Isolation and characterization of the mating-type locus of the barley pathogen *Pyrenophora teres* and frequencies of mating-type idiomorphs within and among fungal populations collected from barley landraces

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**Abstract:** *Pyrenophora teres* f. sp. *teres* mating-type genes (*MAT-1*: 1190 bp; *MAT-2*: 1055 bp) have been identified. Their predicted proteins, measuring 379 and 333 amino acids, respectively, are similar to those of other *Pleosporales*, such as *Pleospora* sp., *Cochliobolus* sp., *Alternaria alternata*, *Leptosphaeria maculans*, and *Phaeosphaeria nodorum*. The structure of the *MAT* locus is discussed in comparison with those of other fungi. A mating-type PCR assay has also been developed; with this assay we have analyzed 150 isolates that were collected from 6 Sardinian barley landrace populations. Of these, 68 were *P. teres* f. sp. *teres* (net form; NF) and 82 were *P. teres* f. sp. *maculata* (spot form; SF). Within each mating type, the NF and SF amplification products were of the same length and were highly similar in sequence. The 2 mating types were present in both the NF and the SF populations at the field level, indicating that they have all maintained the potential for sexual reproduction. Despite the 2 forms being sympatric in 5 fields, no intermediate isolates were detected with amplified fragment length polymorphism (AFLP) analysis. These results suggest that the 2 forms are genetically isolated under the field conditions. In all of the samples of *P. teres*, the ratio of the 2 mating types was consistently in accord with the 1:1 null hypothesis. This ratio is expected when segregation distortion and clonal selection among mating types are absent or asexual reproduction is rare. Overall, sexual reproduction appears to be the major process that equalizes the frequencies of the 2 mating types within populations.

**Key words:** *Pyrenophora teres*, mating-types, AFLPs, sexual reproduction, selection, barley.

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**Résumé :** Les gènes déterminant le type sexuel (*MAT-1*: 1190 pb ; *MAT-2*: 1055 pb) chez le *Pyrenophora teres* f. sp. *teres* ont été identifiés. Les protéines *MAT* prédites, de 379 et de 333 acides aminés respectivement, sont semblables à celles des autres pléosporales telles que *Pleospora* sp., *Cochliobolus* sp., *Alternaria alternata*, *Leptosphaeria maculans* et *Phaeosphaeria nodorum*. La structure du locus *MAT* est comparée à celle chez d’autres champignons. Un test PCR pour le type sexuel a aussi été mis au point permettant d’analyser 150 isolats provenant de 6 populations de variétés de pays d’orge de la Sardaigne. De ce nombre, 68 était du *P. teres* f. sp. *teres* (forme réticulée, NF) et 82 du *P. teres* f. sp. *maculata* (forme maculée, SF). Au sein de chaque type sexuel, les amplicons NF et SF étaient de même taille et très semblables quant à leur séquence. Les 2 types sexuels étaient présents au sein des populations NF et SF à l’échelle du champ, ce qui signifie qu’elles ont maintenu leur capacité à se reproduire sexuellement. Malgré la sympatrie des 2 formes au sein de 5 champs, aucun isolat intermédiaire n’a été observé suite à une analyse AFLP (polymorphisme de longueur des fragments amplifiés). Ces résultats suggèrent que les 2 formes sont isolées génétiquement au champ. Chez tous les échantillons du *P. teres*, le ratio des 2 types sexuels était constamment conforme à l’hypothèse nulle (1:1). Ce ratio est attendu lorsqu’il n’y a pas de distorsion de la ségrégation ou de sélection clonale ou encore
Introduction

Net blotch is a barley disease with a worldwide distribution that can cause substantial yield losses (Jordan et al. 1985; Steffenson et al. 1991). The causal agent is the fungus Pyrenophora teres Drechsler (anamorph: Drechslera teres (Sacc.) Shoemaker). To improve strategies of resistance breeding and to determine the correct management of resistance genes for this pathogen, a better understanding of the evolutionary potential of the pathogen population would be useful (McDonald and Linde 2002). In this regard, it would be of particular interest to study fungal populations from genetically variable barley landraces, that is, pathogen populations that are partners in a scenario of interactions with the host in a dynamic potentially coevolving relationship. In this context, sexual reproduction is of fundamental importance in fungal pathogens, because it allows the generation of genotypic diversity that can adapt to the shifting genetic makeup of host populations.

In ascomycetes, a single regulatory locus, referred to as the mating-type (MAT) locus (Kronstad and Staben 1997; Turgeon 1998), determines cross compatibility. This locus is structurally unusual, because the 2 alternate forms (alleles) encode different transcription factors and consist of completely unrelated sequences, even though they occupy the same chromosomal position (Kronstad and Staben 1997). Metzenberg and Glass (1990) proposed that such unorthodox alleles be named idiomorphs. In heterothallic fungi, the sexual cycle is initiated only when 2 fungal strains of different idiomorphs interact, after each detects the other through the mating-type (Kronstad and Staben 1997).

Despite the lack of significant homology between the 2 idiomorphs, each locus is flanked by (or contains) conserved sequences (Metzenberg and Glass 1990). This allows the use of PCR-based cloning approaches for the analysis of MAT genes (Arie et al. 1997) and the design of idiomorph-specific primers for each species (e.g., Marra and Milgroom 1999; Steenkamp et al. 2000; Cozijnssen and Howlett 2003). MAT genes have thus been studied to determine their molecular structures (Turgeon et al. 1995), to resolve phylogenetic relationships among organisms (Turgeon 1998), and to understand evolutionary trends in fungal mating systems (Yun et al. 1999). MAT genes have been identified and or characterized in an increasing number of ascomycetes (Wirsel et al. 1998; Arie et al. 2000; Yun et al. 2000; Waalwijk et al. 2002; Bennet et al. 2003; Cozijnssen and Howlett 2003; Goodwin et al. 2003).

Mating-type surveys can be used to gauge the potential for sexual recombination. One way to conduct a mating-type survey is to test sexual compatibility with standard mating-type testers. Examples of this approach have been reported for Cochliobolus carbonum (Weltz and Leonard 1993), Magnaporthe grisea (Dayakar et al. 2000), Phytophthora infestans (Hammi et al. 2002), and Phaeosphaeria nodorum (Halama 2002). However, this approach is very time consuming, especially with P. teres. In P. teres, the mating procedure needs 7–15 months (Smedegård-Petersen 1978); therefore, an alternative indirect approach is needed to explore its population structure more efficiently. PCR-based determinations of mating types are reliable and less costly. For Cryphonectria parasitica, PCR-based markers for mating-type alleles correlate perfectly with the phenotypes of individual isolates (Marra and Milgroom 2001). More recently, Zhan et al. (2002) used this technique to study the distribution of mating-type idiomorphs in the wheat pathogen Mycosphaerella graminicola over spatial scales from lesions to continents; their frequency never differed significantly from a 1:1 ratio, as expected for unbiased meiotic segregation at a single locus.

Two morphologically similar intraspecific formae speciales of the net-blotch pathogen are known. The net form (NF) (P. teres f. sp. teres) produces elongated light-brown lesions with dark-brown necrotic reticulations, whereas the spot form (SF) (P. teres f. sp. maculata) produces ovoid dark-brown lesions that are surrounded by a distinct chlorotic area (Smedegård-Petersen 1971). Although these 2 forms can be hybridized in the laboratory (Smedegård-Petersen 1971, 1976; Campbell et al. 1999), whether or not mating occurs between them in the field is not clear. More recently, recombinant between the forms has been seen to occur under field conditions (Campbell et al. 2002). However, Williams et al. (2001) demonstrated that amplified fragment length polymorphism (AFLP) assays distinguished between isolates of P. teres f. sp. teres and P. teres f. sp. maculata. Similarly, Rau et al. (2003) showed that isolates from infected fields of heterogeneous barley landraces clearly split into 2 strongly defined clusters that correspond to the NF and the SF; no intermediate group was seen. Moreover, through both digenic and multilocus linkage disequilibrium (Iₐ test) analysis, Rau et al. (2003) found evidence for sexual reproduction in both the NF and SF populations, and showed that the relative contributions of sexual and asexual reproduction can vary. These observations are consistent with previous data collected on a continental scale (Peever and Milgroom 1994). Despite this indirect genetic evidence of regular cycles of sexual reproduction, the population genetic structure of the mating-type gene has not yet been studied for this fungus.

Our main objectives were to characterize MAT idiomorphs of P. teres and to develop PCR primers for easy mating-type detection. Moreover, because farmers still grow variable landraces of barley on the island of Sardinia (Attene et al. 1996; Papa et al. 1998), our second objective was to determine mating-type idiomorph distribution within the P. teres populations for both NF and SF isolates obtained from 6 populations of Sardinian barley landraces. Equal mating-
type frequencies would support previous tests of linkage disequilibrium (Rau et al. 2003) that indicated the prevalence of sexual reproduction in the pathogen.

**Materials and methods**

Isolation of MAT idiomorphs

**Fungal isolates and media**

The idiomorphs were obtained from the isolates 0-1 and 15A of *P. teres* f.sp. *teres* that were determined to be of opposite mating types by performing classical crossing experiments (Weiland et al. 1999). The *P. teres* strains were stored as dried mycelium at room temperature or in 30 % glycerol at –70 °C. For induction of conidiation, peanut leaf–oatmeal–agar medium was inoculated with a mycelium plug and incubated at 18 °C under near UV light (Philips TLD 36W/08) and white light (Philips TL 40W/33RS), 12 h photoperiod, for 1 week. For extraction of DNA, mycelium was obtained from cultures grown in complete medium in Erlenmeyer flasks on a rotary shaker at 150 rpm and 28 °C.

**DNA extraction, isolation, and manipulation**

Genomic DNA was extracted from *P. teres* mycelium using a DNA Isolation Kit (Genta Systems, Minneapolis, Minn.), in accordance with the instructions of the manufacturer. If not indicated otherwise, all molecular standard techniques were conducted according to Sambrook et al. (1989).

**Cloning strategy**

The strategy followed was essentially that proposed by Arie et al. (1997). As a starting point, genomic DNA of *P. teres* f.sp. *teres* isolates 0-1 and 15A was used for standard PCR amplification, with Pt5 and Pt7 primer pairs (Table 1), which identified the distal end of high-mobility group (HMG) 3′ and 5′ flanks, respectively (Fig. 1) (Arie et al. 1997). PCR amplification with Pt5 and Pt7 primers was successful only on isolate 0-1, suggesting that this isolate has the MAT-2 idiomorph. Additional flanking regions from the MAT-2 isolate 0-1 were obtained using thermal asymmetric interlaced (TAIL) PCR-mediated genomic walking in both directions, using different combinations of specific and degenerated primers (Fig. 1 and Table 1) (Arie et al. 1997). We then moved from flanking regions (assumed to be conserved between the MAT-1 idiomorphs) into the MAT-1 idiomorph sequence by designing new specific primers for the conserved MAT-1 flanking regions (PtMat-fw and PtMAT_rev) (Table 1). The products obtained by PCR amplification of the genomic DNA of both 0-1 and 15A isolates were then sequenced (Fig. 1).

**PCR conditions**

Standard PCR was carried out using the Pt5 and Pt7 and PtMat_fw and PtMat_rev primer pairs (Table 1). These were performed in volumes of 50 µL, containing 1.5 mmol MgCl₂/L, 0.2 mmol each deoxynucleoside triphosphate (dNTP)/L, 150 pmol of each primer, 100 ng of genomic DNA, 1 unit of Taq polymerase (Eppendorf), and 1× PCR buffer (Eppendorf). The PCRs were initiated by denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min.

TAIL-PCR was performed according to Liu and Whittier (1995). A total of 4 PCRs, using different combinations of arbitrary degenerated primers (AD2 and AD4) with nested specific primers (1, 2, 3, and 4), were performed (Table 1) (see Results). All PCR primers were synthesized at Carl Roth GmbH & Co (Karlsruhe, Germany).

**Cloning, sequencing, and analysis of PCR products**

PCR products a and b, as well as the products of primers PtMatfw and PtMatrev from *P. teres* isolates 15A and 0-1, representing the idiomorph and flanking regions, were cloned in pGEM-T Vector System I (Promega, Madison, Wis.). Sequencing was supported by MWG Sequencing Service (MWG-Biotech, Ebersberg, Germany) and AGOWA Sequencing Service (AGOWA GmbH, Berlin, Germany).

Sequences were aligned using the ClustalW algorithm of ClustalX (Thompson et al. 1997). Blastn and Blastp (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1997) searches for nucleotide and peptide sequences, respectively, were completed against the National Center for Biotechnology Information (NCBI)/GenBank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). The search for open reading frames (ORFs) on MAT-1 and MAT-2 sequences was performed using the ORF Finder applet (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The identification of putative splicing sites and introns was also conducted by comparing our sequences with fungal mating-type genes that have already been characterized, deposited in the NCBI/GenBank database, and identified during Basic Local Alignment Search Tool (BLAST) searches. To determine the 3′ end of the idiomorphs (Bennet et al. 2003), sliding-window analysis was performed with a window size of 10 nucleotides, a step of 1 nucleotide, and using DnaSP ver. 4.00 (Rozas et al. 2004). For this analysis, MAT-1 and MAT-2 sequences were aligned and trimmed.

**Phylogenetic analysis**

Representative MAT peptide sequences (of the alpha and HMG-box regions) from different fungi available in GenBank were compared with *P. teres* MAT (Figs. 2 and 3). The sequences were aligned using the Profile Mode option (Goodwin and Zismann 2001; Goodwin et al. 2003) of ClustalX (Thompson et al. 1997).

Sequences from *Schizosaccharomyces pombe* were used as the outgroup. Trees were obtained using the neighbor-joining method (Goodwin et al. 2003). The consistency of each node of the trees was estimated using the bootstrap procedure (1000 replicates). ClustalX and neighbor-joining-plot (Perrière and Gouy 1996) software were used to construct and draw the trees, respectively.

**PCR idiomorph-specific assay**

The primers were designed to amplify both the *P. teres* MAT-1 and MAT-2 idiomorph coding regions. Primers MAT-1 forward (5′-AACAGACTCCTCGTACAACCCG-3′) and MAT-1 reverse (5′-TGACGATGCATAGTTTGTAAGGGTCTC TTC-3′) amplify a fragment of ~1300 bp in MAT-1 isolates (Fig. 4). Primers MAT-2 forward (5′-CAACTTTTCTCTACACGATTCACGCATATCC-3′) and MAT-2 reverse (5′-TGGGGCGACGCTATATTTTTTTTATTTC-3′).
Table 1. Primers used to clone the mating-type idiomorphs of *Pyrenophora teres*.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’→3’</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt5</td>
<td>AGCACCTTCGCCGTAA</td>
<td>Arie et al. 1997</td>
</tr>
<tr>
<td>Pt7</td>
<td>GGC(G)TGACCTGGAAGGGGTTTC</td>
<td>Arie et al. 1997</td>
</tr>
<tr>
<td>1</td>
<td>GTTC(G)CTGCTAGGGAACA</td>
<td>Present study</td>
</tr>
<tr>
<td>2</td>
<td>AGATGA(A)AAATTCGTAA</td>
<td>Present study</td>
</tr>
<tr>
<td>3</td>
<td>AGTCCGCTAAGGAGGAGCAT</td>
<td>Present study</td>
</tr>
<tr>
<td>4</td>
<td>CTTCGACCTCC(A)CTA</td>
<td>Present study</td>
</tr>
<tr>
<td>AD2</td>
<td>AGWGNAGWANCAWAGG</td>
<td>Liu and Whittier 1995</td>
</tr>
<tr>
<td>AD4</td>
<td>TCSTICGNACITWGGA</td>
<td>Liu and Whittier 1995</td>
</tr>
<tr>
<td>PtMat_fw</td>
<td>CAGGACGTTCGCCATCGCAT</td>
<td>Present study</td>
</tr>
<tr>
<td>PtMat_rev</td>
<td>TGTCAGTGTGAAAGCACCCTA</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Note: I, inositol.

\(^a\)Nucleotides N, S, W, according to IUPAC code (Cornish-Bowden 1985).

Fig. 1. Thermal asymmetric interlaced (TAIL)-PCR-mediated genomic walking to clone the *Pyrenophora teres MAT-2* idiomorph flanking regions, and the use of these regions to clone the *P. teres MAT-1* idiomorph. Arrows indicate the primers used (see Table 1 for the sequences). Box, stretch of *MAT-2* idiomorph detectable with the Pt5 and Pt7 primer pair; black, HMG box; gray, regions of the idiomorph flanking the HMG box. Primers 1, 2, 3, and 4 are defined in Table 1. \(a\) and \(b\) indicate the fragments amplified with TAIL-PCR.

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GCATAGTTCGTAC-3’ generate a fragment of ~1150 bp in *MAT-2* isolates (Fig. 4). These specific primers were used in PCR assays to determine mating types (*MAT-1* or *MAT-2*) in a collection of 150 isolates.

**Mating-type frequency survey**

**Collection of isolates**

Leaf samples infected with *P. teres* were collected from each of 6 fields of the barley landrace *S’orgiu sardu* (Attene et al. 1996; Papa et al. 1998), growing in 5 agro-ecological areas of Sardinia (see Table 1 of Rau et al. 2003). The fields were of similar size (2–4 ha) and the sampling strategy was the same in each field: leaves were collected along the diagonal (1 leaf per plant) from plants at least 10 m apart. Only 1 isolate per infected leaf was obtained, to a total of 150 monoconidial isolates. Each was designated as *P. teres* f. sp. *teres* (NF) or *P. teres* f. sp. *maculata* (SF) on the basis of the morphology of the originating lesion (following Smedegård-Petersen 1971), the AFLP analysis, and the controlled reinoculation tests on third-leaf seedlings and on detached leaves (Rau et al. 2003). Of these 150 monoconidial isolates, 68 were NF and 82 were SF. The isolates were labeled using a 3-letter code for each population (SEC, PIR, TER, SIR, BAC, and SES), followed by an identifier number (e.g., PIR 19). For further details about the collection, monoconidial culturing, DNA extraction, and reinoculation tests, see Rau et al. (2003). Three other barley pathogens were also in-
included in the analysis: Pyrenophora graminea Ito & Kuribay. (anamorph: Drechslera graminea (Rabenh. ex Schlecht.) Shoemaker), Cochliobolus sativus Ito & Kuribay. (anamorph: Bipolaris sorokiniana (Sacc.) Shoemaker) and Rhynchosporium secalis (Oud.) Davis.

**Mating-type amplification of field isolates**

PCR amplifications were performed independently for each specific primer pair. Each 20-µL PCR reaction contained 1× PCR buffer (Promega), 2.5 mmol MgCl2/L, 0.2 mmol of each dNTP/L (Promega), 1 µmol of each primer/L, 1 U Taq polymerase (Promega), and 20 ng genomic DNA. Samples of DNA were subjected to 94 °C for 5 min and 30 cycles of the following thermal profile: denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 90 s; this was followed by 72 °C for 10 min. All amplifications were performed in a PE-9700 thermal cycler (Perkin Elmer Corp., Wellesley, Massachusetts). The amplicons were separated by 1.2% agarose gel electrophoresis in 0.5× Tris–borate–EDTA (TBE) buffer (pH 8.0) at 100 V for 3 h.
Fig. 3. Neighbor-joining trees showing the relationships between *P. teres* and other Ascomycetes both for the *MAT-1* (alpha-box; A) and *MAT-2* (HMG-box; B) protein sequences. The consistency of each node is indicated by bootstrap values (>60%) (1000 replicates). Branch lengths are proportional to genetic distance. Fa, the family; Or, the order; Cl, class. Family codes: Le, Leptosphaeriaceae; Ph, Phaeosphaeriaceae; La, Lasiosphaeriaceae; S, Sordariaceae; N, Nectriaceae; D, Dermataceae; M, Mycosphaerellaceae; V, Valsaceae; Sc, Schizosaccharomycetaceae. Order code: Ple, Pleosporales; Sor, Sordariales; Hyp, Hypocreales; Hel, Helotiales; My, Mycosphaerellales; Dia, Diaporthales; Sch, Schizosaccharomycetales. Class code: X, Dothideomycetidae; O, Sordariomycetidae; +, Leotiomycetidae; §, mitosporic ascomycete. Classification on the basis of the Index Fungorum is available at: http://www.indexfungorum.org.
staining with ethidium bromide, the images were captured with a Polaroid camera (Kodak 667). To compare the lengths of the amplicons at higher resolution, a sample of 12 isolates (6 NF and 6 SF, comprising both mating types) was also tested on silver-stained denaturing polyacrylamide gels: 6% acrylamide-bisacrylamide (19:1), 8 mol urea/L in TBE buffer (pH 8.3), at constant power (80 W) for 1.5 h. To check for the reproducibility of the PCR reaction, the complete experiments were conducted twice.

Four amplicons, obtained from isolates PIR b2n and BAC 25N (NF with the MAT-1 and MAT-2 idiomorph, respectively) and SIR 4s and PIR 24s (SF with the MAT-1 and MAT-2 idiomorph, respectively) were purified with GFX PCR DNA and Gel Purification Kit (Amersham Biosciences), and directly sequenced with QiaGen Sequencing Service (Single Read Long). For each isolate, 2 sequences were obtained (forward and reverse), each of ~600–700 bp. Forward and reverse sequences were aligned using ClustalX (Thompson et al. 1997) and trimmed to obtain a final reliable fragment of 1158 bp for the MAT-1 sequence and 1068 bp for the MAT-2 sequence. The 4 sequences were deposited in GenBank with the following codes: AY950581 (PIR b2n), AY950583 (BAC 25n), AY950582 (SIR 4s), and AY950584 (PIR 24s). To confirm their identity, putative P. teres MAT-1 and MAT-2 idiomorph fragments were then subjected to queries using Blastn and Blastp (Altschul et al. 1997).

AFLP analysis

Field isolates were also genotyped using AFLPs (Vos et al. 1995), as described elsewhere (Rau et al. 2003). Rau et al. (2003) reported the results for 2 primer combinations, using 2 EcoRI (E) primers with 2 selective nucleotides (E-AC and E-GC) and 1 Msel (M) primer with a selective nucleotide (M-C). Our study extends the analysis, with data from 2 other primer combinations (E-AC/M-A and E-GC/M-A), to check whether the isolates studied by Rau et al. (2003) that shared the same multilocus genotype are true clones.

Fig. 4. Organization of MAT-1 (A) and MAT-2 (B) idiomorphs of P. teres. Boxes bordered by a thin black continuous line, idiomorphs; white underlined by arrow, coding regions; hatched, introns; gray, 5′ and 3′ noncoding ends. Thick black lines, near identical 3′ flanking regions. Braces, positions of the alpha and HMG boxes of the MAT-1 and MAT-2 idiomorphs, respectively. Short arrows, approximate annealing sites of primers for selective amplification of MAT idiomorphs. Primer names and sequences are given in the Materials and methods. White boxes bordered by a black interrupted line and underlined by an arrow represent the ORF1 stretches.

Statistical analysis

The sample sizes for each single collection field are similar to those of other studies (e.g., Table 3 in Douhan et al. (2002a); Table 4 in Douhan et al. (2002b); Table 4 in Linde et al. (2003); Table 4 in Zhan et al. (2002)). The application of the same statistical methods thus allows direct comparisons to be made. The null hypothesis of the 1:1 ratio of the 2 mating types for each forma specialis of the fungus, both at the regional level and within each field, was evaluated using the χ2 test (Douhan et al. 2002a, 2000b; Zhan et al. 2002; Linde et al. 2003). As it is still not clear whether the 2 formae can hybridize in nature (Campbell et al. 1999, 2002; Williams et al. 2001; Rau et al. 2003), we conducted tests pooling NF and SF isolates at both the regional and field levels. The χ2 values are measures of departure from the 1:1 expectation, but significance tests were performed only on sample sizes 210 (Lewontin and Felsenstein 1965; Douhan et al. 2002a). In addition, an exact binomial test for goodness-of-fit was performed. When sample sizes are small, the exact binomial test is more accurate than the χ2 test (Sokal and Rohlf 1995; Linde et al. 2003). Finally, we calculated the minimum mating-type ratio bias detectable for each given sample size, with a significance level of α = 0.05 and a power (1 – β) of 0.80. Approximating the binomial distribution to a normal distribution, we applied the approximate formula: \( n = \left( \frac{z_\alpha + z_\beta}{\mu_1 - \mu_0} \right)^2 \sigma^2 \), where \( n \) is the sample size, \( z_\alpha \) and \( z_\beta \) are the standardized \( z \) values for \( \alpha = 0.05 \) (1.64) and \( \beta = 0.20 \) (0.84); \( \mu_1 \) and \( \mu_0 \) are the means of success for the alternative and the null hypothesis, respectively; \( \sigma^2 \) is the variance calculated as \( p(1-p) \), where \( p = (\mu_1 + \mu_0) / 2 \). The similarities of mating-type frequencies among the 6 different fields, among the 2 NF populations, among the 3 SF populations, and among the 5 NF and SF populations with larger sample sizes were also evaluated, using the contingency \( \chi^2 \) test with JMP 3.1.5 software (SAS Institute 1995). Clone correction before the analysis of idiomorph frequencies (Douhan et al. 2002b; Zhan et al. 2002; Linde et al. 2003) was unnecessary, because all the isolates had dis-
tinct AFLP genotypes in all cases, except the SF isolates from PIR population, where only 2 individuals shared the same genotype (see Results).

Results

Isolation of MAT idiomorphs

Extending the HMG box flanks by TAIL-PCR

As expected (Arie et al. 1997), PCR amplification with the Ps5 and Ps7 primer pairs produced a fragment of 1611 bp of genomic DNA only on isolate 0-1. The first TAIL-PCR resulted in fragment a (Fig. 1). The PCR for the primary reaction was conducted using the specific primer1 in combination with arbitrary degenerated primer AD2, and the secondary reaction was conducted with the specific nested primer2 in combination with primer AD2. A second TAIL-PCR resulted in fragment b (Fig. 1). This was conducted for the primary reaction using the specific primer3 in combination with arbitrary degenerated primer AD4, and the secondary reaction was conducted with the specific nested primer4 in combination with primer AD4.

Cloning the MAT gene

Fragments a and b were sequenced. Primers PtMat_fw and PtMat_rev (Table 1), nested with respect to the 5′ and 3′ limits of the a and b amplified regions, were then designed to amplify both mating-type idiomorphs (Fig. 1). Using these primers, PCR amplification was successful in both tester isolates 0-1 and 15A. The amplicons were cloned, sequenced, and analyzed. The cloned PCR fragment was 2830 bp in isolate 0-1 (GenBank accession No. AY950586) and 2673 bp in isolate 15A (GenBank acc No. AY950585) (Fig. 1).

Sequence from P. teres isolate 0-1

The ~2.8-kb fragment obtained from isolate 0-1 contained the MAT-2 idiomorph of 2028 bp (or 2076 bp when the degenerated C terminus of ORF1 is considered, see below), as well as 449 bp (or 401 bp, not including the degenerated C terminus of ORF1, see later in the text) and 353 bp of sequence 5′ and 3′ to the idiomorph, respectively (Fig. 4B).

The MAT-2 idiomorph contained a single putative gene of 1055 bp. A putative intron of 56 bp was identified that separated exon I (456 bp) from exon II (542 bp). A lariat sequence, ACTGAC (Brutchez et al. 1993), was found within the intron, beginning 16 nucleotides upstream of the 3′ splice site. After removing the intron, the MAT-2 gene results in an ORF of 999 bp encoding a protein of 333 amino acids (exon I = 152 amino acids; exon II = 180 amino acids). The MAT-2 sequence contained the HMG motif (Fig. 4B). The intron disrupted the HMG domain of MAT-2 after the first base of codon 153 at the same position (serine) as in the HMG domains of all other fungal MAT loci sequenced so far (Fig. 2B).

The Blastp search showed that our entire putative MAT-2 peptide has a strong homology with several MAT-2 mating-type proteins of other fungi, such as Cochliobolus sp. (E = 4 × 10⁻⁷⁸ ÷ 4 × 10⁻⁶⁰), Pleospora sp., Stemphylium sp. (E = 5 × 10⁻⁷⁷ ÷ 2 × 10⁻⁷²), Alternaria sp. (E = 7 × 10⁻⁵⁵ ÷ 2 × 10⁻²³), Leptosphaeria maculans (E = 3 × 10⁻⁵⁵), Phaeosphaeria nodorum (E = 5 × 10⁻³⁵), and Leptosphaeria kor-

rae (E = 2 × 10⁻²¹). Other matches, such as those with Gibberella sp., Fusarium sp., R. secalis, and Phaeosphaeria avenaria, were also found (E = 10⁻⁵ ÷ 10⁻²⁰).

Sequence from P. teres isolate 15A

The analysis of the ~2.7-kb fragment revealed a MAT-1 idiomorph of 1865 bp (or 1919 bp when the degenerated C terminus of ORF1 is considered, see later in the text), as well as 455 bp (or 401 bp, not including the degenerated C terminus of ORF1, see later in the text) and 353 bp of sequence 5′ and 3′ to the idiomorph, respectively (Fig. 4A).

The MAT-1 idiomorph contained a single putative gene of 1190 bp. A putative intron of 53 bp separated exon I (249 bp) from exon II (888 bp). The intron contained a perfect lariat sequence, RCT-RAC, beginning 21 nucleotides upstream of the 3′ splice site. Removal of the intron results in an ORF of 1137 bp encoding a protein of 379 amino acids (exon I = 83 amino acids; exon II = 296 amino acids). The MAT-1 sequence includes the alpha-box motif (Fig. 4A). The introns disrupted the alpha-box domain of MAT-1 after codon 83, at a position corresponding to a cysteine, as in other ascomycetes, such as Pleospora spp., Stemphylium sp., Cochliobolus spp., and Alternaria alternata and L. maculans (Fig. 2A).

The Blastp search showed that our entire MAT-1 putative peptide has a strong homology with several MAT-1 mating-type proteins of other fungi, such as Pleospora sp., Stemphylium sp. (E = 10⁻⁷⁴ ÷ 10⁻⁷⁹), Cochliobolus heterostrophus (E = 5 × 10⁻⁷⁷), Cochliobolus luttrellii (E = 4 × 10⁻⁶⁵), A. alternata (E = 10⁻⁶⁹), A. tenuissima (E = 10⁻⁶⁶), L. maculans (E = 10⁻⁵⁷), and P. nodorum (E = 10⁻⁵⁹). Other matches, such as those with Gibberella sp., Potentilla anserina, Septoria passerinii, Fusarium sp., Sordaria sp., and M. graminicola, were also found (E = 10⁻⁵ ÷ 10⁻²⁰).

Thus, our molecular analysis confirmed that P. teres isolates 0-1 and 15A have opposite mating types, as predicted by classical crossing experiments (Weiland et al. 1999), and that they have MAT-2 and MAT-1 idiomorphs, respectively.

Alignment of sequences of MAT-1 with MAT-2 idiomorphs demonstrated that the idiomorphs are highly dissimilar (similarity of 48.1% and 42.7% excluding and including gaps, respectively) in contrast to the 5′ (similarity of 96.3%) and 3′ (similarity of 98.0%) flanking regions (no gaps observed). Sliding-window analysis revealed an abrupt transition between the flanking regions and the idiomorphs, with no gradual trend (increase of similarity) toward either the 5′ or the 3′ ends of the idiomorphs (Fig. 5).

Open reading frames of 150 amino acids in MAT-1 and 148 amino acids in MAT-2 were found in the 455 bp and 449 bp of sequence 5′ of the idiomorphs, respectively. This is very similar to a stretch of the ORF1 protein, a homolog of a Saccharomyces cerevisiae gene (YLR456W), for which a function is not known. In the BLAST search, the best match was with ORF1 of Pleospora targa (E = 10⁻⁵⁵) for both MAT-1 and MAT-2. Overall, the P. teres ORF1 ortholog is strongly conserved between MAT-1 and MAT-2, except for the last 17 (MAT-1) and 15 (MAT-2) amino-acidic positions. The similarity of the 2 ORF1 sequences degenerates and marks the 5′ border of the idiomorph region (Bennet et al. 2005).
Fig. 5. *P. teres* MAT-1 and MAT-2 were aligned with ClustalX and analyzed with DNA SP ver. 4.00 to view a 10-bp sliding window (step of 1 nucleotide, excluding gap) of the MAT locus.

Fig. 6. Alignment of MAT-1 and MAT-2 open reading frame (ORF)1 peptide sequences of *Phaeosphaeria nodorum*, *P. teres*, and *Cochliobolus heterostrophus*. White arrow, beginning of the *P. nodorum* idiomorph. Black arrow, beginning of the *P. teres* idiomorph. *, amino acids that are conserved among all 3 species; :, and, full conservation of strong and weak groups of amino acids, respectively, as defined by ClustalX. Amino acid positions are indicated below each alignment. Species names (on the left) and accession codes (on the right) are also given.

*Phylogenetic analysis*

The phylogenetic analysis of the alpha-box region indicated that the group of Pleosporales is well separated from the other fungi, and that *P. teres* fits into this group (bootstrap value of 99%). (Fig. 3A). Within the Pleosporales considered, *P. teres* is well separated from *P. nodorum* (Phaeosphaeriaceae) on one side, and from a group that includes *L. maculans* (Leptosphaeriaceae), *A. alternata*, *Cochliobolus* sp., *Pleospora* sp., and *Stemphylium* sp. (all Pleosporaceae) on the other. Similar results were obtained for the HMG-box region (Fig. 3B), except *R. secalis* (mitosporic ascomycete) and *Pyrenopeziza brassicae* (Derma-taceae), which are quite similar to each other and which both belong to Helotiales, clustered with the Pleosporales (bootstrap value of 66%; Fig. 3B) rather than with *M. grami*.
incola and S. passerinii (Mycosphaerellaceae) (bootstrap value of 66%; Fig. 3A).

Mating-type frequency survey

AFLP analysis

Considering all 4 primer combinations, 113 polymorphic markers were considered within the SF and 103 within the NF of the pathogen. All the isolates had distinct AFLP genotypes in all cases, except the PIR-SF population, where PIR 24s(2) (MAT-2) was indistinguishable from PIR 18s (MAT-2).

Mating-type frequency surveys

A total of 150 Sardinian P. teres isolates were assayed, using PCR amplification with mating-type-specific primers, and 147 (66 NF and 81 SF) gave unambiguous results; different primer-pairs never gave 2 different amplification products on the same isolate (Fig. 7). As expected, no amplification products were obtained with R. secalis and B. sorokiniana. However, an amplicon (the same length as that for P. teres) was obtained with the MAT-2 primer pairs and the I$_2$ isolates of P. graminea (not shown). Two NF isolates (BAC 2n and BAC 14n) and 1 SF isolate (PIR 20s) yielded no amplification products. These 3 isolates were excluded from subsequent analyses.

The mating-type alleles of the 2 formae specialae of the fungus were the same size on both agarose (Fig.7) and polyacrylamide (not shown) gels. The sequencing of 2 MAT-1 and MAT-2 genes (1 NF and 1 SF per mating type) confirmed that the stretches of the same mating effectively correspond to MAT genes, that they have the same lengths (MAT-1 = 1158 bp; MAT-2 = 1068 bp), and that they have a high degree of similarity (>99.6%). Tables 2–4 summarize the observed distribution of mating-type idiomorphs across Sardinia. At the regional (metapopulation) level (Table 2), the proportion of the 2 mating types was in accord with the 1:1 null hypothesis in both the NF ($\chi^2$ = 2.18, $P$ = 0.14; exact binomial test, $P_B$ = 0.18) and the SF ($\chi^2$ = 0.17, $P$ = 0.69; exact binomial test, $P_B$ = 0.82). At this level, the sample sizes were large enough to detect, with $\alpha$ = 0.05 mating-type bias, $\geq$1:9:1 (with a power of $1 - \beta$ = 0.80). These results were also confirmed at the population level (Table 2).

Indeed, within each of the 2 NF and the 3 SF populations with larger sample sizes (≥10), the proportion of the 2 mating types did not deviate significantly from the 1:1 null hypothesis (Table 2). Here, the sample sizes were large enough to detect, with $\alpha$ = 0.05 mating-type bias, from $\geq$2.4:1 (BAC-NF) to 3.9:1 (PIR-NF), with a power of $1 - \beta$ = 0.80. When all 147 P. teres isolates were combined, the proportion of the 2 mating types was again in accord with the 1:1 null hypothesis ($\chi^2$ = 1.53, $P$ = 0.22; exact binomial test, $P_B$ = 0.25) (Table 3), as was that within each barley landrace field (Table 3). Again, there were no differences in the distributions of the 2 mating-type idiomorphs among the NF and SF isolates at the regional level, among the 6 fields sampled, among the 2 NF populations, among the 3 SF populations, and among the 5 populations (2 NF and 3 SF) (Table 4). Of note, both mating types were always also found within the remaining NF and SF populations with small sample sizes (Table 2), indicating their presence throughout the island of Sardinia.

Discussion

Isolation of MAT idiomorphs

We isolated and characterized both MAT-1 and MAT-2 idiomorphs of P. teres. The molecular organization of the MAT-1 of P. teres is similar to that of other Dothideomycetidae (Loculoascomycetes) belonging to Pleosporales, such as C. heterostrophus (Wirsel et al. 1998), A. alternata (Arie et al. 2000), L. maculans (Cozijnsen and Howlett 2003), and P. nodorum (Bennet et al. 2003), where only an alpha-containing protein is encoded as well. This organization is different from Sordariomycetidae (Pyrenomycetes) (Turgeon and Yoder 2000) and Leotidiomycetidae (Discomycetes) (Singh and Ashby 1998), which have 3 MAT-reading frames, only 1 of which is homologous to the alpha-domain protein of P. teres. Moreover, the size of the MAT-1 idiomorph of P. teres (~1900 bp) is similar to that of A. alternata (1942 bp), significantly bigger than that of C. heterostrophus (1297 bp), and less than half that of P. nodorum (4282 bp).

In all the ascomycetes studied so far, all MAT-2 genes contain only a single gene encoding a protein with the HMG box as the DNA-binding motif (Turgeon 1998). This is also the case with MAT-2 of P. teres, where its size (2028 bp) is
between that of *C. heterostrophus* (1171 bp) and *P. nodorum* (4505 bp), and closer to that of *A. alternata* (2256 bp).

The MAT-1 (1137 bp) and MAT-2 (999 bp) ORFs and their respective putative encoded proteins (of 379 and 333 amino acids, respectively) were also similar to those of all the abovementioned fungi for both MAT-1 (genes: 1035 ÷ 1323 bp; proteins: 344 ÷ 441 amino acids) and MAT-2 (genes: 1026 ÷ 1191 bp; proteins 342 ÷ 397 amino acids).

The *P. teres* mating type is similar to that examined in other Pleosporales, but some differences in MAT locus organization have also been found.

Unlike *P. nodorum*, where there is a gradual transition from idiomorph to common flanking region at the 3′ end, as also reported for *Cryphonectria parasitica* (McGuire et al. 2001) and *Neurospora crassa* (Randall and Metzenberg 1998), the transition is as abrupt for *P. teres*, as it is, for

### Table 2. Chi-square ($\chi^2$) values for the distribution of mating-type idiomorphs within each net form (NF) and spot form (SF) population. Deviation from the 1:1 ratio was calculated as $\chi^2 = (a - b) \times (a - b) / (a + b)$, where $a$ and $b$ are the number of MAT-1 and MAT-2 genes, respectively. The last column shows the minimum detectable bias of mating-type ratios with $\alpha = 0.05$ (significance level), $1 - \beta$ (power) = 0.80.

<table>
<thead>
<tr>
<th>Population</th>
<th>$N$</th>
<th>MAT-1:MAT-2 ($a:b$)</th>
<th>$\chi^2$</th>
<th>$P_{\chi^2}$</th>
<th>$P_B$</th>
<th>Minimum detectable bias of mating type</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC-NF</td>
<td>2</td>
<td>1:1</td>
<td>0</td>
<td>na</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>SEC-SF</td>
<td>29</td>
<td>15:14</td>
<td>0.03</td>
<td>0.85</td>
<td>1</td>
<td>2.6:1</td>
</tr>
<tr>
<td>PIR-NF</td>
<td>16</td>
<td>7:9</td>
<td>0.25</td>
<td>0.62</td>
<td>0.8</td>
<td>3.9:1</td>
</tr>
<tr>
<td>PIR-SF</td>
<td>7a</td>
<td>1:6</td>
<td>3.57</td>
<td>na</td>
<td>0.13</td>
<td>—</td>
</tr>
<tr>
<td>TER-NF</td>
<td>6</td>
<td>2:4</td>
<td>0.67</td>
<td>na</td>
<td>0.69</td>
<td>—</td>
</tr>
<tr>
<td>TER-SF</td>
<td>20</td>
<td>10:10</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3.3:1</td>
</tr>
<tr>
<td>SIR-NF</td>
<td>3</td>
<td>2:1</td>
<td>0.33</td>
<td>na</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>SIR-SF</td>
<td>18</td>
<td>11:7</td>
<td>0.89</td>
<td>0.35</td>
<td>0.48</td>
<td>3.6:1</td>
</tr>
<tr>
<td>BAC-NF</td>
<td>34a</td>
<td>13:21</td>
<td>1.88</td>
<td>0.17</td>
<td>0.23</td>
<td>2.4:1</td>
</tr>
<tr>
<td>SES-NF</td>
<td>5</td>
<td>2:3</td>
<td>0.2</td>
<td>na</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>SES-SF</td>
<td>7</td>
<td>2:5</td>
<td>1.29</td>
<td>na</td>
<td>0.45</td>
<td>—</td>
</tr>
<tr>
<td>All NF isolates</td>
<td>66</td>
<td>27:39</td>
<td>2.18</td>
<td>0.14</td>
<td>0.18</td>
<td>1.9:1</td>
</tr>
<tr>
<td>All SF isolates</td>
<td>81</td>
<td>39:42</td>
<td>0.17</td>
<td>0.69</td>
<td>0.82</td>
<td>1.8:1</td>
</tr>
</tbody>
</table>

**Note:** $P_{\chi^2}$, probability value for $\chi^2$ with 1 degree of freedom (df); na, $\chi^2$ test not ($a:b$) applicable owing to small sample size (<10) (Lewontin and Felsenstein 1965); $P_B$, likelihood of obtaining the observed result or a more discrepant result, given that the true ratio is 1:1; $N$, the number of isolates with detectable mating-type idiomorphs.

aOne isolate of the 8 originally sampled gave no scorable amplification product for MAT idiomorph.

Two MAT-2 isolates of the 7 SF isolates of the PIR population have the same multilocus genotype, that is, 2 isolates are putative clones. After clone-correction, the 1:1 ratio is 1:5.

bTwo isolates of the 36 originally sampled gave no scorable amplification product for MAT idiomorph.

### Table 2. Chi-square ($\chi^2$) values for the distribution of mating-type idiomorphs within each net form (NF) and spot form (SF) population. Deviation from the 1:1 ratio ($\chi^2$) was calculated as described in Table 2.

<table>
<thead>
<tr>
<th>Field</th>
<th>$N$</th>
<th>MAT-1:MAT-2 ($a:b$)</th>
<th>$\chi^2$</th>
<th>$P_{\chi^2}$</th>
<th>$P_B$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC</td>
<td>31</td>
<td>16:15</td>
<td>0.03</td>
<td>0.86</td>
<td>1</td>
</tr>
<tr>
<td>PIR</td>
<td>23a</td>
<td>8:15</td>
<td>2.13</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>TER</td>
<td>26</td>
<td>12:14</td>
<td>0.15</td>
<td>0.7</td>
<td>0.85</td>
</tr>
<tr>
<td>SIR</td>
<td>21</td>
<td>13:8</td>
<td>1.19</td>
<td>0.28</td>
<td>0.38</td>
</tr>
<tr>
<td>BAC</td>
<td>34b</td>
<td>13:21</td>
<td>1.88</td>
<td>0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>SES</td>
<td>12</td>
<td>4:8</td>
<td>1.33</td>
<td>0.25</td>
<td>0.39</td>
</tr>
<tr>
<td>All isolates (NF + SF)</td>
<td>147</td>
<td>66:81</td>
<td>1.53</td>
<td>0.22</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**Note:** $P_{\chi^2}$, probability value for $\chi^2$ with 1 degree of freedom (df); $P_B$, likelihood of obtaining the observed result or a more discrepant result, given that the true ratio is 1:1; $N$, the number of isolates with detectable mating-type idiomorphs.

aOne isolate of the 24 originally sampled gave no scorable amplification product for MAT idiomorph.

bTwo isolates of the 36 originally sampled gave no scorable amplification product for MAT idiomorph.

### Table 3. Chi-square ($\chi^2$) values for the distribution of mating-type idiomorphs within all collection fields without splitting the 2 forms. Deviation from the 1:1 ratio ($\chi^2$) was calculated as described in Table 2.

<table>
<thead>
<tr>
<th>Field</th>
<th>$N$</th>
<th>MAT-1:MAT-2 ($a:b$)</th>
<th>$\chi^2$</th>
<th>$P_{\chi^2}$</th>
<th>$P_B$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC</td>
<td>31</td>
<td>16:15</td>
<td>0.03</td>
<td>0.86</td>
<td>1</td>
</tr>
<tr>
<td>PIR</td>
<td>23a</td>
<td>8:15</td>
<td>2.13</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>TER</td>
<td>26</td>
<td>12:14</td>
<td>0.15</td>
<td>0.7</td>
<td>0.85</td>
</tr>
<tr>
<td>SIR</td>
<td>21</td>
<td>13:8</td>
<td>1.19</td>
<td>0.28</td>
<td>0.38</td>
</tr>
<tr>
<td>BAC</td>
<td>34b</td>
<td>13:21</td>
<td>1.88</td>
<td>0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>SES</td>
<td>12</td>
<td>4:8</td>
<td>1.33</td>
<td>0.25</td>
<td>0.39</td>
</tr>
<tr>
<td>All isolates (NF + SF)</td>
<td>147</td>
<td>66:81</td>
<td>1.53</td>
<td>0.22</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**Note:** $P_{\chi^2}$, probability value for $\chi^2$ with 1 degree of freedom (df); $P_B$, likelihood of obtaining the observed result or a more discrepant result, given that the true ratio is 1:1; $N$, the number of isolates with detectable mating-type idiomorphs.

aOne isolate of the 24 originally sampled gave no scorable amplification product for MAT idiomorph.

bTwo isolates of the 36 originally sampled gave no scorable amplification product for MAT idiomorph.

### Table 4. Chi-square ($\chi^2$) values for the distribution of mating-type idiomorphs among populations and collection fields.

<table>
<thead>
<tr>
<th>Region</th>
<th>$\chi^2$</th>
<th>df</th>
<th>$P_{\chi^2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All NF isolates versus all SF isolates</td>
<td>1.05</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>Fields</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>All 6 fields</td>
<td>5.25</td>
<td>5</td>
<td>0.39</td>
</tr>
<tr>
<td>Populations</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NF</td>
<td>0.14</td>
<td>1</td>
<td>0.71</td>
</tr>
<tr>
<td>SF</td>
<td>0.55</td>
<td>2</td>
<td>0.76</td>
</tr>
<tr>
<td>NF and SF</td>
<td>2.85</td>
<td>4</td>
<td>0.58</td>
</tr>
</tbody>
</table>

**Note:** $P_{\chi^2}$, probability value for $\chi^2$ with 1 degree of freedom (df).
example, for C. heterostrophus (Dothideomycetidae, Pleosporales) and M. graminicola (Dothideomycetidae, Mycosphaerellales).

The P. teres idiomorph extended further into the 5′ flanking region, into the neighboring upstream ORF1 gene. Indeed, the P. teres ORF1 ortholog is not conserved for the last 15 (MAT-1) or 17 (MAT-2) amino acids at the 3′ end. Comparing P. teres and other Pleosporales, this is more similar to the case of P. nodorum (Bennet et al. 2003) and L. maculans (Cozijnsen and Howlett 2003), where the degeneration of the 3′ part of ORF1 is more extended, and includes 2 regions within the last 84–84 (MAT-1) and 85–80 (MAT-2) amino acids of the C termini, respectively. In A. alternata, the idiomorph extended toward ORF1, but they do not overlap (they are separated by 4 bp), and in C. heterostrophus, the idiomorph and the ORF1 are separated by 1079 bp. The ORFs of both these mating types have a similarity of 100% (see Fig. 6 in Bennet et al. 2003).

In contrast to the situation observed in C. heterostrophus, where the 5′ flanking region of the ORF1 is highly similar in sequence between mating types, in L. maculans, ORF1 is part of the idiomorph (see Fig. 3 in Cozijnsen and Howlett 2003). As consequence, both sides of ORF1 (5′ and 3′) are divergent in opposite mating types. We only sequenced stretches of the P. teres ORF1; therefore, the degree of homology between the 2 mating types of the region upstream of the ORF1 (i.e., if the idiomorph extended beyond the 5′ end of the ORF1) remains unknown.

As pointed out by Cozijnsen and Howlett (2003), the phylogenetic significance of such differences in genomic organization is not clear. Indeed, as noted by these authors, the picture can vary even within 1 genus, as is evident from a comparison of 3 Cochliobolus species: C. kusanoi, C. lutrellii, and C. homomorphus (Yun et al. 1999). Moreover, other Dothideomycetidae, such as M. graminicola, contain DNase upstream (5′) of MAT (Waalwijk et al. 2002) instead of ORF1. Again, in C. heterostrophus, it has been shown that the deletion of ORF1 is not relevant for fertility (Wirsel et al. 1998), the same as for the YLR456 ORF1 homolog in S. cerevisiae (http://sequence-www.stanford.edu/group/yeast_deletion_project).

Phylogenetic analysis revealed that P. teres fits into the Pleosporales group, as expected with the current systematic classification (Index Fungorum; http://www.indexfungorum.org). Moreover, the P. teres that is attributed to the Pleosporaceae family, despite being well separated, tends to be in a position between P. nodorum (Phaeosphaeriaceae) and L. maculans (Leptosphaeriaceae). The clear-cut separation between P. teres and the Cochliobolus subsp. observed in this study is consistent with the observation based on internal transcribed spacers and glyceraldehyde-3-phosphate dehydrogenase DNA sequences (Zhang and Berbee 2001). Some inconsistencies between the alpha-box- and the HMG-box-based trees have also been observed, such as the position of P. brassicae and R. secalis. This could be the result of the different evolutionary histories of MAT-1 and MAT-2 (but see Goodwin et al. 2003).

Mating-type frequency survey

AFLP analysis

Previous analysis of these P. teres fungal populations showed a high AFLP genotypic diversity (78% of unique genotypes) (Rau et al. 2003). In this study, we found only 2 isolates with the same AFLP genotype; they also have the same mating type, suggesting that they are likely true clones. We found a higher genotypic diversity than Rau et al. (2003) because the resolution power of this study was higher.

Mating-type frequency surveys

For sexual reproduction to be possible in P. teres, both mating types must co-occur. Their relative frequencies and distribution among populations is thus an indicator of the likely prevalence of sexual reproduction within and among populations. In Sardinia, both mating types were found in all field populations of both P. teres f. sp. teres and P. teres f. sp. maculata. Moreover, for both NF and SF at the regional level, no general trend of mating-type ratios away from the 1:1 null hypothesis was apparent. These results parallel those of other ascomycete pathogens: of barley, such as C. sativus (Zhong and Steffenson 2001), R. secalis (Linde et al. 2003), and S. passerinii (Goodwin et al. 2003); of wheat, such as C. parasitica (Liu et al. 1996; Marra and Milgroom 2001), M. graminicola (Zhan et al. 2002), and Tapesia yallundae and T. aciformis (Douhan et al. 2002a, 2002b); and of maize, such as C. carbonum (Weltz and Leonard 1993). These are all cases where the impact of sexual reproduction has been either hypothesized or well documented.

The role of evolutionary forces on mating-type distributions

Because of the cropping cycles, fungal plant-pathogen populations in an agricultural field are often founded by a small fraction of the total population. Moreover, many of the previously mentioned fungi, including P. teres, are capable of both sexual and asexual reproduction. The relative contributions of these 2 reproduction modes can vary, depending on the environment. In some cases, there are local populations or geographic areas that exhibit significant inequalities in mating types, such as for the field populations of T. yallundae (12:34) and T. aciformis (12:24) sampled in Washington state in the US or, on a larger scale, for the counties of Grant (7:17) and Lincoln (10:21) (Douhan et al. 2002a). The same applies to field populations of R. secalis sampled in Australia (17:4), Switzerland (25:6), Finland (33:18), and California (6:18) (Linde et al. 2003). So why should the mating-type ratio be “buffered” against any strong variation from the 1:1 ratio within our P. teres samples at the regional and field levels?

The mating-type frequencies determine the probability that 2 random isolates will be compatible. If sexual reproduction is operating, the approximate 1:1 ratio between mating types can be maintained by negative-frequency-dependent selection, a kind of balancing selection (Richman 2000). Under such conditions, a rare allele confers an advantage in encountering compatible matings within a population, so that its frequency tends to increase in succeeding generations, and vice versa (May et al. 1999; Richman 2000). Moreover, it seems unlikely that both mating types would persist at near-equal frequencies within field populations unless the genes were functional, i.e., sexual reproduction occurs (Goodwin et al. 2003).

Gene flow that spreads the 2 mating types over the regional territory could also explain the observed even distri-
bution (Antolin et al. 2003). However, the NF populations appeared to show more structure than the SF populations (Rau et al. 2003). This difference in divergence was attributed to greater genetic drift (or founder effect), with more restricted migration in the NF than in the SF of P. teres (Rau et al. 2003). Thus, under the hypothesis of absence of sexual reproduction, greater inequalities of mating types are expected within and among NF populations than SF populations. However, no differences were observed between the NF and SF in the mating-type ratios.

Finally, there was no evidence of strong selection (other than that essentially due to strict disassortative mating) for one or the other of the mating types of P. teres, at least under Sardinian field conditions. Indeed, if the mating-type idiomorphs (or closely linked loci) differed in viability or clonal fecundity, the mating-type ratio might deviate significantly from the 1:1 null hypothesis (Brasier and Webber 1987), even though the population may be entirely sexual, as, for example, in Microbotryum violaceum (Kaltz and Shykoff 1999). The same can be applied to the wheat pathogen P. nodorum, where 1 mating type unexpectedly predominated (92%; Halama 2002), despite a significant effect of sexual reproduction on its genetic structure (Caten and Newton 2000).

The NF and SF can hybridize in the laboratory (Campbell et al. 1999), although it is not clear if hybridization occurs in the field, where there is evidence both in favor of (Campbell et al. 2002) and against (Williams et al. 2001; Rau et al. 2003) such an event. In our system, isolates of both the NF and SF with opposite mating types coexist within each field. Thus, the opportunity for hybridization between the 2 forms is potentially widespread. However, no intermediate isolates were detected in the AFLP survey in Sardinia (Rau et al. 2003), suggesting that hybridization does not occur, or it is rare, between the 2 forms under field conditions.

To conclude, previous analysis of the P. teres fungal populations here analyzed for mating-type frequency were characterized by high AFLP diversity and low or no disequilibrium (Rau et al. 2003). Here, we also observed that when 2 additional AFLP primer combinations are used, only 2 individuals share the same multilocus genotype. The most likely explanation for low linkage disequilibrium is sexual reproduction, whereas high pathogen diversity could be explained by selection in a highly variable landrace of a strictly selfing host or with sexual reproduction. The main advance in this study is that both mating-type idiomorphs are present and coexist at similar frequencies in each field; sexual reproduction in P. teres is therefore likely important in generating the observed pathogen diversity.

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