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FULL PAPER

Relationship between intraspecific variations and host insects of *Ophiocordyceps nutans* collected in Japan

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Abstract To investigate the host specificity of *Ophiocordyceps nutans* against hemipteran insects in the wild, we determined the relationship between host species and rDNA-internal transcribed spacer (ITS) variation in *O. nutans*. The analyzed fungal specimens infected 16 host species belonging to four families of Hemiptera. The molecular phylogenetic analysis revealed that *O. nutans* can be classified into two types corresponding to their host families. The genetic distance values between the two types were very remote (>0.084), and the strains of *O. nutans* that parasitized *Halyomorpha halys* and *Plautia crossota stali*, well-known insect pests, formed a subclade. The results suggest that *O. nutans* should have host specificity which can be valuable for developing biological control agents against specific hemipteran insects.

Keywords Entomogenous fungus · Host specificity

Introduction

Ophiocordyceps nutans (Pat.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora is an entomopathogenic fungus that was originally described as *Cordyceps nutans* based on Japanese material (Patouillard 1887). It is an ascomycetous fungus which can form ascostromata. In general, *Cordyceps* s. l., including *O. nutans*, shows host specificity (Ito and Hirano 1997; Sato et al. 1997; Evans et al. 1999; Nikoh and Fukatsu 2000). *Ophiocordyceps nutans* parasitizes only stink bugs (Hywel-Jones 1995; Fukatsu 1999) and is found in Japan, Taiwan, China, New Guinea as well as other locations (Shimizu 1994). In Korea, *O. nutans* is one of the most common species of *Cordyceps* s. l. (Sung 1996).

The stink bug hosts of *O. nutans* are classified in the order Hemiptera, suborder Heteroptera, which includes major insect pests in agriculture and forestry. These stink bugs cause damage to agriculturally important crops, such as pear, rice, and sugar beet, as well as serious damage to cedar and sun trees by sucking sap from the cones (Tomokuni et al. 1993). Chemical control of fruit-damaging stink bugs is very difficult due to the concern for chemical residues in agricultural products. Hence, appropriate pest control procedures are required in orchards (Tsuda et al. 1997). The use of entomopathogenic fungi as biological control agents is regarded as being less harmful to living creatures and to the environment than the use of chemical insecticides (Fukuhara 1989). *Ophiocordyceps nutans* has been shown to have pathogenic properties (Sung et al. 1993) and is considered to be a candidate biological insecticide against stink bugs.

A number of studies have been carried out to identify hosts of *O. nutans* with the aim of determining its range of parasitism. The results revealed that the hosts of *O. nutans* cover at least 22 species of stink bugs, including seriously harmful insects in agriculture and forestry (Esaki 1929; Moureau 1949; Samson and Evans 1975; Sung et al. 1993; Sasaki et al. 2008). However, the relationship between intraspecific variation and host species of *O. nutans* is still unknown.

Intraspecific variations related to host species in other entomopathogenic fungi have been reported. For example, Jensen et al. (2001) demonstrated intraspecific variation in

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the internal transcribed spacer (ITS) ITS1-5.8S rDNA-ITS2 regions of *Entomophthora muscae*, a fly pathogen, using PCR–restriction fragment length polymorphism (RFLP) analysis. The study suggested that the host specificity of *E. muscae* should be correlated with the phylogenic clusters. Wada et al. (2003) also reported that *Beauveria brongniartii*, an entomopathogenic fungus utilized as a bioinsecticide against longicorn beetles, could be classified into two types based on differences in genetic sequence of the ITS1-5.8S rDNA-ITS2 region, with the type correlating to whether the host is longicorn beetles or scarab beetles. These results studies suggest that it is possible to find more selective entomopathogenic fungal strains that can target specific harmful insects.

In this study, specimens of *O. nutans* in several parts of Japan were collected and their host species determined. Based on the results, we analyzed the relationships between host species and genetic variation in the ITS1-5.8S rDNA-ITS2 region of the fungus and discussed the potential capabilities of *O. nutans* as a biological control agent.

Materials and methods

Samples

Between August 2003 and August 2006, specimens of *O. nutans* were collected in various parts of Japan (5 in Ebetsu, Hokkaido Island, 5 in Yamagata Prefecture, 7 in Fukushima Prefecture and 15 in Kyoto Prefecture of Honshu Island, and 10 in Kagoshima Prefecture of Kyushu Island) and their hosts identified (Table 1; Fig. 1).

The host insects were identified with reference to "A field Guide to Japanese Bugs" (Tomokuni et al. 1993). Isolation from the fruiting bodies of *O. nutans* was conducted according to the method described by Sasaki et al. (2004), and the remaining fruiting bodies were oven-dried at 60°C for 3 days. The fruiting bodies were identified as *O. nutans* based on original and recent descriptions (Patouillard 1887; Hywel-Jones 1995), using the methods of Sasaki et al. (2008). The specimens have been deposited in the Laboratory of Forest Resource Biology, Hokkaido University, Hokkaido, Japan.

DNA extraction, PCR, and sequencing

Isolates were transferred onto Sabouraud-glucose agar medium (pH 8.0) and incubated in darkness at 20°C (Sasaki et al. 2005). The cultured mycelia were cut into about 3 × 3 × 3-mm cubes for DNA extraction. Some samples were prepared to extract DNA directly from 3-mm pieces of stipe cut from oven-dried fruiting bodies. Total DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR was conducted using a set of primers, ITS1f (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and G-Taq DNA polymerase for general PCR analysis (Cosmo Genetech, Seoul, Korea) with a GeneAmp PCR System 2720 (Applied Biosystems, Foster City, CA). The cycling program consisted of an initial denaturation for 30 s at 94°C, followed by 25 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, extension for 2 min at 72°C, and a final extension for 10 min at 72°C.

The PCR products were electrophoresed for 55 min at 100 V on a 2% AMRESCO Agarose SFR agarose gel (Amresco, Solon, OH) in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1.0 mM EDTA–2Na–$2H_2O$, pH 8.0). After staining with 1 μg/mL ethidium bromide for 20 min, the band patterns were evaluated under UV irradiation.

The samples were purified using the LaboPass PCR Purification kit (Cosmo Genetech) following the manufacturer's instructions. The sequencing of the obtained templates was performed at Hitachi High-Tech Science Systems Co. Ltd. (Tokyo, Japan) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an Auto Sequencer 3730 (Applied Biosystems). The DNA sequences of the ITS1-5.8S rDNA-ITS2 region of the samples were determined using ITS1f and ITS4 primers. All sequences were registered in the DNA Databank of Japan (DDBJ) under accession numbers AB544450–AB544491.

Phylogenetic analysis

In addition to the DNA templates obtained in this study, sequences of *O. nutans* deposited in GenBank (AJ309367, AJ536558, AJ536560 from China and AJ786583 from Thailand) and those of 19 previously used strains collected in Tomakomai, Hokkaido Island (Sasaki et al. 2008) were also used in the phylogenetic analysis. Alignments of the obtained sequences of the ITS1-5.8S rDNA-ITS2 region were adjusted using Clustal W (Thompson et al. 1994). The distance values of samples were calculated using the Kimura two-parameter model (Kimura 1980), and a phylogenetic tree was created with the neighbor-joining method (Saitou and Nei 1987), based on the results of the gene analysis software MEGA ver. 4.1 (Tamura et al. 2007). To estimate the reliability of the clades formed on the tree, bootstrap analysis was run for 1,000 replicates.

Results

All specimens had the same taxonomical morphotypes in their fruiting body parts. Table 1 shows the host species of *O. nutans* specimens used in our study. Altogether, 11

Table 1 Host species of *Ophiocordyceps nutans* collected in different parts of Japan

Host	Strain	Location
Acanthosomatidae		
Acanthosoma denticaudum Jakovlev	03Y1, 03Y4, 06Yaka1	Yamagata
	06Fuka2	Fukushima
	04Yak3, 05Yak1, 05Yak2	Kagoshima
A. forficula Jakovlev	03Y3	Yamagata
	06Fuka1, 06Fuka4, 06Fuka5, 06Fuka6	Fukushima
A. labiduroides Jakovlev	04Yak2	Kagoshima
Sastragala esakii Hasegawa	06Fuka7	Fukushima
S. scutellata Scott	04Yak1, 05Yak4	Kagoshima
Pentatomidae		
Halyomorpha halys Stal	06Fuka3	Fukushima
	03Y2	Yamagata
Pentatoma rufipes Linnaeus	06N10, 06N11, 06N12, 06N13, 06N14	Hokkaido-Ebetsu
Plautia crossota stali Scott	05Yak3, 06Yak1, 06Yak3	Kagoshima
Coreidae		
Hygia lativentris Motschulsky	06Tank2, 06Tank5, 06Tank6, 06Tank10, 06Tank14, 06Tank15, 06Tank17, 06Tank20, 06Tank22, 06Tank23	Kyoto
H. opaca Uhler	06Tank11, 06Tank13, 06Tank19, 06Tank21	Kyoto
Homoeocerus sp.	06Tank1	Kyoto
	06Yak2	Kagoshima

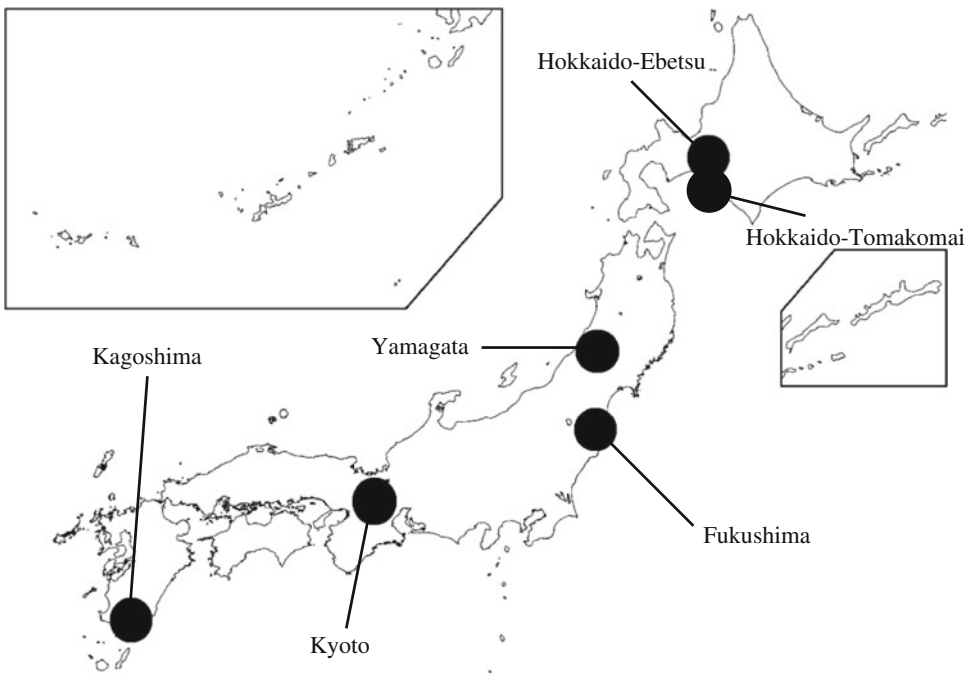


Fig. 1 Geographical locations of *Ophiocordyceps nutans* specimens used in this study

species belonging to three families were identified. In total, 61 ITS sequences of *O. nutans* parasitizing 16 hemiptera species in four families were used for the phylogenetic analysis.

The results of phylogenetic analysis are shown in Fig. 2. The sequences of all of the Kyoto strains and one Kagoshima strain (06Yak2) were very different from those of the other fungal strains, and all host species of these strains

belonged to family Coreidae. We therefore concluded that the type distinctions were: Type 1, which includes specimens whose hosts belong to Coreidae, and Type 2, which includes specimens parasitizing other hemipterans, with the exception of Coreidae. Homology in the sequence of the ITS1-5.8S rDNA-ITS2 region between Type 1 and Type 2 was <85% based on a comparison of ungapped alignments. The distance values between Types 1 and 2 was very remote (>0.084), resulting in the formation of different clades.

In Type 1, almost no variation was observed among the fungal strains. Consequently, the homology of ungapped alignments of sequences of Type 1 was >98%, and the distance value was <0.003, which supported the formation of one complete clade (BS 95). In Type 2, the distance value among the strains was <0.032, but their variations were frequently observed. The strains only parasitizing *Halyomorpha halys* and *Plautia crossota stali* formed subclade 1 (BS 99) in the Type 2 clade.

The sequences of the samples collected by the authors and GenBank sequences of *O. nutans* collected in East Asia were also compared. The distance values between Type 1 and the East Asian strains (excluding Japan) were <0.027, which indicates that they are more closely related to each other than to Type 2.

Discussion

Two types of *O. nutans* strains based on phylogenetic analysis parasitize different host insects. In this study, *O. nutans* strains parasitizing four different host families were used in the phylogenetic analysis, and only the strain parasitizing Coreidae (Type 1) created a genetically remote cluster (Fig. 2).

This genetic remoteness is associated with host ecologies and phylogeny. The host insects were classified into two types based on feeding habit and microhabitats: Type 1 insects are leaf-hoppers that suck juice mainly from grass and herbaceous plant stems, and Type 2 insects live on trees and feed on fruits, including cones (Tomokuni et al. 1993). The natural habitat of Type 1 insects is grassland, while that of Type 2 is forest (Tomokuni et al. 1993).

Li et al. (2006) conducted a nuclear 18S rDNA phylogenetic analysis of 21 putative families of hemipteran insects. According to their research, Acanthosomatidae, Pentatomidae, and Urostylidae, the host families of *O. nutans* Type 2, form one clade as Pentatomoidea, a superfamily of the Hemiptera, and are separated from Coreidae.

Tian et al. (2010) reported that most species of the order Hymenoptera, one of the hosts of subgenus *Neocordyceps* of *Cordyceps*, are rather narrowly adapted to specific habitats and are gregarious, which may easily cause the rapid parasitic infection of the host family. *Ophiocordyceps nutans* also belongs to *Neocordyceps*. However, host insects of each *O. nutans* type have different feeding habits and microhabitats and are not gregarious in same location. Therefore, two types of *O. nutans* have less of a chance to interact, although parasitic infection may easily occur in the host family in each type.

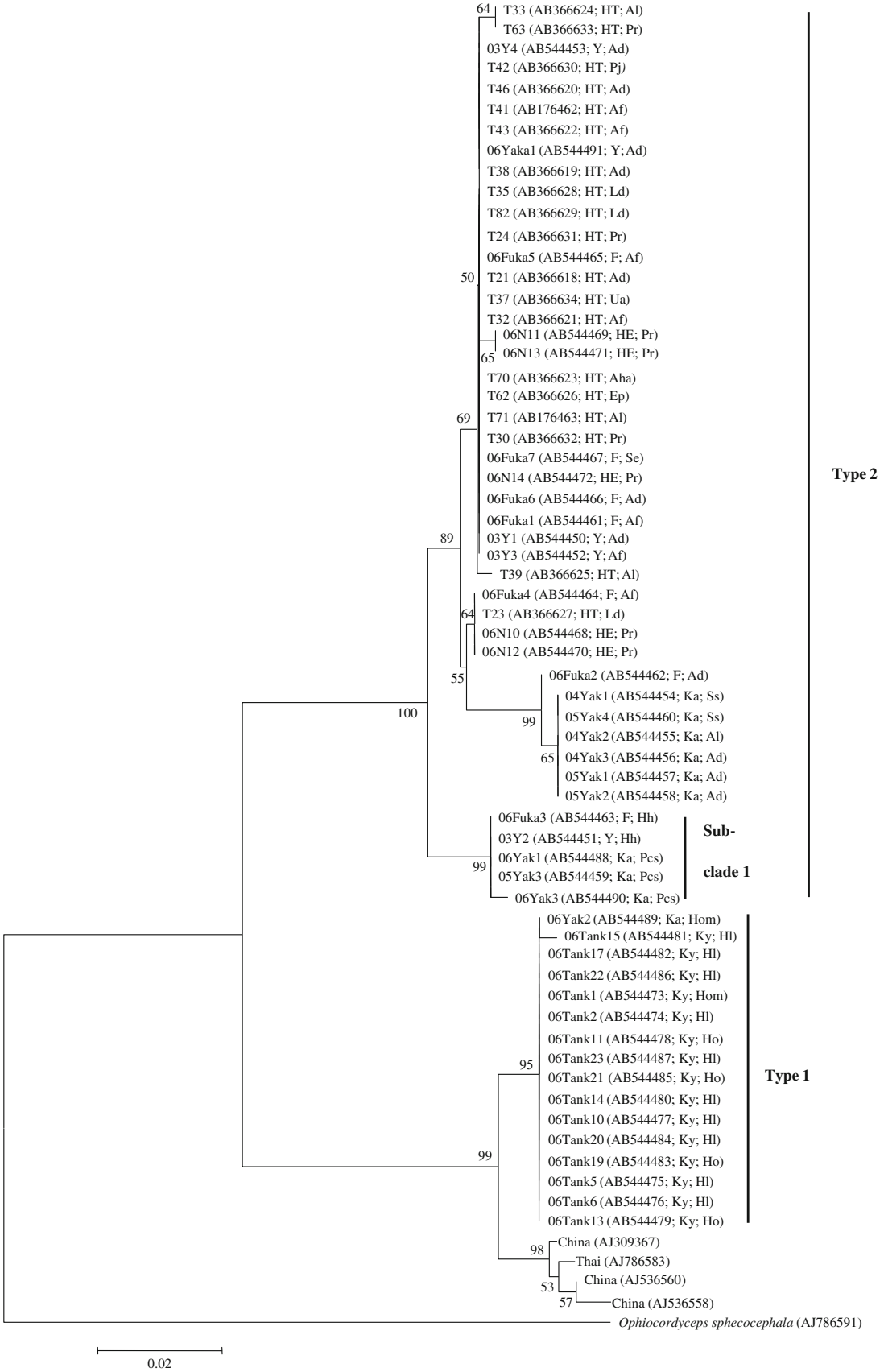
In conclusion, genetic divergence with the consequences of ecological differences between Coreidae and Pentatomoidea have led to decreased gene flow between two types of *O. nutans* and caused genetic remoteness.

In general, the sequence distance of the rDNA-ITS is between 0.000 and 0.050 for one fungal species, according to the Kimura two-parameter model (Chen et al. 2001, 2004). The distance value between Type 1 and Type 2 of *O. nutans* was obviously more remote (0.083), suggesting that these two types are different species. We also examined the genetic differences inside types. In contrast to Type 1 strain, in which almost no variation was found, minor genetic variation was observed in Type 2 strains. However, the distance value was <0.050. Thus, the variation observed in Type 2 might only reflect the geographical distance or minor phylogenetic differences.

Sequences of *O. nutans* Type 1 strain are genetically close (distance value <0.027) to the GenBank strain sequences from China and Thailand (Fig. 2). Accordingly, we consider that these four East Asian strains can be classified as Type 1, although their host species were not provided in GenBank and it is not clear whether the host insects are Coreidae or not.

Although differences between the two types in terms of perithecia size and ascospores in the fruiting bodies were not observed, some other differences were observed. Primarily, the beak in the perithecia was curved, and abdominal hyphae in insects were usually not compact in Type 1, although the beak was usually straight and the abdomen was filled with compact sclerotium in Type 2. A hollow abdomen in hosts of *O. nutans* was reported in Taiwan (Chou and Chang 2005). This supports the suggestion that *O. nutans* Type 1 in Japan is closely related to East Asian strains. However, it is necessary to investigate the shape and size of perithecia and abdominal hyphae as

Fig. 2 Phylogenetic relationship of *O. nutans* based on rDNA-internal transcribed spacer (ITS) sequences. All sequences were edited to the 379 sites and subjected to neighbor-joining (NJ) analysis. The values shown at *nodes* (>50) are confidence levels from 1,000 replicate bootstrap samplings. *HE* Hokkaido-Ebetsu, *HT* Hokkaido-Tomakomai, *Y* Yamagata, *F* Fukushima, *Ky* Kyoto, *Ka* Kagoshima, *Ad Acanthosoma denticaudum*, *Af A. forficula*, *Aha A. haemorrhoidale anglatum*, *Al A. labiduroides*, *Se Sastragala esakii*, *Ss S. scutellata*, *Ep Elasmucha putoni*, *Ld Lelia decempunctata*, *Hh Halyomorpha halys*, *Pj Pentatoma japonica*, *Pr P. rufipes*, *Pcs Plautia crossota stali*, *Ua Urostylis annulicornis*, *Hl Hygia lativentris*, *Ho H. opaca*, *Hom Homoeocerus* sp. ▶


64
T33 (AB366624; HT; Al)
T63 (AB366633; HT; Pr)
03Y4 (AB544453; Y; Ad)
T42 (AB366630; HT; Pj)
T46 (AB366620; HT; Ad)
T41 (AB176462; HT; Af)
T43 (AB366622; HT; Af)
06Yaka1 (AB544491; Y; Ad)
T38 (AB366619; HT; Ad)
T35 (AB366628; HT; Ld)
T82 (AB366629; HT; Ld)
T24 (AB366631; HT; Pr)
06Fuka5 (AB544465; F; Af)
50
T21 (AB366618; HT; Ad)
T37 (AB366634; HT; Ua)
T32 (AB366621; HT; Af)
06N11 (AB544469; HE; Pr)
06N13 (AB544471; HE; Pr)
65
T70 (AB366623; HT; Aha)
T62 (AB366626; HT; Ep)
69
T71 (AB176463; HT; Al)
T30 (AB366632; HT; Pr)
06Fuka7 (AB544467; F; Se)
06N14 (AB544472; HE; Pr)
06Fuka6 (AB544466; F; Ad)
06Fuka1 (AB544461; F; Af)
89
03Y1 (AB544450; Y; Ad)
03Y3 (AB544452; Y; Af)
T39 (AB366625; HT; Al)
06Fuka4 (AB544464; F; Af)
64
T23 (AB366627; HT; Ld)
06N10 (AB544468; HE; Pr)
06N12 (AB544470; HE; Pr)
55
06Fuka2 (AB544462; F; Ad)
04Yak1 (AB544454; Ka; Ss)
100
99
05Yak4 (AB544460; Ka; Ss)
04Yak2 (AB544455; Ka; Al)
65
04Yak3 (AB544456; Ka; Ad)
05Yak1 (AB544457; Ka; Ad)
05Yak2 (AB544458; Ka; Ad)
06Fuka3 (AB544463; F; Hh)
03Y2 (AB544451; Y; Hh)
Sub-
clade 1
99
06Yak1 (AB544488; Ka; Pcs)
05Yak3 (AB544459; Ka; Pcs)
06Yak3 (AB544490; Ka; Pcs)
Type 2
06Yak2 (AB544489; Ka; Hom)
06Tank15 (AB544481; Ky; Hl)
06Tank17 (AB544482; Ky; Hl)
06Tank22 (AB544486; Ky; Hl)
06Tank1 (AB544473; Ky; Hom)
06Tank2 (AB544474; Ky; Hl)
06Tank11 (AB544478; Ky; Ho)
95
06Tank23 (AB544487; Ky; Hl)
06Tank21 (AB544485; Ky; Ho)
Type 1
06Tank14 (AB544480; Ky; Hl)
06Tank10 (AB544477; Ky; Hl)
06Tank20 (AB544484; Ky; Hl)
99
06Tank19 (AB544483; Ky; Ho)
06Tank5 (AB544475; Ky; Hl)
06Tank6 (AB544476; Ky; Hl)
06Tank13 (AB544479; Ky; Ho)
98
China (AJ309367)
Thai (AJ786583)
53
China (AJ536560)
57
China (AJ536558)
Ophiocordyceps sphecocephala (AJ786591)
0.02

well as the host species of East Asian strains in detail in future investigations.

Our results suggest that *O. nutans* Type 1 may be effective as a selective biocontrol agent against pests belonging to the Coreidae due to its host specificity in terms of parasitism. *O. nutans* Type 2 parasitizes a wide variety of host species, including Acanthosomatidae, Pentatomidae, and Urostylidae, which also suggests the possibility of using *O. nutans* Type 2 as a candidate biocontrol agent against these hemipteran species.

The host insects of subclade 1 shown in Fig. 2 are exclusive to *Halyomorpha halys* and *Plautia crossota stali.* This subclade contains the fungal strain collected in Yamagata, Fukushima and Kagoshima Prefectures, indicating that they can occur in both northern and southern parts of Japan. *Halyomorpha halys* and *P. crossota stali* are well-known pest insects that cause serious damage to agriculture crops and forest stands (Tomokuni et al. 1993; Tsutsumi 2003), suggesting that these strains have host specificity and have the potential to be effective biocontrol agents, especially against *H. halys* and *P. crossota stali.*

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