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Distribution of the antifungal agents sordarins across filamentous fungi

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ABSTRACT

Sordarins are a class of natural antifungal agents which act by specifically inhibiting fungal protein synthesis through their interaction with the elongation factor 2, EF2. A number of natural sordarins produced by diverse fungi of different classes have been reported in the literature. We have run an exhaustive search of sordarin-producing fungi using two different approaches consecutively, the first one being a differential sensitivity screen using a sordarin-resistant mutant yeast strain run in parallel with a wild type strain, and the second one an empiric screen against *Candida albicans* followed by early detection of sordarins by LC–MS analysis. Using these two strategies we have detected as many as 22 new strains producing a number of different sordarin analogues, either known (sordarin, xylarin, zofimarin) or novel (isozofimarin and 4′-O-demethyl sordarin). Sordarin and xylarin were the most frequently found compounds in the class. The producing strains were subjected to sequencing of the ITS region to determine their phylogenetic affinities. All the strains were shown to belong to the *Xylariales*, being distributed across three families in this order, the *Xylariaceae*, the *Amphisphaeriaceae*, and the *Diatrypaceae*. Despite being screened in large numbers, we did not find sordarin production in any other fungal group, including those orders where sordarin producing fungi are known to exist (i.e., *Sordariales*, *Eurotiales*, and *Microascales*), suggesting that the production of sordarin is a trait more frequently associated to members of the *Xylariales* than to any other fungal order.

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Introduction

Protein synthesis has been considered as one of the most attractive targets for the development of antibacterial agents (Hall *et al.* 1992). Although the application of this idea to the field of antifungal therapy is not trivial because of the high similarity between the fungal and the mammalian protein synthesis machineries, fungal translation has evolved as a desirable target. The most important family of antifungal agents acting at the protein synthesis level are the sordarins.

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Publications from Merck and GSK groups demonstrated that the sordarins are potent and selective inhibitors of translation in fungi, which act *via* a specific interaction with the fungal elongation factor 2 (EF2), stabilizing the EF2-ribosome complex. The specificity of the sordarins for the fungal EF2 makes this an attractive antifungal target, despite the high homology in amino acid sequence exhibited by the EF2 from various eukaryotes (Justice *et al.* 1998; Dominguez and Martin 1998). All compounds in this class inhibit *in vitro* translation in *Candida albicans*, *C. tropicalis*, *C. kefyr* and *Cryptococcus neoformans,* but to varying degrees. The lack of activity of the sordarins against *Candida krusei, C. glabrata* and *C. parapsilosis*, in comparison with their high potency against *C. albicans*, suggests that these compounds have a highly specific binding site, which may also be the basis for the greater selectivity of these compounds against fungal *vs.* mammalian protein synthesis.

After the discovery of sordarin (Hauser & Sigg 1971), several compounds structurally related in sharing the common aglycone of sordarin, sordaricin, were isolated from diverse fungal species (Fig 1, Table 1): zofimarin (Ogita *et al.* 1987; Sato *et al.* 1995); BE31405 (Okada *et al.* 1998); SCH57404 (Coval *et al.* 1995), also known as xylarin (Schneider *et al.* 1995); hypoxysordarin (Daferner *et al.* 1999); hydroxysordarin and neosordarin (Davoli *et al.* 2002); and compound GR135402 and derivatives (Kennedy *et al.* 1998).

During recent years, multiple patents for sordarin derivatives have been filed by diverse companies, including GSK (Martin *et al.* 2000), Merck (Balkovec and Tse, 2000), Banyu (Hirano *et al.* 2000), Sankyo (Kaneko *et al.* 2003) and BMS (Serrano-Wu *et al.* 2002), evidencing the interest that these molecules have raised across drug discovery groups in industry. Efforts directed toward the development of new sordarin antifungal agents with improved activity against pathogenic fungi and better pharmacological properties have resulted in the discovery of new series of derivatives where the sugar moiety of sordarin has been replaced by other substitutents, such as a morpholinyl ring, in the azasordarins (Herreros *et al.* 2001), an oxazepane ring (Kamai *et al.* 2005) or a sulphur-containing side chain (Serrano-Wu *et al.* 2002). The potent broad-spectrum *in vitro* activity and their oral efficacy in animal models are significant advantages of these compounds, justifying the ongoing efforts into developing the full clinical potential of the sordarin class.

Besides the therapeutic potential of these molecules, sordarins are likely to play an ecological role in nature. Studies have shown that sordarins are produced by *Podospora pleiospora* in its natural substrate (dung) at sufficiently high doses to produce antibiosis against yeasts (Weber *et al.* 2005).

In our own laboratory we have performed across the years exhaustive screening of antifungal agents, including strategies specifically designed to search for compounds with the same mode of action as sordarins. Our search for other members of this class resulted in the discovery of the novel sordarin analog moriniafungin (Basilio *et al.* 2006). In addition, this continued effort, using different screening approaches, allowed us to detect sordarin and several known derivatives in diverse fungal strains. These fungi have been subjected to rDNA sequencing to assess their phylogenetic affinities. The results obtained have provided interesting information about the taxonomic distribution of sordarin and related compounds across filamentous fungi.

Material and methods

Fungal isolation

Fungal strains were isolated from environmental sources following methods described in the literature (Bills & Polishook 1993, 1994; Collado *et al.* 1996, 2007). The majority were preferentially isolated from living or decaying plant material and minor proportions from other sources, such as soil, dung, freshwater, or marine samples, or directly isolated from fungal fruitbodies. Substrates for microbial isolations were collected from both tropical and temperate regions, including all continents. After isolation, dereplication of redundant strains was carried out using macro- and micro-morphological criteria (Peláez & Genilloud 2003). The in-house isolated strains were complemented with strains purchased to culture collections or received from external collaborators for the screening.

Fungal fermentations

Fungal fermentations for the screening process using the differential sensitivity test (see below) were carried out as follows: Seed flasks were prepared from fresh slants as described (Bills *et al.* 1992). Two-ml portions of the resulting cultures were used to inoculate 250 ml unbaffled Erlenmeyer flasks containing 50 ml of diverse production media, as indicated in Table 4. Production flasks were incubated at 220 rpm, at 22 °C and 50 % relative humidity for different time periods (14, 21 and 28 d) before harvesting.

For the screening process using a *Candida albicans* liquid growth assay, liquid inocula for fungal fermentations were prepared in tubes containing 8 ml of seed medium from frozen agar plugs of the selected strains. The tubes were incubated in a rotary shaker for 4–6 d at 22 °C. Microscope cover glasses were placed at the bottom of the tubes to shear mycelium and obtain a homogeneous suspension of hyphae in grown cultures. Inoculum suspensions were arrayed in a master plate by transferring 1 ml aliquots from the tubes into the wells of a 96-square deep-well plate. A Duetz cryoreplicator pin tool (Duetz *et al.* 2000) was used to transfer minute inoculum aliquots from the master plate to 8–12 production plates. Production plates consisted of 96-deep well plates each filled with 1 ml of a production medium, as indicated in Table 6. Production plates were incubated statically for 3 weeks at 22 °C. The preparation of inoculum and production cultures is described in detail in Bills *et al.* (2008).

Extraction of fungal fermentations for screening

Secondary metabolites produced by the cultures grown in flasks were extracted with methyl ethyl ketone (MEK) or methanol (MeOH), as described (Cabello *et al.* 2001). In short, for the semisolid media, extracts were prepared by adding 50 ml of MEK or MeOH to the flasks, disrupting the mycelium with a spoon and shaking for 1 h. Aliquots of the organic phase (0.8 ml) were taken, dried out completely in a Savant SpeedVac (GMI Inc., Ramsey, Minnesota) or under N_2 flow, and the solid residue reconstituted in 0.5 ml of 25 % DMSO. For the liquid media, 2 ml aliquots were taken from the whole broth and

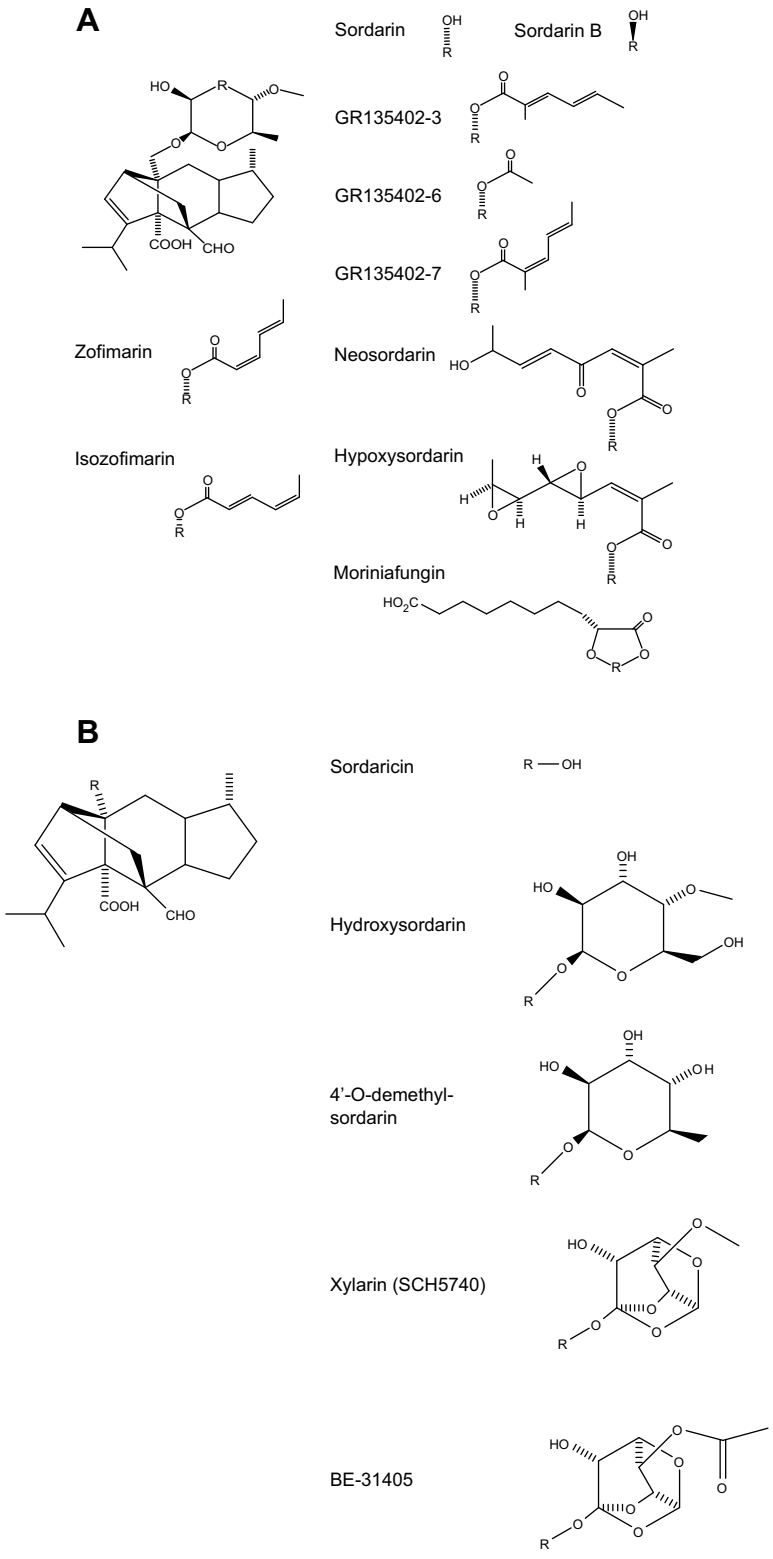

Fig 1 – Structure of the naturally occurring sordarin derivatives. A. Analogues sharing the sugar moiety of sordarin, differing in the radical in position 3′. B. Analogues with other sugar moieties.

subjected to extraction with an equivalent amount of either MeOH or with 2.8 ml of MEK.

For the cultures grown in 96-well plates we used acetone (1 ml per well) to extract the metabolites produced. The culture-solvent mixture was agitated for 1 h on a shaker board and centrifuged for 5 min. A total 900 μl of the supernatant were taken from each well and transferred to a recipient 96-well plate with 2-ml wells. 100 μl per well of DMSO were added

Table 1 – Natural sordarins reported in the literature

Fungal species (strain code)	Family, Order	Strain origin	Compound reported	Reference
Sordaria araneosa Cain (ATCC36386)	*Sordariaceae, Sordariales*[a]	Unknown	Sordarin	Hauser & Sigg 1971
Sordaria araneosa Cain (ATCC36386)	*Sordariaceae, Sordariales*	Unknown	Neosordarin, hydroxysordarin	Davoli *et al.* 2002
Hypoxylon croceum (M97-25)	*Xylariaceae, Xylariales*	Driftwood, mangrove estuary, Everglades (Florida, US)	Hypoxysordarin, sordarin	Daferner *et al.* 1999
Graphium putredinis (F13302/F13310)	*Microascaceae, Microascales*	Soil, excavated site, Leeds (UK)	GR135402 (and steroisomers), zofimarin, acetyl-sordarin, 6-hydroxy-GR135402	Kinsman *et al.* 1998; Kennedy *et al.* 1998
Penicillium minioluteum (F31405)	*Trichocomaceae, Eurotiales*	Soil, Saitama prefecture (Japan)	BE-31405	Okada *et al.* 1998
Podospora pleiospora (D01035)	*Lasiosphaeriaceae, Sordariales*	Rabbit dung, Braunton Burrows, Devon (UK)	Sordarin, sordarin B, hydroxysordarin, sordaricin	Weber *et al.* 2005
Xylaria sp. (PSU-D14)	*Xylariaceae, Xylariales*	*Garcinia dulcis* leaves, Songkhla Prov. (Thailand)	Sordaricin	Pongcharoen *et al.* 2008
Unidentified fungus (SCF1082A)	Unknown	Unknown	SCH57404	Coval *et al.* 1995
Xylaria sp. (A19-91)	*Xylariaceae, Xylariales*	Wood, Lescun (France)	Xylarin (=SCH57404)	Schneider *et al.* 1995
Zopfiella marina (SANK 21274, CBS 155.77)	*Lasiosphaeriaceae, Sordariales*	Marine mud, Chinese Sea (Taiwan)	Zofimarin	Ogita *et al.* 1987
Morinia pestalozzioides (MF6856)	*Amphisphaeriaceae, Xylariales*	*Sedum sediforme*, Sierra Alhamilla, Almería (Spain)	Moriniafungin	Basilio *et al.* 2006; Collado *et al.* 2007

a This strain is shown to belong to the *Lasiosphaeriaceae* in this work.

to minimize precipitation. The volume of the mixture was reduced by 50 % under a N_2 stream to get a final 500 μl aqueous extract with 20 % DMSO that was used for antifungal tests (Bills *et al.* 2008).

Differential susceptibility test against sordarin resistant and sensitive Saccharomyces cerevisiae *strains*

A screening assay was designed to identify antifungal compounds targeting the sordarin-sensitive step of fungal protein synthesis (EF2). The screen consisted of a two-plate differential zone size determination comparing the sensitivity of an erg6 *S. cerevisiae* mutant (WT EFT2) to that of an erg6 sordarin-resistant mutant (Sord^R). In this strain, the mutation A562 → P is located in the genomic copy of EFT2 (MAT alpha ade2 lys2 ura3 erg6::LEU2 eft1::HIS3 eft2-sordarin resistant), conferring resistance to sordarin at levels >100 μg ml^{-1}, compared to a sensitivity of 1 μg ml^{-1} for WT EFT2 (MAT alpha ade2 lys2 ura3 erg6::LEU2 eft1::HIS3 EFT2) when assayed in YPAD medium (Justice *et al.* 1998). In both strains the ERG6 gene was inactivated, a trait that confers increased sensitivity to a variety of agents (Gaber *et al.* 1989). The yeast gene ERG6 is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol (Gaber *et al.* 1989). Both strains are deposited in the Merck Culture Collection (strain codes MY2302 and MY2303 for the wild type and resistant strains, respectively).

A frozen stock of each strain was thawed in a flask containing YPAD medium and incubated overnight at 28 °C with shaking at 220 rpm (to mid or late log phase) to obtain the inoculum for the assay plates. The cultures were diluted 1:100 with water, and the cells counted in a Neubauer camera. YPAD medium containing 1.5 % agar, equilibrated at 45 °C in a water bath, were seeded with each culture at 3×10^5 CFU ml^{-1}. Aliquots of 100 ml of the inoculated agar media were poured into Nunc plates (24 × 24 cm).

Twenty-five μl aliquots of the MEK or MeOH extracts were applied to the surface of the seeded assay plates. Sordarin (2 μg) was used as positive control, amphotericin B (62.5 μg) and nystatin (25 μg) were used as negative controls in each assay plate. The plates were incubated at 28 °C and inhibition zones were scored 24 h later. Diameters of the zones were read to the outermost edge and recorded in mm. The haziness of the zone was reported as clear, hazy, or very hazy. Those extracts producing a difference of more than 5 mm between in the size of the inhibition zone in the sensitive *vs.* the resistant strain, or a clear zone of inhibition *vs.* a hazy/very hazy zone, were selected for follow up as they could contain potential inhibitors of fungal protein synthesis.

Candida albicans *liquid growth screening assay*

For the empiric screening of antifungal activity in natural products extracts, a strain of *C. albicans* MY1055 (Merck Culture Collection) was used. Frozen stocks of this strain were used to inoculate Sabouraud Dextrose Agar (SDA) plates for confluent growth. Plates were incubated for 24 h, at 35 °C. The grown colonies were harvested from the SDA plates and suspended in RPMI-1640 modified medium, which was prepared as follows: 20.8 g of RPMI powder (Sigma) were poured into a 2 l flask, together with 13.4 g of YNB, 1.8 l of milliQ water, 80 ml of Hepes 1 M, and 72 ml of glucose 50 %. The volume was adjusted at 2 l and filtered. The OD_{660} was adjusted to 0.25 (~1.0×10^8 CFU ml^{-1}) using RPMI-1640 modified as diluent and blank. This inoculum was diluted 1:10 and kept on ice until used to inoculate 96-well microtiter

plates. For the screening assay, 90 μl of the 1:10 diluted inoculum were mixed with 10 μl of the samples to screen. Amphotericin B and Penicillin G were used as positive and negative controls at each plate, respectively. After dispensing the inoculum, the assay plates were read in a Tecan Ultraevolution spectrophotometer (Männedorf, Switzerland) at 612 nm for T_0 (zero time). Then, the plates were statically incubated at 37 °C for 18–24 h. After incubation, the plates were shaken in a DPC Micromix-5 (Flanders, New Jersey) and read again for T_f (final time). Percentage of growth inhibition was calculated using the following normalization: % Inhibition = $[1 - [(T_f\text{Sample} - T_0\text{Sample}) - (T_f\text{Blank} - T_0\text{Blank})/(T_f\text{Growth} - T_0\text{Growth}) - (T_f\text{Blank} - T_0\text{Blank})] \times 100$. An extract was considered to have activity when its percentage of inhibition was superior to 50 %.

Determination of sordarin titers

Whole broth samples from the strains selected as active in the differential sensitivity screen were extracted with either MEK or MeOH as described above. MEK extracts were concentrated to dryness *in vacuo* and dissolved in MeOH:H_2O (50:50 v/v) containing 0.1 N NaOH and shaken overnight at room temperature. MeOH extracts were adjusted to 0.5 N NaOH by the addition of concentrated (5 N) NaOH and shaken overnight. The base-treated extracts were neutralized by the addition of concentrated H_2SO_4 and clarified using high speed microcentrifugation. Sordarin titers in the samples were determined using analytical RP-HPLC on Phenomenex Primesphere (Torrance, California) C8, with a mobile phase of acetonitrile:H_2O (45:55 v/v) containing 0.1 % H_3PO_4 at 1.0 ml min^{-1}, 40 °C and detection at 210 nm.

Liquid chromatography–mass spectrometry (LC–MS) analysis and database matching of known microbial metabolites

Aliquots of the extracts (2 μl) selected from the empiric screen against *Candida albicans* were analyzed by LC–MS. Analysis was performed on an Agilent (Santa Clara, California) 1100 single Quadrupole LC–MS, using an Agilent Zorbax SB-C8 column (2.1 × 30 mm), maintained at 40 °C and with a flow rate of 300 μl min^{-1}. Solvent A consisted of 10 % acetonitrile and 90 % water with 1.3 mM trifluoroacetic acid and ammonium formate, while solvent B was 90 % acetonitrile and 10 % water with 1.3 mM trifluoroacetic acid and ammonium formate. The gradient started at 10 % B and went to 100 % B in 6 min, kept at 100 % B for 2 min and returned to 10 % B for 2 min to initialize the system. Full diode array (DAD) UV scans from 100 to 900 nm were collected in 4 nm steps at 0.25 s $scan^{-1}$.

Ionization of the eluting solvent was obtained using the standard Agilent 1100 ESI source adjusted to a drying gas flow of 11 l min^{-1} at 325 °C and a nebulizer pressure of 40 psig. The capillary voltage was set to 3500 V. Mass spectra

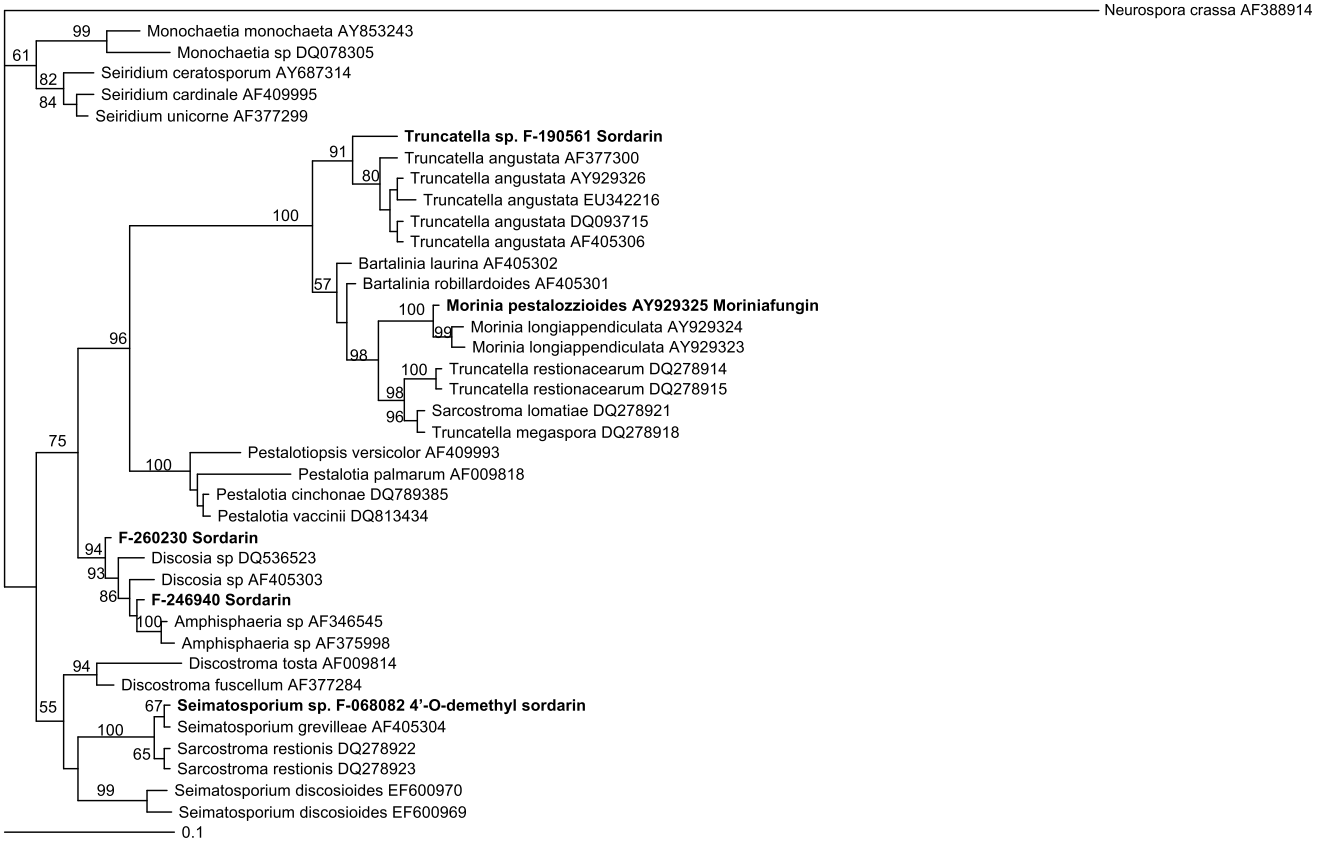

Fig 2 – Phylogenetic tree of selected species of the *Amphisphaeriaceae* including sordarin producers, generated from Bayesian analysis based on ITS sequences. Tree length: 294 steps. Clade credibility values are indicated at the branches. Sordarin-producing strains are marked in bold in the tree, with information on the sordarin analogues detected.

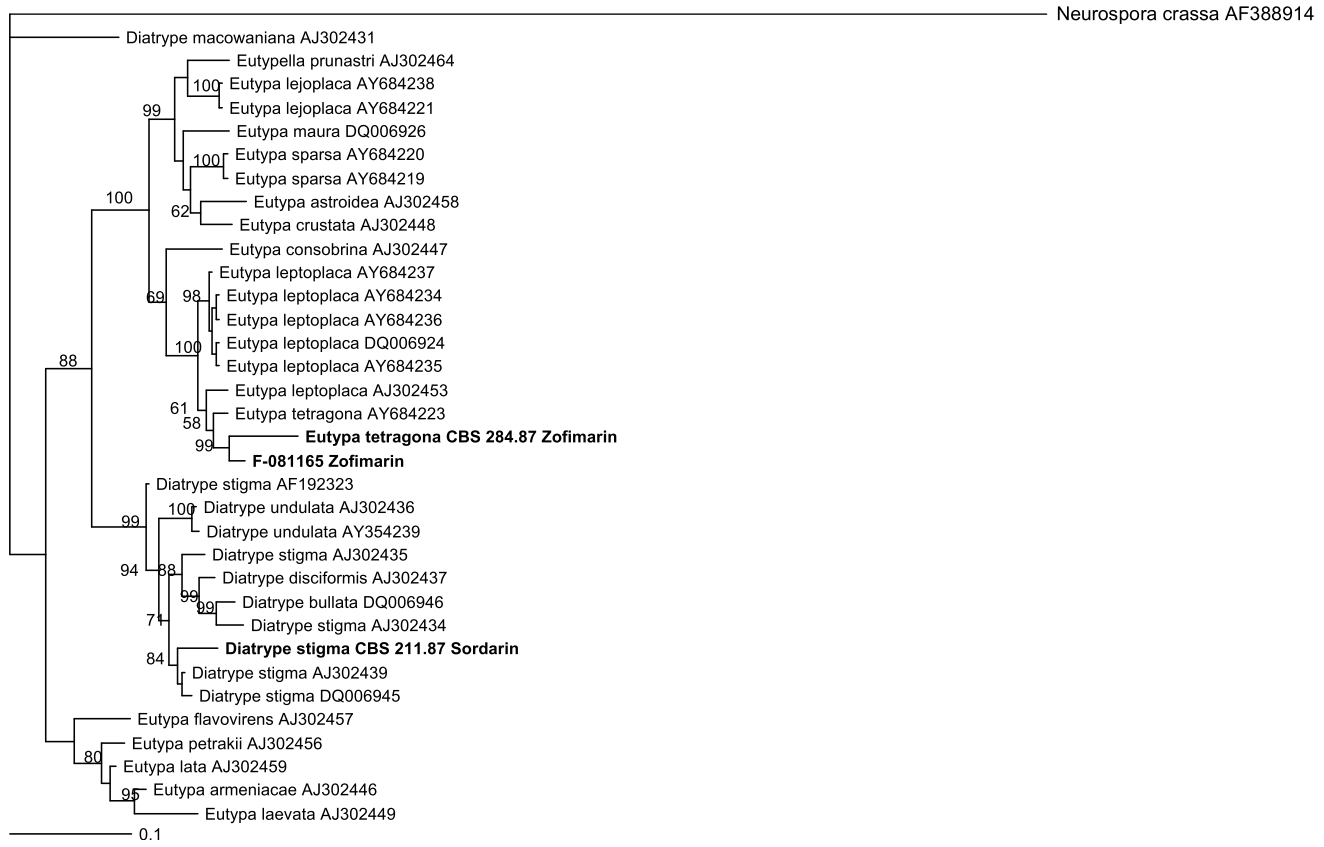

Fig 3 – Phylogenetic tree of selected species of the *Diatrypaceae* including sordarin producers, generated from Bayesian analysis based on ITS sequences. Tree length: 429 steps. Clade credibility values are indicated at the branches. Sordarin-producing strains are marked in bold in the tree, with information on the sordarin analogues detected. Strain CBS 211.87, labeled in the tree as *Diatrype stigma*, is found in CBS catalog as *Selenosporella falcate* (see text).

were collected as full scans from 150 *m/z* to 1500 *m/z*, with one scan every 0.77 s, in both positive and negative modes.

Database matching was performed using an in-house developed application against a Merck proprietary reference library (Zink *et al.* 2002, 2005) where the DAD, retention time, POS and NEG mass spectra of the active samples were compared to the UV–LC–MS data of known metabolites stored in a proprietary database. This contained metabolite standard data obtained under identical conditions as for the samples under analysis.

rDNA sequencing and phylogenetic analysis

DNA isolation, PCR amplification of the ITS1-5.8S-ITS2 region and sequencing were performed as previously described (Bills *et al.* 1999). Sequence alignment was made using the multiple alignment program CLUSTALW (Thompson *et al.* 1997) (IntelliGenetics, Inc., Mountain View, California). Due to the high variability found in the ITS1 region of *Xylariales* in previous studies (Platas *et al.* 2001), the section of the ITS1 subunit containing tandem repeats was removed from the analysis.

All the sequences obtained in this work have been deposited in GenBank (Table 3, Figs 2–5). In addition to the sordarin-producing strains, some additional sequences were obtained from strains belonging to species related to some of the sordarin producers. These are indicated in the legends of the phylogenetic trees.

The phylogenetic analysis of the aligned sequences was performed using a Bayesian analysis based on Markov chain Monte Carlo (MCMC) approach run as implemented in the computer program MrBayes 3.01 (Ronquist & Huelsenbeck 2003). To improve mixing of the chain, four incrementally heated simultaneous Monte Carlo Markov chains were run over 2 000 000 generations. MrModeltest 2.2 (Nylander 2004) was used to perform hierarchical likelihood ratio tests to calculate the Akaike information criterion (AIC) values of the nucleotide substitution models. The model selected by AIC for the alignment of the ITS1-5.8S-ITS2 gene fragment was the HKY + G model for *Amphisphaeriaceae, Xylariaceae* and *Diatrypaceae*, and the GTR + I+G for *Lasiosphaeriaceae*. In all cases six classes of substitution types were allowed, a portion of invariant alignment positions, and mean substitution rates varying across the remaining positions according to a gamma distribution. Priors used for the MCMC process were a Dirichlet distribution for substitution rates and nucleotide frequencies, and a uniform prior for the rate parameter of the gamma distribution. The MCMC analysis for both trees were carried out using the following parameters: sampling frequency = 100, the number of trees discarded for the consensus tree was 1000; and the resulting consensus tree was a majority rule consensus tree including

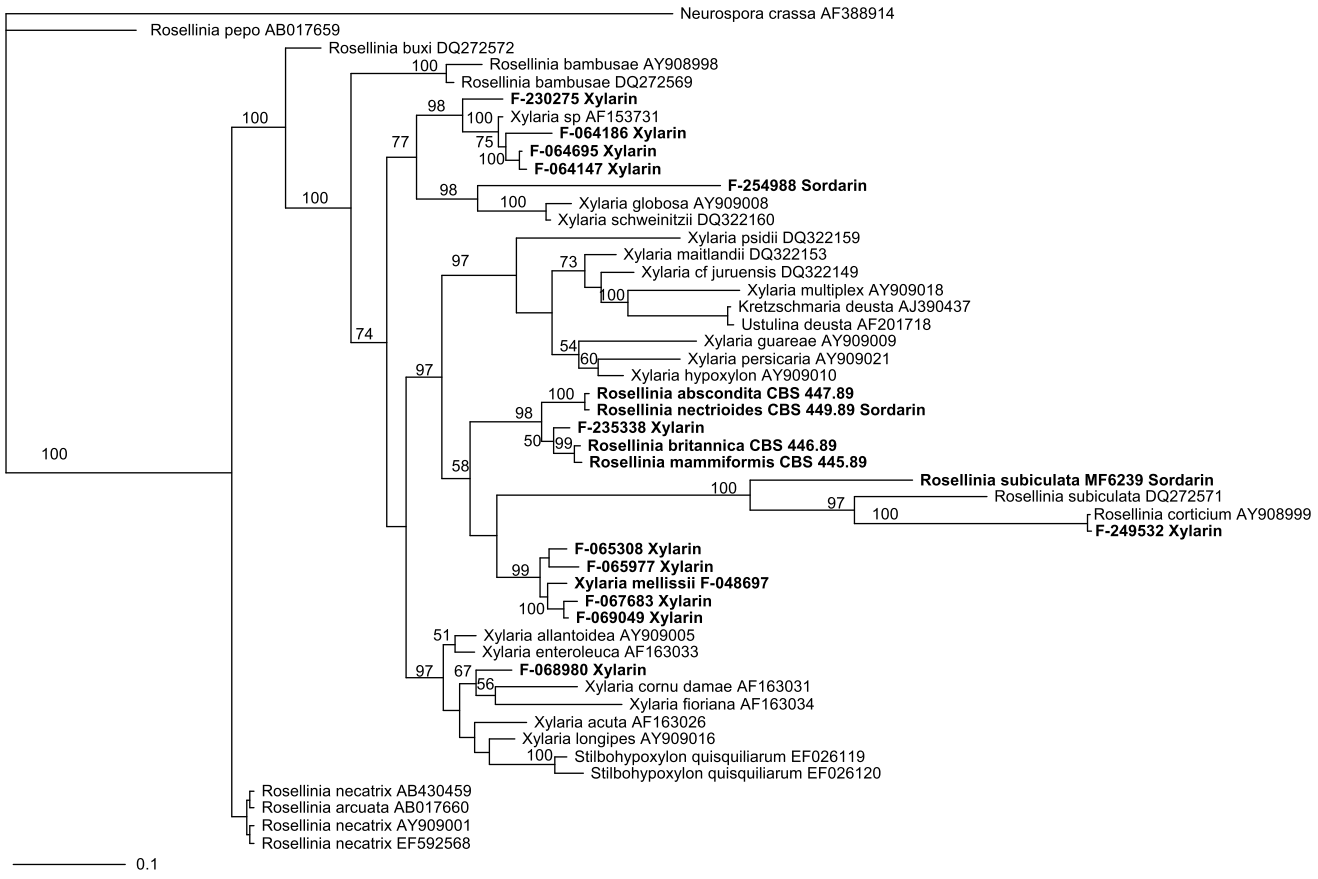

Fig 4 – Phylogenetic tree of selected species of the *Xylariaceae* including sordarin producers, generated from Bayesian analysis based on ITS sequences. Tree length: 928 steps. Clade credibility values are indicated at the branches. All the ITS sequences obtained in this work are marked in bold, including the sordarin-producing strains, with information on the sordarin analogues detected. The ITS sequences of the *Rosellinia* species obtained from CBS have been deposited in GenBank with the accession numbers FJ175180 (*R. abscondita* CBS 447.89), FJ175182 (*R. britannica* CBS 446.89) and FJ175183 (*R. mammiformis* CBS 445.89), as well as the sequence of *Xylaria mellissii* F-048,697 (FJ175173).

compatible groups of lower frequencies. The alignments have been submitted to Treebase with the accession number SN4079-19725, SN4079-19726, SN4079-19727 and SN4079-19728.

Results and discussion

Detection of sordarin-like compounds from the screening of fungal natural products

Two different strategies were used in our laboratory across the years that allowed us to detect the production of sordarins and sordarin derivatives. In our first approach we run an exhaustive screening for microbial secondary metabolites specifically searching for inhibitors of fungal protein synthesis, using a differential susceptibility test based on a mutant resistant to sordarin as described in Methods. The number of microbial strains tested as well as those found active in the differential susceptibility assay, distributed by fungal orders, are shown in Table 2.

A total of 45 987 fungal isolates representing 45 fungal orders from both *Ascomycota* and *Basidiomycota*, were screened. The best represented in number of strains tested were the *Xylariales*, followed by *Pleosporales*, and *Hypocreales*, but at least ten further orders were represented by large numbers of isolates (several hundred), guaranteeing that the chances of finding sordarin production in members of those groups, if they exist, would be reasonably high. Overall, all the groups with previous records of sordarin production (i.e., *Xylariales, Sordariales, Microascales* and *Eurotiales*) were well represented.

Only 86 of those isolates tested produced activity in the primary screening. Eleven of these 86 active isolates confirmed the activity in the differential susceptibility agar assay and were worth of further study. The remaining activities were discarded due to lack of sufficient potency, small differential between the two strains, lack of reproducibility or other reasons. Extracts from these 11 cultures were fractionated by HPLC and found to contain different members of the sordarin class, including sordarin, xylarin, zofimarin and the new derivatives isozofimarin and 4′-O-demethyl sordarin. (Table 3, Fig 1). Isozofimarin is a new geometric isomer of zofimarin around the sugar ester side chain double bond (2″–3″ E/4″–5″ Z in isozofimarin *vs.* 2″–3″ Z/4″–5″ E in zofimarin).

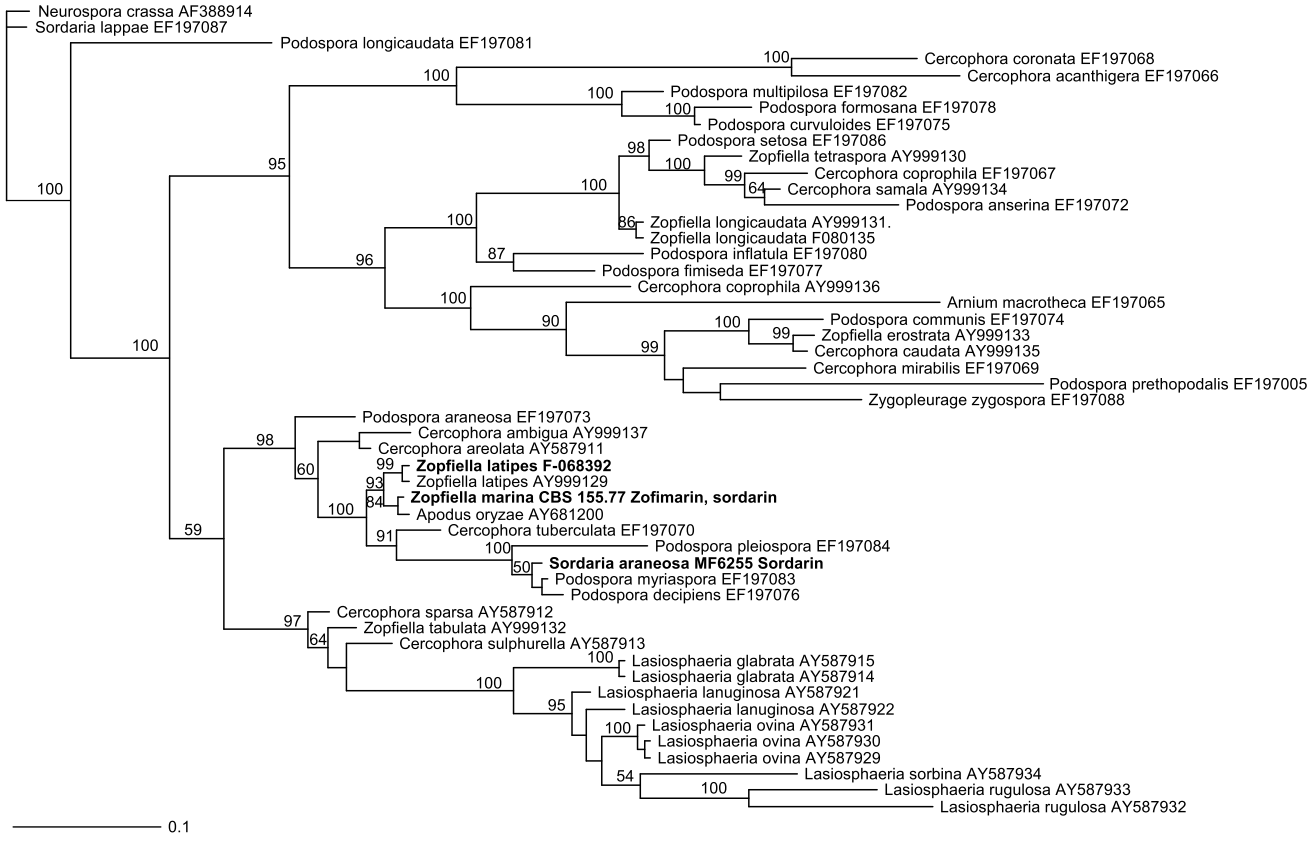

Fig 5 – Phylogenetic tree of selected species of the *Lasiosphaeriaceae* including sordarin producers, generated from Bayesian analysis based on ITS sequences. Tree length: 775 steps. Clade credibility values are indicated at the branches. All the ITS sequences obtained in this work are marked in bold, including the sordarin-producing strains, with information on the sordarin analogues detected. The sequence of *Zopfiella latipes* F-068,392 has been deposited in GenBank with the accession number FJ175158.

As shown in Table 4, sordarins were detected indistinctly in MEK or MeOH extracts, although only in one case was the compound detected in both extracts from the same culture. The titers of the compounds isolated varied widely from a few micrograms per ml (as in F-064,188) to more than 1 mg ml^{-1} (in F-068,082).

In this screening effort all the strains were grown in five production media combining different nutrients, complex and/or synthetic sources of carbon, nitrogen and phosphorous, and liquid or semi-solid formulations, to maximize the chances of having the strains expressing their secondary metabolite potential. The five media were selected from 10 different formulations, and they were periodically replaced. Interestingly, 10 out of the 11 strains that were detected as sordarin-like producers showed activity in a specific medium, FFL (Table 4). In three of the cases, the production was also observed in a second medium, CYS80-7 or BRFT. The strain F-081,165 was not grown in FFL but it produced sordarin in a different medium, OP26-NLW, which had been observed to support the production of the sordarin analog moriniafungin from *Morinia pestalozzioides* (Basilio *et al.* 2006). Interestingly, both media OP26-NLW and FFL have in common a high content in glycerol (>100 g l^{-1}), compared to any other media used.

These results are a good example of the importance of selecting the right growth conditions for the production of secondary metabolites in lead discovery programs. The relevance of this factor has been well known from the early days of antibiotic discovery (Peláez & Genilloud 2003). However, there are few examples of secondary metabolites systematically studied from the perspective of the influence of environmental and nutritional factors on their biosynthesis (e.g. Connors & Pollard 2005). Little is known about the biosynthesis of sordarins (Mander & Robinson 1991; Jenny & Borschborg 1995; Fukushima *et al.* 2006; Yao 2007), and there is no information on which nutritional factors may be driving the synthesis of sordarins. The observation that all these strains produced sordarin in media with a high concentration of glycerol suggests that this metabolite might play a role, but obviously many more studies should be done to really understand how nutrients or other environmental parameters may affect sordarin biosynthesis. Medium FFL was originally designed to enhance the production of nodulisporic acids, a family of antiparasitic compounds produced by a *Nodulisporium* sp. (Polishook *et al.* 2001), which are indole diterpenes. Considering that sordarins are also diterpenes, it is tempting to speculate about whether glycerol could be an important factor for the synthesis of other fungal diterpenoids as well.

More recently, we have used a different antifungal screening strategy with a broader scope regarding the potential targets of the compounds discovered. This strategy combined the use of an empiric screen against *Candida albicans* followed

Table 2 – Strains tested in a differential sensitivity screen looking for antifungal agents with the same mode of action as sordarins, sorted according to their adscription to fungal orders, following Index Fungorum complemented as per Hibbett et al. (2007)

Class	Order	Isolates tested
Ascomycetes	Xylariales	8053
	Pleosporales	7780
	Hypocreales	5052
	Dothideales	2074
	Helotiales	2011
	Diaporthales	1953
	Eurotiales	1620
	Sordariales	886
	Phyllachorales	761
	Pezizales	637
	Mycosphaerellales	592
	Microascales	472
	Trichosphaeriales	350
	Chaetothyriales	144
	Onygenales	111
	Ophiostomatales	84
	Calosphaeriales	28
	Others/Undetermined	2533
	Anamorphic fungi (incertae sedis)	5192
Basidiomycetes	Agaricales	1624
	Polyporales	832
	Russulales	155
	Hymenochaetales	128
	Boletales	96
	Thelephorales	54
	Dacrymycetales	48
	Others/Undetermined	1009
	Anamorphic fungi (incertae sedis)	72
Others		87
Sterile isolates and unidentified fungi		1621
Total		45 987

by early dereplication of known compounds by LC–MS and subsequent use of a genomic-wide platform using *C. albicans* heterozygous strains to prioritize antifungal extracts with interesting modes of action (Jiang *et al.* 2008; Parish *et al.* 2008). This new paradigm was coupled with the use of a system of mini-fermentations in nutritional arrays using microtiter plates that allowed generating microbial extracts in high throughput mode, facilitating the cultivation of each strain in multiple conditions with minimum additional effort. This system has been shown to increase the probabilities of detecting biological activities from any defined fungal set (Bills *et al.* 2008).

Across 11 569 fungal strains screened under this new paradigm, nearly 25 % provided a positive signal in the empiric antifungal screen against *C. albicans*, but only 11 strains from this pool were detected as producing sordarin or sordarin analogues by LC–MS analysis (Table 3). Dereplication of natural products using different analytical methods (HPLC, UV spectroscopy, MS or even NMR) and reference databases has become one of the most useful and widely used approaches to minimize the redundant isolation of known metabolites (Bitzer *et al.* 2007).

About half of the strains tested were subjected to some level of taxonomic identification, usually by morphological analysis, and a number of isolates were also subjected to rDNA sequencing. These strains were distributed across more than 60 fungal orders, mostly from the *Ascomycota*. *Pleosporales, Helotiales*, and *Hypocreales* were the groups represented with the largest number of isolates (Table 5). Again, the screening included all the groups known to contain sordarin-producing fungi (*Xylariales, Sordariales, Eurotiales*, and *Microascales*).

In this case, and unlike our previous experience with the differential sensitivity screen, the production of sordarins was not associated with any particular growth condition, and as many as 9 different production media supported the synthesis of sordarins (Table 6). Consistently with previous observations though, production of sordarins was observed in very few conditions for each strain (only in 1–3 out of the 8 media used for each strain). It is important to note that during this time we did not use the media FFL and OP26-NLW, due to the technical inconveniences associated with their preparation, which requires the sterilization of some components separately. The control strain *Zopfiella marina* produced a strong antifungal activity in three out of the four media tested, and LC–MS analysis detected the presence of both zofimarin and sordarin in the extracts.

The striking association of sordarin biosynthesis with medium FFL in the first screening period sharply contrasts with the lack of correlation with any particular medium in the second screening effort. It is worth noting that none of the media used during the latter period contained high concentrations of glycerol, what could explain this lack of preference for any particular medium. The screening would detect sordarins only in those strains able to produce sufficiently high titers in any other culture media. However, other differences between the two screening strategies may also contribute to the dissimilar results. First, the different fermentation formats (flasks *vs.* microtiter plates), secondly the different extraction process; and probably even more important, the sensitivity of the screening process itself. It is questionable whether the antifungal extracts in which sordarins were detected by LC–MS, would have given a positive result if tested in the differential sensitivity test using the *Saccharomyces cerevisiae* mutants.

It is interesting to note that the two screening strategies identified the same number of sordarin producing strains, but starting from a very different number of tested strains (45 000 *vs.* 11 000). Thus, the empiric screening against *C. albicans* followed by LC–MS analysis was more efficient in the detection of sordarin-producing strains. However, it seems likely that this second screening strategy, not specifically designed for the detection of antifungal agents with the same mode of action as sordarins but with a much broader scope, may have actually underestimated the potential of the strains tested to produce sordarins. Probably the frequency of detection of sordarins would have been even higher if a glycerol-rich medium had been included among the media selected.

Table 3 – Fungal strains found to produce sordarin and sordarin analogues

Strain code	Taxonomy (morphology)	Taxonomy (rDNA sequencing)	Collection place	Substrate	Compounds detected	GenBank accession
Control strains described in previous literature						
F-116,360[a] (MF-6239)	*Rosellinia subiculata*	Xylariaceae, *Rosellinia subiculata* (?)	Navesink River, New Jersey (USA)	Decayed wood	Sordarin	FJ175163
F-116,361 (MF-6255)	*Sordaria araneosa*	Lasiosphaeriaceae, related to several *Podospora* spp.	Original strain from Sandoz, no data on origin	Unknown	Sordarin	FJ175160
F-262,723 (CBS 155.77)	*Zopfiella marina*	Lasiosphaeriaceae, closely related to *Apodus oryzae*	Chinese Sea (Taiwan)	Marine mud	Zofimarin, sordarin	FJ175159
Strains discovered through a differential sensitivity screening using a sordarin-resistant mutant *vs.* wild type yeast						
F-068,082	*Seimatosporium* sp.	Amphisphaeriaceae, consistent with *Seimatosporium grevillae*	Sierra Villuercas, Cáceres (Spain)	Twigs and leaves of *Quercus faginea*	4′-O-dimethyl sordarin	FJ175156
F-064,147	Sterile isolate	Xylariaceae, likely *Xylaria* sp.	Curepipe (Mauricio Is.)	Leaf litter	Xylarin	FJ175166
F-064,695	Sterile isolate	Xylariaceae, likely *Xylaria* sp.	Le Morne (Mauricio Is.)	Twigs from conifer	Xylarin	FJ175165
F-064,186	Sterile isolate	Xylariaceae, likely *Xylaria* sp.	Grandes Gorges (Mauricio Is.)	Twigs from unidentified bush	Xylarin	FJ175167
F-068,980	Sterile isolate	Xylariaceae, likely *Xylaria* sp.	Acan-Bot Esaveg (Bata, Eq. Guinea)	Bark of *Plagiostylis* Africana	Xylarin	FJ175169
F-067,683	Sterile isolate	Xylariaceae, likely *Xylaria mellissii*	Three Okumes, Road Bata-Mbini Km. 27 (Eq. Guinea)	Mixed leaf litter	Xylarin	FJ175171
F-069,049	Sterile isolate	Xylariaceae, likely *Xylaria mellissii*	Three Okumes, Road Bata-Mbini Km. 27 (Eq. Guinea)	Leaves of *Monopetalanthus microphyllus*	Xylarin	FJ175172
F-065,308	Sterile isolate	Xylariaceae, likely *Xylaria mellissii*	Tepui Ruraima (Venezuela)	Leaves from unidentified bush	Xylarin	FJ175174
F-065,977	Sterile isolate	Xylariaceae, likely *Xylaria mellissii*	Punta Cana (Rep. Dominicana)	Undetermined plant	Xylarin	FJ175175
F-064,188	Sterile isolate	Xylariaceae	Grandes Gorges (Mauricio Is.)	Twigs from unidentified bush	Sordarin, zofimarin, isozofimarin	FJ175177
F-081,165	Sterile isolate	Diatrypaceae, likely *Eutypa tetragona*	Santa Elena, Jaén (Spain)	*Phellodon melaleucus* fruitbody	Zofimarin	FJ175178
Strains discovered through empiric screening against *C. albicans* followed by LC/MS analysis						
F-190,561	*Truncatella* sp.	Amphisphaeriaceae, closely related to *Truncatella angustata*	Guatemala	Mixed leaf litter	Sordarin	FJ175157
F-230,275	Sterile isolate	Xylariaceae, likely *Xylaria* sp.	El Calafate, Santa Cruz (Argentina)	Undetermined plant	Xylarin	FJ175168
F-130,895	Sterile isolate	Xylariaceae	Santa Marina, Taramundi, Asturias (Spain)	*Taxus baccata*	Sordarin	FJ175176
F-235,338	Sterile isolate	Xylariaceae, likely *Rosellinia* sp.	Alcantud, Cuenca (Spain)	Leaf litter from *Pinus* sp.	Xylarin	FJ175170
F-246,940	Sterile isolate	Amphisphaeriacae, likely *Amphisphaeria* sp.	Salazie (Reunion Is.)	Leaf litter	Sordarin	FJ175155
F-260,230	Sterile isolate	Amphisphaeriacae, likely *Amphisphaeria* sp.	Turku (Finland)	Lichen	Sordarin	FJ175154
F-254,988	Sterile isolate	Xylariaceae	Huelva (Spain)	*Scirpus holoschoenus*	Sordarin	FJ175164
F-247,493 (CBS 284.87)	*Eutypa tetragona*	Diatrypaceae, consistent with *Eutypa tetragona*	Tourrettes/Loup, Alpes Maritimes (France)	*Sarothamnus scoparius*	Zofimarin	FJ175179
F-249,532 (IZ-1295)	Sterile isolate	Xylariaceae, likely *Rosellinia corticium*	La Coruña (Spain)	*Thinopyrum junceiforme*	Xylarin	FJ175162

(*continued on next page*)

Table 3 – (continued)

Strain code	Taxonomy (morphology)	Taxonomy (rDNA sequencing)	Collection place	Substrate	Compounds detected	GenBank accession
F-249,628 (CBS 449.89)	*Rosellinia nectrioides*	Xylariaceae, closely related to *Rosellinia abscondita*	Jerusalem, Dalby Par. Uppland (Sweden)	*Trifolium medium*	Sordarin	FJ175181
F-266,831 (CBS 211.87)	*Diatrype stigma*	Diatrypaceae, consistent with *Diatrype stigma*	Loch Dan, Wicklow Co. (Ireland)	*Quercus* cf. *robur*	Sordarin	AJ302438[b]

a Strains with codes other than F numbers in parenthesis were either obtained from culture collections or received from external collaborators. MF: Merck Culture Collection (Rahway, NJ, USA); CBS: Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands); IZ: Iñigo Zabalgogeazcoa (Instituto de Recursos Naturales y Agrobiología, Salamanca, Spain).
b This strain had been already sequenced by our group years ago (Acero *et al.* 2004). We sequenced it again for confirmation, but the original GenBank access code is indicated.

Phylogenetic analysis of the sordarin-producing organisms

All the fungal strains detected to produce sordarin and sordarin-like compounds were subjected to a preliminary taxonomic analysis based on the morphology of the sporulation structures. Unfortunately, most of the strains remained sterile even after prolonged incubation, as frequently observed when working with fungi isolated from plant materials using indirect methods (e.g., Guo *et al.* 2000; Collado *et al.* 2001). With the aim of getting more meaningful information on their taxonomy, all of them were subjected to sequencing of the ITS region. The sequences obtained were compared with sequences in GenBank using BLAST analysis, and the closest matches found were retrieved, complemented with other related species and used to build the phylogenetic trees shown in Figs 2–5. This analysis included several strains previously known to produce sordarins, included in our screening systems for quality control purposes.

As shown in Table 3, the strains detected as sordarin-producing fungi were all of them from the *Xylariales*, being distributed across three different families: *Xylariaceae*,

Table 4 – Production media supporting the production of sordarins detected using a differential sensitivity screen. Black boxes indicate combinations of strain/medium that resulted in the production of sordarins, grey boxes are combinations that were inactive. All the strains were tested in five different media. The figures in the boxes indicate the concentration of sordarin analog produced (in μg ml^{-1}). Those strains where less than five media were tested correspond to cases of contaminations or lack of growth of the production culture. Strains MF 2639 and MF6255 were control strains known to produce sordarin (see Table 3). Media formulations are described in [a]Peláez *et al.* (2001), [b]Singh *et al.* (2003), [c]Basilio *et al.* (2006), [d]Polishook *et al.* (2001), or taken from the Merck proprietary database ([e])

	Production media										
Strain codes	**CYS80[a]**	**FFL[d]**	**TG106[a]**	**MV8[b]**	**BRFT[b]**	**AD2M2BM[b]**	**CYS80-7[a]**	**OAT[e]**	**STP[a]**	**OP26NLW[c]**	**Solvent**
F-068,082		1200									MeOH
F-064,147		~590									MeOH
F-064,695		156									MeOH
F-064,186		~590									MeOH
F-068,980		34									MeOH
F-067,683		110					ND*				MeOH/MEK
F-069,049		27									MeOH
F-065,308		13					ND				MEK
F-065,977		590									MEK
F-064,188		74**			ND						MEK
F-081,165										ND	MEK
MF 6239						ND					MeOH
MF 6255						ND					MeOH

*ND = Not determined. ** This strain produced 74 μg ml^{-1} of sordarin and minor amounts (~10 μg ml^{-1}) of zofimarin and isozofimarin.

Table 5 – Strains tested in an empiric screen looking for antifungal agents with activity against *Candida albicans*, sorted according to their adscription to fungal orders, following Index Fungorum complemented as per Hibbett *et al.* (2007)

Class	Order	Isolates tested
Ascomycetes	Pleosporales	1361
	Hypocreales	964
	Helotiales	687
	Diaporthales	343
	Capnodiales	361
	Xylariales	309
	Sordariales	216
	Chaetothyriales	133
	Eurotiales	83
	Ophiostomatales	77
	Botryosphaeriales	73
	Dothideales	56
	Chaetosphaeriales	50
	Coniochaetales	35
	Hysteriales	32
	Microascales	32
	Onygenales	28
	Others/undetermined	372
Basidiomycetes	Agaricales	42
	Polyporales	35
	Hymenochaetales	20
	Others/undetermined	72
Sterile isolates and unidentified fungi		6188
Total		11 569

Diatrypaceae and *Amphisphaeriaceae*. Although sordarins have been reported previously from members of the *Sordariales, Microascales* and *Eurotiales* (Table 1), we have not found any new sordarin producing strain from these three orders.

Amphisphaeriaceae

Within this family we observed production of sordarins in four strains. Fig 2 shows the phylogenetic relationships of these four strains with a number of species of the *Amphisphaeriaceae*, based on ITS sequences. The sequence alignment used to build this tree contained 471 characters, 309 of which were constant.

The strain F-068,082 could be identified morphologically as a *Seimatosporium* sp., and the ITS sequence was identical to a sequence deposited in GenBank as *S. grevilleae*, strongly suggesting these two strains to be conspecific. Accordingly, the phylogenetic tree showed these two strains grouped in a branch, as a sister group of two sequences of *Sarcostroma restionis*. Interestingly, this strain produced a new natural analog of sordarin (4′-O-demethyl sordarin), never reported before. The remaining strains of the family produced sordarin.

Another strain (F-190,561) was identified as a *Truncatella* sp. by morphology, and the ITS sequence analysis showed it to be closely related to a group of *T. angustata* sequences, placed as a sister group rooted at the base of the branch harboring these sequences. This topology would be consistent with this strain being either *T. angustata* or at least an intimately related species.

The remaining two strains belonging to the *Amphisphaeriaceae* (F-260,940 and F-260,230) showed almost identical ITS sequences (99 % homology) and were grouped together in a branch that contained sequences of *Discosia* sp. and *Amphisphaeria* sp., being also highly related to those ones (nucleotide homology >97 %). Despite their high similarity, which suggest their conspecificity, those two strains came from very distant geographic origins (Finland and Reunion Island), suggesting they would belong to a species with a cosmopolitan distribution.

We had described previously the production of another sordarin analog, moriniafungin, from *Morinia pestalozzioides*, another member of the *Amphisphaeriaceae* (Basilio *et al.* 2006; Collado *et al.* 2006). We have not found this particular sordarin derivative in any other member of this or any other fungal family. Putting all these findings together we conclude that the production of sordarin-like compounds is a trait relatively widespread across different lineages within the *Amphisphaeriaceae*.

The topology of the phylogenetic tree was consistent with previously reported phylogenies of the *Amphisphaeriaceae* (Jeewon *et al.* 2002; Lee *et al.* 2006), showing well supported monophyletic clades correlating with conidial morphology.

Diatrypaceae

We observed production of sordarins in three strains ascribed to species of this family. The phylogenetic tree originated from the alignment of ITS sequences of these isolates and a number of species from the family is shown in Fig 3. The dataset consisted of 551 characters, 349 of which were constant.

Two of the strains were shown to produce zofimarin. One of these strains (F-247,493) was obtained from CBS (*Eutypa tetragona* CBS 284.87), whereas the other one (F-081,165) was isolated from a fruitbody of *Phellodon melaleucus* collected in Spain. These two strains were grouped together in the phylogenetic tree in a branch with very high statistical support, the closest neighbor being another sequence labeled as *E. tetragona*. The nucleotide homology among these three sequences was very high (>93 %). Although the statistical support for the whole group was weak (58 %), it seems reasonable to conclude that the two zofimarin producing strains belong to the species *E. tetragona*.

The third strain (F-266,831; CBS 211.87) produced sordarin, not zofimarin, and it appears in CBS catalog under the name *Selenosporella falcata*. This strain fell within a monophyletic group with two strains of *Diatrype stigma*. Actually, this strain was originally described by Rappaz (1987) as *D. stigma*, and neither this author nor others who have studied *D. stigma* in culture (Glawe & Rogers 1982) assigned the name *S. falcata* to its anamorph. Likewise, the original description of *S. falcata* did not mention this apparent connection with the teleomorph (Sutton 1973).

To our knowledge, this is the first report of the production of sordarins in members of the *Diatrypaceae*, a fungal group poorly known with respect to their potential to produce biologically active metabolites, compared to other related groups such as the *Xylariaceae* (Stadler & Hellwig 2005).

Xylariaceae

By far the group where the production of sordarins seems to be the most common is the *Xylariaceae*. As many as 15 strains were detected producing sordarin or related compounds. The ITS derived phylogenetic affinities of 13 of these strains can be

Table 6 – Production media supporting the production of sordarins detected from *C. albicans* empiric screen followed by LC–MS analysis. Black boxes indicate combinations of strain/medium that resulted in the production of sordarins, grey boxes are combinations that were inactive. All the strains were grown in eight different media. Those strains where less than eight media were tested correspond to cases of contaminations or lack of growth of the production culture. The strain of *Zopfiella marina* CBS 155.77 (last raw in the table) was used as control in this experiment. Media formulations described in [a]Peláez *et al.* (2001), [b]Singh *et al.* (2003), [c]Scott *et al.* (1970), or taken from the Merck proprietary database ([d])

	Production media														
Strain codes	BRFT[b]	CMK[d]	CYS80[a]	MED1[d]	MMK[d]	MMK2[d]	MSCM[d]	MV8[b]	STP[a]	SCAS[d]	SUPM[d]	TG106[a]	WHEAT-1[d]	WS80[d]	YES[c]
F-190,561															
F-230,275															
F-130,895															
F-235,338															
F-246,940															
F-260,230															
F-254,988															
F-247,493															
F-249,532															
F-249,628															
F-266,831															
CBS 155.77															

tracked in the tree shown in Fig 4, which contains exclusively sequences from the *Xylarioideae*, essentially *Xylaria* and *Rosellinia* species. The sequence dataset used to build the tree contained 482 characters, 261 of which were constant.

The two remaining strains (F-064,188 and F-130,895) had their closest matches in GenBank with a sequence of *Anthostomella eucalyptorum* and other sequences ascribed to several xylariaceous fungi, but the homology and the quality of the alignments was too low to allow for any reliable conclusion about the phylogenetic relationships of these strains, and they were excluded from this analysis.

Xylarin was the compound the most frequently found in this group, being produced by 11 of the xylariaceous strains. Xylarin has been previously reported from a *Xylaria* sp. and from one unidentified fungus (Schneider *et al.* 1995; Coval *et al.* 1995). The remaining strains produced only sordarin, except for the strain F-064,188, which produced sordarin, zofimarin and the new isomer isozofimarin.

The strain CBS 449.89 (*Rosellinia nectrioides*) was found to produce sordarin. This strain showed an ITS sequence identical to another CBS strain identified as *R. abscondita*, and they appeared together in the tree in a branch with 100 % clade credibility. This strongly suggests that these two isolates would be conspecific. Actually, both species are morphologically extremely similar or even indistinguishable (Petrini & Petrini 2005; Bahl *et al.* 2005), but their synonymy has not been formally proposed. However, we did not detect any antifungal activity in the extracts derived from the strain of *R. abscondita* CBS 447.89, and therefore these extracts were not subjected to LC–MS analysis. This negative result might be due to a real lack of sordarin production, or to the titers of the compound being too low to produce an antifungal signal.

We isolated another phylogenetically related strain (F-235,338) from pine leaf litter, but producing xylarin instead of sordarin. This strain fell in a clade with high statistical support together with *R. nectrioides*, *R. abscondita*, and two other *Rosellinia* species; *R. britannica* and *R. mammiformis*. These four species are morphologically closely related, sharing the presence of a *Geniculosporium* anamorph, ellipsoidal ascospores, germ slit straight or sigmoid covering most of the length of the ascospore, and rim of ascus apical structure rounded. *Rosellinia britannica* and *R. mammiformis* differ from the other two species in the morphology of the slimy sheath covering the ascopore. They all belong to subgenus *Calomastia*, and fell together in a clade in a UPGMA cluster analysis based on morphological characters (Petrini & Petrini 2005). Again, we did not detect any antifungal activity in the strains of *R. britannica* and *R. mammiformis*, but it would be interesting to confirm with additional studies whether these related species are really unable to produce sordarins, since this character could represent an additional feature to discriminate species across this group. In any case, the position of the strain F-235,338 in the tree strongly suggests that it would belong to a closely related *Rosellinia* species.

Another xylarin-producing strain (F-249,532) was isolated as an endophyte of *Thinopyrum junceiforme* and shown to have a sequence 99 % identical to a sequence of *R. corticium*, suggesting these two strains to be conspecific. However, we did not find any sordarin analog production in the strain of *R. corticium* F-160,845 (corresponding to the sequence AY908999) when this was subsequently tested. These strains were relatively close to strains of *R. subiculata*, one of which (MF6239, ATCC 74386) at least has been reported to produce sordarin in the patent literature (Balkovec & Tse 2000).

Three xylarin producing strains (F-064,186; F-064,695 and F-64,147) were closely related (nucleotide homology > 95%) and grouped together in the tree. All of them were isolated from plant material collected in Mauritius Island, and they could well be conspecific. The closest match (99 % homology) in GenBank was a sequence (AF153731) labeled as *Xylaria* sp.,

isolated as an endophyte of *Livistonia chinensis* in Hong Kong (Guo *et al.* 2000). These four strains were grouped in a clade with total statistical support. Another xylarin-producing strain (F-230,275) isolated from plant material in Argentina appeared rooted to the base of this branch, and all these five sequences were grouped in a clade with very high clade credibility (98 %).

Other four xylarin-producing strains were also grouped in a monophyletic clade, together with a sequence from a strain identified as *X. mellissii*. Those strains came from diverse tropical areas, including Equatorial Guinea (F-067,683 and F-069,049), Venezuela (F-065,308), and Republica Dominicana (F-065,977). The two strains from Equatorial Guinea were almost identical in sequence (99 % homology), strongly suggesting they would be conspecific, and all the sequences in the clade shared >93 % homology, what could be interpreted as all of them being also conspecific with *X. mellissii*. Unfortunately, we have not been able to check whether the strain identified as *X. mellissii* produces xylarin as well, due to the lack of viability upon preservation at −70 °C.

The xylarin-producing strain F-068,980, isolated from bark collected in Equatorial Guinea, was relatively close to a branch containing two *Xylaria* species, *X. cornudamae* and *X. fioriana*, although the support for the clade containing these three sequences was rather weak (67 %). This small clade fell within a branch containing a number of other *Xylaria* species (*X. allantoidea*, *X. enteroleuca*, *X. acuta*, *X. longipes*) and *Stilbohypoxylon quisquiliarum* with very good statistical support (97 %). This suggests this strain would most likely belong to a related *Xylaria* species.

The sordarin-producing strain F-254,988, an endophyte of *Scirpus holoschoenus* from Spain, was clustered together with two *Xylaria* species (*X. globosa* and *X. schweinitzii*) in a branch with high clade credibility, although the nucleotide homology with those sequences was low (77 %).

It is interesting to remark that all these strains showed affinities with the *Xylarioideae*. Actually, we did not find any strains clearly related to the *Hypoxyloideae*, although *Hypoxylon croceum* has been reported as producing sordarin and hypoxysordarin (Daferner *et al.* 1999).

Lasiosphaeriaceae

Although we did not find any new isolate from this family producing sordarin, we subjected to ITS sequencing some strains from culture collections that have been reported as sordarin producers, which were used as controls for the screening process. These strains were shown to belong to the *Lasiosphaeriaceae*. The phylogenetic tree in Fig 5 shows the relationships of those strains with other members of the family. The size of the alignment was 456 characters, 243 of which were constant.

The fungal strain MF6255 is a duplicate of the strain deposited by Sandoz as the original producer of sordarin, obtained from ATCC (ATCC36386/NRRL3196). The strain was originally identified as *Sordaria araneosa*, and its taxonomic adscription gave name to this family of antifungal compounds. Interestingly, our ITS sequence analysis showed that this strain is not a member of genus *Sordaria*, bearing very little homology to any sequence of other *Sordaria* species in GenBank. However, the strain was found to be phylogenetically closely related to a number of *Podospora* species (*P. myriaspora*, *P. decipiens* and *P. pleiospora*), as shown in Fig 5. Nucleotide homology with *P. myriaspora* and *P. decipiens* in particular was very high (97 %). *Sordaria araneosa* had been synonymized with *Podospora araneosa* (Cain 1962), therefore our molecular data would appear consistent with this synonymy. However, the sordarin-producing strain is not conspecific with the only sequence of *P. araneosa* in GenBank (EF197073). This may be either because the latter sequence is incorrectly labeled or because the sordarin-producing strain is not really *S. araneosa*/*P. araneosa*. In the absence of other sequences of this species to provide further verification, our data just suggest that the original sordarin producing strain is akin to several *Podospora* species. Interestingly, a strain of *P. pleiospora*, a species that appears to be close to the original sordarin producer, has been also reported to produce sordarin and a number of analogues (Table 1; Weber *et al.* 2005).

An obvious implication of these findings is that the name of sordarins applied to this class of compounds, is somewhat misleading. The production of sordarins is not apparently a feature associated to genus *Sordaria* or to the *Sordariaceae* but to the *Lasiosphaeriaceae*, and even more frequent in other fungal families, as shown above.

Within the *Lasiosphaeriaceae*, we also confirmed the production of sordarins from the strain of *Zopfiella marina* CBS 155.77 (=SANK 21274, reported to produce zofimarin (Ogita *et al.* 1987, Sato *et al.* 1995);=NHL 2731, type of the species). Our results showed that this strain produced both zofimarin and sordarin. In the phylogenetic tree, the type strain of *Z. marina* appeared very closely related (98 % nucleotide homology) with the type strain of *Apodus oryzae* (CBS 376.74), and as a sister group of two strains of *Z. latipes*, within a clade with high credibility support (93 %). Cai *et al.* (2006a) reported that genus *Apodus* fits better in the *Lasiosphaeriaceae* than in the *Sordariaceae*, where it was originally ascribed; and later on they reported a close relationship between *A. oryzae* and *Z. latipes* based on multi-gene molecular phylogenies (Cai *et al.* 2006b), but this finding was not subjected to any interpretation. Our results confirm this relationship, and include an additional dimension by placing *Z. marina* as another tightly related species. The high nucleotide homology with *A. oryzae* even suggests the potential synonymy between these two species. Actually, their original descriptions (Furuya & Udagawa 1975; Arx 1975) reported remarkably similar characters, and the minor differences could probably be attributed to strain to strain (and lab to lab) variations. However, we have not compared morphologically these two strains *vis-à-vis*, neither tested any strain of *A. oryzae* to check for the production of sordarin analogues.

It is worth noting that all the strains producing sordarins, although belonging to different genera, were grouped together in a clade with total statistical support.

The phylogenetic tree in Fig 5, on the other hand, is fully consistent with previous molecular analysis on the *Lasiosphaeriaceae*. Except for the species of *Lasiosphaeria*, which appeared clustered together in a branch with total statistical support as in Miller & Huhndorf (2004), the rest of genera in the tree (*Podospora, Cercophora* and *Zopfiella*) appeared to be polyphyletic, in agreement with previous reports (Cai *et al.* 2005, 2006b).

Sordarins as chemotaxonomic markers

The data reported here can be used to explore potential correlations between the production of specific sordarin derivatives

and the taxonomic placement and phylogenetic affinities of the producing organisms. In this regard, a few points are noteworthy. Thus, sordarin and xylarin were the derivatives more frequently found in our screening (8 and 11 strains respectively), but while the production of sordarin appeared to be spread across all the fungal families reported here, xylarin was confined to the *Xylariaceae*, where it is most frequent in *Xylaria* spp. Zofimarin was far less frequent, with only three strains, two of them identified as *Eutypa tetragona*, within the *Diatrypaceae*. The new compounds 4′-O-demethyl sordarin and isozofimarin were found only in one strain. Last, there seem to be some rather infrequent sordarin derivatives (e.g., moriniafungin, BE-31405, GR135402), for which our extensive screening program did not yield any further producing strains beyond those reported in the literature.

One of the main conclusions from this study is a confirmation and extension of scattered historical data of the distribution of sordarins across the *Xylariales*. Sordarins are particularly frequent in the *Xylariaceae*, but extend also to the *Amphisphaeriaceae* and the *Diatrypaceae*, the latter being implicated for the first time in the present report.

Despite including in our screen significant numbers of isolates from the *Sordariales, Eurotiales* and *Microascales*, orders where the production of sordarins has been reported, we did not find any producing strain, suggesting that sordarin production is a rare feature in these groups. Although it is possible that our screening systems (including cultivation techniques) could favor sordarin production in *Xylariales*, we did detect sordarins also in control strains from the *Lasiosphaeriaceae* (*S. araneosa* and *Z. marina*). Finally, other fungal groups that have been massively subjected to screen, such as the *Pleosporales, Hypocreales* or *Helotiales*, seem to lack the ability to synthesize this class of compounds.

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REFERENCES

Acero FJ, González V, Sánchez-Ballesteros J, Rubio V, Checa J, Bills GF, Salazar O, Platas G, Peláez F, 2004. Molecular phylogenetic studies on the Diatrypaceae based on rDNA–ITS sequences. *Mycologia* **96**: 249–259.

Arx JA von, 1975. On *Thielavia* and some similar genera of ascomycetes. *Studies in Mycology* **8**: 1–29.

Bahl J, Jeewon R, Hyde KD, 2005. Phylogeny of *Rosellinia capetribulensis* sp. nov. and its allies (*Xylariaceae*). *Mycologia* **97**: 1102–1110.

Balkovec JM, Tse B, 2000. Sordarin derivatives. US patent No 6,040,463.

Basilio A, Justice MC, Harris GH, Bills GF, Collado J, de la Cruz M, Díez MT, Hernández P, Liberator PA, Kahn JN, Peláez F, Platas G, Schmatz DM, Shastry M, Tormo JR, Vicente MF, 2006. The discovery of moriniafungin, a novel sordarin derivative produced by *Morinia pestalozzioides*. *Bioorganic Medicinal Chemistry* **14**: 560–566.

Bills GF, Polishook JD, 1993. Selective isolation of fungi from dung of *Odocoileus hemionus* (mule deer). *Nova Hedwigia* **57**: 195–206.

Bills GF, Polishook JD, 1994. Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. *Mycologia* **86**: 187–198.

Bills GF, Giacobbe RA, Lee SH, Peláez F, Tkacz JS, 1992. Tremorgenic mycotoxins paspalitrem A and C, from a tropical *Phomopsis*. *Mycological Research* **96**: 977–983.

Bills GF, Platas G, Peláez F, Masurekar P, 1999. Reclassification of a pneumocandin-producing anamorph, *Glarea lozoyensis* gen. et sp. nov., previously identified as *Zalerion arboricola*. *Mycological Research* **103**: 179–192.

Bills GF, Platas G, Fillola A, Jimenez R, Collado J, Vicente MF, Martín J, González del Val A, Bur-Zimmerman J, Tormo JR, Peláez F, 2008. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *Journal of Applied Microbiology* **104**: 1644–1658.

Bitzer J, Köpcke B, Stadler M, Hellwig V, Ju Y-M, Seip S, Henkel T, 2007. Accelerated dereplication of natural products, supported by reference libraries. *Chimia International Journal for Chemistry* **61**: 332–338.

Cabello MA, Platas G, Collado J, Díez MT, Martín I, Vicente F, Meinz M, Onishi JC, Douglas C, Thompson J, Kurtz MB, Schwartz RE, Bills GF, Giacobbe RA, Abruzzo GK, Peláez F, 2001. The discovery of arundifungin, a novel antifungal compound produced by fungi. Biological activity and taxonomy of the producing organisms. *International Microbiology* **4**: 93–102.

Cai L, Jeewon RA, Hyde KD, 2005. Phylogenetic evaluation and taxonomic revision of *Schizothecium* based on ribosomal DNA and protein coding genes. *Fungal Diversity* **19**: 1–21.

Cai L, Jeewon RA, Hyde KD, 2006a. Phylogenetic investigations of Sordariaceae based on multiple gene sequences and morphology. *Mycological Research* **110**: 137–150.

Cai L, Jeewon RA, Hyde KD, 2006b. Molecular systematics of *Zopfiella* and allied genera: evidence from multi-gene sequence analysis. *Mycological Research* **110**: 359–368.

Cain RF, 1962. Studies on coprophilous ascomycetes VIII. New species of *Podospora*. *Canadian Journal of Botany* **40**: 447–490.

Collado J, Platas G, Peláez F, 1996. Fungal endophytes in leaves twigs and bark of *Quercus ilex* from central Spain. *Nova Hedwigia* **63**: 347–360.

Collado J, Platas G, Peláez F, 2001. Identification of an endophytic *Nodulisporium* sp. from *Quercus ilex* in Central Spain as the anamorph of *Biscogniauxia mediterranea* by rDNA sequencing analysis and effect of different ecological factors on distribution of the fungus. *Mycologia* **93**: 875–886.

Collado J, Platas G, Bills GF, Basilio A, Vicente MF, Tormo JR, Hernández P, Díez MT, Peláez F, 2006. Studies on *Morinia*: recognition of *Morinia longiappendiculata* sp. nov. as a new endophytic fungus, and a new circumscription of *Morinia pestalozzioides*. *Mycologia* **98**: 616–627.

Collado J, Platas G, Paulus B, Bills GF, 2007. High-throughput culturing of fungi from plant litter by a dilution-to-extinction technique. *FEMS Microbiology Ecology* **60**: 521–533.

Connors N, Pollard D, 2005. Pneumocandin B_0 production by fermentation of the fungus *Glarea lozoyensis*: physiological and engineering factors affecting titer and structural analogue formation. In: An Z (ed), *Mycology Series*. *Handbook of Industrial Mycology*, vol. 22. Marcel Dekker Inc., New York, pp. 515–538.

Coval SJ, Puar MS, Phife DW, Terracciano JS, Patel M, 1995. SCH 57404 an antifungal agent possessing the rare sordaricin skeleton and a tricyclic sugar moiety. *Journal of Antibiotics* **48**: 1171–1172.

Daferner M, Mensch S, Anke T, Sterner O, 1999. Hypoxysordarin, a new sordarin derivative from *Hypoxylon croceum*. *Zeitschrift für Naturforschung* **54c**: 474–480.

Davoli P, Engel G, Werle A, Sterner O, Anke T, 2002. Neosordarin and hydroxysordarin, two new antifungal agents from *Sordaria araneosa*. *Journal of Antibiotics* **55**: 377–382.

Dominguez JM, Martin JJ, 1998. Identification of elongation Factor 2 as the essential protein targeted by sordarins in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **42**: 2279–2283.

Duetz WA, Rüedi L, Hermann R, O'Connor K, Büchs J, Witholt B, 2000. Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Applied and Environmental Microbiology* **66**: 2641–2646.

Fukushima M, Watanabe E, Eguchi T, Kakinuma K, 2006. Biosynthesis of zofimarin diterpene glycoside antifungal compound. *Nippon Kagakkai Koen Yokoshu* **86**: 1314.

Furuya K, Udagawa S, 1975. Two new species of cleistothecial Ascomycetes. *Journal of Japanese Botany* **50**: 249–254.

Gaber RF, Copple DM, Kennedy BK, Vidal M, Bard M, 1989. The yeast gene ERG6 is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. *Molecular and Cellular Biology* **9**: 3447–3456.

Glawe DA, Rogers JD, 1982. Observations on the anamorphs of six species of *Diatrype* and *Diatrypella*. *Canadian Journal of Botany* **60**: 245–251.

Guo LD, Hyde KD, Liew ECY, 2000. Identification of endophytic fungi from Livistona chinensis based on morphology and rDNA sequences. *New Phytologist* **147**: 617–630.

Hall CC, Bertasso AB, Watkins JD, Georgopapadakou NH, 1992. Screening assays for protein synthesis inhibitors. *Journal of Antibiotics* **45**: 1697–1699.

Hauser D, Sigg HP, 1971. Isolierung und abbau von sordarin. *Helvetica Chimica Acta* **54**: 1178–1190.

Herreros E, Almela MJ, Lozano S, Gómez de las Heras F, Gargallo-Viola D, 2001. Antifungal activities and cytotoxicity studies of six new azasordarins. *Antimicrobial Agents and Chemotherapy* **45**: 3132–3139.

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson K-H, Lichtwardt R, Longcore J, Miądlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J, Zhang N, 2007. A higher-level phylogenetic classification of the. *Fungi*. *Mycological Research* **111**: 509–547.

Hirano A, Torigoe K, Ogawa H, Kamiya S, Okada H, Nagashima M, Kojiri K, Suda H, 2000. Derivatives of antifungal substance BE-31405 and process for producing the same. US Patent No 6087390.

Jenny L, Borschborg H-J, 1995. Synthesis of the dolabellane diterpene hydrocarbon (±)-*δ*- araneosene. *Helvetica Chimica Acta* **78**: 715–731.

Jeewon R, Liew ECY, Hyde KD, 2002. Phylogenetic relationships of *Pestalotiopsis* and allied genera inferred from ribosomal DNA sequences and morphological characters. *Molecular Phylogenetics and Evolution* **25**: 378–392.

Jiang B, Xu D, Allocco J, Parish C, Davison J, Veillette K, Sillaots S, Hu W, Rodríguez-Suárez R, Trosok S, Zhang L, Li Y, Rahkoodaee F, Ransom T, Martel N, Wang H, Gauvin D, Wiltsie J, Wisniewski D, Salowe SP, Kahn JN, Hsu M-J, Giacobbe R, Abruzzo G, Flattery A, Youngman P, Wilson K, Bills G, Platas G, Peláez F, Díez MT, Kauffman S, Becker J, Harris G, Liberator P, Roemer T, 2008. PAP inhibitor with *in vivo* efficacy identified by *Candida albicans* genetic profiling of natural products. *Chemistry and Biology* **15**: 363–374.

Justice M, Hsu MJ, Tse B, Ku T, Balkovec J, Schmatz D, Nielsen J, 1998. Elongation factor 2 as a novel target for selective inhibition of fungal protein synthesis. *Journal of Biological Chemistry* **273**: 3148–3151.

Kamai Y, Kakuta M, Shibayama T, Fukuoka T, Kuwahara S, 2005. Antifungal activities of R-135853, a sordarin derivative, in experimental candidiasis in mice. *Antimicrobial Agents and Chemotherapy* **49**: 52–56.

Kaneko S, Arai M, Uchida T, Konosu T, 2003. Antimycotic agent containing zofimarin derivative having oxazepane ring. Japan Patent No JP2003261450.

Kennedy TC, Webb G, Cannell RJP, Kinsman OS, Middleton RF, Sidebottom PJ, Taylor NL, Dawson MJ, Buss AD, 1998. Novel inhibitors of fungal protein synthesis produced by a strain of *Graphium putredinis*. Isolation, characterization and biological properties. *Journal of Antibiotics* **51**: 1012–1018.

Kinsman OS, Chalk PA, Jackson HC, Middleton RF, Shuttleworth A, Rudd BAM, Jones CA, Noble HM, Wildman HG, Dawson MJ, Stylli C, Sidebottom PJ, Lamont B, Lynn S, Hayes MV, 1998. Isolation and characterisation of an antifungal antibiotic (GR135402) with protein synthesis inhibition. *Journal of Antibiotics* **51**: 41–49.

Lee S, Crous PW, Wingfield MJ, 2006. Pestalotioid fungi from Restionaceae in the Cape Floral Kingdom. *Studies in Mycology* **55**: 175–187.

Mander LN, Robinson RP, 1991. Studies on the synthesis of the diterpenoid mold metabolite sordaricin. Exploration of a prospective biogenetic intramolecular [4 + 2] cycloaddition. *Journal of Organic Chemistry* **56**: 3595–3601.

Martin JJ, Chicharro-Gonzalo J, Gómez JRR, Garcia-Ochoa S, Gómez de las Heras F, Hayes MV, Dawson MJ, Wildman HG, Hall RM, 2000. Antifungal sordaridin derivatives. US patent No 6054478.

Miller AN, Huhndorf SM, 2004. Using phylogenetic species recognition to delimit species boundaries within *Lasiosphaeria*. *Mycologia* **96**: 1106–1127.

Nylander JA, 2004. *MrModeltest v2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Ogita T, Hayashi A, Sato S, Furaya K, 1987. Antibiotic zofimarin. Japan Kokai Tokkyo Koho JP 62-40292.

Okada H, Kamiya S, Shina Y, Suwa H, Nagashima M, Nakajima S, Shimokawa H, Sugiyama E, Kondo H, Kojiri K, Suda H, 1998. BE-31405, a new antifungal antibiotic produced by *Penicillium minioluteum*. I. Description of producing organism, fermentation isolation, physico-chemical and biological properties. *Journal of Antibiotics* **51**: 1081–1086.

Parish CA, Smith SK, Calati KB, Zink DL, Wilson KE, Roemer T, Jiang B, Xu D, Bills GF, Platas G, Peláez F, Díez MT, Tsou NN, McKeown AE, Ball RG, Powles MA, Yeung L, Liberator PA, Harris GH, 2008. Isolation and structure elucidation of parnafungins, antifungal natural products that inhibit mRNA poly-adenylation. *Journal of the American Chemical Society* **130**: 7060–7066.

Peláez F, Collado J, Platas G, Díez MT, Basilio A, González A, Vicente F, Dai P, Harris G, Rosenbach M, Dreikorn S, Thompson J, 2001. Studies on *Tricellulortus pepoformis*, a tropical mitosporic fungus. *Nova Hedwigia* **72**: 217–230.

Peláez F, Genilloud O, 2003. Discovering new drugs from microbial natural products. In: Barredo JL (ed), *Microorganisms for Health Care, Foods and Enzyme Production*. Research Signpost, Trivandrum, India, pp. 1–22.

Petrini LE, Petrini O, 2005. Morphological studies in *Rosellinia (Xylariaceae)*: the first step towards a polyphasic taxonomy. *Mycological Research* **109**: 569–580.

Platas G, Salazar O, Acero J, Sánchez-Ballesteros J, Rubio V, González V, Portal MA, Borkowski J, Peláez F, 2001. Presence of a simple tandem repeat DNA sequence in the ITS1 region of the Xylariales. *Current Microbiology* **43**: 43–50.

Polishook JD, Peláez F, Platas G, Ondeyka JG, Dombrowskii A, Terán AM, 2001. Biogeography and relatedness of *Nodulisporium* strains producing nodulisporic acid. *Mycologia* **93**: 1125–1137.

Pongcharoen W, Rukachaisirikul V, Phongpaichit S, Kühn T, Pelzing M, Sakayarog J, Taylor WC, 2008. Metabolites from the endophytic fungus *Xylaria* sp. PSU-D14. *Phytochemistry* **69**: 1900–1902.

Rappaz F, 1987. Taxonomie et nomenclature de *Diatrype stigma, D. decorticata* et *D. undulata* (Diatrypaceae, Ascomycetes). *Mycotaxon* **30**: 209–219.

Ronquist F, Huelsenbeck JP, 2003. MRBAYES 3: bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Sato A, Takahashi S, Ogita T, Sugano M, Kodama K, 1995. Marine natural products. *Annual Reports of Sankyo Research Laboratories* **47**: 1–58.

Schneider G, Anke H, Sterner O, 1995. Xylarin, an antifungal *Xylaria* metabolite with an unusual tricyclic uronic acid moiety. *Natural Products Letters* **7**: 309–316.

Scott PM, Lawrence JW, van Walbeek V, 1970. Detection of mycotoxins by thin-layer chromatography: applications to screening of fungal extracts. *Applied Microbiology* **20**: 839–842.

Singh SB, Jayasuriya H, Dewey R, Polishook JD, Dombrowski AW, Zink DL, Guan Z, Collado J, Platas G, Peláez F, Felock PJ, Hazuda DJ, 2003. Isolation, structure and HIV-1-integrase inhibitory activity of structurally diverse fungal metabolites. *Journal of Industrial Microbiology and Biotechnology* **30**: 721–731.

Stadler M, Hellwig V, 2005. Chemotaxonomy of the Xylariaceae and remarkable bioactive compounds from Xylariales and their associated asexual stages. *Recent Research Developments in Phytochemistry* **9**: 41–93.

Serrano-Wu M, Du X, Balasubramanian N, St Laurent DR, 2002. Thio derivatives of sordarin as antifungal agents. US Patent No 6436930.

Sutton BC, 1973. Hyphomycetes from Manitoba and Saskatchewan, Canada. *Mycological Papers* **132**: 1–143.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.

Weber RWS, Meffert A, Anke H, Sterner O, 2005. Production of sordarin and related metabolites by the coprophilous fungus *Podospora pleiospora* in submerged culture and in its natural substrate. *Mycological Research* **109**: 619–626.

Yao Q, 2007. Biosynthetic studies on fungal diterpene antibiotics. MSc. thesis, Oregon State University.

Zink D, Dufresne C, Liesch J, Martin J, 2002. Automated LC–MS analysis of natural products: extraction of UV, MS and retention time data for component identification and characterization. Poster presented at the American Society for Mass Spectrometry (ASMS) 50th Annual Meeting.

Zink D, Dufresne C, Liesch J, Martin J, 2005. Identification/dereplication of natural products by LC–UV–MS Spectral search parameters. Poster presented at the Small Molecule Science Conference (COSMOS).