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FULL PAPER

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Species separation in *Curvularia* "geniculata" group inferred from *Brn1* gene sequences

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Abstract *Brn1*, a reductase gene involved in the melanin biosynthetic pathway, was adopted for species delimitation among members in the "geniculata" group of *Curvularia* species and proved to be useful for this purpose. Phylogenetic trees of these fungal members were constructed from nucleotide sequences of this region. The so-called geniculata group of *Curvularia* was separated into several clusters. The conidial morphology of the members in each cluster is closely similar but clearly different among discrete clusters. The phylogenetic groups almost concurred with the morphological grouping. Thus, the synonymous treatment of *Curvularia affinis*, *C. fallax*, and *C. senegalensis* to *C. geniculata* in a previous study was supported. The isolates with warping hilum conidia were clearly different from *C. geniculata* and separated into two clusters. *C. geniculata* ATCC 6671 made an independent cluster situated near these clusters. The protuberant hilum species were located separately in the phylogenetic trees. For sound taxonomic treatment of these isolates, we should accumulate more information and retain our species determination for them.

Key words *Curvularia robusta* · Genetic distance · Intron · Phylogenetic trees · Protuberant hilum conidia

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Introduction

Some members of the "geniculata" group in the genus *Curvularia* have 4-distoseptate conidia somewhat geniculated or inflated at the middle part by dispropotional development or enlargement at the third cell from the base. *C. geniculata* (Tracy et Earle) Boedijn had been considered to be a representative species having these tendencies, and the "geniculata" group was established by Boedijn (1933). Subsequently, many 4-distoseptate conidial species that were clearly different from *C. geniculata* as well as similar to *C. geniculata* were added to this group (Groves and Skolko 1945; Subramanian 1953; Corbetta 1963, 1964; Somal 1976; Tsuda 1992). Species separation in this group has been principally based on conidial morphology and some cultural characteristics (Ellis 1966, 1971; Sivanesan 1987). However, the conidial morphology available for species separation is sparse, and the practical identification of the species is difficult mainly because of the inconsistency and variability within an isolate and among isolates depending on conditions. Thus, the species delimitation is sometimes arbitrary and vague (Upsher 1975; Tsuda and Ueyama 1982, 1983; Hosokawa et al. 2003).

When the authentic isolates of *C. affinis* Boedijn, *C. fallax* Boedijn, and *C. senegalensis* (Speg.) C.V. Subram. were crossed with tester isolates of *C. geniculata*, they produced fertile ascocarps (Hosokawa et al. 2003); this means that these species are conspecific because there is no reproductive isolation. This method is considered to be useful for the delimitation of the species (Tsuda and Ueyama 1987). However, teleomorph induction is sometime difficult because maternal factors are easily lost in culture conditions and with prolonged subculturing. Worse, most of the members in the "geniculata" group are still only known as anamorphic fungi. Thus, some isolates that were not fertile with typical *C. geniculata* are left unidentified. The conidia of these isolates share some morphological characteristics in common and also have some peculiar characteristics.

In our previous study, the 1,3,8-trihydroxynaphthalene reductase gene *Brn1* in the melanin biosynthetic pathway in

a related fungus, *Bipolaris maydis* (Nisikado) Shoemaker (teleomorph; *Cochliobolus heterostrophus* (Drechsler) Drechsler), was determined (Shimizu et al. 1997) and proved to be a useful taxonomic criterion in *Bipolaris* systematics (Shimizu et al. 1998). Consequently, the *Brn1* gene might be also adopted for species separation in "geniculata" groups of the genus *Curvularia*.

The present study was conducted on *Curvularia* species of the "geniculata" group (Boedijn 1933) to make phylogenetic comparisons based on the *Brn1* gene to examine species delimitation in the group. Next, a justification of the previous treatment of three *Curvularia* species, namely *C. affinis*, *C. fallax*, and *C. senegalensis*, which were reduced to synonyms of *C. geniculata*, was conducted.

Materials and methods

Isolates and media used

Curvularia species of the "geniculata" group obtained from the field and culture collections were used in this study (Table 1). They were grown on modified V-8 juice agar

Table 1. List of isolates used in this study and their clustering based on *Brn1* sequences

Strains	Species[a]	Host plant	Locality	Cluster	DDBJ accession no.
NC3-cu2	A	Unidentified[b]	New Caledonia	VI	AB096757
NC7-cu2-2	A	Unidentified	New Caledonia	VI	AB096758
B71	B	*Oryza sativa*	Burma	V	AB096759
B77	B	*Oryza sativa*	Burma	V	AB096760
B77-1	B	*Oryza sativa*	Burma	V	AB096761
Awa-Kenjyo	B	*Setaria italica*	Kumamoto	V	AB096762
Red top	C	*Agrostis alba*	Kumamoto	I	AB096763
NC3-cu1	C	Unidentified	New Caledonia	VII	AB096764
Tojin 1-1	D	*Pennisetum glaucum*	Kyoto	IV	AB096765
Tojin 1-2	D	*Pennisetum glaucum*	Kyoto	IV	AB096766
Ohi-Akatorii	D	*Pennisetum alopecuroides*	Tokushima	IV	AB096767
Kobayashi-1[c]	D	Graminea plant (lawn)		III	AB096768
Kobayashi-4[c]	D	Graminea plant (lawn)		III	AB096769
Host-Kuma 3	D	Unidentified	Kagoshima	III	AB096770
Shiba 91-5	D	*Zoisia japonica*		III	AB096771
Zoi cg 7.6	D	*Zoisia japonica*		III	AB096772
Lemon g-1	D	*Cymbopogon citratus*	Kyoto	III	AB096773
Cyp ina-g 2-1	D	*Cyperus iria*	Kyoto	III	AB096774
Cyp ina-g 2-2	D	*Cyperus iria*	Kyoto	III	AB096775
Karukaya-Dazai	D	*Cymbopogon* sp.	Fukuoka	III	AB096776
Ascos.g-4	E	ascospore isolate		VIIIa	AB096777
Ascos.g 49-5	E	ascospore isolate		VIIIc	AB096778
Blue Stem 3	E	*Andropogon furcatus*	Kumamoto	VIIIb	AB096779
Niigata 5	E	*Oryza sativa*	Niigata	VIIIa	AB096780
Clover 84-3	E	*Trifolium repens*	Kumamoto	VIIIa	AB096781
Awa Hito 81	E	*Setaria italica*	Kumamoto	VIIIc	AB096782
NC29-1-2	E	Unidentified	New Caledonia	VIIIc	AB096783
Myoga cu4-2	E	*Zingber mioga*	Kumamoto	VIIIc	AB096784
19A4-1	E	*Oryza sativa*	Burma	VIIIc	AB096785
19A5	E	*Oryza sativa*	Burma	VIIIa	AB096786
B80-1-2	E	*Oryza sativa*	Burma	VIIIc	AB096787
Cyp Hito	E	*Cyperus iria*	Kumamoto	VIIIc	AB096788
914 chijimi	E	*Oryza sativa*	Kyoto	VIIIc	AB096789
Byou cu7-2	E	*Oryza sativa*	Burma	VIIIc	AB096790
KokibiMatsu	E	*Panicum miliaceum*	Kumamoto	VIIIc	AB096791
KU195	E	Unidentified	China	VIIIc	AB096792
KU197	E	Unidentified	China	VIIIc	AB096793
Yaseiine g	E	*Oryza sativa*	Indonesia	VIIIb	AB096803
Isolates from culture collections					
Curvularia geniculata IMI 69539				VIIIc	AB096794
Curvularia geniculata ICMP 6140-78				VIIIc	AB096795
Curvularia geniculata ATCC 6671				II	AB096796
Curvularia affinis IMI 38975				VIIIa	AB096797
Curvularia fallax IMI 79732				VIIIc	AB096798
Curvularia fallax IMI 58645				VIIIa	AB096799
Curvularia fallax IMI 170211				III	AB096800
Curvularia senegalensis IMI 80285b				VIIIc	AB096801
Curvularia senegalensis ATCC 24154				VIIIc	AB096802

[a] Species abbreviations: A, *C. robusta*; B, *C. matsushimae*; C, unidentified species with protuberant conidia; D, unidentified species with hilum-warping conidia; E, *C. geniculata*

[b] Unidentified, unidentified graminea plant

[c] Isolated from unidentified turf grass by Dr. T. Kobayashi

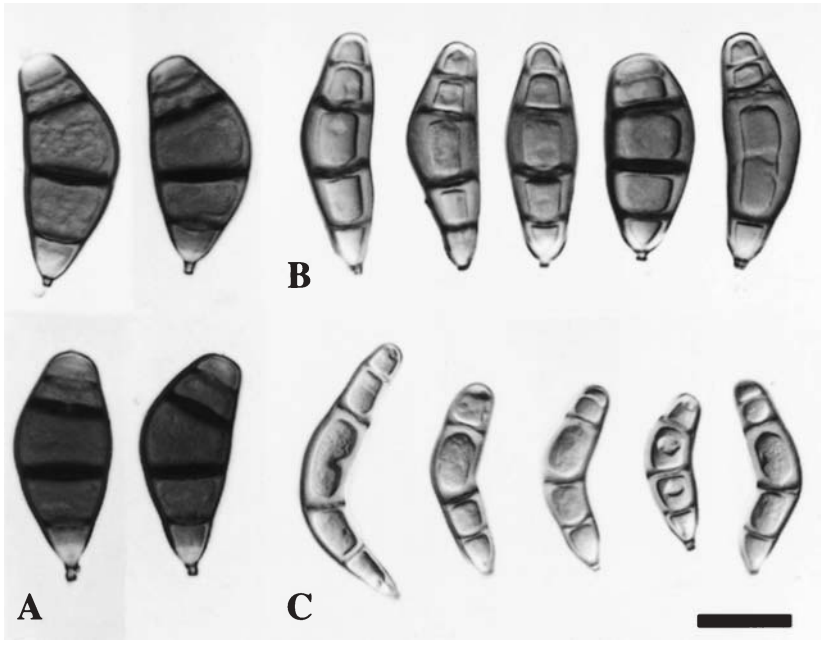

Fig. 1. Conidial morphology of isolate NC3-cul on different media. **A** On natural substrate; **B** on V-8 agar medium; **C** on soil agar medium. *Bar* 20 μm

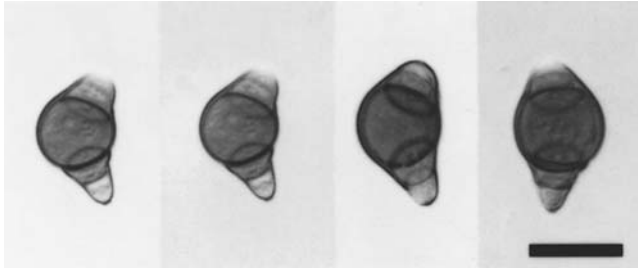

Fig. 2. Conidia of isolate NC3-cu2 on natural substrate. *Bar* 20 μm

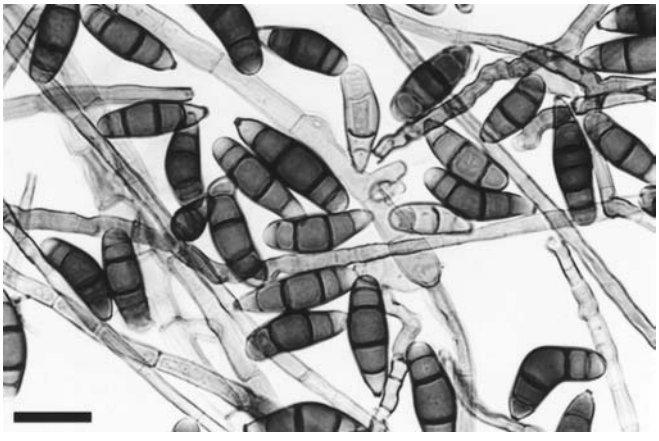

Fig. 3. Conidial of isolate Red top on soil agar medium. *Bar* 20 μm

medium and soil decoction agar medium (Tsuda and Ueyama 1987) or complete agar medium (CM) (Shimizu et al. 1997) in slant cultures maintained at 25°C. Conidial morphology of representative isolates was shown in the previous report (Hosokawa et al. 2003). Species or isolates with protuberant hilum conidia and *C. robusta* isolates used in this study are shown in Figs. 1–3.

DNA preparation and PCR

To obtain the mycelium, each isolate was inoculated in 3 ml modified V-8 liquid medium (180 ml V-8 juice and 2 g $CaCO_3$ in 5 l distilled water) in a glass test tube. Cultures were incubated at 25°C on a rotary shaker for 3 days. Mycelium was harvested in a 1.5-ml microtube, and liquid medium was removed by centrifugation. Total DNA was extracted by the method of Nakada et al. (1994). PCR primers used in this study were 5′-GCCAACATCGAGCAAA CATGG-3′ and 5′-GCAAGCAGCACCGTCAATACC AAT-3′. PCR amplification was performed with KOD Dash (Toyobo, Osaka, Japan) according to the manufacturer's direction. For nucleotide sequencing, two additional internal primers were designed: 5′-GAGGAGTTTGACC GTGTCTTC-3′ and 5′-GAAGACACGGTCAAACTCCT C-3′.

Phylogenetic analysis

The sequence data were edited with the software package Seq-Man II (version 5; Dnastar, Madison, WI, USA). Nucleotide sequences were aligned and analyzed using the Clustal W multiple alignment program (Thompson et al. 1994). The alignment was deposited in TreeBASE (http://www.treebase.org/treebase). Phylogenetic trees were constructed by a neighbor-joining method (Saitou and Nei 1987), and maximum likelihood and parsimony with a heuristic search. Phylogenetic analyses were performed with the software PHYLIP (version 3.72; Felsenstein 1993), using DNADIST, NEIGHBOR, SEQBOOT, DNAML,

and CONSENSE, and with PAUP 3.12 (Swofford 1993). For the neighbor-joining method, the distance matrix was calculated using DNADIST with the Kimura two-parameter method with NEIGHBOR (transition to transversion rate, 2.0). To determine the confidence levels of the inferred phylogenies, bootstrap analyses were carried out using 1000 replicate samplings for neighbor joining and 1000 replicates of heuristic searches for parsimony (Felsenstein 1985). *Bipolaris maydis* (AB001564) was used as an outgroup.

Results

Brn1 DNA sequences

The *Brn1* region of all isolates used in this study was amplified and sequenced. The amplified region corresponds to *C. heterostrophus* nucleotide position at 122–819. DDBJ accession numbers for the nucleotide sequences reported here are listed in Table 1. Sequences are highly conserved among these isolates except for introns. The intron position is identical to that reported for the *C. heterostrophus Brn1* gene (Shimizu et al. 1997). The deduced amino acid sequence showed high homology throughout the entire region. Insertion and deletion were not shown in this deduced exon.

Intraspecific variation within the *C. geniculata* population

Intraspecific variation was found within the *C. geniculata* population, including groups formerly treated as the separate species *C. affinis*, *C. fallax*, and *C. senegalensis* (Hosokawa et al. 2003). Among *C. geniculata* populations, the maximum intraspecific genetic distance was 0.0137. No intraspecific length mutation or mutation in the amino acid sequence was found.

Phylogenetic analysis

The DNA sequence data were used to infer phylogenetic relationships via neighbor joining (Fig. 4). Within this tree, each morphologically distinctive isolate formed an exclusive group and was clearly separated from other species or isolate populations. A maximum-likelihood tree was also used to examine phylogenetic relationships (see Fig. 6). Each analysis supported these phylogenetic groups.

Each phylogenetic tree is composed of eight clusters (Figs. 4–6). Cluster I is an unidentified protuberant hilum species and cluster II is *C. geniculata* ATCC 6671 with clearly different conidia from *C. geniculata*. Cluster III is composed of isolates having hilum warping conidia shown in the previous report, represented by Cyp ina-g 2-1 (Fig. 1G in Hosokawa et al. 2003) and also *C. affinis* 170211. Cluster IV is composed of unidentified species with warping hilum conidia, Tojin 1-1 and Ohi-Akatorii (Fig. 6H, I in Hosokawa et al. 2003). Cluster V is composed of *C. matsushimae*, which has pale conidia with a rough surface,

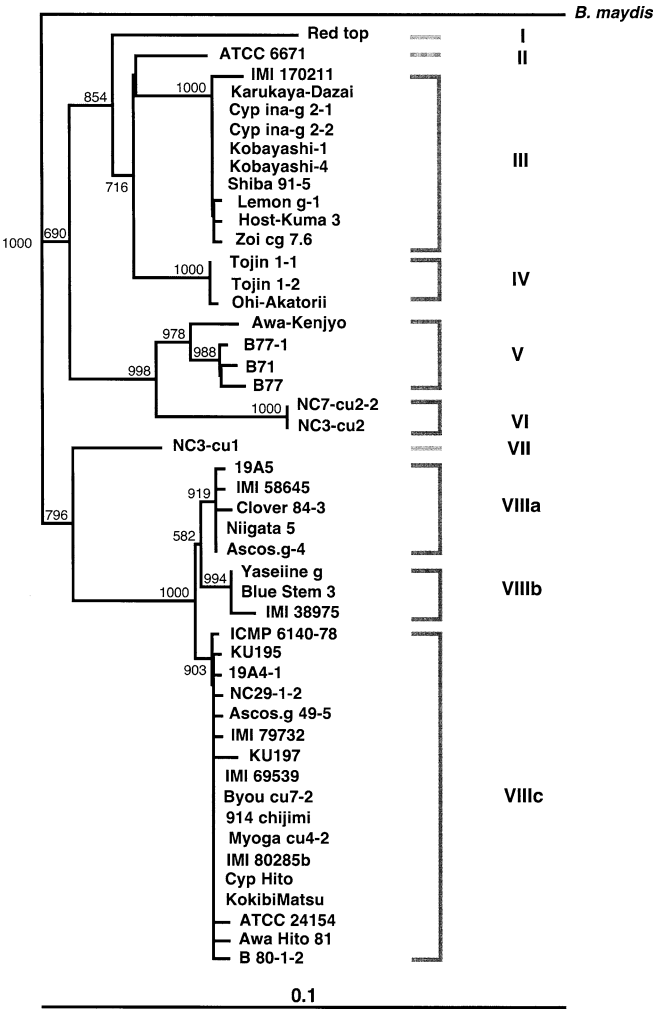

Fig. 4. Phylogram of *Curvularia* "geniculata" group species resulting from the neighbor-joining analysis of *Brn1* region. Values shown at the *nodes* are the confidence levels from 1000 replicate bootstrap samplings. *Scale bar* at bottom is the distance corresponding to 10 base changes per 100 nucleotide positions

isolated from *Oryza sativa* and *Setaria itatica* isolate Awa-Kenjyo. Cluster VI is composed of isolates assignable to *C. robusta* (see Fig. 2). Most of the species assignable to *C. geniculata* are in cluster VIII. In this cluster, *C. geniculata* IMI 69539, ICMP 6140-78, *C. affinis* IMI 38975, *C. fallax* IMI 58645, IMI 79732, and *C. senegalensis* IMI 80285b, ATCC 24154 were included. Cluster VII is composed of protuberant hilum isolate NC3-cu1, the conidia of which vary drastically depending on culture conditions (see Fig. 1). Parsimony and maximum-likelihood analyses also supported the eight clusters.

Discussion

For construction of a practically useful key for species identification in *Curvularia* species, many efforts have been conducted. An attempt based on the reproductive isolation is biologically most desirable. However, the teleomorph

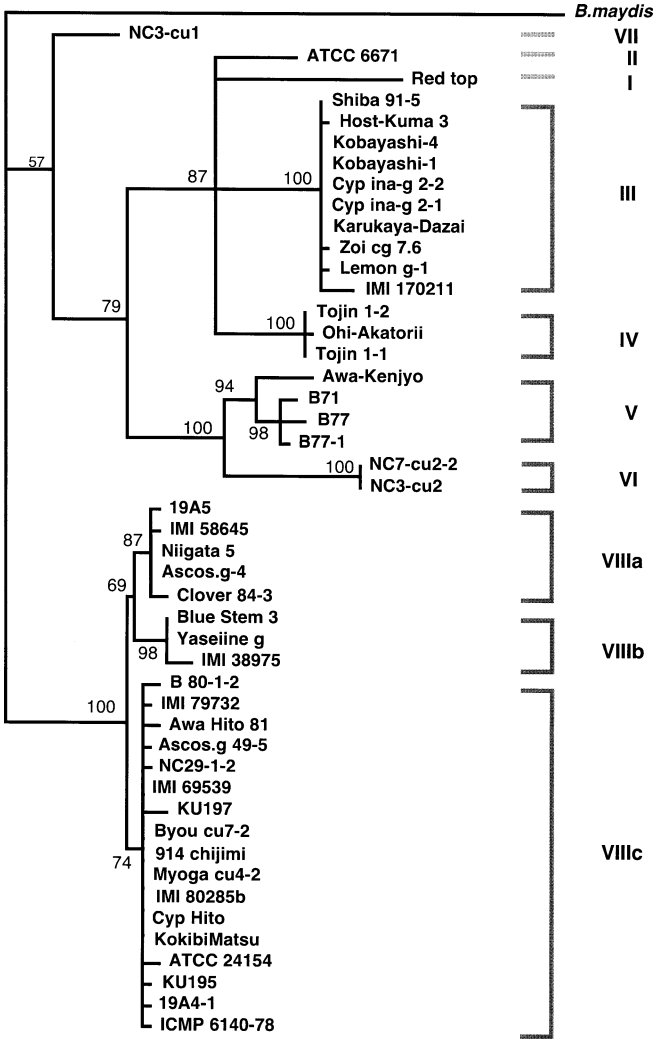

Fig. 5. One of ten equally parsimonious trees of *Curvularia* "geniculata" group species based on *Brn1* region. Values shown at the *nodes* are the confidence levels from 1000 replicate bootstrap samplings. Tree length was 260, consistency index (CI) was 0.796, homoplasy index (HI) was 0.204, retention index (RI) was 0.939, and rescaled consistency index (RC) was 0.747. *Scale bar* at the bottom refers to branch length

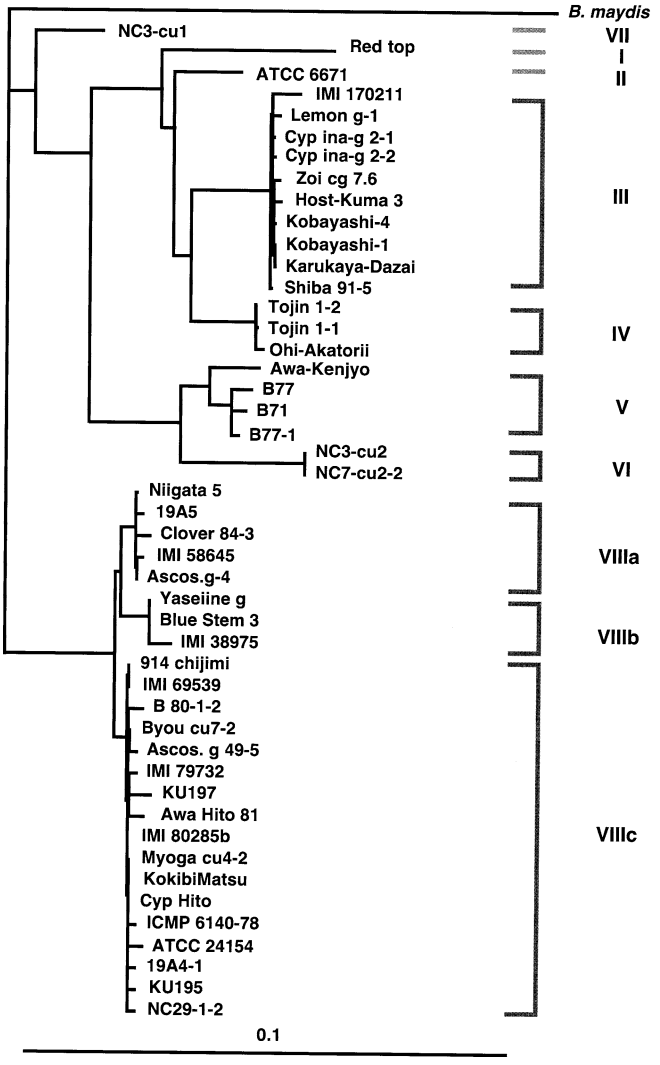

Fig. 6. A phylogenetic relationship of *Curvularia* "geniculata" group species inferred from a maximum-likelihood analysis of the *Brn1* region. The Ln likelihood was −2426.95192 and the estimated transition/transversion ratio was 1.545302

production in these groups is somewhat difficult because of lack or early decline of maternal factors. Clearly, new approaches are needed to improve the taxonomy of *Curvularia*, which should reflect their genetic relations. Recently, phylogenetic relationships based on DNA base sequences have been adopted for comparison among many phenotypically uncertain organisms (e.g., Baum 1992). To evaluate such similarities, Nakada et al. (1994) adopted the differences of restriction fragment length polymorphism (RFLP) patterns given by the combination of DNA fragments produced by digestion with specific enzymes and identified by Southern hybridization for some *Curvularia* and *Bipolaris* species. This method gave good results for taxonomic considerations of some members of the "geniculata" group of *Curvularia* (Hosokawa et al. 2003). Shimizu et al. (1998) demonstrated that the *Brn1* gene sequence was useful for species determination for *Cochliobolus* and related anamorphic fungi.

Phylogenetic trees of *Curvularia* species analyzed and constructed by the *Brn1* gene in this study separate into several different clusters (Fig. 4), corresponding to each morphologically distinguishable species or population. Thus, the *Brn1* sequence analyses would be useful to distinguish these species.

The previous treatment of three *Curvularia* species reduced to synonyms of *C. geniculata* (Hosokawa et al. 2003) was confirmed and justificated. They also formed one cluster (cluster VIII) with some variations in DNA sequences in the *Brn1* regions. The maximum intraspecific genetic distance value is 0.0137. The isolates belonging this cluster have the common sequence "CGCTTCACTnTAT AnCATCC" in the second intron. Thus, the treatment of *C. geniculata* as a morphologically variable species is desirable.

We also checked some populations that are difficult to assign to appropriate species in the "geniculata" group.

Isolates with hilum warping conidia were separated to two clusters (clusters III and IV). The conidia of these isolates were larger and longer than other members in the "geniculata" group (cf. Hosokawa et al. 2003; Sivanesan 1987). These conidial sizes and shapes with relative septal positions concurred with *C. ribaldii*. However, which cluster is attributable to this species is unclear.

An isolate named *C. geniculata* ATCC 6671, which formed cluster II, is considered to be a different species from *C. geniculata* or *C. inaequalis* on the basis of this study. This result is also supported by the morphological differences of conidia and RFLP analyses (Hosokawa et al. 2003). Problematic isolates that are now treated under the name of *C. inaequalis* were not used in this study. Unfortunately, the conidial morphology of *C. inaequalis* depicted by the already existing literature has been different or vague as pointed out previously (Hosokawa et al. 2003). Thus, we must retain our comments on *C. inaequalis* status and the precise taxonomic position of hilum warping isolates.

There are some species with protuberant hilum conidia in the "geniculata" group. In this study, we analyzed two types of isolates clearly protuberant under microscopy. They are situated in separate clusters (clusters I and VII), which means that the protuberancy in this member did not reflect the phylogenetic relationship. That is, protuberancy in the conidia is an independent event. The nature of the hilum is clearly different from that of *Exserohilum* species (Luttrell 1977). Incidentally, the two populations used in this study are clearly different and independent species that we can retain in already known species. In particular, the conidia of isolate NC3-cu1 were unexpectedly changed from those shown on a natural substrate under different culture conditions. Three separate species might be described for each condition, if one examined these conidia.

The genetic distance in the *Curvularia* "geniculata" group is considered to be somewhat larger than in *Bipolaris* species (Shimizu et al. 1998). Explanation of this fact is that they are sexually segregated in nature. Even the members in *C. geniculata* population are fertile only in artificial conditions by crossing experiments. We could not find teleomorph production in nature. Some fertile populations such as *C. verruculosa* and *C. pallescens* also showed the same tendencies (Tsuda and Ueyama 1982, 1983). Therefore, more precise and detailed morphological observations are needed for approaches to natural populations.

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