFR*) (Fig. 1).

Table 3 contains the summary statistics for genetic diversity. Within *SFR* the percentage of polymorphic bands was slightly lower than in *NFR* (27% versus 33% respectively). The percentage of polymorphic bands in the *NFR* (33%) was similar to that found among the reference isolates that fitted into the same cluster (33%). The diversity index (H) calculated for all the Sardinian

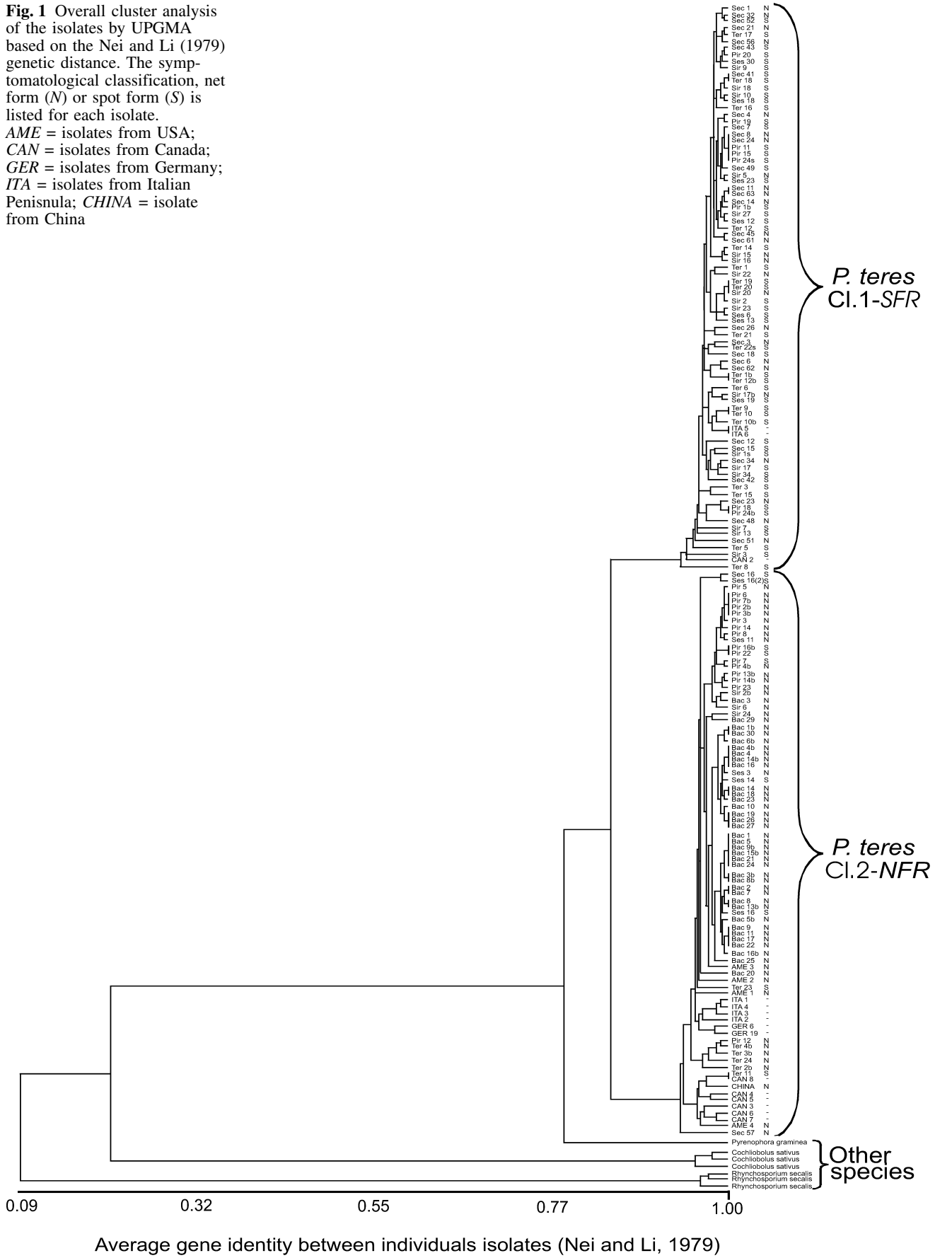

Fig. 1 Overall cluster analysis of the isolates by UPGMA based on the Nei and Li (1979) genetic distance. The symptomatological classification, net form (*N*) or spot form (*S*) is listed for each isolate. *AME* = isolates from USA; *CAN* = isolates from Canada; *GER* = isolates from Germany; *ITA* = isolates from Italian Penisnula; *CHINA* = isolate from China

Table 3 Overall polymorphism and gene diversity for AFLPs in the two *Pyrenophora teres formae speciales* for the Sardinian and reference samples

Isolates	Number of		Number of polymorphic markers (%)	Average gene diversity (*H*)		Shannon index (*I*)[b]
	Isolates	Haplotypes		Isolates	Haplotypes	
Sardinian *SFR*	82	71	33 (27%)	0.045 ± 0.025	0.047 ± 0.025	0.077
Sardinian *NFR*	68	46	40 (33%)	0.046 ± 0.025	0.054 ± 0.029	0.077
All Sardinian	150	117	51 (42%)1	0.136 ± 0.067	0.150 ± 0.072	0.200
Reference SFR	3	–[a]	–[a]	–[a]	–[a]	–[a]
Reference NFR	18	18	40 (33%)	0.083 ± 0.042	0.083 ± 0.042	0.133

[a] Descriptive statistics for *SFR* reference isolates were not calculated because the sample size was too small
[b] Shannon index was calculated only for samples with all isolates

Table 4 Diversity estimates of *Pyrenophora teres SFR* and *NFR* isolates by population

Population	Form	Number of		Polymorphic markers (%)	Average gene diversity (*H*)		Shannon index (*I*)[a]
		Isolates	Haplotypes		Individuals	Haplotypes	
SEC	*SFR*	29	27	22 (19)	0.041 ± 0.023	0.042 ± 0.024	0.065
PIR	*SFR*	8	5	7 (6)	0.022 ± 0.015	0.026 ± 0.019	0.030
TER	*SFR*	20	17	24 (21)	0.056 ± 0.031	0.058 ± 0.032	0.086
SIR	*SFR*	18	18	19 (17)	0.045 ± 0.025	0.045 ± 0.025	0.067
BAC	*SFR*	0	0	–	–	–	–
SES	*SFR*	7	7	10 (9)	0.031 ± 0.020	0.031 ± 0.020	0.041
SEC	*NFR*	2	2	9 (8)	0.074 ± 0.078	0.074 ± 0.078	0.052
PIR	*NFR*	16	12	14 (12)	0.025 ± 0.016	0.030 ± 0.019	0.042
TER	*NFR*	6	6	18 (16)	0.058 ± 0.037	0.058 ± 0.037	0.078
SIR	*NFR*	3	3	9 (8)	0.050 ± 0.040	0.050 ± 0.040	0.047
BAC	*NFR*	36	18	16 (14)	0.021 ± 0.015	0.031 ± 0.019	0.039
SES	*NFR*	5	5	11 (9)	0.040 ± 0.027	0.040 ± 0.027	0.048

[a] Shannon index was calculated only for samples with all isolates

Table 5 Difference in the genetic structure of Sardinian *Pyrenophora teres SFR* and *NFR* populations. F_{ST} standard deviations are calculated by jack-knifing over loci; lower and upper limits (in brackets) of the confidence intervals are for and from bootstrap (99%) over loci, and jack-knife (99.73%) over populations (10,000 replications), respectively

Population		F_{ST} values	C.I. (Bootstrap over loci)	C.I. (Jack-knife over populations)
NFR	Isolates	0.43 ± 0.08	(0.25 to 0.62)	(0.32 to 0.56)
	Haplotypes	0.37 ± 0.06	(0.21 to 0.52)	(0.26 to 0.47)
SFR	Isolates	0.09 ± 0.02	(0.03 to 0.14)	(0.01 to 0.16)
	Haplotypes	0.06 ± 0.02	(0.02 to 0.11)	(–0.01 to 0.14)

isolates fitting in the *SFR* or in the *NFR* were similar (H = 0.045 and H = 0.046 respectively). If clone-corrected samples were considered, *NFR* has gene diversity slightly higher than *SFR* (H = 0.054 versus H = 0.047) but the difference is still not significant. The diversity index calculated for the *NFR* (H = 0.045) is about half that in the group of 18 samples from different countries that fitted in the same group (H = 0.083), but the difference is not significant. When Shannon's diversity indices are considered, the results are consistent.

Comparison among populations

Splitting the Sardinian isolates into different groups according to the source field and AFLP cluster results in 11 different samples (5 in the *SFR* and 6 in the *NFR*), which we hereafter treat as different populations. Table 4 lists the diversity statistics estimated at the population level. The diversity index (H) ranged between 0.021 (BAC-*NFR*) and 0.074 (SEC-*NFR*) in the Cl.2-*NFR*, and from 0.022 (PIR-*SFR*) to 0.056 (TER-*SFR*) in the *SFR*. If clone-corrected samples were used, the ranking of populations as to their gene diversity was substantially unaffected (Table 4). The correlation (Spearman rank) between the diversity indices for the two forms was positive but not significant, both using all isolates (r = 0.71; P = 0.18; n = 5) and clone-corrected samples (r = 0.66; P = 0.22; n = 5). Differences among populations for the average level of gene diversity were not significant because of the magnitude of their standard errors.

The molecular analysis of variance (AMOVA) showed that all the F_{ST} values were significantly different from zero ($P \leq 0.001$). Moreover, genetic divergence between populations was marked within *NFR* (F_{ST} = 0.43), whereas it was low within *SFR* (F_{ST} = 0.09). To test

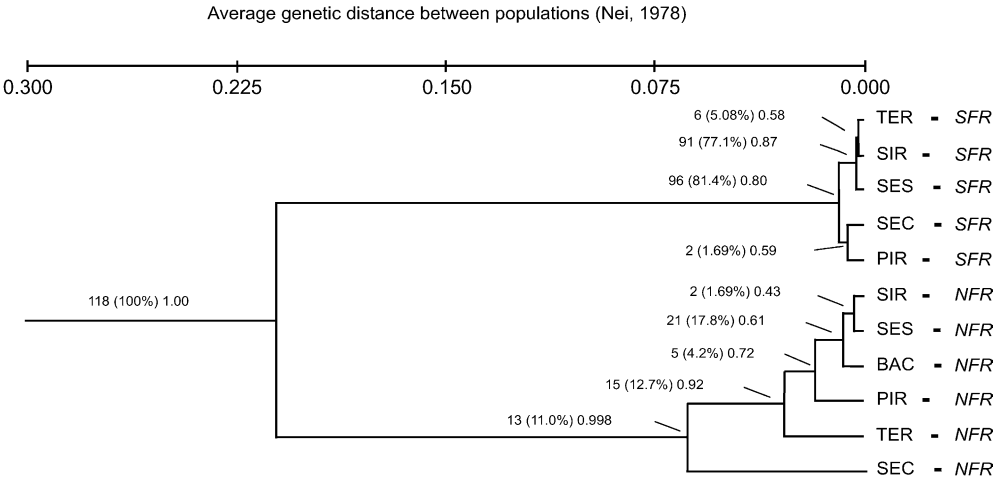

Fig. 2 Relationship between Sardinian *SFR* and *NFR* populations using samples with all individuals, based on Nei's genetic distance. For each node are reported the number and (percentage) of loci individually supporting the topology of the tree and the proportion of permuted datasets that resulted in the formation of that node

Table 6 Number and percentage of significant ($P \leq 0.05$) pairwise linkage disequilibria found with the Fisher exact test in Sardinian *Pyrenophora teres NFR* and *SFR* groups, and some Sardinian *Pyrenophora teres* populations, with sufficient sample size using all polymorphic markers. Values were calculated both using all individuals and clone-corrected samples

Form	Population	Number of individuals and (haplotypes)	Number of comparisons	Number and (percentage) of pairwise linkage disequilibria	
				Individuals	Haplotypes
SFR	SEC	29 (27)	231	26 (11)	33 (14)
SFR	TER	20 (17)	276	29 (11)	29 (11)
SFR	SIR	18 (18)	171	19 (11)	19 (11)
NFR	PIR	16 (12)	91	6 (7)	12 (13)
NFR	BAC	36 (18)	120	17 (14)	10 (8)
Sardinian *SFR*		82 (74)	528	92 (17)	63 (12)
Sardinian *NFR*		68 (46)	780	275 (35)	294 (38)

whether the F_{ST} value between *NFR* populations is significantly higher than that calculated for *SFR* populations, both bootstrap over loci and jack-knife resampling over populations were performed (Table 5). The F_{ST} values differed for both tests ($P < 0.01$). The Wilcoxon non-parametric test of all pairwise F_{ST} values also found the two groups to differ ($P = 0.0027$). The average F_{ST} values in clone-corrected samples decreased both between the *NFR* ($F_{ST} = 0.37$) and *SFR* ($F_{ST} = 0.06$) populations (Table 5), but that for the *NFR* population is still significantly higher than for the *SFR* populations (Table 5). Also in this case, the result of the Wilcoxon non-parametric test indicates greater divergence between *NFR* populations than between *SFR* populations ($P = 0.0013$).

The relationship between Sardinian populations based on Nei's genetic distances (Nei 1978) using samples comprising all the individuals is shown in Fig. 2. The higher divergence between *NFR* populations than between *SFR* populations and a North-South gradient within the *NFR* group is evident in the dendrogram. The correlation between geographic and genetic distances (Nei 1978) was significant ($r = 0.69$, Mantel test $P = 0.022$) for Sardinian populations belonging to the *NFR*, but not for those belonging to *SFR* ($r = 0.41$, Mantel test $P = 0.15$).

Digenic linkage disequilibria

The proportion of significant linkage disequilibria is higher in a composite sample of all six Sardinian *NFR* populations than it is in the sample built using all five *SFR* populations (35% versus 17% using all the isolates; 38% versus 12% using clone-corrected samples) (Table 6). One possible reason is the different population structures within the two main groups (i.e. a greater effect of population admixture for *NFR* because of more divergence between *NFR* populations than between *SFR* populations).

Indeed, within populations in each group, similar low levels of linkage disequilibrium were found. At the population level, estimates of linkage disequilibrium were made only for the larger sample sizes (SEC-*SFR*, TER-*SFR*, SIR-*SFR*, PIR-*NFR*, BAC-*NFR*). Considering all the isolates in each population, the level of linkage disequilibrium ranged from 7% to 14% in the *NFR* populations and was 11% in all three *SFR* populations (Table 6). Apart from the PIR-*NFR* and BAC-*NFR* population, the clone-corrected samples showed essentially similar estimates of linkage disequilibrium.

Table 7 Multilocus statistics for five *Pyrenophora teres* Sardinian populations with larger sample size both considering all isolates and clone-corrected samples. I_A = index of multilocus association (Brown et al. 1980). r_d = index of multilocus association (Burt et al. 1999). I_{nor} = normalized Shannon-Weaver information index (Liu et al. 1996; Kumar et al. 1999).

Form	Population	Distribution of multilocus genotype frequencies								Ratio Hap./Ind.	I_{nor}	Indices of multilocus association (I_A)			
		N	1	2	3	4	5	6	Number of genotypes			All isolates		Clone corrected	
												I_A	r_d	I_A	r_d
SFR	SEC	29	25	2	0	0	0	0	27	0.93	0.97	0.477**	0.024**	0.42*	0.021*
SFR	TER	20	14	3	0	0	0	0	17	0.85	0.93	0.526*	0.023*	0.48*	0.021*
SFR	SIR	18	18	0	0	0	0	0	18	1.00	1.00	0.269	0.016	0.269	0.016
NFR	PIR	16	10	1	0	1	0	0	12	0.75	0.84	0.466	0.037	0.285	0.022
NFR	BAC	36	9	5	1	2	0	1	18	0.50	0.75	0.471***	0.039***	0.230	0.017
Sardinian SFR		82	64	5	1	0	1	0	71	0.87	0.92				
Sardinian *NFR*		68	35	6	1	3	0	1	46	0.68	0.86				
Total		150	99	11	2	3	1	1	117	0.78	0.92				

Significance levels ***P<0.001; **P<0.01; *P<0.05

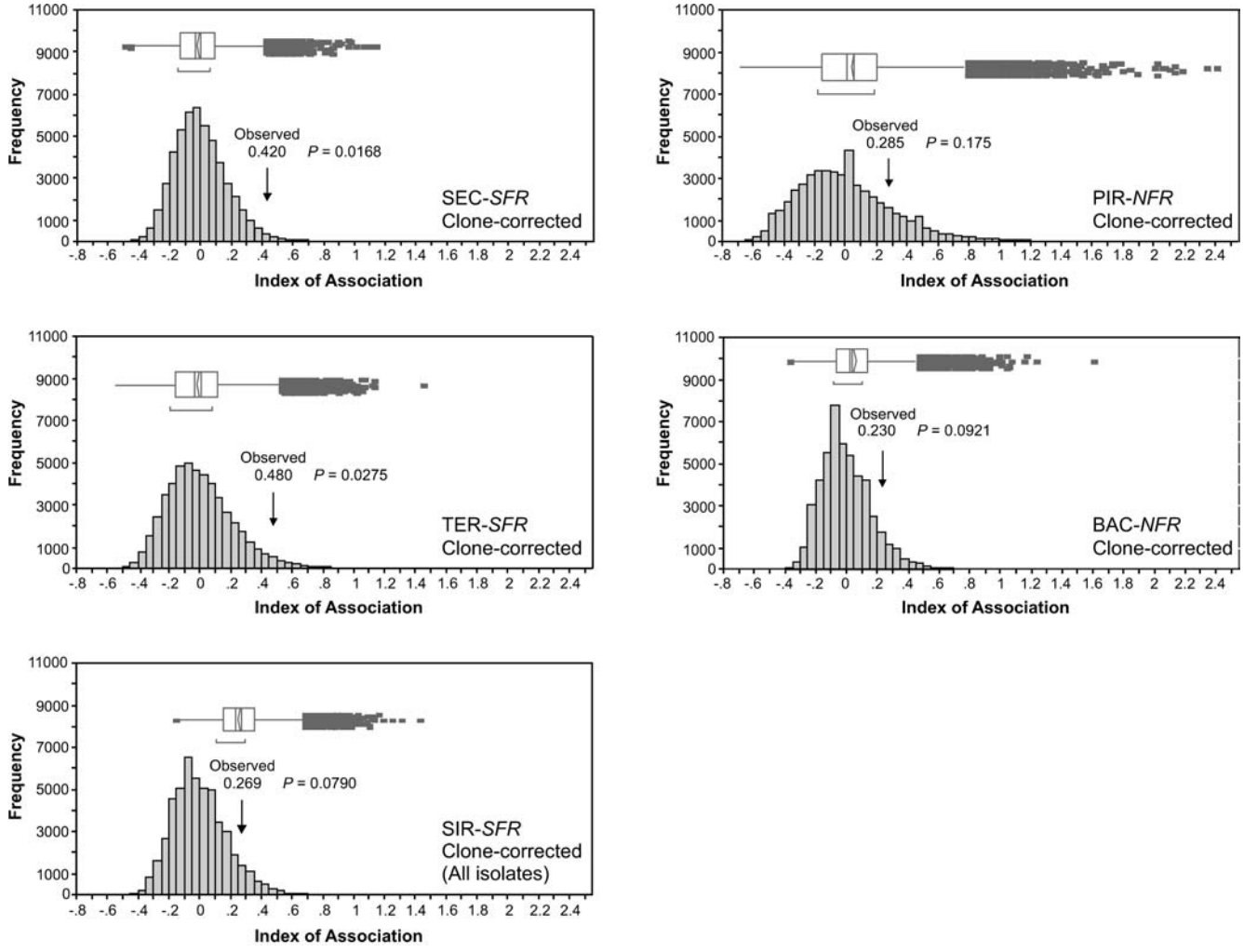

Fig. 3 Randomization tests comparing the observed values for the Index of Association (*arrows*) with datasets in which alleles in clone-corrected samples have been randomly shuffled across isolates for each locus separately (*histograms*). Histograms are based on 100,000 randomizations. Extreme values and outlier box plots (drawn from Mahalanobis distance) show more detail of sample distribution for each simulation. The end of the boxes are the 25th and the 75th quantiles, the mid line identifies the median sample value, and the end of the whiskers are the outer-most data points from their respective quartiles that fall within the distance computed as 1.5× the interquantile range. The bracket along the edge of the box identifies the shortest half, which is the densest half of the observations (JMP software, 1985–1999, SAS Institute, Inc.)

Multilocus analysis

Overall, considering all the Sardinian isolates classified as *P. teres*, 117 unique multilocus genotypes were identified in the sample of 150 individuals (Table 7) giving an average number of 1.3 individuals per haplotype. The number of individual isolates per genotype ranged from one to six in the *NFR* group and from one to five in the *SFR*. The sample of 68 individuals in the *NFR* group had 46 haplotypes whereas the 82 individuals in *SFR* had 71 haplotypes (Table 7).

Table 7 also gives the results of the multilocus analysis of the five populations with larger sample size. The number of multilocus genotypes per individual was lower in BAC-*NFR* (0.5) and PIR-*NFR* (0.75) populations than in TER-*SFR* (0.85), SEC-*SFR* (0.93), and SIR-*SFR* (1.00). The SIR-*SFR* population was the only one in which all individuals had a unique genotype. The sample-size-corrected Shannon's genotypic diversity (I_{nor}) follows the same pattern, being lower in BAC-*NFR* (0.75) and PIR-*NFR* (0.84) populations than in TER-*SFR* (0.93) SEC-*SFR* (0.97) and SIR-*SFR* (1.00).

The distribution of genotypes appears to differ in the two groups. In fact, no multilocus genotype was shared among *NFR* populations. In contrast, shared haplotypes were observed among the *SFR* populations. Indeed, one multilocus genotype occurred in SEC-*SFR* (two isolates) and PIR-*SFR* (three isolates) populations, a second genotype occurred in SEC-*SFR* (one isolate) and TER-*SFR* (one isolate), and a third genotype was found in TER-*SFR* (two isolates) and SIR-*SFR* (one isolate).

The observed values of the index of multilocus association, I_A, and their significance levels for the total samples and for clone-corrected samples are presented in Table 7. Figure 3 displays the simulated distributions in the clone-corrected analyses. In all cases, except the BAC-*NFR* population, the significance levels of I_A for the total samples are consistent with those for the clone-corrected samples. The I_A value was significantly different from zero in SEC-*SFR* and TER-*SFR* ($P < 0.01$ and $P < 0.05$ respectively), and not significantly different from zero in the PIR-*NFR* and SIR-*SFR* populations. In the BAC-*NFR* population the "test" with all the isolates was significant, but when clone-mates were eliminated, the random mating hypothesis could not be rejected using I_A. This reflects the observation that BAC-*NFR* population has the lowest proportion of individuals (25%) with unique genotypes (Table 7). Multilocus linkage disequilibria, r_d, were also estimated and, in clone- corrected samples, these values were similar (0.016 ÷ 0.022; Table 7).

Discussion

AFLP genotypes clearly differentiate between *P. teres* and the other three barley leaf pathogen species in this analysis, *P. graminea*, *C. sativus* and *R. secalis*. Additionally, intraspecific variation clearly resolves two clusters of *P. teres* isolates (Fig. 1). Given the presence in both clusters of isolates classified on a symptomatological basis as spot and net form, and the specific placement of known reference isolates from other researchers, we named these two main clusters as the Spot-Form Related cluster (*SFR*) and the Net-Form Related (*NFR*) cluster, reflecting the predominant form in each cluster. On this basis, and considering that the AFLP assay can be a better predictor of *forma specialis* than symptom expression (Williams et al. 2001), isolates belonging to the *NFR* and *SFR* clusters are tentatively reclassified as net or spot form, respectively.

The imperfect correlation between field diagnosis and AFLP classification may arise from misdiagnosis, or because genetic isolation (sexual or parasexual) between the net and spot forms of *P. teres* is not absolute. The joint occurrence of both forms in the field at five sites makes possible that both forms were growing in close proximity on the same leaf, and that the genotype(s) responsible for a particular lesion sampled in the field may not be the ones isolated and purified in vitro. The observation that the BAC population, where only the Net form was isolated, is the only population where the correlation between field diagnosis and the AFLP genotype cluster is fully congruent versus superficially supports this inference. A third explanation for the imperfect correlation between the AFLP cluster and the net versus spot phenotype, is that both phenotypes are present in each genetically isolated population.

Several studies have reported successful mating between the *P. teres* net and spot forms in the laboratory (Smedegård-Petersen 1971, 1976; Peever and Milgroom 1994; Campbell et al. 1999). Yet the frequency of the mating between the two forms in the field has not been established (Campbell et al. 1999). However, if hybridization was the explanation, we would expect that "misclassified" genotypes would not cluster tightly with the predominant conforming isolates in each group, but that segregants of various degrees of relationship would occur. Moreover, if the two AFLP clusters have recently diverged from an ancestral population that had both spot and net types, it is feasible that both AFLP clusters have maintained this particular polymorphism.

On the other hand, the controlled inoculation test of ten *SFR* isolates from Net-type lesions and with two *NFR* isolates from Spot-type lesions, found that isolates produced symptoms in accord with their AFLP classification. This result resembles that of Williams et al. (2001), who found that AFLP assays distinguished between isolates of *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* more reliably than did leaf symptoms.

The expectation of more polytomy would follow if the molecular polymorphisms actually predated the evolutionary divergence of the *formae speciales* under the 'lineage sorting' hypothesis. In our study, if relationships between all individuals are considered, the two forms of the pathogen appear strongly differentiated without any intermediate group (Fig. 1). The strong divergence between the two clusters in Sardinia indicates that

hybridization is very rare or absent in the field. Despite the success of artificial crosses, Campbell et al. (1999) reported the difficulty in obtaining viable progenies from matings of some *P. teres* strains, and suggested that one reason for strain incompatibility could be the heterothallic biallelic mating system of *P. teres* (McDonald 1963).

Misclassification could also arise from the fact that many host genotypes have different reactions to various pathogen isolates and therefore the morphology of the lesion is not determined entirely by the fungal genotype, but reflects an interaction between the host and the pathogen (Afanasenko et al. 1995). Our pathogen collection was made by sampling infected leaves from different populations (i.e. fields) of a Sardinian barley landrace. Previous studies showed the presence of significant genetic variation between and within populations of this landrace both for molecular and phenotypic traits (Papa et al. 1998). It is likely that genetic variation for host response against *P. teres* f. sp *maculata* and *P. teres* f. sp. *teres* in these landrace populations is similarly present. In the light of these potentially confounding processes, the agreement between provisional lesion diagnosis and the AFLP cluster is remarkable.

Despite the clear overall genetic discrimination evident in the cluster analysis between the two forms, only one band was "diagnostic", i.e. had one allele present in all Sardinian isolates in one group and absent from the other group. The usefulness of this band to discriminate between the *SFR* and the *NFR* groups is lost, however, when all the reference isolates are added into the analysis. This suggests that, despite the high divergence between the two forms, validation of molecular assays requires a large (and diversified) reference sample, as pointed out also by Williams et al. (2001).

Considering the *SFR* or *NFR* groups separately, the level of divergence found in our study merits consideration. Peever and Milgroom (1994) found a continent-scale G_{ST} value of 0.46 for samples of *P. teres* from America, Canada and Germany. Remarkably, the Sardinian *NFR* populations had a similar population differentiation (F_{ST} = 0.43) at much smaller scale. Peltonen et al. (1996), working with *P. teres* f. sp. *teres* populations collected in Finland, found that the average genetic identity between *P. teres* f. *teres* isolates was 0.63, a lower level than that observed in the Sardinian *NFR* group (Fig. 1). Moreover we also observed that the degree of differentiation is much stronger in the *NFR* group than in the *SFR* group (F_{ST} = 0.43 versus F_{ST} = 0.09 respectively) (Table 5 and Fig. 2). In order to explain this difference in divergence between populations of the two forms, the potential role of evolutionary forces such as selection, migration, drift, mutation and recombination require appraisal.

Selection

In general, AFLP markers are expected to be selectively neutral, so selection should not be an obvious factor here. On the other hand, barley genotypes can differ in their seedling reaction to individual pathotypes of *P. teres* f. *teres* and *P. teres* f. sp. *maculata*, and these reactions may change further in mature plants (Richter et al. 1998; Williams et al. 1999; Tekauz 2000). The genetic basis of resistance to the two pathogens is at least partially independent (Richter et al. 1998; Williams et al. 1999), which would allow different patterns of variation for resistance genes against the two forms of the pathogen to evolve in barley landrace populations.

In addition, as pointed out by Peever and Milgroom (1994), the role of alternate hosts in the epidemiology of the two *formae* could differentially affect pathogen variation structure. In artificial inoculation tests and field trials, *P. teres* f. sp. *teres* can infect many grasses from different genera and families (Brown et al. 1993), but comparable information on alternate hosts of the spot form is lacking.

Migration, drift and mutation

We found that the genetic divergence of *NFR* populations was significantly associated with geographic distance (r = 0.69; P = 0.022), whereas the correlation between these two variables was not significant in the *SFR* group. The correlation between genetic distance and geographic distance depends largely on the dispersal ability of an organism (McDermott and McDonald 1993). Under this hypothesis, more gene flow has occurred between populations of *P. teres* f. sp. *maculata* than between *P. teres* f. sp. *teres* populations. Wright (1951) showed that for neutral alleles, $F_{ST} = 1/(1 + 4Nm)$, where N is the local population size and m is the average rate of immigration in an "island" model of population structure. Correcting for haploidy, and the number of populations, the estimated migration rate for populations in the *NFR* and *SFR* group, respectively, was 0.11 and 1.01 individuals per generation, i.e. an order of magnitude difference. Interestingly, the multilocus data supported this difference in that shared haplotypes were found between *SFR* populations but not between *NFR* populations. Thus, in the absence of evidence for selection, the most-likely explanation for the higher genetic differentiation in the *NFR* group than in the *SFR* group is that genetic drift (and/or founder effect) combined with restricted migration play a more prominent role in *P. teres* f. sp. *teres* populations than in *P. teres* f. sp. *maculata* populations.

This result raises the question: why should the migration rate of the two forms differ? The simplest explanation is that the two forms have two different dispersal systems. Long-distance dispersal is possible by wind (Shipton 1973; Martin and Clough 1984) or by infested seed exchange among farms, whereas short distance is by air turbulence and water splashing (Deadman and Cooke 1989). Under this hypothesis, perhaps *P. teres* f. sp. *maculata* is better at infesting seeds or to be dispersed by wind than *P. teres* f. sp. *teres*. Further study

of dispersal in *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* is required to resolve this question.

Recombination

Since *P. teres* is known to have a sexual stage, recombination is likely to have a crucial role in shaping the population genetic structure within the two forms. Following the framework suggested by Milgroom (1996) we address two questions:

(1) *Is there evidence for recombination*? Tybarenc et al. (1991) point out that with multilocus genotypes, associations among alleles at different loci and over-represented genotypes provide evidence of clonality. The level of digenic linkage disequilibrium detected in these populations was low, ranging from 7% to 14%, as expected for a sexually reproducing organism. These disequilibrium values are similar to those found by Peever and Milgroom (1994) in five populations of net blotch.

AFLP fingerprints resolved 117 distinct haplotypes or multilocus genotypes among 150 isolates sampled. The *NFR* populations have a lower ratio of haplotypes to individuals, and less genotypic diversity than the *SFR* populations (Table 7). The high numbers of genotypes detected in both groups suggests that recombination plays a major role in both forms. Even so, the *NFR* group seems to have marginally more clonality than the *SFR* group. In both groups, no genotype was common to all populations. In the *NFR* group, the multiple occurrences of the same haplotype were confined to the one population, and isolates from different populations were always distinct. In contrast, in *SFR* populations, clonal occurrences of the same haplotype were fewer than in *NFR* populations, but there were instances of shared haplotypes between pairs of *SFR* populations in three cases. This contrast is in line with *SFR* populations showing greater migration.

It is interesting to compare these Sardinian estimates with those of Peever and Milgroom's (1994) North American and European populations obtained by random sampling leaves from commercial barley fields. In that study, the normalised Shannon Information index calculated from published multilocus genotypes frequencies, was noticeably lower (0.34 to 0.49) compared to values here (0.75 to 1). Assuming technical comparability, for example, in field-sampling procedures and in the ability to discriminate clone mates, such a marked difference could arise from many factors. The environments may favour clonality in one case compared with the other, or the pathogen populations from relatively uniform host varieties may contrast with those sampled from diverse landrace populations (Teshome et al. 2001). Indeed, the proportionate level of sexual recombination might be elevated in diverse populations.

If the presence of clones is a direct consequence of clonal reproduction, this does not rule out recombination in relationships among the different genotypes (Taylor et al. 1999a, b). For this reason, it is very important to apply statistical tests that can index the extent of clonality within fungal populations (Bucheli and Leuchtmann 1996). With genotypes made from several discrete loci (as in the case of our AFLP genotypes), this can be done by using a statistics test known as the Index of Association test (I_A test) originally developed for barley (Brown et al. 1980), adapted for microbes (Maynard Smith et al. 1993) and applied extensively in fungi (Taylor 1999a, b). We performed the I_A test on single populations, choosing those with larger sample sizes, and analyzed for association among loci both in the sample with all isolates and in the sample with clone-mates removed. This is a well-tested method for recognising "an epidemic structure" (Maynard-Smith 1993, 2000) and of distinguishing between the null hypothesis of recombination and the alternate hypothesis of clonality. We observed I_A (and r_d) values not significantly different from zero in two populations, SIR-*SFR* and PIR-*NFR* (in this latter case, both considering all individuals and the clone corrected sample). Thus, it appears that in SIR-*SFR* and PIR-*NFR* populations, recombination may occur regularly. In two other populations, SEC-*SFR* and TER-*SFR*, the I_A is significantly different from zero ($P < 0.01$ and $P < 0.05$ respectively). This was consistent between the two types of samples (all individuals versus clone corrected), and is suggestive of an asexual component. However, causes other than clonal reproduction can explain non-zero values of I_A, such as epistatic selection, population admixture, genetic drift and slow asymptotic decay of historic disequilibrium (Milgroom 1996).

In the BAC-*NFR* population, the sample size decreased from 36 to 18 when clone-mates were eliminated, and the random mating hypothesis could not be rejected using I_A. This could be indicative of an "epidemic" population structure, i.e. a population structure composed of two populations: a limited number of frequent closely related genotypes superimposed on a background population of relatively rare and unrelated genotypes recombining at high frequency (Maynard-Smith 2000). On the other hand, removing clone-mates from the sample may result in the loss of statistical power for the test (Lenski 1993). Repeating the I_A test on artificial samples of 18 isolates from the original sample (Kumar et al. 1999) showed that disequilibrium in the population can be detected reliably even at these lower sample sizes (data not shown). This confirms the validity of the statistic and suggests that departure from random mating in the BAC-*NFR* population is due to an epidemic structure of the BAC-*NFR* population, and is not an artefact of sample size.

Giraud et al. (1997) characterized 356 isolates of *Botrytis fuckeliana* with four RFLP and two retrotransposon-like elements. They observed two genetically isolated group of the fungus on *Vitis vinifera* with most pairs of loci in equilibrium supporting the hypothesis of two recombining populations. As in our case, how two sympatric species with similar life histories exist on the same host poses a puzzle.

(2) *How frequently does recombination occur*? This question is pertinent in intermediate situations where

evidence suggests some recombination but populations are not randomly mating (Milgroom 1996). To assess the frequency of recombination, we used the model derived by Burt et al. (1996) as an analogue of the infinite neutral allele model of single locus diversity in equilibrium under mutation and drift (Crow and Kimura 1970). Assuming that unique genotypes can be distinguished with AFLP markers, and that each new genotype arises from meiosis (not mutation), it is possible to formulate a relationship between clonal diversity under the opposing forces of recombination and random drift. At equilibrium, the number of sexually derived individuals (tN; where N is the census population size and t is the proportion of individuals that are derived by outcrossing) is related to the probability that two random isolates are clone-mates (f) as follows:

$$tN \approx (1-f)/2f,$$

where $f = \sum_i^n p_i^2$, p_i is the frequency of the ith multilocus genotypes and n is the number of multilocus genotypes.

This formula gives estimates of tN of 3.8 in the PIR-*NFR* and 5.6 in the BAC-*NFR* populations, and $tN = 12.2$ in SEC-*SFR* and 7.2 in TER-*SFR* populations. That is, relatively few sexually derived individuals per generation are needed to generate the observed diversity in these populations.

Overall, the AFLP genotypes indicate a significant level of sexual reproduction in Sardinia for both forms of *P. teres*. The relative importance of sexual versus asexual reproduction could vary both in *NFR* and *SFR* populations depending on the environment, and in some cases a genetic structure consistent with high clonal reproduction may occur.

Conclusion

The pattern of AFLP detected in *P. teres* populations infecting local barley landraces in Sardinia indicates that: (1) there is sufficient DNA divergence to distinguish between the two forms of the pathogen, presumed to be those causing the net or spot symptoms, (2) the two forms appear genetically isolated under the field conditions, (3) the *NFR* populations were more structured than *SFR* populations, probably due to their having a lower migration rate, and (4) in both the *NFR* and *SFR* groups, sexual and asexual reproduction occur and may have different relative importance depending on the location.

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