Surculiseries rugispora gen. et sp. nov., a new endophytic mitosporic fungus from leaves of Bruguiera gymnorrhiza

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Surculiseries rugispora gen. et sp. nov. is described as an endophytic fungus from leaves of *Bruguiera gymnorrhiza* in mangrove forests in the Iriomote Is., Okinawa, Japan. This fungus develops peculiar conidiogenous cells that resemble octopus legs with obvious sucker-like scars, and produces lentiform conidia with lines on surface. Sequence analysis of 18S rDNA places this new fungus in the family Xylariaceae and shows its close affinity to the genus *Ascotricha* (anamorph: *Dicyma*).

Assemblages of endophytic fungi were investigated on leaves of *Bruguiera gymnorrhiza* Lamk. growing at the mouth and banks of the Shiira River, Iriomote Is., Okinawa, Japan. Leaves were collected from the upper reach to the river mouth. In the course of the study, an unknown mitosporic fungus was found from leaves collected at the stations in the middle reach of the river. Its conidiophores were morphologically similar to those of species of *Dicyma* Boulanger which are known as anamorphs of *Ascotricha* Berk., Xylariaceae, Xylariales. Some branches on the conidiophores remained very short and non-septate as clavate, hyaline, and sterile processes. However, the conidiogenous cells clearly differed from those of *Dicyma* and the other related species. Conidiogenous cells of this fungus recurve and have obvious scars: they resemble octopus legs. The present fungus produces lentiform conidia with lines on the surface. And with these morphological characteristics, it cannot properly be accommodated in any existing genus. Therefore, we here describe the new fungus and establish a new genus to accommodate it. To infer the phylogenetic position of this fungus, we carried out sequence analysis of 18S rDNA. The results showed that this new fungus is phylogenetically close to the members of Xylariaceae. Xylariaceous fungi have been isolated from several plant families and are considered to appear commonly in the census list of endophytes (Petrini and Dreyfuss, 1981; Petrini and Petrini, 1985).

Materials and Methods

Isolation Asymptomatic leaves of *B. gymnorrhiza* were collected from the Shiira River in Iriomote Is, Okinawa, Japan in July 1997 and February 1998. Disks were punched out with an 8-mm cork borer from leaves. The surfaces of the leaf disks were sterilized by immersion in 70% solution of ethanol for 1 min and sodium hypochlorite solution (1% available chlorine) for 2 min.

The leaf disks were then rinsed in sterile distilled water and put into sterile paper towels for 3 h to remove surface water. These were placed on half-strength corn meal seawater agar medium (CMSWA) in 90-mm diam plates and incubated at room temperature (17–25°C) for over three months. The two strains (IFO 33167 and IFO 33168) isolated from the leaf disk were studied by morphological observation.

Morphological observations Morphological observations were made under a light microscope and a scanning electron microscope. Spores were mounted in one drop of lactophenol solution on glass slides for light microscopic observation. For measurement of spore dimensions, 50 spores were measured under the light microscope.

DNA isolation Two strains of the present fungus, which were isolated from leaves collected in July 1997 and February 1998, and four other strains were examined: *Ascotricha guamensis* L. M. Ames IFO 9991, *A. lusitanica* R. G. Kenneth IFO 9992, *A. chartarum* Berk. IFO 32635, and *Dicyma olivacea* (Emoto & Tubaki) Arx IFO 9178.

Fungal strains were incubated for 3–4 wk at 24°C on a half-strength malt extract medium solution. The mycelium was harvested by vacuum filtration, washed with sterilized distilled water, and frozen at −20°C. DNA was extracted by the method of Marmur (1961) and Saito and Miura (1963) with some modifications. To extract total genomic DNA, 0.7–1.5 g (fresh weight) of mycelium was placed with liquid nitrogen in a mortar and ground with a pestle into a fine powder. The mycelium powder was suspended in 7 ml of TNE buffer (pH 7.5) and transferred to a 30-ml centrifuge tube. Then 350 μl of 10% SDS and 70 μl of proteinase K solution (20 mg/ml) were added, and the mixture was incubated at 60°C for 30 min. The lysate was extracted with 7 ml of phenol-chloroform-isoamylalchohol (25:24:1, v/v). The same volume of ice-cold isopropanol was added to the

aqueous layer to precipitate DNA. The precipitate was rinsed with 70% ethanol, dried, and dissolved in 900 μl of sterilized distilled water in a microtube. The DNA was purified by treatment with 4 μl of RNase solution at 37°C for 30 min, then 40 μl of 10% SDS and 4 μl of proteinase K solution were added, and the mixture was incubated at 37°C for 1 h. The solution was extracted two times or more with the same volume of phenol-chloroform-isoamylalchohol. The DNA was isopropanol-precipitated from aqueous layers in the presence of 60 μl of 3 M ammonium acetate, washed in 70% ethanol, dried, and dissolved in 300 μl of sterilized distilled water. The concentration of DNA solution was measured by using a photometer (Beckman DU-65, Beckman Coulter Co. Ltd., Tokyo). DNA samples having an A260/A280 ratio of approximately 1.8 were used.

Phylogenetic analysis based on 18S rDNA 18S rDNA was amplified by PCR using TaKaRa Taq and a pair of primers, 18-F (5'-ATCTGGTTGATCCTGCCAGT-3') and 18-R (5'-GATCCTTCCGCAGGTTCACC-3'). DNA sequences were determined using a Thermo Sequenase™ Cycle Sequence Kit (US78500/Amersham) and a model LIC-4200L sequencer (ALOKA, Tokyo) according to the manufacturer's protocols, with the following primers: 1300-F (5'-TTGGTGGAGTGATTTGTCTG-3'), 550-R (5'-GAATTACCGCGGCTGCTGGC-3'), 18-F and 18-R. These primers used were designed by Ueda and Mikata (1999). Sequence data were analyzed in an Image Analyzer (ALOKA). The CLUSTAL W ver. 1.7 software (Thompson et al., 1994) package was used to generate the evolutionary distances (the K_{nuc} value of Kimura (Kimura, 1980)), the similarity values, the neighbor-joining (NJ) analysis (Saitou and Nei., 1987) from K_{nuc} values, and the bootstrap resampling method of Felsenstein (Felsenstein, 1985) with 1000 replicates for evaluation of the topology of the phylogenetic tree. The TREEVIEW (Page, 1996) was used for plotting the phylogenetic tree.

The sequence data of ascomycetous fungi employed in phylogenetic analysis were obtained from a database of DNA Data Bank of Japan (DDBJ). Their accession numbers are shown in Fig. 15. The nucleotide sequence data of 18S rDNA of fungal strains obtained in this study have been deposited with DDBJ nucleotide sequence database as follows: *S. rugispora* IFO 33167/ AB048279, IFO 33168/AB048280; *A. guamensis* IFO 9991/AB048281; *A. lusitanica* IFO 9992/AB048282; *A. chartarum* IFO 32635/AB048283; *D. olivacea* IFO 9178 /AB048284.

Results

The present fungus was found from leaves of *B. gymnorrhiza* collected from the middle reach of the river. It produces dark, long, erect conidiophores. Some branches of conidiophores remain very short and non-septate as clavate, hyaline, and sterile processes. A hyaline cell develops on the end of these dark conidiophores (Fig. 1), which subsequently branches off, grows, and produces conidiogenous cells on the end (Figs. 2, 4, 8 and 9).

Conidiogenous cells curve exteriorly while sporulating (Figs. 5, 10 and 11). After conidia separate, obvious scars remain on the upper side of the recurved conidiogenous cell (Fig. 12). These scars are arranged in a zig-zag, not in a straight line, and they suggest sympodial conidiogenesis in this fungus. Conidia are produced successively in holoblastic proliferation. Conidia are lentiform or elliptical, olivaceous to pale brown, single-celled, and have a longitudinal ridgeline on the surface (Figs. 6, 7 and 13). Up to 15 conidia were produced on a conidiogenous cell in an incubation study on medium. Morphology of the present fungus suggests its affinity with genus *Dicyma* (anamorphs of *Ascotricha*, Xylariaceae) and *Pleurothecium* Höhn. Species of *Dicyma* are similar to the present fungus in producing short, non-septate as clavate, hyaline, and sterile processes on conidiophores. However, the present fungus is clearly distinguished from the species of *Dicyma*, because conidiogenous cells of the latter species are polyblastic, sympodial, cylindrical to clavate and acropleurogenously produce obovoid to ellipsoidal or subspherical conidia on the cylindrical conidiogenous cells (Ellis, 1971; Hawksworth, 1971; Kenneth, 1971; Roberts et al., 1984). *Pleurothecium recurvatum* (Morgan) Höhn., which is the only species of the genus *Pleurothecium*, does not produce clavate, hyaline, sterile branches on conidiophores and conidiogenous cells with sucker-like scars. This fungus produces three-septum conidia (Goos, 1969). Thus, no extant genus accommodates the new fungus properly, so that the establishment of the following new genus is warranted.

Taxonomy

*Surculiseries* Okane, Nakagiri et Tad. Ito, gen. nov. Coloniae effusae, cremeae vel fulvescentes. Hyphae immersae vel superficiales. Stromata nulla. Setae et hyphopodia absentia. Conidiophora macronematica, mononematica, fusca, laevia, sympodialia, ramis brevibus aseptalis clavatis hyalinis sterilibus praedita. Cellulae conidiogenae polyblasticae, recurvae, supra cicatricibus surculioidibus claris uniserialibus instructae. Conidia solitaria, siccata, lenticularia vel elliptica, aseptata.

Species typica: *Surculiseries rugispora* Okane, Nakagiri et Tad. Ito.

Etymology: *Surculus*=sucker, *series*=row; referring to the shape of conidiogenous cells with a row of sucker-like scars: they resemble the legs of an octopus.

Colonies effuse, cream to whitish-brown. Hyphae immersed and superficial. Stroma none. Setae and hyphopodia absent. Conidiophores macronematous, mononematous, dark brown, smooth, sympodially, some of the branches remaining very short and non-septate as clavate, hyaline, sterile processes. Conidiogenous cells polyblastic, bent backward, retaining obvious sucker-like scars. Conidia solitary, dry, lentiform to elliptical, olivaceous, non-septate.

Type species: *Surculiseries rugispora* Okane, Nakagiri et Tad. Ito.
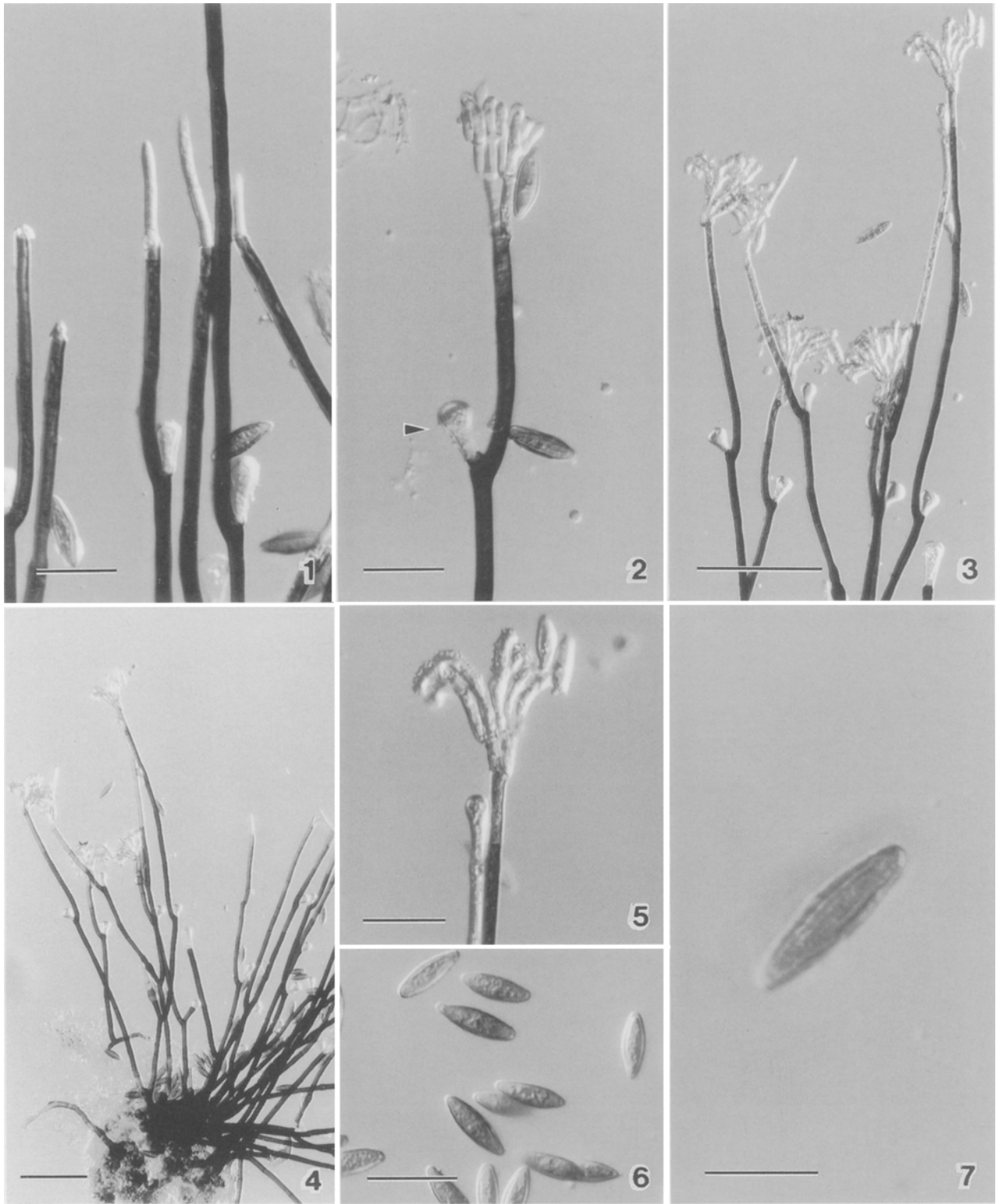
Figs. 1–7. *Surculiseries rugispora.* 1. Dark conidiophores having a hyaline cell on the end. 2. Short and non-septate branch as ampullate, hyaline, and sterile process on conidiophore (arrow) and branched hyaline part. 3 & 4. Conidiophores and conidiogenous cells. 5. Conidiogenous cell producing conidia. 6. Pigmented conidia. 7. Lentiform conidium. Bars: 1, 2, 5, 6=20 μm, 3, 4=50 μm, 7=10 μm.
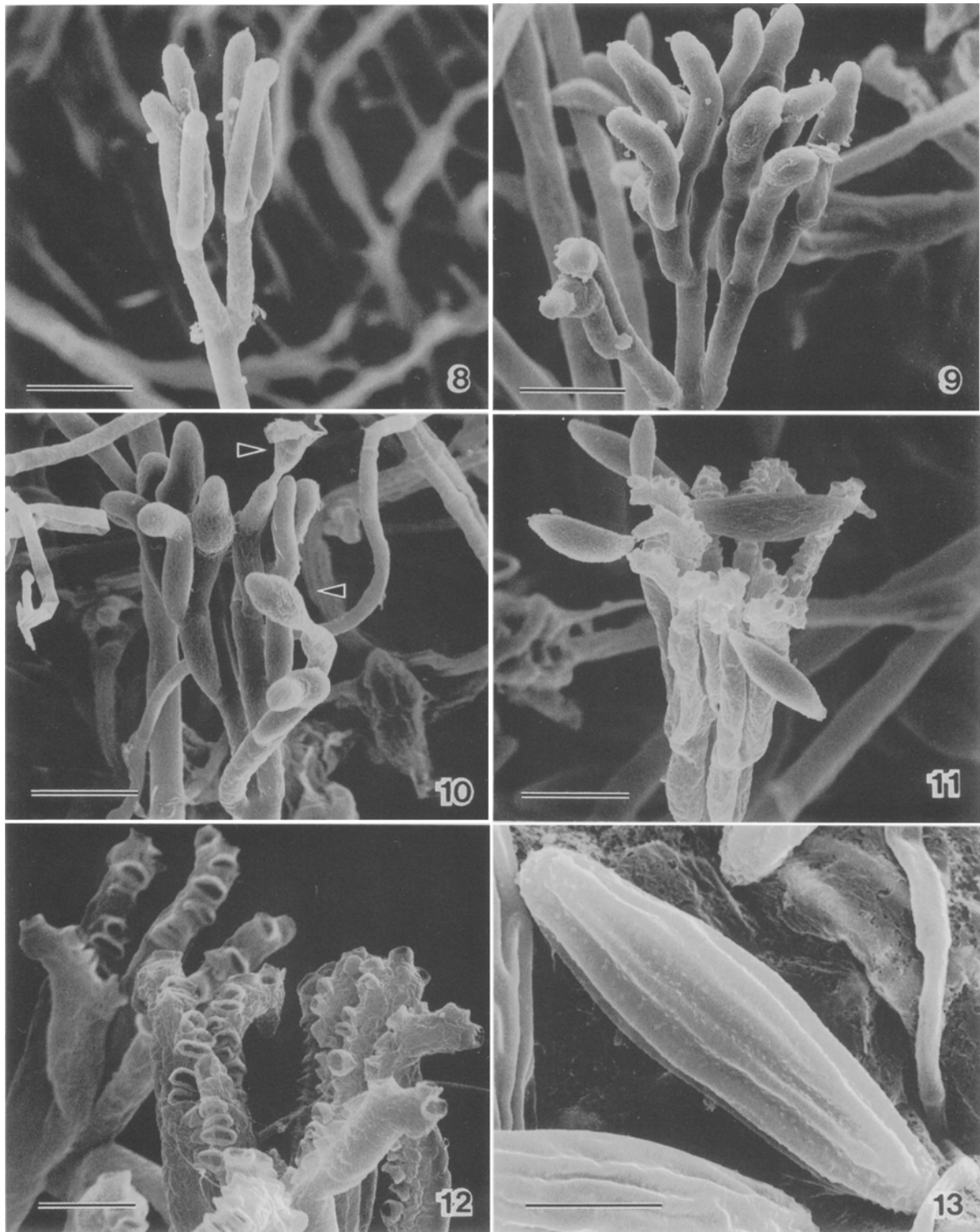
Figs. 8–13. *Surculiseries rugispora*. Scanning electron microscope views. 8. Initial stage of conidiogenous cell. 9. Branched conidiogenous cell. 10. The first conidia arising from conidiogenous cells (arrows). 11. Conidia and conidiogenous cells with remaining scars. 12. Obvious scars remaining on the upper side of recurved conidiogenous cells. 13. Conidia with lines on the surface. Bars: 8–11=10 μm, 12, 13=5 μm.
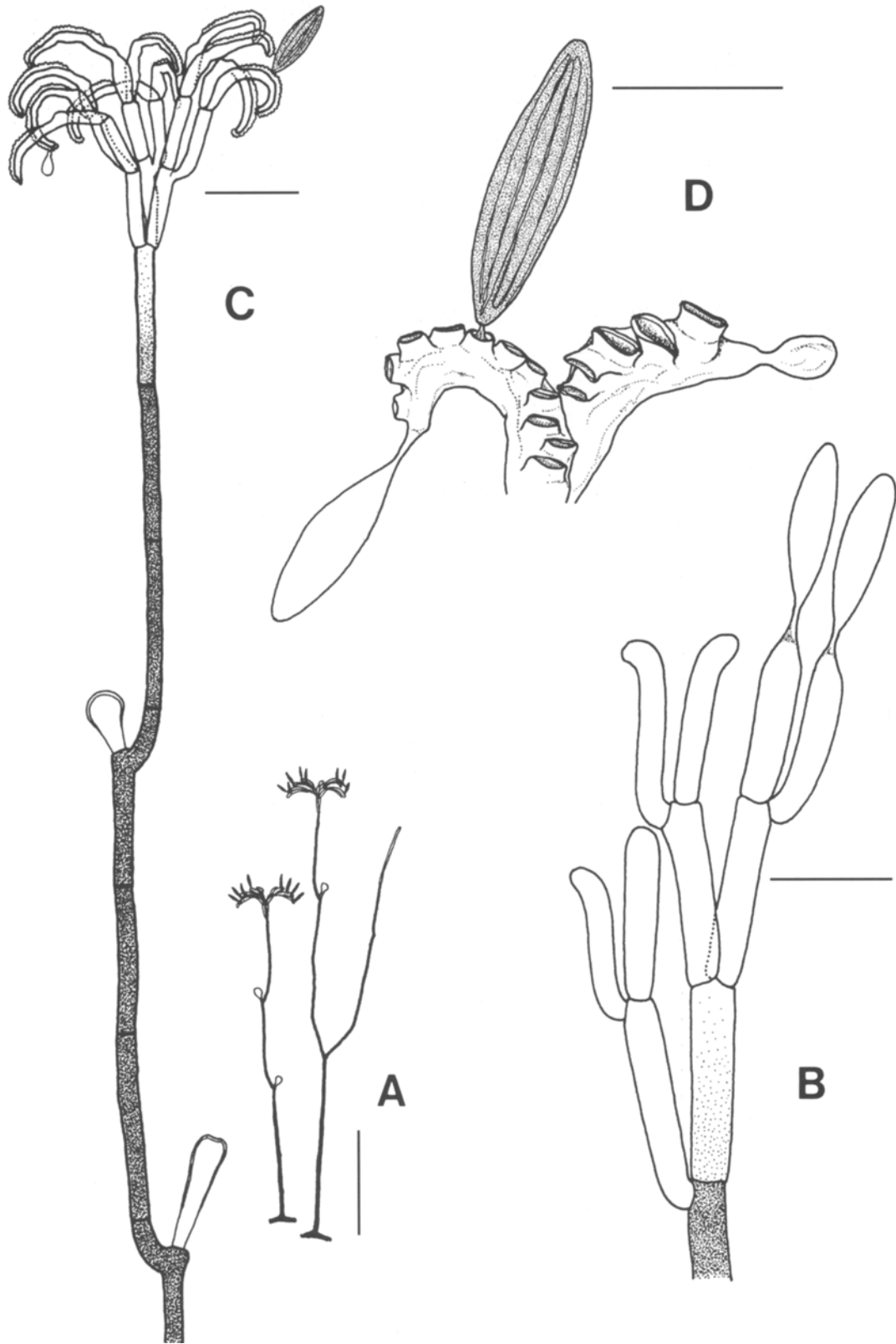
Fig. 14. *Surculiseries rugispora*. A. Erect conidiophores. B. Initial stage of development of conidiogenous cells. C. Conidiophore and conidiogenous cells. D. Conidiogenous cells with obvious scars remaining and conidium. Bars: A＝100 μm, B, D＝10 μm, C＝20 μm.

A

B

S. rugispora IFO 33167 /
AB048279

998

S. rugispora IFO 33168 /
AB048280

Dicyma olivacea IFO 9178 / AB048284

658

Surculiseries rugispora IFO 33167 / AB048279

Surculiseries rugispora IFO 33168 / AB048280

A. lusitanicum IFO 9992 / AB048282

986

A. guamansis IFO 9991 / AB048281

A. chartarum IFO 32635 / AB048283

777

Dicyma olivacea IFO 9178 / AB048284

109

964

Rosellinia necatrix / AB014044

800

Xylaria sp. / AB014042

469

Xylaria polymorpha / AB014043

Xylaria carpophila / Z49785

244

189

Obolarina dryophila / Z49784

Ascotricha chartarum IFO 32635 / AB048283

993

Hypoxylon fragiforme / AB014046

850

Hypoxylon fragiforme / AB014045

208

367

Daldinia concentrica / U32402

Ascotricha lusitanicum IFO 9992 / AB048282

570

Hypoxylon atroroseum / U32411

819

825

Pestalosphaeria sp. / AF104356

Ascotricha guamensis IFO 9991 / AB048281

Diatrype disciformis / U32403

Chaetomium elatum / M83257

514

Chaetomium globosum IFO 6310 /

912

AB048285

Xylaria carpophila / Z49785

604

Chaetomium globosum / U20379

0.01 K_{nuc}

Sordaria fimicola / X69851

1000

994

Podospora anserina / X54864

669

Ophiostoma stenoceras / M85054

999

Ophiostoma schenckii / M85053

992

Ophiostoma ulmi / M83261

Pseudallescheria boydii / U43912

Pseudallescheria ellipsoidea / U43911

959

Pseudallescheria ellipsoidea / PEU43913

Pseudallescheria boydii / M89782

933

Lementospora prolificans / U43909

959

Lementospora prolificans / U43910

757

Microascus cirrosus / M89994

999

Halosarpheria retorquens / AF050486

634

930

Aniptodera chesapeakensis / AF050484

Colletotrichum gloeosporioide / M55640

Paecilomyces tenuipes / D85136

997

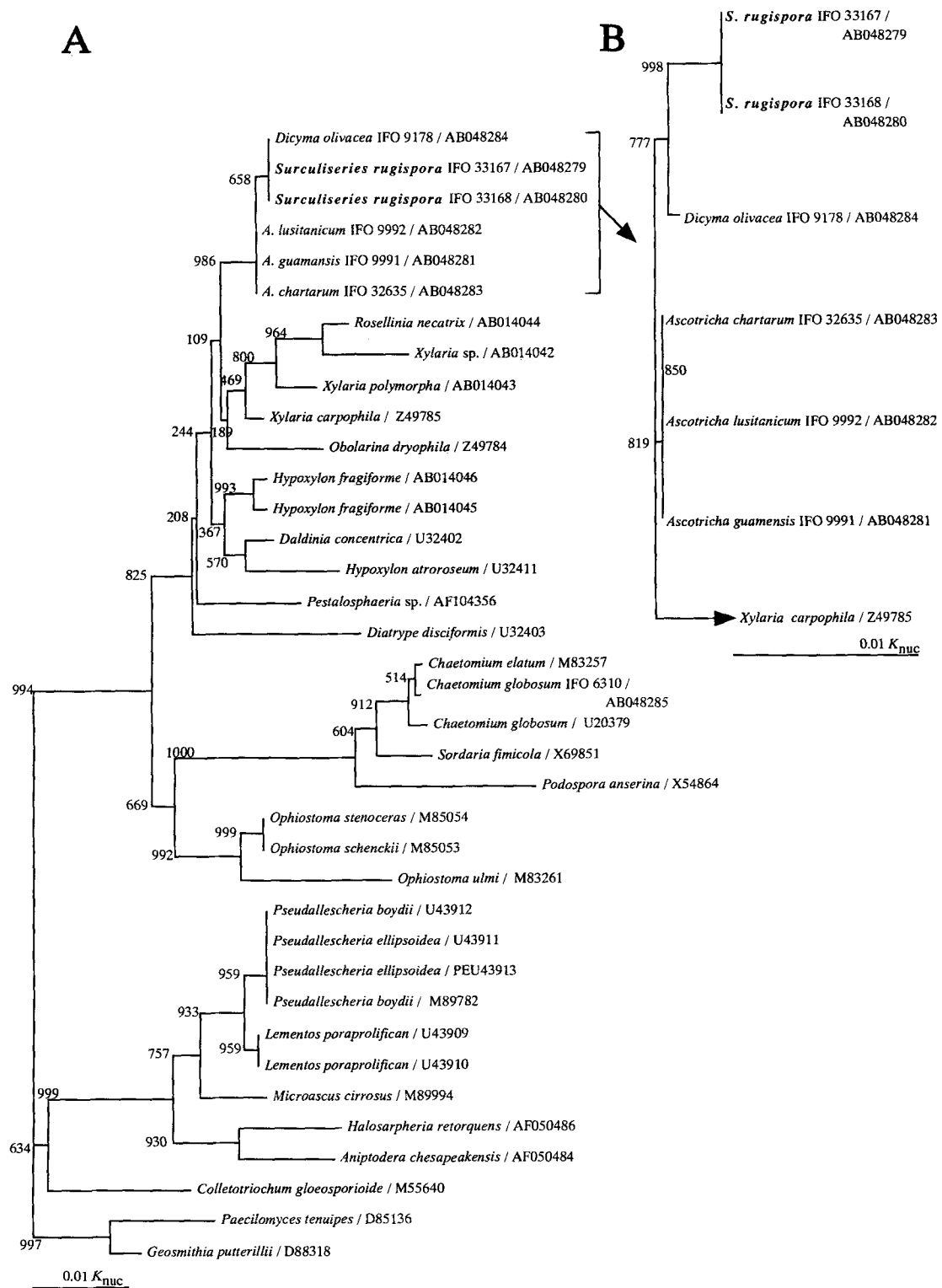*Geosmithia putterillii* / D88318

0.01 K_{nuc}

Fig. 15. Phylogenetic tree, based on neighbour-joining, derived from 18S rDNA sequences. A: The tree for members of Asco-
mycetes based on approximately 800 bases. *Paecilomyces tenuipes* and *Geosmithia putterilli* were used as the outgroup. B: The
tree for the new species and related species of *Dicyma* and *Ascotricha* based on approximately 1700 bases. *Xylaria carpophila*
was used as the outgroup. Bars: 0.01 K_{nuc} in nucleotide sequences. The number on the branches are confidence limits estimat-
ed from bootstrap analysis with 1000 replicates.

Surculiseries rugispora Okane, Nakagiri et Tad. Ito, sp. nov.   Figs. 1–14

Coloniae effusae, cremeae vel fulvescentes. Hyphae immersae vel superficiales, 2–3 μm diam. Stromata nulla. Setae et hyphopodia absentia. Conidiophora macronematica, mononematica, fusca, laevia, sympodialia, 170–520 μm alta, 4 μm diam, ramis brevibus aseptalis clavatis hyalinis sterilibus praedita. Cellulae conidiogenae polyblasticae, recurvae, supra cicatricibus surculioidibus claris uniserialibus 14–26 × 3 μm instructae. Conidia solitaria, siccata, lenticularia vel elliptica, olivacea, aseptata, in maturitate rugosa, 17–22 × 6–8 μm.

Habitatio: In frondibus *Bruguierae gymnorrhizae* Lamk.

Holotypus: IFO H-12236, colonia exsiccata in cultura ex fronde *Bruguierae gymnorrhizae* Lamk., ad flouvium Shiira, Ins Iriomote, Okinawa Pref., Japonia, a I. Okane isolata, in Herbario IFO conservata.

Colonies effuse, whitish brown. Mycelium immersed and superficial, septate, hyaline to whitish brown 2–3 μm in diam. In culture on corn meal agar, colony spreading slowly. Stroma none. Setae and hypodia absent. Conidiophores macronematous, mononematous, dark brown, smooth, sympodially, 170–520 μm in length and 4 μm in diam. Some of the branches remaining very short and non-septate as clavate, hyaline, sterile processes. Conidiogenous cells polyblastic, bent backward, remaining obvious sucker-like scars, 14–26 μm in length and 3 μm in diam. Conidia solitary, dry, lentiform to elliptical, olivaceous, none septum, rugose in mature, 17–22 × 6–8 μm.

Host: Leaves of *Bruguiera gymnorrhiza*.

Holotypus: IFO H-12236, dried colony on oatmeal agar of IOC-1246 (=IFO 33167) isolated from leaf of *B. gymnorrhiza* in Shiira River, Iriomote Is., Okinawa Pref., July 6, 1997, collected and isolated by I. Okane; deposited in the herbarium of IFO.

Strain examined: IOC-1247 (=IFO 33168, IFO H-12237) isolated from leaf of *B. gymnorrhiza* in Shiira river, Iriomote Is., Okinawa Pref., February 12, 1998.

Etymology: *ruga*=line, *spora*=spore; referring to conidia having lines on surfaces.

Phylogeny

In phylogenetic analysis based on the partial sequence of 18S rDNA (approximately 800 bases), *S. rugispora* located in a cluster consisting of the members of the family Xylariaceae in the NJ tree (Fig. 15A). This new fungus is close to strains of *Dicyma* and *Ascotricha*. In comparison based on approximately 1700 bases of the 18S rDNA, characteristic sequences of *S. rugispora* are found at several positions, and they distinguish this fungus from the species of *Dicyma* and *Ascotricha* examined. According to the helix numbering of the secondary structure model for eukaryotic 18S rRNAs by de Rijk et al. (1992), one transition in helix E10-1, two deletions in the stem of helix 34, and two transversions in helices 43 and 49 were observed in 18S rDNA sequence data of

S. rugispora. The phylogenic tree of these related species is shown in Fig. 15B.

Discussion

In our ecological study on endophytic fungi of *B. gymnorrhiza* conducted from 1996 to 1998, several hundred leaf disks were examined, but *S. rugispora* was isolated from only two leaf disks. Each isolate (IFO 33167 and IFO 33168) of this new fungus was found from one leaf disk, in studies of July 1997 and February 1998, respectively. Thus, the isolation frequency of *S. rugispora* was exceedingly low. Furthermore, sporulation of this fungus was observed over 3 mo after incubation of the leaf disks on CMSWA plates. In an incubation study, sporulation of this fungus was observed in 3 weeks after inoculation on oatmeal agar, and hyphae grew slow. Colonies on oatmeal agar attained a diam of 40 mm in 28 d at 24°C. Around the research site where the present fungus was found, several subtropical plants grow as back-mangrove plants. Of these subtropical plants, leaves of *Pandanus tectorius* Park., *Barringtonia racemosa* Blume, and *Machilus thunbergii* Sieb. & Zucc. were also examined for *S. rugispora*, but this fungus was not detected. It is considered that the other fungi living in *B. gymnorrhiza* extend faster than *S. rugispora* on medium and interfere with its detection from the leaves. It is possible that *S. rugispora* lives inside *B. gymnorrhiza* as an endophytic fungus, and acts as a decomposer after the host tissues wither and die.

Surculiseries rugispora is similar to species of *Dicyma* (anamorphs of *Ascotricha*, Xylariaceae) in producing short sterile hyaline branches on the conidiophore (Hawksworth, 1971). But, as mentioned above, the species of *Dicyma* are clearly distinct from this new fungus in morphology of conidiogenous cells and conidia. According to a monograph of the genus *Ascotricha* by Hawksworth (1971), *Dicyma* anamorphs of *Ascotricha* are often produced on ascomata as terminal hairs. To induce development of the teleomorph, we inoculated *S. rugispora* onto autoclaved leaves of *B. gymnorrhiza* on agar plate, but it did not appear. Conidiophores of this new species develop independently.

Phylogenetic analysis based on a partial sequence of 18S rDNA (approximately 800 bases) placed the two strains of *S. rugispora*, IFO 33167 and IFO 33168, in a cluster with *Dicyma* and *Ascotricha* in the NJ tree (Fig. 15A). Though development of the teleomorph of *S. rugispora* has not been confirmed, this phylogenic analysis suggests that this fungus is a new member of Xylariaceae. In the analysis based on approximately 1700 bases of the 18S rDNA, nucleotide diversifications between this new fungus and the species of *Dicyma* and *Ascotricha* were observed at several sites in this nucleotide region. In addition to morphological differences, genetic differences in 18S rDNA are found between *S. rugispora* and the species of *Dicyma* and *Ascotricha*.

In this study, *Dicyma olivacea*, IFO 9178, which was derived from the type specimen of this species, was employed for phylogenetic analysis. *Dicyma olivacea* IFO

9178 was located closest to *S. rugispora* in the phylogenetic tree, but these two fungi are clearly distinguished in morphology. *Dicyma olivacea* has needle-shaped erect conidiophores, and the conidia are produced pleurogenously over the fertile laterals. This fungus was described as a new combination based on the transfer of *Gonytrichella olivacea* Emoto & Tubaki to *Dicyma* (Arx, 1981). *Gonytrichella olivacea* was described as the type species of the genus *Gonytrichella* Emoto & Tubaki (Emoto and Tubaki, 1970), and it was subsequently synonymised with the genus *Dicyma* (Arx, 1981). *Dicyma olivacea* also includes *Puciola spinosa* de Bert. as a synonym, which is the type species of the genus *Puciola* (de Bertoldi, 1976). Thus, though already known species of *Dicyma* and *Ascotricha* are phylogenetically close to *S. rugispora*, the morphological characteristics clearly distinguish this new fungus from the other species, and warrant the establishment of the new genus, *Surculiseries*.

Xylariaceous fungi have been isolated from several plant families and are considered to appear commonly in the census list of endophytes (Petrini and Dreyfuss, 1981; Petrini and Petrini, 1985). Though xylariaceous fungi are known as saprobes or weak parasites on wide range of plants, they are considered to relate in harmony with some hosts as endophytes. Discovery of the present fungus suggests that undiscovered xylariaceous fungi are living inside their host plants as endophytes.

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