Studies on ecology and population genetics of *Microdochium nivale* and *M. majus*, causal agents of Fusarium head blight on wheat

（コムギ赤かび病菌*Microdochium nivale* と*M. majus*の

生態学および集団遺伝学的研究）

Hokkaido University　Graduate School of Agriculture

Division of agrobiology　Doctor Course

Yusaku Hayashi

SUMMARY

Fusarium head blight (FHB) is an economically important disease of small grains of wheat and barley in warm and humid areas worldwide and can result in significant yield losses. FHB is commonly caused by *Fusarium graminearum* and *Microdochium* spp., such as *M. nivale* and *M. majus*, especially in Hokkaido, Japan.

In CHAPTER III, I developed the selective medium, LATTS, to isolate airborne spores of *Microdochium* spp. In developing the selective medium, carbon, nitrogen sources and vitamin B as basal components to increase the formation of aerial mycelium as well as the mycelial growth was selected. A significant effect ($p < 0.05$) of carbon, nitrogen and vitamin B sources on the mycelial growth was observed among three *M. majus* isolates, respectively. The mycelial growth of *M. majus* was increased on the medium containing lactose as the carbon source and L-asparagine as the nitrogen source. The formation of aerial mycelium increased on the medium containing thiamine hydrochloride as the vitamin B source. In the investigation for selection of the antimicrobial components, the growth of only *Fusarium* spp. was selectively inhibited by the addition of thiophanate methyl, to which *Microdochium* spp. were resistant. Besides, the colony formation of other filamentous fungi was greatly inhibited by the addition of spiroxamine, a kind of ergosterol biosynthesis inhibitor fungicides. Spore trapping was performed using the LATTS in a wheat field to confirm if airborne spores of *M. majus* can be isolated. Airborne spores of *M. majus* could be trapped and *M. majus* isolates formed characteristic pinkish colonies, regardless of more or less of the contamination with other filamentous fungi. The characteristic pinkish colonies, therefore, facilitated the identification of other filamentous fungi. There are differences in dynamics of the spore dispersal between *M. majus* and *F. graminearum*. Primary, the number of spores of *M. majus*

trapped was much higher than that of *F. graminearum.* Besides, the spore dispersal of *M. majus* was earlier than that of *F. graminearum.* To confirm the growth of the two species in *Microdochium* spp., suspensions of each species were plated on LATTS medium respectively. After incubation for 7 days, *M. majus* isolates formed characteristic centroclinal and deep pinkish colonies, whereas *M. nivale* isolates formed flat and white colonies. From the 12 days on, *M. nivale* isolates also formed flat and light pinkish colonies. LATTS medium, therefore, could distinguish *M. nivale* and *M. majus* based on the difference in the colony morphology between *M. nivale* and *M. majus*.

In CHAPTER IV, with the aim of the distribution of the two species in *Microdochium* spp. obtained from different host tissue, 361 isolates of *Microdochium* species were first classified into each species using polymerase chain reaction (PCR) amplification with species-specific primers. In total 344 (95.3 % of all isolates) and 17 isolates were identified as *M. majus* and *M. nivale*, respectively, These results indicated that *M. majus* was the dominant species regardless of origins of the host. The levels of genetic variation and structure in populations of *Microdochium* spp. obtained from infected wheat seeds originating from geographically distant regions in Hokkaido were analyzed using two inter-simple sequence repeat (ISSR) markers. ISSR analysis showed that the total genetic diversity was 0.023 when estimated by Nei's gene diversity index within the five populations dominated by *M. majus*. An analysis of molecular variance (AMOVA) analysis also showed that 86.74% of the total genetic variation was within populations and 13.26% among populations. These results indicated that little genetic differentiation occurred among the five populations of *M. majus*. Based on the unweighted pair group method of cluster analysis using the ISSR data, all isolates were identified as one of eight haplotypes in *M. majus* or six haplotypes in *M. nivale*, allowing the construction of a dendrogram with two clades

corresponding to each species. There was no correlation between the clustering of isolates and their geographic distribution on the tree. These findings show that migration is likely playing an important role in the population biology of *M. majus*, providing some support for the prediction of epidemics of fungicide resistant strains within populations of the FHB pathogen.

In CHAPTER V, I investigated the distribution and frequency of strobilurins-resistant strains of *Microdochium* spp. that were obtained from geographically distant regions in Hokkaido in 2011. According to the sensitive test, the strobilurins-resistant strains have been already widespread in Hokkaido with 52.6% (Tokachi) and 51.1% (Abashiri) of tested isolates. This finding would, therefore, suggest that the resistant strains developed rapidly in a short time after their appearance. No significant difference in fitness parameters, such as the mycelial growth, conidia germination, and sporulation ability, was found between resistant and sensitive strains *in vitro*. Moreover, strobilurins resistance in *M. nivale* and *M. majus* was not significantly reduced from the first generation to the fifth generation that were grown and preserved on a strobilurin-free medium. It suggested that strobilurins resistance in *M. nivale* and *M. majus* is stable in the absence of the fungicide. The sequencing of partial regions in cytochrome *b* (*CYTB*) indicated that all strobilurins-resistant strains but isolate T35 had the G143A mutation (the substitution of glycine for alanine at position 143), the typical amino acid mutations related to strobilurins resistance, in *CYTB* gene. *M. nivale* isolate T35 resistant to strobilurins, however, had not the F129L and G137R mutations as well as G143A. Based on ISSR fingerprinting and *CYTB* sequences, genetic diversity of *Microdochium* populations were analyzed. The analyses showed that *M. majus* isolates had a high genetic uniformity compared with *M. nivale* isolates due to high gene flow. Furthermore, AMOVA and the coefficient of gene differentiation (G_{ST}) showed that most of the mutations associated with

strobilurins resistance had occurred and spread within *Microdochium* population. Phylogenetic trees constructed based on haplotypes identified by the *CYTB* sequences and ISSR fingerprinting data showed the strobilurins-resistant strains tested emerged independently through a single amino acid mutation, G143A, that had occurred in populations of sensitive isolates.

In CHAPTER VI, I investigated the mode of sexual reproduction, either heterothallic or homothallic, and evaluated the possibility of the interspecific hybridization within isolates of *M. majus* and *M. nivale.* According to the test for confirming whether isolates of each species are self-fertile, 75.0% of *M. majus* isolates produced perithecia, while 31.3% of *M. nivale* only produced perithecia. These findings, therefore, suggested that both *M. majus* and *M. nivale* had a potential to reproduce homothallically although there was a difference in the proportion of the mode of predominant sexual reproduction between the two species. 93.3% of all combinations of six isolates in *M. majus* produced perithecia, which was more frequent than that (23.6%) of all combinations of eleven isolates in *M. nivale.* These results suggested that there might be many opportunities for sexual reproduction of *M. majus* than *M. nivale* in nature because *M. majus* had a higher rate of production of perithecia in either alone or crossing. PCR fragments of *MAT1-2-1* allele were found in all isolates of *M. nivale* and *M. majus*, either self-fertile or self-sterile. Moreover, mature perithecia were also obtained by crossing of self-sterile isolates of *M. majus* or *M. nivale* that carried *MAT1-2-1* allele, respectively. This phenomenon might be the atypical mating, which were called “unbalanced heterothallism.” Interspecific hybridization between *M. majus* and *M. nivale* was proved by the crossing of self-sterile isolates of the two species. These results indicated that interspecific hybridization might play a role as means for the development of strobilurins resistant strains within populations of *M. majus* and *M. nivale*. However, the frequency of

interspecific hybridization was considered to be low in nature because the presence of perithecia without matured ascospore were observed at high frequency in the crossing test between the two species.

Knowledge about biology, ecology and genetics of *M. nivale* and *M. majus* in this study could be useful in the improvement of FHB management and would lead to the effective control of FHB worldwide.

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CONTENTS

CHAPTER I
GENERAL INTRODUCTION

The stable production of food is essential in the modern times that world population have been increasing dramatically and is a problem to be solved beyond the country. Agriculture is one of the major industries in Japan as well as other countries. Hokkaido, the northernmost of Japan, is the leading agricultural producer in Japan. According to 2014 fiscal year the Ministry of Agriculture, Forestry and Fisheries Statistics Department “Crop Statistics”, the total producing area of winter wheat in Hokkaido is 123,400 hectares (about 58.0% of the country), and the total harvest weight is about 551 tons (about 64.7% of the country). These statistics supports that agriculture in Hokkaido plays an important role as a source of food.

Fusarium head blight (FHB) is a plant disease that occurs in the ear of wheat. Especially in Hokkaido, FHB is the most economically important disease because it can result in a significant yield loss for the reason of much rainfall during the anthesis, which encourages the FHB infection. FHB is caused by several pathogens in Hokkaido, such as *Fusarium* spp. (*F. graminearum, F. avenaceum* and *F. asiaticum*) and *Microdochium* spp. (*M. nivale* and *M. majus*). *Microdochium* spp. have a character different from *Fusarium* spp.; that is a broad range of viable temperature. *Microdochium* spp. were able to grow from -6 to 28°C, with optimum growth between 18 to 21°C (Årsvoll, 1975). This character is suitable for the occurrence of pink snow mold of wheat under the snow, subsequently causing pre-emergence damping-off after wintering. *Microdochium* spp. is, therefore, an important control target of wheat diseases in Hokkaido.

In this study, I focused on *Microdochium* spp. and aimed at obtaining the principal findings of their biology, ecology and epidemiology. In CHAPTER III, I developed the selective medium, LATTS, for isolation of airborne spores of *Microdochium* spp. Spore trapping using the LATTS medium was performed in several times in a wheat field, and I discussed the characteristic of spore dispersal of *Microdochium* spp. through comparison

with that of *F. graminearum* trapped using a selective medium. This new tool enabled various subsequent analyses such as the genetical (CHAPTER IV) and ecological (CHAPTER V) study. In CHAPTER IV, I analyzed the genetic structure in populations of *Microdochium* spp. obtained from infected wheat seeds originating from geographically distant regions in Hokkaido. Besides, the distribution of the two species of *Microdochium* spp. in Hokkaido was investigated. In CHAPTER V, I investigated the frequency and fitness of the strobilurins-resistant strains in populations of *Microdochium* spp., which were identified in Hokkaido in 2011. Furthermore, we discussed the evolution of the strobilurins-resistant strains through phylogenetic relationships constructed by nuclear genomic fingerprinting and mitochondrial cytochrome *b* sequences. In CHAPTER VII, I discussed the effect of the mode of sexual reproduction, which was determined by the crossing test among isolates, on the spread of fungicide-resistant strains of *Microdochium* spp.

CHAPTER II
LITERATURE REVIEW

2-1. Introduction of Fusarium head blight

2-1-1. Fusarium head blight

Fusarium head blight (FHB) is an economically important disease of small grains of wheat and barley in warm and humid areas worldwide. It can result in a significant yield loss (Bechtel *et al.*, 1985; McMullen *et al.*, 1997; Tekauz *et al.*, 2000). Some FHB pathogens including *Fusarium* species produce a range of toxic secondary metabolites called mycotoxins. The mycotoxins have a potential harmful risk in human and animal food products (D'Mello & Macdonald, 1997; Placinta *et al.*, 1999) as well as cause a loss of grain quality (Bai & Shaner, 1994; Placinta *et al.*, 1999). In 2002, the provisional standards concerning contamination of grain with DON was set at below 1.1 ppm. In 2003, the Japanese Agricultural Products Inspection Law was amended to less than 0.049%. FHB is very damaging to wheat producers because contaminated grains are regulated on the market.

2-1-2. FHB pathogens

FHB is commonly caused by various *Fusarium* spp. such as *F. graminearum*, *F. avenaceum*, *F poae*, *F. culmorum* and *F. asiaticum,* and *Microdochium* spp. such as *M. nivale* and *M. majus* (Sutton, 1982; Parry *et al.*, 1995; Brennan *et al.*, 2003). In Hokkaido, *Fusarium graminearum* and *Microdochium* species are primary pathogens of FHB (Souma & Kozawa, 2006). *Fusarium* spp. produce various mycotoxins; for example, *F. graminearum* produce deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) (Greenhalgh *et al.,* 1983). On the other hand, *Microdochium* spp. are not thought to produce any mycotoxins (Nakajima & Naito, 1995).

2-1-3. Control of FHB

FHB is usually controlled by fungicide treatments, and several kinds of fungicides that have different modes of action are applied on wheat spikes during the flowering period. For example, demethylation inhibitor (DMI) (e.g. thiophanate-methyl, tebuconazole, and proconazole), inhibitior of membrane lipid biosynthesis (e.g. iminoctadine triacetate), and respiration inhibitor (e.g. kresoxim-methyl) have been used. These fungicides were applied in the rotation for effective control because the effectiveness of each fungicide varies depending on the type of FHB pathogens, either *Fusarium* spp. or *Microdochium* spp. It has been required to be highly effective in the reduction in DON accumulation and the growth inhibition of *Microdochium* spp. when using the fungicides.

2-2. *Microdochium* species

2-2-1. Classification into two species based on the genetic differences

The identification of two varieties (previously called *Fusarium nivale* var. *nivale* and *majus*) was first reported by Wollenweber & Reinking (1935), followed by the reclassification of *F. nivale* as *Microdochium* by Samuel & Hallett based on ascospore septation, perithecial structures, and rRNA sequence (Samuels & Hallett, 1983). The subspecies within *Microdochium* were subsequently classified into two varieties, var. *nivale* and var. *majus*. The presence of the two varieties has been proven by some molecular techniques, such as random amplified polymorphic DNA (RAPD) analysis (Lees *et al.*, 1995); restriction fragment length polymorphism (RFLP) analysis based on the ITS region (Parry *et al.*, 1995); sequence analysis based on the gene encoding elongation factor 1-a (EF-1a) (Maurin *et al.*, 1995); esterase isozymes and ITS polymorphisms (Simpson *et al.*, 2000); DNA sequences based on RNA polymerase II (RPB2), β-tubulin, and EF-1a (Jewell & Hsiang, 2013). These DNA analyses, therefore, supported that *Microdochium* spp. are classified as the two species, *M. nivale* sensu strict and *M. majus* sensu strict. Several researchers have reported that there is a high degree of variation between *M. nivale* and *M. majus* (Lees *et al.*, 1995; Maurin *et al.*, 1995; Parry *et al.*, 1995). Moreover, *M. nivale* has a high number of polymorphisms within individuals (Lees *et al.*, 1995; Parry *et al.*, 1995), indicating that this subspecies can reproduce sexually in nature (Lees *et al.*, 1995). On the other hand, *M. majus* has been described as having a high level of genetic uniformity, indicating that this variety generally reproduces homothallically in nature (Lees *et al.*, 1995).

2-2-2. Biological differences between two species

Several studies showed differences in biological and ecological properties between *M. nivale* and *M. majus*; for example, the mating system (either homothallic or heterothallic) (Lees *et al.*, 1995; Maurin *et al.*, 1995; Parry *et al.*, 1995; Mahuku *et al.*, 1998), host preference (Lees *et al.*, 1995; Diamond & Cooke, 1997; Mahuku *et al.*, 1998; Simpson *et al.*, 2000), growth temperature preference in vitro (Simpson *et al.*, 2002), greater pathogenicity of *M. nivale* var. *nivale* on grasses (Grosch & Schumann, 1993) versus greater pathogenicity of *M. nivale* var. *majus* on cereals (Maurin *et al.*, 1995; Diamond & Cooke, 1997), and the higher frequency of isolation of *M. nivale* var. *majus* from grain (Lees *et al.*, 1995; Parry *et al.*, 1995).

2-3. Fungicide-resistance of *Microdochium nivale* and *M. majus*

2-3-1. History of fungicide resistant strains

Several researchers reported the emergence of resistant strains of *Microdochium* spp. to several fungicides worldwide. Tanaka *et al.* (1983) collected the resistant strains to thiophanate-methyl, which had been widely used as a common fungicide, from symptoms of pink snow mold in northern Japan. Besides, Pennucci *et al.* (1990) identified dicarboximide-resistant strains from golf courses in the northern half of the North Island, New Zealand. Recently, the decrease of efficiency of strobilurins was reported in wheat fields both in France (Walker *et al.*, 2009) and in Hokkaido, Japan (Kozawa *et al.*, 2011), though the application of strobilurins at flowering time are highly effective against *Microdochium* spp. (Ioos *et al.*, 2005; Mullenborn *et al.*, 2007).

2-3-2. Resistant strains to strobilurin fungicide

Kresoxim-methyl is a member of strobilurins fungicides (QoIs), which are widely used for the control of fungal plant pathogens all over the world. Strobilurins interacts with the mitochondrial bc_1 (or III) complex in mitochondrial DNA (mtDNA), specifically inhibiting cytochrome *b* (*CYTB*) by binding to the outer quinol oxidation site (Qo) in the respiratory electron transfer chain. Although strobilurins were highly effective against *Microdochium* spp. (Ioos *et al.*, 2005; Müllenborn *et al.*, 2007), they had a high risk of resistance for having a single-site mode of action (Brent & Hollomon, 2007). It was already reported that several plant pathogenic fungi showed the less effectiveness of strobilurins (Sierotzki *et al.*, 2000; Steinfeld *et al.*, 2001; Fraaije *et al.*, 2005; Chen *et al.*, 2007; Leroux *et al.*, 2007). The resistance was usually responsible for G143A mutation (the substitution of glycine for

alanine at position 143), F129L mutation (the substitution of phenylalanine for leucine at position 129), and G137R mutation (the substitution of glycine for arginine at position 137) in the quinol oxidation site of *CYTB*, encoded by the mitochondrial gene (Bartlett *et al.*, 2002; Fernández-Ortuño *et al.*, 2008).

2-4. Sexual reproduction

2-4-1. Sexual reproduction in ascomycetes fungi

Sexual reproduction in ascomycetes fungi is controlled by a single locus (*MAT*) with two idiomorphic alleles, called *MAT-1-1-1* and *MAT-1-2-1* (Coppin *et al.*, 1997: Turgeon, 1998). The *MAT1-1-1* allele encodes proteins with a α-box motif, while the *MAT1-2-1* allele contains an HMG box (Arie *et al.*, 1997). Heterothallic individuals are self-sterile, and require a partner, which has an alternate *MAT* allele because they carry a single *MAT* allele, either *MAT-1-1-1* or *MAT-1-2-1*. On the other hand, homothallic individuals are self-fertile, and require no partner for the sexual reproduction because they carry both *MAT* alleles.

Detailed information on *MAT* genes was available for only three filamentous ascomycete genera, *Neurospora* (Glass & Smith, 1994), its close relative *Podospora* (Picard *et al.*, 1991) and *Cochliobolus* (Turgeon *et al.*, 1995). The reason was because it was thought to be difficult to identify the *MAT* gene, which highly varied between species (Cisar *et al.*, 1994; Arie *et al.*, 1997; Turgeon, 1998). However, Turgeon (1998) reported that *MAT-1-1-1* and *MAT-1-2-1* contained strongly conserved regions of DNA binding protein, α-box and HMG box, among species in common. Arie *et al.* (1997) developed a simple and fast procedure for cloning *MAT* genes of various species in both sexual and asexual fungi. That technique is based on PCR amplification of the conserved regions with degenerate primers. The PCR-based technique was already used to amplify the *MAT* genes in a range of species in ascomycetes, such as *F. oxysporum* and *Alternaria alternata* (Arie *et al.*, 2000), *Giberella fujikuroi* and *G. zeae* (Yun *et al.*, 2000) and *Ryncosporium secalis* (Celeste *et al.*, 2003).

2-4-2. Sexual reproduction in *Microdochium nivale* and *M. majus*

M. nivale and *M. majus* belong to ascomycetes and can reproduce asexually and sexually (Lees *et al.*, 1995; Litschko & Burpee, 1987; Parry *et al.*, 1995). Sexual reproduction has significant advantages of generating the novel phenotypes that parents have not possessed, through mating with others having a different genetic background. Several genetic studies suggested that *M. nivale* isolates reproduced sexually in nature because they had a high number of polymorphisms within individuals (Lees *et al.*, 1995; Parry *et al.*, 1995). On the other hand, *M. majus* isolates generally reproduce homothallically in nature because they have a high level of genetic uniformity (Lees *et al.*, 1995; Hayashi *et al.*, 2013). Although both *M. nivale* and *M. majus* alone produce perithecia *in vitro* (Lees *et al.*, 1995; Litschko & Burpee, 1987; Parry *et al.*, 1995), these reports showed *M. majus* produce perithecia more readily than *M. nivale*.

Development of *Microdochium*-specific primers to amplify the *MAT* genes was performed by Jewell (2013) who was able to amplify the putative *MAT1-2-1* and the region between SLA2 and APN2, but no amplification of *MAT1-1-1* could be observed in any sequences of *M. majus* and *M. nivale* isolates.

CHAPTER III
DEVELOPMENT OF SELECTIVE MEDIUM

3-1. Introduction

The primary inoculum of FHB is considered to be airborne ascospores released from perithecia that were produced on crop residues (Parry *et al.*, 1995; Sutton, 1982). The development of FHB is mainly affected by weather conditions, such as air temperature, relative humidity, and rainfall. Information about the numbers of airborne spores in fields is necessary to understand the level of FHB development. To obtain the information of the number of airborne spores, we needed to count the number of airborne spores on the glass slides coated with glycerin jelly and exposed in fields. However, this method requires both time-consuming and labor-intensive investigations as well as enough experience in the microscopic operation. Togawa (1994) developed a FG medium as the selective medium to identify of *F. graminearum,* and this tool was used to understand the fundamental studies, such as its biology, ecology and epidemiology. However, Kozawa (2006) reported that the growth of *Microdochium* spp. was very slow on the FG medium, and there is a need to develop novel selective media to understand the fundamental characters of *Microdochium* spp.

The FG medium is an excellent tool to isolate the spores of *F. graminearum* for the following reasons. Firstly, xylose as the carbon source of the medium increased the formation of aerial mycelium more vigorously. Secondly, L-glutamic acid (Na) as the nitrogen source turned the colony of *F. graminearum* bright red. These unique characteristics of the colony made it easy to distinguish the colony of *F. graminearum* from that of other filamentous fungi in the case of contamination. Thirdly, antimicrobial components of the FG medium efficiently depressed the growth of other filamentous fungi. In fact, the growth of filamentous fungi except for *Fusarium* spp. was not seen on the FG medium.

The purposes of this chapter were (i) to develop a selective medium for isolation of airborne spores of *M. majus*, and (ii) to confirm whether the medium developed was suitable for counting colonies grown on the medium, and (iii) to compare the colony morphology between *M. nivale* and *M. majus* on the medium. In developing the selective medium, we selected carbon and nitrogen sources to increase the formation of aerial mycelium as well as the mycelial growth. Besides, we examined the addition of vitamin B because Yamauchi *et al.* (2003) reported that the formation of the aerial mycelium of *Fusarium* spp. was increased by the addition of biotin to the medium.

3-2. Investigation of basal components

Materials & Methods

Fungal isolates

Three *M. majus* isolates, MN-2T, MN406 and MN451, were used in this examination. They were obtained from infected winter wheat grown in Hokkaido between 2002 and 2004. Until use, all isolates were stored on slant culture containing 1.5% agar at 5°C in darkness.

Selection of carbon and nitrogen source

The basal medium (BM) was prepared based on the basal composition of Fusarium-selective medium (Komada, 1975), and used for investigation of the basal components of the selective medium of *Microdochium* spp. The overall components of the BM medium were as following: K_2HPO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Fe-EDTA, 0.01 g; carbon source, 20 g; nitrogen source, 2.0 g; agar, 15 g; and distilled water, 1 l adjusted to pH 6.8. Ten carbon sources tested were sucrose, trehalose, melitose, lactose, maltose, glucose, fructose, sorbitol, xylose, and galactose. Eleven nitrogen sources tested were L-serine, glycine, L-α-alanine, L-histidine, DL-aspartic acid, D-aspartic acid, L-glutamic acid (Na), L-asparagine, $NaNO_2$ and urea. Each plate was inoculated with a 5 mm mycelium plug cut from the margin of a five-day-old colony on 1.5% water agar (WA; agar, 15 g; distilled water, 1 l) of *M. majus*. The plates were incubated at 20°C for 7 days in darkness. Colony diameter was measured manually with a rule and assessed as the mycelial growth. The degree of aerial mycelium formation measured visually with 0 being the minimum and 3 the maximum, and assessed as

the aerial mycelium index. The mean values of the three replicates were assessed for each treatment.

Selection of a combination of carbon and nitrogen source

Based on the results of the examination of the respective carbon and nitrogen source, I examined different combinations of the selected carbon sources (trehalose, melitose and lactose) and nitrogen sources (L-asparagine, glycine, and L-histidine) and assessed the mycelial growth and aerial mycelium index on the medium. Procedures were the same as the above.

Selection of Vitamin B source

Based on the results of the above, the BM medium was prepared with lactose as the carbon source and L-asparagine as the nitrogen source. Eight sources of vitamin B tested were pyridoxine hydrochloride, 500 mgl^{-1}; thiamine hydrochloride, 500 mgl^{-1}; biotin, 500 mgl^{-1}; calcium pantothenate, 2000 mgl^{-1}; riboflavin, 500 mgl^{-1}; myo-inositol, 10000 mgl^{-1}; folic acid, 2000 mgl^{-1}; and nicotinic acid, 2000 mgl^{-1}. Each vitamin B was added in the BM medium containing lactose and L-asparagine, and sterilized at 121°C for 20 min. Examination of vitamin B was performed as above for the assessment of carbon and nitrogen sources.

Results

Selection of carbon and nitrogen source

The mycelial growth and the aerial hyphal index were examined by inoculation of a mycelium plug on BM media containing eleven carbon sources, respectively (Table 3-2-1). A significant effect ($p < 0.05$) of carbon sources on the mycelial growth was observed among three *M. majus* isolates. Xylose, the carbon source of the FG medium, prevented the growth of all *M. majus* isolates. Although galactose, the carbon source of Komada's *Fusarium*-selective medium, showed a high aerial mycelium index, the mycelial growth was suppressed considerably in comparison to other carbon sources. The mycelial growth was most prominent with sucrose, followed by trehalose and melitose. However, the results of the aerial mycelium index did not correspond with those of the mycelial growth. Lactose and galactose had the best effect on the aerial mycelium index followed by trehalose, melitose and sorbitol. Therefore, trehalose and melitose that showed both a high mycelial growth and aerial mycelium index, and lactose that showed the highest aerial mycelium index were selected as the carbon sources of selective medium. None of them showed the formation of a colored colony.

The mycelial growth and the aerial mycelium index were examined by inoculation of a mycelium plug on BM media containing ten nitrogen sources, respectively (Table 3-2-2). Each nitrogen source had a significant effect ($p < 0.05$) on the mycelial growth of three *M. majus* isolates. The nitrogen source used in Komada's *Fusarium*-selective medium, L-asparagine, showed the highest mycelial growth, followed by glycine and L-serine. The nitrogen source used in the FG medium, L-glutamic acid (Na), suppressed the mycelial growth considerably. On the other hand, the aerial mycelium index was highest with

L-histidine and L-asparagine, followed by L-serine. Therefore, L-asparagine and glycine that showed a high mycelial growth, and L-histidine that showed a high aerial mycelium index were selected as the nitrogen sources. None of the nitrogen sources showed the formation of a colored colony.

Selection of a combination of carbon and nitrogen source

A significant effect ($p < 0.05$) of various combinations on the mycelial growth was observed among three *M. majus* isolates. Trehalose showed the highest mycelium growth in all the combinations although it showed the low level of the aerial mycelium index. Melitose showed comparatively high mycelium growth regardless of the nitrogen source, but the combination with trehalose did the low level of the aerial mycelium index. Lactose showed the highest aerial mycelium index regardless of the nitrogen source. The combination of lactose and L-histidine showed the highest aerial mycelium index in all the combinations and formed slightly centroclinal colonies. Because mycelium growth was most suppressed in all combinations, the combination of lactose and L-histidine was deemed unsuitable for selective isolation of *M. majus*. Therefore, the combination of lactose and L-asparagine showed the second highest aerial mycelium index and reasonable mycelium growth was selected as the carbon and nitrogen source of the selective medium. None of the combinations of carbon and nitrogen sources showed the formation of colored colonies.

Selection of vitamin B

Differences of vitamin B had a significant effect ($p < 0.05$) on the mycelial growth among three *M. majus* strains (Table 3-2-4). Pyridoxine hydrochloride, thiamine hydrochloride, and biotin increased the mycelium growth to 1.5, 1.4, and 1.3-times, respectively, compared with

the growth on the medium without vitamin B. Besides, thiamine hydrochloride, pyridoxine hydrochloride, biotin, and folic acid clearly increased the aerial mycelium index compared with the growth on medium without vitamin B. In particular, thiamine hydrochloride showed the aerial hyphal index of 3.0 (Fig. 3-2-1). Therefore, thiamine hydrochloride, pyridoxine hydrochloride, and biotin were deemed to be effective for further promoting both mycelium growth and the formation of the aerial mycelium of *M. majus*.

In addition, different amounts of the selected vitamin B were examined to determine optimal concentration (Table 3-2-5). The effect of pyridoxine hydrochloride on the mycelial growth differed with concentration, but thiamine hydrochloride and biotin showed very little change. Thiamine hydrochloride showed an extreme aerial hyphal index of 3.0 regardless of concentration and formed characteristic centroclinal colonies (Fig. 3-2-1). However, the mycelial growth was significantly lower ($p < 0.05$) in isolate MN-2T than the other two strains when 1000 mgl^{-1} of thiamine hydrochloride was used. Mean mycelium growth was highest with 500 mgl^{-1} of thiamine hydrochloride. Therefore, thiamine hydrochloride at 500 mgl^{-1} was selected as the vitamin B source. No concentration of any of the three vitamin B selected showed the formation of colored colonies.

Table 3-2-1 Effects of different carbon sources on the mycelial growth of three *Microdochium majus* isolates.

Carbon source	Mycelial growth (mm)							Aerial mycelium index[z]
	MN2T		MN406		MN451		Average	
Sucrose	57.6[x]	(±1.71) a[y]	61.9	(±4.90) a	72.6	(±0.49) a	64.0	0.7
Trehalose	50.4	(±1.99) a	45.2	(±1.85) b	50.0	(±5.41) b	48.5	1.0
Inositol	41.5	(±1.58) ab	53.2	(±2.83) ab	50.7	(±3.24) b	48.5	0.7
Melitose	41.7	(±1.73) ab	31.8	(±5.87) b	43.4	(±2.91) bc	39.0	1.0
Lactose	39.2	(±2.30) b	38.7	(±1.33) b	33.6	(±1.62) c	37.1	1.7
Maltose	35.7	(±0.56) b	32.6	(±2.84) b	36.0	(±1.25) c	34.7	0.7
Glucose	31.1	(±3.44) b	31.6	(±3.18) b	33.1	(±2.79) c	31.9	0.7
Fructose	21.7	(±0.39) c	12.0	(±1.04) c	13.4	(±0.44) d	15.7	0.3
Sorbitol	18.3	(±2.20) c	11.7	(±1.43) c	11.4	(±0.90) de	13.8	1.0
Garactose	12.7	(±0.49) c	12.8	(±1.89) c	10.2	(±0.34) de	11.9	1.7
Xylose	0.0	(±0.00) d	0.0	(±0.00) c	0.0	(±0.00) e	0.0	0.0

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($p < 0.05$)

[z] The levels of the amount of aerial mycelium

Table 3-2-2 Effects of different nitrogen sources on the mycelial growth of three *Microdochium majus* isolates.

Nitrogen source	Mycelial growth (mm)				Aerial mycelium index[z]
	MN2T	MN406	MN451	Average	
L-asparagine	53.9[x] (±0.60) a[y]	52.3 (±2.74) a	49.0 (±2.02) a	51.8	2.0
L-serine	59.2 (±1.63) a	43.1 (±1.59) a	42.6 (±0.57) a	48.3	1.7
Glycin	47.6 (±1.36) b	46.9 (±3.88) a	36.9 (±7.00) a	43.8	1.3
L-α-alanine	46.7 (±1.13) b	35.0 (±3.96) a	26.9 (±2.71) ab	36.2	1.3
L-histidine	41.0 (±1.11) b	23.7 (±2.87) b	22.0 (±0.91) b	31.9	2.0
Dl-aspartic acid	48.2 (±1.65) c	26.2 (±1.12) b	21.3 (±1.04) b	28.9	1.0
D-aspartic acid	38.2 (±0.61) c	24.9 (±7.58) bc	16.2 (±3.19) bc	26.4	1.3
L-glutamic acid (Na)	38.8 (±0.79) c	16.4 (±2.98) bc	16.7 (±2.12) bc	24.0	1.3
$NaNO_3$	26.7 (±1.30) d	16.0 (±1.95) bc	15.0 (±0.94) bc	19.2	1.0
Urea	12.5 (±0.34) e	6.5 (±0.77) c	7.0 (±2.33) c	8.7	0.7

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($p < 0.05$)

[z] The levels of the amount of aerial mycelium

Table 3-2-3 Effects of different combinations carbon and nitrogen sources on the mycelial growth of three *Microdochium majus* isolates.

Carbon source	Nitrogen source	Mycelial growth (mm)				Aerial mycelial index[z]
		MN2T	MN406	MN451	Average	
Lactose	Glycin	37.1[x] (±1.20) c[y]	50.9 (±0.77) b	44.3 (±3.59) d	44.1	2.3
	L-asparagine	47.7 (±3.05) bc	48.6 (±0.73) b	44.9 (±2.61) d	47.1	2.3
	L-hystidine	20.2 (±1.98) d	13.7 (±0.80) d	13.7 (±1.45) e	15.9	3.0
Trehalose	Glycin	63.9 (±1.16) a	52.1 (±5.10) b	53.8 (±1.75) bc	56.6	1.3
	L-asparagine	66.7 (±1.63) a	70.2 (±1.41) a	78.0 (±0.40) a	71.6	1.3
	L-hystidine	52.6 (±2.32) ab	26.8 (±0.92) c	35.6 (±0.57) d	38.3	1.7
Melitose	Glycin	57.6 (±2.72) ab	72.5 (±3.22) a	66.3 (±0.72) b	65.4	1.3
	L-asparagine	57.3 (±4.69) ab	71.2 (±1.00) a	72.8 (±0.64) ab	67.1	1.7
	L-hystidine	58.3 (±1.46) ab	64.8 (±0.74) a	59.1 (±1.53) bc	60.7	1.7

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($p < 0.05$)

[z] The levels of the amount of aerial mycelium

Table 3-2-4 Effects of different vitamin B on the mycelial growth of three *Microdochium majus* isolates.

Vitamine B	Hyphal growth (mm)				Aerial mycelial index[z]
	MN2T	MN406	MN451	Average	
Pyridoxine hydrochloride	63.2[x] (±0.73) a[y]	56.1 (±0.68) a	52.7 (±0.58) a	57.3	2.7
Thiamine hydrochloride	58.9 (±1.09) a	51.2 (±0.86) a	56.8 (±0.97) a	55.6	3.0
Biotin	58.1 (±1.51) a	48.5 (±1.64) a	42.8 (±2.25) b	49.8	2.3
Calcium pantothenate	48.9 (±1.15) b	46.1 (±1.63) ab	38.3 (±1.06) b	44.5	1.3
Riboflavin	45.7 (±2.86) b	42.2 (±1.08) b	37.6 (±0.75) b	41.8	1.7
Myo-inositol	37.6 (±2.35) bc	38.3 (±0.72) bc	37.4 (±1.58) b	37.8	1.3
Folic acid	40.1 (±0.11) bc	34.8 (±0.87) bc	32.0 (±1.10) bc	35.6	2.3
Nicotinic acid	33.0 (±1.31) c	31.4 (±0.48) c	30.1 (±2.27) bc	31.5	1.3
Control	39.4 (±0.56) c	36.8 (±1.45) c	38.0 (±1.77) c	38.1	1.6

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($p < 0.05$)

[z] The levels of the amount of aerial mycelium

Table 3-2-5 Effects of different vitamin B on the mycelial growth of three *Microdochium majus* isolates.

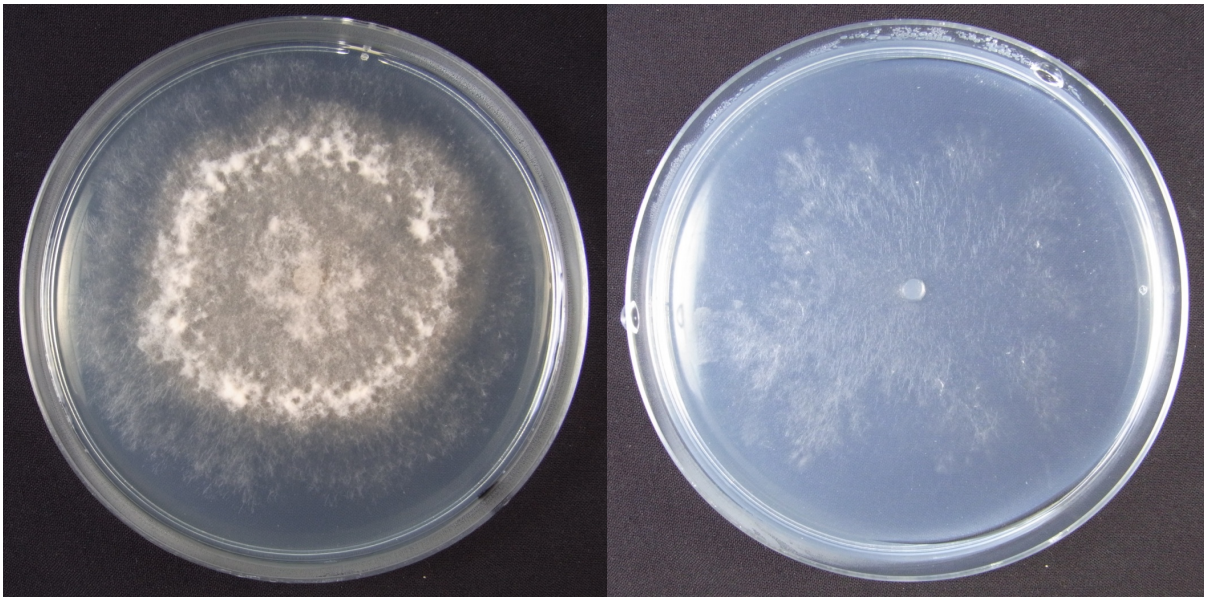
Vitamin B	Concentration (mg/L)	Mycelial growth (mm)				Aerial mycelial index[z]
		MN2T	MN406	MN451	Average	
Thiamine hydrochloride	100	61.2[x] (±0.29) a[y]	49.7 (±3.01) a	58.5 (±1.45) a	56.5	3.0
	500	57.7 (±1.01) a	57.8 (±2.46) a	61.1 (±0.20) a	58.9	3.0
	1000	52.8 (±0.77) b	46.7 (±1.67) ab	60.0 (±1.62) a	53.2	3.0
Pyridoxine hydrochloride	100	46.0 (±0.22) bc	37.9 (±2.30) b	38.1 (±1.52) b	40.7	1.3
	500	41.5 (±1.42) cd	33.2 (±1.49) b	30.9 (±1.51) bd	35.2	1.7
	1000	57.1 (±2.35) a	58.6 (±2.42) ac	63.5 (±1.67) a	59.7	2.0
Biotin	100	59.2 (±2.37) a	48.2 (±1.47) abd	44.4 (±1.42) b	50.6	1.3
	500	58.6 (±2.32) a	45.4 (±0.90) abd	44.8 (±1.96) b	49.6	1.0
	1000	54.3 (±1.25) a	48.3 (±0.73) abd	49.4 (±1.98) bc	50.7	1.3
Control		53.1 (±2.15) ad	48.6 (±1.07) abd	51.1 (±1.20) ab	51.0	1.3

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($p < 0.05$)

[z] The levels of the amount of aerial mycelium

Fig. 3-2-1 The comparison in the amount of aerial mycelium of *Microdochium majus* isolate MN2T on the BM medium (A) with thiamine hydrochloride (500 mgL^{-1}) and (B) without vitamin B



3-3. Investigation of the antimicrobial components

Materials & Methods

Fungal isolates

Three *M. majus* isolates, MN-2T, MN406 and MN451, used in this examination were the same with those used in session 3-2. Three isolates of *Fusarium* spp. used were TYK101-1 (*F. graminearum*), TW0414 (*F. asiaticum*) and TW0425 (*F. avenaceum*). All isolates of *M. majus* and *Fusarium* spp. used were obtained from Local Independent Administrative Agency, Hokkaido Research Organization, Tokachi Agricultural Experiment Station. Until use, all isolates were stored on slant culture containing 1.5% agar at 5°C in darkness.

Effect of thiophanate methyl on the growth of *M. nivale* and *Fusarium* spp.

The modified BM consisted of 2% lactose as the carbon source, 0.2% L-asparagine as the nitrogen source and 500 mgl^{-1} thiamine hydrochloride as vitamin B source. The medium was sterilized at 121°C for 20 min, and then 300 mgl^{-1} streptomycin sulfate was added.

Thiophanate methyl was added to the modified BM to inhibit the growth of *Fusarium* spp. A 5-mm diameter plug of potato dextrose agar culture was placed on oatmeal agar (OMA; oatmeal, 40 g; agar, 15 g; distilled water, 1 l) medium and incubated at 20°C for 10 days. Conidia were harvested and suspended in 10 ml of sterile distilled water containing 0.05% Tween 80 then filtered through two layers of cheesecloth. The final concentration was determined using a hemacytometer. Conidia suspensions of *M. majus* strains (MN2T, MN406 and MN451) and *Fusarium* spp. (TYK101-1, TW0425 and TW0414) were prepared and adjusted to 2×10^2 conidia/ml, respectively. Each suspension was plated on the modified BM

medium containing thiophanate methyl at 5 or 10 mgl^{-1} before sterilization. After incubation at 20°C for 10 days, the number and diameter of colonies (mm/day) of *M. majus* and *Fusarium* spp. were measured. Triplicate plates were inoculated per treatment for each isolate.

Effects of different ergosterol biosynthesis inhibitor (EBI) fungicides on the growth of *M. majus* and other filamentous fungi

Each ergosterol biosynthesis inhibitor (EBI) fungicides were added to the modified BM medium to inhibit the growth of other filamentous fungi. EBI fungicides used were imibenconazole, simeconazole, myclobutanil, miconazole nitrate, econazole nitrate, thiabendazole and spiroxamine. Conidia suspensions of *M. majus* (MN-2T) were prepared as described above and adjusted to 2×10^2 conidia/ml. The modified BM medium containing 0.5 ml of each EBI-fungicide was then plated on the conidia suspension. Debieu *et al.* (1999) suggested that the EC_{50} (50% reduction in the rate of the mycelial growth) of *M. nivale* against spiroxamine was 84 mgl^{-1}. Therefore, 80 mgl^{-1} of spiroxamine in this examination was added, while others were added at the concentration of 5 mgl^{-1}. After incubation at 20°C for 7 days, the number and diameter of colonies (mm/day) of *M. majus* were measured, and the effect of each EBI-fungicide on the mycelial growth of *M. majus* was assessed. Triplicate plates were inoculated per treatment for each isolate.

The modified BM medium containing each EBI-fungicide was placed in a wheat field in Memuro, Hokkaido to assess antimicrobial activity to other filamentous fungi. Spore trapping was performed from 17:00 to 9:00 on May 9, 2011. Each plate was placed on a device that was just above the level of the heads, about 80 cm, in the center of the field and was exposed all night. The plates were then recovered and incubated at 20°C for 10 days in the darkness.

The number of colony formations and diameter of observed colonies (mm/day) of other filamentous fungi was measured, and the effect of each EBI-fungicide was assessed. Triplicate plates were inoculated per treatment for each isolate.

Results

Effect of thiophanate methyl on the growth of *M. majus* and *Fusarium* spp.

The effects of thiophanate methyl on the mycelial growth (mm/day) and the colony formation of *M. majus* and *Fusarium* spp. were examined by inoculation of each conidia suspension (Table 3-3-1). In *M. majus*, there was little difference in the colony formation and the mycelial growth on the medium containing 5 and 10 mgl^{-1} thiophanate methyl. No concentration resulted in the formation of colored colonies. On the other hand, the colony formation of *F. graminearum* and *F. asiaticum* was completely inhibited on the medium even containing 5 mgl^{-1} thiophanate methyl. Although *F. avenaceum* could grow on the medium containing 5 mgl^{-1}, the colony formation was completely inhibited on the medium containing 10 mgl^{-1}. Consequently, addition of thiophanate methyl at a concentration of 10 mgl^{-1} inhibited the growth of *Fusarium* spp. alone, with no effect on the colony formation of *M. majus*.

Effects of different ergosterol biosynthesis inhibitor fungicides on the growth of *M. majus* and other filamentous fungi

The number of colony formations and diameter of observed colonies (mm/day) of *M. majus* (MN-2T) and other filamentous fungi were examined to assess antimicrobial activity to respective fungi (Table 3-3-2). Miconazole nitrate and econazole nitrate significantly inhibited ($p < 0.05$) the colony formation of *M. majus* in comparison to the control. The remaining fungicides, however, did not inhibit colony formation of *M. majus.* On the medium containing spiroxamine and simeconazol, the colony diameter (mm/day) of *M. majus* was larger than that of other filamentous fungi. However, the colony formation of other

filamentous fungi was most strongly inhibited by the addition of spiroxamine followed by econazole nitrate and miconazole nitrate. Furthermore, the addition of imibenconazole and spiroxamine caused the formation of characteristic pinkish colonies visible from the underside of the petri plate.

Table 3-3-1 Effects of thiophanate metyl on the colony formation and mycelial growth of *Microdochium majus* and three isolates of *Fusarium* spp.

Antimicrobial agent (mgl^{-1})		The number of colony formation						Mycelial growth (mm/day)					
		M. majus			FG[z]	Fa	FA	*M. majus*			FG	Fa	FA
		MN2T	MN406	MN451	TYK101-1	TW0414	TW0425	MN2T	MN406	MN451	TYK101-1	TW0414	TW0425
Thiophanate metyl	0	50[x] a[y] (±2.19)	41 a (±1.00)	46 a (±2.85)	44 b (±4.49)	33 b (±1.73)	49 c (±1.20)	2.6 a (±0.26)	3.2 a (±0.12)	3.1 a (±0.02)	5.5 a (±0.56)	5.1 a (±0.17)	4.9 a (±0.10)
	5	56 a (±3.25)	34 a (±0.67)	56 a (±3.51)	0 a (±0.00)	0 a (±0.00)	8 b (±0.88)	2.9 a (±0.28)	3.0 a (±0.07)	3.3 b (±0.06)	0.0 b (±0.00)	0.0 b (±0.00)	0.1 b (±0.02)
	10	56 a (±1.45)	44 a (±4.10)	60 a (±3.61)	0 a (±0.00)	0 a (±0.00)	0 a (±0.00)	3.4 a (±0.19)	3.3 a (±0.15)	3.4 b (±0.04)	0.0 b (±0.00)	0.0 b (±0.00)	0.0 b (±0.00)

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($p < 0.05$)

[z] Abbreviation: FG, *F. graminearum*; Fa, *F. asiaticum*; FA, *F. avenaceum*

Table 3-3-2 Effects of diffrent EBI fungicides on the colony formation and mycelial growth of *Microdochium majus* and other fungus.

Antimicrobial agent	The number of colony formation				Mycelial growth（mm/day）			
	MN-2T		other fungus		MN-2T		other fungus	
Imibenconazol	62[x]	(±3.67)c[y]	9	(±2.96)a	0.41	(±0.02)b	0.74	(±0.01)a
Simeconazol	41	(±3.79)b	34	(±3.84)b	2.64	(±0.09)e	1.47	(±0.16)b
Myclobutanil	65	(±7.64)c	30	(±2.65)b	0.43	(±0.02)b	0.83	(±0.09)ab
Miconazol nitrate	0	(±0.00)a	2	(±0.00)a	0.00	(±0.00)a	0.33	(±0.08)a
Econazol nitrate	0	(±0.00)a	1	(±1.33)a	0.00	(±0.00)a	0.17	(±0.17)a
Thiabendazol	44	(±3.48)bc	78	(±2.08)c	1.89	(±0.03)d	2.55	(±0.10)c
Spiroxamin[z]	52	(±4.81)bc	1	(±0.58)a	0.77	(±0.03)c	0.46	(±0.09)a
Control	39	(±1.67)b	89	(±8.41)c	2.50	(±0.02)e	1.83	(±0.25)b

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($p < 0.05$)

[z] The levels of the amount of aerial mycelium

3-4. Spore trapping using the developed medium

Materials & Methods

Spore trapping was performed using the developed medium, LATTS (initials of components) medium, in a field to confirm if the medium was able to isolate airborne spores of *M. majus*. The components of LATTS medium were K_2HPO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Fe-EDTA, 0.01 g; lactose, 20 g; L-asparagine, 2.0 g; thiamine hydrochloride, 500 mg; streptomycin sulfate, 300 mg; thiophanate methyl, 10 mg; spiroxamine, 80 mg; agar, 15 g; and distilled water, 1 l. Spore trapping with FG medium was also used to identify *F. graminearum*. The components of FG medium were as following: K_2HPO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Fe-EDTA, 0.01 g; D-(+)-xylose, 20 g; L-glutamic acid monosodium salt, 2.0 g; oxgall, 0.5 g; $Na_2B_4O_7 \cdot 10H_2O$, 1.0 g; chloramphenicol, 0.25 g; triazine (50%-wp), 1.0 g; PCNB (75%-wp), 0.2 g; agar, 15 g; pH was adjusted to 11.0 with NaOH. Spore trapping was performed 15 times in total in a wheat field in Memuro from 17:00 to 9:00 between June 4 and July 6, 2011. LATTS and FG medium put into 9 cm petri plate was placed on a device that was just set above the level of the heads, about 80 cm, in the center of the field. The plates were exposed all night, and then incubated at 20°C for 10 days in darkness. The number of colony of *M. majus* and *F. graminearum* on respective plates was then measured. Colonies were randomly transferred to fresh WA medium and incubated at 20°C for 14 days before identification by microscopic examination of conidia and conidiophore morphology.

Results

Airborne spores of *M. majus* or *F. graminearum* could be trapped using LATTS medium or FG medium, respectively (Fig. 3-4-1 and Fig. 3-4-2). The number of trapped spores varied depending on the date trapped. Each colony could be counted easily because colonies of *M. majus* were dominant and larger than those of other filamentous fungi on LATTS medium. On the other hand, when a large number of spores were trapped on LATTS medium, the colonies were much smaller (Fig. 3-4-3). In this case, the number of colonies could be counted easily because the little growth of other filamentous fungi was observed. In addition, *M. majus* isolates formed characteristic pinkish colonies, regardless of more or less of the contamination with other filamentous fungi. The characteristic pinkish colonies, therefore, facilitated the identification of other filamentous fungi.

There were differences in the dynamics of airborne spores between *M. majus* and *F. graminearum*. The number of spores of *M. majus* that were trapped in each day was much higher than that of *F. graminearum.* Airborne spores of *M. majus* were more trapped around on June 10, whereas those of *F. graminearum* were more trapped around on June 25.

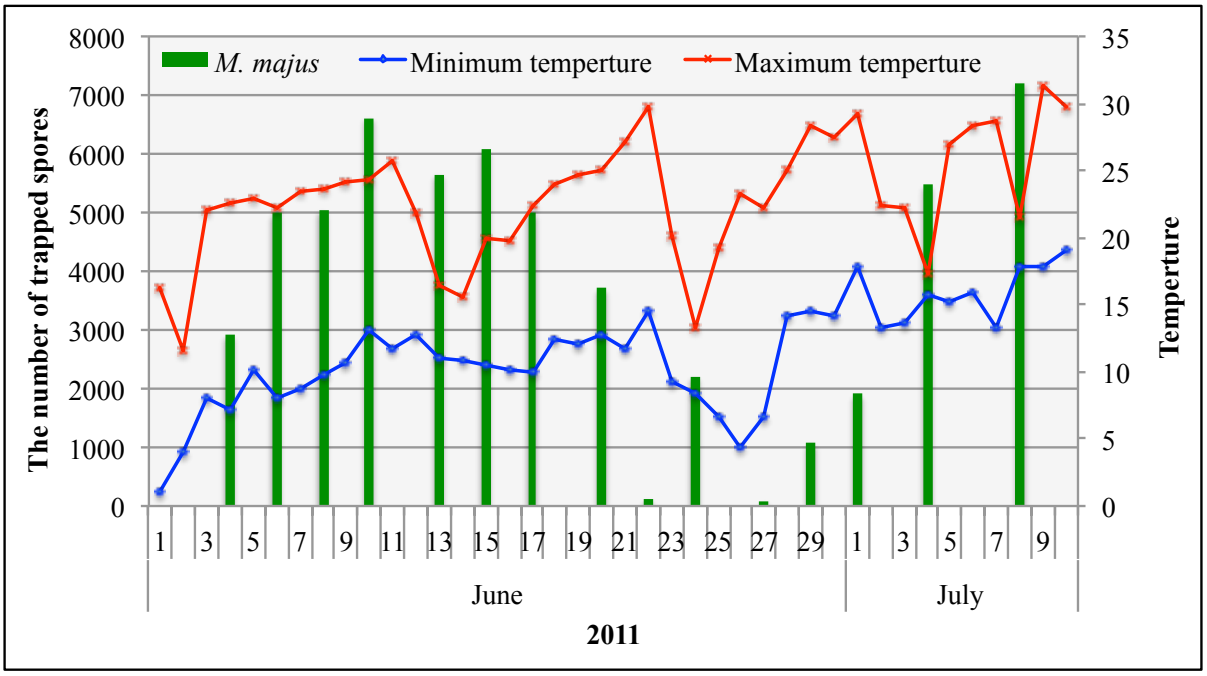


Fig. 3-4-1 The number of trapped airborne spores of *Microdochium majus* in a field using LATTS medium.

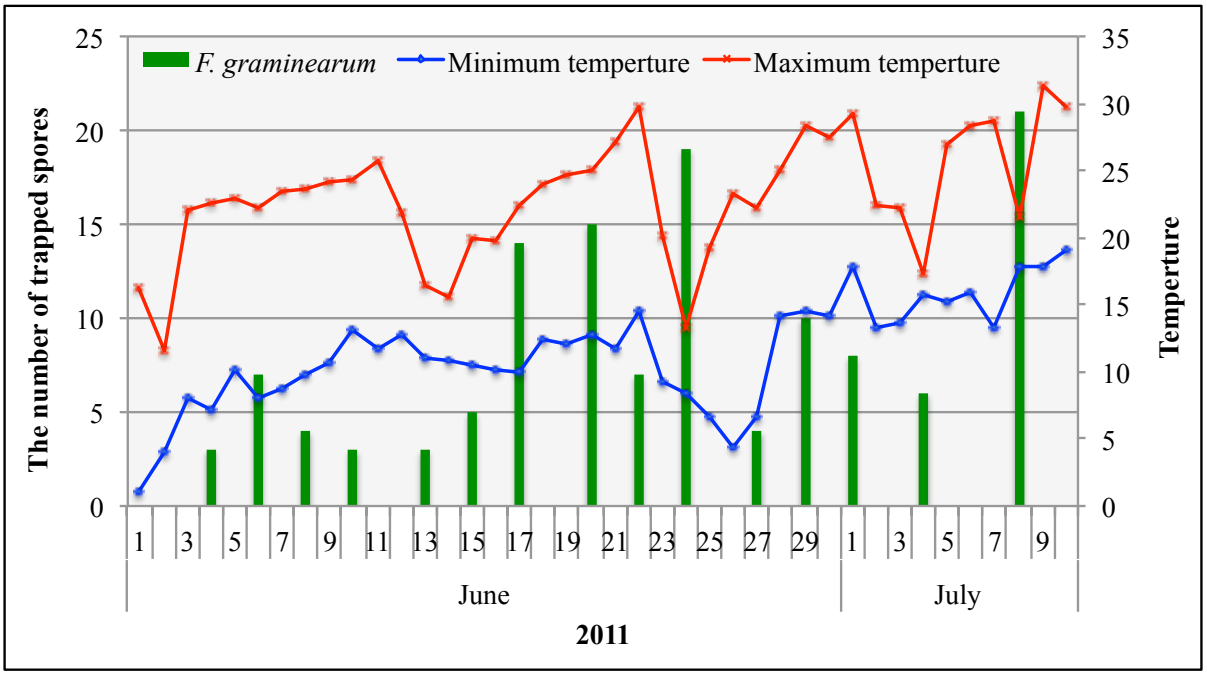


Fig. 3-4-2 The number of trapped airborne spores of *Fusarium graminearum* in a field using FG medium.

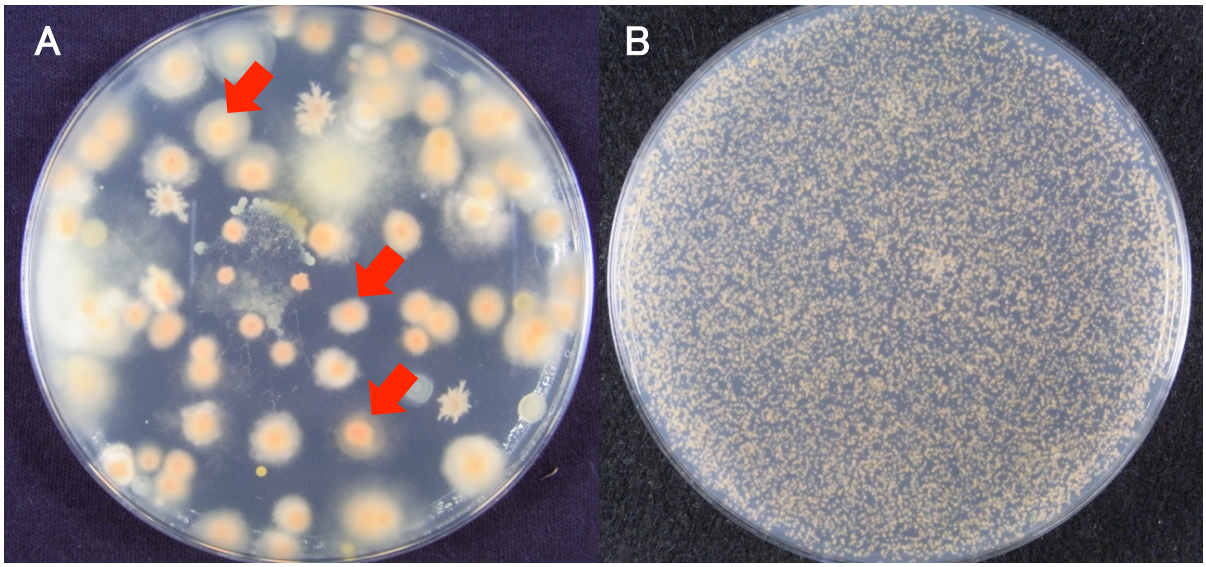


Fig. 3-4-3 Colonies of *Microdochium majus* observed on LATTS medium exposed in a field.

A) LATTS medium on which only a small number of spores were trapped.

B) LATTS medium on which abundant spores were trapped.

Both plates were incubated at 20°C for 10 days after exposure.

The red arrows indicate a part of colonies of *M. nivale*, showing characteristic pinkish color.

3-5. Classification of the two species based on the colony morphology

Materials & Methods

Fungal isolates

Three *M. majus* isolates, T34, T152 and K013, and three *M. nivale* isolates, T35, T99 and K014, were used in this examination. These were obtained from infected wheat seeds originating from geographically distant regions in Hokkaido, Japan, in 2011. Until use, all isolates were stored on slant culture containing 1.5% agar at 5°C in darkness.

Classification using LATTS medium

Each conidia suspension of *M. majus* and *M. nivale* was prepared as described above (session 3-3), and adjusted to 1.0×10^2 conidia/ml, respectively. Each 0.5 ml of single or mixed suspensions with species type was plated on LATTS medium, respectively. Triplicate plates were inoculated per treatment for each suspension. After incubation at 20°C for 7 and 12 day, the colony diameter (mm/day) and the colony morphology of the two species were assessed. Five colonies per a plate were picked up in each species and confirmed the species type using PCR amplification with species-specific primer pairs. DNA extraction and PCR amplification were performed as described in session 4-2, CHAPTER IV.

Results

Classification using LATTS medium

Conidia suspensions of respective species were plated on the LATTS medium to confirm whether both species were able to grow on the medium. After incubation for 7 days, *M. majus* formed characteristic centroclinal and deep pinkish colonies, whereas *M. nivale* formed flat and white colonies (Fig. 3-5-1). From the 12 days on, *M. nivale* formed light pinkish colonies. In the case of plating mixed suspensions with species type on the LATTS medium, the colony morphology of the both species was the same with in case of plating single suspension. Species classification on the medium agreed with that using PCR amplification with species-specific primer pairs.

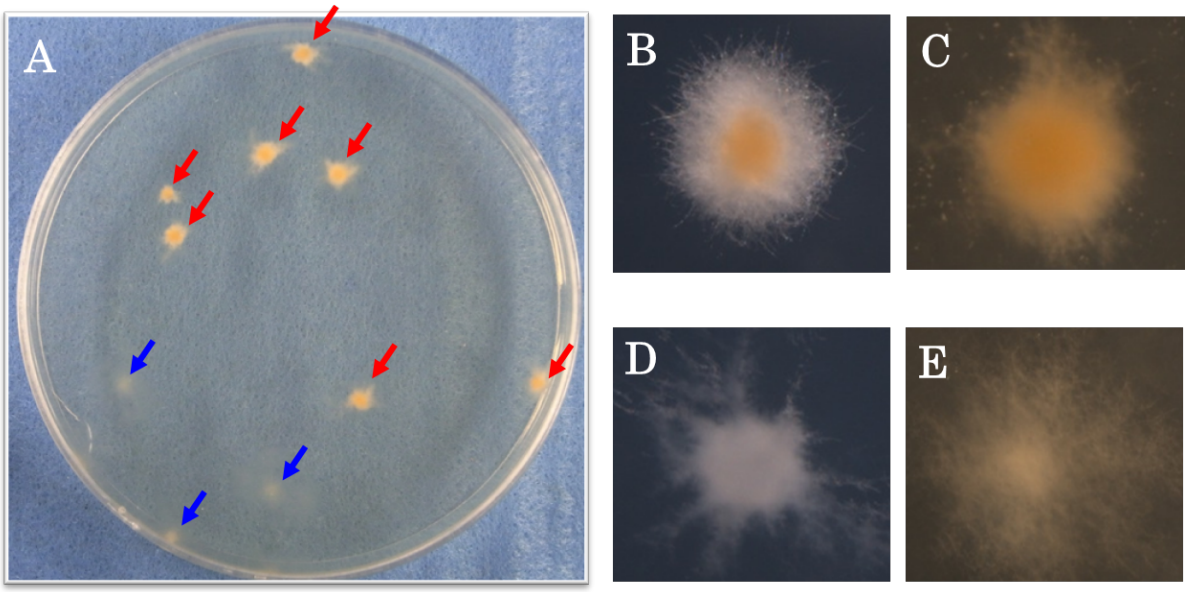


Fig. 3-5-1 Comparison in the colony morphology between *Microdochium nivale* and *Microdochium majus* observed on LATTS medium after incubation at 20 °C for 7 days.

A) LATTS medium mixed the two species types of *Microdochium* spp.

B and C) Colonies of *M. majus* observed by using stereomicroscope.

D and E) Colonies of *M. nivale* observed by using stereomicroscope.

The red arrows indicate colonies of *M. majus*, showing characteristic pinkish color.

The blue arrows indicate colonies of *M. nivale*, showing white color.

3-6. Discussion

This chapter demonstrated that LATTS medium, the selective medium developed in this study, was highly effective for selective detection of airborne spores of *Microdochium* spp. I had needed the selective medium of *Microdochium* spp. to obtain more information on its biology, ecology and epidemiology. FG medium is an excellent selective medium for isolation of *F. graminearum*, whereas it was not suitable for isolation of *Microdochium* spp. (Kozawa, 2006). This result was estimated to be due to the incompatibility of components of the FG medium, and we firstly investigated that carbon and nitrogen sources of the selective medium.

There is a significant effect ($p < 0.05$) of different carbon and nitrogen sources on the mycelial growth among three *M. majus* isolates. The mycelial growth of these isolates was prevented on the medium containing xylose as the carbon source, which suggested that the addition of xylose was one of the reasons why FG medium was not suitable for isolation of *Microdochium* spp. Furthermore, the kind of carbon and nitrogen sources to increase the aerial mycelium index did not necessarily correspond with those to increase the mycelial growth. This result supported that the components of the selective medium should be selected by evaluating both the aerial mycelium index and the mycelial growth.

The mycelial growth and the aerial mycelium formation of *Fusarium oxysporum* f. sp. *lactucae* race 2, causal agent of root rot in lettuce, were increased by the addition of biotin, a kind of vitamin B, to the medium (Yamauchi *et al.*, 2003). Therefore, the effect of eight vitamin B on the mycelial growth of *M. majus* was investigated. By the addition of thiamine hydrochloride, the aerial mycelium index showed an extreme of 3.0 and formed the

characteristic centroclinal colonies. This character of the colony morphology would be suitable to isolate easily in the case of contamination of other filamentous fungi.

Thiophanate methyl has been widely used as a fungicide for a treatment of FHB in Hokkaido. *Microdochiun* spp. isolates resistant to thiophanate methyl were identified in Hokkaido in 1981 (Tanaka *et al.*, 1983) and were thought to be widely distributed throughout the prefecture. On the other hand, *F. graminearum* isolates resistant to thiophanate-methyl were firstly identified in Oita prefecture (Yoshimatsu *et al.*, 2005) and Mie prefecture (Kuroda & Suzuki, 2009). These reports, however, showed that the frequency of the resistant strains was very low. So far, the rapid development of the resistant strains to thiophanate-methyl was never seen in each area.

It is difficult to identify the FHB pathogens, either *Microdochium* spp. or *Fusarium* spp., from the observation of the symptoms alone. Therefore, identification of two species using selective medium was needed. The addition of thiophanate methyl at a concentration of 10 mgl^{-1} inhibited the growth of *Fusarium* spp. alone, with no effect on the colony formation of *M. majus*. Also, the selective medium needed a high antimicrobial activity against other filamentous fungi. The addition of spiroxamine effectively inhibited the growth of other filamentous fungi with no effect on the colony formation of *M. majus*. Moreover, the characteristic pinkish color of the *M. majus* colonies made them easy to differentiate from those of other filamentous fungi. Spiroxamine was, therefore, selected as the antimicrobial component of the selective medium.

Spore trapping in a wheat field demonstrated the LATTS medium was highly effective for selective detection of airborne spores of *M. majus*. The formation of characteristic pinkish colonies of *M. majus* contributed to a high selectivity and made it possible to differentiate *M. majus* isolates from other filamentous fungi without using a microscope. Furthermore, the

LATTS medium could classify the two species, *M. majus* and *M. nivale*, based on the colony morphology. This unique characteristic might be useful for classification of the two species when investigating the frequency of *M. majus* and *M. nivale* isolates obtained from infected seeds or crop residues.

According to the results of the spore trapping, dynamics of airborne spores of *M. majus* seemed to be different from that of *F. graminearum*. Primary, the number of spores of *M. majus* that were trapped in each day was much higher than that of *F. graminearum.* Besides, the period of the spore dispersal of *M. majus* was earlier than that of *F. graminearum*. These differences might be caused by the different response to weather factors, such as air temperature, relative humidity, and rainfall.

Overall, the findings show that the LATTS medium developed in this study was highly effective for selective detection of airborne spores of *M. majus*. More information on biological, ecological and epidemiological studies of *M. majus* was expected to obtain using LATTS medium.

CHAPTER IV
ANALYSIS OF GENETIC POPULATION STRUCTURE

4-1. Introduction

Inter-simple sequence repeat (ISSR) is a PCR-based technique that involves the amplification of DNA sequences between simple sequence repeats (SSR) using anchored or non-anchored SSR homologous primers (Zietkiewicz *et al*., 1994). ISSR analysis does not require genome sequence information in advance and can detect a greater number of polymorphisms than those detected with RFLP or RAPD markers (Godwin *et al.*, 1997). ISSR is, therefore, an efficient tool for analyzing genetic diversity within closely related species (Yu *et al.*, 2008) and also for studying genetic populations of plant pathogenic fungi (Menzies *et al*., 2003; Chadha *et al*., 2007). The genetic diversity within or between pathogen populations will lead to an understanding of how the pathogen is likely to adapt or evolve with environmental change, such as exposure to abiotic stresses, fungicides, and plant resistance (McDonald *et al.*, 2002). Thus, understanding the genetic variation in populations of the causal agent of disease would be useful in the improvement of disease management systems or in helping to develop cultivars with resistance to disease (Stenglein & Balatti, 2006; Bayraktar *et al.*, 2008).

4-2. The distribution and frequency of the two species

Materials & Methods

Fungal isolates

The location and number of all 361 isolates used in this study are listed in Table 4-2-1 and shown on the map (Fig. 4-2-1). 172 isolates were obtained from infected wheat seeds originating from geographically distant regions (Abashiri, Iburi, Shiribeshi, Sorachi, and Tokachi) in Hokkaido in 2011. 165 isolates were obtained from symptoms of pink snow mold originating from the six regions (Abashiri, Iburi, Ishikari, Shiribeshi, Sorachi, and Tokachi). 12 isolates were obtained by trapping airborne spores with LATTS medium in Tokachi region in 2013. 12 isolates were also obtained in Abashiri region in 1991. Symptomatic seeds and leaves were collected from several wheat fields in each region, and then were plated on 1.5% WA medium containing 300 mg/l streptomycin sulphate. Airborne spores were trapped using LATTS medium, the selective medium for isolation of *M. majus* and *M. nivale* (Hayashi *et al.*, 2013), in a winter wheat field in Tokachi region between May and July. LATTS medium put into 9 cm petri plate was placed on a device, which was just above the level of the heads, about 80 cm, in the center of the field. The plates were exposed all night, and then incubated at 20°C for 10 days in darkness. All isolates were purified by conventional single spore isolation. Until use, all isolates were stored as spore suspensions containing 10% skim milk in 0.2 ml tube at -80 °C.

DNA extraction

Genomic DNA was extracted from 14-day-old cultures in potato dextrose broth (PDB, Difco) based on Saitoh's protocol (Saitoh *et al.*, 2006). Mycelium was harvested from PDB, and 500 µl lysis buffer (400 mM Tris-HCl, pH 8.0; 60 mM EDTA, pH 8.0; 150 mM NaCl; 1% SDS) was added to a 1.5-ml tube. Each tube was incubated at room temperature for 10 min, and then 150 µl of potassium acetate (pH 4.8) was added (60 ml 5 M potassium acetate plus 11.5 ml glacial acetic acid) and each tube was vortexed briefly. Each tube was centrifuged for 5 min at 15,000 rpm and then 300 µl of the supernatant was transferred to a new tube. 750 µl of ethanol was added and the tube was centrifuged for 3 min at 15,000 rpm. After decanting the supernatant, 300 µl of 70% ethanol was added and centrifuged for 5 min at 15,000 rpm. After decanting the supernatant, the precipitate was dried for 20 min and then resuspended in 50 µl TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0).

PCR amplification

(i) Singleplex PCR for identification of the two species

The two species of population isolated in 2011 and 2012 was identified by the singleplex PCR. The PCR reactions were performed in a total volume of 20 µl containing 10 × PCR buffer, 2.5 mM of dNTPs, 0.4 µM of each primer, 1.25 U of Taq DNA Polymerase (Takara, Japan), and 10-30 ng of genomic DNA. Primers used were those for *M. nivale* (Y13NF/R: CCAGCCGATTTGTGGTTATG/GGTCACGAGGCAGAGTTCG) (Nicholson *et al.*, 1996) and *M. majus* (Mnm2F/R: TGCAACGTGCCAGAAGCT/ AATCGGCGCTGTCTACTAAAAGC) (Nicholson & Parry, 1996). Amplification was performed in a Program Temp Control System PC-818 (ASTEC, Japan) with an initial denaturation step of 94°C for 2 min; followed by 32 amplification cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final step of 72°C for 3 min. Negative controls with

no target DNA were included to test for the presence of DNA contamination in the reagent and reaction mixtures. Aliquots (4 µl) of amplification products were electrophoresed on 2% agarose gels, and separated fragments were visualized under 300 nm UV light after staining with ethidium bromide.

(ii) Multiplex PCR for identification of the two species

The two species in population isolated in 1991 and 2013 was identified by the multiplex PCR. The PCR reactions were performed in a total volume of 10 µl containing each 0.2 µM primers for *M. nivale* (Y13NF/R) (Nicholson *et al.*, 1996) and for *M. majus* (Mnm2F/R) (Nicholson & Parry, 1996), 2 × GoTaq Green Master Mix (Promega, USA), and 10-30 ng of genomic DNA. Amplification was performed in a Program Temp Control System PC-320 (ASTEC, Japan) with an initial denaturation step of 94°C for 2 min; followed by 28 amplification cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 45 s, with a final step of 72°C for 2 min. Aliquots (4 µl) of amplification products were electrophoresed on 1.2% agarose gels, and then the separated fragments were visualized under 300 nm UV light after staining with ethidium bromide.

Results

Identification of the two species

Singleplex PCR with respective primers Y13NF/R or Mnm2F/R amplified a single band of approximately 300 or 750 bp, respectively (Fig. 4-2-2). Multiplex PCR with both primer pairs also showed the same pattern of bands produced by singleplex PCR (Fig. 4-2-3). These bands were the same size as expected by Nicholson & Parry (1996), respectively. Thus, the former was identified as *M. nivale*, and the latter was identified as *M. majus*.

Of the 361 isolates, 344 isolates were identified as *M. majus*, and 17 isolates was identified as *M. nivale*. The isolates of *M. majus* were widely detected in five regions, whereas the isolates of *M. nivale* were detected within only Tokachi and Abashiri populations.

Table 4-2-1 The number, location, origin and species in population of *Microdochium nivale* and *Microdochium majus* used in this examination.

Year	Population	Location	Origin	No. of isolates	Species	
					M. nivale	*M. majus*
1991	Abashiri	Unknown	Unkhown	12	10	2
2011	Tokachi	Asyoro, Makubetsu, Memuro, Obihiro, Shihoro, Shikaoi, Shintoku, Taiki, Toyokoro	Seed[a]	101	6	95
	Abashiri	Bihoro,Engaru, Ozora, Kunneppu, Memanbetsu, Saroma	Seed	33	1	32
	Sorachi	Fukagawa, Iwamizawa, Naganuma	Seed	19	0	19
	Iburi	Abira, Date	Seed	12	0	12
	Shiribeshi	Kyogoku	Seed	7	0	7
2012	Tokachi	Ikeda, Makubetsu, Memuro, Nakasatsunai, Obihiro, Otofuke, Sarabetsu, Shihoro, Shimizu	lesion[b]	55	0	55
	Abashiri	Bihoro, Okido, Kitami, Kiyosato, Syari, Tsubetsu,	lesion	29	0	29
	Ishikari	Abira, Chitose, Ebetsu, Kitahiroshima	lesion	24	0	24
	Sorachi	Iwamizawa, Naganuma, Nanporo, Shinshinotsu, Tobetsu,Tsukikage	lesion	23	0	23
	Kamikawa	Biei, Nayoro, Furano, Nakafurano, Shibetsu	lesion	22	0	22
	Shiribeshi	Makkari, Niseko, Kyogoku	lesion	12	0	12
2013	Tokachi	Obihiro	Air trap	12	0	12

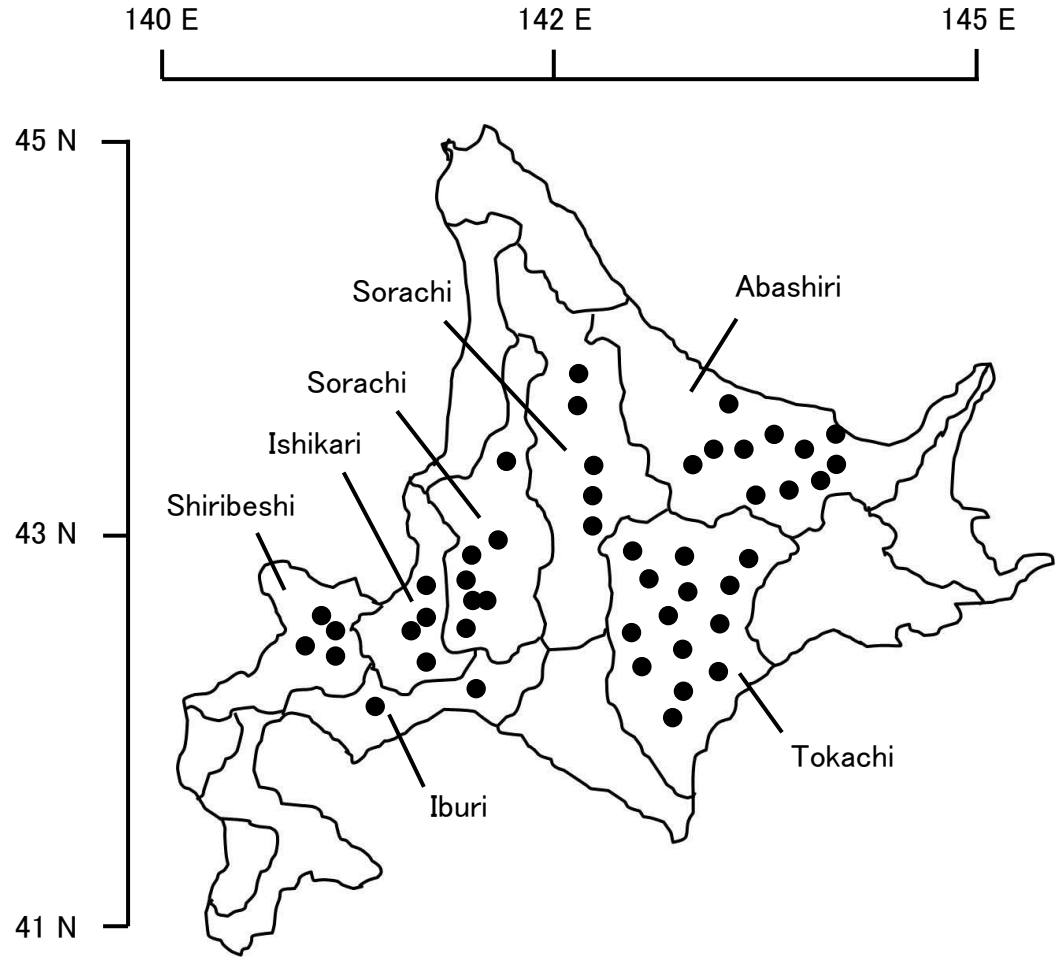


Fig. 4-2-1 Map showing geographical location of population of *Microdochium nivale* and *Microdochium majus* isolates used in this study.

The locations of isolates sampled from each population are represented by a black circle.

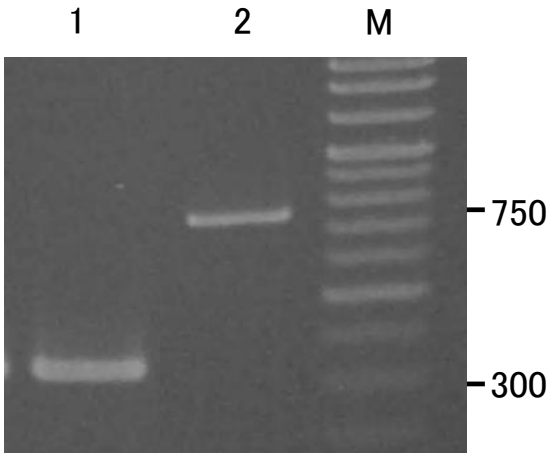


Fig. 4-2-2 Example of ethidium bromide-stained gel of fragments obtained by singleplex PCR with respective primer Y13NF/R and Mnm2F/R to classify *Microdochium* spp. into *M. majus and M. nivale.*

1: *Microdochium nivale*

2: *Microdochium majus*

M: 100 bp DNA ladder

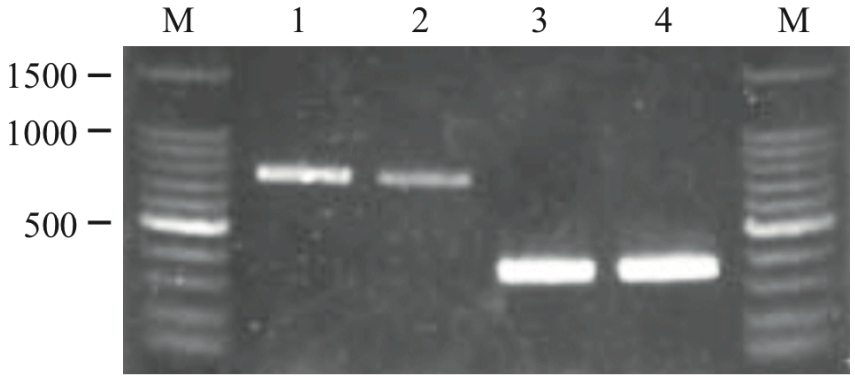


Fig. 4-2-3 Example of ethidium bromide-stained gel of fragments obtained by multiplex PCR with primer Y13NF/R and Mnm2F/R pairs to classify *Microdochium* spp. into *M. majus and M. nivale.*

Lane M, 100 bp DNA ladder

Lane 1, *Microdochium majus* T24

Lane 2, *Microdochium majus* TKSW002

Lane 3, *Microdochium nivale* T106

Lane 4, *Microdochium nivale* K014.

4-3. Analysis of genetic population structure

Materials & Methods

Fungal isolates

ISSR analysis was performed using only 172 isolates, which were obtained from infected wheat seeds originating from geographically distant regions (Abashiri, Iburi, Shiribeshi, Sorachi, and Tokachi) in Hokkaido in 2011. Detailed information was shown in Table 4-2-1.

Genetic diversity using ISSR markers

Extracted DNA was amplified using ISSR primers to assess the levels of genetic variation and structure in populations. Two primers, $(GTG)_6$ and $(ACA)_6$, that showed good repetition, special bands, and distinct polymorphisms, were chosen from 16 pre-screened primers, which are $(GAT)_6$, $(TGTC)_4$, $(GTG)_6$, $(GAA)_6$, $(CA)_8CT$, $(CT)_8$, $(CA)_8GT$, $(GCC)_6$, $(ACA)_6$, $(TG)_8RC$, $(GACA)_4$, $HVH(TG)_6$, $(AC)_8YC$, $(CATA)_4$, $(TGTC)_4$, and $(AG)_8YC$. The PCR reactions were performed in a total volume of 20 µl containing 10 × PCR buffer, 2.5 mM of dNTPs, 0.4 µM of each primer, 1.25 U of ExTaq DNA Polymerase (Takara, Japan), and 10-30 ng of genomic DNA. Amplification was performed in a Program Temp Control System PC-818 with an initial denaturation step of 94°C for 2 min; followed by 35 amplification cycles of 94°C for 30 s, 55°C for 30 s for $(ACA)_6$ or 60°C for 30 s for $(GTG)_5$, and 72°C for 45 s; with a final extension at 72°C for 5 min. Negative controls with no target DNA were included to test for the presence of DNA contamination of the reagent and reaction mixtures. Aliquots (4 µl) of amplification products were electrophoresed through 2%

agarose gels, and separated fragments were visualized under 300 nm UV light after staining with ethidium bromide.

Data analysis

Because ISSR markers are dominant, I assumed that each band represented the phenotype at a single biallelic locus. The bands that were repeatable and clearly visible with high intensity generated by ISSR primers were scored manually for the presence (1) or absence (0) of bands in each isolate. These data were analysed using the POPGENE software version 1.31 (Yeh *et al.*, 1999) to estimate genetic diversity and the following population structure parameters: the percentage of polymorphic bands (PPB), effective number of alleles per locus (A_E) and average gene diversity (H_E). The coefficient of gene differentiation (G_{ST}; McDermott & McDonald, 1993) was measured using Nei's method (Nei, 1973). The level of gene flow (Nm) was estimated as 0.5 $(1-G_{ST})(G_{ST})$-1 (McDermott & McDonald, 1993). An analysis of molecular variance (AMOVA) was also used to partition the total phenotypic variance within and among populations using the software Arlequin version 3.5 (Excoffier & Lischer, 2010). To visualize the genetic relationships among populations, a phylogenetic tree was also constructed by the neighbour-joining (NJ) method based on genetic distance using the MEGA software ver. 6.0 (Tamura *et al.*, 2013).

Results

Genetic diversity

Two ISSR primers selected by pre-screening were used to investigate genetic diversity and population structure within 165 isolates of *M. majus* originating from different regional origins. The ISSR primers produced a total of 22 replicated bands, of which 20 were polymorphic (Table 4-3-1). The size of amplified fragments ranged from 400 to 2000 bp (Fig. 4-3-1). All 172 isolates in the five populations were identified as 14 ISSR haplotypes, named as H1 to H14 (Table 4-3-2). The predominant haplotype in all populations was H2, representing 59.3% of the total isolates.

The percentage of polymorphic bands (PPB) was 27.3%, whereas that of a single population ranged from 0% (Shiribeshi) to 15.0% (Tokachi and Sorachi), with an average of 10.0% (Table 4-3-3). Thus, PPB showed relatively high uniformity among populations. The average effective number of alleles per locus (A_E) and the average gene diversity (H_E) of the Tokachi population were relatively high compared with those of other populations. Pairwise comparisons of Nei's genetic identity and genetic distance in five populations were uniformly high (ranging from 0.9924 to 0.9994) and uniformly low (0.0006 to 0.0125), respectively (Table 4-3-4).

Population structure

The total gene diversity within the five populations of *M. majus* was 0.023 and the coefficient of gene differentiation (G_{ST}) was 0.13. This value indicates that 13% of the observed genetic variation was due to gene differentiation among regional populations (Table 4-3-3). The average number of individuals exchanged among populations per generation (Nm) was 3.334.

The AMOVA for regional populations showed that there were 0.05 variance components among populations and 0.32 within populations; 13.26% of variance occurred among populations and 86.74% occurred among individuals within populations (Table 4-3-5). No significant ($P < 0.05$) genetic differentiation was observed among the five populations.

Dendrogram analysis

The dendrogram was constructed by the UPGMA from Nei's genetic distance values based on the ISSR data (Fig. 4-3-2). All haplotypes formed two clades that belonged to either *M. majus* or *M. nivale*. No significant correlation was found between geographic distribution and genetic distance on the UPGMA tree.

Table 4-3-1 ISSR primers used in this study.

Primer	No. of amplified bands	No. of polymorphic bands	PPB (%)[a]
$(ACA)_6$	13	12	92.3
$(GTG)_5$	9	8	88.9

[a] Percentage of polymorphic bands

Table 4-3-2 Haplotype identified within five populations of *Microdochium nivale* and *Microdochium majus* based on ISSR fingerprinting.

Haplotype		Population					Species	binary code
		Tokachi (101)	Abashiri (33)	Sorachi (19)	iburi (12)	Shiribeshi (7)		
H1	(12)	12	0	0	0	0	*M. majus*	01011010101010100000
H2	(102)	44	28	14	9	7	*M. majus*	01011010101010001000
H3	(41)	33	3	3	2	0	*M. majus*	01011010101010101000
H4	(1)	1	0	0	0	0	*M. nivale*	10100000000111001011
H5	(5)	4	0	0	1	0	*M. majus*	01011010101010000000
H6	(1)	1	0	0	0	0	*M. nivale*	10011000000111010111
H7	(2)	2	0	0	0	0	*M. majus*	01011011101010001000
H8	(1)	1	0	0	0	0	*M. nivale*	00111000010111011111
H9	(2)	1	1	0	0	0	*M. nivale*	00100110010111011111
H10	(1)	1	0	0	0	0	*M. nivale*	10011000000111011111
H11	(1)	1	0	0	0	0	*M. nivale*	00100010100111010111
H12	(1)	0	0	1	0	0	*M. majus*	01011010001010001000
H13	(1)	0	0	1	0	0	*M. majus*	01001010101010001000
H14	(1)	0	1	0	0	0	*M. majus*	01010010101010101000

The number in parentheses indicates the number of isolates.

Table 4-3-3 Genetic diversity of 165 isolates in five populations of *Microdochium majus* based on ISSR fingerprinting.

Population	No. of isolates	PPB (%)[a]	A_E[b]	H_E[c]	H_T[d]	H_S[e]	G_{ST}[f]	Nm[g]
Tokachi	95	15.0	1.071	0.041				
Abashiri	32	10.0	1.017	0.014				
Sorachi	19	15.0	1.029	0.023				
Iburi	12	10.0	1.028	0.022				
Shiribeshi	7	0.0	1.000	0.000				
Species level	-	27.3	1.054	0.034	0.020	0.023	0.130	3.334

[a] Percentage of polymorphic bands

[b] Effective number of alleles per locus

[c] Nei's gene diversity

[d] Total gene diversity of populations

[e] Average gene diversity within populations

[f] Coefficient of gene differentiation

[g] Level of gene flow

Table 4-3-4 Pairwise calculations of Nei's genetic identity (above diagonal) and genetic distance (below diagonal) of 165 isolates in *Microdochium majus* population based on ISSR fingerprinting.

Populations	Tokachi	Abashiri	Sorachi	Iburi	Shiribeshi
Tokachi	****	0.9924	0.9932	0.9945	0.9876
Abashiri	0.0076	****	0.9993	0.9989	0.9988
Sorachi	0.0069	0.0007	****	0.9994	0.9985
Iburi	0.0055	0.0011	0.0006	****	0.9983
Shiribeshi	0.0125	0.0012	0.0015	0.0017	****

Table 4-3-5 Analysis of molecular variance (AMOVA) of 165 isolates in five populations of *Microdochium majus* based on ISSR fingerprinting.

Source of variation	d.f.[a]	MSD[b]	Variance component	Total variance (%)	P-value[b]
Among populations	4	1.53	0.05	13.26	< 0.001
Within populations	160	0.32	0.32	86.74	< 0.001

[a] Degree of freedom

[b] Mean squared deviation

[c] Probabilities were calculated by 1023 random permutations of individuals across population

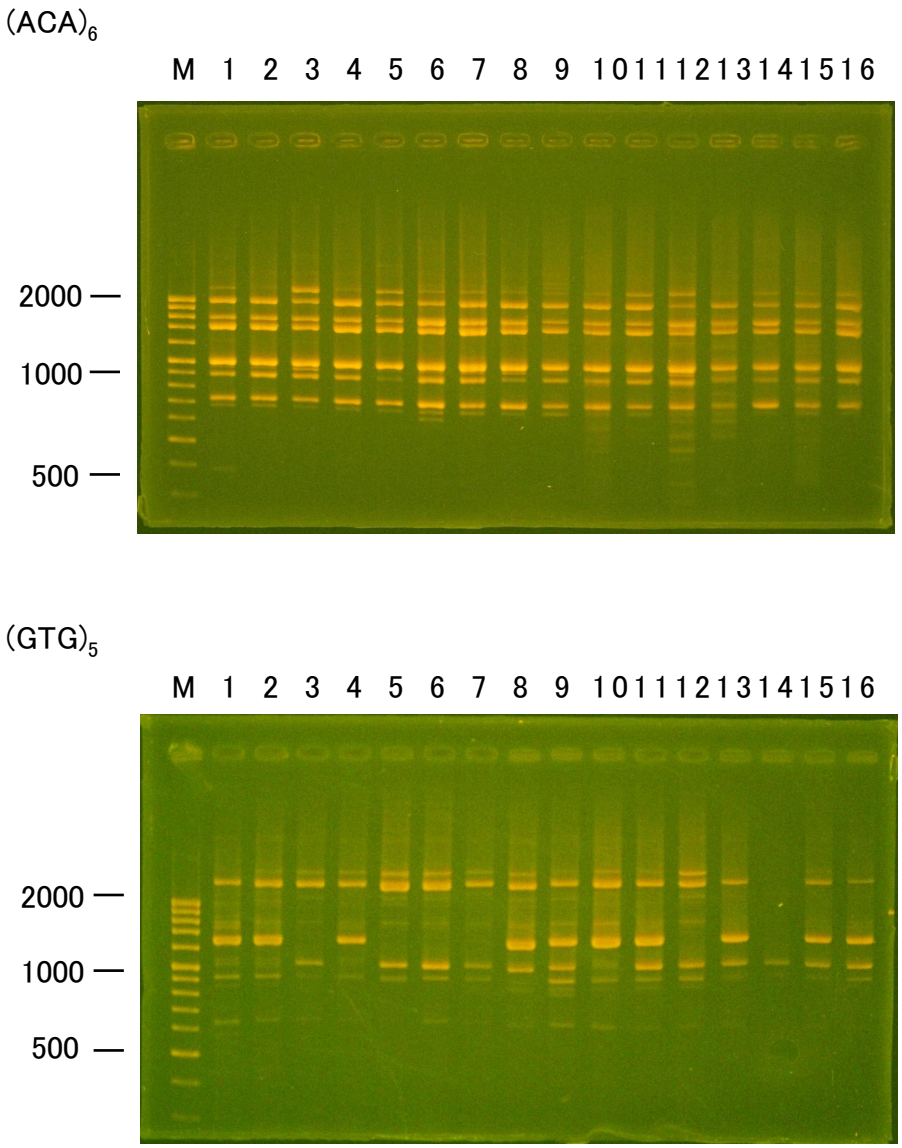


Fig. 4-3-1 Example of PCR amplification with two ISSR primers, $(ACA)_6$ and $(GTG)_5$.

M: 100 bp DNA ladder

1–16: *Microdochium majus* isolates

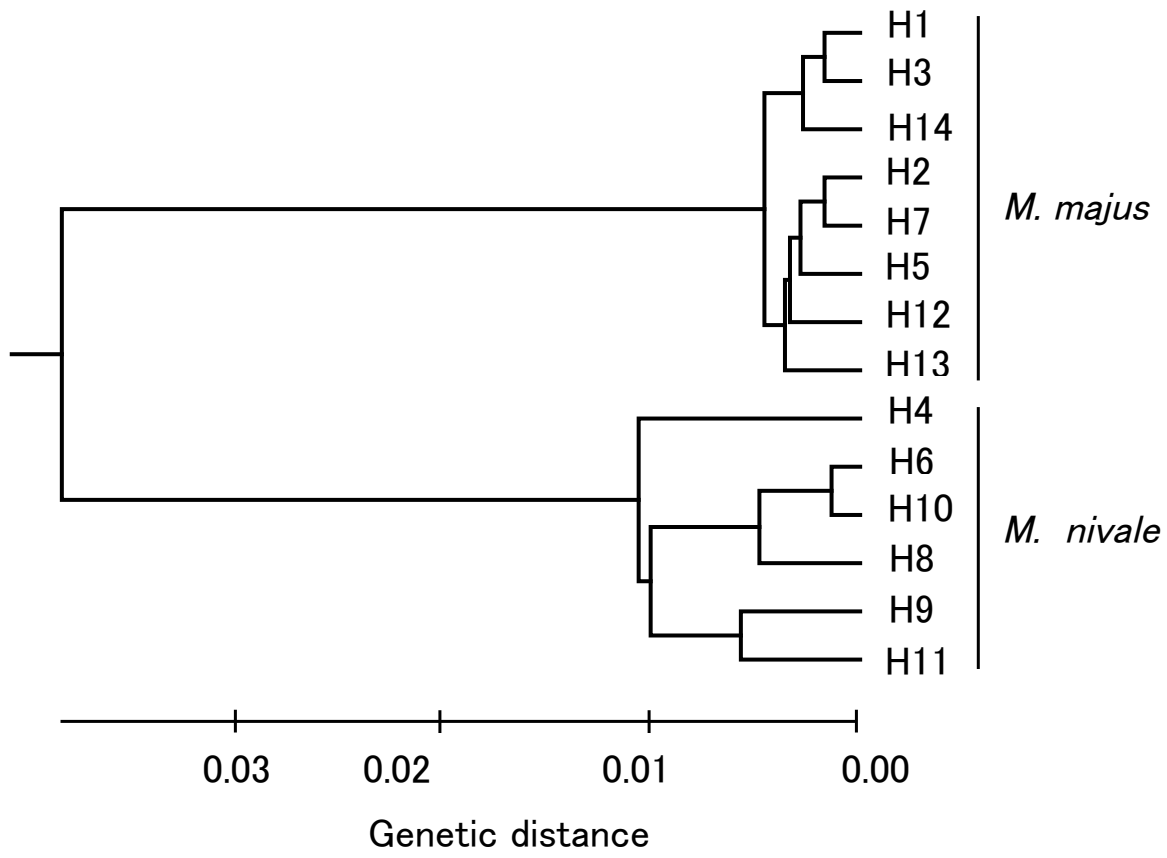


Fig. 4-3-2 Dendrogram of 14 haplotypes identified from 172 isolates of *Microdochium nivale* and *Microdochium majus* based on UPGMA cluster analysis based on ISSR data.

4-4. Discussion

This is the first report on the ISSR analysis of *Microdochium* species and investigations of genetic diversity and population structure. ISSR markers are capable of the detection of a higher number of polymorphisms than are other molecular markers, such as RFLP or RAPD (Godwin *et al.*, 1997). High-performance results with ISSR markers have been reported by several studies on population genetics. For example, ISSR analysis showed high intraspecific variability within the population of *Fusarium poae*, causing FHB from infected grains (Dinolfo *et al.*, 2010). Therefore in this study, ISSR analysis was performed to investigate genetic diversity and population structure within 172 isolates of *Microdochium* species in winter wheat from five different geographical origins in Hokkaido.

This study demonstrated that multiplex PCR was useful to identify the two species in *Microdochium* spp. effectively. All 361 isolates were identified as either of two species with specific primer pairs, Y13NF/R and Mnm2F/R. The majority (95.3%) of isolates were identified as *M. majus* and each population but the population in 1991 showed a marked predominance of *M. majus*. It was also reported in some studies that *M. nivale* var. *majus* (currently *M. majus*) was much more abundant than var. *nivale* (currently *M. nivale* sensu strict) in both grains and stem bases of winter wheat (Lees *et al.*, 1995; Parry *et al.*, 1993; Nielsen *et al.*, 2013). In contrast, Terami & Kamikawa (2006) previously reported that 94.3% of the isolates obtained from pink snow mold of winter wheat in Hokkaido were classified as *M. nivale*. This opposite result might indicate a correlation between the origin of host tissues and the frequency of species isolated under the environmental or ecological conditions in Hokkaido. However, this study indicated that *M. majus* was the dominant species regardless of origins of host tissues. This finding, therefore, suggested that the frequency of the species

isolated may have been affected by other factors as well as the origin of host tissue. For example, weather conditions, competition for nutrients and space, and fungicide treatments might have affected it.

Genetic diversity in all populations of *M. majus* showed few differences and a high level of genetic uniformity among populations. The overall diversity within and among the five populations of *M. majus* in Hokkaido was assessed using Nei's gene diversity index and AMOVA. The coefficient of gene differentiation (G_{ST}), based on Nei's gene diversity index, was very low. In addition, AMOVA showed that most molecular variations were found within rather than among the populations, indicating little genetic differentiation among the five populations. A high occurrence of polymorphisms within isolates of *M. nivale* than *M. majus* (described as *M. nivale* var. *nivale* and var. *majus*, respectively) has been reported (Lees *et al.*, 1995) and all isolates originating from turfgrass were shown to be *M. nivale* with very high levels of genetic diversity (Mahuku *et al.*, 1998). Further surveys for genetic diversity in *M. nivale* as well as *M. majus* in Hokkaido would clarify the genetic structure of *Microdochium* species.

Some studies have reported that seed dispersal was a primary factor influencing variation in gene flow and population structure (Kalisz *et al.*, 1999). Besides seed dispersal, I hypothesized that the high level of gene flow observed in this study might be responsible for the dispersal ability of airborne spores of the fungi to distant areas. Little is known about how far these spores can be dispersed in air. Moreover, this dispersal ability might have contributed to the rapid development of thiophanate methyl-resistant strains of *Microdochium* species in Hokkaido in which the fungicide has been used widely for the control of FHB complex. These resistant strains were first reported in Hokkaido in 1981 (Tanaka *et al.*, 1983) and have been spreading rapidly throughout the prefecture. Therefore, further research on the

dispersal ability of airborne spores will be needed in the future. In addition, the emergence and spread of fungicide resistance including strobilurins in *Microdochium* species should also be examined through more detailed population structure studies within and among populations of *Microdochium* species in different geographical origins.

Based on the ISSR data, there is a distinct resolution between *M. majus* and *M. nivale* as in the previous reports (Glynn *et al.*, 2005; Jewell & Hsiang, 2013), and each of all 172 isolates from the five populations was identified as one of eight ISSR haplotypes of *M. majus* or six ISSR haplotypes of *M. nivale*. The Tokachi population showed the highest haplotype diversity, followed by Sorachi and Abashiri. According to the dendrogram, there was no correlation between the clustering of isolates and the geographic distribution on the tree. These findings confirmed previous reports that ISSR was an efficient tool for analyzing genetic diversity within closely related species (Yu *et al.*, 2008), and that it provides a rapid and easy method for DNA fingerprinting (Bornet & Branchard, 2001).

In conclusion, this study demonstrated that ISSR was an efficient tool for genetic diversity and population structure studies of *M. nivale* and *M. majus*. The high level of genetic uniformity among *M. majus* populations and the higher gene flow showed that migration was likely playing an important role in the population biology of *M. majus*, providing some support for the prediction of epidemics of fungicide resistant strains within populations of FHB pathogens.

CHAPTER V
ANALYSIS OF STROBILURINS-RESISTANT STRAINS

5-1. Introduction

There are some reports on the emergence of resistant strains to several fungicides of *Microdochium* spp. worldwide (See session 2-3-1 in CHAPTER II). For example, resistant isolates to thiophanate-methyl (Tanaka *et al*., 1983), dicarboximide (Pennucci *et al*., 1990), and strobilurins (Walker *et al.*, 2009) have been ever identified. Recently, a decrease in the effectiveness of strobilurins fungicides against *Microdochium* spp. was also reported in Hokkaido (Kozawa *et al.*, 2011). In Japan, strobilurins have been usually used for the control of FHB because they are highly effective against *Microdochium* spp. (Ioos *et al.*, 2005; Mullenborn *et al.*, 2008). It has been, however, already reported that several plant pathogenic fungi showed the less effectiveness of strobilurins (Sierotzki *et al.*, 2000; Steinfeld *et al.*, 2001; Fraaije *et al.*, 2005; Chen *et al.*, 2007; Leroux *et al.*, 2007). It has been also reported that amino acid mutations involved in strobilurins resistance at the quinol oxidation site of cytochrome *b* (*CYTB*) encoded by the mitochondrial gene (See session 2-3-2 in CHAPTER II).

In CHAPTER IV, I have reported that the majority of isolates (95.9%) in all populations were identified as *M. majus.* This result indicated that *M. majus* isolates were the primary FHB pathogen than *M. nivale* isolates. Furthermore, ISSR analysis showed the high level of genetic uniformity and gene flow in *M. majus* populations. The high level of gene flow might have contributed to the rapid development of thiophanate methyl-resistant strains of *Microdochium* species in Hokkaido. Knowledge about the emergence and the spread of fungicide resistant strains will lead to an understanding of how the pathogen has adapted to fungicide selection. Moreover, detailed information about fungicide resistance may reduce the risk of the spread of FHB and improve disease management strategy.

It is important to understand the fitness of fungicide-resistant strains because the effectiveness of disease control depends on the frequency of resistant strains. If resistant strains carry fitness costs, the frequency of them will decrease in the absence of the fungicide. Fitness studies have been usually performed using laboratory-induced mutants or field-resistant strains, and the fitness between the resistant and sensitive strains has been compared. Fitness has been often assessed by measuring fundamental traits, such as mycelial growth, sporulation and conidial germination, in the life cycle of organisms. Markoglou *et al.* (2006) reported that pyraclostrobin-resistant strains carried fitness costs in sporulation, conidial germination, and sclerotia production, and were less competitive than the sensitive strain in the absence of the fungicide.

The purpose of this study, therefore, were (i) to investigate the frequency of strobilurins-resistant strains, (ii) to analyze genetic structures based on ISSR fingerprinting and *CYTB* sequences, and (iii) to compare the fitness between the strobilurins-resistant and sensitive strains of *M. majus* and *M. nivale* originating from geographically distant regions in Hokkaido.

5-2. Diagnosis of the resistant strains

Materials & Method

Fungal isolates

The location and number of all 84 isolates used in this study are listed in Table 5-2-1. 60 isolates were obtained from infected wheat seeds collected from Tokachi and Abashiri in northern Japan in 2011. 12 isolates were obtained by trapping airborne spores in Tokachi region in 2013. Furthermore, 12 isolates were obtained in Abashiri region in 1991. Procedures for the isolation and preservation of each sample are the same as the session 4-2 in CHAPTER IV.

Sensitivity test

33 isolates consisted of isolates of 17 *M. majus* and 16 *M. nivale* were randomly chosen from all isolates, and used to assess the level of strobilurins sensitivity (Table 5-2-2). The level of strobilurins sensitivity was assessed on potato dextrose agar (PDA) medium containing some concentrations of kresoxim-methyl. Concentrations of kresoxim-methyl (0.001, 0.01, 0.1, 1.0, and 100 mgl^{-1}) were adjusted to determine minimal inhibitory concentration (MIC) values. 1.0 mM *n*-propyl gallate was supplemented to inhibit the alternative oxidase (AOX) pathway which is partially involved in strobilurins resistance. The plates were inoculated with 5 mm mycelium plugs, cut from the margin of a five-day-old colony on 1.5% WA medium. Effects of the fungicide on mycelial growth were assessed by measuring the diameter of fungal colonies after incubation at 20°C for 7 days in darkness. Three replicates were used per treatment. Judging from the above results, 1 mgl^{-1} kresoxim-methyl was used to assess the

sensitivity of all isolates. The sensitivity test of all isolates was performed as well as given above.

PCR amplification of *CYTB* gene

Genomic DNA was extracted using Cica Geneus DNA Extraction Reagent ST (Cica, Japan). The PCR fragments including codons 129, 137, and 143 in the *CYTB* genes were amplified with a *Microdochium*-specific primer N2/N4 pair (GTACCTATTGTAGGTAAAGA/GGGTAGCTGAAATGCTGCTT) (Walker *et al.*, 2009). PCR reactions were performed in a total volume of 30 µl containing 5 × PrimeSTAR Buffer (Mg^{2+} plus), 2.5 mM dNTPs, 0.4 µM of the each primer, 1.25 U PrimeSTAR HS DNA Polymerase (Takara, Japan), and 10-30 ng of genomic DNA. Amplification was performed by a Program Temp Control System PC-320 (ASTEC, Japan) with an initial denaturation step of 94°C for 1 min; followed by 35 amplification cycles of 98°C for 10 s, 58°C for 5 s and 72°C for 60 s.

PCR-RFLP was performed to detect the strobilurins resistant A143 alleles of all isolates. PCR products with the primer N2/N4 pair were digested with 1U *Fnu*4HI (isoschizomer; *Sat*I) for 2 h at 37°C according to the manufacturer's recommendations. Aliquots (4 µl) of the digested products were electrophoresed through 1.2% agarose gels, and then separated fragments were visualized under 300 nm UV light after staining with ethidium bromide.

Results

Sensitivity test

The sensitivity test divided 33 isolates into three groups (sensitive, Str S; moderate-resistant, Str r; and high-resistant, Str R), which showed the different level of strobilurins resistances (Table 5-2-1). Strobilurins Str S group (MIC < 0.01 mgl^{-1}) consisted of 22 isolates including 12 isolates obtained in 1991, strobilurins Str r (0.01 mgl^{-1} < MIC < 1 mgl^{-1}) is isolate K015 alone, and Str R group (MIC > 1 mgl^{-1}) includes 10 isolates.

PCR amplification of *CYTB* gene

The PCR amplifications with the N2 and N4 primer pair produced approximately 630 bp fragments, and then the fragments were aligned with 522 bases (Fig. 5-2-1 A), and a part of these alignments was shown in Fig. 5-2-2. All resistant strains but *M. majus* isolate T35 had the single nucleotide substitutions from GGA or GGT to GCA at codon 143, which lead to the G143A mutation. The F129L and G137R mutation were not observed in any of the sequences. Although the isolate T35 did not have the G143A mutation, it showed high strobilurins resistance in the sensitivity test (Table 5-2-1). The resistant strains with the G143A mutation were also detected by PCR-RFLP because *Fnu*4HI recognized 5'-GC|NGC-3' sequence generated by the mutation. Digestion of the N2-N4 fragment with *Fnu*4HI produced a two-bands pattern (Fig. 5-2-1 B), which was the same as expected by Walker *et al.* (2009). This digestion pattern was observed in all resistant strains but isolate T35.

Table 5-2-1 Location, Year, Origin, fungicide phenotype and number of isolates of *Microdochium nivale* and *M. majus* used.

Location	Year	Origin	QoI phenotype					
			Str S[a]		Str r[b]		Str R[c]	
			M. majus	*M. nivale*	*M. majus*	*M. nivale*	*M. majus*	*M. nivale*
Tokachi	2011	grain	13	3	0	0	14	2
	2013	air trap	6	0	0	0	6	0
Abashiri	1991	unknown	2	10	0	0	0	0
	2011	grain	4	1	1	0	22	0

[a] Str S, sensitive to strobilurins.

[b] Str r, moderate-resistant to strobilurins.

[c] Str R, high-resistant to strobilurins.

Table 5-2-2 Characteristics of 33 isolates of *Microdochium nivale* and *M. majus* used to assess the level of strobilurins sensitivity.

Isolate	Location	Year	Origin	Species	QoI fungicide				
					Sensitivity test		at position 143		Digestion with *Fnu4*HI
					MIC mgL^{-1}	phenotype	Codon	Amino acid	
T18	Tokachi	2011	seed	*M. nivale*	<0.01	Str S	GGT	G	-
T24	Tokachi	2011	seed	*M. majus*	>100	Str R	GCA	A	-
T35	Tokachi	2011	seed	*M. nivale*	>100	Str R	GGA	G	-
T56	Tokachi	2011	seed	*M. majus*	>100	Str R	GCA	A	+
T99	Tokachi	2011	seed	*M. nivale*	>100	Str R	GCT	A	+
T106	Tokachi	2011	seed	*M. nivale*	<0.01	Str S	GGT	G	-
T107	Tokachi	2011	seed	*M. nivale*	<0.01	Str S	GGT	G	-
TKSW002	Tokachi	2013	air trap	*M. majus*	<0.01	Str S	GGA	G	-
TKSW006	Tokachi	2013	air trap	*M. majus*	<0.01	Str S	GGA	G	-
TKSW056	Tokachi	2013	air trap	*M. majus*	>100	Str R	GCA	A	+
TKSW081	Tokachi	2013	air trap	*M. majus*	<0.01	Str S	GGA	G	-
TKSW131	Tokachi	2013	air trap	*M. majus*	<0.01	Str S	GGA	G	-
TKSW142	Tokachi	2013	air trap	*M. majus*	>100	Str R	GCA	A	+
TKSW177	Tokachi	2013	air trap	*M. majus*	>100	Str R	GCA	A	+
TKSW197	Tokachi	2013	air trap	*M. majus*	<0.01	Str S	GGA	G	-
TKSW219	Tokachi	2013	air trap	*M. majus*	>100	Str R	GCA	A	+
TKSW252	Tokachi	2013	air trap	*M. majus*	>100	Str R	GCA	A	+
TKSW265	Tokachi	2013	air trap	*M. majus*	<0.01	Str S	GGA	G	-
TKSW277	Tokachi	2013	air trap	*M. majus*	>100	Str R	GCA	A	+
7-1	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
11-1	Abashiri	1991	unknown	*M. majus*	<0.01	Str S	GGT	G	-
11-2	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
11-5	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
11-6	Abashiri	1991	unknown	*M. majus*	<0.01	Str S	GGT	G	-
11-7	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
11-8	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
13-5	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
82-5-1	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
Bi-3	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
Bi-8	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
Me-1	Abashiri	1991	unknown	*M. nivale*	<0.01	Str S	GGT	G	-
K014	Abashiri	2011	seed	*M. nivale*	<0.01	Str S	GGT	G	-
K015	Abashiri	2011	seed	*M. majus*	<1.0	Str r	GCA	A	+

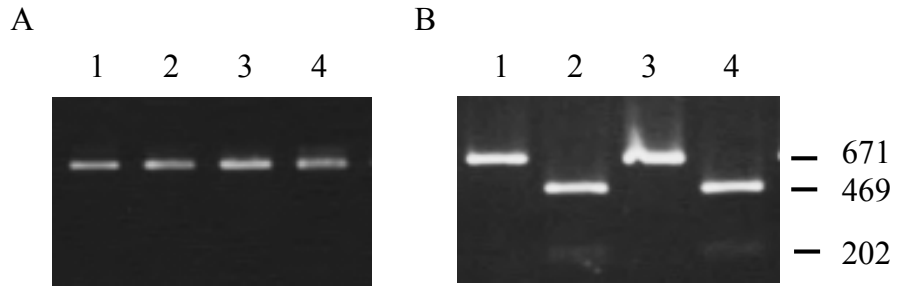


Fig. 5-2-1 Example of ethidium-bromide-stained gel of (A) PCR fragments amplified with the primers N2 and N4 pair and (B) *Fnu*4HI-digested fragments.

1: *Microdochium nivale* T18

1: *Microdochium nivale* T99

2: *Microdochium majus* TKSW002

2: *Microdochium majus* T56

Fig. 5-2-2 Nucleotide sequence alignments for *CYTB gene* of strobilurins resistant and sensitive strains of *Microdochium nivale* and *Microdochium majus*, respectively.

Strains 18 is *M. nivale* sensitive to strobilurins.

Strains 99 is *M. nivale* resistant to strobilurins.

Strains 106 is *M. majus* sensitive to strobilurins.

Strains 24 is *M. majus* resistant to strobilurins.

Red-colored nucleotides indicate the there is the G143A mutation (the substitution of glycine for alanine at position 143) whereas blue-colored nucleotides indicate there is no amino acid mutation at position 143.

5-3. Phylogenetic analysis of the resistant strains

Materials & Method

Fungal isolates

All 84 isolates used in this examination are same as the session 5-2 in CHAPTER V (Table 5-3-1). Procedures for the isolation and preservation of each sample are the same as the session 4-2 in CHAPTER IV.

CYTB sequences analysis

The PCR products including codons 129, 137, and 143 in the *CYTB* genes were extracted from agarose gels, and then purified using FastGene Gel/PCR extraction kit (Nippon Genetics, Japan). Sequencing of the purified products was performed by Hokkaido System Science Co., Ltd (Sapporo, Japan). Multiple alignments of the *CYTB* sequences were constructed with ClustalW program (Thompson *et al.*, 1994) in MEGA software ver. 6.0 (Tamura *et al.*, 2013). Haplotypes were identified using DnaSP software ver. 5.10 (Librado & Rozas, 2009) based on aligning sequence data. Furthermore, haplotype diversity (Hd) and nucleotide diversity (Nd) were also calculated using the DnaSP software ver. 5.10. The coefficient of gene differentiation (G_{ST}; McDermott & McDonald, 1993) was evaluated using Nei's method (Nei, 1973). The level of gene flow (*Nm*) was estimated as $0.5\ (1\text{-}G_{ST})(G_{ST})^{-1}$ (McDermott & McDonald, 1993). Nei's gene diversity (Gd) and analysis of molecular variance (AMOVA) were also used to partition the total phenotypic variance within populations and among populations using Arlequin software version 3.5 (Excoffier & Lischer, 2010). To visualize the genetic relationships among isolates, a phylogenetic tree was

constructed by the maximum-likelihood (ML) methods using the MEGA software ver. 6.0 (Tamura *et al.*, 2013). The robustness was tested by the bootstrap method (1000 replicates).

ISSR fingerprinting analysis

PCR amplification and fingerprinting analysis using the two ISSR markers were performed as described in the session 4-3 in CHAPTER IV.

Results

Genetic diversity

Based on the *CYTB* sequences data, 84 isolates were divided into 14 haplotypes, consisting eight haplotypes with a single isolate and six with more than two isolates (Table 5-3-2). Three most frequent haplotypes represented 72.4% of all isolates, including 70.9% of the strobilurins resistant isolates and 57.1% of the sensitive isolates. Nucleotide diversity (Nd) and Nei's gene diversity (Gd) of *M. nivale* were 0.005 and 0.2121, respectively, which were higher than that (0.002 and 0.077) of *M. majus*. On the other hand, haplotype diversity (Hd) of *M. majus* was 0.736, which was slightly higher than that (0.575) of *M. nivale*. Comparing the two populations, the Tokachi population showed a little higher haplotype diversity (Hd) than the Abashiri population. Furthermore, an extremely low gene diversity was found in the Abashiri population of *M. nivale* isolated in 1991.

Based on the ISSR fingerprinting data, all 84 isolates were divided into 18 haplotypes. These ISSR-haplotypes consisted of 10 haplotypes with a single isolate and eight with more than two isolates. The highest frequent haplotype represented 69.3% of all isolates including 84.4% of the strobilurins resistant isolates and 33.3% of the sensitive isolates. The average effective number of alleles per locus (A_E) and the average gene diversity (Gd) of *M. nivale* were 1.397 and 0.249, respectively, which were relatively higher than those of *M. majus* (A_E, 1.067 and Gd, 0.056) (Table 3). There was little difference in these genetic parameters between the Tokachi and Abashiri populations, regardless of the difference of species.

Structure of genetic variation

AMOVA and G_{ST} based on two genetic markers, regardless of the species, showed a similar pattern of the genetic structure. These results indicated that most of the total variation occurred within populations, much higher than that between populations. For example, the AMOVA for *M. majus* populations based on *CYTB* sequences showed that there were 0.079 variance components between populations and 0.605 within populations; 11.61% of the variance occurred between populations and 88.39% occurred among individuals within populations (Table 5-3-3). No significant ($P < 0.001$) genetic differentiation was found between the two populations.

On the other hand, G_{ST} based on Nei's gene diversity (Gd), regardless of the two markers, was different between the two species. G_{ST} of *M. majus* based on the *CYTB* sequences and ISSR fingerprinting data were 0.097 and 0.093, respectively, which was much lower than that (0.594 and 0.238) of *M. nivale* (Table 5-3-4). These results suggest that 90.3% and 90.7% of the observed genetic variation in *M. majus* population occurred within regional populations. On the other hand, G_{ST} of *M. nivale* based on *CYTB* sequences was 0.594, which suggests that 40.6% of the observed genetic variation occurred within regional populations. Furthermore, the level of gene flow (*Nm*) based on Nei's gene diversity (Gd) was 4.67 and 4.86 based on *CYTB* sequences and ISSR fingerprinting, respectively, which was higher than that (0.34 and 1.60) of *M. nivale*.

Phylogenetic relationships

The phylogenetic trees were respectively constructed based on haplotypes identified by the *CYTB* sequences and ISSR fingerprinting data (Fig. 5-3-1). The *CYTB* sequences-based tree was not able to divide into the two species because only *M. nivale* isolate T35 resistant to strobilurins belonged to exceptionally the clade consisted of *M. majus* isolates. On the other

hand, the ISSR fingerprinting-based tree clearly divided into the two clades that belong to either *M. majus* or *M. nivale. M. nivale* clade of the *CYTB* sequences-based tree consisted of 12 haplotypes, much more than that (three haplotypes) of ISSR fingerprinting-based tree. Conversely, *M. majus* clade of the *CYTB* sequences-based tree consisted of eight haplotypes, less than that (11 haplotypes) of ISSR fingerprinting-based tree.

Moreover, the *CYTB* sequences-based tree consisted of haplotypes that had same codons at position 143 in the *CYTB* gene. On the other hand, the ISSR fingerprinting-based tree showed that some haplotypes had different codons at the position. Although the sensitive isolates of both *M. majus* and *M. nivale* had in common the codons GGT and GGA, in the case of the resistant strains, the codon GCA was found in the *CYTB* sequences of only *M. majus*, and the codon GCT was found in the *CYTB* sequences of only *M. nivale*. No significant correlation was found between geographic distribution and genetic distance on the both trees.

Table 5-3-1 The location, Year, Origin and fungicide phenotype of 68 isolates of *Microdochium majus*.

Location	Year	Origin	QoI phenotype					
			Str S[a]		Str r[b]		Str R[c]	
			M. majus	*M. nivale*	*M. majus*	*M. nivale*	*M. majus*	*M. nivale*
Tokachi	2011	grain	13	3	0	0	14	2
	2013	air trap	6	0	0	0	6	0
Abashiri	1991	unknown	2	10	0	0	0	0
	2011	grain	4	1	1	0	22	0

Table 5-3-2 Genetic diversity of 84 isolates of *Microdochium* spp. in the two populations based on the *CYTB* sequences and ISSR markers.

Population	Isolate	*CYTB* sequence				ISSR fingerprinting		
		haplotype	Hd[a]	Nd[b]	Gd[c]	haplotype	A_E[d]	Gd
M. majus	68	11	0.736	0.002	0.077	8	1.067	0.056
Tokachi	39	8	0.754	0.003	0.100	6	1.041	0.029
Abashiri	29	6	0.496	0.002	0.055	4	1.111	0.079
M. nivale	16	4	0.575	0.005	0.212	12	1.397	0.249
Tokachi	5	3	0.700	0.012	0.400	5	1.376	0.213
Abashiri	11	2	0.182	0.001	0.024	7	1.292	0.187

a Haplotype diversity

b Nucleotide diversity

c Nei's gene diversity

d Effective number of alleles per locus

Table 5-3-3 Analysis of molecular variance (AMOVA) of 84 isolates of *Microdochium* spp. in the two populations based on the *CYTB* sequences and ISSR markers.

Species	Source of variation	d.f[a]	MSD[b]	Variance component	Total variance	P-value[c]
M. majus	*CYTB* squences					
	Between population	1	3.25	0.079	11.61	<0.001
	Within population	66	0.01	0.605	88.39	<0.001
	ISSR fingerprinting					
	Between population	1	0.80	0.010	2.1	<0.005
	Within population	66	0.01	0.465	97.9	<0.005
M. nivale	*CYTB* squences					
	Between population	1	5.24	0.619	38.55	<0.005
	Within population	14	0.07	0.987	61.45	<0.005
	ISSR fingerprinting					
	Between population	1	9.04	0.974	29.33	<0.005
	Within population	14	0.17	2.345	70.67	<0.005

a Degree of freedom

b Mean squared deviation

c Probabilities were calculated by 1023 random permutations of individuals across populations

Table 5-3-4 Genetic structure of 84 isolates of *Microdochium* spp. in the two populations based on the *CYTB* sequences and ISSR markers.

Population	*CYTB* sequence		ISSR fingerprinting	
	G_{ST}[a]	*Nm*[b]	G_{ST}[a]	*Nm*[b]
M. majus	0.097	4.67	0.093	4.86
M. nivale	0.594	0.34	0.238	1.60

a Coefficient of gene differentiation

b Level of gene flow

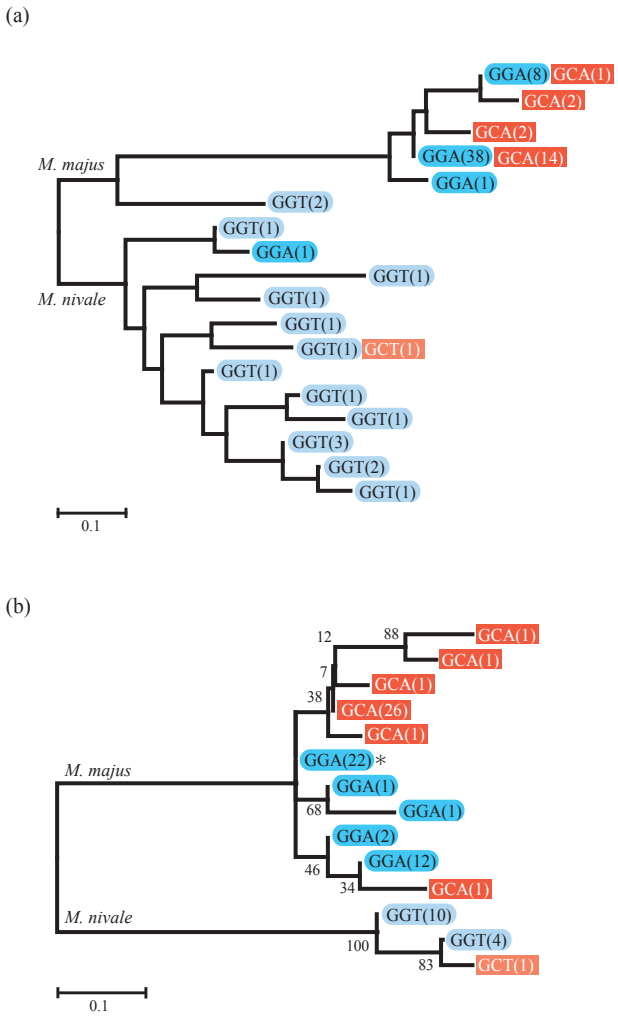


Fig. 5-3-1 Phylogenetic trees of 84 isolates of *Microdochium majus* and *Microdochium nivale* based on (A) ISSR fingerprinting with NJ method and (B) *CYTB* sequences with ML method.

Strobilurins-resistant strains of *M. majus* and *M. nivale* were surrounded by a square, and codons GCA and GCT were represented by filling red and orange respectively. Strobilurins-sensitive strains of them were surrounded by an ellipse, and codons GGA and GGT were represented by filling blue and light blue, respectively. The asterisk indicates the presence of strobilurins resistant *M. nivale* isolate T35, which is alone belonged to exceptionally the clade consisted of *M. majus* isolates. Numbers within parentheses indicate the number of strains. Numbers on branches indicate bootstrap values.

5-4. Fitness of the resistant strains

Materials and Methods

Fungal isolates

The location, year, species, and sensitivity to strobilurins of 18 isolates used in this examination are listed in Table 5-4-1. Eight isolates were obtained from infected wheat seeds collected from Tokachi and Abashiri regions in 2011. Ten isolates were obtained by trapping airborne spores with LATTS medium in Tokachi region in 2013. All isolates were purified by conventional single spore isolation. Identification of two species was determined based on PCR amplification in session 4-2, CHAPTER IV. The level of strobilurins sensitivity in isolates was determined based on the sensitivity test in session 5-2.

Fitness parameters

The mycelial growth, conidia germination, sporulation ability, and stability of resistance were compared *in vitro* between field-resistant strains and sensitive strains.

(i) Mycelial growth

Mycelial growth was assessed by measuring the radial growth on yeast extract sucrose agar (YES; yeast extract, 0.3 g; sucrose 20 g; agar, 15 g; distilled water, 1 l). Each mycelial plug (5 mm diameter), cut from the margin of a five-day-old colony on WA medium, was placed in the center of YES medium. After incubation at 20°C for 5 days in darkness, the diameter of fungal colonies was measured with a ruler. Two replicates were used per treatment.

(ii) Sporulation ability

Sporulation ability was assessed by counting spores produced on OMA medium. Each 5-mm diameter mycelial plug of WA medium was placed on OMA medium containing 1 mgl^{-1} or without a fungicide, and incubated at 20°C for 7 days. Conidia produced on OMA medium were harvested and suspended in 10 ml of sterile distilled water containing 0.05% Tween 80 then filtered through two layers of cheesecloth. The final concentration was determined with a haemacytometer. Two replicates were used per treatment.

(iii) Conidia germination

Conidia were obtained as described above. Conidial suspension of each isolate was adjusted to 1.0×10^5 conidia/ml and was plated on a 1.5% WA plate. After incubation at 20°C for 6 h or 24 h in darkness, germination rate was determined by examining 100 conidia per plate. Three plates per replicate were used for each isolate. A conidium was considered germinated if the germ tube is at least the length of the conidia.

(iv) Stability of resistance

Each resistant isolate was subcultured on YES medium, fungicide-free medium, for at least fifth transfers. The sensitivity to strobilurins was assessed by measuring the radial growth on PDA medium containing 1 mgl^{-1} strobilurins after third and fifth subculture of resistant isolates. Two replicates were used per treatment.

Statistical analysis

Statistical tests were carried out by multiple comparison tests in Microsoft Excel 2011 software. The means of each fitness parameter (mycelial growth, conidia germination,

sporulation ability, and stability of resistance) of each group with different fungicide-resistance phenotypes were compared using a Tukey-Kramer test.

Results

Fitness parameters

(i) Mycelial growth

There was no significant difference ($P < 0.01$) in the mycelial growth of strobilurins-resistant and sensitive strains on YES medium. The average of the mycelial growth of resistant and sensitive strains showed 0.59 (minimum; 0.47, maximum; 0.74) and 0.56 (minimum; 0.51, maximum; 0.65), respectively.

(ii) Conidia germination

There was no significant difference ($P < 0.01$) in the conidia germination between strobilurins-resistant and sensitive strains after being incubated for both 6 and 24 hour.

(iii) Sporulation ability

There was no significant difference ($P < 0.01$) in sporulation ability of strobilurins-resistant and sensitive strains on the medium with or without 1 mgl^{-1} strobilurins. In all isolates, the average of conidial production on the medium containing 1 mgl^{-1} strobilurins was 9.8×10^6, which was much lower than that (1.2×10^8) on the strobilurins-free medium.

(iv) Stability of resistance

Strobilurins resistance was not significantly reduced from the first generation to the fifth generation that were grown and preserved on a strobilurin-free medium. On the medium containing 1 mgl^{-1} strobilurins, the mycelial growth of all isolates at fifth generations was equal to or increased compared to that at the 1st generation. Furthermore, the mycelial

growth of strobilurins-resistant strain excluding two isolates, ABOK1 and ABOK5, was highest at the fifth generations on the medium containing 1 mgl^{-1} strobilurins.

Table 5-4-1 Fungicide phenotype, year and origin of isolates of *Microdochium nivale* and *Microdochium majus* used.

Species	Isolates	Year	Location	Sensitivity to strobirulin		
				Phenotype	MIC (mgL^{-1})	Codon
M. majus	T126	2011	Sorachi	S	<0.01	GGA
	T162	2011	Ishikari	S	<0.01	GGA
	TKOB02	2013	Tokachi	S	<0.01	GGA
	TKOB04	2013	Tokachi	S	<0.01	GGA
	ABOK03	2013	Abashiri	S	<0.01	GGA
	ABOK04	2013	Abashiri	S	<0.01	GGA
	T24	2011	Tokachi	R	>100	GCA
	T56	2011	Tokachi	R	>100	GCA
	TKOB01	2013	Tokachi	R	>100	GCA
	TKOB03	2013	Tokachi	R	>100	GCA
	TKOB05	2013	Tokachi	R	>100	GCA
	ABOK01	2013	Abashiri	R	>100	GCA
	ABOK02	2013	Abashiri	R	>100	GCA
	ABOK05	2013	Abashiri	R	>100	GCA
M. nivale	T18	2011	Tokachi	S	<0.01	GGT
	T106	2011	Tokachi	S	<0.01	GGT
	T35	2011	Tokachi	R	>100	GGA
	T99	2011	Tokachi	R	>100	GCT

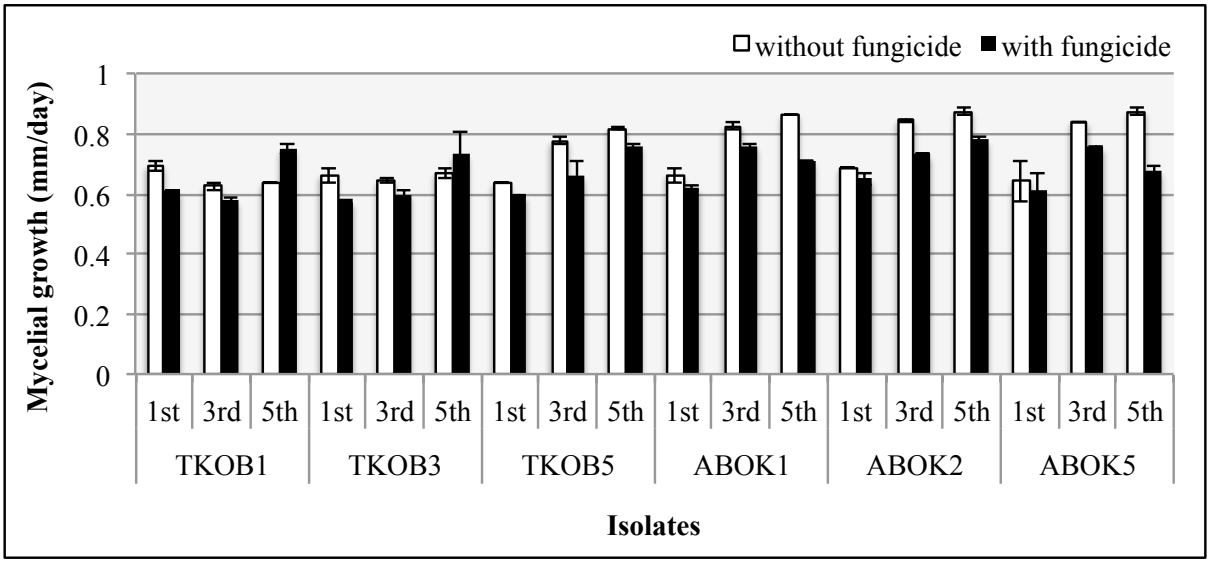
Table 5-4-2 Fitness parameters of strobilurins resistant and sensitive strains of *Microdochium majus* and *Microdochium nivale.*

Species	Isolates	Phenotype	Fitness parameters					
			Mycelium growth (mm/day)		Conidia germination (%)		Conidia production (spore/ml)	
			Strobilurins		6 h	24 h	Strobilurins	
			1mgl^{-1}	Free			1mgl^{-1}	Free
M. majus	T126	S	0[x] e[y]	0.67 ab	68.0 b	72.5 b	NT a	9.8×10^6 a
	T162	S	0 e	0.72 a	75.0 a	89.0 b	NT a	5.2×10^8 a
	TKOB02	S	0 e	0.46 g	5.5 c	100.0 a	NT a	7.0×10^7 a
	TKOB04	S	0 e	0.47 fg	0.5 c	99.5 a	NT a	8.0×10^7 a
	ABOK03	S	0 e	0.51 efg	14.5 c	100.0 a	NT a	8.3×10^7 a
	ABOK04	S	0 e	0.54 defg	2.0 c	100.0 a	NT a	1.4×10^8 a
	T24	R	0.85 d	0.62 bcd	87.5 a	95.5 a	6.5×10^7 a	6.5×10^7 a
	T56	R	0.95 c	0.59 cdef	85.0 a	94.0 a	1.5×10^7 a	6.7×10^6 a
	TKOB01	R	3.05 a	0.54 defg	2.5 c	98.5 a	NT a	3.6×10^5 a
	TKOB03	R	2.88 a	0.53 defg	2.5 c	99.0 a	2.2×10^6 a	9.8×10^7 a
	TKOB05	R	2.98 a	0.51 efg	10.0 c	99.5 a	3.6×10^6 a	6.8×10^7 a
	ABOK01	R	3.10 a	0.53 defg	12.0 c	100.0 a	7.8×10^5 a	2.2×10^8 a
	ABOK02	R	3.25 a	0.55 cdefg	10.5 c	100.0 a	4.8×10^5 a	2.1×10^8 a
	ABOK05	R	3.05 a	0.52 efg	15.0 b	100.0 a	6.3×10^5 a	3.4×10^8 a
M. nivale	T18	S	0 e	0.59 cde	13.5 c	65.0 c	NT a	7.0×10^7 a
	T106	S	0 e	0.74 a	2.5 c	97.0 a	NT a	3.5×10^6 a
	T35	R	1.30 b	0.65 abc	12.5 c	98.5 a	2.7×10^5 a	2.1×10^8 a
	T99	R	1.40 b	0.56 cdef	7.0 c	55.0 d	9.7×10^4 a	4.4×10^6 a

[x] Mean (± standard error) values of three replicates.

[y] Mean values with the same letter were not significantly different according to the Tukey test ($P < 0.01$)

Fig. 5-4-1 Mycelial growth of strobilurins-resistant isolates of *Microdochium majus* on PDA medium containing at 1 mgl^{-1}, after third and fifth sub-culturing on strobilurins-free medium.



5-5. Discussion

This chapter has proved the strobilurins-resistant strains of *Microdochium* spp. were already widespread in Hokkaido with 52.6% (Tokachi) and 51.1% (Abashiri) of tested isolates. These regions are principal producing areas of winter wheat in Japan, in which the strobilurins fungicide has been used for control of FHB since the late 1990s. It is reasonable for the year 1991 isolates to be sensitive to strobilurins because they had never been exposed to the selection of strobilurins fungicides. After that, Kozawa *et al*. (2011) reported that the effectiveness of strobilurins against *Microdochium* spp. decreased in some areas of Hokkaido. This finding would, therefore, suggested that the resistant strains developed rapidly in a short time after their appearance.

All strobilurins-resistant strains but isolate T35 were found that there was the G143A mutation in the *CYTB* gene. The G143A mutation has already been reported in a broad range of plant pathogenic fungi, usually leading to high level of QoI-resistance (Bartlett *et al.*, 2002; Fernández-Ortuño *et al.*, 2008). However, a highly strobilurins-resistant strain T35 had neither G143A mutation nor the F129L and G137R mutations. The absence of the typical amino acid mutations such as G143A, F129L, and G137R was reported in the resistant strains of *Venturia inaequalis* (Steinfeld *et al.*, 2001) and *Podosphaera fusca* (Fr.) Braun & Shishkoff (Ishii *et al.*, 2001; Fernández-Ortuño *et al.*, 2008). Walker *et al*. (2009) reported that the atypical resistant strain of *M. majus*, isolate 71, was detected in French populations. To the author's knowledge, this study is the first report on the occurrence of the resistant *M. nivale* isolate without the typical amino acid mutations related to QoI-resistance. Several researchers have discussed the mechanism of the atypical QoI-resistant strains. Steinfeld *et al.* (2001) suggested that the atypical resistance might be due to compensation for the energy

deficiency following QoI treatment upstream from NADH dehydrogenase in the respiratory chain. Furthermore, Fernández-Ortuño *et al.* (2008) proposed that the stability of the Rieske FeS protein (ISP) might be involved in QoI-resistance because the Qo site was related to the mobile extrinsic domain of the ISP as well as the *CYTB* gene (Zhang *et al.*, 1998).

According to the sensitivity test, the levels of strobilurins resistance of *Microdochium* spp. were divided into the two resistant groups, which were Str R and Str r. How did the differences in the levels of strobilurins resistance arise? Several studies have been trying to answer the question. Firstly, it was already reported that F129L and G137R mutations were usually responsible for a moderate level of strobilurins resistance in plant pathogenic fungi, such as *Alternaria solani* (Pasche *et al.*, 2005; Leiminger *et al.*, 2014), *Pyricularia grisea* (Kim *et al.*, 2003), *Pyrenophora teres,* and *P. tritici-repentis* (Sierotzki *et al.*, 2007). Secondly, it was considered to be the effects of mitochondrial heteroplasmy, which was the coexistence of wild-type and mutated mitochondria in single cells. Lesemann *et al.* (2006) reported that the differences in the levels of trifloxystrobin sensitivity were clearly correlated with the relative proportion of wild-type and mutated mitochondria of *Podosphaera leucotricha* causing the apple powdery mildew. In this study, however, sequence analysis observed no overlapping guanine and cytosine peaks at position 143 in the *CYTB* gene of the resistant isolates.

No significant difference in fitness parameters, such as the mycelial growth, conidia germination, and sporulation ability, was found between resistant and sensitive strains *in vitro*. This study, therefore, proved that strobilurins resistance of *Microdochium* spp. was not associated with the fitness cost. Fitness cost associated with QoI-resistance was reported in several plant pathogenic fungi, such as *Plasmopara viticola* and *P. grisea* (Avila-Adame & Köller, 2003), *Botrytis cinerea* (Markoglou *et al.*, 2006), *Aspergillus carbonarius*

(Malandrakis *et al.*, 2013). Avila-Adame and Köller showed that the conidia production of azoxystrobin-resistant strain that had the G143S (substitution guanine for serine at position 143) mutation associated with QoI-resistance was significantly lower under both saprophytic and pathogenic conditions of reproduction. On the other hand, several studies reported that fitness cost was not found in QoI-resistant strains, such as *Blumeria graminis* (Heaney *et al.*, 2000), *Alternaria alternata* (Avenot & Michailides, 2007) and *B. cinerea* (Kim & Xiao, 2011). Kim and Xiao showed that there were no significant differences in the mycelial growth, osmotic sensitivity, conidial germination, and sporulation between Pyraclostrobin- and Boscalid-resistant and sensitive strains although some boscalid-resistant strain produced fewer conidia *in vitro* than sensitive strains. There is a high interest about the stability of fungicide-resistance because, in the absence of the fungicide, use of the fungicide depends on the frequency of the resistant strains. In this study, strobilurin resistance of *M. nivale* and *M. majus* was not significantly reduced from the first generation to the fifth generation that were grown and preserved on a strobilurin-free medium. It suggested that strobilurin resistance of *M. nivale* and *M. majus* was stable in the absence of the fungicide. This finding was supported by several reports, which were intended for *Blumeria graminis* (Heaney *et al.*, 2000)*, B. cinerea* (Kim & Xiao, 2011), *Erysiphe graminis* f.sp. *tritici* (Chin *et al.*, 2001) and *Alternaria alternata* (Avenot & Michailides, 2007).

The *CYTB* sequence and ISSR fingerprinting-based trees suggested that the strobilurins-resistant strains used in this study emerged independently through a single amino acid mutation (G143A), occurred in sensitive strains, and then rapidly developed through high gene flow. In plant pathogenic fungi, the polyphyletic origins of resistant strains were reported by Chen *et al.* (2007), who showed that two QoI-resistant haplotypes of *Plasmopara viticola* Berl. & de Toni, grapevine downy mildew pathogen, emerged independently by one

mutational step from two sensitive haplotypes. Furthermore, Torriani *et al*. (2008) also reported that QoI-resistant *Mycosphaerella graminicola* isolates emerged independently through at least four recurrent mutations at the position 143 of the *CYTB* gene in different genetic and geographic backgrounds. The parallel evolution of the resistant strains in these reports had in common a high gene flow in pathogen populations. The high gene flow may, therefore, play a significant role in the rapid development of fungicide resistant strains.

A significant advantage of sexual reproduction is to be able to generate the novel phenotypes that parents do not have. Although this character could have contributed to the species persisting in populations in the long period, especially in the case of only a particular phenotype such as fungicide resistant strains had a chance for survival in extraordinary environments, asexual reproduction could gain an advantage in spreading rapidly in populations compared with sexual reproduction. That is because the simple system as asexual reproduction may be suitable for increasing the frequency of acquired mutations. This hypothesis may explain the higher isolation frequency of *M. majus* in Hokkaido. Understanding the inheritance and mechanisms of strobilurins resistance could be useful for the control of *Microdochium* spp.

In conclusion, this chapter demonstrated that the parallel evolution of the resistant strains of *M. nivale* and *M. majus* isolated occurred in Hokkaido. Furthermore, the finding of genetic diversity and structure showed that the higher gene flow was likely playing an important role as means for migration in the population biology of *Microdochium* spp., and providing some support for the prediction of epidemics of fungicide resistant strains within populations of the FHB pathogens.

CHAPTER VI
THE MODE OF SEXUAL REPRODUCTION

6-1. Introduction

In CHAPTER IV, I have reported that *M. majus* was the dominant species regardless of the differences of host tissue and there was a high gene flow in populations of *M. majus* isolates. In CHAPTER V, I have reported that the strobilurins-resistant strains of *Microdochium* spp. were already widespread in Hokkaido. Furthermore, I have suggested that the strobilurins-resistant strains emerged independently through a single amino acid mutation (G143A), occurred in sensitive strains, and then rapidly developed through high gene flow. The rapid development of the strobilurins-resistant strains was affected by the mode of sexual reproduction as well as the high gene flow.

Both *M. nivale* and *M. majus* belong to ascomycetes and can reproduce asexually and sexually (Lees *et al.*, 1995; Litschko & Burpee, 1987; Parry *et al.*, 1995). According to some genetic studies, *M. nivale* isolates reproduce sexually in nature because they have a high number of polymorphisms within individuals (Lees *et al.*, 1995; Parry *et al.*, 1995; Mahuku *et al.*, 1998). On the other hand, *M. majus* isolates generally reproduce homothallically in nature because they have a high level of genetic uniformity (Lees *et al.*, 1995; Hayashi *et al.*, 2013). Sexual reproduction has an advantage that can generate novel phenotypes that parents have not possessed, through mating with others that had a different genetic background. Sexual reproduction, therefore, might have a significant role on the evolution of fungicide-resistant strains of plant pathogenic fungi.

There is little information on genetic mechanism of sexual reproduction in *Microdochium* spp. Jewell (2013) showed that the putative *MAT1-2-1* was found, but the amplification of *MAT1-1-1* could not be observed in any sequences of *M. majus* and *M. nivale* isolates.

The purpose of this study, therefore, was (i) to investigate the mode of sexual reproduction of *M. majus* and *M. nivale* and sexual fertility by crossing among self-sterile isolates, (ii) to evaluate the possibility of the interspecific hybridization between *M. majus* and *M. nivale*, and (iii) to discuss the effect of sexual reproduction on the development of fungicide-resistant strains of *Microdochium* spp.

6-2. PCR amplification of *MAT1-2-1* gene

Materials and Method

Fungal isolates

The location, year, species and sensitivity to strobilurins of all 34 isolates used in this examination are listed in Table 6-2-1. 22 isolates were obtained from infected wheat seeds collected from Tokachi and Abashiri regions in 2011 and 2013. 20 isolates were obtained in Abashiri region in 1991. All isolates were purified by conventional single spore isolation. Identification of two species was determined based on PCR amplification in session 4-2, CHAPTER IV. The level of strobilurins sensitivity in isolates was determined based on the sensitivity test in session 5-2.

Amplification and sequencing of the putative *MAT1-2-1* gene

Genomic DNA was extracted using Cica Geneus DNA Extraction Reagent ST (Cica, Japan). The PCR fragments including the putative *MAT1-2-1* gene were amplified with *Microdochium*-specific primers Mic_MAT2_198F (CGAAGCGYGAGRCGAAG) and Mic_MAT2_676R (AAGCTCTGRTCTTGAGTGT) pair (Jewell *et al.*, 2013). PCR reactions were performed in a total volume of 6 μL containing 0.4 μM of each primer, 2 × GoTaq Green Master Mix (Promega, USA), and 10-30 ng of genomic DNA. Amplification was performed by a Program Temp Control System PC-320 (ASTEC, Japan) with an initial denaturation step of 94°C for 2 min; followed by 30 amplification cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s with a final step of 72°C for 2 min. Aliquots (4 μL) of

amplification products were electrophoresed on 1.2% agarose gels, and then the separated fragments were visualized under 300 nm UV light after staining with ethidium bromide.

Each PCR product was extracted from agarose gels, and then purified using FastGene Gel/PCR extraction kit (Nippon Genetics, Japan). Sequencing of the purified products was performed by Hokkaido System Science Co., Ltd (Sapporo, Japan). Multiple alignments of the *MAT1-2-1* sequences were constructed with ClustalW program (Thompson *et al.*, 1994) in MEGA software ver. 6.0 (Tamura *et al.*, 2013). *MAT1-2-1* sequences of *F. graminearum* FG-13a (GenBank accession no. HM474067.1) and *F. asiaticum* NRRL 28720 (GenBank accession no. AY452891) were retrieved from GenBank. Haplotypes were identified using DnaSP software ver. 5.10 (Librado & Rozas, 2009) based on the aligning sequence data. To visualize the genetic relationships among isolates, a phylogenetic tree was constructed by the maximum-likelihood (ML) methods using the MEGA software ver. 6.0 (Tamura *et al.*, 2013). The robustness was tested by the bootstrap method (1000 replicates).

Results

Amplification and sequencing of the putative *MAT1-2-1* gene

PCR amplification with primer Mic_MAT2_198F and Mic_MAT2_676R pair produced a single band of approximately 500 bp in all isolates of *M. nivale* and *M. majus* (Fig. 6-2-1). According to the comparison of *MAT1-2-1* sequences between two species, three genetic deletions were observed in downstream of HMG-box of *Microdochium majus* isolates (Fig. 6-2-2). These deletions were not found in *MAT1-2-1* sequences of both *F. graminearum* isolate FG-13 and *F. asiaticum* isolate NRRL28720.

Table 6-2-1 Production of perithecia on wheat straw inoculated a isolate of *Microdochium majus* or *Microdochium nivale*, respectively.

Species	Isolates	Location	Year	Perithecia		
				Production[a]	Size (nm)	
					Height	Width
M. majus	11-1	Abashiri	1991	++ [b]	276	240
	11-6	Abashiri	1991	++	290	210
	T24	Tokachi	2011	++	218	196
	T56	Tokachi	2011	-	-	-
	T126	Tokachi	2011	++	144	134
	T129	Tokachi	2011	++	316	272
	T162	Tokachi	2011	++	262	224
	K015	Abashiri	2011	++	282	246
	TKOB01	Tokachi	2013	-	-	-
	TKOB02	Tokachi	2013	-	-	-
	TKOB03	Tokachi	2013	-	-	-
	TKOB04	Tokachi	2013	-	-	-
	TKOB05	Tokachi	2013	++	208	186
	ABOK01	Abashiri	2013	++	262	220
	ABOK02	Abashiri	2013	++	334	268
	ABOK03	Abashiri	2013	++	248	214
	ABOK04	Abashiri	2013	-	-	-
	ABOK05	Abashiri	2013	++	258	222
M. nivale	7-1	Abashiri	1991	-	-	-
	11-2	Abashiri	1991	-	-	-
	11-5	Abashiri	1991	-	-	-
	11-7	Abashiri	1991	-	-	-
	11-8	Abashiri	1991	-	-	-
	13-5	Abashiri	1991	-	-	-
	82-5-1	Abashiri	1991	-	-	-
	Bi-3	Abashiri	1991	+	152	124
	Bi-8	Abashiri	1991	+	178	158
	Me-1	Abashiri	1991	++	220	210
	T18	Tokachi	2011	-	-	-
	T35	Tokachi	2011	-	-	-
	T99	Tokachi	2011	+	178	156
	T106	Tokachi	2011	+	188	176
	T107	Tokachi	2011	-	-	-
	K014	Abashiri	2011	-	-	-

[a] Mean values of four replicates.

[b] The perithecia production score: - , no production; + , less than 50; and ++ , 50 or more.

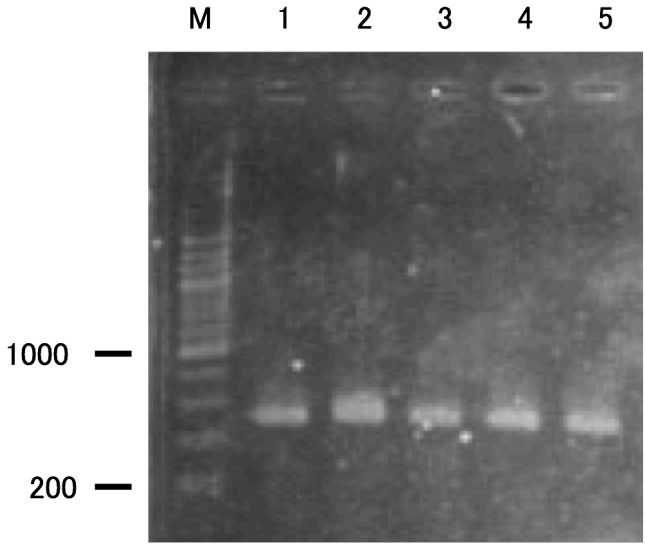


Fig. 6-2-1 Example of PCR amplification of *MAT1-2-1* with *Microdochium*-specific primers Mic_MAT2_198F and Mic_MAT2_676R pair.

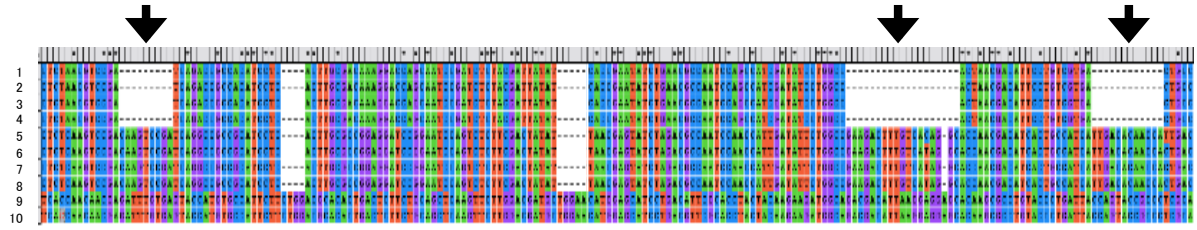


Fig. 6-2-2 The differences in nucleotide sequences of *MAT1-2-1* gene among (1-4) *Microdochium nivale*, (5-8) *Microdochium majus* and (8-10) *Fusarium* spp.

Three genetic deletions observed in *MAT1-2-1* gene of *Microdochium majus* isolates are indicated by black narrows.

1: TKOB1

2: TKOB3

3: TKOB5

4: T129

5: T18

6: 7-1

7: Bi-8

8: Me-1

9: *F. graminearum* FG-13a

10: *F. asiaticum* NRRL 28720

6-3. Mating test

Materials & Methods

Fungal isolates

Isolates used in this examination were the same as the session 6-2 (Table 6-2-1).

Production of perithecia *in vitro*

(i) Self-fertility

Each fungal plug (5 mm diameter) cut from the margin of a five-day-old colony on WA medium was placed in the center of YES medium. After incubation at 20°C for 4 days in darkness, two straw segments (2 cm) were placed on the margin of each fungal colony. The straw segments were sterilized by autoclaving two times at 121°C for 20 min with 24 h between each sterilization. Each plate was incubated at 20°C for 14 days in darkness. The production of perithecia in each plate was measured visually as below; -, no production; +, less than 50; and ++, 50 or more (Fig. 6-3-1).

The sizes (height and width) of five perithecia were measured under a microscope using a micrometer. Five perithecia were transferred to a 1.5 ml tube containing 500 µl distilled water and then ground with a plastic pestle. The tube was vortexed briefly and centrifuged for 1 min at 500 rpm to remove a shell of perithecia. The maturity of perithecia was assessed by confirming the presence of ascospores in the tube. Four replicates were used per treatment.

(ii) Intraspecific hybridization

Isolates used in this crossing test were self-sterile isolates that could not produce perithecia by itself. They include six isolates (TK56, TKOB1, TKOB2, TKOB3, TKOB4, and ABOK4) of *M. majus* and 11 isolates (7-1, 11-2, 11-5, 11-7, 11-8, 13-5, 82-5-1, T18, T35, T107, and K014) of *M. nivale.* All 70 combinations of isolates tested consist 15 combinations of six isolates within *M. majus* and 55 combinations of 11 isolates within *M. nivale.* Two mycelial plugs were placed at a distance of 2 cm each other on YES medium. After incubation at 20°C for 4 days in darkness, two straw segments (2 cm) were placed on the border of two fungal colonies. Assessment of the production of perithecia was performed as described as above.

(iii) Interspecific hybridization

All 66 combinations of six self-sterile *M. majus* isolate*s* and 11 self-sterile *M. nivale* isolates were tested. Methods and assessment of the production of perithecia were performed as described above.

Of all combinations produced perithecia, three combinations (T56 × 11-7, TKOB3 × 11-7*,* TKOB3 × 13-5) were randomly selected and used to confirm whether perithecia are produced by interspecific hybridization. A perithecium of each combination was transferred to a 1.5 ml tube containing 500 μl distilled water and then ground with a plastic pestle. Each tube was vortexed briefly and centrifuged for 1 min at 500 rpm to remove a shell of perithecia. After that, 400 μl of the supernatant was transferred to a WA medium plate containing 150 mgl^{-1} streptomycin sulphate. After incubation at 20°C for 24 hours in darkness, 20 germinated spores were picked up and transferred on a YES medium plate, respectively. After incubation at 20°C for 24 hours in darkness, each genomic DNA was extracted from mycelium using Cica Geneus DNA Extraction Reagent ST (Cica, Japan). Identification of two species was

determined based on PCR amplification in session 4-2, CHAPTER IV. Sensitivity of strobilurins was determined as described in session 5-2, CHAPTER V.

Results

(i) Self-fertility

Of 18 *M. majus* isolates, 12 isolates produced a lot (++) of perithecia with mature ascospores on wheat straw (Table 6-2-1) and an example of perithecia production was shown in Fig 6-3-2(A) and (B). Of 16 *M. nivale* isolates, five isolates produced perithecia with mature ascospores. Only a single isolate, Me-1, produced a lot of (++) perithecia whereas others produced moderate (+) perithecia.

Perithecia of both *M. majus* and *M. nivale* isolates were commonly spherical to subspherical and brown to black. There were slight differences in size of perithecia between the two species. The mean size of perithecia, height 258.2 μm (144-334) × width 219.3 μm (134-268), of *M. majus* isolates were bigger than those, height 183.2 μm (152-220) × width 164.8 μm (124-210), of *M. nivale* isolates (Table 6-2-1).

(ii) Intraspecific hybridization

Six isolates of *M. majus* and 11 isolates of *M. nivale* that could not produce perithecia in single culture were used in the crossing test. Of 15 combinations of the six *M. majus* isolates, 14 combinations produced perithecia with mature ascospores on wheat straw (Table 6-3-1). Of the 14 combinations, seven combinations produced a lot of (++) perithecia and an example of perithecia production was shown in Fig 6-3-2(C). Only the combination of T56 and TKOB1 produced no perithecia. Of 55 combinations of the 11 *M. nivale* isolates, 13 combinations produced perithecia with mature ascospores (Table 6-3-2). Of the 13 combinations, no combination produced a lot of (++) perithecia.

(iii) Interspecific hybridization

Of 66 combinations of the six *M. majus* and the 11 *M. nivale* isolates, 58 combinations produced perithecia with mature ascospores (Table 6-3-3). Of the 58 combinations, 27 combinations produced a lot of (++) perithecia. No difference in the color and shape of the produced perithecia was not observed, compared to those produced by a single isolate or combinations of isolates in respective species. All combinations with TKOB2, TKOB4 or ABOK4 of *M. majus* isolates produced perithecia, respectively.

The progeny was obtained from perithecia produced in three combinations (T56 × 11-7, TKOB3 × 11-7, TKOB3 × 13-5), respectively (Fig. 6-3-3). No ascospore was observed in some perithecia in all combinations. These infertile pericethia were found highest in T56 × 11-7, followed by TKOB3 × 11-7, TKOB3 × 13-5. 15 and eight of 23 single spores obtained from a combination between two isolates, TKOB3 and 13-5, were identified as *M. majus* and *M. nivale*, respectively and were the same with strobilurins phenotype of each parental species (Table 6-3-4). 20 single spores obtained from combinations between TKOB3 and 11-7, or TKOB3 and 13-5, were identified as *M. majus*.

Table 6-3-1 Perithecia formation on the different combination of six self-sterile isolates in *Microdochium nivale*.

Species	Isolate	The production of perithecia[a]					
		TK56	TKOB1	TKOB2	TKON3	TKOB4	ABOK4
M. majus	T56		-[b]	++	+	+	++
	TKOB1			+	+	+	++
	TKOB2				+	++	++
	TKOB3					+	++
	TKOB4						++
	ABOK4						

[a] Mean values of four replicates.

[b] The perithecia production was assessed as the below; - , no production; + , less than 50; and ++ , 50 or more.

Table 6-3-2 Perithecia formation on the different combination of eleven self-sterile isolates in *Microdochium nivale*.

Species	Isolate	The production of perithecia[a]										
		7-1	11-2	11-5	11-7	11-8	13-5	82-5-1	T18	T35	T107	K014
M. nivale	7-1		+[b]	+	-	-	-	+	-	-	+	+
	11-2			-	-	-	-	-	-	-	-	-
	11-5				-	-	-	-	-	-	-	+
	11-7				-	-	-	-	-	-	-	-
	11-8					-	-	-	-	-	-	+
	13-5						-	-	-	-	-	-
	82-5-1							-	+	-	-	+
	T18									+	-	+
	T35										-	+
	T107											+
	K014											

[a] Mean values of four replicates.

[b] The perithecia production was assessed as the below; - , no production; + , less than 50; and ++ , 50 or more.

Table 6-3-3 Perithecia formation on the different combination of six and eleven self-sterile isolates in *Microdochium majus* and *Microdochium nivale*, respectively.

Species	Isolate	The production of perithecia[a]										
		M. nivale										
		7-1	11-2	11-5	11-7	11-8	13-5	82-5-1	T18	T35	T107	k014
M. majus	T56	+[b]	+	+	+	+	+	+	-	+	+	+
	TKOB1	-	-	+	-	+	-	+	+	-	-	+
	TKOB2	+	++	++	++	+	+	++	+	++	++	+
	TKOB3	++		++	++	+	++	+	+	+	+	+
	TKOB4	++	++	++	++	++	+	+	+	++	+	+
	ABOK4	++	++	++	++	++	++	++	++	++	++	++

[a] Mean values of four replicates.

[b] The perithecia production was assessed as the below; - , no production; + , less than 50; and ++ , 50 or more.

Table 6-3-4 Sensitivity of strobilurins and species type of isolates of parents and their progeny obtained by crossing between two isolates, TKOB3 and 13-5.

Phenotype	Isolates	Species		Sensitivity of strobilurins[a]
		M. majus	*M. nivale*	
Parents	TKOB3	○		R
	13-5		○	S
Progeny	Pr1	○		R
	Pr2	○		R
	Pr3		○	S
	Pr4	○		R
	Pr5	○		R
	Pr6	○		R
	Pr7	○		R
	Pr8		○	S
	Pr9	○		R
	Pr10	○		R
	Pr11		○	S
	Pr12	○		R
	Pr13	○		R
	Pr14	○		R
	Pr15	○		R
	Pr16		○	S
	Pr17		○	S
	Pr18	○		R
	Pr19		○	S
	Pr20		○	S
	Pr21	○		R
	Pr22		○	S
	Pr23	○		R

[a] Sensitivity of strobilurins was assessed on PDA medium containing 1.0 mgl^{-1} kresoxim-methyl and 1.0 mM n-propyl gallate.

(−) (+) (++)

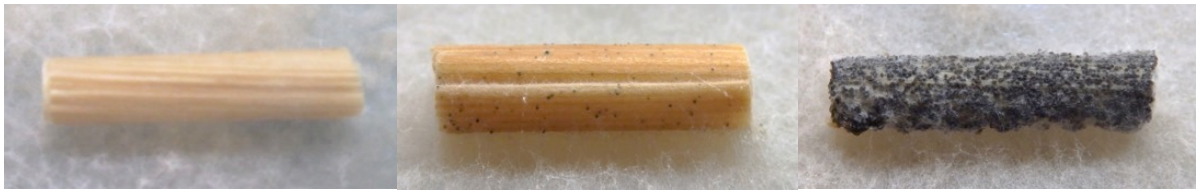


Fig. 6-3-1 An example of assessment in the production of perithecia on a wheat straw segment (2 cm).

The perithecia production was assessed as below; -, no production; +, less than 50; and ++, 50 or more.

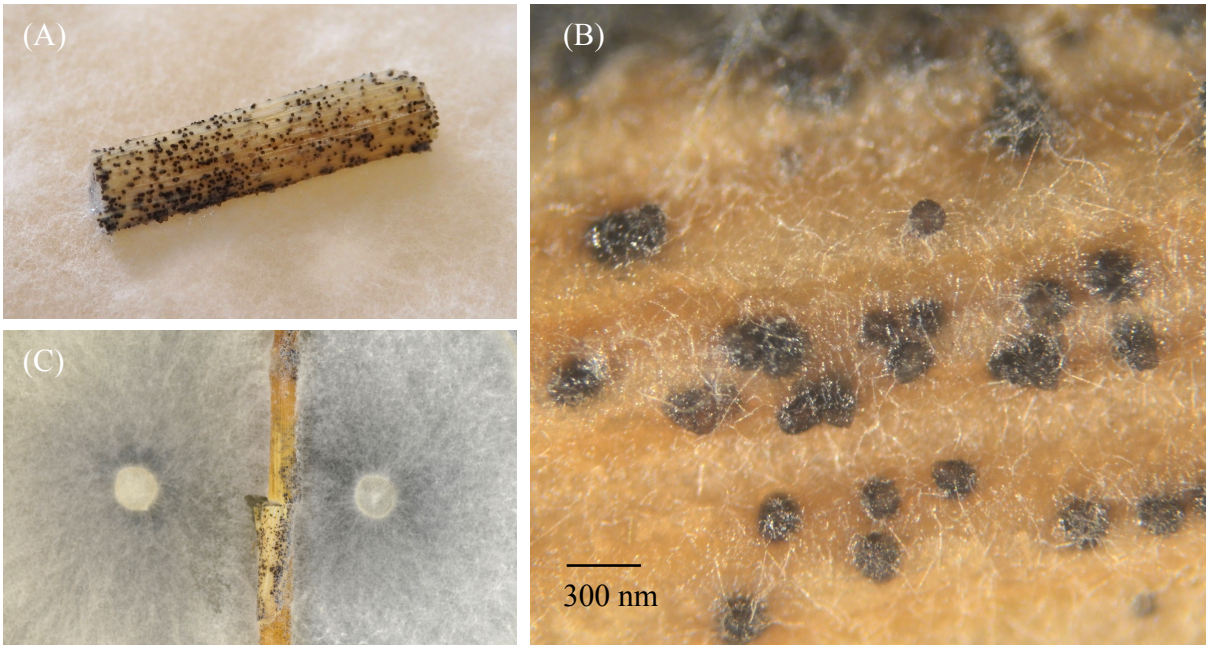


Fig.6-3-2 An example of perithecia production on a wheat straw segment (2 cm).

(A) Perithecia produced on a wheat straw cultured with a mycelial plug of *Microdochium majus* isolate T24.

(B) Perithecia shown in (A) under a stereoscopic microscope.

(C) Perithecia produced on a wheat straw cultured between two mycelial plugs of isolate T56 and TKOB4 of *M. majus*.

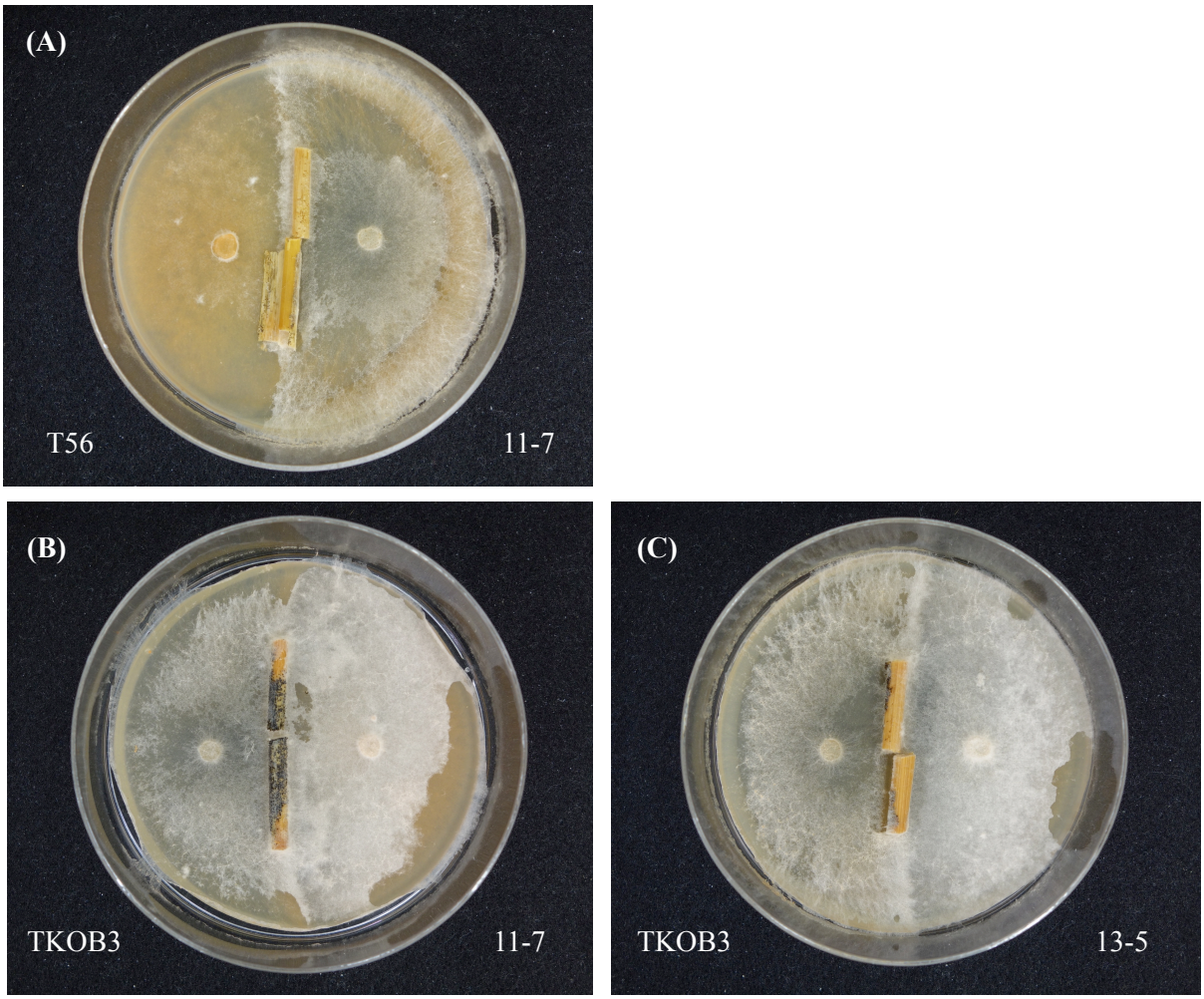


Fig. 6-3-3 Perithecia produced by crossing of isolates of (A) T56 and 11-7, (B) TKOB3 and 11-7, (C) TKOB3 and 13-5, respectively.

6-4. Phylogenetic analysis

Materials & Methods

Fungal isolates

The isolates used in this examination were the same as session 6-2 (Table 6-2-1).

Sequencing analyzes using two genetic markers

The PCR fragments including β-tubulin and RPB2 were amplified with primers Btub526_F (CGAGCGYATGAGYGTYTACTT) and Btub1332_R (TCATGTTCTTGGGGTCGAA) pair for β-tubulin, fRPB2-7cF (GAYGAYMGWGATCAYTTYGG) (Jewell *et al.*, 2013) and fRPB2-7cR (CCCATRGCTTGYTTRCCCAT) pair for RPB2 (Liu *et al.*, 1999). The PCR reactions were performed in a total volume of 11 μl containing 10 × PCR buffer, 2.5 mM of dNTPs, 0.3 μM of each primer, 1.25 U of ExTaq DNA Polymerase (Takara, Japan), and 10-30 ng of genomic DNA. Amplification was performed in a Program Temp Control System PC-320 (ASTEC, Japan) with an initial denaturation step of 94°C for 2 min; followed by 32 amplification cycles of 94°C for 30 s, 55°C for 30 s for β-tubulin or 60°C for 30 s for RPB2, and 72°C for 60 s; with a final extension at 72°C for 2 min. Aliquots (4 μl) of amplification products were electrophoresed on 1.2% agarose gels, and then the separated fragments were visualized under 300 nm UV light after staining with ethidium bromide.

Each PCR product including β-tubulin and RPB2 was extracted from agarose gels, and then purified using FastGene Gel/PCR extraction kit (Nippon Genetics, Japan). Sequencing of the purified products was performed by Hokkaido System Science Co., Ltd (Sapporo, Japan).

Multiple alignments of the each sequence were constructed with ClustalW program

(Thompson *et al.*, 1994) in MEGA software ver. 6.0 (Tamura *et al.*, 2013). To visualize the genetic relationships among isolates, a phylogenetic tree was constructed by the maximum-likelihood (ML) methods using the MEGA software ver. 6.0 (Tamura *et al.*, 2013). The robustness was tested by the bootstrap method (1000 replicates).

Results

PCR amplification and sequencing of β-tubulin and RPB2

PCR with respective primer Btub526_F/Btub1332_R or fRPB2-7cF/fRPB2-7cR pairs amplified a single band of approximately 900 or 1000 bp, respectively (Fig. 6-4-1). These fragments were aligned with 693 or 847 bases respectively, and a part of these alignments was shown in Fig. 6-4-2.

Phylogenetic relationships

To visualize the relationships between the genetic distance and the mode of sexual reproduction, phylogenetic trees were constructed based on the β-tubulin and RPB2 sequences, respectively (Fig. 6-4-1). There was no significant correlation between the genetic distance and the mode of sexual reproduction, either homothallic or heterothallic, on the both sequences-based trees.

The both sequences-based trees were not able to divide into the two clades consisted of the respective species. In β-tubulin sequence-based tree, a single *M. majus* isolate TKOB4 belonged to exceptionally the clade consisted of *M. nivale* isolates. In RPB2 sequence-based tree, three *M. majus* isolates, K015, T129 and T162, belonged to exceptionally the *M. nivale* clade.

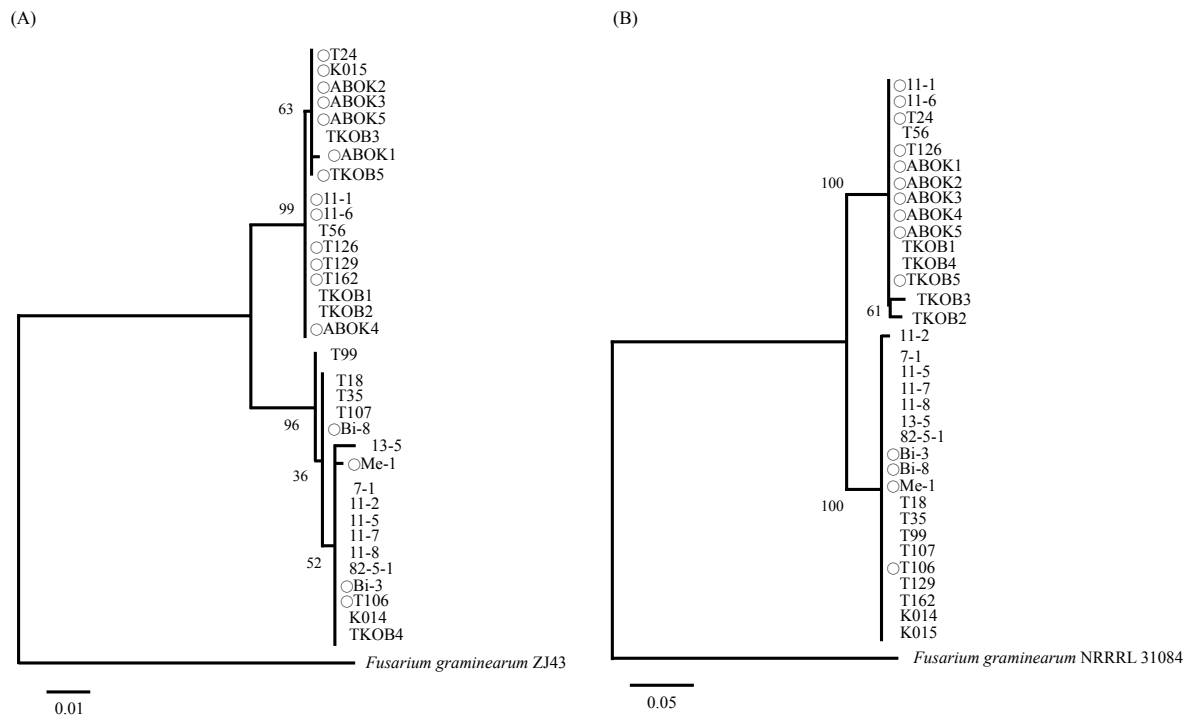


Fig. 6-4-1 Phylogenetic trees 34 isolates of *Microdochium majus* and *Microdochium nivale* based on the (A) β-tubulin and (B) RPB2 sequences with ML method.

Homothallic isolates were indicated by circle.

6-1. Discussion

In this chapter, I investigated the mode of sexual reproduction, either heterothallic or homothallic, and evaluated the possibility of the interspecific hybridization between isolates of *M. majus* and *M. nivale.* Both *M. majus* and *M. nivale* are members of ascomycetes and produce perithecia through the process of sexual reproduction. It is considered that homothallic isolates can produce perithecia by itself whereas heterothallic isolates require a partner of the complementary mating type for sexual reproduction. According to the test for confirming whether isolates of each species are self-fertile, 75.0% of *M. majus* isolates produced perithecia, which was higher than that (31.3%) of *M. nivale.* This finding suggests that both *M. majus* and *M. nivale* can reproduce homothallically. There was, however, a difference in the proportion of the mode of predominant sexual reproduction between the two species. In short, most of *M. majus* isolates was homothallic (self-fertile) whereas most of *M. nivale* isolates was heterothallic (self-sterile). These results were consistent with the previous report by Lee *et al.* (1995) who showed that 24 of 38 isolates of *M. majus* and six of ten isolates of *M. nivale* produced perithecia by itself *in vitro*. These findings, therefore, suggested that both *M. majus* and *M. nivale* had a potential to reproduce homothallically. In β-tubulin and RPB2 sequences-based trees, no significant correlation was found between the genetic distance and the mode of sexual reproduction, homothallic or heterothallic.

In the comparison of the mean size of perithecia, the perithecium of *M. majus* was bigger and more variable than that of *M. nivale*. However, it would be difficult to distinguish visually between isolates of *M. majus* and *M. nivale* under a microscope because there was an overlapping range of the size of the two species. On the other hand, Hernández-Restrepo *et al.* (2016) reported that there was no difference in the size of perithecia between *M. majus* and *M.*

nivale, which were 300 × 170 μm (height × width). This discrepancy might have caused because the genetic variation among individuals originating from different geographic regions.

18 self-sterile isolates, six isolates of *M. majus* and 11 isolates of *M. nivale*, were crossed each other in all possible combinations within respective species. 93.3% of all combinations of six isolates in *M. majus* produced perithecia more frequently than that (23.6%) of all combinations of 11 isolates in *M. nivale.* This result suggests that there might be more opportunities of sexual reproduction in *M. majus* than *M. nivale* in nature because *M. majus* have a higher rate of production of perithecia in either alone or crossing. If so, the difference in sexual reproduction between the two species may lead to our finding that *M. majus* was the dominant species regardless of the origin of host tissues.

Sexual reproduction in ascomycetes is controlled by two alleles, *MAT-1-1-1* and *MAT-1-2-1* (Coppin *et al.*, 1997: Turgeon, 1998). However, Jewell *et al.* (2013) reported that *MAT1-1-1* was not found in any sequences of *M. majus* and *M. nivale*. This study confirmed whether each isolate carried *MAT1-2-1* allele using PCR amplification with primers Mic_MAT2_198F and Mic_MAT2_676R pair. Amplified fragments, approximately 500 bp, of *MAT1-2-1* allele were found in all isolates of *M. nivale* and *M. majus*, regardless of either self-fertile or self-sterile. Surprisingly, matured perithecia were also obtained by crossing of self-sterile isolates of *M. majus* or *M. nivale* that carried *MAT1-2-1* allele each other, respectively. Similar results have been reported in several *Glomerella* species; for example, *G. graminicola* (Vaillancourt *et al.*, 2000), *G. lindemuthiana* (Rodríguez-Guerra *et al.*, 2005) and *G. truncata* (Menat *et al.*, 2012). These atypical mating, which was called “unbalanced heterothallism” were firstly proposed by Wheeler (1954), who explained the unbalanced heterothallism that each partner of a fertile cross carried mutated fertility genes that could

complement each other. Cisar & TeBeest (1999) also suggested that the unbalanced heterothallism was regulated by a single mating-type locus with multiple alternate alleles through the mating among self-sterile isolates of *G. cingulate*. On the other hand, Vaillancourt *et al*. (2000) suggested that the unbalanced heterothallism was controlled by two unlinked loci because the presence of the recombinant progeny, which could not cross with either parent, were identified by the backcross with their parents. In either hypothesis, there was, however, no molecular evidence for genetic mechanisms that controled the unbalanced heterothallism.

Interspecific hybridization was observed in 58 of all 66 combinations of *M. majus* and *M. nivale.* To the author's knowledge, this study was the first report that proved interspecific hybridization between *M. majus* and *M. nivale* in the crossing tests using self-sterile isolates of the two species. There is a possibility that interspecific hybridization between the two species occurs in natural because both species have been isolated from a population of the infected grains (session 4-2 in CHAPTER IV). Furthermore, this result indicates there might be no reproductive isolation between two *Microdochium* species. However, the presence of perithecia without matured ascospore was observed at high frequency in the crossing test between the two species. In natural, therefore, the frequency of interspecific hybridization was considered to be low.

In conclusion, this chapter demonstrated that *M. majus* had the opportunity for sexual reproduction compared to *M. nivale* in either alone or crossing. Furthermore, interspecific hybridization between *M. majus* and *M. nivale* was proved in the crossing tests using self-sterile isolates of the two species. These findings showed that sexual reproduction played a significant role in the development of strobilurins resistant strains within populations of *M. majus* and *M. nivale*.

CHAPTER VII
GENERAL DISCUSSION & CONCLUSIONS

FHB is an economically devastating disease of wheat and in warmer regions is often caused by *F. graminearum* and other *Fusarium* spp., whereas *Microdochium* spp. are tended to be the dominant causal agents in the cooler regions. (Daamen *et al.*, 1991; Parry *et al.*, 1995). In addition, *Microdochium* spp. cause pink snow mold, resulting in seedlings with brown lesions on roots and leaves of winter cereals (Millar & Colhoun, 1966). Despite a potential to cause significant damage to the production of wheat, there is little information on biology, epidemiology or ecology of *Microdochium* spp. compared with *Fusarium* spp associated with FHB. In this study, we have focused on the control of *Microdochium* spp. and aimed at obtaining the significant findings on their biology, ecology and genetics. Firstly, I developed LATTS medium, the selective medium for isolation of *Microdochium* spp., to obtain epidemiological information on the numbers of airborne spores as the primary FHB inoculum. According to the spore trapping using the LATTS medium, it was highly effective for selective detection of airborne spores of *Microdochium* spp. Furthermore, there are differences in the dynamics of spore dispersal between *M. majus* and *F. graminearum*; for example, the period of spore dispersal of *M. majus* was earlier than that of *F. graminearum*, and the number of spores trapped of *M. majus* was more abundant more than that of *F. graminearum.* These findings would be useful for effective control of FHB in cooler regions. The LATTS medium could distinguish the two species, *M. nivale* and *M. majus,* based on the morphology of their colonies. This unique characteristic might be useful for investigation of the isolation frequency of *M. majus* and *M. nivale* isolates obtained from air trapping, infected seeds and crop residues.

Several researchers reported that there were differences in the isolation frequency between *M. nivale* and *M. majus*. For example, the higher frequency of isolation of *M. majus* from grain was observed (Lees *et al*., 1995; Parry *et al.*, 1995), whereas *M. nivale* was the

dominant species in symptoms of pink snow mold (Terami & Kamikawa, 2006). According to the investigation of the distribution and frequency of the two species, the majority of isolates tested were identified as *M. majus*, regardless of the origin of host tissues. Furthermore, according to the mating test, *M. majus* had a high opportunity for sexual reproduction compared to *M. nivale* although both species can reproduce homothallically. These results indicated that there was a difference in the proportion of the mode of predominant sexual reproduction, either homothallic or heterothallic, between the two species. The differences in sexual reproduction might explain the higher frequency of isolation of *M. majus*, regardless of the origin of host tissues. For a deeper understanding, it would be needed further study about seasonal distribution and population dynamics of the each species in hosts or fields.

It was already reported that several plant pathogenic fungi showed the less effectiveness of strobilurins (Sierotzki *et al.*, 2000; Steinfeld *et al.*, 2001; Fraaije *et al.*, 2005; Chen *et al.*, 2007; Leroux *et al.*, 2007). A decrease of efficiency of strobilurins was firstly reported in wheat fields in France (Walker *et al.*, 2009) and was also identified in Hokkaido, Japan (Kozawa *et al.*, 2011). According to the test of strobilurins sensitivity *in vitro*, the levels of strobilurins resistance of *Microdochium* spp. were divided into the two resistant groups, Str R and Str r. All strobilurins-resistant strains but isolate T35 have been found that there was the G143A mutation in *CYTB* gene. The highly strobilurins-resistant strain T35 had neither G143A mutation nor the F129L and G137R mutations. The atypical mechanism of strobilurins-resistance was reported in several plant pathogenic fungi (Ishii *et al.*, 2001; Steinfeld *et al.*, 2001, Fernández-Ortuño *et al.*, 2008), and has been found in a single isolate 71 of *M. majus* in French populations. To the author's knowledge, this study is the first report

on the occurrence of the resistant *M. nivale* isolate without the typical amino acid mutations related to QoI-resistance.

According to the comparison of fitness between the strobilurins sensitive and resistant strains, there was no significant difference in fitness parameters, such as the mycelial growth, conidia germination, and sporulation ability. This study, therefore, proved that strobilurins resistance of *Microdochium* spp. was not associated with the fitness cost. Moreover, strobilurin resistance of *M. nivale* and *M. majus* was not significantly reduced from the first generation to the fifth generation that were grown and preserved on a strobilurin-free medium. It suggested that strobilurins resistance of *M. nivale* and *M. majus* was stable in the absence of the fungicide. Therefore, there will be a need to continue the monitoring of spore dispersal to confirm the frequency of the resistant strains.

A difference of the mode of sexual reproduction between the two species might have the effects on the levels of development of resistant strains within populations. Several studies reported that there was no obvious difference in fungicide sensitivity between the two species (Glynn *et al.*, 2008; Walker *et al.*, 2009). Several researchers reported that *M. nivale* had a high number of polymorphisms within individuals (Lees *et al.*, 1995; Parry *et al.*, 1995) whereas *M. majus* has a high level of genetic uniformity (Lees *et al.*, 1995). These genetic findings indicated that *M. nivale* could reproduce heterothallically whereas *M. majus* generally reproduced homothallically in nature, which was also supported by our findings based on the mating test *in vitro* as well as genetic analyses using ISSR fingerprinting and *CYTB* sequences. According to the test for confirming whether isolates of each species were self-fertile, 75.0% of *M. majus* isolates produced perithecia by itself, which showed higher than that (31.3%) of *M. nivale.* Moreover, 93.3% of all combinations of six heterothallic isolates in *M. majus* produced perithecia, which was more frequently than that (23.6%) of all

combinations of 11 heterothallic isolates in *M. nivale.* Theses results suggested that *M. majus* isolates had many opportunities for sexual reproduction compared to *M. nivale* isolates in nature. The rapid development of the strobilurins resistant strains in a short period might be due to many opportunities of sexual reproduction in populations of *M. majus*. Furthermore, *CYTB* sequence and ISSR fingerprinting-based trees suggested that the strobilurins-resistant strains tested emerged independently through a single amino acid mutation (G143A), occurred in sensitive strains, and then rapidly developed through high gene flow. The parallel evolution of the resistant strains was also reported in QoI-resistant isolates in populations of *Mycosphaerella graminicola* with a high gene flow (Torriani *et al.*, 2008). In this study, both *CYTB* sequence and ISSR fingerprinting analyses showed that there was a high gene flow in populations of *M. majus*. In the case of a particular phenotype such as fungicide resistant strains having a chance for survival in extraordinary environments, asexual reproduction could gain the advantage in spreading rapidly in populations compared with sexual reproduction because it may be suitable for increasing the frequency of acquired mutations. Therefore, the combination of sexual and asexual reproduction would play a significant role in the rapid development of the strobilurins resistant strains in *M. majus* populations.

A significant advantage of sexual reproduction is to be able to generate the progeny with novel phenotypes that their parents do not possess, through mating with others that have a different genetic background. Understanding the sexual reproduction is necessary to clarify the ecologic, epidemic and genetic characteristics of plant disease pathogens, information of which would lead to the effective control of plant disease. Interspecific hybridization between *M. majus* and *M. nivale* was proved by crossing self-sterile isolates of the two species. These results indicated that interspecific hybridization might have a role as means for the development of strobilurins resistant strains within populations of *M. majus* and *M. nivale*.

However, the frequency of interspecific hybridization was considered to be low because the presence of perithecia without matured ascospore was observed at high frequency in the crossing test between the two species.

In this study, I obtained much new knowledge about biology, ecology and genetics of *M. nivale* and *M. majus*. These findings could be useful for the improvement of FHB management and would lead to the effective control of FHB worldwide.

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