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A New Species of Hyphomycetes, *Aspergillus coreanus* sp. nov., Isolated from Traditional Korean *Nuruk*

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Abstract Strain NR 15-1^T isolated from traditional Korean *Nuruk* is described as a new species and named as *Aspergillus coreanus* NR 15-1^T sp. nov. Strain NR 15-1^T grew rapidly to form yellow-green colonies whose surfaces were velvety on Czapek solution agar. Conidial heads were yellow to light and elliptical, whereas the conidiophore was colorless and typically long. In addition, vesicles were from flask-shaped to globose, and sterigmata are uniseriate. Conidia were spherical and deep yellow-green, and their surfaces were lightly roughened. The G+C content of strain NR 15-1^T was 51 mol% and strain NR 15-1^T contained a dihydrogenated ubiquinone with Q9 (94.9%) as a major quinone. The nucleotide sequences of strain NR 15-1^T in the two Internal Transcribed Spacers (ITS 1 and 2) and 5.8S rDNA showed highest similarity when compared with that of *A. tubingensis* and *A. phoenicis* NRRL 365^T. However, based on morphological and chemotaxonomic characteristics, this strain was different from *A. tubingensis* and *A. phoenicis* NRRL 365^T. On the basis of the data presented, it is proposed that strain NR 15-1^T should be placed in the genus *Aspergillus* as a new species, *Aspergillus coreanus* sp. nov. Therefore, the type strain of the new species is strain NR 15-1^T (=KCTC 18075P^T, =KCCM 80006^T).

Key words: *Aspergillus coreanus*, hyphomycetes, taxonomy, Korean traditional *Nuruk*, phylogenetic tree

We isolated a glucoamylase-producing microorganism, strain NR 15-1^T, by using a screening program for new enzymes. It is considered to be an important enzyme due to its formation of good taste and ethyl alcohol from Korean folk wines. The production of an active enzyme depends on the selection of a suitable mold for the purpose. Traditional Korean *Nuruk* exists in unboiled raw barley and various grains. These are ground to a paste and moistened, and then naturally inoculated by airborne microorganisms. Therefore, many kinds of microorganisms such as fungi, yeasts, and some bacteria grow in *Nuruk*. *Nuruk* has been used for brewing traditional wines in Korea [2, 8, 12, 13, 17]. Useful fungi from various Korean *Nuruks* with high dextrinogenic and saccharogenic activity have been isolated [9]. Among the isolated *Aspergillus* species, fungal strains NR 3-6 and NR 17-6 were identified as *A. oryzae* based upon morphological characteristics [10, 19]. However, strain NR 15-1^T differed from that of *A. oryzae* ATCC 1011^T and *A. fumigatus* in colony color and other characteristics. Therefore, strain NR 15-1^T and a typical *A. oryzae* ATCC 1011^T, *A. tubingensis*, and *A. phoenisis* NRRL 365^T were investigated to determine their chemotaxonomic characteristics and phylogenetically analyzed by the nucleotide sequences of ITS 1, ITS 2, and 5.8S rDNA genes. The aim of the present study was to determine the taxonomic status of this isolate using a polyphasic approach. In this study, strain NR 15-1^T is designated as a new species, *Aspergillus coreanus* sp. nov. Strain NR 15-1^T used for morphological and genetic comparisons was isolated from traditional Korean *Nuruk* (obtained from the traditional market of Boun-up, Boun-kun, Chungbuk Province, Korea) [9]. Cultural, morphological, and phylogenetic characteristics of strain NR 15-1^T were investigated according to the standard methods from "The Genus *Aspergillus* [14]", "Illustrated Genera of Imperfect Fungi [1]", "Textbook of Fungi [16]", and "A Manual of the *Aspergilli* [22]". For the cultivation of the fungus, Czapek agar, Potato dextrose agar (PDA, Difco), and Sabouraud agar (Difco) were used. Morphological observations were made to evaluate slides or plates of cultures grown on Czapek agar at 25°C for 14 h by light microscope (Olympus Co. Tokyo, Japan) and scanning electron microscope (SEM JSM 5140, JEOL Co. Japan). Morphological characteristics of strain NR 15-1^T and a typical species of

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Table 1. Morphological characteristics of strain NR 15-1^T and three *Aspergillus* species.

Portions observed	Strain NR 15-1^T	*A. oryzae* ATCC 1011^T	*A. tubingensis*	*A. phoenicis* NRRL 365^T
Front color				
Czapek agar	yellow-green	white-green	slightly grayish	gray
Sabouraud agar	yellow-gray	yellow-gray	black-brown	-
PDA*	green	yellow-green	-	-
Reverse color	yellow-white	yellow	brown	slightly gray
Colony characteristics (Czapek agar)				
Texture	velvety	floccose	deeply velvety	velvety
Conidial heads stage				
Color	yellow (very light)	white-green	chocolate brown	very dark brown-black
Form	elliptical	radiate	radiate	elliptical
Dimensions	90-100 μm	100-300 μm	200-300 μm	300-500 μm
Vesicle				
Shape	flask shape to globose	globose	globose	globose or nearly so
Color	colorless	colorless	colorless	colorless
Dimensions	10-11 μm	20-30 μm	40-60 μm	45-65 μm
Sterigmata				
Arrangement	uniseriate	uniseriate	-	uniseriate
Conidiophore				
Length	4.0-5.0 mm	4.0-5.0 mm	2.0-3.0 mm	1.0-2.5 mm
Diameter	7.0-8.0 μm	-	15-20 μm	10-20 μm
Shape	coarsely roughened	-	smooth, long, and coarse	smooth
Conidia				
Dimensions	3.0-3.5 μm	2.0-4.0 μm	3.0-3.5 μm	3.0-3.5 μm
Shape	spherical	spherical	globose	globose
Color	deep yellow-green	white-green	darker	slightly gray
Wall character	delicately roughened	smooth	horizontally flattened	horizontally flattened

PDA*; Potato Dextrose Agar.

A. oryzae ATCC 1011^T, *A. tubingensis*, and *A. phoenisis* NRRL 365^T are shown in Table 1. Strain NR 15-1^T grew rapidly to form yellow-green colonies on Czapek agar. The colony surfaces of strain NR 15-1^T and *A. oryzae* ATCC 1011^T were velvety and floccose on Czapek agar. Conidial heads of strain NR 15-1^T were elliptical; and 90 to 100 μm in diameter (Fig. 1A). On the other hand, conidophores of strain NR 15-1^T were coarsely roughened, and typically 4 to 5 mm long and 7 to 8 μm wide. In addition, vesicles were from flask-shaped to globose, colorless, and 10 to 11 μm in diameter (Fig. 1B). Sterigmata were uniseriate. Furthermore, conidia were spherical, deep yellow-green and 3 to 3.5 μm in diameter, and their surfaces were delicately roughened (Fig. 1C). Strain NR 15-1^T developed straight spore chains (conidia, Fig. 2A), and contains Aspergilla (Fig. 2B). Based on these morphological characteristics, strain NR 15-1^T was thought not to be identical to species of *A. oryzae* ATCC 1011^T. Strain NR 15-1^T was also similar to *A. niger* NRRL 326^T in morphology. However, strain NR 15-1^T was yellowish in colony color, in contrast to *A. niger* NRRL 326^T, which forms dark or gray brown colonies. The optimum growth temperature and pH of strain NR 15-1^T were at 30°C and 5.0, respectively.

The ubiquinones were extracted and isolated according to the method of Yamada and Kondo [26]. The purified ubiquinones were identified by HPLC with their retention times [21]. The analytical systems were as previously described [18] with slight modifications: instrument, Hitachi L-5000 LC Controller; pump, Hitachi L-6000; column, YMC-Pack ODS-AM (4.6x250 mm, YMC Co., Ltd., Japan); column temperature, 30°C; elutent, methanol/ isopropyl ether (3:1); flow rate, 1 ml/min. Ubiquinones were detected by monitoring at 275 nm with a Tosoh UV-8011 detector. Strain NR 15-1^T contained two types of dihydrogenated ubiquinones such as Q9 (94.9%) as the major quinone and Q8 (5.1%) as the minor quinone. Three major ubiquinones, Q9, Q10, and Q10 (H_2), occur in *Aspergillus* [20]. Therefore, the genus *Aspergillus* is divided into three subgroups according to the types of ubiquinones. Ubiquinone analysis provides a very useful criterion of classifying *Aspergillus* taxa and identifying their isolates. The heterogeneity of the ubiquinone system suggests that the genus of *Aspergillus* is in need for revision. Fungal DNA was isolated from each sample by the benzyl chloride method [5]. In addition, the concentration and quality of the DNA preparation were determined by spectroscopic measurement and by agarose gel electrophoresis,

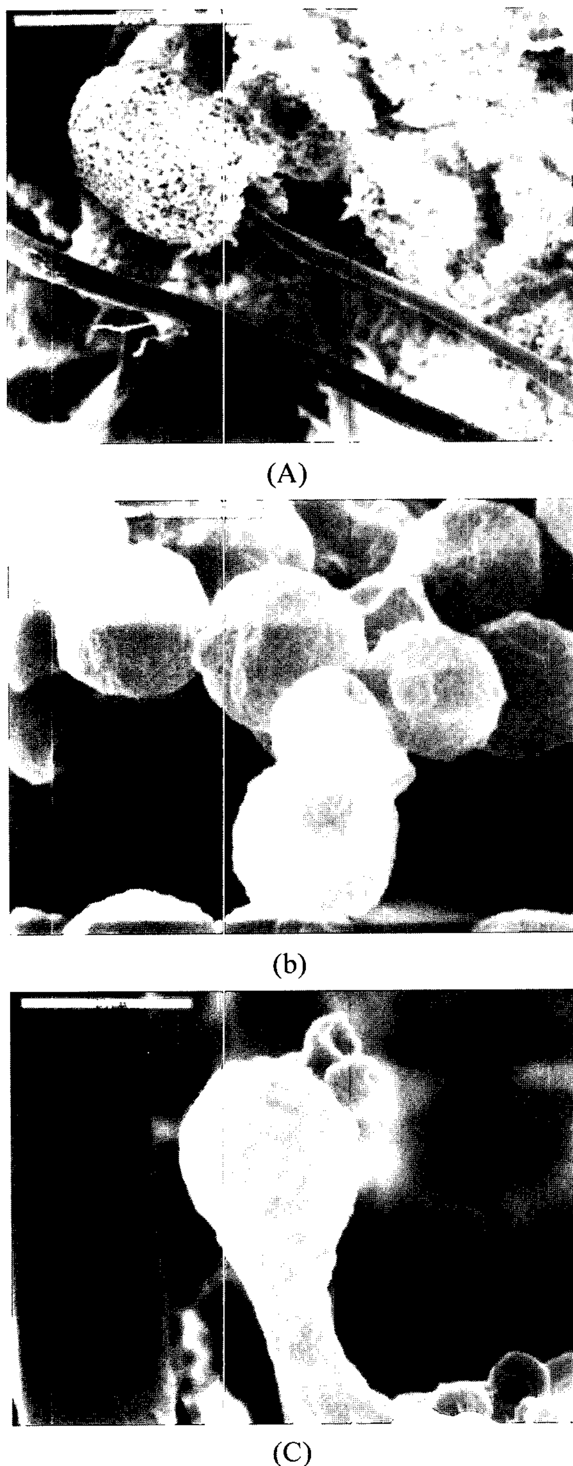

Fig. 1. Scanning electron micrographs of strain NR 15-1^T. Cultivation was performed with Czapek agar at 25°C for 14 d. A, conidial head (×500, bar=100 µm); B, vesicle (×3,500, bar=10 µm); C, spores (×10,000, bar=5 µm).

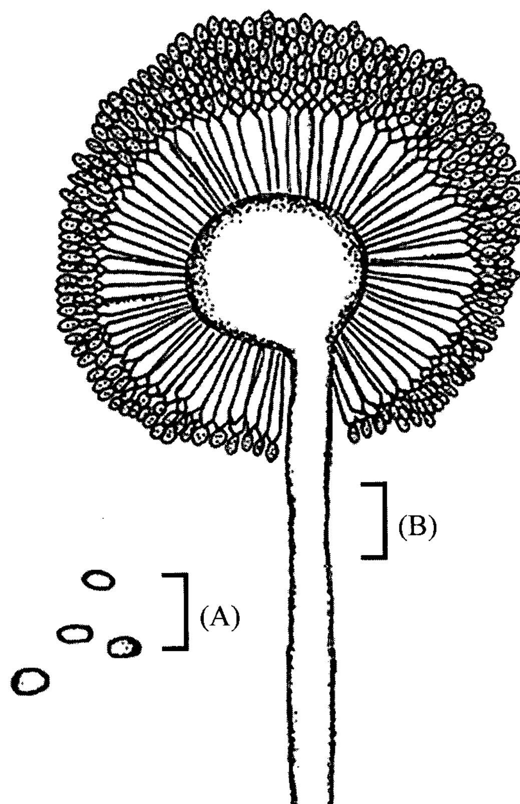

Fig. 2. Morphology of *Aspergillus coreanus* NR 15-1^T. A, conidia (bar=10 µm); B, Aspergilla (bar=10 µm).

respectively. Furthermore, the G+C content was determined by HPLC [7] under slightly different conditions from the ubiquinone system analysis. The conditions were as follows; mobile phase, [$(NH_4)H_2PO_4$:acetonitrile=40:1], and UV absorbance, 260 nm. The G+C content in other strains of the genus *Aspergillus* range from 48 to 76 mol% (*A. clavatus*, *A. clavatonanicus*, *A. giganteus*, *A. longivesica* =48 to 50 mol%, and *A. awamori*, *A. wentii*=66 to 76 mol%). On the other hand, strain NR 15-1^T had 51 mol% (as determined by HPLC) of G+C content. Ubiquinone systems and G+C content appear to be objective and useful indicators for the precise identification of *Aspergillus* spp.

The internal transcribed spacers (ITS) 1 and 2, and 5.8S rDNA were amplified by using ITS 5F primer (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS 4R primer (5'-TCCTCCGCTTATTGATATGC-3'), which were derived from the conserved regions of the 18S and 28S rDNA, respectively [11, 24]. The polymerase chain reaction (PCR) was carried out with a Perkin-Elmer model 480 thermocycler using the following program: initial denaturation for 3 min at 95°C, 30 cycles of amplification (denaturation for 30 s at 95°C, annealing for 30 s at 50°C, and extension for 1 min at 72°C), and final extension of 5 min at 72°C. The amplified PCR products were subjected to preparative electrophoresis in a 1.6% agarose gel in TBE buffer. The PCR products were excised from the ethidium bromide stained gel and purified by using a QIAGEN gel extraction

kit (Qiagen, Wartworth, CA, U.S.A.). The sequences of the ITS 1, ITS 2, and 5.8S rDNA were determined with an Applied Biosystems Image (CA, U.S.A.) model 377 with automatic DNA sequencer using a PRISM dye dideoxy terminator cycle sequencing kit (Perkin-Elmer, CA, U.S.A.). The ribosomal DNA sequences have been deposited in the NCBI (National Center for Biotechnology Information) data library. The DNA sequences were aligned with representative sequences of the genus *Aspergillus* and related taxa from the NCBI data library with the multiple alignment program CLUSTAL W [23]. Gaps at 5' and 3' ends of the alignment were omitted for further analyses. Evolutionary distance matrices were calculated by using the algorithm of *Mammalian Protein Metabolism* [6] with the DNADIST program within the PHYLIP package [3]. A phylogenetic tree was constructed by the neighbor-joining method [15] as implemented within the NEIGHBOR program of the same package. The stability of relationships was assessed by bootstrap analysis of 1000 data sets by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENS of the PHYLIP package. The GenBank, EMBL, and DDBJ accession numbers for reference sequences used in this analysis are as follows: AJ280008 (*A. tubingensis*), U65307 (*A. phoenicis* NRRL 365^T), AF048739 (*G. cibotii* JCM 9206^T), AF048741 (*V. bulbillosum* JCM 9214^T), U65306 (*A. niger* NRRL 326^T), AF176662 (*A. fumigatus*), AF149752 (*A. wentii* ATCC 1023^T), AB008417 (*A. oryzae* ATCC 1011^T), AB008415 (*A. flavus* NRRL 11612^T), AB008419 (*A. sojae* IFO 4386^T). In our study, a BLAST search of all GenBank sequences was conducted using ITS 1, ITS 2, and 5.8S rDNA region sequences of the ten reference strains. The DNA segment was 511 bp long consisting of the ITS 1 (185 bp), the 5.8S rDNA (127 bp), and the ITS 2 (199 bp). The sequence of strain NR 15-1^T was compared with a data set consisting of 10 reference strains obtained from the database. Since the morphology, ubiquinone system, and G+C content suggested that strain NR 15-1^T is a member of the genus *Aspergillus* as described above, the reference sequences were selected among the species belonging to this phylogenetic group. The phylogenetic analysis in Fig. 3 shows that strain NR 15-1^T is a member of the genus *Aspergillus*, although it is most closely related to *V. bulbillosum* JCM 9214^T (99.8%) and *A. niger* NRRL 326^T (99.6%). And *G. cibotii* JCM 9206^T was identical (100%, for partial sequences) to strain NR 15-1^T in the phylogenic analysis. Colonies of *A. tubingensis* had a wrinkled dark band in the center and those of *A. phoenicis* NRRL 365^T were characterized by long and occasionally septate primary sterigmata. In addition, colonies on Czapek solution agar produced a conspicuous white zone that is irregular at the margin and granual in texture [14]. However, it is clear that strain NR 15-1^T is different from *V. bulbillosum* JCM 9214^T, *A. niger* NRRL 326^T, and *G. cibotti* JCM 9206^T. In *Aspergillus* species, *A. tubingensis* and *A. phoenicis* NRRL 365^T showed the highest similarity (100%) to strain NR 15-1^T (Table 2). However, these three species were different in the front color and colony characteristics (Table 1). Furthermore, strain NR 15-1^T is thought to be a new *Aspergillus* species. In particular, this strain is expected to be use in a variety of industrial applications used for brewing traditional wines in Korea. In a previous report [10], general characteristics of strain NR 15-1^T were shown to be similar to those of *A. oryzae* NR 3-6 and NR 17-6 except for the colony color on various media. In addition, strain NR 15-1^T showed some morphological properties that were different from those of other *Aspergillus* species (Table 1). In particular, strain NR 15-1^T was different from the type strains of *A. tubingensis* and *A. phoenicis* NRRL 365^T in colony color on Czapek agar, conidial head dimension, vesicle dimension, and

Table 2. Levels of DNA similarity between strain NR 15-1^T and reference strains used in the phylogenetic analysis.

Strain	Sequence similarity (%)									
	1	2	3	4	5	6	7	8	9	10
1. *Aspergillus sojae* IFO 4386^T										
2. *Aspergillus fumigatus*	91.1									
3. *Aspergillus oryzae* ATCC 1011^T	99.3	91.6								
4. *Aspergillus flavus* NRRL 11612^T	99.1	90.6	99.8							
5. *Aspergillus wentii* ATCC 1023^T	91.4	93.3	91.9	91.7						
6. *Aspergillus niger* NRRL 326^T	92.7	92.8	92.2	91.4	91.8					
7. *Aspergillus phoenicis* NRRL 365^T	92.3	92.8	92.0	90.5	91.3	99.5				
8. *Verticillium bulbillosum* JCM 9214^T	92.2	92.5	91.1	90.5	91.3	99.1	99.6			
9. ***Aspergillus* sp. NR 15-1^T**	91.6	92.5	91.2	90.2	91.3	99.6	100	99.8		
10. *Aspergillus tubingensis*	92.6	93.0	92.3	90.9	91.3	99.5	100	99.6	100	
11. *Gliocladium cibotii* JCM 9206^T	91.8	92.3	91.4	89.8	91.3	99.4	100	99.6	100	100

The following sequences were given under the Methods section (accession no. in parentheses: *A. sojae* AB008419, *A. fumigatus* AF176662, *A. oryzae* AB008417, *A. flavus* AB008415, *A. wentii* AF149752, *A. niger* U65306, *A. phoenicis* U65307. *V. bulbillosum* AF048741, *A. tubingensis* AJ280008, and *G. cibotii* AF048739).

conidial wall character. The colony color on Czapek agar of strain NR 15-1^T was yellow-green, but *A. tubingensis* and *A. phoenicis* NRRL 365^T were slightly grayish and gray, respectively. The dimensions of the conidial head and vesicle of strain NR 15-1^T were 90-100 µm and 10-11 µm, respectively, and these values were smaller than those of *A. tubingensis* and *A. phoenicis* NRRL 365^T (200-300 µm and 40-60 µm, 300-500 µm and 45-65 µm, respectively). Furthermore, the conidia wall of this strain was delicately roughened, but *A. tubingensis* and *A. phoenicis* NRRL 365^T were horizontally flattened. The genus *Aspergillus* can be taxonomically distinguished from other ubiquinones-containing *Aspergillus* genera by a combination of chemotaxonomic and phylogenetic data. Three major ubiquinones, Q9 (in *A. tubingensis*, *A. phoenicis* NRRL 365^T, and *Aspergillus niger* NRRL 326^T), Q10 (in *A. alliaceus*, *A. fumigatus*, and *A. longivesica* JCM 10186^T), Q9 plus Q10 (*A. longivesica* JCM 1720^T), and Q10 (H_2) (in *A. wentii* ATCC 1023^T, *A. oryzae* ATCC 1011^T, *A. flavus* NRRL 11612^T, *A. sojae* IFO 4386^T, and *A. tamarii*) occur in *Aspergillus* [20]. These compounds are important carriers in the electron transport chain of respiratory systems. The number of isoprene units attached to the quinone nucleus varies, and such differences in ubiquinone structure are excellent indicators in the classification of genera and subgeneric taxa in bacteria and yeasts. Although less common, these techniques are also being used in the taxonomy of black yeast [27] and filamentous fungi [25, 28, 29]. The results provided by these techniques sometimes correlate with those provided by molecular techniques, although conclusions based purely on ubiquinone systems are debatable and, depending on the method used, can provide different sets of data. The strain contained a dihydrogenated ubiquinone with Q9 (94.9%) as a major quinone (Fig. 3), and had 51 mol% of G+C content. Strain NR 15-1^T exhibited the closest phylogenetic affinity to *Aspergillus* species from ITS 1, ITS 2, and 5.8S rDNA sequence comparison. Phylogenetic inference based on ITS 1, ITS 2, and 5.8S rDNA sequences places strain NR15-1^T within a phyletic cluster comprising *Aspergillus* species (Fig. 3). The ITS regions have been used as targets for phylogenetic analysis because they generally display sequence variation between species, but only minor variation within strains of the same species. Gaskell *et al.* [4] investigated sequence variation in ITS regions to distinguish *Aspergillus* from other allergenic mold. They found little variation between *Aspergillus* and *Penicillium* within the ITS 2 region but concluded that the ITS 1 region may be sufficient for identification. The comparison of ITS 1-5.8S rDNA-ITS 2 region sequences among NR 15-1^T and reference strains of ten species revealed several areas of sequence variation. Strain NR 15-1^T is a member of the genus *Aspergillus* as described above; it is most closely related to *V. bulbillosum* JCM 9214^T (99.8%) and *A. niger* NRRL 326^T (99.6%). And *G. cibotii* JCM 9206^T was identical (100%) to strain NR 15-1^T in sequence homology (Table 2). By contrast, *V. bulbillosum* JCM 9214^T, *G. cibotii* JCM 9206^T, *A. tubingensis*, and *A. phoenicis* NRRL 365^T have morphological features distinct from those of NR 15-1^T. Gaskell *et al.* [4] have previously shown that *Alternaria*, *Penicillium*, *Cladosporium*, and *Aspergillus* could be differentiated at the genus level on the basis of ITS sequence analysis. The question remained, however, whether ITS sequences could be used to identify any fungus that may be recovered clinically, including those that may be environmental contaminants. Molecular phylogenetic analysis based on the nucleotide sequences of small subunit 5.8S rDNA and the internal transcribed spacer region indicated that the strain NR 15-1^T was closely related to the described Q9 containing *Aspergillus* species. The results obtained in the chemotaxonomic analysis are consistent with the results of sequence similarity and phylogenetic inference. Its differences in some phenotypic characteristics and its genetic distinctiveness indicate that strain NR 15-1^T is separate from *Aspergillus* species. On the basis of the data described above, strain NR 15-1^T represents a new species of the genus *Aspergillus*, for which we propose the name *Aspergillus coreanus* (co. re. a. nus. M. L. adj. *corea*, pertaining to corea (Korea); Gr. adj. *nus*, of) sp. nov. This study demonstrates the power of combined molecular and biochemical methods and provides criteria for precise identification for this economically important group of fungi. We therefore concluded that ITS 1, ITS 2, and 5.8S rDNA region sequences were necessary for species level identification. The ITS 1, 5.8S rDNA, and ITS 2 nucleotide sequences of strain NR15-1^T have been submitted to the DDBJ database under the following accession numbers: AB055392 (ITS 1), AB055393 (5.8S rDNA), and AB055394 (ITS 2).

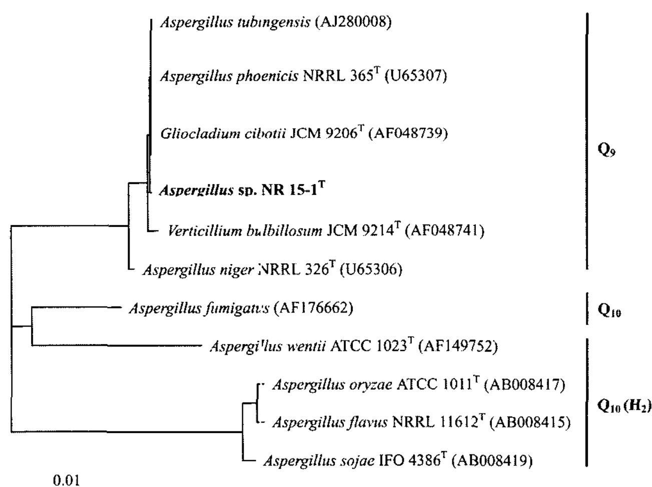

Fig. 3. Phylogenetic tree showing the relationship among strain NR 15-1^T and related species of the fungi.

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