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SHORT COMMUNICATION

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Isolation and identification of mycorrhizal fungi associated with *Stigmatodactylus sikokianus* (Maxim. ex Makino) Rauschert (Orchidaceae)

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Abstract The mycorrhizal fungi of *Stigmatodactylus sikokianus* (Orchidaceae) were isolated and identified to be nearly related to *Sebacina* spp. in Sebacinaceae (Basidiomycota) by a neighbor-joining phylogenetic analysis based on the sequences of the ITS region of nuclear rDNA. In spite of the geographically separated samplings, high sequence similarity was found among the obtained DNA sequences, which suggested that *S. sikokianus* might be highly specialized to the group of fungi. It is known that *Sebacina* spp. are saprobes or ectomycorrhiza-forming fungi. The mycorrhizal fungi of *S. sikokianus* were regarded to be saprobic from the environment of their habitats.

Key words Critically Endangered species · *Cryptomeria japonica* · Orchid mycorrhizal fungi · Saprobic fungi · Sebacinaceae

Stigmatodactylus (= *Pantlingia*) *sikokianus* (Maxim. ex Makino) Rauschert is a tiny terrestrial orchid distributed in Shikoku Island, the south of Kyushu Island, and Kii Peninsula in Honshu Island in Japan (Maekawa 1971). This orchid is usually found in the understory of *Cryptomeria japonica* plantations, and its underground organs grow in litter. In habitats in Kochi and Tokushima Prefectures in Shikoku Island, we observed that the shoots emerge around late August, and shoot growth, flowering, fructification, and seed dispersal occur in sequence in the next 2 months.

This orchid is categorized as a Critically Endangered species (CR) in the Red Data Book of the Ministry of Environment in Japan. "Critically Endangered" is the highest risk of extinction in categories of the Red Data Book; therefore, conservation of this orchid is urgently required in some habitats. However, little is known about its ecological characteristics.

It is well known that orchids depend on the associated mycorrhizal fungi for carbon supply in the protocorm stage. For green orchids, dependency on mycorrhizal fungi for the carbon supply would be lower or might be lost in the adult stage. Because *S. sikokianus* grows in heavily shaded understory, and the leaves are tiny and few, it was suggested that dependency on the carbon supply from mycorrhizal fungi would be high throughout the life cycle. Clarification of the mycorrhizal fungi is, therefore, important to reveal the carbon source for this orchid, which would be helpful when considering its conservation and propagation.

Underground organs of *S. sikokianus* were collected from one individual (YA1) in a habitat (YA) in Mt. Yokokura in Kochi Prefecture, about 700 m above sea level, in September 2004, and three individuals (NA1, NB1, and NB2) were from two habitats (NA and NB) in Nakamachi, Naka-gun, in Tokushima Prefecture, about 750 m above sea level, in September 2005 (Table 1). The collected individuals were in the flowering stage with about 6–10 cm of shoot height. The annual mean temperature and the annual total precipitation recorded at the nearest meteorological stations for each of the sampling sites are 16.6°C and 2627 mm in Kochi Meteorological Station in Kochi Prefecture, and 13.2°C and 3036.8 mm in Kito Meteorological Station in Tokushima Prefecture, respectively (averages from 1971–2000). All habitats are *Cryptomeria japonica* plantations, and shoots of *S. sikokianus* were sparsely found on heaps of litter. The underground organ of *S. sikokianus* consists of two parts, a tuber and a rhizome (Fig. 1). The tuber, covered by a brownish skin, was formed at the base of the plant body. Colonization of mycorrhizal fungi was not found in the tuber. The rhizome, in which mycorrhizal colonization was observed in the cortical cells, was formed between the tuber and the flowering shoot. The characteristics are similar to those of Australian terrestrial orchids in tribe Diurideae, *Eriochilus* spp. and *Caladenia* spp. (David 1988), to which *Stigmatodactylus* also belongs.

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Table 1. Isolates of mycorrhizal fungi from *Stigmatodactylus sikokianus* sequenced in this study

Isolate number	Sampling date	DDBJ/EMBL/GenBank accession no.
YA1-1	10 Sept. 2004	AB370308
YA1-2*	10 Sept. 2004	AB370309
YA1-3	10 Sept. 2004	AB370310
YA1-4	10 Sept. 2004	AB370311
YA1-5	10 Sept. 2004	AB370312
YA1-6	10 Sept. 2004	AB370313
NA1-1	19 Sept. 2005	AB370314
NA1-2	19 Sept. 2005	AB370315
NA1-3	19 Sept. 2005	AB370316
NA1-4	19 Sept. 2005	AB370317
NA1-5	19 Sept. 2005	AB370318
NA1-6	19 Sept. 2005	AB370319
NA1-7	19 Sept. 2005	AB370320
NA1-8	19 Sept. 2005	AB370321
NB1-1	19 Sept. 2005	AB370322
NB2-1	19 Sept. 2005	AB370323
NB2-2	19 Sept. 2005	AB370324
NB2-3	19 Sept. 2005	AB370325
NB2-4	19 Sept. 2005	AB370326
NB2-5	19 Sept. 2005	AB370327
NB2-6	19 Sept. 2005	AB370328

*The isolate numbers indicate sampling sites (YA), plant numbers (1) and fungal isolate numbers (2). YA, Mt. Yokokura in Kochi Pref.; NA and NB, Naka Machi in Tokushima Pref.

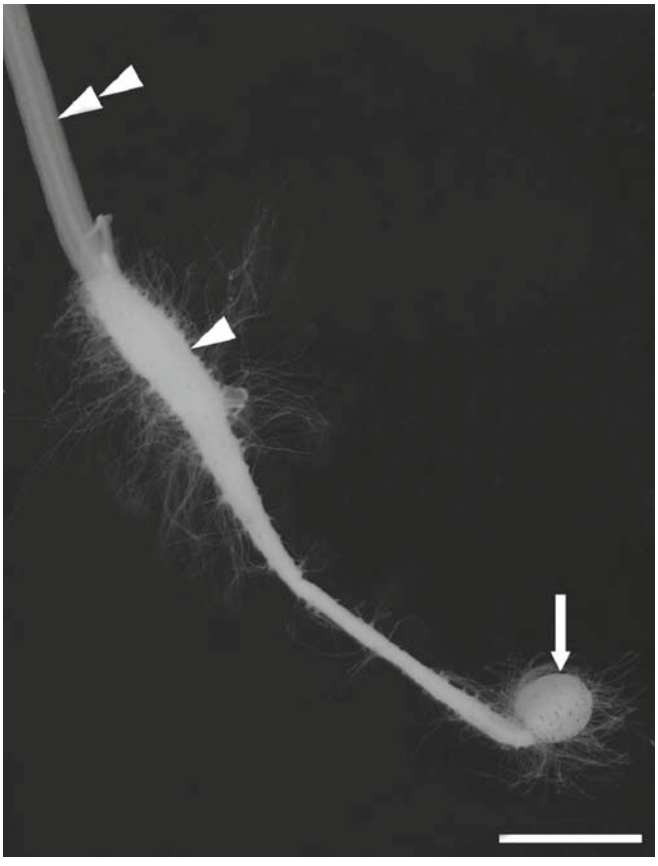

Fig. 1. Underground organs of *Stigmatodactylus sikokianus* consisted of tuber (*arrow*) and rhizome (*arrowhead*). A shoot (*double arrowheads*) grows from the rhizome. *Bar* 5 mm

The mycorrhizal fungi were isolated from the collected rhizomes according to Warcup and Talbot (1967) with slight modification as follows. The surface of the rhizome was washed in tap water and sterilized by immersion in 70% ethanol for 30 s and in sodium hypochlorite solution containing 1% available chlorine for 30 s. The surface-sterilized rhizome, cut into pieces about 5 mm in length, was put into 1 ml sterilized distilled water in a Petri dish (9 cm in diameter) and crushed by a sterilized glass rod to disperse the intracellular hyphal coils (pelotons). About 20 ml autoclaved modified Czapek Dox agar medium (sucrose 0.5 g, $NaNO_3$ 0.33 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, KCl 0.1 g, yeast extract 0.1 g, agar 15 g, distilled water 1000 ml), cooled to 45°C, was poured into the Petri dish and mixed before solidification to disperse the pelotons in the medium. The plates were incubated at 25.0° ± 0.5°C in the dark for 3 days. The fungal colonies growing from the pelotons were isolated by a sterilized scalpel and cultured on potato dextrose agar (PDA) medium.

DNA was extracted from each of the cultured fungi using PrepMan Ultra Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The internal transcribed spacer (ITS) region of nuclear rDNA was amplified from the extracted DNA by polymerase chain reaction (PCR) with primers ITS1-F and ITS4 (Gardes and Bruns 1993) using TaKaRa Ex Taq Hot Start Version (Takara Bio, Otsu, Japan). The PCR reaction mixture contained 5 µl template DNA, 0.75 units *Taq* polymerase, 0.25 µM each primer, 200 µM each dNTP, and 3 µl supplied PCR buffer in 30 µl total amount. The reaction was performed on a TaKaRa PCR Thermal Cycler 480 (TaKaRa Bio) as follows: initial denaturation step at 94°C for 2 min, a subsequent step of 35 cycles at 94°C for 20 s, at 55°C for 30 s, and at 72°C for 1 min, and a final elongation step at 72°C for 5 min. The PCR products were cloned using a pT7Blue Perfectly Blunt Cloning Kit (Novagen, Madison, WI, USA) according to the manufacturer's instructions, and plasmid DNAs were extracted from the cloned products with MagExtractor Plasmid (Toyobo, Osaka, Japan). The plasmid inserts were sequenced by the dye terminator method using sequencing primers M13–47 and RV-M. Obtained sequence data were deposited to the DDBJ/EMBL/GenBank database with accession numbers AB370308–AB370328. All the sequences were subjected to BLAST searches (Altschul et al. 1997), and related sequences were downloaded from the DDBJ/EMBL/GenBank nucleotide sequence database. For the sequenced and the downloaded data, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). Multiple sequence alignments were carried out using CLUSTAL W (Thompson et al. 1994), and the evolutionary history was inferred using the neighbor-joining (NJ) method (Saitou and Nei 1987). The alignments were deposited in TreeBASE (http://www.treebase.org/) under the accession number of S2198. The evolutionary distances were computed using the Kimura two-parameter model (Kimura 1980), and bootstrap analysis (Felsenstein 1985) was performed with 1000 replications.

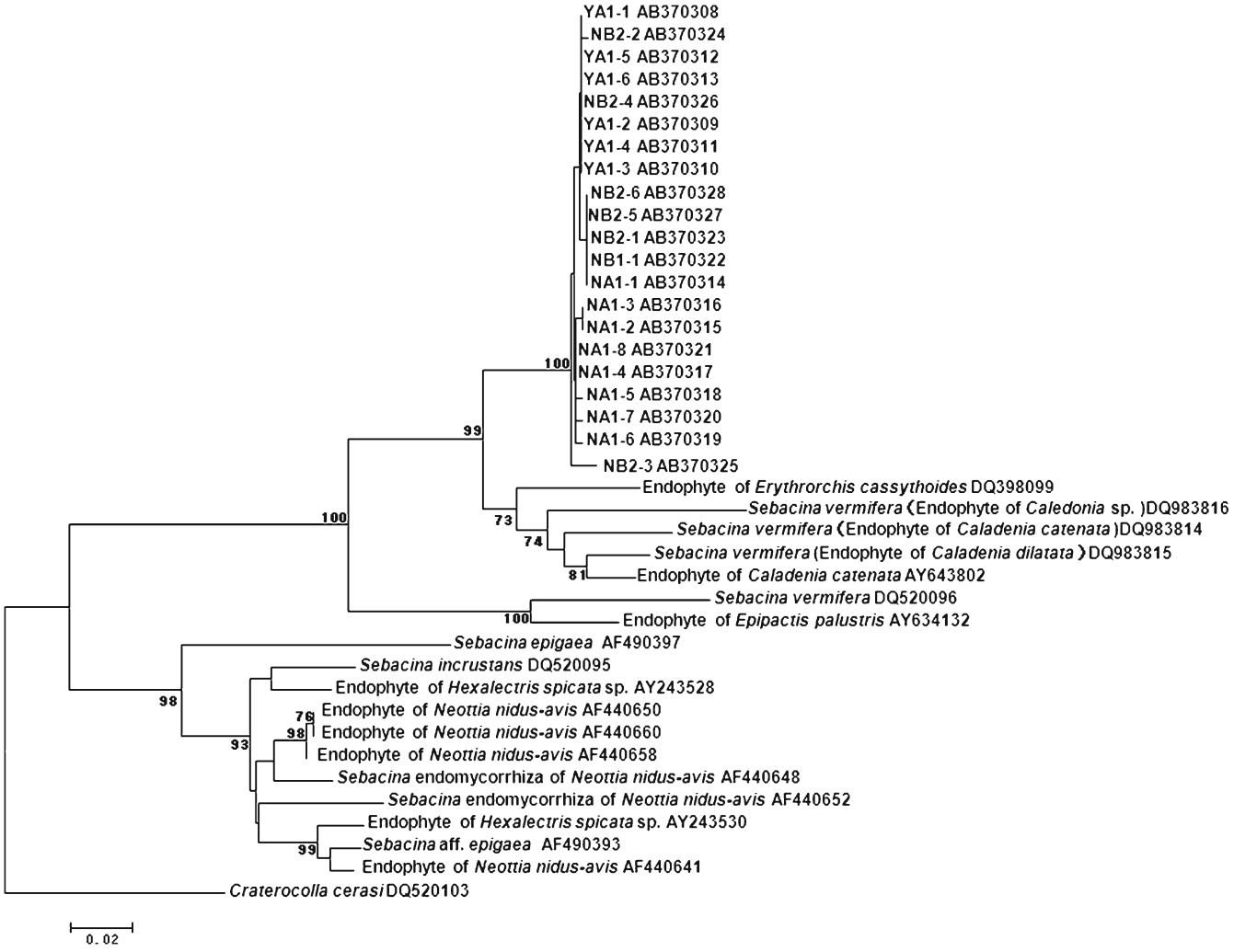

Fig. 2. A neighbor-joining phylogenetic tree based on the sequences of the internal transcribed spacer (ITS) region of nuclear rDNA showing the relationship between mycorrhizal fungi isolated from *Stigmatodactylus sikokianus* and the related fungi in Sebacinaceae. *Craterocolla cerasi* (DQ520103) in Sebacinales is used as the outgroup. Bootstrap values more than 70% are shown. Accession numbers of the DDBJ/EMBL/GenBank nucleotide database are shown on the figure

The classification of the fungi corresponds to Kirk et al. (2001).

A total of 21 fungal isolates were obtained from four individuals in the three sampling sites. DNA sequences of the ITS region were obtained from the isolates, and the phylogenetic analysis showed that they were homologous to those of *Sebacina* spp. and mycorrhizal fungi of Australian endemic terrestrial orchids *Erythrorchis cassythoides*, *Caladenia catenata*, and *Caladenia dilatata* (Fig. 2). The fungi in Sebacinaceae (Basidiomycota) are known to form many kinds of mycorrhizae, i.e., orchid mycorrhizae, ectomycorrhizae, ericoid mycorrhizae, and jungermannioid mycorrhizae (Weiß et al. 2004). For orchids, *Sebacina vermifera* were reported as mycorrhizal fungi in Australian terrestrial orchids: *Acianthus* spp., *Caladenia* spp., *Elythranthera* spp., *Eriochilus* spp., *Glossodia major* R. Br., *Microtis* spp. and *Prasophyllum* spp. (Roberts 1999). These orchids grow on grassland with no ectomycorrhiza-forming trees; therefore, the mycorrhizal fungi were regarded to be saprobic. On the other hand, mycorrhizal fungi of achlorophyllous orchids *Neottia nidus-avis* and *Hexalectris* spp., identified to be *Sebacina* spp., were found to be ectomycorrhiza-forming fungi (McKendrick et al. 2002; Selosse et al. 2002; Taylor et al. 2003). *Stigmatodactylus sikokianus* usually grows in *C. japonica* plantations, and this tree species is known to form arbuscular mycorrhiza. Therefore, it was considered that the mycorrhizal fungi of this orchid have saprobic features.

Because high sequence identity (99.9%–99.6%) was found among the mycorrhizal fungi isolated from three sampling sites, it was suggested that *S. sikokianus* is highly specialized to this group of fungi. To reveal the specificity and diversity of mycorrhizal fungi of this orchid, further studies are required using more samples in various sites. Such studies would be useful to consider the conservation and propagation of *S. sikokianus* in natural habitats.

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