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Genetic relationships in the natural population of *Pholiota nameko* from Japan based on DNA polymorphisms

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Abstract This study characterized the genetic relationships in the natural population of *Pholiota nameko* (Strophariaceae) from Japan based on the RFLPs of two regions of nuclear rDNA (ITS and IGS) and mitochondrial DNA and the RAPD profile of nuclear DNA. No intraspecific polymorphism in rDNA was found among 36 isolates of *P. nameko* used. By contrast, digests of mtDNAs by endonucleases *Hin*dIII and *Bgl*II produced RFLP patterns that distinguished all isolates except for 2, and clustered 36 wild isolates phenetically into three major similarity groups. However, these groups as obtained by analysis of mtDNA RFLPs did not reflect the geographic origin of the isolates. In RAPD analysis of nuclear DNA using three kinds of primers, every isolate showed its own distinct RAPD profile, but all isolates were clustered only into a large similarity group by phylogenetic analysis based on the RAPD profile. From these results, it is suggested that wild isolates of *P. nameko* distributed in Japan form a continuous genetic population that has conserved the genetic diversity.

Key words Genetic diversity · mtDNA · PCR-RFLP · RAPD · rDNA

Introduction

Pholiota nameko (T. Ito) S. Ito and Imai (Strophariaceae), commonly called "Nameko," is one of the most popular edible mushrooms in Japan. It has been cultivated since the 1930s, and its annual volume of commercial production was 1000 tons (fresh weight) in 2000, followed by *Lentinula edodes* (Berk.) Pegler, *Hypsizygus marmoreus* (Peck) Bigelow, *Flammulina velutipes* (Curt.: Fr.) Sing., and *Grifola frondosa* (Dicks.: Fr.) S.F. Gray. Because *P. nameko* has economic importance in Japan, efforts have been devoted to improvement of its cultivation procedure (Arita 1978) and to basic research on its cytology, genetics, physiology, etc. (Arita 1979). However, improvement of cultivars has received little attention. For example, it is speculated that most cultivars offered so far by the mushroom spawn suppliers were used without further improvement of the wild strains (Arita 1997). It is important, for breeding programs for the future market of cultivated *P. nameko*, to genetically improve various characteristics such as size, color, texture, and nutritional value. Thus, breeding programs introducing its wide genetic resources will have to be actively promoted.

Wild isolates of basidiomycetes are useful breeding materials that accumulate various genetic variations, and information on genetic relatedness among the wild isolates is necessary for their utilization for effective breeding. However, very little work has been performed on the genetic structure of the natural population of *P. nameko*. Previously, we surveyed the distribution of incompatibility factors, a gene cluster that controls sexual reproduction, in the natural population of this fungus in Japan, and found many cases with a common factor among the isolates collected in the different regions (Matsumoto et al. 1999). However, more work is needed to make a definitive argument on the genetic structure of *P. nameko* natural populations in Japan.

Molecular approaches are now considered to be powerful tools to investigate inter- and intragenetic relatedness of species of basidiomycetes, supporting results obtained from conventional methods such as morphological, physiological and biochemical studies. Many different molecular approaches have been developed for genetic analysis of various organisms. The technique of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to analyze nuclear ribosomal DNA (rDNA) has become increasingly popular for the identification and phy-

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logenetic studies of genera or species in many fungi (Gardes et al. 1990; Vilgalys and Gonzalez 1990; Henrion et al. 1994). The coding regions of the rDNA are usually found in multiple copies with regions that are very highly conserved. Furthermore, the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions of the rDNA are highly variable, thus permitting assessment of genetic variation at the intraspecific level using RFLP analysis (Hintz et al. 1989; Walsh et al. 1990; Bruns et al. 1991; Vilgalys and Sun 1994; Hibbett et al. 1995; Iraçabal et al. 1995). For population genetics, the use of RFLP of mitochondrial DNA (mtDNA) has become increasingly popular because of its rapid rate of sequence divergence (Taylor 1986). RFLPs in mtDNA have been used to estimate the genetic relationships among natural populations in various taxa including basidiomycetes (Anderson et al. 1987; Fukuda et al. 1994; Matsumoto and Fukumasa-Nakai 1995). Another approach for genetic diversity analyses has been the use of the technique of random amplified polymorphic DNA (RAPD), resulting in amplification of DNA from the entire genome based on arbitrary 10-base oligonucleotide primers (Welsh and McClelland 1990; Williams et al. 1990). RAPD analysis has become increasingly popular to explore genetic variability in basidiomycetes (Khush et al. 1992; Chiu et al. 1996) and other fungi (Francis et al. 1994; Munau et al. 1998; Morris et al. 2000). The RAPD technique permits amplification of all genomic DNA, so it may provide a better representation of the genome structure than analysis targeting a single locus.

In this study, we used DNA polymorphic markers such as PCR-RFLP of nuclear rDNA (ITS and IGS), mtDNA RFLP, and RAPD to investigate the genetic relationships within natural populations of *P. nameko* across its geographic range in Japan.

Materials and methods

Isolates used

Thirty-six wild isolates of *P. nameko* from different regions in Japan (Fig. 1) were used in this study. Detailed description of each isolate has been given previously (Matsumoto et al. 1999). Cultures of all isolates were deposited in the Tottori Mycological Institute Culture Collection (TMIC), and before use, the absence of dedikaryotization (Arita 1979) was verified.

DNA preparation

Fungal isolates were cultured for 2 weeks at 25°C on MYG liquid medium (2% malt extract, 0.2% yeast extract, 2% glucose). Mycelia were harvested onto nylon cloth (300-μm pore size), washed with distilled water, and lyophilized. Both nuclear DNA and mtDNA were purified from lyophilized mycelia according to the procedure of Fukumasa-Nakai et al. (1992) with RNase treatment and fractionation by centrifugation with cesium chloride.

rDNA-RFLP analysis

The 5.8S ribosomal RNA gene intervening ITS region of the nuclear genome was amplified using the primer pairs

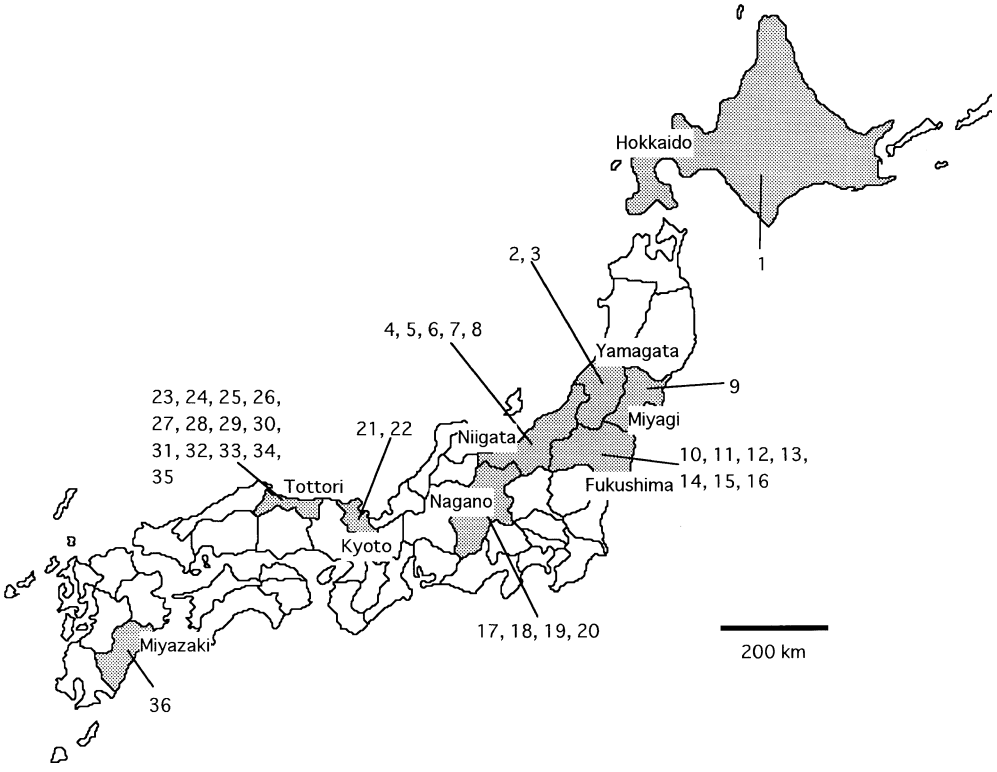

Fig. 1. Map of Japan showing locations of collecting areas (*dotted outlined areas*) for *Pholiota nameko* isolates used in this study

ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and the IGS region was amplified by the primer pairs LR12R (5′-CTGAACGCCTCTAAGTCAGAA-3′) and M1 (5′-AACCACAGCACCCAGGATTCCC-3′). Each primer sequence was based on the known sequence of the rDNA repeat from *Saccharomyces cerevisiae* (Georgiev et al. 1981; Walker and Doolittle 1982; White et al. 1990). The 50-μl reaction mixture for PCR amplification contained the following: 0.1 μg nuclear DNA, 1× amplification buffer for Ex Taq DNA polymerase (Takara, Kusatsu, Japan), 200 μM deoxynucleoside triphosphates (dNTPs), 0.4 μM each primer, and 0.5 U Ex Taq DNA polymerase. Amplifications were performed in a Mp 100 Thermal cycler (Takara), with an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 10 min.

Aliquots (4 μl) of amplification products were electrophoresed through 1.5% (w/v) agarose gels in TBE buffer [0.09 M Tris-borate, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.3], stained with ethidium bromide (0.5 μg/l), and visualized on a UV transilluminator. When fragments of the appropriate size for each target region were obtained, 4-μl aliquots of the PCR products were digested separately with 20 restriction enzymes, *Alu*I, *Ava*II, *Bam*HI, *Bgl*II, *Bsm*I, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*cII, *Hin*dIII, *Hin*fI, *Hpa*I, *Msp*I, *Nde*I, *Pst*I, *Rsa*I, *Sac*I, *Taq*I (Nippon Gene, Toyama, Japan), and *Mbo*I (Takara) according to the respective manufacturer's instructions. Electrophoresis of all digests was carried out on a 3.0% (w/v) agarose slab gel in TBE buffer, and gels were stained with ethidium bromide. The sizes of restriction fragments were scored for each isolate by comparing the fragment size with a molecular size standard.

mtDNA-RFLP analysis

mtDNA derived from each isolate of *P. nameko* was digested separately with two restriction enzymes, *Hin*dIII and *Bgl*II, according to the manufacturer's instructions (Nippon Gene). RFLP analysis of digested DNAs was carried out with 0.7%–1.0% agarose gels (w/v) in the same manner as the rDNA-RFLP analysis.

RAPD analysis

To select PAPD primers, the nuclear DNAs from five isolates of *P. nameko* were chosen to screen 25 primers. Three primers, OPA-01, OPA-02, and LAR-B, were selected for their large number of amplified DNA fragments; their sequences (5′–3′) were CAGGCCCTTC (OPA-01), GTGACGTAGG (OPA-2), and GAGTCCGCAA (LAR-B), respectively. RAPD reactions were performed in a 50-μl reaction mixture containing 0.1 μg nuclear DNA, 1× amplification buffer for Ex Taq DNA polymerase, 200 μM dNTPs, 0.4 μM primer, and 0.5 U Ex Taq DNA polymerase (Takara). Amplifications were carried out in a MP 100 Thermal cycler (Takara), with an initial denaturation step 94°C for 3 min, followed by 45 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 10 min. Detection of DNA amplifications products was performed with 1.0% agarose gels (w/v) in the same manner as the rDNA-RFLP analysis. There were at least three replications per reaction.

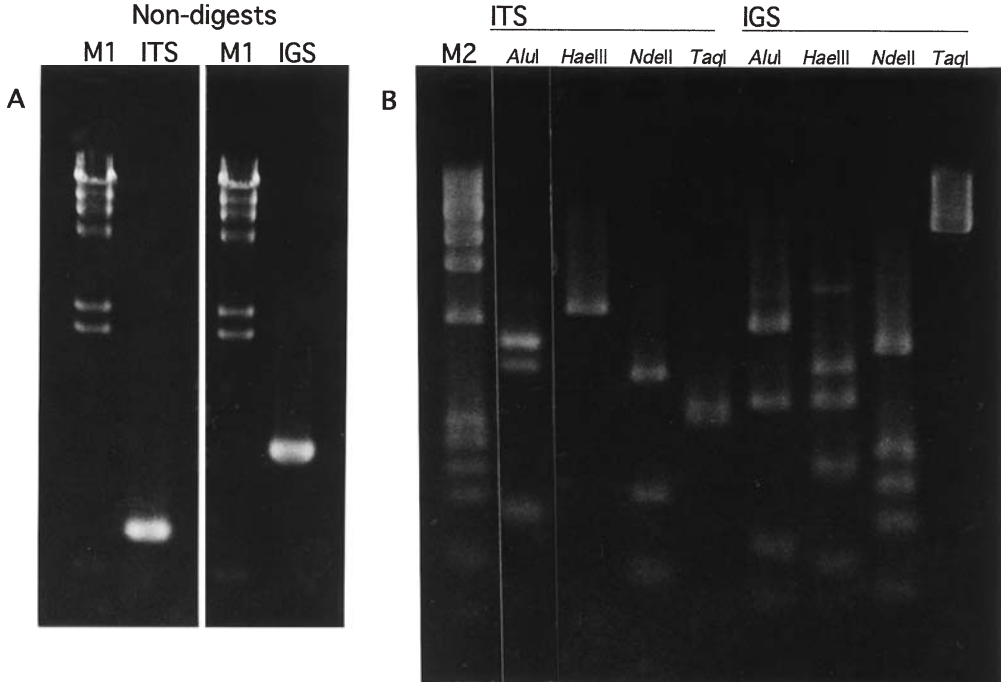

Fig. 2. Representative amplification (**A**) by polymerase chain reaction (PCR) using primer pairs ITS1/ITS4 (internal transcriber spacer, ITS) and LR12R/M1 (intergenic spacer, IGS) of *Pholiota nameko* isolate (TMIC 30783) and its restriction fragment length polymorphism (RFLP) patterns (**B**) as digested by *Alu*I, *Hae*III, *Nde*II, and *Taq*I. *M1*, λ/*Hin*dIII digest DNA size marker; M2, ϕX174/*Hae*III digest DNA size marker

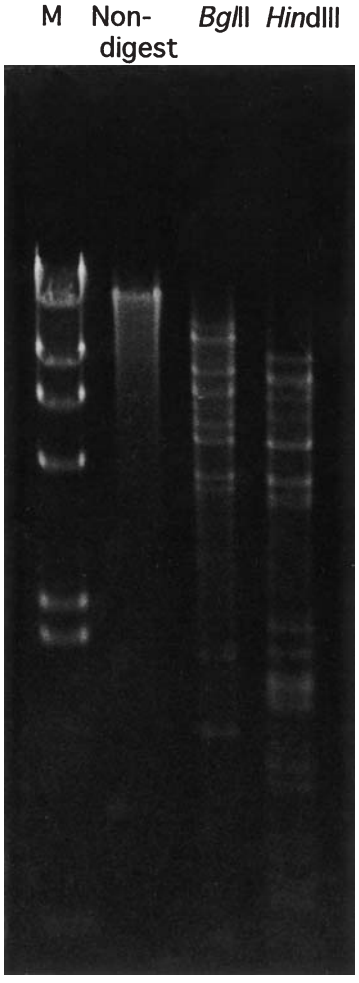

Fig. 3. Representative mtDNA extracted from *Pholiota nameko* (TMIC 30783) and its restriction fragment patterns with *Bgl*II and *Hin*dIII. *M*, λ/*Hin*dIII digest DNA size marker

Table 1. Mitochondrial DNA (mtDNA) size among *Pholiota nameko* wild isolates from different geographic origins

Isolate no.	Source (TMIC)	Size (kb)		Mean
		*Bgl*II	*Hin*dIII	
1	30815	98.6	95.3	96.9
2	30776	85.6	85.5	85.6
3	31786	91.4	91.0	91.2
4	30187	82.8	83.4	83.1
5	30793	95.7	96.9	96.3
6	30794	88.6	86.7	87.6
7	30795	90.6	89.6	90.1
8	30799	90.1	94.9	92.5
9	30774	95.2	95.2	95.2
10	30717	91.7	95.5	93.6
11	30719	89.1	87.5	88.3
12	30721	90.0	92.5	91.3
13	30722	91.5	94.6	93.1
14	30724	89.9	96.4	93.1
15	30727	88.3	92.1	90.2
16	30728	87.0	93.3	90.2
17	30783	90.2	95.2	92.7
18	30787	87.4	93.2	90.3
19	30789	87.4	93.2	90.3
20	30792	87.1	86.8	86.9
21	30767	88.3	85.4	86.8
22	30768	87.9	90.0	89.0
23	30078	87.5	94.5	91.0
24	30732	89.0	88.2	88.6
25	30734	92.2	93.4	92.8
26	30737	84.7	85.6	85.1
27	30739	92.1	87.9	90.0
28	30741	93.7	87.0	90.3
29	30742	89.0	94.3	91.6
30	30745	87.0	91.4	89.2
31	30748	91.1	88.4	89.8
32	30753	88.4	87.6	88.0
33	30758	89.8	91.2	90.5
34	30762	90.2	93.6	91.9
35	30765	84.8	89.6	87.2
36	30773	83.0	83.9	83.5

Average size mtDNA, 90.1 kb

Phenetic analysis

The presence or absence of reproducible polymorphic bands from RFLP and RAPD analysis was scored 1 and 0, respectively. The distance matrix based on a Dice coefficient (Sneath and Sokal 1973) was calculated between pairs of different RAPD or RFLP phenotypes as $D = 1 - 2(n_{xy})/(n_x + n_y)$, where n_{xy} is the number of shared fragments and n_x and n_y are the number of fragments in the fingerprints x and y, respectively. Trees based on this matrix were constructed using the UPGMA (unweighted pair-group with arithmetic averaging) method (Sokal and Michener 1958) via the PHYLIP program (Phylogeny Inference Package, version 3.5c; Felsenstein 1994). The tree was drawn with the Treeview (Page 1996) software package.

Results

rDNA-RFLP analysis

A single product of about 730 bp resulted from PCR amplification of nuclear DNA using the primers ITS1 and ITS4 for ITS region in all isolates (Fig. 2A). Likewise, a single product of about 1.0 kb was amplified for all isolates using the primers LR12R and M-1 for the IGS region (Fig. 2A). Of 20 restriction enzymes examined, only 4, *Alu*I, *Hae*III, *Nde*II, and *Taq*I, produced 4–9 bands in the sum of two regions, respectively (Fig. 2B). However, no RFLP within 36 isolates of *P. nameko* was shown in both regions of rDNA gene.

mtDNA-RFLP analysis

By digestion with *Bgl*II and *Hin*dIII, *P. nameko* mtDNA was found to be highly polymorphic (Fig. 3). Endonuclease digests of mtDNAs from 36 isolates of *P. nameko* produced 35 *Bgl*II RFLP patterns and 35 *Hin*dIII patterns. When all these RFLP patterns were combined, the 34 isolates, except for 2, i.e., TMIC-30787 and −30789, were distinct from each other. Based on the summation of restriction fragment sizes from two endonucleases, *Bgl*II and *Hin*dIII, the sizes of mtDNAs were estimated to range from 83.1 kb for TMIC-30187 to 96.9 kb for TMIC-30815. The

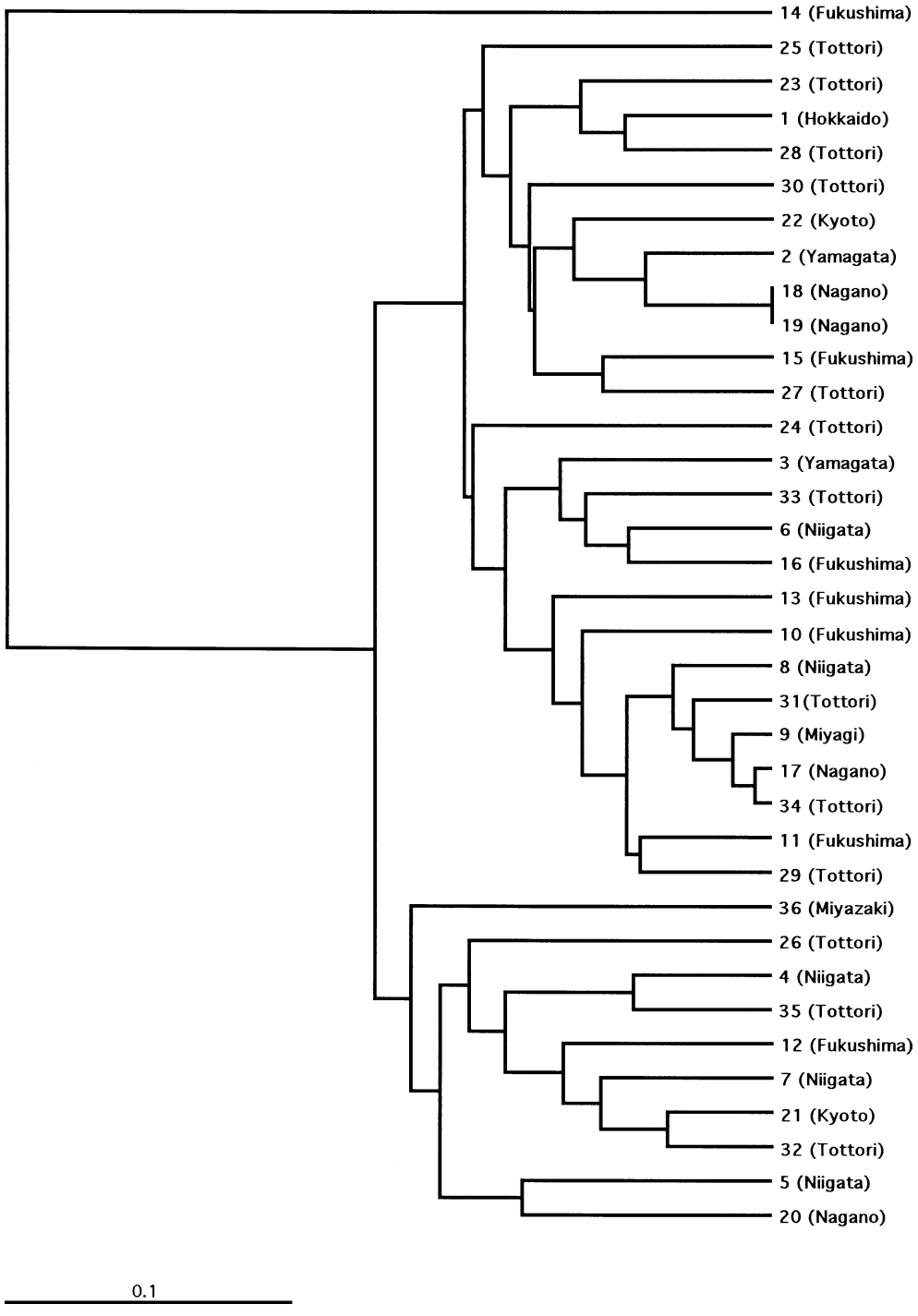

Fig. 4. Unweighted pair-group with arithmetic averaging (UPGMA) cluster diagram of relationships among 36 *Pholiota nameko* isolates. The dendrogram is based on genetic dissimilarity coefficients from RFLP patterns of mtDNA. Geographic origin of isolates is shown in parentheses

average size of mtDNA was 90.1 kb (Table 1). This value agrees with that described previously in *P. nameko* by Babasaki (1999).

Pairwise genetic distance among the isolates from different geographic origins in Japan ranged from 0.011 to 0.610. No tendency for lower distance values to be obtained among the mtDNA phenotypes of wild isolates from the same geographic region (prefecture) compared to values among isolates from different regions was observed. Cluster analysis based on genetic distance separated the isolates into three major groups, each of which consisted of 1, 10, and 25 mtDNA phenotypes (Fig. 4). However, each dendrogram group was not correlated to a geographically distinct population.

RAPD analysis

A total of 53 reproducible polymorphic bands were generated with three primers, OPA-01, OPA-08, and LAR-B, on the 36 isolates of *P. nameko*. Figure 5 shows representative RAPD profiles of *P. nameko* illustrating the different

RAPD fragment patterns detected. By combining all RAPD fragment patterns obtained with the three primers, every isolate showed its own distinct RAPD profile. A pairwise comparison among RAPD profiles of 36 isolates revealed that the values of genetic distance ranged from 0.04 to 0.43. However, the extent of these distance values was not correlated to a geographically distinct population. In addition, all the RAPD profiles were grouped into the same large similarity group by cluster analysis with the UPGMA method (data not shown).

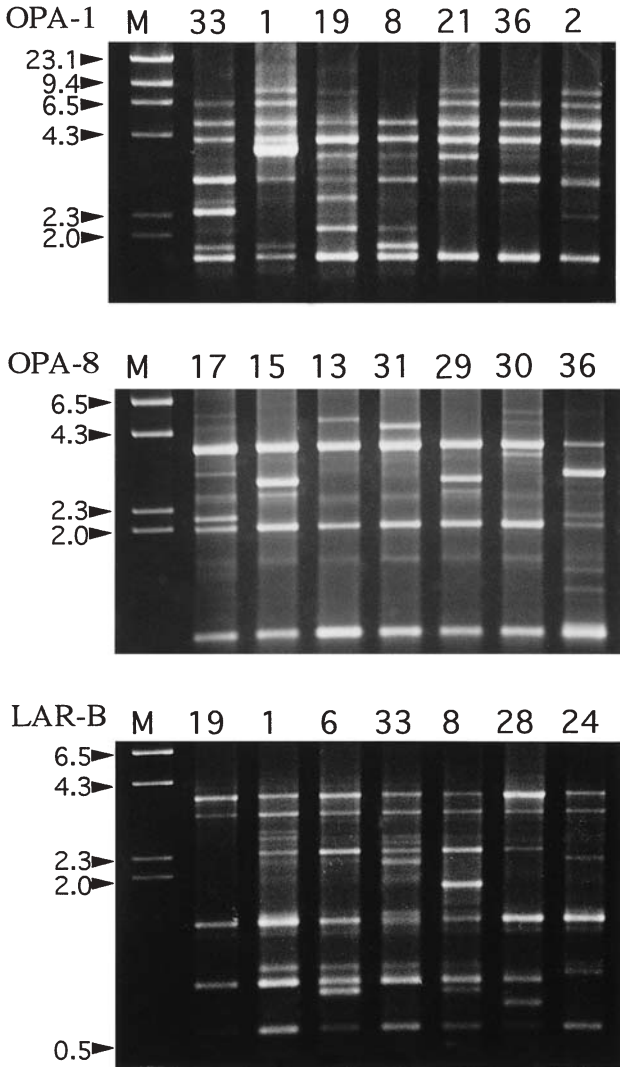

Fig. 5. Representative random amplified polymorphic DNA (RAPD) profiles produced from selected isolates of *Pholiota nameko* using primers OPA-01, OPA-08, and LAR-B, respectively. Isolate number is shown above each lane. *M*, λ/*Hin*dIII digest DNA size marker (band size given in kilobase pairs)

Discussion

The analysis of polymorphisms in the rDNA gene provides reliable information for estimating not only the interspecific but also the intraspecific relationship because conserved and highly variable regions coexist in the rDNA repeat (Vilgalys and Sun 1994; Hibbett et al. 1995; Iraçabal et al. 1995). For example, in *L. edodes* (Hibbett et al. 1995) and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Vilgalys and Sun 1994), which have worldwide distribution and are cultivated extensively in many countries including Japan, polymorphisms in the ITS region of rDNA revealed genetic variation that was correlated with the geographic distance. In this study, by amplification of two regions of nuclear rDNA for *Pholiota nameko*, ITS and IGS, there was no difference in size of any region of rDNA and no polymorphism was found in restriction patterns from 36 isolates, although restriction patterns of the two regions were apparently different from those of the related species, *Pholiota adiposa* (Fr.) Kummer etc. (data not shown). The intraspecific genetic diversity of *P. nameko* may be small as compared with the species of those basidiomycetes with worldwide distribution. To detect more detailed intraspecific variations within the population of this fungus, more sensitive analysis using mtDNA or nuclear DNA rather than rDNA is needed.

Two polymorphic DNA markers, mtDNA-RFLP and RAPD, have been considered as useful markers for detecting intraspecific variability in basidiomycetes (Anderson et al. 1987; Khush et al. 1992; Fukuda et al. 1994; Matsumoto and Fukumasa-Nakai 1995; Chiu et al. 1996). In this study, also, high polymorphic results were obtained by analyses based on both markers. Cluster analyses for natural populations of this fungus based on both mtDNA-RFLP and RAPD markers revealed that any similarity group correlated with geographic origins was not formed, suggesting that geographic differentiation did not occur among natural populations of this fungus from Japan. This observation may show that gene flow frequently occurs among populations from distinct regions in Japan.

In a previous study for the distribution of incompatibility factors in the natural population of *P. nameko*, many incompatibility factors were shared among the isolates from different locations in Japan, and the allele number of incompatibility factors recognized was lower than that expected in theoretical calculations (Matsumoto et al. 1999). In general, decrease of allele frequency in the natural population indicates the progress of genetic homogeneity. However, mtDNA-RFLP and RAPD analyses, which can estimate the genetic variation based on the structure of whole genome DNA, genetically differentiated all most isolates of this fungus. Additionally, with analyses of mtDNA-RFLP of *P. nameko*, dissimilarity values ranged from 0.011 to 0.610. This range of dissimilarity values was rather large as compared to those of *L. edodes* (Fukuda et al. 1994) and *Pleurotus ostreatus* (Matsumoto and Fukumasa-Nakai 1995), among natural populations in Japan, ranging from 0.026 to 0.300, and from 0.170 to 0.542, respectively. With RAPD analysis on nuclear DNA, dissimilarity values ranged from 0.040 to 0.430. The level of this range seems to be high, because the dissimilarity values among Japanese wild isolates of *P. ostreatus*, which were calculated using isozyme analysis of 5 enzymes (total of 121 isozyme bands),

ranged from 0.009 to 0.267 (Matsumoto et al. 1995). These results indicated that heterogeneity in the natural population of *Pholiota nameko* in Japan was well maintained as compared with those of *L. edodes* and *Pleurotus ostreatus*.

For breeding *Pholiota nameko*, as well as other cultivated mushrooms, the high genetic variability present in the population of this fungus is an advantage because it is necessary for improvement programs. In this study, rDNA-RFLP, mtDNA-RFLP, and RAPD analyses indicated that the natural population of this fungus in Japan is a continuous genetic population that has maintained a certain genetic variation. This finding suggests that the natural population of this fungus could offer valuable genetic resources for breeding.

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