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Full Length Article

Identification and Characterization of a Rare Species of *Neurospora*

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Abstract

An isolate (ZZS4408) of *Neurospora* was isolated from a water sample of Xinye county, Henan, China. The isolate was identified as *Neurospora brevispora*, a rare species of the genus with less information on biology, based on its morphological characteristics and ribosomal DNA internal transcribed spacer (rDNA-ITS) sequence. To clarify its basic biological characteristics, the relationship between growth of the isolate and temperature, pH and nutrition was studied. The temperature suitable for growth of the isolate was 28-37°C with 31°C as the optimum. The growth rate of hyphal tips reached to 19.1-42.5 (av.31.9) μm min^{-1} at 32°C. The pH suitable for vegetative growth was 5-7 with 5.5 as the optimum. Of the 10 carbon sources tested, two heterodisaccharides (sucrose and lactose) were most suitable for vegetative growth. Of the 7 nitrogen sources tested, D-alanine was most favorable for growth of *N. brevispora*. The vegetative growth of the fungus was more significantly influenced by nitrogen sources compared to carbon sources. *N. brevispora* was identified as a fast-growing fungal species, and could be considered a desirable material in morphodifferentiation study and demonstration. © 2015 Friends Science Publishers

Keywords: Biological characteristic; Identification; *Neurospora brevispora*; Rare species

Introduction

Neurospora is a genus of Ascomycete fungi with high academic and economical values, and consists of at least 26 species. The best known species in this genus is *Neurospora crassa*, a common model organism in biology (http://en.wikipedia.org/wiki/Neurospora). *N. crassa* has been used as a model organism in molecular genetics, physiology and biochemistry researches (Chung *et al*., 2001; He and Liu, 2005), and has great 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 high-activity dietary fiber (Tu *et al*., 2008). Other *Neurospora* spp. such as *N. intermedia* (Chen *et al*., 2009) and *N. stophila* (Wu *et al*., 2008; Deng *et al*., 2009) has also shown strong abilities in cellulose production.

Since 1998, the Neurospra International Symposium has been held every two years in California Asilomar Conference Center. The information presented and discussed in the symposium reflects the advancing status in *Neurospora* research. The current research on *Neurospora* is predominantly focused on *N. crassa* worldwide. Little is known of most of the other *Neurospora* spp., making the knowledge system in the genus dramatically unbalanced among different species. For most of the rare species such as *N. brevispora* (= *Galasinospora brevispora*), the biological characteristics are even unclear. Studies on the rare species of *Neurospora* undoubtedly may contribute to a better understanding on genetic diversity of *Neurospora*, and to a further utilization of the genus. In recent years, the authors isolated a rare species of *Neurospora* (isolate ZZS4408) from a river water sample in Henan, China. The objective of the present study was to identify and characterize the rare *Neurospora* species.

Materials and Methods

Experimental Details and Treatments

Experimental materials: An isolate (ZZS4408) of *N. brevispora* was isolated from a river water sample of Xinye county, Nanyang city, China (112°35′E, 32°52′N), using a conventional dilution plate method. The water sample was spread on PDA plate (200 g potato, 20 g glucose, 16 g agar, 1000 mL water), and incubated at 28°C for 24 h. A rapid-growing fungal colony (isolate ZZS4408) was isolated from the water sample and used for the present study.

Hyphal Growth Rates and Morphological Observations

The isolate ZZS4408 was grown on PDA plates at 28-32°C for 20 days to observe its morphological characteristics and to test its single hyphal growth rate. The determination of the single hyphal growth rate was conducted within 24 h of colony development at 32°C under a microscope. Two photographs of a hyphal tip were taken with 10-15 min interval. The hyphal growth rates were determined based on

the hyphal length increased to the time elapsed. After sporulation occurred, the dimensions of the developed perithecia, asci and ascospores were separately measured.

Phylogenetic Analysis

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 PDB liquid medium (the same components as in PDA except agar), and incubated at 28°C for 7 days. The mycelia were harvested and rinsed with sterile water to remove the residual medium components, and used for genomic DNA extraction after being dried at 80°C for 12h.

The genomic DNA of isolate ZZS4408 was extracted using the liquid nitrogen method (Lu *et al*., 2011). Dry mycelial sample was placed in a sterile mortar and rapidly ground in liquid nitrogen to obtain mycelial powder. The mycelial powder was transferred to a sterilized centrifuge tube with 800 µL lysis buffer, and bathed in hot water (65°C) for 2 h, in which the tube was turned reversely once every 10 min. Subsequently the DNA solution was centrifuged at 4°C under 12 000 rpm for 10 min. The supernatant was added to an equal volume of the mixture of three organic solvents (phenol: chloroform: isoamyl alcohol =25:24:1). After gentle blending, the DNA solution was centrifuged at 4°C under 12 000 rpm for 10 min. The extraction process was repeated three times. Two volumes of precooled ethanol and 1/10 volume of 3 mol/L sodium acetate solution were added into the DNA solution and shaken well. After being maintained at -20°C for 1.5 h, the DNA solution was centrifuged at 4°C under 12 000 rpm for 10 min. After removing 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 samples were appropriately diluted with PCR buffer according to the brightness of the genomic DNA bands in electrophoresis (Qi *et al*., 2009). PCR reaction components consisted of 1 µL genomic DNA, 15 µL mixture (PCR buffer, dNTP, *Taq* DNA polymerase, $MgCl_2$), 1.2 µL of each of 10 pmol/L ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and 10 pmol/L ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'), and ddH_2O was added to a final volume of 30 µL. 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 extending at 72°C for 30 s, and extending at 72°C for another 10 min. After completion of the reaction, the PCR products were analyzed with 1% agarose gel electrophoresis, and send to Takara Biotechnology (Dalian) Co., Ltd. for sequencing (both-strand sequenced) after purification. Sequences homology comparison was conducted using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Related rDNA-ITS sequences of other *Neurospra* spp. from 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 (Lin *et al*., 2011). Both pairwise and multiple aligments of bases were performed using Clustal W(1.6) with gap opening penalty 15 and gap extension penalty 6.66. Bootstrap test of the phylogenetic tree was performed by the Neighbor-Joining method under 10000 replicates (seed=64238).

Tests on Cultural Conditions

Effect of temperature on vegetative growth of isolate ZZS4408: Isolate ZZS4408 was grown on PDA plates at 25°C for 2 days. Mycelial plugs (6 mm diameter) were cut out with sterilized punch from the actively growing edges of the colonies, and transferred on PDA plates. After incubation at different temperatures (4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40 and 43°C) for 3 days, the diameters of the developed colonies were cross-measured. Three replicates were set up for each treatment.

Effect of pH on vegetative growth of isolate ZZS4408: The pH of PDB was adjusted at different levels (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) with NaOH/HCl. Mycelial plugs of isolate ZZS4408 were inoculated to 100 mL PDB with different pHs. After incubation at 25°C for 6 days, mycelia were harvested, and dried overnight at 60°C. The dry weights of the mycelia were separately measured (Lu *et al*., 2011). Three replicates were set up for each treatment.

Effect of different carbon and nitrogen sources on vegetative growth of isolate ZZS4408: A basic medium (BM: 2 g 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, 1000 mL water) was used for nutrition tests. To determine the effect of carbon sources on vegetative growth of isolate ZZS4408, the sucrose of 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 effect of nitrogen sources on vegetative growth of isolate ZZS4408, the D-alanine of BM medium was substituted with equal amounts of glycine, carbamide, L-cystine, L-histidine, sodium nitrate and ammonium dihydrogen phosphate, respectively. The media were sterilized using a conventional method. Mycelial plugs of isolate ZZS4408 were prepared as mentioned above, and inoculated on the BM plates containing different carbon and nitrogen sources. The plates were incubated at 25°C for 3 days prior to measuring the diameters of developed colonies. Three replicates were set up for each treatment.

Statistical Analysis

Data obtained from the experiments were subjected to analysis of variance (ANOVA) using a SPSS statistical software (version 16.0; 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. Within 3 days of incubation on the plates at 28°C, the diameter of colonies was greater than 7 cm. The growth rate of 8 randomly selected hyphal tips was 19.1-42.5 (mean 31.9) μm min^{-1} at 32°C. Before the colonies covered the PDA plates, they barely produced aerial mycelia. As the colonies fully covered the plates, aerial mycelia were vigorously produced, with white to dark gray color (Fig. 1A). The diameter of the hyphae was 3.0-16.0 μm. The mycelia overgrew on whole the inner glass surface of the lid of Petri dish. After 10 days of incubation, black perithecia developed on the inner surface of the lid of Petri dish as well as on the PDA plates. The perithecia were spherical, oval or flask-shaped (Fig. 1B), with a dimension of 117.6-454.9 μm × 156.0-498.0 μm. After 60 days of incubation, mature ascospores were found in the developed asci. The asci were rod-shaped, with a dimension of approximately 160×16 μm, containing 8 linearly aligned ascospores in an ascus (Fig. 1C). The ascospores were oval-shaped, unicellular, black-brown with a dimension of 18.7-22.7 μm × 13.1-15.4 μm (mean 20.9 ×14.3 μm)(Fig. 1D).

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* (=*Gelasiaospora brevispora*) (Fig. 3) as well as the isolate IR353 reported by Li *et al*. (2011). The phylogenetic tree supported that isolate ZZS4408 is a member of *N. brevispora*.

Effects of Temperature, pH and Carbon and Nitrogen Sources on the Growth of Isolate ZZS4408

The temperature range for the growth of isolate ZZS4408 was 10-40°C (Table 1). The mean diameter of colonies developed at temperatures 28-37°C for 5 days was greater than 80 mm. Although no difference was observed among the temperatures at 0.05% level, the maximum growth was observed at 31°C with a mean colony diameter of 89.7 mm. Therefore, the optimum growth temperature range could be considered between 28-37°C, and the optimum growth temperature as 31°C (Table 1). Taking the peak growth temperature 31°C as a dividing point, significant difference was observed in the variation of colony diameter between the low temperature side and high temperature one: colony diameters gradually increased with the increase of temperatures in the former, and sharply decreased in the latter. At the temperatures lower than 31°C, the increase of 1°C in average came with the mean increased diameter of 3.74 mm, while at the temperatures higher than 31°C,

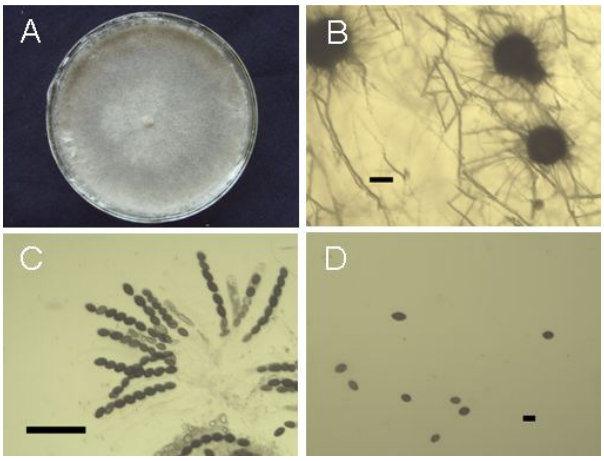

Fig. 1: Morphological characteristics of isolate ZZS4408
A, a colony incubated at 31°C on PDA plate for 14 days; B, perithecia developed on BM plate after 15 days of incubation at 28°C (scale bar=100 μm); C, an ascus containing 8 linearly aligned ascospores on BM plate after 15 days of incubation at 28 °C (scale bar=100 μm); D, ascospores on BM plate after 15 days of incubation at 28 °C (scale bar=20 μm)

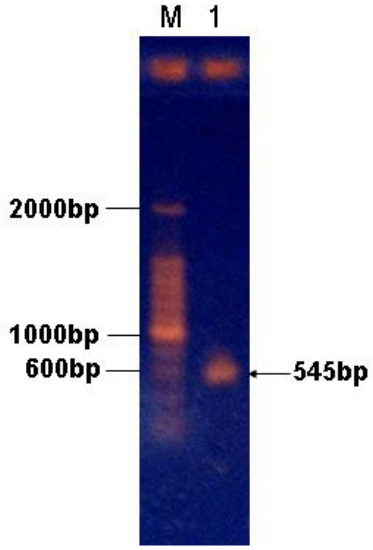

Fig. 2: Electrophoretogram for PCR products of the rDNA-ITS of isolate ZZS4408
1, isolate ZZS4408; M, DNA marker

the increase of 1°C came with 7.48 mm colony diameter reduced. As the temperature rose from 40°C to 43°C, the mean colony diameter sharply dropped from 73.6 mm to 0 mm.

Vegetative growth of isolate ZZS4408 was significantly influenced by pH (Table 2). The isolate could grow at pH 4-9 as indicated by mycelial dry weight, 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 0.12 g in the other pH treatments.

Table 1: Effects of temperature on vegetative growth of isolate ZZS4408

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

*Data are the means of three replicates. The means followed by the same capital and lower case letters are not significantly different at $P=0.05$ and $P=0.01$, respectively

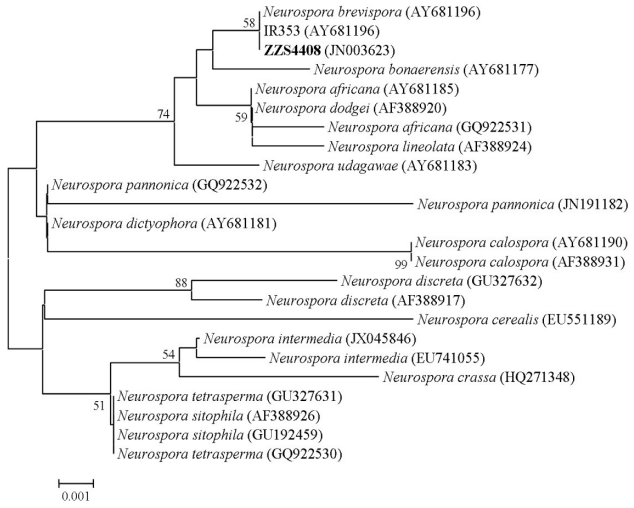

Fig. 3: A rDNA-ITS-based phylogenetic tree of *Neurospora* spp. showing the position of isolate ZZS4408
Note: The numbers in parentheses represent the accession numbers of *Neurospora* spp. in GenBank. The numbers in each branch points denote the percentages supported by bootstrap (values lower than 50% are not shown). The scale bar represents 0.001 substitutions per nucleotide position

Therefore, pH 5-7 could be considered as the favorable pH range for vegetative growth of isolate ZZS4408 and pH5.5 is the optimum pH for its growth.

The effect of carbon sources on vegetative growth of isolate ZZS4408 is shown in Table 3. Among 10 carbon sources tested, favorable growth was found on the medium plates containing heterodisaccharides (sucrose/lactose) with a colony diameter of greater than 80 mm, compared to the other carbon sources with a reduced colony diameter. A weak vegetative growth was detected on the plates containing galactose as carbon source with a colony diameter less than 70 mm. A moderate growth was observed 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 for the vegetative growth of the isolate compared to the monosaccharides. Among the 10 tested carbon sources, the deviation between the maximum mean colony diameter and minimum mean colony diameter was of 17.4 mm.

The effect of nitrogen sources on the vegetative growth of isolate ZZS4408 is shown in Table 4. Among 7 nitrogen sources tested, D-alanine treatment had the maximum mean colony diameter (87.7 mm) compared with the other nitrogen sources, followed by L-histidine and L-cystine with a colony diameter of 70-80 mm. The colony diameter on the plates containing the other nitrogen sources was less than 70 mm. The difference between the maximum mean colony diameter and the minimum one reached up to 55.3 mm.

Discussion

Dowding established the genus *Gelasinospora* in 1933. The ascospores with surface depression were considered as the principal morphological characteristic of *Gelasinospora* (Dowding, 1933). Thereafter, *Gelasinospora* was once accepted as a valid genus and used for description of new fungal species (Alexopoulos and Sung, 1950; Khan and Krug, 1989). In recent years, García *et al.* (2004) systematically conducted a comparative study between *Neurospora* spp. and *Gelasinospora* spp. using molecular and morphological methods, and reported that no difference in the cell wall patterns of ascospores existed between *Gelasinospora* and *Neurospora* species, and that no difference was found between the two genera in specific DNA sequences. As *Neurospora* was established earlier than *Gelasinospora*, the latter was treated as a synonym of *Neurospora* (García *et al*., 2004).

G. brevispora (=*N. brevispora*) was reported as a new species of *Gelasinospora* by Khan and Krug (1989). Li *et al.* obtained isolate IR353 (GenBank accession number: AB640864) from a wild cardamine sam in Funiu Mountain, Henan, China, which was identified as *G. udagawae* (Li *et al*., 2011). In the phylogenetic tree established in the present study, both isolates IR353 and ZZS4408 clustered with *N. brevispora* (GenBank accession number: AY681196), clearly separating from *N. udagawae* (=*G. udagawae*, accession number: AY681183), and could be considered as the same species (Fig. 3). In the phylogenetic tree established by Li *et al.* (2011), only 9 isolates were used for the establishment of the tree. To establish the phylogenetic tree in the present study, 22 isolates of *Neurospora* spp. were used as the reference isolates besides the isolate ZZS4408 (Fig. 3). The inadequateness of isolates of *Neurospora* spp. used in the establishment of the phytogenetic tree by Li *et al*. (2011) might lead to the isolate IR353 misidentified as *G. udagawae.* The Funiu Mountain is one of the water sources for the river from which the

Table 2: Effects of pH on vegetative growth of isolate ZZS4408

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

*Data are the means of three replicates. The means followed by the same capital and lowercase letters are not significantly different at $P=0.05$ and $P=0.01$, respectively

Table 3: Effects of carbon sources on vegetative growth of isolate ZZS4408

Carbon source	Colony diameter(mm)*
Sucrose	85.2 Dc
Lactose	82.7 CDc
Glucose	70.7 ABab
Fructose	72.6 ABab
Xylitole	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

*Data are the means of three replicates. The means followed by the same capital and lowercase letters are not significantly different at $P=0.05$ and $P=0.01$, respectively

Table 4: Effects of nitrogen sources on vegetative growth of isolate ZZS4408

Nitrogen source	Colony diameter(mm)*
D-alanine	87.7 Ed
Carbamide	32.4 Aa
L-histidine	70.9 CDc
Glycine	54.5 Bb
Sodium nitrate	69.5 CDc
L-cystine	77.0 Dcd
Ammonium dihydrogen phosphate	65.7 Cbc

*Data are the means of three replicates. The means followed by the same capital and lowercase letters are not significantly different at $P=0.05$ and $P=0.01$, respectively

isolate ZZS4408 was obtained. Although Xinye county is more than 100 km away from Funiu Mountain, the *N. brevispora* might be carried from the Funiu Mountain to the county through the river partially originating from the mountain.

As a few researchers identified isolates of *N. brevispora* (Khan and Krug, 1989; Li *et al*., 2011), little is known about its biological characteristics. The temperature suitable for the growth of isolate ZZS4408 was 28-37°C with 31°C as the optimum (Table 1). Both the Funiu Mountain and the Xinye county locate in the northern subtropical and warm temperate transition zone in which the summer temperatures frequently fluctuates between 28-33°C, substantially supporting the rapid growth of *N. brevispora* in this season. Among the pH levels tested, the fungus showed the maximum dry mycelial weight at pH5.5 (Table 2), indicating that the acidic environment was more suitable for the growth of *N. brevispora*, like some other plant pathogenic fungi (Lu *et al*., 2011; Wang *et al*., 2013).

In the present work, the growth rate of isolate ZZS4408 was significantly enhanced by heterodisaccharides (sucrose, lactose) compared to its constituent monosaccharides (glucose, fructose, galactose) (Table 3). The results suggested that two pathways of metabolizing two different monosaccharides might be activated in the case of utilizing heterodisaccharides as carbon sources, resulting in more rapid growth compared to the monosacchardes. 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 7 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). The influence of the nitrogen sources on the growth of isolate ZZS4408 was significantly greater than that of the carbon sources. This same has been reported in *Athelia rolfsii* causing sesame southern blight (Wang *et al*., 2013), and might be due to the difference in the basic biological functions between carbon and nitrogen sources. The former mainly act as an energy supplier, while the latter as a supplier of nitrogen during synthesis of biologically important macromolecules such as proteins and DNAs. On the other hand, *N. brevispora* might be considered a desirable fungal material in morpho-differentiation study due to its rapid growth on PDA plates (Table 1). Our results enhance the understanding on the biological characteristics of *N. brevispora*.

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