



# Muscodor albus MOW12 an Endophyte of Piper nigrum L. (Piperaceae) Collected from North East India Produces Volatile Antimicrobials

Authors: Debdulal Banerjee, Akhil Pandey, Maloy  
Jana, and Gary Strobel

This is a postprint of an article that originally appeared in [Indian Journal of Microbiology](#) in March 2014.

The final publication is available at Springer via <http://dx.doi.org/10.1007/s12088-013-0400-5>

Banerjee, D., Pandey, A., Jana, M., and Strobel, G. (2014). Muscodor albus MOW12 an endophyte of Piper nigrum L. (Piperaceae) collected from North East India produces volatile antimicrobials. *Indian Journal of Microbiology* 54: 27-32.  
<http://dx.doi.org/10.1007/s12088-013-0400-5>

Made available through Montana State University's [ScholarWorks](#)  
[scholarworks.montana.edu](http://scholarworks.montana.edu)

# Muscodor albus MOW12 an Endophyte of Piper nigrum L. (Piperaceae) Collected from North East India Produces Volatile Antimicrobials

Debdulal Banerjee<sup>1</sup>, Akhil Pandey<sup>1</sup>, Maloy Jana<sup>1</sup>, and Gary Strobel<sup>2</sup>

<sup>1</sup> Microbiology Laboratory, Department of Botany and Forestry, Vidyasagar University, Midnapore 721 102, West Bengal, India

<sup>2</sup> Department of Plant Science, Montana State University, Bozeman, MT, USA

## Abstract

Muscodor albus MOW12, an endophytic fungus isolated from Piper nigrum in Mawlong, Meghalaya, India, resembles some cultural and hyphal characteristics of previous isolates of Muscodor sp. In addition, it possesses about 99 % similarity in its ITS rDNA with other M. albus isolates and thus is nicely centered within the genetic tree to other Muscodor spp. This xylariaceae fungus effectively inhibits and kills certain plant pathogenic fungi by virtue of a mixture of volatile compounds that it produces. The majority of these compounds were identified by gas chromatography/mass spectrometry as small molecular weight esters, alcohols, and acids. The main ester components of this isolate of M. albus in its volatile mixture are acetic acid, ethyl ester; propanoic acid, 2-methyl-, methyl ester and acetic acid, 2-methylpropyl ester. This appears to be the first report of any M. albus strain from India.

**Keywords** Endophyte · Fungi · *Muscodor albus* · *Piper nigrum* · Volatile organic compound

## Introduction

Muscodor albus, Woropong, Strobel & Hess strain CZ620, a volatile organic compound (VOC)-producing fungus, was first isolated from Cinnamomum zeylanicum from a rain-forest of Honduras in the late 1990s [1]. It produces several volatile compounds which, as a mixture, are both inhibitory and, for the most part, lethal to a broad range of pathogenic fungi and bacteria [2–4]. Different isolates of Muscodor sp. produce unique sets of volatile compounds [2]. There are many reports on VOC production by microorganisms, many of these VOCs are common to many microorganisms, whereas others seem to be unique for one species [5–8]. There are a limited number of VOC-producing fungi possessing any biological activity with their volatile compounds. As isolates of M. albus can inhibit and kill a broad range of both bacterial and fungal pathogens in practical test situations, they are being considered for use in agriculture, medical and industrial applications [2]. Mycofumigation with M. albus against a pathogen was already reported in smut-infected barley seeds and 100 % disease control was reported [1]. It was also applied for the treat-

ment of fruits in storage and in transit [9]. Soil treatments have also been effectively used in both field and greenhouse situations [10]. Patents with this organism were granted to control harmful microbes in human and animal wastes [11] and to use in agriculture [12] and also in seed and grain sanitation [13]. Muscodor albus is completely sterile; spores or fungal fruiting bodies were never observed when the fungus was examined under different laboratory conditions [14, 15]. Until the present most of the Muscodor species are reported from tropical locations of Asia and South America, but there is no report of this organism in India. In this communication, we report a new strain of M. albus, designated as MOW12, having genetic, biological and morphological similarities with M. albus strain I41-3s, which inhibits and kills many plant pathogens [16]. Muscodor albus strain MOW12 also has a unique set of VOCs and biological activities and, for the first time, this organism has been isolated from a plant existing in India. Presently there are several patents that have issued

on *M. albus* as related to its commercial exploitation. The demonstration that *M. albus* exists in the natural environment of the India has enormous implications for governmental regulation of this organism and for its practical biological uses in agriculture and industry.

## Materials and Methods

### Collection of Plant Samples

*Piper nigrum* L. was collected from Mawlong (East Khasi Hill district) area of Meghalaya (25°12' North and 91°41' East). Plantlets were sealed in a zip lock plastic pack immediately after collection to resist dehydration. Samples were transported to laboratory within 72 h after collection. The packet containing plant samples were kept refrigerated (at 4 °C) until endophyte isolation.

### Isolation of Endophyte

Plant parts were washed with tap water. Explants are cut into pieces, and then subjected to surface sterilization with 70 % ethanol for 45 s. Explants were flamed to evaporate alcohol. Woody stems were cut into several layers of tissue with a sterile scalpel. Explants were placed on 2 % water agar plate and incubated at 25 °C until endophytes became visible around the samples.

### Pure Culture of Isolated Fungi

When endophytes were visible around the samples, hyphal tips of the fungus were transferred with a sterile needle tip to a Potato Dextrose Agar plate. Plates were properly marked, are sealed with parafilm and incubated at 25 °C. Plates were checked regularly for growth of endophytes.

### Screening of Fungal Strains for VOC Production

Pure cultures of fungi were tested against *M. albus* GBA strain since *M. albus* produces potent volatile antibiotic compounds. If any endophyte strain remains alive when it cultured with *M. albus*, then there is a possibility that it may be a related species of *Muscodor* which may also produce VOCs. So to screen for VOCs; PDA media was poured in a plate and allowed to cool. A simple bioassay test system was devised which allowed for VOCs only being the agents for any microbial inhibition being assessed. Initially, an agar strip of 1.0 cm wide is completely removed from the mid portion of PDA plate. The act of removing a strip of agar from the mid portion of the plate effectively precluded the diffusion of any inhibitory soluble compounds emanating from *M. albus*. Now *M. albus*

was cultured in one side of the plate and plate is properly sealed. The plate was kept in an incubator at 25 °C for 4–5 days prior to testing. When the colony diameter of *M. albus* became 1–2 cm then test fungi are placed on the other side of the plate. The plate was again sealed and kept in incubator at 25 °C. After 2 days, the plates were checked for growth of test organisms. The fungal species that survived were tested against fungal plant pathogens such, *Pythium* sp., *Geotrichum* sp., *Aspergillus* sp., *Trichoderma* sp., *Cercospora* sp., *Botrytis* sp., *Fusarium* sp., *Phytophthora palmivora*, *Sclerotinia* sp., *Colletotrichum leginerium*.

### Confirmation Tests for Volatile Antimicrobial Production

First PDA is poured in plates and allowed to cool. An agar channel at the center of the plate is cut to resist diffusion of non volatiles. Some plates were retained as control plates for pathogens. Endophytes were cultured at one of half of the plate and marked at the back side. Plates were sealed and kept in an incubator at 25 °C until endophytes became 1.5–2.0 cm diameter size. Then pathogens were inoculated on the other side of the plate. A control plate for each pathogen was made to measure the percent of inhibition. Plates were observed daily and any inhibition of growth was noted. After few days, if any pathogen/s is/had not grown, the block/s is/are transferred into fresh PDA plates to confirm whether the pathogen was fully inhibited or killed by the endophyte.

### Scanning Electron Microscopy of Endophytes

The fungi were grown on PDA plates and then processed for SEM. The samples were slowly dehydrated in ethanol, then critically point dried, coated with gold and examined under a scanning electron microscope (Zeiss) at 10.00–20.00 kv ETH.

### GC–MS Analysis of Volatiles

The analytical conditions are: instrument: Agilent 6890 GC with 5973 Network MSD and G1888 static Headspace sampler; column: ZB-624, 6 % cynopropyl phenyl polydimethylsiloxane, 30 m × 0.25 mm × 1.4 µ; oven temperature program: initial 40 °C, hold time 2 min, 8 °C/min ramp, final 240 °C, hold time 2 min; carrier gas: He @ 1.0 mL/min, constant flow (36.7 cm/s velocity); injection mode: split less for 1 min, 220 °C; head space conditions: vial temperature—85 °C, loop temperature—95 °C, transfer line temperature—100 °C; vial pressure 10 psi, pressurization time 0.5 min, loop fill time—0.05 min, loop

equilibration time—0.01 min; injection time—1 min, vial equilibration time 30 min; transfer line temperature: 220 °C; MS conditions: ion source—EI—230 °C; quadrupole—150 °C; library search reports: NIST and WILEY library databases; The data is presented in the following way: 1. Each sample TIC (top) is accompanied by the control sample TIC (bottom), 2. The peaks that were found extra in the cultured samples were identified by comparison with the control sample TIC and the data for only those extra peaks associated with the fungus are presented.

## Results and Discussion

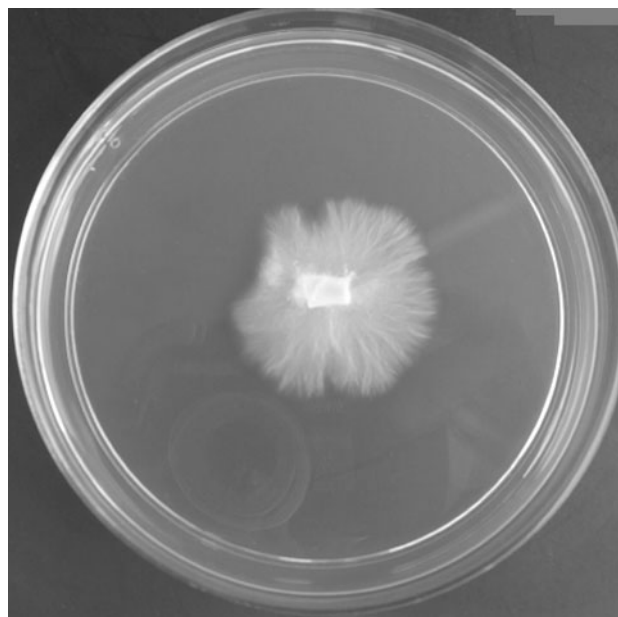
### Identification of *M. albus* MOW12

This isolate was obtained by using the *M. albus* selection technique on small pieces of limb tissue of *Piper longum* placed on split PDA plates. The organism appeared to have a whitish mycelium with heavily intertwining hyphae (Fig. 1). When trying to transfer it to other plates, the mycelial mat did not lift of the surface of the agar (Fig. 2) as previous *M. albus* isolates [17]. The SEMs showed hyphae as intertwined and appearing in rope-like and coiled strands which is similar to other *M. albus* isolates (Fig. 3) [3]. Under no circumstances was it ever possible to observe any fruiting bodies or spores being produced by this fungal isolate.

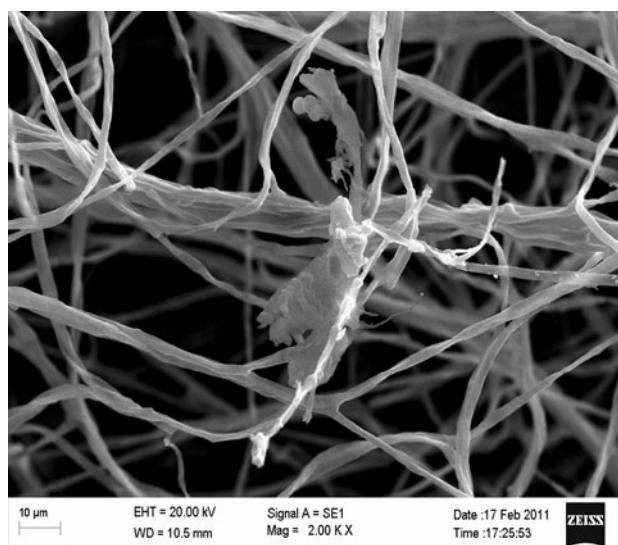
The ITS-5.8S rDNA-ITS sequence data of isolate MOW12 were obtained and deposited as JX469138 in GenBank. A BLAST search of the database indicated at



**Fig. 1** *Piper* sp. collected from rain forest of Mawlong, Meghalaya. From this host the *M. albus* MOW12 strain was isolated



**Fig. 2** MOW12 in plate culture



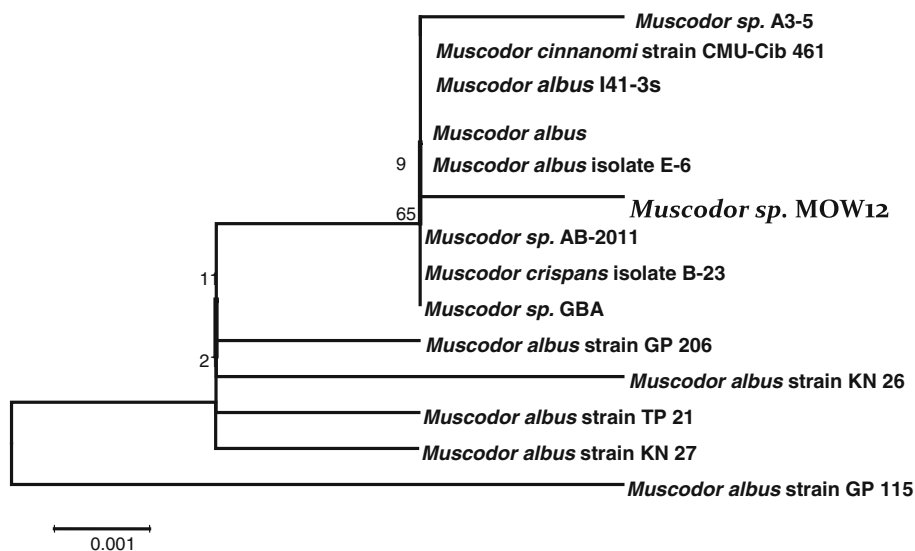
**Fig. 3** SEM of MOW12 at  $\times 2,000$  magnification

least 99 % sequence identity to the previous isolate of *M. albus* I41-3s [16] and a close genetic relationship to other isolates of this fungus including the original *M. albus* isolate CZ620 [1], as per the phylogenetic tree (Fig. 4).

### Chemical Composition of the Volatiles

The VOCs produced by *M. albus* MOW12 were tentatively identified by the initial GC/MS method. These compounds ultimately fell into several classes of chemical substances. Present in the mixture of a 2-week-old culture were esters, alcohols, acids, lipids and ketones (Table 1). Comparable

**Fig. 4 a** Phylogenetic tree to show the relationship of *M. albus* MOW12 with other *M. albus* strains. The evolutionary history was inferred using the neighbor-joining method [21]. The optimal tree with the sum of branch length = 0.02559860 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 471 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [22]

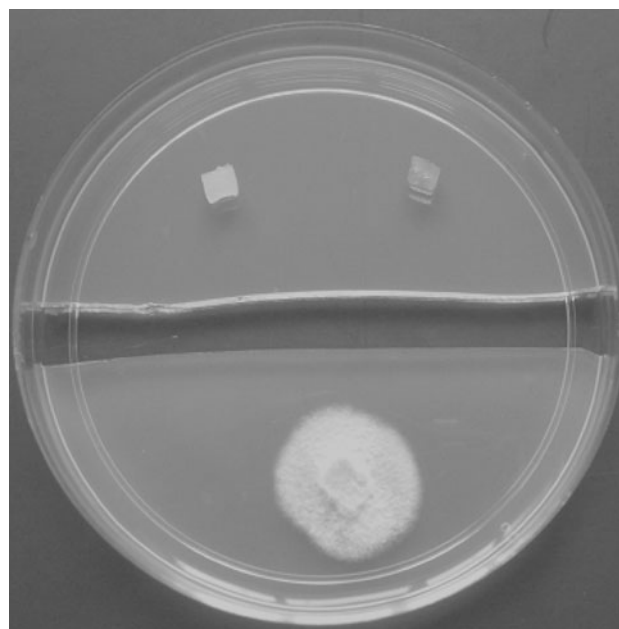


**Table 1** GC/MS analysis of the VOCs of *M. albus* MOW12 after 10 days incubation at 23 °C on PDA

RT	Compound	Relative %
4.102	Ethanol	71.2
6.153	Acetic acid, ethyl ester	4.7
6.981	2-Methyl-1-propanol	2.4
7.598	2-Methyl-propanoic acid, methyl ester	2.4
8.179	Pentanal	1.0
9.427	2-Methyl-1-butanol,	8.0
9.724	Acetic acid, 2-methylpropyl ester	3.0
10.613	Hexanal	5.1
12.108	3-Methyl-1-butanol acetate	0.8
12.182	2-Methyl-1-butanol	0.8
12.467	Hexanol	0.5
12.640	Styrene	0.5

RT retention time, VOC volatile organic compound

analyses were done on the gas phase above a regular PDA petri plate and several compounds were identified and subsequently eliminated from the analysis done on the petri plate containing *M. albus* MOW12. Final identification of 10 compounds was done and compounds have only been tentatively identified on the basis of the data base information (Table 1). The most abundant compound, based on the total area of the GC analysis, was ethanol followed by hexanal and acetic acid ethyl ester (Table 1). Collectively, the alcohols comprised the greatest percentage of compounds present in the gas phase of the *M. albus* MOW12 culture followed by esters and acids (Table 1). The VOC data also follow a distinct pattern for this fungal isolate since no other *Muscodor* isolate ever studied revealed a pattern identical to this one (Table 1) [1–4].



**Fig. 5** The *M. albus* split-plate test for assaying test organisms in the presence of fungal VOCs. In this particular assay, none of the test organisms grew in the presence of *M. albus* MOW12

#### Biological Effects of *M. albus* MOW12 Volatiles on Various Pathogenic Fungi

A wide range of freshly growing pathogenic fungi were tested in the standard bioassay test (Fig. 5). The test organisms were selected on the basis of a broad taxonomic representation of major plant fungal pathogens. Most test organisms were completely inhibited, and in fact killed, after a 2-day exposure to the *M. albus* MOW12 gases (Table 2). A time frame of 4 days was used as the exposure period for all test fungi and bacteria. However, a few microbes, including *Fusarium*

**Table 2** Response of several test fungi to the volatiles of *M. albus* strain MOW-12

Pathogens	Kill	Inhibit	% of Inhibition
<i>Sclerotinia</i> sp.	+	+	100
<i>Pythium</i> sp.	+	+	100
<i>Geotrichum</i> sp.	+	+	100
<i>Botrytis</i> sp.	+	+	100
<i>Fusarium</i> sp.	–	+	40
<i>Trichoderma</i> sp.	–	+	10
<i>Aspergillus</i> sp.	–	+	100
<i>Colletotrichum lagenarium</i>	+	+	100
<i>Cercospora</i> sp.	+	+	100
<i>Phytophthora palmivora</i>	+	+	100

The test organism was exposed to the VOCs of these *Muscodor* strain for 48 h. Percentage inhibition was measured relative to the growth of the control culture. Viability was tested after the original inoculum was replaced on a regular PDA plate and checked for growth. The experiment was repeated twice with comparable results

+ yes, – no

*solani*, and *Trichoderma* sp. (10%) were only partially inhibited after a 4-day exposure to *M. albus* MOW12, and in fact, for more prolonged periods, these test organisms never seemed to expire (Table 2). Earlier complete inhibition of pathogenic fungi were also reported in other strains of *M. albus* [3, 4, 16]. *Trichoderma* sp., an agriculturally important fungi used as a biocontrol agent was 20–70 % inhibited with *M. albus* in earlier reports [4, 16].

## Conclusion

Endophytic fungi may exist in their host plants in a range of biological associations from near pathogenic to symbiotic [18]. In the latter case, a number of endophytes have been discovered that make products that are extremely biologically active and selective against certain microbes that may be a potential threat to the host plant [19]. In this manner the endophyte seems to have the potential to contribute to the benefit of the host by providing protection to it from a major biological threat—a plant pathogen. Some protective compounds recently isolated from endophytes are exemplified by taxol, oocydin A, cryptocin, ambuic acid and jesterone [20]. Each is active against a select group of pathogens and each is soluble in organic solvents. While this mechanism for the protection of the host plant may exist with endophytes producing such compounds, it seems that no comparable situation involving inhibitory and lethal volatile inhibitors has been previously demonstrated except for the various isolates of *Muscodor* spp. This is the only *Muscodor* sp. producing ethanol as major volatile component. Only 10 % inhibition of *Trichoderma*

sp. was found with the volatiles of this organism. So it can be applied in the field with *Trichoderma* to prevent soil borne pathogens. This is the first report of *M. albus* isolated from India and also from *Piper nigrum* family-Piperaceae. The organism produces more than 70 % ethanol which is unique with this strain of *Muscodor albus*. Only 10 % inhibition of *Trichoderma* sp. was observed with this organism which was about 20–70 % with other strains. The demonstration that *M. albus* exists in the natural environment of the India has enormous implications for governmental regulation of this organism and for its practical biological uses in agriculture and industry.

**Acknowledgments** DB is thankful to University Grants Commission, New Delhi for financial assistance and Mr. Pallab Pattanayak for his help during this study. Dr Strobel is grateful for DoE and NSF project support.

## References

1. Strobel GA, Dirksie E, Sears J, Markworth C (2001) Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. *Microbiology* 147:2943–2950
2. Strobel G (2006) *Muscodor albus* and its biological promise. *J Ind Microbiol Biotechnol* 33:514–522
3. Mitchell AM, Strobel GA, Moore E, Robison R, Sears J (2010) Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. *Microbiology* 156:270–277
4. Banerjee D, Strobel G, Geary B, Sears J, Ezra D, Liarzi O, Coombs J (2010) *Muscodor albus* strain GBA, an endophytic fungus of *Ginkgo biloba* from United States of America, produces volatile antimicrobials. *Mycology* 1:179–186
5. Schnurer J, Olsson J, Borjesson T (1999) Fungal volatiles as indicators of food and feeds spoilage. *Fung Genet Biol* 27:209–217
6. Schulz S, Dickschat JS (2007) Bacterial volatiles: the smell of small organisms. *Nat Prod Rep* 24:814–842
7. Korpi A, Jarnberg J, Pasanen AL (2009) Microbial volatile organic compounds. *Crit Rev Toxicol* 39:139–193
8. Banerjee D, Strobel GA, Booth E, Geary B, Sears J, Spakowicz D, Busse S (2010) An endophytic *Myrothecium inundatum* producing volatile organic compounds. *Mycosphere* 1:229–240
9. Mercer J, Jimenez JI (2004) Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus*. *Post Harvest Biol Tech* 31:1–8
10. Mercier J, Manker D (2005) Biocontrol of soil-borne disease and plant growth enhancement in green house soilless mix by the volatile-producing fungus *Muscodor albus*. *Crop Prot* 24:355–362
11. Strobel GA, Ezra D (2008) Application of *Muscodor albus* to control harmful microbes in human and animal wastes. United States Patent 7341862
12. Strobel GA, Mankar DC, Mercier J (2012) Novel endophytic fungi and methods of use. United States Patent 8093024
13. Jimenez JI, Margolis JS, Biard JK, Lego SF (2012) Compounds derived from muscodor fungi. United States Patent 0058058
14. Woropong J, Strobel GA, Ford EJ, Li JY, Baird G, Hess WM (2001) *Muscodor albus* anam. nov., an endophyte from *Cinnamomum zeylanicum*. *Mycotaxon* 79:67–79
15. Ezra D, Hess WM, Strobel GA (2004) Unique wild type endophytic isolates of *Muscodor albus*, a volatile antibiotic producing fungus. *Microbiology* 150:4023–4031

16. Atmosukarto I, Castillo U, Hess WM, Sears J, Strobel G (2005) Isolation and characterization of *Muscodora albus* I-41.3s, a volatile antibiotic producing fungus. *Plant Sci* 169:854–861
17. Strobel G, Knighton B, Kluck K, Ren Y, Livinghouse T, Griffen M, Spakowicz D, Sears J (2008) The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum*. *Microbiology* 154:3319–3328
18. Bacon CW, White JF Jr (2000) *Microbial endophytes*. Marcel Dekker, New York
19. Yang X, Strobel GA, Stierle A, Hess WM, Lee J, Clardy J (1994) A fungal endophyte tree relationship: *Phoma* sp. in *Taxus wallichiana*. *Plant Sci* 102:1–9
20. Strobel GA, Daisy BH, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. *J Nat Prod* 67:257–268
21. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol and Evol* 28:2731–2739