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FULL PAPER

Internal transcribed spacer haplotype diversity and their geographical distribution in *Dasyscyphella longistipitata* (Hyaloscyphaceae, Helotiales) occurring on *Fagus crenata* cupules in Japan

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Abstract Phylogeographic analysis of *Dasyscyphella longistipitata* (Hyaloscyphaceae, Helotiales), which occurs on decaying *Fagus crenata* cupules, was carried out. The ITS-5.8S regions of the 120 isolates from 12 sites in areas across Japan revealed 38 haplotypes, the majority of which comprised the haplotype designated H12 (42.5%). H12 was found in isolates from all sites, followed by H28 and H10, which were detected in isolates from 10 and 7 sites, respectively. Thirty-two haplotypes were obtained in single isolates. In the haplotype network, H12 was the root, and it formed interior clades with H28 and H10. Genetic diversity was higher in northern and southern Japan, but genetic distance was not correlated with geographical distance, nor with the phylogenetic clades of *F. crenata*. Therefore, it was concluded that *D. longistipitata* forms a genetic continuum that covers all of the areas in which it is distributed in Japan, with variations being generated in local populations from the major haplotypes.

Keywords Genetic diversity · Genetic structure · Haplotype network · Host distribution · Phylogeography

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Introduction

Although fungi are fundamentally microscopic organisms that are not easily detected by the naked eye, they have their own geographical distributions, like plants and animals (Arnolds 1997). The limiting factors of a fungal distribution are climate, host selectivity, geographical history, etc. Distribution also depends on the mode of nutrition and dispersal mechanisms (Wicklow 1981). For example, climatic factors (temperature, precipitation) are the major factors to limit the distributions of the saprophytic fungi that grow on various substrata (e.g., Tokumasu 2001). On the other hand, fungi with host selectivity largely depend on the distributions of their hosts.

Dasyscyphella longistipitata Hosoya (Hyaloscyphaceae, Helotiales) is a fungus that forms macroscopic apothecia on decaying *Fagus crenata* cupules in spring (May–June). Although *D. longistipitata* was described recently (Ono and Hosoya 2001), it has been known for years in Japan (e.g., Imazeki and Hongo 1989), but only from *F. crenata*, indicating its substrate specificity. The distribution of *D. longistipitata* is presumed to be identical to that of its host, because we have collected *D. longistipitata* across Japan, from south Hokkaido to southern Kyushu. *Dasyscyphella longistipitata* can easily be identified based on several characteristic morphological features, and is easily culturable in artificial media (Ono and Hosoya 2001).

Because *F. crenata* is a species endemic to Japan, its distribution and phylogeographical background have been studied (Kurata 1964; Murai et al. 1991). *F. crenata* populations are distributed continuously across northeastern areas, while they become more sparsely distributed in southwestern areas (Fig. 1). Tsukada (1982a, b) postulated that *F. crenata* was distributed along the shore line in southern areas at 38°N at the end of the last glacial period (refugia). Tomaru et al. (1997) indicated that the genetic diversity of 11 loci encoding nine enzymes is greater in southwestern areas than in northeastern areas. They postulated that this difference was due to the ancestry of the southwestern populations. Based on chloroplast DNA analysis, Fujii et al. (2002) elucidated that *F. crenata* in Japan comprises three clades: clade I, distributed along the coast of the Sea of Japan and extending to the Pacific region in central Japan; clade II, distributed along two areas of the Kii peninsula and along the Pacific coast in northeastern Japan; and clade III, found mainly in Kyushu and Shikoku as well as southeastern Japan (Fig. 1). Based on the genetic structure, Fujii et al. (2002) postulated that *F. crenata* expanded from the refugia to the northern area along two routes, leading to two major phylogenetic clades. It is also hypothesized that *F. crenata* started to expand its distribution into mid- and northeastern Japan about 12,000–10,000 years ago along two routes, one along the western and the other along the eastern sea shore, and the present distribution was established about 6,000 years ago (Tsukada 1982a, b). It is worth examining if this historical background of the host affected the genetic diversity of the ecologically closely related fungus.

Genetic analyses of populations of an organism not only elucidate its distribution patterns, but they also help when determining factors that affect this distribution (Anderson and Kohn 1995; James et al. 1999, 2001; Carbone and Kohn 2001; Johannesson et al. 2001; Iwamoto et al. 2005; Franzén et al. 2007). Based on the background information described above, this paper presents a qualitative and quantitative analysis of the genetic diversity of internal transcribed spacer (ITS) regions of *D. longistipitata* populations, and discusses its genetic structure in relation to its host. The ITS region was selected for evaluation because (1) ITS is known to be the barcode region that contains genetic polymorphisms which can resolve intra- and interspecies rank, and (2) techniques have already been established for amplification and sequencing (White et al. 1990).

Materials and methods

Collection and isolation

Samples were collected at 12 sites covering a range of areas within the distribution of *F. crenata*, and included three phylogenetic clades of *F. crenata* (Fujii 2002) in Japan during 2005–2007 (Table 1). Five to 20 cupules with apothecia of *D. longistipitata* were collected at least 5 m

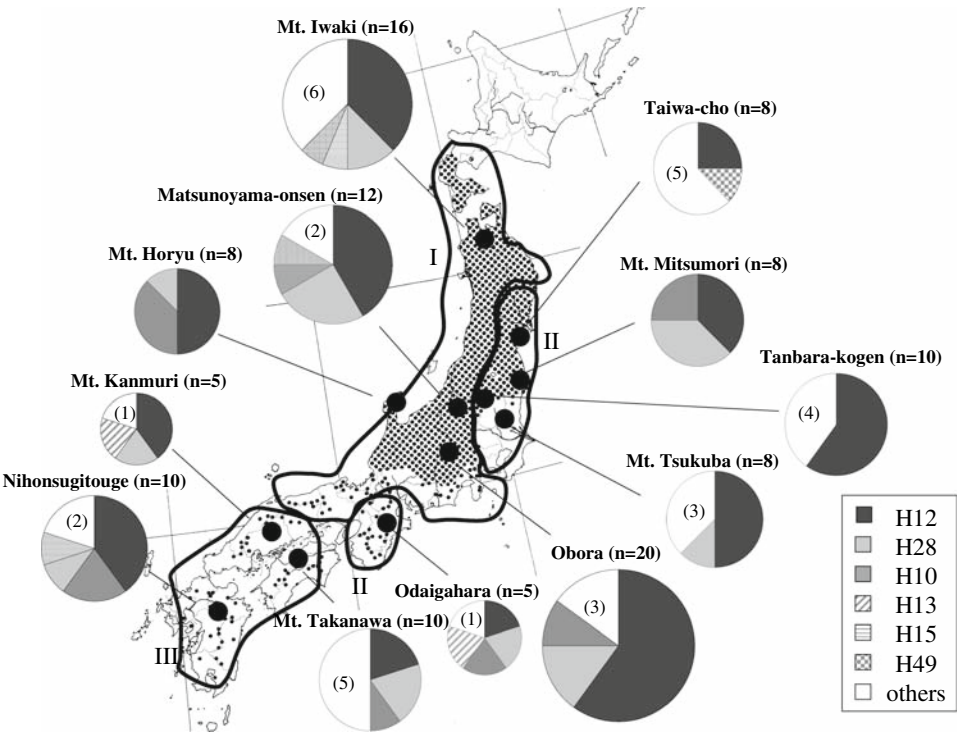

Fig. 1 Haplotype diversity in *Dasyscyphella longistipitata* populations sampled across Japan. Relative abundances of the detected haplotypes are shown in the *pie chart*. Haplotypes obtained from more than two sites (*H10*, *12*, *13*, *15*, *28* and *49*) are indicated by *colored sectors*. Site-specific (obtained only once) haplotypes are indicated by *white sectors*, and their numbers are indicated in *parentheses*. The radius of the chart is proportional to the number of isolates examined. *Dotted areas* on the map show the distribution of *Fagus crenata* (after Kurata 1964). The phylogenetic clades elucidated by Fujii et al. (2002) are indicated by areas surrounded by curves with *Roman letters* (*I*, *II*, and *III*)

Table 1 Sampling sites for *Fagus crenata* cupules with Dasyscyphella longistipitata and geographical and phylogenetic information for them

Site #	Site	Clade[a]	Latitude	Longitude	Altitude (m)	Sampling date
1	Mt. Iwaki, Hirosaki-shi, Aomori Pref.	I	40°39′1.7″N	140°16′23.7″E	777	24 May 2006
2	Taiwa-cho, Kurokawa-gun, Miyagi Pref.	II	38°28′N	140°41′E	1500	2 May 2007
3	Mt. Mitsumori, Yaguki, Yotsukura-machi, Iwaki-shi, Fukushima Pref.	II	37°11′37″N	140°54′36″E	640	29 April 2006
4	Matsunoyama-onsen, Matsunoyama, Tokamachi-shi, Niigata Pref.	I	37°6′5.3″N	138°37′12.5″E	340	13 May 2006
5	Mt. Tsukuba, Sakuragawa-shi, Ibaraki Pref.	II	36°13′48.4″N	140°6′29.9″E	684	2 May 2006
6	Tanbara highland, Numata-shi, Gunma Pref.	I	36°47′N	139°4′E	1300	22 May 2007
7	Mt. Horyu, Horyu-machi, Suzu-shi, Ishikawa Pref.	I	37°25′N	137°10′E	467	6 May 2006
8	Obora, Ueda-shi, Nagano Pref.	I	36°30′13.7″N	138°19′44.1″E	1314	16 May 2005
9	Odaigahara, Kamikitayama-mura, Yoshino-gun, Nara Pref.	II	34°10′58.6″N	136°6′10.4″E	1585	24 April 2006
10	Mt. Kanmuri, Yoshiwa, Hatsukaichi-shi, Hiroshima Pref.	III	34°28′N	132°4′E	1000	29 April 2006
11	Mt. Takanawa, Matsuyama-shi, Ehime Pref.	III	33°57′N	132°51′E	966	17 April 2007
12	Nihonsugitouge, Misato-cho, Shimomakishi-gun, Kumamoto Pref.	III	32°34′31″N	130°54′1″E	1100	10 May 2006

[a] Phylogenetic clade of *Fagus crenata* in Fujii et al. (2002)

apart from each other at each site. Single ascosporous cultures were obtained from each cupule using Skerman's micromanipulator (Skerman 1968). In the present paper, isolates obtained from a single site are defined as a local population. Dried herbarium specimens were preserved in the mycological herbarium of the National Museum of Nature and Science (TNS).

DNA extraction and sequencing

Isolates were cultivated in 2 ml of 2% malt extract for 2 weeks and the mycelia were harvested and frozen at −80°C. About 50 mg of mycelium were mechanically lysed by a Qiagen TissueLyser using ceramic beads. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada), following the manufacturer's instructions. Primer pairs ITS1 and ITS4 were used to amplify the internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal region (White et al. 1990).

DNA was amplified using 1 μl template DNA, 0.2 μM of each primer, 1 U of TaKaRa Ex Taq DNA polymerase (Takara, Tokyo, Japan), and a deoxynucleoside triphosphate (dNTP) mixture containing 2.5 mM of each dNTP, and ExTaq buffer containing 2 mM Mg^{2+}. The mixture was adjusted to 40 μl by dH_2O. Polymerase chain reaction (PCR) was carried out using a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The process involved 2 min of denaturing at 94°C, followed by 30 cycles of annealing at 55°C for 30 s, extension at 72°C for 1.5 min, denaturing at 94°C for 30 s, then a final extension at 72°C for 7 min.

Polymerase chain reaction products were purified using an ExoSAP-IT purification kit (USB Corporation, Cleveland, OH, USA). Total DNA samples were deposited in the Molecular Biodiversity Research Center in the National Museum of Nature and Science, and are available for research upon request. Sequencing was carried out using a BigDye Terminator v 3.1 Cycle Sequencing Kit on a DNA autosequencer (3130x, Applied Biosystems, Inc., Foster City, CA, USA), following the manufacturer's instructions. The sequence obtained was assembled and edited by SeqMan II (DNASTAR, Madison, WI, USA), and the congruence of the sequences obtained from both strands was confirmed. The obtained sequences representing each haplotype were deposited in GenBank, where they are registered as the conitunous series of registration numbers AB508099–AB508166.

Population genetic analysis

Haplotypes of *D. longistipitata* were numbered in order of their appearance in the analysis. To visualize the relationship between the haplotypes, a haplotype network was constructed using TCS1.21 (Clement et al. 2000) based on statistical parsimony (Templeton et al. 1992). ARLEQUIN ver. 3.1 (Excoffier et al. 2005) was used to carry out analyses of molecular variance (AMOVAs) within each local population and among the 12 populations in order to analyze the degree of genetic diversity. To evaluate genetic diversity within the local population, haplotype diversity (h) was calculated by Dnasp, version 4.20.2 (Rozas et al. 2003), according to the following equation (Nei 1987): $h = (2n/2n-1)\left(1-\sum f_i^2\right)$ where f_i is the frequency of the ith haplotype and n is the number of samples.

To analyze the relationship between genetic distance and geographical distance, Slatkin's genetic distance (Slatkin 1995) was calculated using ARLEQUIN ver. 3.1 (Excoffier et al. 2005). Cluster analysis based on the

unweighted pair-group method with arithmetic mean analysis (UPGMA) was carried out using MEGA 4 (Tamura et al. 2007).

Results and discussion

Quantitative and qualitative analysis of the haplotype diversity

In the present paper, the prefix "H" has been added to each numbered haplotype for identification purposes. Sequencing resulted in the alignment of 490 base pairs. No differences were found in the 5.8S rDNA region; all of the haplotype diversity occurred in the ITS1 and ITS2 regions.

In total, 38 haplotypes were obtained from 120 isolates. H12 corresponded to 42.5% of the isolates, followed by H28 (15%), and H10 (10%) (Table 2; Fig. 1). H12 was found in all of the local populations. H28 and H10 were found in 10 and 7 local populations, respectively, out of the 12 populations. These three haplotypes comprised the majority in almost all of the local populations. In addition, H13 (3%) was found in all of the clades of *F. crenata* designated by Fujii et al. (2002). H15 (1.6%) and H49 (1.6%) were each obtained from two isolates, and the remaining 32 haplotypes were obtained from single isolates. No geographic pattern was found in the sequence variation.

On the other hand, the haplotype diversity (h) of *D. longistipitata* for the whole population was 0.79006. It was the lowest in Obora (0.63158) and the highest in Odaigahara (1.0000) (Fig. 2). Haplotype diversity tended to be higher in northeastern areas (Tohoku district) and southeastern areas of Japan, and could be approximated by the equation $y = 0.00093x^2 - 0.6893x + 13.595$ ($R^2 = 0.2677$), where x is the haplotype diversity and y is the latitude.

When the haplotype frequency was compared to the phylogenetic clades of *F. crenata* (Fujii et al. 2002), the trend in frequency (H12 > H28 > H10) was found to be identical, and the frequency did not differ in any of the two clades according to the χ^2 test (>0.05). In the haplotype network generated by TCS 2.1, H12 was designated as the root. The major haplotypes H12, H28 and H10 found in multiple sites formed interior clades, and they were thought to be more ancestral than the other haplotypes (Fig. 3). Most of the haplotypes obtained only once were positioned in one step away from the major three haplotypes, suggesting that they were derived from the major haplotypes.

AMOVA showed that the genetic variation was attributable to the variation within the population (Table 3), indicating no clear difference among populations. UPGMA revealed five clusters based on Slatkin's genetic distance (Fig. 4), and no relationship was found between genetic

Table 2 Summary of the haplotype diversity of *Dasyscyphella longistipitata* at each sampling site

Site #	Number of isolates	Number of detected haplotypes	Number of site-specific haplotypes	Haplotype number																																			
				12	28	10	13	15	49	19	21	22	23	25	26	31	32	37	45	50	1	52	34	35	36	18	53	54	55	30	43	44	48	29	51	56	57	61	62	59	60
1	16	10	6	6	2		1	1		1	1	1	1	1	1																										
2	8	7	5	2					1							1	1	1	1	1																					
3	8	3	0	3	3	2																																			
4	12	6	3	5	3	1		1													1	1																			
5	8	5	3	4	1																		1	1	1																
6	10	5	4	6																						1	1	1	1												
7	8	3	0	4	1	3																																			
8	20	6	3	12	3	2																								1	1	1									
9	5	5	1	1	1	1	1																										1								
10	5	4	1	2	1		1																											1							
11	10	8	5	2	2	1																													1	1	1	1	1		
12	10	6	2	4	1	2			1																															1	1
			Total	51	18	12	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

distance and geographical distance or phylogenetic clade of *F. crenata*. Therefore, it is concluded that *D. longistipitata* forms a genetic continuum that covers almost all of the areas in which it is distributed in Japan, with variations in local populations generated from the major haplotypes.

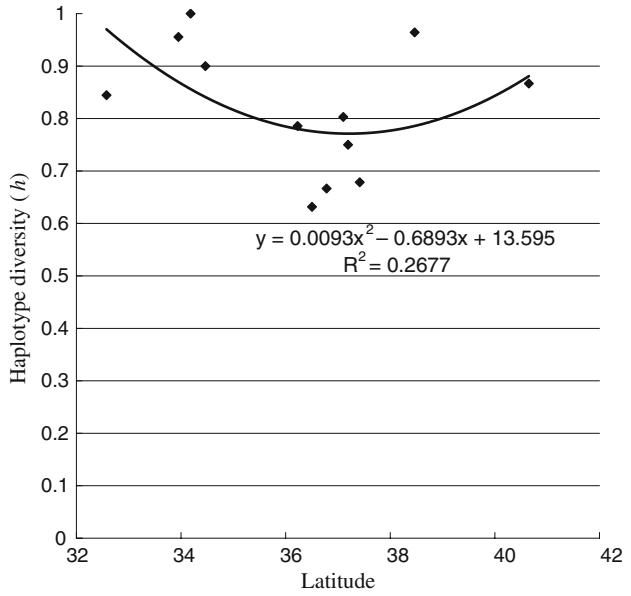

Fig. 2 Genetic diversity of *Dasyscyphella longistipitata* populations by latitude. The *x* axis shows the latitude, while the *y* axis shows the genetic diversity (*h*). Note that the approximate curve is higher in southern and northern Japan

Haplotype diversity in *D. longistipitata* with respect to its host distribution

Although a genetic structure based on three major haplotypes was found for *D. longistipitata*, as shown in Fig. 3, this does not directly explain how the evolution of the distribution of *D. longistipitata* relates to the distribution of *F. crenata*. However, an analysis of genetic diversity in relation to geographical factors may reflect the historical background. As suggested by Tomaru et al. (1997) and Fujii et al. (2002), *F. crenata* originated in southwestern Japan and expanded into northeastern areas. This was followed by a reduction in its distribution in central to southwestern areas. The number of site-specific haplotypes (Table 2) shows a similar trend to the haplotype diversity. The lower genetic diversity observed in mid-Japan populations (Fig. 2) may be explained by a bottleneck effect due to the reduced distribution of *F. crenata* in this region, while the high genetic diversity observed in southwestern Japan may be explained by the populations in this region being more ancestral. However, the proper genetic structure may not be represented in southwestern populations because populations with fewer samples are included

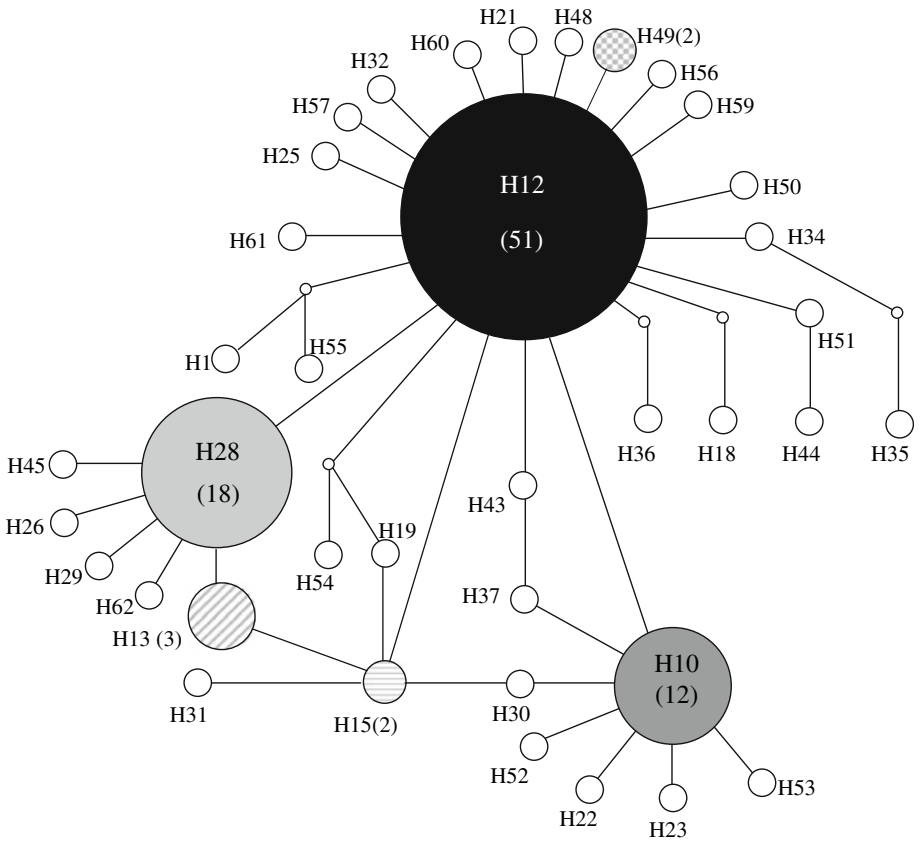

Fig. 3 Haplotype network of *Dasyscyphella longistipitata* based on ITS sequences. Radius of the circle is proportional to the numbers of isolates obtained, as shown in the *parentheses*. *Small circles* are hypothesized haplotypes. Note that the major haplotypes (*H12*, *28*, and *10*) are placed in the *inner clades*

Table 3 Analysis of molecular variance (AMOVA) of ITS sequence variation for *Dasyscyphella longistipitata* populations

	df	Sum of squares	Variance components	Percentage of variation	*P* value
Among populations	11	7.638	0	0	0.49
Within populations	108	75.471	0.698	100	
Total	119	83.108	0.698	100	

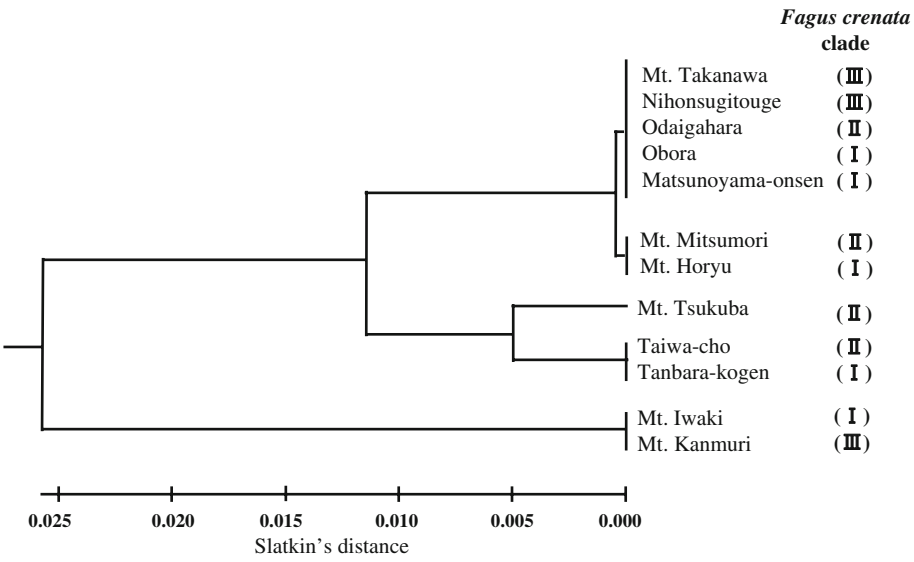

Fig. 4 A cladogram generated by UPGMA based on genetic distance among the local populations of *Dasyscyphella longistipitata*, supplemented with the phylogenetic groups of *Fagus crenata*

(Table 2). Further analysis based on increased numbers of samples and new markers with high evolutionary rates, e.g., the IGS region (James et al. 2001) or EF-1α (Carbone and Kohn 2001), may improve our knowledge of how the genetic structure of *D. longistipitata* depends on its historical background.

Iwamoto et al. (2005) reported that areas close to each other showed similar genetic structures of *Thysanophora penicillioides* (Roum.) W. B. Kendr, an *Abies* leaf-inhabiting hyphomycete, suggesting gene flow between geographically close areas via conidia. *Thysanophora penicillioides* produces conidia by asexual production repeatedly under suitable conditions at all times of the year, and the conidia are dispersed by the wind. The high mobility of *T. penicilloides* contributed to the formation of the current genetic structure. In contrast, the genetic diversity of *D. longistipitata* is relatively site-limited (Table 2). According to studies of cultures, there is no anamorph of *D. longistipitata*, so long-distance distribution solely depends on ascospore dispersal. Although the ascospores are as minute as those in some hyphomycetes, the limited fruiting period may limit the dispersal ability of *D. longistipitata*. Sexual reproduction also contributes to the diversification of the haplotypes based on recombination.

The haplotype diversity in ITS of *D. longistipitata* (38 haplotypes in 120 isolates) exceeds those reported for other ascomycetes or their anamorphs, such as *T. penicilloides* (20 in 347; Iwamoto et al. 2005) or *Tuber melanosporum* Vittad. (10 in 188; Murat et al. 2004). The variation was restricted to a relatively narrow range, usually with one substitution from the major haplotypes (H10, 12, and 28), and they differed by only five nucleotides at the most from each other. These facts suggest that the derivation occurred relatively recently. This is also supported by the fact that no clear morphological differences in ascospore and ascus morphology were observed among populations (data not shown).

Analysis based on ITS showed that *D. longistipitata* forms a genetic continuum in Japan, but it also suggested genetic heterogeneity. Using genes with faster evolutionary rates, genetically discontinuous groups may be found in sparsely distributed areas, which would suggest geographical isolation in terminal populations.

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