 Doi: 10.1111/j.1365-3059.2006.01439.x

Phylogenetic analysis based on ITS sequences and conditions affecting the type of conidial germination of *Bipolaris oryzae*

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One taxonomic characteristic of *Bipolaris* species is the bipolar germination of conidia, but conidia of *Bipolaris oryzae*, the causal pathogen of brown spot in rice, are regularly observed to show intercalary germination, a characteristic of *Drechslera* species. The effect of selection, culture media and culture age on type of conidial germination was determined for three brown spot isolates from Cavinti, San Pablo and Palawan in the Philippines, obtained from infected leaves showing typical disease symptoms. Based on the analyses of their ITS1, ITS2 and 5·8S rDNA nucleotide sequences, the local isolates were clearly identified as *B. oryzae*. Selection for colonies of the three isolates derived from single conidia with either bipolar or intercalary germination had no effect on the number of spores showing bipolar germination in subsequent cultures. Germination on seven different culture media was tested; of these, rabbit food agar and water agar increased the percentage of bipolar germination of conidia, although this varied between isolates. Incubation of the cultures of all three isolates for longer periods prior to harvesting conidia increased the percentage of bipolar-germinating conidia from *c*. 40 to *c*. 90% with 5-day-old and 30-day-old cultures, respectively.

Keywords: bipolar germination, *Bipolaris oryzae*, brown spot of rice, *Cochliobolus*, conidial germination, *Oryza sativa*

Introduction

Brown spot is a fungal disease of rice (*Oryza sativa*) with a worldwide distribution in the rice-growing countries in Asia, America and Africa. It is prevalent in rainfed lowlands and uplands, or in situations of abnormal or poor soil conditions. Brown spot has been noted to reduce yields from 6 to 90% in Asia (Padmanabhan, 1973; Estrada, 1984; Mew & Gonzales, 2002). In the Philippines brown spot is still considered a minor disease, but its occurrence has been observed repeatedly in many rice-growing areas of the country (Estrada, 1984). Ocfemia (1924) first reported 10–58% seedling mortality associated with brown spot, and originally identified it under the name *Helminthosporium oryzae*.

The taxonomy of graminicolous species of *Helminthosporium sensu lato* has long been controversial (Subramanian & Jain, 1966). Shoemaker (1959) suggested that the name *H. oryzae* be changed to *Bipolaris oryzae* because of the bipolar nature of the conidial germination. However, various workers observed that the conidia of the fungus also germinated in an amphigenous manner, thus Subramanian & Jain (1966) described the fungus as *Drechslera oryzae*. Drechsler (1934) considered that the age or maturity of conidia affects the type of conidial germination. In addition, Alcorn (1983) also observed that germination substrate greatly affected the manner by which *Bipolaris maydis* conidia germinated.

Berbee *et al*. (1999); Olivier *et al*. (2000) and Zhang & Berbee (2001) conducted phylogenetic analyses of the glyceraldehyde-3-phosphate, ITS1, ITS2 and 5·8S rRNA sequences of a number of pleosporaceaeous fungi, including one isolate of *B. oryzae*. Sequence comparisons indicated that *B. oryzae* clustered with fungi with a perfect stage of *Cochliobolus*, which had been divided into two groups. The perfect stage of the fungus was originally classified by Ito & Kuribayashi (1927) as *Ophiobolus miyabeanus*, but the fungus was later transferred to the genus *Cochliobolus* as *Cochliobolus miyabeanus* by Drechsler (1934).

After Ito (1930) proposed the name *Drechslera* for *Helminthosporium* species having cylindrical conidia germinating from any cell, Shoemaker (1959) redefined the genus and segregated the fusoid-spored forms with bipolar germination to create a new genus, *Bipolaris*.

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Accepted 6 March 2006

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Leonard & Suggs (1974) later erected *Exserohilum* to separate species that germinated in a bipolar fashion with a protruding hilum at the base of the conidium, from *Bipolaris* species that germinated in a bipolar fashion with only a slightly protruding hilum. However, there are differing opinions: for example, Subramanian & Jain (1966) placed the former *Helminthosporium* species from *Bipolaris* back to *Drechslera*.

This study considers the taxonomy of the organism infecting rice, and the plasticity of the germination type of *B. oryzae*, in order to correctly identify and classify the organism causing brown spot of rice. A quantitative examination was made of several factors affecting germination type (bipolar, intercalary and monopolar) exhibited by three different isolates of *B. oryzae* collected from different locations in the Philippines. The factors examined for their effect on type of conidial germination were: (i) selection for a particular conidial phenotype to determine whether it affected germination type in subsequent cultures; (ii) the effect of culture media used to germinate the conidia; and (iii) the effect of the age of culture used to harvest the conidia. In addition, a phylogenetic analysis was undertaken of ITS1, ITS2 and 5·8S rRNA sequences of a number of *B. oryzae* isolates with different germination types. A number of sequences of additional fungi in the Pleosporaceae, not previously analysed for these sequences, were also included and the results were compared with the phylogenetic placement of the isolate of *B. oryzae* described by Berbee *et al.* (1999); Olivier *et al.* (2000) and Zhang & Berbee (2001).

Materials and methods

Isolates

Leaves infected with brown spot disease were collected from a field at San Pablo and a field at Cavinti in Laguna Province on the island of Luzon, and from one field on the island of Palawan, the Philippines.

Lesions were cut and surface sterilized with 1% chlorox for 3 min, rinsed twice with sterile distilled water, and dried with a sterile paper towel. Surface-sterilized lesions were incubated on rabbit food agar (RFA; Hau & Rush, 1980: 50 g L^{-1} steeped filtrate of rabbit food pellets plus 15 g agar) under alternating 12 h light and 12 h dark for 7 days, to allow for conidial production. Light sources used were near-ultraviolet (nUV, 320–400 nm lamp), Philips TLD 36 W/08 and daylight fluorescent tube Philips TL40W/54.

Conidia were transferred from 7-day-old cultures onto water agar (WA; Alcorn, 1983: 20 g L^{-1} agar). After 12 h at 28°C under alternating 12 h light and 12 h darkness, conidia were observed under the dissecting microscope. A block of agar with either a single bipolar, intercalary or monopolar-germinating conidium was transferred aseptically to potato dextrose agar (PDA, Difco) slants with a flame-sterilized scalpel to create monoconidial cultures. The isolates were allowed to grow for 10–14 days on sterile filter paper pieces placed on the agar. The filter paper pieces were then removed from the agar and stored in air-tight containers at 5°C.

Effect of selection on germination type

A single conidium with either a bipolar, intercalary or monopolar germination type, following 7 days' incubation under alternating 12 h light/dark at 28°C on RFA, was transferred to RFA medium using a heat-flamed, fine-pointed capillary tubing. The culture was then incubated for 7 days at 28°C under alternating 12 h light/dark, and conidia were washed from the plates with sterile distilled water. After adjusting to $2{\cdot}0 \times 10^3$ conidia mL^{-1}, 0·1 mL was spread onto RFA in three Petri dishes per isolate. Following incubation at 28°C for 12 h, random samples of 100 conidia were examined under the microscope, and the number of conidia showing bipolar, intercalary and monopolar germination was counted.

Effect of culture media on germination type

A suspension of $2{\cdot}0 \times 10^3$ conidia mL^{-1} was prepared from the original isolations prior to single-spore isolation, and 0·1 mL was spread onto several media selected because they had been used previously for culturing *B. oryzae* or related fungi: PDA (Noble, 1965); malt extract agar (MEA) (Shoemaker, 1962); rice leaf agar (RLA), a modification of wheat straw on water agar (Alcorn, 1983); rice polish agar (RPA) (Imam & Schroeder, 1966); RFA or WA. The same suspension was also deposited on top of a glass slide (WG) (Alcorn, 1983). After incubation of inoculated media at 28°C for 12 h, random samples of three sets of 100 conidia were examined under the microscope, and the mean number of conidia showing bipolar, intercalary and monopolar germination was counted.

Effect of conidial maturation on germination type

A 0·1-mL aliquot of $2{\cdot}0 \times 10^3$ conidia mL^{-1}, prepared from the original isolations prior to single-spore isolation, was spread onto three RFA plates per isolate. Plates were incubated for 5, 10, 20 and 30 days under alternating near-UV light and darkness at 28°C. Conidia were then harvested and plated onto RFA. After incubation at 28°C for 12 h, random samples of three sets of 100 conidia were examined under the microscope, and the mean number of conidia showing bipolar, intercalary and monopolar germination was counted.

The effects on conidial germination of selection, culture media and conidial maturation were subjected to ANOVA for a completely randomized design, and treatment means were compared using least significant difference ($P = 0{\cdot}05$) for the data in Table 2, and Tukey–Kramer tests ($P = 0{\cdot}05$) for the data in Table 3.

DNA isolation for ITS sequencing

Scraped mycelium from a monoconidial PDA culture was added to 20 mL potato dextrose broth (PDB),

and incubated for 2–3 days with continuous shaking at 100 r.p.m. at room temperature (20°C). The mycelium was harvested by filtration through filter paper and immediately freeze-dried.

DNA was isolated following the method of Dr Mostafa Kamal, Bangladesh Rice Research Institute, Banani, Dhaka, Bangladesh (personal communication, 2003). Frozen mycelia were ground in liquid nitrogen using a sterile mortar and pestle. About 420 μg ground, desiccated mycelia were placed in 2-mL microcentrifuge tubes and homogenized with *c.* 840 μL extraction buffer (100 mM Tris–HCl, 100 mM EDTA, 250 mM NaCl). 10% Sodium dodecyl sulphate (84 μL) was then added and the solution gently mixed. Tubes were incubated at 65°C for 30 min, and 370 μL 3 M sodium acetate was then added, followed by incubation at 4°C for 15 min. Each centrifugation was at 8750 *g*. After centrifugation for 15 min, the aqueous phase was transferred to another microcentrifuge tube and 0·5 mL chloroform was added. Following centrifugation for 30 min, the supernatant was collected, and absolute ethanol at twice the volume of the supernatant was added and incubated at 4°C for 10 min. After centrifugation for 5 min, the DNA pellet was washed with 200 μL 70% ethanol, centrifuged for 2 min and the supernatant removed. The pellet was resuspended in 50–100 μL Tris-EDTA (Sambrook *et al.*, 1989). Each centrifugation was at 8750 *g*.

PCR and sequencing for phylogenetic analysis

The isolates used in the phylogenetic analyses are described in Table 1. The nucleotide sequences for ITS1, ITS2 and 5·8S rDNA were obtained following amplification using primers ITS4 and ITS5 (White *et al.*, 1990). Each 25-μL reaction contained 1 ng fungal DNA, sterile distilled water, 10 × PCR buffer (Mg^{2+}-free) (Promega), 2 mM dNTPs (Roche Diagnostics), 25 pmol each of primers ITS4 and ITS5, and 1 U Taq DNA polymerase. Reactions were carried out in a PTC-100 Programmable Thermal Controller (MJ Research) with 30 s at 94°C followed by 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 30 s, with a final extension at 72°C for 30 s. All amplification reactions were carried out in Costar microtitre plates (Corning Corp.). Sterile distilled water was included as negative control to ensure no other DNA template was present in the PCR mixture.

Analysis of PCR products was carried out by gel electrophoresis using 2% agarose–Synergel (Laboratorios Conda/Diversified Biotech; 0·5 : 0·75%, w : w) in 0·5 × Tris-borate buffer. DNA fragments were stained with ethidium bromide, and an Alpha Imager system (Alpha Innotech) was used to photograph the gels. Purification and sequencing of the PCR products was done by Macrogen Inc.

ITS sequence alignments and analyses

Sequences were determined in both directions, and consensus sequences were constructed using SEQUENCHER 4·2 (Gene Codes). As the alignment of the sequences of the different germination types from the isolates of *B. oryzae* from Cavinti, Palawan and San Pablo using the program CLUSTALW (Thompson *et al.*, 1994) showed that they were all identical, only one sequence from each of these locations was included in the phylogenetic analyses. The ITS1, ITS2 and 5·8S rDNA sequences from a set of 46 other fungal isolates were collected from GenBank (Table 1), including *Leptosphaeria bicolor*, which was selected as an outgroup based on a previous study of *Helminthosporium* species (Olivier *et al.*, 2000).

Maximum parsimony analysis (Saitou & Nei, 1987) was used to construct a phylogenetic tree using the program DNA Pars in PHYLIP ver. 3·6. The robustness of the branching patterns was determined by bootstrap analysis using the program SeqBoot in PHYLIP ver. 3·6.

Results

Bipolaris oryzae isolates obtained from single, characteristic leaf lesions, collected at Cavinti, Palawan and San Pablo, the Philippines, produced three different germination types (bipolar, intercalary and monopolar) when conidia were washed from 7-day-old RFA cultures and germinated on RFA (Fig. 1). Bipolar germination was defined as formation of germ tubes from each of the terminal (polar) cells on agar medium; intercalary germination as the formation of germ tubes from intermediate and polar cells; and monopolar germination as the formation of a germ tube from only one polar cell.

Effect of selection on conidial germination

For the Cavinti, Palawan and San Pablo isolates, the second-generation germination type-selected cultures described above were given the name of the original isolate (location) followed by the code B, I or M (Table 2). Germination types of conidia from the three germination type-selected cultures were then observed on RFA (Table 2). A χ^2 test of those results ($P < 0{\cdot}001$) indicated that selection for a certain conidial germination phenotype to start a culture had an effect on the frequency distribution of the different germination types. However, this effect was often negative, resulting in lower levels of a particular germination type being selected for, compared with selection for a different germination type (Table 2). The percentage of different germination types varied across all selected isolates, but regardless of whether the culture originated from a bipolar or nonbipolar germinating conidium, it was the bipolar germination type that predominated over the other two types.

For the Cavinti isolates, selection for bipolar germination (Cavinti-B) did not result in significantly higher percentage bipolar germination than selection for intercalary germination in the next generation (Table 2). Selection for intercalary germination (Cavinti-I) resulted in less intercalary germination in the next fungal generation, compared with isolate Cavinti-M, selected for monopolar germination. The latter isolate produced the lowest (2%) monopolar germination after 12 h incubation on RFA.

For the Palawan isolates, Palawan-M produced significantly more bipolar germinating conidia than the other

Table 1 List of fungi used in this study and their isolate strain designation, GenBank accession number and depositor

Anamorph[a]	Teleomorph[a]	Isolate/strain	GenBank	Source/depositor
Bipolaris australis	*Cochliobolus* sp.	77139	AF081448	Berbee[d]
B. crustacea	*Cochliobolus* sp.	8225-1	AF163070[b]	Goh and Hyde[e]
B. eleusines	*C. eleusines*	BRIP 15826	AF163071[c]	Goh and Hyde[e]
B. heveae 1	*C. heveicola*	Cyn-1	AB179834[c]	Tsukiboshi, Chung and Yoshida[f]
B. heveae 2	*C. heveicola*	Zoy-1	AB179831[c]	Tsukiboshi, Chung and Yoshida[f]
B. iridis	*Cochliobolus* sp.	DAR29777	AF163062[b]	Goh and Hyde[e]
B. oryzae	*C. miyabeanus*	24/89	X78122	Rollo[g]
B. oryzae CB	*C. miyabeanus*	Cavinti-Bipolar	DQ300202	This study
B. oryzae CI	*C. miyabeanus*	Cavinti-Intercalary	DQ300203	This study
B. oryzae CM	*C. miyabeanus*	Cavinti-Monopolar	DQ300204	This study
B. oryzae PB	*C. miyabeanus*	Palawan-Bipolar	DQ300199	This study
B. oryzae PI	*C. miyabeanus*	Palawan-Intercalary	DQ300200	This study
B. oryzae PM	*C. miyabeanus*	Palawan-Monopolar	DQ300201	This study
B. oryzae SB	*C. miyabeanus*	San Pablo-Bipolar	DQ300205	This study
B. oryzae SI	*C. miyabeanus*	San Pablo-Intercalary	DQ300206	This study
B. oryzae SM	*C. miyabeanus*	San Pablo-Monopolar	DQ300199	This study
B. ovariicola	*Cochliobolus* sp.	8842-1	AF163092[b]	Goh and Hyde[e]
B. papendorfii	*Cochliobolus* sp.	9084c	AF163075[b]	Goh and Hyde[e]
B. portulacae	*Pyrenophora portulacae*	DAOM 208494	AY004780	Zhang and Berbee[d]
B. sacchari 1	*Cochliobolus* sp.	Macko HS4	AF071318	Berbee, Pirseyedi and Hubbard[d]
B. sacchari 2	*Cochliobolus* sp.	ATCC13447	AB179836[c]	Tsukiboshi, Chung and Yoshida[f]
B. sorghicola	*Cochliobolus* sp.	MAFF 511378	AF071332	Berbee, Pirseyedi and Hubbard[d]
B. sorokiniana	*C. sativus*	BS11	AY372677[c]	Goh and Hyde[e]
B. spicifera	*C. spicifer*	BRIP12529	AF163076[c]	Goh and Hyde[e]
B. stenospila	*C. stenospilus*	CBS156·36	AB179837[b]	Tsukiboshi, Chung and Yoshida[f]
B. tetramera	*C. spicifer*	CBS 371·72	AY004777[b]	Zhang and Berbee[d]
B. urochloae	*Cochliobolus* sp.	DAOM 171970	AF071334	Berbee, Pirseyedi and Hubbard[d]
B. zeae	*C. zeae*	Alcorn 8641a	AF081452[c]	Berbee[d]
Curvularia affinis	*C. affinis*	DAOM 46365	AF071335	Berbee, Pirseyedi and Hubbard[d]
C. clavata	*C. eragrostidis*	DAOM 148084	AF071336	Berbee, Pirseyedi and Hubbard[d]
C. cymbopogonis	*C. cymbopogonis*	88109–2	AF163079[c]	Goh and Hyde[e]
C. eragrostidis	*C. eragrostidis*	95/1857a	AF163077[b]	Goh and Hyde[e]
C. gladioli	*Cochliobolus* sp.	DAOM 164725	AF071337	Berbee, Pirseyedi and Hubbard[d]
C. gudauskasii	*Cochliobolus* sp.	DAOM 165085	AF071338	Berbee, Pirseyedi and Hubbard[d]
C. heteropogonicola	*Exserohilum heteropogonicola*	IMI268958	AF163080[b]	Goh and Hyde[e]
C. inaequalis	*Cochliobolus* sp.	BRIP14448	AF163081[c]	Goh and Hyde[e]
C. intermedia	*C. intermedius*	BRIP15932	AF163078[c]	Goh and Hyde[e]
C. lunata	*C. lunatus*	95/1937b	AF163082[c]	Goh and Hyde[e]
C. oryzae	*Cochliobolus* sp.	MRL1089	AF163083[b]	Goh and Hyde[e]
Drechslera avenae	*P. avenae*	DAR33699	AF163055[c]	Goh and Hyde[e]
D. biseptata	*Pyrenophora* sp.	WU353	AF163056[c]	Goh and Hyde[e]
D. brizae	*Cochliobolus* sp.	PDDCC6183	AF163057[b]	Goh and Hyde[e]
D. campanulata	*P. semeniperda*	BRIP15927	AF163058	Goh and Hyde[e]
D. teres	*P. teres*	8712	AF163061[c]	Goh and Hyde[e]
D. triseptata	*Pyrenophora* sp.	NZ6120	AF163059	Goh and Hyde[e]
D. tritici-repentis	*P. tritici-repentis*	DAOM 208990	AF071348	Berbee, Pirseyedi and Hubbard[d]
D. tuberosa	*P. japonica*	DAOM 169286	AF071347	Berbee, Pirseyedi and Hubbard[d]
Exserohilum fusiforme	*Setosphaeria* sp.	8822b	AF163063[b]	Goh and Hyde[e]
E. gederafense	*Setosphaeria* sp.	8307	AF163068[b]	Goh and Hyde[e]
E. longirostratum	*Setosphaeria* sp.	7728	AF163064[b]	Goh and Hyde[e]
E. minor	*S. minor*	81100b	AF163065[c]	Goh and Hyde[e]
E. monoceras	*S. monoceras*	DAOM 208990	AF071340	Berbee, Pirseyedi and Hubbard[d]
E. rostratum	*S. rostrata*	BRIP23191	AF163066[c]	Goh and Hyde[e]
E. turcicum	*S.turcica*	94/1823	AF163067[b]	Goh and Hyde[e]
	Leptosphaeria bicolor	42652	LBU04203[b]	Morales, Jasalavich, Pelcher, Petrie and Taylor[h]

[a]Fungal names obtained from GenBank accession information (http://www.ncbi.nlm.nih.gov) or Index Fungorum (http://www.indexfungorum.org).
[b]Fungal species with ITS sequence not previously published.
[c]Fungal species with ITS sequence previously published, but sequence used in this study not previously published.
[d]M. L. Berbee, Department of Botany, University of British Columbia, 6270 University Blvd, Vancouver, BC V6T 1Z4, Canada.
[e]K. D. Hyde, Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, China.
[f]T. Tsukiboshi, National Institute of Floricultural Science, Laboratory of Plant Pathology, Fujimoto 2-1, Tsukuba, Ibaraki 305-8519, Japan.
[g]F. Rollo, Dipartimento Biologia Moleculare, Cellulare Animale, Via F. Camerini 2, I-62032 Camerino, Italy.
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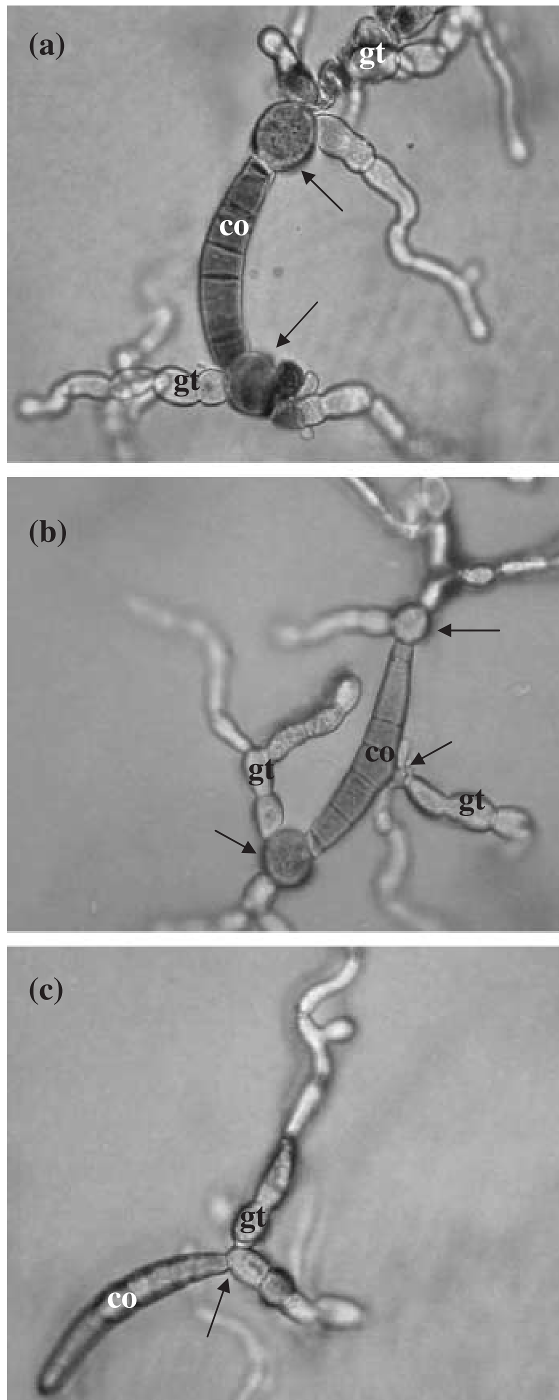

Figure 1 Types of germination, (a) bipolar; (b) intercalary; (c) monopolar, exhibited by conidia (co) of *Bipolaris oryzae*. Arrows, origin of germ tubes (gt) during germination (×400).

two isolates from this location (Table 2). Overall, more intercalary germination was observed with all three isolates from Palawan than from the other two locations, in contrast to monopodial germination which was the least common phenotype.

Table 2 Effect of selection on percentage of bipolar, intercalary and monopolar conidial germination by isolates of *Bipolaris oryzae* from Cavinti, Palawan and San Pablo, the Philippines, on germination type in the subsequent germination

Isolate selection[a]	Germination type[b]		
	Bipolar	Intercalary	Monopolar
Cavinti-B	72·33[a]	21·33[c]	6·33[a]
Cavinti-I	70·00[a]	26·67[b]	3·33[b]
Cavinti-M	63·33[b]	34·67[a]	2·00[c]
Palawan-B	61·00[b]	38·00[b]	1·00[b]
Palawan-I	54·00[c]	45·00[a]	1·00[b]
Palawan-M	80·00[a]	17·33[c]	2·67[a]
San Pablo-B	71·67[c]	19·67[b]	8·67[a]
San Pablo-I	82·33[a]	15·67[c]	2·00[b]
San Pablo-M	75·67[b]	24·00[a]	0·33[c]

[a]Second-generation culture from the Cavinti, Palawan or San Pablo isolate where a single conidium showing bipolar (B), intercalary (I) or monopolar (M) germination was used to start the culture.
[b]Mean percentage from three replicates of 100 conidia per replicate. Germination type was determined based on the location of the germ tube of the conidium 12 h after being placed on rabbit food agar. Means followed by the same letter in a column for each location are not significantly different ($P = 0·05$, LSD).

When the results for subsequent germination phenotypes of bipolar, intercalary and monopolar-selected starter isolates from Cavinti, Palawan and San Pablo were combined, there was no significant effect of original selection of conidial germination phenotypes. For example, selection for bipolar germination never resulted in significantly higher levels of bipolar germination in the next fungal generation compared with both other germination types.

Effect of substrate on conidial germination

Conidia from 7-day-old cultures of *B. oryzae* from the original single lesions exhibited different percentages of conidial germination phenotypes after 12 h incubation (Table 3).

For example, the percentage bipolar germination of conidia of the Cavinti isolate predominated in each medium, compared with intercalary and monopolar germination (Table 3). Bipolar germination was highest on RFA, PDA and WA, which were not significantly different from each other, and was lowest for WG and RLA. Intercalary germination was highest on RLA and WG, which were not significantly different from each other, and lowest on RFA, PDA and WA. Monopolar germination varied between media, but there were no significant differences.

Overall, bipolar germination of conidia predominated irrespective of media, but substrate effects were apparent. For example, WA and RFA were the most consistent in yielding relatively high percentage bipolar germination compared with relatively low intercalary germination (Table 3).

Table 3 Effect of medium on percentage bipolar, intercalary and monopolar conidial germination by the original cultures of *Bipolaris oryzae* from Cavinti, Palawan and San Pablo, the Philippines

Isolate	Medium[a]	Germination type[b] Bipolar	Intercalary	Monopolar
Cavinti	MEA	69·33[bc]	23·33[b]	7·33[a]
	PDA	80·00[a]	16·67[bc]	3·33[a]
	RFA	83·33[a]	10·33[c]	6·33[a]
	RLA	56·33[d]	43·00[a]	0·67[a]
	RPA	69·33[bc]	24·00[b]	6·67[a]
	WA	78·00[ab]	15·00[bc]	7·00[a]
	WG	61·33[cd]	36·67[a]	2·00[a]
Palawan	MEA	80·00[a]	12·00[a]	8·00[b]
	PDA	84·33[a]	9·00[a]	7·00[b]
	RFA	79·33[a]	11·00[a]	10·00[b]
	RLA	78·67[a]	12·00[a]	9·33[b]
	RPA	75·33[a]	15·33[a]	9·33[b]
	WA	82·67[a]	6·33[a]	11·00[b]
	WG	53·33[b]	10·33[a]	36·33[a]
San Pablo	MEA	73·67[bc]	10·33[b]	17·67[ab]
	PDA	63·33[c]	22·00[a]	14·67[abc]
	RFA	71·33[bc]	15·00[ab]	13·67[abc]
	RLA	84·00[a]	9·33[b]	7·33[bc]
	RPA	83·33[a]	13·00[ab]	3·67[c]
	WA	78·67[ab]	10·00[b]	13·67[abc]
	WG	68·33[c]	13·67[ab]	18·33[ab]

[a]MEA (malt extract agar), PDA (potato dextrose agar), RFA (rabbit food agar), RLA (rice leaf agar), RPA (rice polish agar), WA (water agar), WG (distilled water droplet on a glass slide).
[b]Mean percentage from three replicates of 100 conidia per replicate. Germination type was determined based on the location of the germ tube of the conidium 12 h after being placed on each medium. Means followed by the same letter in a column for each location are not significantly different ($P = 0·05$, Tukey–Kramer).

Effect of conidial maturity on germination

The age of the culture from which conidia were harvested had a significant effect on the percentage bipolar, intercalary or monopolar germination on RFA for all three single-lesion isolates. A χ^2 test ($P < 0·0001$) indicated that there was an association between the different germination types and culture age for all three isolates, and the results for the Cavinti, Palawan and San Pablo isolates were combined (Fig. 2). Bipolar germination increased significantly and intercalary germination decreased significantly as source cultures for conidia increased from 5 to 30 days incubation (Fig. 2). Bipolar germination was significantly less than intercalary germination at 5 days incubation, but became significantly greater by 10 days, and differences continued to increase for conidia from 20-day-old source cultures. From 30-day-old source cultures, bipolar germination was 88% while both intercalary and monopolar germination phenotypes were 6% each of the total.

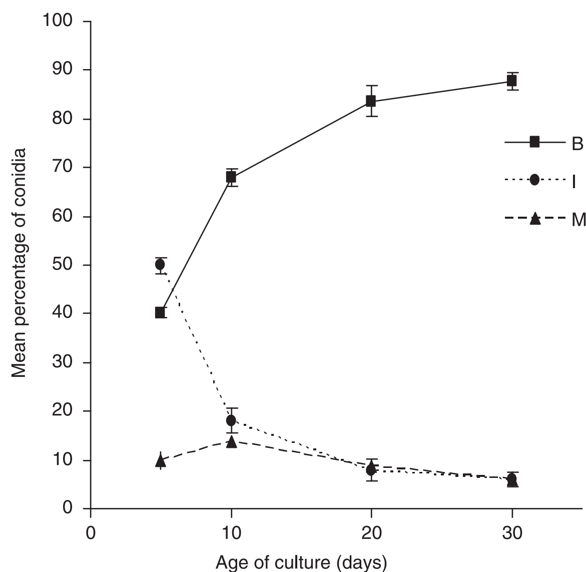

Figure 2 Effect of conidial maturation on mean number of bipolar (B), intercalary (I) and monopolar (M) germination patterns from different age cultures of *Bipolaris oryzae* across all isolates from Cavinti, Palawan and San Pablo, the Philippines. Bars show SEM.

Phylogenetic analysis

For the Cavinti, Palawan and San Pablo isolates, single conidia with each germination type were selected to grow second-generation cultures, which were then named germination type-selected cultures. The sequence of the ITS1, ITS2 and 5·8S rDNA for these nine germination type-selected cultures were all identical, indicating that the different germination types did not reflect a mixed infection of different fungal species at each location. A BLASTn search of the GenBank nonredundant database showed that the sequence was most similar (98% identity) to the ITS1, ITS2 and 5·8S rDNA sequences of *B. oryzae* (accession no. X78122), and they were different by six bases, all located within the ITS1 region. Cultures of *B. oryzae* from single-spore isolations that had bipolar germination were used in all subsequent experiments unless otherwise noted.

A parsimony consensus tree of the ITS1, ITS2 and 5·8S rDNA regions (640 nucleotides) was constructed for the 49 fungal isolates listed in Table 1 (Fig. 3). The outgroup was *Leptosphaeria bicolor*. Three clades representing *Drechslera/Pyrenophora*, *Exserohilum/Setosphaeria* and *Curvularia/Bipolaris/Cochliobolus* were observed in this tree. Both the *Drechslera/Pyrenophora* and *Exserohilum/Setosphaeria* clades were supported by 100% bootstrap values, and the *Curvularia/Bipolaris/Cochliobolus* clade was supported by a 95% bootstrap value.

The *Bipolaris* species were divided into two subclades within the *Curvularia/Bipolaris/Cochliobolus* clade: a collection of only *Bipolaris* isolates in the *Cochliobolus* Group 1 subclade, and a collection of intermingled *Bipolaris* and *Curvularia* isolates in the *Cochliobolus*

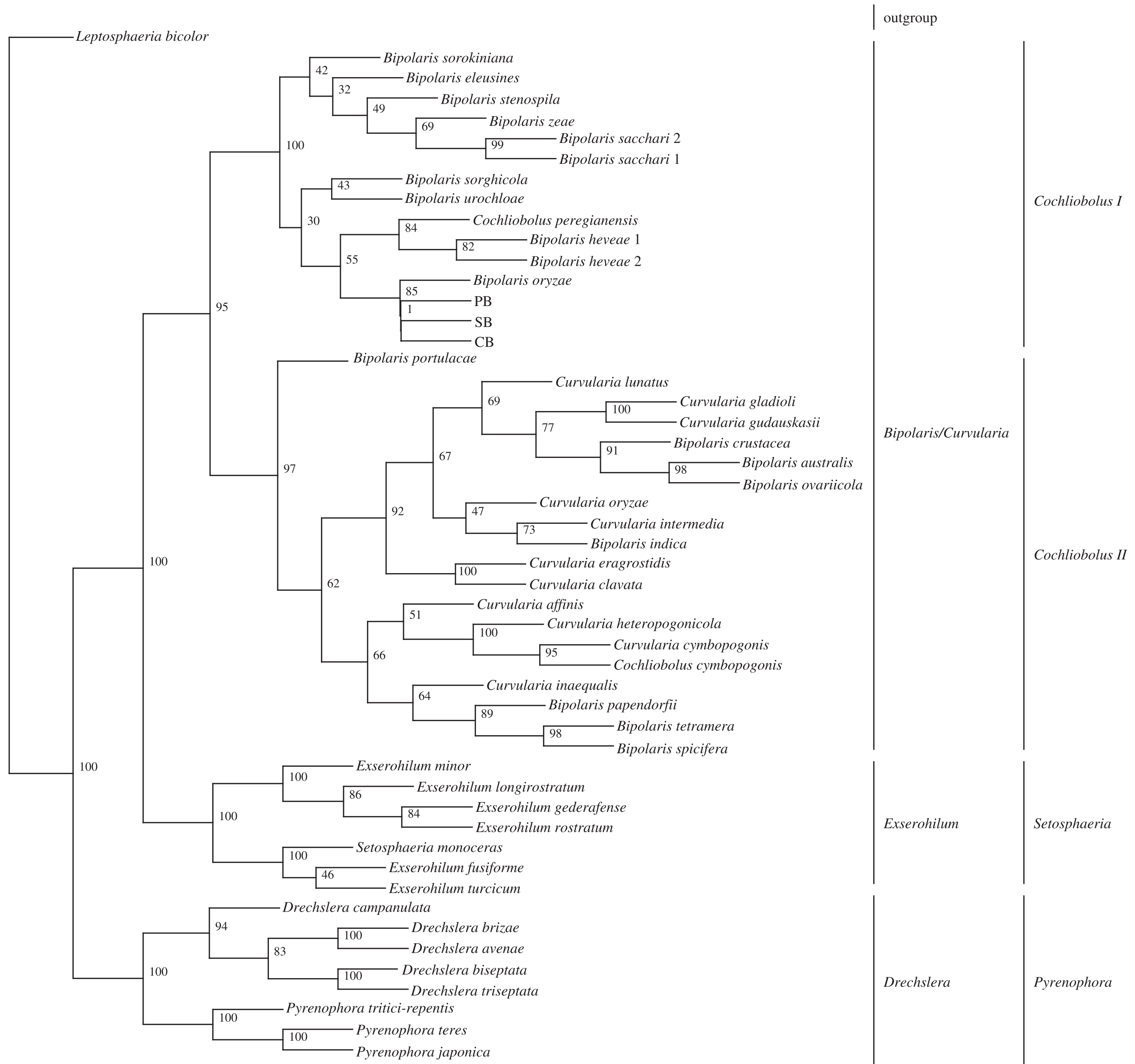

Figure 3 An extended majority rule consensus of 12 equally parsimonious trees (1394 steps) based on complete sequence of internal transcribed spacer 1, 5·8S ribosomal RNA gene and internal transcribed spacer 2 of 51 sequences with *Leptosphaeria bicolor* as the outgroup. GenBank accession numbers and species are given in Table 1. Tree topology was derived from parsimony analysis of a multiple sequence alignment from CLUSTALW. Numbers near each branch represent percentages out of 100 bootstrap replications. The three isolates (CB, PB, SB) identified in this study represent the nine isolates from Cavinti, Palawan and San Pablo, the Philippines, that germinated in a bipolar (B), intercalary (I) and monopolar (M) fashion. The other 48 taxa were retrieved from GenBank.

Group 2 subclade. The isolates of *B. oryzae* from Cavinti, Palawan and San Pablo used in this study were in the *Bipolaris*/*Cochliobolus* Group 1 subclade, specifically clustering with *B. oryzae* (GenBank X78122) supported with an 85% bootstrap value. In addition, the amount of sequence variation between the different *B. oryzae* isolates is comparable with that of other species, such as *Bipolaris heveae* and *Bipolaris sacchari* (Fig. 3). From this analysis, the isolates from Cavinti, Palawan and San Pablo are all *B. oryzae*, irrespective of whether the culture originated from a conidium with a bipolar, intercalary or monopolar germination type. A highly similar clustering was obtained using neighbour-joining analysis (data not shown).

As many of the sequences included in the dendrogram had not been previously analysed, some unexpected relationships were observed. At the Index Fungorum (http://www.indexfungorum.org), *Drechslera brizae* is described as synonymous with *Bipolaris brizae* with a teleomorph of a *Cochliobolus* species. However, the clustering indicates that *D. brizae* is the correct name, and the teleomorph should be a *Pyrenophora* species (Fig. 3). Also at the Index Fungorum, *Curvularia heteropogonicola* is

described as synonymous with *Exserohilum heteropogonicola*, with a teleomorph of a *Setosphaeria* species. However, the clustering shows that *C. heteropogonicola* appears to be the correct name, and the teleomorph should be a *Cochliobolus* species (Fig. 3). The *Bipolaris tetramera* sequence also has not been previously analysed, and that species is described at the Index Fungorum as having a teleomorph of *Cochliobolus spicifer*. This is consistent with the clustering of *B. tetramera* with *B. spicifera*, which also has a teleomorph of *C. spicifer* (Fig. 3).

Discussion

Bipolar germination of conidia is one feature of the genus *Bipolaris* compared with *Drechslera*, conidia of which germinate from any cell (Shoemaker, 1959, 1962; Leonard & Suggs, 1974; Luttrell, 1977, 1978). Another distinguishing feature is that conidia of *Drechslera* are cylindrical, whereas conidia of *Bipolaris* are fusiform–ellipsoidal (Domsch, 1980). Although bipolar germination should be a convenient conidial feature used as part of the identification of *Bipolaris*, Alcorn (1983) stated that bipolar germination is not a reliable generic criterion.

Commenting on brown spot of rice, Ou (1985) observed that the pathogen was not typical of *Helminthosporium* because the conidia are slightly curved, fusiform or obclavate, have an inconspicuous hilum, are borne acropleurogenously, and are brown when mature: consequently, he named the fungus *Drechslera oryzae*. However, this fungus has also been described as *B. oryzae* by Subramanian (1974); Luh (1991); Mathur & Kongsdal (2003). In addition to morphological traits, Alcorn (1988) noted that differences in germination were important in segregating certain genera from *Helminthosporium*, such as *Drechslera* and *Bipolaris* (Shoemaker, 1959). Luttrel (1963) also indicated that aspects of basal cell germination are more useful criteria for classification at the generic level. These traits can also be compared with differences or similarity in conserved DNA sequences to provide a more reliable classification system at the generic and species levels.

Berbee *et al.* (1999) used the ITS1, ITS2, 5·8S rDNA and a portion of the glyceraldehyde-3-phosphate dehydrogenase sequences to show that *Helminthosporium* species can be segregated into three distinct clades: *Cochliobolus* (anamorphs *Bipolaris* and *Curvularia*); *Pyrenophora* (anamorph *Drechslera*); and *Setosphaeria* (anamorph *Exserohilum*) (Berbee *et al.*, 1999). These workers proposed that *Bipolaris* be divided further, separating species with large, canoe-shaped, gently curving conidia (*Cochliobolus* Group 1) from those with short, either straight or curved conidia without a gentle curve along the whole spore length (*Cochliobolus* Group 2), which are intermixed with *Curvularia* species. Although some species in this study are the same as those analysed by Berbee *et al.* (1999); Olivier *et al.* (2000); Zhang & Berbee (2001), an additional 11 species of *Bipolaris*, seven species of *Curvularia*, three species of *Drechslera*, six species of *Exserohilum* and one species of *Pyrenophora*, for which the ITS1, ITS2 and 5·8S rDNA have not yet been analysed, were included. Even with these 28 additional species from GenBank deposited as unpublished data and included in the analysis, the dendrogram of this study agrees well with the phylogenetic groupings described by Berbee *et al.* (1999); Olivier *et al.* (2000); Zhang & Berbee (2001), thus extending and confirming their conclusions about the relationships between these fungal species.

A comparison of the isolates from Palawan, Cavinti and San Pablo showed that all had completely identical overlapping ITS1, ITS2 and 5·8S rDNA sequences, and were most similar to the ITS1, ITS2 and 5·8S rDNA sequences previously reported for *B. oryzae* (Olivier *et al.*, 2000). All the *B. oryzae* isolates clustered in the *Cochliobolus* Group 1 subclade, which contains only large, canoe-shaped *Bipolaris* species, both by parsimony and neighbour-joining analysis. This agrees with visual observations of the conidia from Cavinti, Palawan and San Pablo, which had large, canoe-shaped morphology. The conidia of these isolates also lacked the presence of a hilum, as described by Ou (1985).

A comparison of the ITS1, ITS2 and 5·8S rDNA sequences from the isolates from Cavinti, Palawan, and San Pablo with different germination types revealed no differences in sequences between the bipolar, intercalary and monopolar versions of the brown spot isolate. Differences between fungal species are normally found in the highly variable ITS sequences between the 5·8S rRNA gene and the small (18S) and large (28S) rRNA genes (White *et al.*, 1990; Berbee *et al.*, 1999; Olivier *et al.*, 2000). The lack of any sequence differences indicates that there is no evidence that the bipolar, intercalary and monopolar-germinating conidia come from different species. This is also supported by other data in this study showing that environmental and developmental factors play a major role in determining whether the majority of spores derived from *B. oryzae* cultures would show bipolar, intercalary or monopolar germination.

Selecting conidia with a particular germination type (bipolar, intercalary or monopodial) to start another culture never resulted in a greater percentage of the same germination type among the conidia produced from that culture, except for the bipolar phenotype, which was predominant irrespective of the initial phenotype.

Alcorn (1983) germinated six isolates of *B.* maydis (type species of *Bipolaris*) on host maize leaves, WA and water placed on glass slides (WG) as germination substrates. The latter two substrates were also used in this study, while the former was used in a modified form by mixing rice leaves in a thin layer of water agar (RLA). For conidia obtained from 5–7-day-old V8 agar cultures grown at 24–26°C, Alcorn (1983) observed *c.* 90% bipolar germination type on WA or leaves of *Zea mays*, but bipolar germination decreased to *c.* 50% in water placed on slides, while total monopolar germination increased correspondingly. Intercalary germination occurred in only 3% of germinated conidia. However, neither a description of the degree of variation between isolates nor any statistics were given. In the present study, WA was always better

than WG for bipolar germination of *B. oryzae*, but the testing of additional media in this study showed that none provided any significant improvement over WA. However, isolates do vary noticeably in their response to the type of medium on which spores germinate. An indication of differences in germination between *B. oryzae* and *B. maydis* is that the percentage intercalary and monopolar germination of *B. oryzae* was usually much greater than that reported for *B. maydis* by Alcorn (1983).

The age of culture from which spores were harvested had a very large effect on germination phenotypes. At 30 days, bipolar germination had increased to 88%. Ou (1985) noted that subhyaline spores showed intercalary germination, and he believed these were less mature conidia. More mature conidia had more bipolar germination and appeared to be fuliginous or brownish, with a moderately thin peripheral wall, further attenuated at the apex as well as immediately around a rather inconspicuous hilum that was visible within the contour of the base. However, Ou (1985) gave no description of the variation within and between isolates.

A random search for subhyaline conidia in cultures of the three Philippine isolates showed that they comprised over 50% of the conidia in 5-day-old cultures, but fewer than 10% of the conidia in 30-day-old cultures, showing that the number of less-mature spores declined over the incubation period. It appears that, as conidia become older, the cells in the centre of the spores become more inhibited in their germination (perhaps by deposition of melanized material in the cell wall), or become less viable, whereas cells on the ends of spores become more favoured for germination. Germination from only one end cell of the conidium (monopolar) appears always to occur in a low number of conidia, and does not appear to be greatly affected by the maturity of conidia.

This study provides the first quantitative data demonstrating that developmental factors play a particularly significant and consistent role in determining the type of conidial germination. If this is taken into consideration and appropriate conditions are selected, bipolar germination can be a reliable characteristic for identification of *Bipolaris* species.

Acknowledgements

This work was supported by an affliate scholarship from the International Rice Research Institute to M.A.G.D.P. We thank Maria Ymber Reveche and Ellen Silab for technical assistance, and Violeta Bartolome for advice on statistical analysis.

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