Full Paper

ITS haplotypes 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. ITS-5.8S region of the 120 isolates from 12 sites in wide areas in Japan revealed 38 haplotypes, the majority of which comprised the haplotype designated H12 (42.5%). H12 was found from all sites, followed by H28 and H10, detected from 10 and 7 sites, respectively. Thirty-two haplotypes were obtained for single isolates. In the haplotype network, H12 was the root, and 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 is concluded that *D. longistipitata* forms a genetic continuum that covers all the distributed areas in Japan, generating variations in their local populations from their major haplotypes.

Key Words Genetic diversity, Genetic structure, Haplotype network, Host distribution, Phylogeography

Introduction

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

56 *Dasyscyphella longistipitata* Hosoya (Hyaloscyphaceae, Helotiales) is a fungus that forms
57 macroscopic apothecia on decaying *Fagus crenata* cupules in spring (May to June). Although
58 *D. longistipitata* was described recently (Ono and Hosoya 2001), it has been known for years
59 in Japan (e. g., Imazeki and Hongo 1989) only from *F. crenata*, indicating its substrate
60 specificity. The distribution areas of *D. longistipitata* is presumed to be identical to that of its
61 hosts, because we have collected *D. longistipitata* widely in Japan from south Hokkaido to
62 southern Kyushu. *Dasyscyphella longistipitata* can be easily identified based on several
63 characteristic morphological features, and easily culturalble in artificial media (Ono and
64 Hosoya 2001).
65 Because *F. crenata* is an endemic species in Japan, the distribution and its
66 phylogeographical background has been studied (Kurata 1964; Murai et al. 1991). The *F.*
67 *crenata* populations distribute continuously in northeastern areas, while in southwestern
68 areas becoming more sparsely distributed (Fig. 1). Tsukada (1982a, b) postulated *F. crenata*
69 distributed along the sea shore in southern area at 38°N in the end of the last glacial
70 period (refugia). Tomaru et al. (1997) indicated that genetic diversity of 11 loci encoding
71 nine enzymes is increased in southwestern areas than those in northeastern areas. They
72 postulated this difference because of the ancestry of southwestern populations. Based on
73 chloroplast DNA analysis, Fujii et al. (2002) elucidated that *F. crenata* in Japan comprises
74 three clades: clade I distributed along coast of the Japan sea, and extending to the Pacific
75 region in central Japan; clade II distributed along two areas of Kii peninsula and along the
76 Pacific ocean coast in north east Japan; clade III distributed mainly in Kyushu, Shikoku
77 islands and southeast Japan (Fig. 1). From the genetic structure, Fujii et al. (2002) postulated
78 that *F. crenata* expanded from the refugia to the northern area along two routes, deriving two
79 major phylogenetic clades. It is also hypothesized that *F. crenata* started to expand its
80 distribution to mid- to northeastern Japan about 12,000 to 10,000 years ago along two
81 routes, one along the western and the other along eastern sea shores, and the present

distribution was established about 6,000 years ago (Tsukada 1982a, b). It is worthwhile to examine if this historical background of the host affected the genetic diversity of the ecologically closely related fungus.

Genetic analyses of the population not only elucidate distribution patterns of the organisms, but also contributes to estimate factors affecting their distribution (Anderson et al. 1995; James et al. 1999; Carbone and Kohn 2001; James et al. 2001; Johannesson et al. 2001; Iwamoto et al. 2005; Franzén et al. 2007). Based on the background above, this paper presents qualitative and quantitative analysis of genetic diversity of internal transcribed spacer regions (ITS) 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 which contains genetic polymorphisms that have resolution of intra- to inter- species rank, and 2) techniques are already established for amplification and sequencing (White et al. 1990).

Materials and methods

Collection and isolation

Samples were collected at 12 sites covering wide areas of *F. crenata* distribution to include 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 apart from each other in 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 is defined as a local population. Dried herbarium specimens were preserved in the mycological herbarium of 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 –80C. About 50 mg of mycelium was mechanically lysed by Qiagen TissueLyser using ceramic beads. DNA was extracted using DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, Ontario, Canada) following the manufacturer's instruction. To amplify internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal region, primer pairs ITS1 and ITS4 (White et al. 1990) were used.

DNA was amplified using 1 μl template DNA, 0.2 μM of each primer, 1 unit of TaKaRa Ex Taq DNA polymerase (Takara, Tokyo, Japan), and 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, California, USA). The process involved 2 minutes of denature at 94C, followed by 30 cycles of annealing at 55C for 30 seconds, extension at 72C for 1.5 minutes, denature at 94C for 30 seconds, then final extension at 72C for 7 minutes.

PCR products were purified using a ExoSAP-IT purification kit (USB Corporation, Cleveland, Ohio, USA). Total DNA samples were deposited in the Molecular Biodiversity Research Center in National Museum of Nature and Science and available for research upon request. Sequencing was carried out using BigDye Terminator v 3.1 Cycle Sequencing Kit on a DNA auto sequencer 3130x (Applied Biosystems, Inc., Foster City, California, USA) following the manufacturer's instructions. The obtained sequence was assembled and edited by SeqMan II (DNASTAR, Wisconsin, USA), and the congruence of sequences obtained from both strands were confirmed. The obtained sequences representing each haplotypes were deposited to GenBank, registrated as AB508099 to AB508166 in continuous registration number.

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135 Population genetic analysis

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137 Haplotypes of *D. longistipitata* were numbered in the order of their appearance in the
138 analysis. To visualize the relationship between the haplotypes, a haplotype network was
139 constructed using TCS1.21 (Clement et al. 2000) based on statistical parsimony (Templeton et
140 al. 1992). ARLEQUIN ver. 3.1 (Exocoffier et al. 2005) was used to carry out for analysis of
141 molecular variance (AMOVA) within each local population and among 12 populations to
142 analyze the degree of genetic diversity. To evaluate genetic diversity within the local
143 population, haplotype diversity (h) was calculated by Dnasp version 4.20.2 (Rozas et al. 2003)
144 according to the following equations: $h = (n/n\text{-}1)(1\text{-}\Sigma f_i^2)$ (Nei 1987) where f_i is the frequency of
145 the ith haplotype and n is the number of samples.
146 To analyze the relationship between genetic distance and geographical distance, Slatkin's
147 genetic distance (Slatkin 1995) was calculated using ARLEQUIN ver. 3.1 (Exocoffer et al.
148 2005). Cluster analysis based on unweighted pair-group method with arithmetic mean
149 analysis (UPGMA) was carried out using MEGA 4 (Tamura et al. 2007).

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151

152 **Results and discussion**

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154 Quantitative and qualitative analysis of the haplotype diversity

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156 In the present paper, prefix "H" is added to each numbered haplotype for identification.
157 Sequence resulted alignments of 490 base pairs. No difference was found in 5.8S rDNA region,
158 and all the haplotype diversity was found in the ITS1 and ITS2 regions.
159 In total, 38 haplotypes were obtained from 120 isolates. H12 consisted of 42.5% of all the

160 isolates, followed by H28 (15%), and H10 (10%) (Table 2, Fig. 1). H12 was found from all the
161 local populations. H28 and H10 were found from 10 and 7 local populations, respectively, out
162 of 12 populations. These three haplotypes comprised the majority in almost all the local
163 populations. In addition, H13 (3%) was found from all the clades of *F. crenata* designated by
164 Fujii et al. (2002). Two isolates were obtained for H15 (1.6%) and H49 (1.6%), respectively, and
165 the remaining 32 haplotypes were obtained from single isolates. No geographic pattern was
166 found in the sequence variation.
167 On the other hand, haplotype diversity (*h*) of *D. longistipitata* of whole the population
168 was 0.79006, being the lowest in Obora (0.63158) and highest in Odaigahara (1.0000)(Fig. 2).
169 Haplotype diversity tended to be higher in northeastern areas (Tohoku district) and
170 southeastern areas of Japan, and could be approximated by the equation $y = 0.00093x^2 -$
171 $0.6893x + 13.595$ ($R^2 = 0.2677$) where x is haplotypes diversity and y is the latitude.
172 In the comparison of haplotype frequency in relation to the phylogenetic clades of *F.*
173 *crenata* (Fujii et al. 2002), the tendency of frequency (H12>H28>H10) was identical, and no
174 significance was recognized between the clade pairs by Chi square test (>0.05). In the
175 haplotype network generated by TCS 2.1, H12 was designated as the root. The major
176 haplotypes H12, H28 and H10 found from multiple sites formed interior clades, and they were
177 thought to be more ancestral compared to other haplotypes (Fig. 3). Most of the haplotypes
178 obtained only once were placed in one step away from the major three haplotypes, suggesting
179 their derivation from the major haplotypes.
180 AMOVA showed that the genetic variation was attributable to the variation within the
181 population (Table 3), indicating no clear difference among populations. UPGMA revealed five
182 clusters based on Slatkin's genetic distance (Fig. 4), and no relationship was found in genetic
183 distance and geographical distance, nor in the phylogenetic clades of *F. crenata*. Therefore, it
184 is concluded that *D. longistipitata* forms a genetic continuum that covers almost all the
185 distributed areas in Japan, generating variations in their local populations from their

major haplotypes.

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

Although the genetic structure based on three major haplotypes were clarified as in Fig. 3, it does not directly explain historical background in relation to the distribution of *F. crenata*. However, analysis of genetic diversity in relation to geographical factor may refrect the historical background. As suggested by Tomaru et al. (1997) and Fujii et al (2002), *F. crenata* originated in southwestern area in Japan, and expanded towards northeastern areas, followed by reduction of its distribution in central to southwestern areas. Number of site specific haplotypes (Table 2) also shows the similar tendency as the haplotypes diversity. The lower genetic diversity in mid- Japan populations (Fig. 2) may be explained by a bottleneck effect due to the reduction of the distribution areas of *F. crenata*, while the high genetic diversity in southwestern Japan may be explained that the populations in these sites are more ancestral. However, proper genetic structure may not be represented in southwestern populations because populations with fewer samples are included (Table2). Further analysis based on increased number of samples and new markers with high evolutionary rate, e.g. IGS region (James et al. 2001) or EF-1α (Carbone and Kohn 2001) may improve the resolution in genetic structure based on historical background.

Iwamoto et al. (2005) reported similarity of genetic structures in closer areas in *Thysanophora penicillioides* (Roum.) W. B. Kendr, an *Abies* leaf inhabiting hyphomycete, suggesting the gene flow among geographically close areas by distribution by conidia. *Thysanophora penicillioides* produces conidia by asexual production repeatedly under suitable conditions at all times of the year, and the conidia are dispersed by wind. The high mobility of *T. penicilloides* contributed to the formation of the current genetic structure. In contrast, the genetic diversity of *D. longistipitata* was relatively site limited

(Table 2). Based on cultural studies, an anamorph of *D. longistipitata* is lacking, hence long-distance distribution solely depends on ascospore dispersal. Although ascospores are as minute as those in some hyphomycetes, the limited fruiting period may set limits to the dispersal ability of *D. longistipitata*. Sexual reproduction also contributes to diversification of the haplotypes based on recombination.

The haplotype diversity in ITS of *D. longistipitata* (38 haplotypes out of 120 isolates) exceeds those reported for other ascomycetes or their anamorphs, such as *T. penicilloides* (20 out of 347; Iwamoto et al. 2005) or that in *Tuber melanosporum* (10 out of 188; Murat et al. 2004). The variation was restricted to relatively narrow range, having mostly one substitution from the major haplotypes (H10, 12, and 28), and differed only by five nucleotides at 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 were observed in ascospore and ascus morphology among populations (data not shown).

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

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309 **Figure legends**

310

311 **Fig. 1.** Haplotype diversity in *Dasyscyphella longistipitata* populations sampled in wide areas
312 in Japan. Relative abundances of detected haplotypes are shown in pie chart. Haplotypes
313 obtained from more than 2 sites (H10, 12, 13, 15, 28 and 49) are indicated by colored sectors.
314 Site specific (obtained only once) haplotypes were indicated by white sectors and their
315 numbers are indicated in the parenthesis. The radius of the chart is proportional to the
316 numbers of examined isolates. Dotted areas on the map show distribution of *Fagus crenata*.
317 Phylogenetic clades elucidated by Fujii et al. (2002) are indicated by areas surrounded by
318 curves with roman letters (I, II, and III)

319

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

323

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

328

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

332