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CHARACTERIZATION OF A RARE SPECIES OF *NEUROSPORA* ISOLATED FROM A RIVER WATER SAMPLE IN CHINA

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Abstract: A fungal isolate (ZZS4408) was obtained from a river water sample in Henan, China, and identified as a member of *Neurospora brevispora*, a rare species of *Neurospora* based on its morphological characteristics and ribosomal DNA internal transcribed spacer (rDNA-ITS) sequence. The temperatures suitable for growth of the isolate were 28-37°C with 31°C as the optimum. The growth rates of hyphal tips were 19.1-42.5 (av. 31.9) μm min^{-1} at 32°C. The pH suitable for vegetative growth ranged from 5 to 7, with pH 5.5 as the optimum. The heterodisaccharides (sucrose and lactose) and D-alanine were found to be most favorable for vegetative growth of the isolate, as carbon and nitrogen sources, respectively. The vegetative growth of the isolate was more significantly influenced by nitrogen sources compared to carbon sources. *N. brevispora* could be considered a desirable fungal species for morphodifferentiation studies due to its rapid growth rates under favorable conditions.

Keywords: Biological characteristic; identification; *Neurospora brevispora*

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INTRODUCTION

Neurospora is a genus of ascomycete fungi with high academic and commercial values, and consists of at least 26 species. As a well-known species in the genus, *Neurospora crassa* has been used as a model organism in biology (Chung et al., 2001; He and Liu, 2005), and has greater potential in the production of valuable metabolites such as cellulase (Feng et al., 2004), laccase (Chen et al., 2005), ethanol and xylitol (Zhang et al., 2003), carotenoids (Li et al., 2009), and highly reactive dietary fiber (Tu et al., 2008). Other *Neurospora* spp. such as *N. intermedia* (Chen et al., 2009) and *N. sitophila* (Wu et al., 2008; Deng et al., 2009) also had stronger abilities in cellulose production.

Since 1998, the Neurospora International Symposium has been held every two years in the California Asilomar Conference Center. The information presented at the symposium could reflect the latest advances in *Neurospora* research. Biological and molecular studies on *Neurospora* were focused predominantly on a few species (especially *N. crassa*) worldwide. Little is known

about most of the other *Neurospora* spp. (particularly the rare species), making the knowledge system dramatically unbalanced among different species in this genus. For most of the rare species such as *N. brevispora* (=*Gelasinospora brevispora*), their basic biological characteristics are unclear. Biological characterization of rare species could not only contribute to a better understanding of genetic diversity in *Neurospora*, but to an effective utilization of the related fungal species. In recent years, the authors isolated a rare species of *Neurospora* (isolate ZZS4408) from a river water sample in Henan, China. The goal of the present study is to identify and characterize the rare *Neurospora* species.

MATERIALS AND METHODS

Isolation and growth rate tests of isolate ZZS4408

The isolate (ZZS4408) of *Neurospora brevispora* was isolated from a river water sample in Xinye (112°35′E, 32°52′N), a southwestern county in Henan province, China, using a conventional dilution plate method. The water sample was spread on PDA plates (200 g potato, 20 g glucose, 16 g agar, 1000 ml water) with a sterilized glass spreader, and incubated at 28°C for 24h. A rapid-growing fungal colony (isolate ZZS4408) was obtained from the water sample and used for the present study.

The isolate ZZS4408 was grown on PDA plates at 28-32°C for 20 days to observe its morphological characteristics. The determination of single hyphal growth rates of the isolate was conducted using 24-hour-old colonies grown on PDA plates at 32°C under a microscope. Two photographs of a hyphal tip were separately taken at 10-15 min interval under a microscope. A hyphal growth rate was determined based on the hyphal length increased over a certain time (10-15 min) period. After sporulation occurred, the dimensions of fully-developed perithecia, asci and ascospores were separately measured.

Molecular identification

The isolate ZZS4408 was incubated on PDA plates for 2 days, and its mycelial plugs (6 mm diameter) were cut out from actively growing colonies. A mycelial plug was transferred to potato dextrose broth (PDB) liquid medium (the same components as PDA except agar), and incubated at 28°C for 7 days. The resultant mycelia were harvested and rinsed with sterile water to remove residual medium components, and subjected to DNA extraction after being dried at 80°C for 12h.

Genomic DNA of isolate ZZS4408 was extracted using the liquid nitrogen method (Lu et al., 2011). The dry mycelial sample was placed in a sterile mortar and quickly ground in liquid nitrogen to obtain mycelial powder. The mycelial powder was transferred to 800 μl lysis buffer in a sterilized centrifuge tube, and bathed in hot water (65°C) for 2h. The tube was turned reversely once every 10 min during the water-bath treatment. The DNA solution was subsequently centrifuged at 4°C under 12000 rpm for 10 min. The supernatant was added to an equal volume of the mixture of four organic solvents (phenol:chloroform:isoamyl alcohol = 25:24:1). After being gently blended, the DNA solution was centrifuged at 4°C at 12000 rpm for 10 min. The extraction process was repeated three times. Two volumes of pre-cooled ethanol and one-tenth volume of 3 mol/L sodium acetate solution were added into the DNA solution and well shaken. After being maintained at -20°C for 1.5 h, the DNA solution was centrifuged at 4°C

at 12000 rpm for 10 min. After removal of the supernatant, the precipitate was rinsed three times with 75% alcohol by centrifugation as mentioned above. The DNA sample was dissolved in 25 µl ddH_2O and maintained at -20°C for 30 min prior to electrophoresis analysis.

The DNA sample was diluted properly with PCR buffer according to the brightness of DNA bands in electrophoresis (Qi et al., 2009). The PCR reaction system consisted of 1 µl genomic DNA, 15 µl mixture (PCR buffer, dNTP, *Taq* DNA polymerase, $MgCl_2$), 1.2µl of each of 10 pmol/L ITS4 (5'-tcctccgcttat tgatatgc-3') and 10 pmol/L ITS5 (5'-ggaagtaaaagtcgtaacaagg-3'), and ddH_2O was added to a final volume of 30 µl. The PCR program was set as follows: predenaturing at 95°C for 5 min, subsequently denaturing at 94°C for 40 s, annealing at 55°C for 40 s, and 30 cycles of extension at 72°C for 30 s followed by extension at 72°C for another 10 min. PCR products were analyzed with 1% agarose gel electrophoresis, and sent to Takara Biotechnology (Dalian) Co. Ltd. for sequencing (both strands sequenced) after

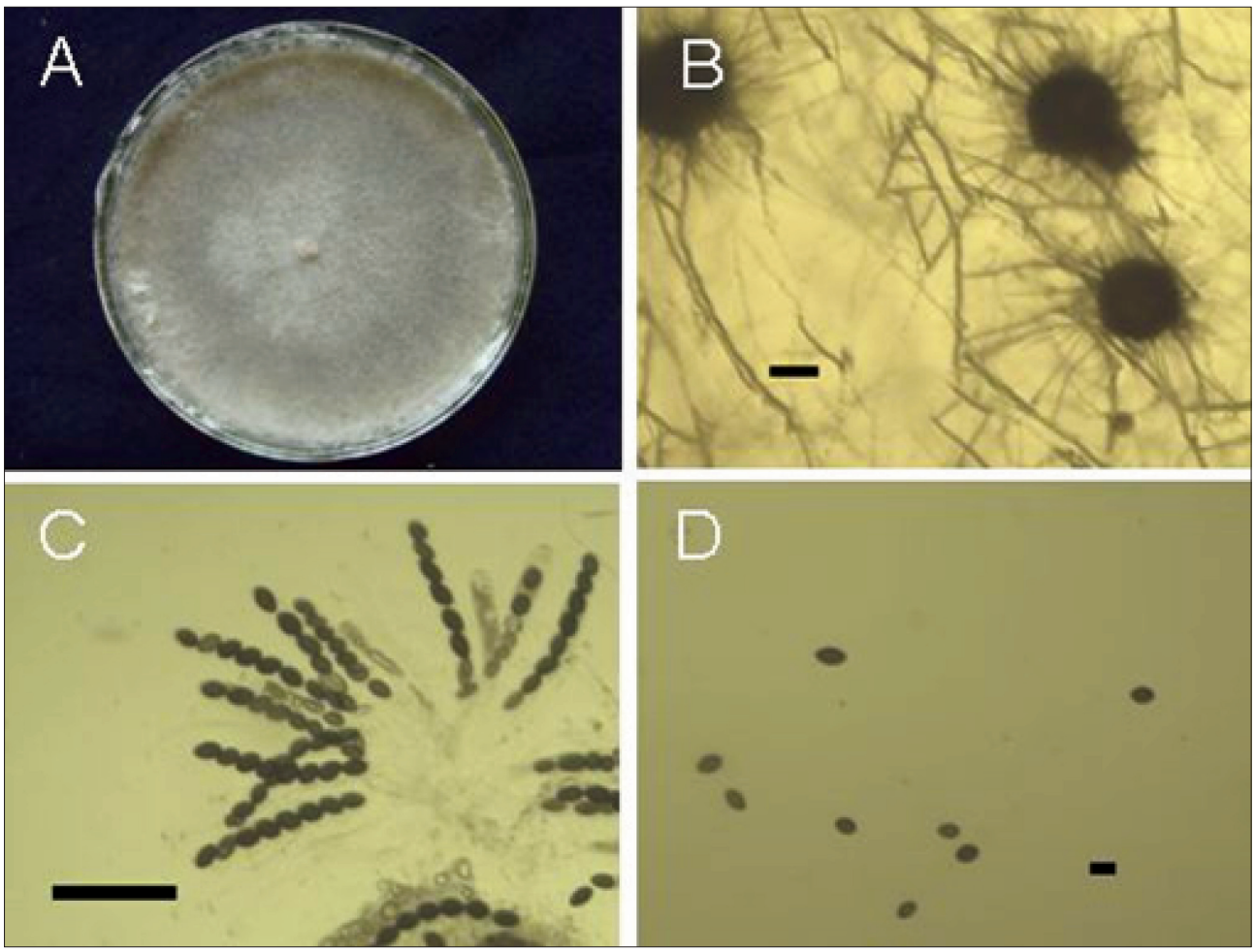

Fig. 1. Morphological characteristics of isolate ZZS4408 (A: a colony incubated at 31°C on PDA plate for 14 days; B: perithecia developed on BM plate after 15 days of incubation at 28°C; C: eight linearly aligned ascospores within an ascus developed on BM plate after 15 days of incubation at 28°C; D: ascospores on BM plate after 15 days of incubation at 28°C. The scale bars represent 100 µm and 20 µm for B and C, respectively).

being purified. Sequence homology comparison was conducted using the BLAST program (http://blast.ncbi.nlm. nih.gov/Blast. cgi). The rDNA-ITS sequences of other *Neurospora* spp. from the GenBank database were used as reference sequences. A phylogenetic tree was constructed with MEGA4 software (Tamura et al., 2007) to determine the taxonomic hierarchy of isolate ZZS4408 under 1000 bootstrap replicates.

Tests on biological characteristics of isolate ZZS4408

To clarify the effects of temperatures on colony growth, isolate ZZS4408 was grown on PDA plates at 25°C for 2 days. Mycelial plugs (6 mm diameter) were cut out with a sterilized punch from actively growing colonies, and transferred onto PDA plates. After being incubated at a series of temperatures (4°C, 7°C, 10°C, 13°C, 16°C, 19°C, 22°C, 25°C, 28°C, 31°C, 34°C, 37°C, 40°C, 43°C) for 3 days, the diameters of the developed colonies were cross-measured. Three replicates were set up for each temperature.

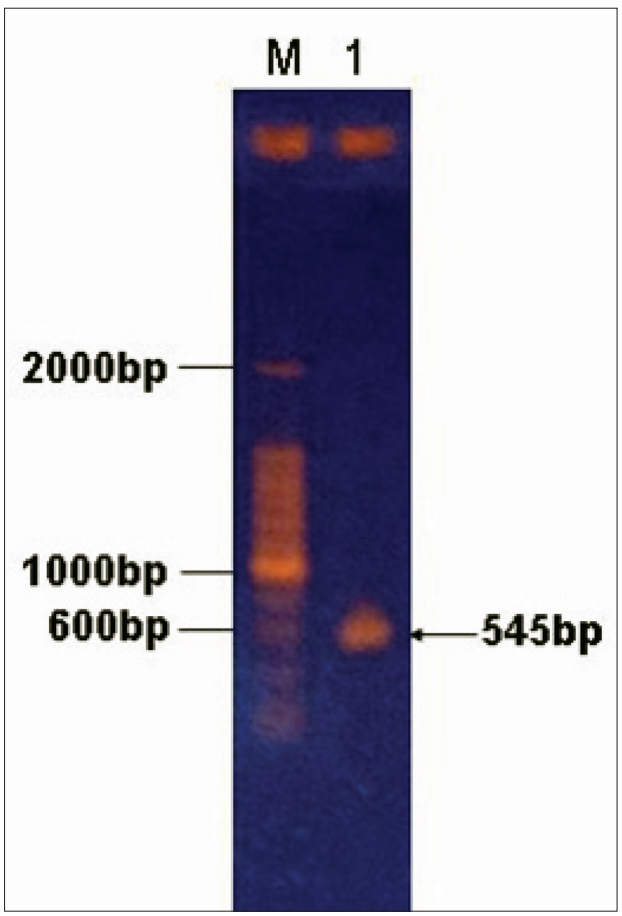

Fig. 2. Electrophoretogram for PCR products of the rDNA-ITS of isolate ZZS4408 (1: isolate ZZS4408; M: DNA marker).

To know the effects of pH on the vegetative growth of isolate ZZS4408, the pH of PDB was adjusted to different levels (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 8.0, 8.5, 9.0) with 1mol NaOH/HCl. Mycelial plugs of isolate ZZS4408 were separately inoculated in 100 ml PDB with different pHs. After being incubated at 25°C for 6 days, the resultant mycelia were harvested, and dried overnight at 60°C. The dry weights of the mycelia were separately measured (Lu et al., 2001). Three replicates were set up for each pH treatment.

A basic medium (BM: 2g D-alanine, 1 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 30 g sucrose, 17 g agar, water 1000 ml) was used for tests of the effects of nutrients on the vegetative growth of isolate ZZS4408. To determine the effects of carbon sources on the vegetative growth of the isolate, the sucrose in the BM medium was substituted with equal amounts of glucose, fructose, lactose, galactose, D-mannose, xylitol, maltose, L-arabinose and D-sorbitol, respectively. To determine the effects of nitrogen sources on the vegetative growth of the isolate, the D-alanine of the BM medium was substituted with equal amounts of glycine, carbamide, L-cysteine, L-histidine, $NaNO_3$ and NH_4Cl, respectively. Mycelial plugs of isolate ZZS4408 were prepared as mentioned above, and inoculated on the BM plates containing different carbon/nitrogen sources. The plates were incubated at 25°C for 3 days prior to measuring the diameters of the

developed colonies. Three replicates were set up for each treatment.

Statistical analyses of experimental data

Data obtained from the experiments were subjected to analysis of variance (ANOVA) using SPSS statistical software (version 17.0, IBM Corporation, New York, USA). Multiple comparisons of means were performed using one-way ANOVA at two significance levels (P=0.05 and P=0.01).

RESULTS

Identification of isolate ZZS4408

Isolate ZZS4408 grew rapidly on PDA plates under favorable conditions. Its colony diameters were greater than 7 cm after 3 days of incubation at 28°C. The growth rates of eight randomly selected hyphal tips were 19.1-42.5 (mean 31.9) μm min^{-1} at 32°C. The colonies produced less aerial mycelia before they fully covered the plates. After the plates were completely covered by the colonies, aerial mycelia vigorously occurred, with white to dark gray color (Fig. 1A). Hyphal diameters varied from 3.0 to 16.0μm. Mycelia could overgrow far away from the PDA plates, even spreading over the inner glass surface of the Petri dish. After 10 days of incubation, black perithecia developed on the inner glass surface of the Petri dish as well as on the PDA plates. Mature perithecia were spherical, oval or flask-shaped (Fig. 1B), with a dimension of 117.6-454.9 μm × 156.0-498.0 μm. Ascospores were found in the 60-day-old asci, but not in the 14-day-old ones. Mature asci were rod-shaped with a dimension of approximately 160μm×16μm, with eight linearly aligned ascospores within an ascus (Fig. 1C). The ascospores were oval-shaped, unicellular, black-brown in color with a dimension of 18.7-22.7 μm×13.1-15.4 μm (mean 20.9 μm×14.3 μm) (Fig. 1D).

Table 1. Effects of temperatures on vegetative growth of isolate ZZS4408 (The values followed by the same capital/lowercase letters are not significantly different at P=0.05 and P=0.01, respectively).

Temperature (°C)	Colony diameter (mm)
4	0 .0 Aa
7	0 .0 Aa
10	7.7 Bab
13	12.5 Cb
16	25.0 Db
19	40.6 Ec
22	64.6 Fd
25	68.8 FGd
28	87.6 He
31	89.7 He
34	85.6 He
37	83.8 He
40	73.6 Dd
43	0.0 Aa

The rDNA-ITS sequence of isolate ZZS4408 was amplified using the primers ITS4 and ITS5. PCR products were visualized by 1% agarose gel electrophoresis (Fig. 2). After purification and sequencing, a 545-bp rDNA-ITS fragment (GenBank accession number: JN003623) was obtained. In the established phylogenetic tree based on rDNA-ITS sequences of *Neurospora* spp, isolate ZZS4408 clustered with *Neurospora brevispora* (= *Gelasinospora brevispora*) (Fig. 3) as well as the isolate IR353 reported by Li et al. (2011). The phylogenetic tree indicated that both isolates ZZS4408 and IR353 belonged to *N. brevispora*.

Effects of environmental factors on the growth of isolate ZZS4408

The temperature range for the growth of isolate ZZS4408 was 10-40°C (Table 1). The mean diameters of colonies developed at temperatures

28-37°C for 5 days were greater than 80 mm. Although no difference in colony size was observed among the temperatures at P=0.05 level, the maximum growth occurred at 31°C with a mean colony diameter of 89.7mm. The temperatures suitable for active growth of isolate ZZS4408 were 28-37°C with 31°C as the optimum (Table 1). Taking the peak growth temperature 31°C as a dividing line, significant difference was observed in colony diameters between the lower temperature side and higher temperature: colony diameters gradually rose with the increase of temperatures in the former, and sharply decreased with the enhancement of temperatures in the latter. At temperatures lower than 31°C, the increase of 1°C came with an increased mean diameter of 3.74 mm, while at temperatures higher than 31°C, the increase of 1°C came with a reduced mean colony diameter of 7.48 mm. As temperatures rose from 40°C to 43°C, the mean colony diameters sharply declined from 73.6 mm to 0 mm.

Vegetative growth of isolate ZZS4408 was significantly influenced by the pH levels of the media (Table 2). The isolate could grow at pH 4-9 as shown by mycelial dry weights, wherein a greater mycelial dry weight was found at pH 5-7 (greater than 0.16 g) and the maximum mycelial dry weight occurred at pH5.5 (0.993 g). The mean mycelial dry weight was less than 0.12g in the other pH treatments. Therefore, pH 5-7 could be considered as the favorable pH range for active growth of isolate ZZS4408 with 5.5 as the optimum.

The effects of carbon sources on the vegetative growth of isolate ZZS4408 is shown in Table 3. Of 10 carbon sources tested, the fastest growth occurred on the plates containing heterodisaccharides (sucrose/lactose) with a colony diameter greater than 80 mm, compared to the other carbon sources with a reduced colony diameter.

Table 2. Effects of pH on vegetative growth of isolate ZZS4408 (the values followed by the same capital/lowercase letters are not significantly different at P=0.05 and P=0.01, respectively).

pH	Dry mycelial weight (g)
4.0	0.055 ABa
4.5	0.106 Ba
5.0	0.177 CDb
5.5	0.993 Ec
6.0	0.197 CDb
6.5	0.168 Cb
7.0	0.227 Db
7.5	0.111 BCa
8.0	0.073 ABa
8.5	0.066 ABa
9.0	0.038 Aa

Table 3. Effects of carbon sources on vegetative growth of isolate ZZS4408 (the values followed by the same capital/lowercase letters are not significantly different at P=0.05 and P=0.01, respectively).

Carbon source	Colony diameter(mm)
Sucrose	85.2 Dc
Lactose	82.7 CDc
Glucose	70.7 ABab
Fructose	72.6 ABab
Xylitol	74.5 Bab
L-arabinose	76.5 Bb
D-sorbitol	78.5 BCbc
Galactose	67.8 Aa
D-mannose	75.0 Bb
Maltose	75.1 Bb

Table 4. Effects of nitrogen sources on vegetative growth of isolate ZZS4408 (The values followed by the same capital/lowercase letters are not significantly different at P=0.05 and P=0.01, respectively).

Nitrogen source	Colony diameter(mm)
D-alanine	87.7 Ed
Carbamide	32.4 Aa
L-histidine	70.9 CDc
Glycine	54.5 Bb
$NaNO_3$	69.5 CDc
L-cysteine	77.0 Dcd
NH_4Cl	65.7 Cbc

The slowest growth occurred on the plates containing galactose as a carbon source with a colony diameter less than 70 mm. Moderate growth occurred on the plates containing the other carbon sources with a colony diameter of 70-80 mm. In general, the disaccharides (especially heterodisaccharides) were more beneficial to the growth of isolate ZZS4408 compared to the monosaccharides. Among the 10 carbon sources tested, the deviation between the maximum mean colony diameter and minimum mean colony diameter was of 17.4 mm.

The effects of nitrogen sources on the growth of isolate ZZS4408 are shown in Table 4. Of seven nitrogen sources tested, the D-alanine treatment had the largest mean colony diameter (87.7 mm) compared with the other nitrogen sources, followed by the L-histidine and L-cysteine treatments with colony diameters of 70-80 mm. The colony diameters on the plates containing the other nitrogen sources were less than 70 mm. The difference between the maximum mean colony diameter and the minimum one reached up to 55.3 mm.

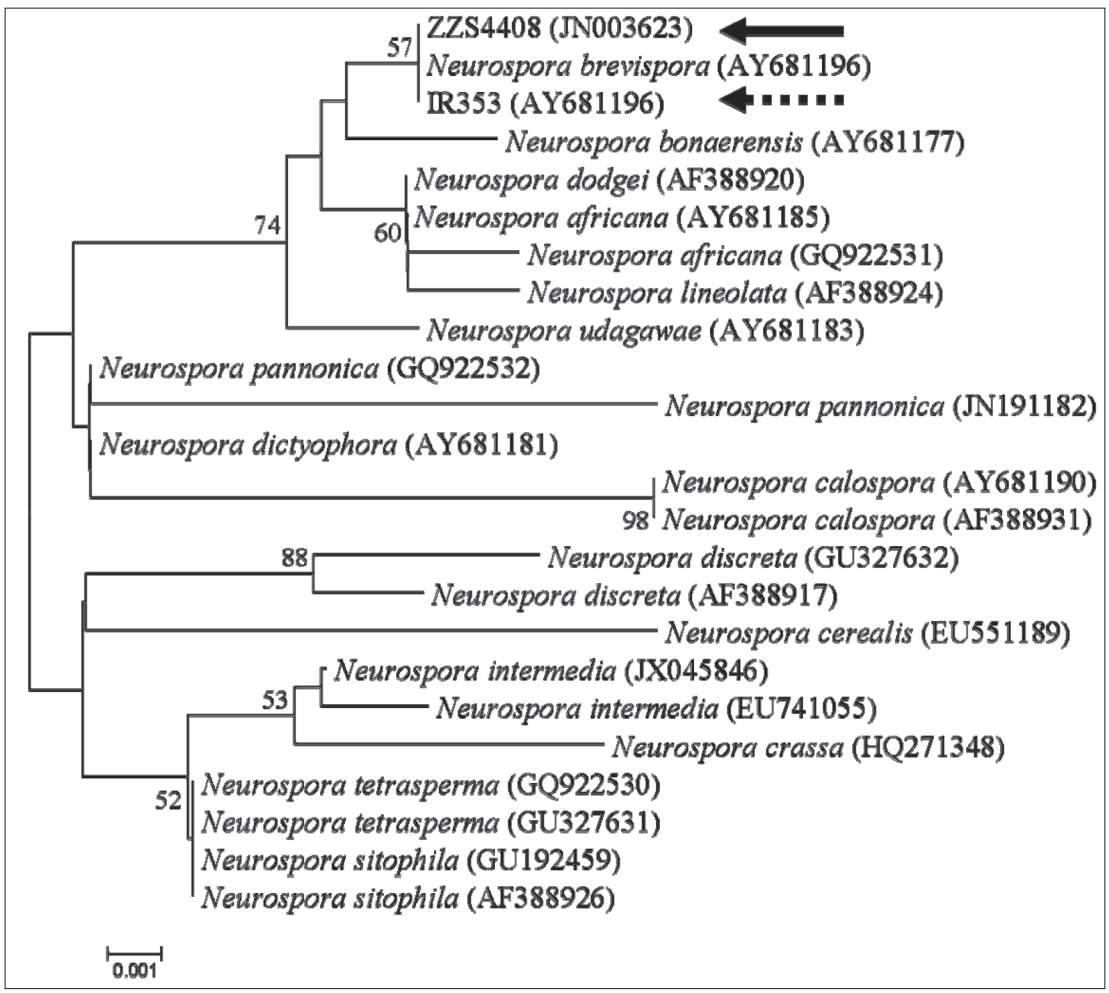

Fig. 3. A rDNA-ITS-based phylogenetic tree of *Neurospora* spp. showing the position of isolate ZZS4408. The numbers in parentheses represent the accession numbers of the *Neurospora* spp. in GenBank. The numbers in each branch points denote the percentages supported by bootstrap (values lower than 50% were not shown). The scale bar represents 0.001 substitutions per nucleotide position. The real line arrowhead indicates the isolate ZZS4408. The broken line arrowhead indicates the isolate IR353 (Li et al., 2011).

DISCUSSION

Dowding established *Gelasinospora* in 1933. The ascospores with surface depression were considered as the principal morphological characteristic of the genus (Dowding, 1933). Thereafter, *Gelasinospora* was accepted as a valid genus and used for descriptions of new fungal species (Alexopoulos and Sung, 1950; Khan and Krug, 1989). In 2004, García et al. systematically conducted a comparative study between *Neurospora* spp. and *Gelasinospora* spp. using molecular and morphological methods, and found that no differences existed in the cell wall patterns of ascospores as well as conserved DNA sequences between *Gelasinospora* and *Neurospora* spp., indicating that *Gelasinospora* and *Neurospora* might be regarded as the same taxon. As *Neurospora* was established earlier than *Gelasinospora*, the latter was treated as a synonym of *Neurospora* (García et al., 2004).

Gelasinospora brevispora (=*Neurospora brevispora*) was reported as a new species of *Gelasinospora* (Khan and Krug, 1989). Li et al. (2011) obtained isolate IR353 (GenBank accession number AB640864) from a wild cardamine shoot apical meristem (SAM) on Funiu Mountain, Henan, China, and treated it as a member of *G. udagawae*. In the phylogenetic tree established in the present study, both isolates IR353 and ZZS4408 clustered with *N. brevispora* (GenBank accession number: AY681196), clearly separated from *N. udagawae* (=*G. udagawae*, accession number: AY681183), and could be regarded as the same species (Fig. 3). In the phylogenetic tree established by Li et al. (2011), only nine isolates were used for establishing the phylogenetic tree. To establish the phylogenetic tree in the present study, a total of 22 isolates of *Neurospora* spp. were used as the reference isolates besides the isolate ZZS4408 (Fig. 3). The inadequateness of reference isolates used in the phylogenetic tree established by Li et al. (2011) might lead to the isolate IR353 being misidentified as *G. udagawae*. The river from which the isolate ZZS4408 was obtained originated from Funiu Mountain. It was reasonable to speculate that *N. brevispora* might be transmitted from Funiu Mountain to the county by the river originating from the mountain.

Although a few researchers identified isolates of *N. brevispora* (Li et al. 2011; Khan and Krug, 1989), so far little is known on its biological characteristics. The temperatures suitable for the growth of isolate ZZS4408 were 28-37°C with 31°C as the optimum (Table 1). Both Funiu Mountain and Xinye County are located in the northern subtropical and warm temperate transition zone where the summer temperatures frequently fluctuate between 28-33°C, fully meeting the needs for active growth of *N. brevispora* in this season. Among the pHs tested, the fungus showed the highest mycelial dry weight at pH5.5 (Table 2), indicating that an acidic environment might be more suitable for active growth of *N. brevispora*, which agreed with the other fungi (Lu et al., 2011; Wang et al., 2013).

In the present work, the growth rates of isolate ZZS4408 were significantly enhanced by heterodisaccharides (sucrose, lactose) compared to its constituent monosaccharides (glucose, fructose, galactose) (Table 3). The results suggested that two pathways metabolizing two different constituent monosaccharides might be activated in the case of utilizing each of the heterodisaccharides as a carbon source, resulting in more active growth compared to the monosaccharides. Among the 10 carbon sources tested, the deviation between the maximum mean colony diameter

and the minimum mean colony diameter was 17.4 mm (Table 3). On the other hand, among the seven nitrogen sources tested, the deviation between the maximum mean colony diameter and the minimum mean colony diameter reached up to 55.3 mm (Table 4), indicating that the influence of the nitrogen sources on the growth of isolate ZZS4408 was significantly greater than that of the carbon sources. This was agreed with *Athelia rolfsii* causing sesame southern blight (Wang et al., 2013), and might be due to the differences in the basic biological functions between carbon and nitrogen sources. The former mainly acts as an energy supplier, while the latter as a supplier of nitrogen for synthesis of biologically important macromolecules such as proteins and DNAs. Based on the biological characteristics of the isolate ZZS4408 in the present study, *N. brevispora* might be considered a desirable fungal species in morphodifferentiation studies due to its rapid growth feature. Our results increase understanding of the biological characteristics of *N. brevispora*.

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