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Detection and distribution of six linear mitochondrial plasmids in the shiitake mushroom, *Lentinula edodes**

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The presence of plasmids was surveyed in 90 wild isolates of *Lentinula edodes* collected from geographically different world regions. DNA plasmids of different sizes were found in about 80% of the isolates. The plasmids detected were of six kinds, designated as pLE1 (9.0 kb), pLE2 (11.1 kb, =pLLE1 described by other authors), pLE3A (9.8 kb), pLE3B (10.8 kb), pLE3C (12.1 kb), and pLE3D (12.3 kb). Hybridization analysis suggested that pLE1 and pLE2 were distinct plasmid types of different homology groups to each other, and the four other plasmids were variant types belonging to a third homology group. These plasmids had no homology with their host's and non-host's nuclear and mitochondrial genome DNAs. Restriction analysis and electron microscopy indicated that the plasmids are linear in form. Since all six plasmids were transmitted uniparentally in sexual crosses and were consistently associated with the DNA preparations from mitochondria fractionated from mycelia of representative isolates, they were suggested to be located in mitochondria, similar to many other known fungal DNA plasmids. Geographically, pLE1 and pLE2 were widely distributed in natural populations of *L. edodes*, while the remaining four plasmids were uniquely present in delimited natural populations.

Key Words——characterization; geographical distribution; *Lentinula edodes*; linear mitochondrial DNA plasmids.

Small extragenomic deoxyribonucleic acid elements, namely, DNA plasmids, are known to be associated with many fungi in a wide range of taxonomic groups (Meinhardt et al., 1990; Griffiths, 1995). They are linear or circular in form, and most of them appear to be localized in mitochondria and therefore to be inherited uniparentally. The linear plasmids also generally share the following properties; they have terminal inverted repeat sequences, carry a 5′-linked terminal protein, and show no sequence homology to their host's nuclear and mitochondrial genome DNA (Meinhardt et al., 1990; Griffiths, 1995). Nevertheless, the functional role of most of these plasmids is not clear. Exceptions to this include the kalilo or maranhar DNA of *Neurospora* species, which elicits the expression of the senescence syndrome in the hosts (Bertrand et al., 1985, 1986; Court et al., 1991), and the pGKL1 plasmid of the yeast *Kluyveromyces lactis* (Dombrowski) van der Walt and the pPac-1 plasmid of the yeast *Pichia acaciae* van der Walt, which mediate the killer activity of the host against other yeasts (Gunge et al., 1981; Worsham and Bolen, 1990).

Among the higher basidiomycetes, such as commercially important cultivated mushrooms, some linear mitochondrial DNA plasmids have been detected: pEM and pMPJ plasmids from *Agaricus bitorquis* (Quél.) Sacc. (Mohan et al., 1984); pLPO1, pLPO2 and pLPO3 plasmids from *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Yui et al., 1988; Nakajima et al., 1993); and pLLE1 plasmid from *Lentinula edodes* (Berk.) Pegler (Katayose et al., 1990). Of these plasmids the pLPO1 has been found widely distributed among *P. ostreatus* natural populations (Nakaya et al., 1991; Katayose et al., 1992). However, little information is available on the distribution of pLLE1 and the occurrence of other types of plasmids in *L. edodes*. The aim of this paper is to report on five new linear DNA plasmids associated with mitochondria of *L. edodes*, together with the pLLE1, and to describe their distribution and general properties.

Materials and Methods

Strains and culture conditions Ninety wild isolates (dikaryons) of *L. edodes* were collected from geographically different regions of the world as listed in Table 1. Twenty Japanese commercial strains were also employed. All isolates and strains were deposited in the culture collection of the Tottori Mycological Institute and maintained on 2% malt extract agar. Preparation of lyophilized mycelium for total cellular DNA (total DNA) extraction and plasmid isolation was done according to the method of Fukumasa-Nakai et al. (1992).

Detection and isolation of plasmids Plasmids were detected by a modified procedure based on the methods of Hirt (1967) and Cummings et al. (1979). A 0.3 g portion of lyophilized mycelium of each isolate was ground to a fine powder in a mortar and pestle with a 1 g of fine sea sand, suspended in 5 ml of 0.1 M citrate/phosphate buffer (pH 6.0) containing 0.1 M EDTA, 2% N-lauroylsar-

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Table 1. A survey of *Lentinula edodes* wild isolates for plasmids.

Isolate	Geographical source	Plasmid[a)]	Size of plasmids (kb)
1. J933	Teshio-gun, Hokkaido, Japan	+	11.1
2. J938	Teshio-gun, Hokkaido, Japan	−	
3. J941	Teshio-gun, Hokkaido, Japan	+	11.1
4. J951	Nakagawa-gun, Hokkaido, Japan	+	11.1
5. J952	Teshio-gun, Hokkaido, Japan	+	11.1
6. J1315	Uryu-gun, Hokkaido, Japan	+	11.1, 12.1
7. J1346	Karachi-gun, Hokkaido, Japan	+	11.1
8. J1350	Horosen-gun, Hokkaido, Japan	+	11.1
9. J1012	Morioka-city, Iwate, Japan	+	11.1, 12.1
10. J960	Utsunomiya-city, Tochigi, Japan	+	12.1
11. J-SA11	Minamiado-mura, Nagano, Japan	+	11.1, 12.1
12. J-SA12	Ina-city, Nagano, Japan	+	12.1
13. J797	Obama-city, Fukui, Japan	+	11.1
14. J855	Shizuoka-city, Shizuoka, Japan	+	11.1, 12.1
15. J866	Nakaizu-cho, Shizuoka, Japan	+	11.1
16. J1156	Mt. Amagi, Shizuoka, Japan	+	11.1
17. J1299	Aichi, Japan	+	11.1, 12.1
18. J709	Minamimuroguchi-gun, Mie, Japan	+	12.1
19. J725	Takatsuki-city, Osaka, Japan	+	11.1
20. J1069	Kawachinagano-city, Osaka, Japan	−	
21. J917	Uda-gun, Nara, Japan	−	
22. J1440	Yoshino-gun, Nara, Japan	−	
23. J406	Wakayama, Japan	+	11.1, 12.1
24. J832	Hashimoto-city, Wakayama, Japan	+	11.1, 12.1
25. J724	Ibo-gun, Hyogo, Japan	+	11.1, 12.1
26. J1003	Shisou-gun, Hyogo, Japan	+	11.1, 12.1
27. J1455	Mt. Hachibuse, Hyogo, Japan	+	11.1, 12.1
28. J679	Tottori-city, Tottori, Japan	+	11.1, 12.1
29. J730	Mt. Daisen, Tottori, Japan	−	
30. J780	Yazu-gun, Tottori, Japan	+	11.1, 12.1
31. J860	Mt. Daisen, Tottori, Japan	−	
32. J879	Mt. Daisen, Tottori, Japan	+	12.1
33. J1067	Tottori-city, Tottori, Japan	−	
34. J1162	Hino-gun, Tottori, Japan	−	
35. J667	Shimane, Japan	−	
36. J699	Oki-gun, Shimane, Japan	+	12.1
37. J766	Ouchi-gun, Shimane, Japan	−	
38. J777	Oki-gun, Shimane, Japan	+	11.1, 12.1
39. J778	Oki-gun, Shimane, Japan	+	11.1, 12.1
40. J956	Oki-gun, Shimane, Japan	+	12.1
41. J1261	Hiba-gun, Hiroshima, Japan	+	11.1, 12.1
42. J1425	Yamaguchi, Japan	+	11.1, 12.1
43. J571	Ozu-city, Ehime, Japan	+	11.1, 12.1
44. J782	Kita-gun, Ehime, Japan	+	11.1, 12.1
45. J965	Kitauwa-gun, Ehime, Japan	+	11.1
46. J1158	Ehime, Japan	+	11.1, 12.1
47. J712	Hada-gun, Kochi, Japan	+	11.1, 12.1
48. J773	Hada-gun, Kochi, Japan	+	11.1, 12.1
49. J820	Hada-gun, Kochi, Japan	+	11.1, 12.1
50. J707	Yamakuni-cho, Oita, Japan	+	11.1, 12.1

Table 1. (continued)

51. J1157	Takeda-city, Oita, Japan	+	11.1
52. J1201	Ono-gun, Oita, Japan	+	11.1, 12.1
53. J1442	Minamikaifu-gun, Oita, Japan	+	11.1, 12.1
54. J1456	Oita-gun, Oita, Japan	−	
55. J693	Tagawa-gun, Fukuoka, Japan	+	11.1, 12.1
56. J1454	Yame-gun, Fukuoka, Japan	+	11.1, 12.1
57. J695	Kuma-gun, Kumamoto, Japan	+	11.1, 12.1
58. J751	Kamimashiki-gun, Kumamoto, Japan	+	11.1
59. J1439	Aso-gun, Kumamoto, Japan	+	11.1, 12.1
60. J763	Kamigata-gun, Nagasaki, Japan	+	12.1
61. J646	Miyazaki, Japan	−	
62. J719	Nishiusuki-gun, Miyazaki, Japan	+	11.1, 12.1
63. J1079	Higashiusuki-gun, Miyazaki, Japan	+	11.1
64. J1270	Izumi-city, Kagoshima, Japan	+	12.1
65. J1403	Amamioshima, Kagoshima, Japan	+	12.1
66. J-SA13	Yakushima, Kagoshima, Japan	+	12.1
67. J818	Kunigami-son, Okinawa, Japan	+	11.1
68. J1148	Kunigami-son, Okinawa, Japan	+	11.1
69. J1149	Kunigami-son, Okinawa, Japan	+	12.1
70. NP1814	Nepal	+	11.1
71. TH1632	Thailand	+	11.1
72. TH1634	Thailand	+	11.1
73. BO689	Borneo	+	9.8
74. PN1465	Mt. Albert-Edward, Papua New Guinea	−	
75. PN1476	Mt. Albert-Edward, Papua New Guinea	−	
76. PN1485	Mt. Albert-Edward, Papua New Guinea	+	11.1
77. PN1490	Mt. Kaindi, Papua New Guinea	+	9.0
78. PN1492	Mt. Missim, Papua New Guinea	+	9.0
79. PN1499	Mt. Wilhelm, Papua New Guinea[b)]	+	9.0, 10.8, 11.1
80. PN1500	Mt. Wilhelm, Papua New Guinea[b)]	+	9.0, 10.8, 11.1
81. PN1502	Mt. Wilhelm, Papua New Guinea[b)]	+	9.0, 10.8
82. PN1503	Mt. Wilhelm, Papua New Guinea[b)]	+	9.0, 10.8
83. PN1667	Mt. Kaindi, Papua New Guinea	+	11.1
84. PN1668	Mt. Kaindi, Papua New Guinea	+	11.1
85. PN1669	Mt. Kaindi, Papua New Guinea	−	
86. PN1671	Mt. Kaindi, Papua New Guinea	−	
87. NZ1172	Lake Matheson, New Zealand	+	9.0, 12.3
88. NZ1448	Nelson-city, New Zealand	+	9.0, 12.3
89. NZ1569	New Zealand	+	9.0, 12.3
90. NZ1570	New Zealand	+	9.0, 12.3

a) +, detected; −, not detected.
b) These four Papua New Guinean wild isolates were collected from highlands at above 3,000 m elevation.

cosine sodium salt, and 200 μg/ml proteinase K, and incubated at 37°C for 2 h, then 65°C for 30 min to lyse cellular organella. This preparation was adjusted to 1 M NaCl, kept on ice overnight, then centrifuged at 0°C and 17,000$\times g$ for 30 min. The supernatant was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, by volume) and several times with chloroform/isoamyl alcohol (24:1, v/v). The aqueous phase was digested with 100 μg/ml RNase A and 200 μg/ml α-amylase at 37°C for 2 h, deprotenized again with phenol and chloroform, and from it total DNA was precipitated with ethanol. A portion of the total DNA was subjected to electrophoresis on 0.7% agarose (Nippon Gene Type S, Japan) slab gel in TAE (40 mM Tris/acetate and 10 mM EDTA) or TBE (89 mM Tris/borate, 89 mM boric acid, and 2 mM EDTA) at 5 V/cm for 2.5–3 h for plasmid detection. Plasmids were fractionated from the total DNAs by CsCl/bisbenzimide density gradient equilibrium centrifugation (1.0 g/ml and 0.2 mg/ml bisbenzimide) in a vertical rotor at 20°C and 140,000$\times g$ for 16 h. The dis-

crete bands of plasmid DNAs were always formed above a mitochondrial DNA band located in the middle portion of the density gradients. For more complete isolation, each plasmid sample removed from the bands was purified through agarose gel electrophoresis and elution from the gel. The mitochondrial location of plasmids was determined using DNA preparations from mitochondria purified from fresh mycelial cultures by flotation in sucrose density gradients, based on the procedure of Lambowitz (1979).

Southern hybridization and restriction analysis The total DNAs extracted from *L. edodes* wild isolates were electrophoresed on 1% agarose slab gel in TBE at 5 V/cm for 3 h, then transferred to nylon membranes (Atto, Japan) by the general method of Southern (Maniatis et al., 1982). The Southern blot was probed with each of Digoxigenin-labelled plasmids according to the manufacturer's instructions (Boehringer Mannheim Yamanouchi, Japan). For restriction analysis of plasmid DNAs, the purified plasmids were digested separately with several restriction endonucleases (Nippon Gene, Japan) according to the supplier's specifications. The restricted plasmid DNAs were electrophoresed on 0.7–2.0% agarose slab gel in TAE or TBE at 5 V/cm for 2.5–3 h, and the gels were stained with ethidium bromide (0.5 μg/ml). Restriction patterns were recorded by photographing the gels on a UV transilluminator.

Other enzymatic treatments For RNase and DNase digestion test, a portion of plasmid samples suspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 10 mM $MgCl_2$ were treated with 50 μg/ml RNase A or 50 μg/ml DNase at 37°C for 1 h, then analyzed by agarose gel electrophoresis. Restriction end fragments of plasmids were determined by a procedure combining the methods of labeling the 3'-end of their whole molecules using Digoxigenin 3'-end labeling kit (Boehringer Mannheim Yamanouchi, Japan) and subsequent restriction analysis.

Electron microscopy To examine plasmid morphology, purified preparations of plasmids suspended in TE buffer containing 0.01% cytochrome c were applied to carbon-coated Formvar grids according to the procedure of Kleinschmidt et al. (1962). These grids were shadowed with platinum-palladium, then observed in an JEM-100CX II electron microscope at 80 kV.

Results

Detection of plasmids Total DNAs extracted from 90 wild isolates were fractionated by agarose gel electrophoresis to detect the presence of plasmids. The results are shown in Table 1 and Fig. 1. In addition to high-molecular-weight nuclear and mitochondrial DNAs, six low-molecular-weight nucleic acid elements of differ-

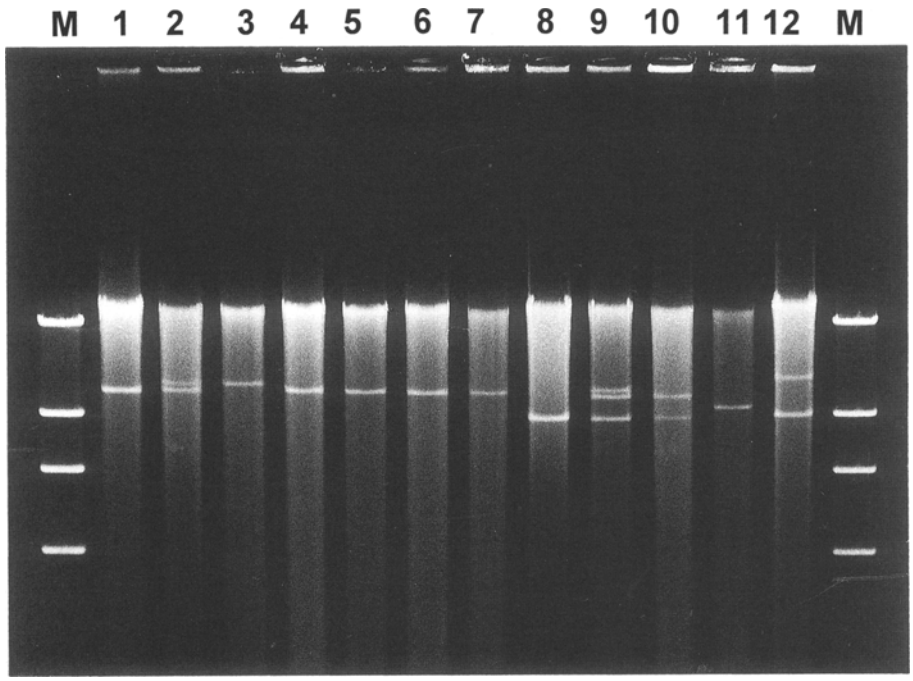

Fig. 1. Electrophoretic analysis of total DNA samples derived from 12 representative isolates of *Lentinula edodes* for the presence of plasmids.
Lane M, *Hin*d III-digested λDNA size markers; lane 1, Me-1610 commercial strain with 11.1 kb plasmid; lane 2, J571 isolate with 12.1 and 11.1 kb plasmids; lane 3, J879 isolate with 12.1 kb plasmid; lane 4, J1148 isolate with 11.1 kb plasmid; lane 5, NP1814 isolate with 11.1 kb plasmid; lane 6, TH1632 isolate with 11.1 kb plasmid; lane 7, PN1485 isolate with 11.1 kb plasmid; lane 8, PN1492 isolate with 9.0 kb plasmid; lane 9, PN1500 isolate with 11.1, 10.8 and 9.0 kb plasmids; lane 10, PN1502 isolate with 10.8 and 9.0 kb plasmids; lane 11, BO689 isolate with 9.8 kb plasmid; lane12, NZ1569 isolate with 12.3 and 9.0 kb plasmids.

Table 2. Southern hybridization analysis of sequence homology among six plasmid DNAs of different sizes from *Lentinula edodes*.

Isolate	Size of plasmid (kb)	Probe[a]					
		9.0 kb plasmid from PN1492	9.8 kb plasmid from BO689	10.8 kb plasmid from PN1502	11.1 kb plasmid from J1148 or pLLE1[b]	12.1 kb plasmid from J879	12.3 kb plasmid from NZ1569
Me1610	11.1 (pLLE1)	−	−	−	+	−	−
J571	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J679	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J719	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J777	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J820	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J832	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J855	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J879	12.1	−	+	+	−	+	+
J1003	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J1012	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J1148	11.1	−	−	−	+	−	−
J1149	12.1	−	+	+	−	+	+
J1201	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J1315	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
J1439	11.1	−	−	−	+	−	−
	12.1	−	+	+	−	+	+
NP1814	11.1	−	−	−	+	−	−
TH1632	11.1	−	−	−	+	−	−
BO689	9.8	−	+	+	−	+	+
PN1485	11.1	−	−	−	+	−	−
PN1492	9.0	+	−	−	−	−	−
PN1500	9.0	+	−	−	−	−	−
	10.8	−	+	+	−	+	+
	11.1	−	−	−	+	−	−
PN1502	9.0	+	−	−	−	−	−
	10.8	−	+	+	−	+	+
NZ1448	9.0	+	−	−	−	−	−
	12.3	−	+	+	−	+	+
NZ1569	9.0	+	−	−	−	−	−
	12.3	−	+	+	−	+	+

a) +, strong sequence homology was detected; −, no sequence homology was detected.
b) pLLE1 plasmid reported previously from a commercial strain, Me1610, of *Lentinula edodes* by Katayose et al. (1990).

ent in sizes were found singly or in combinations of two or three in about 80% of the isolates. The six elements were constantly observed as clear bands of uniform intensity on stained agarose gels and had constant molecular sizes of about 9.0, 9.8, 10.8, 11.1, 12.1, and 12.3 kb even after repeated subcultures. Many of the Japanese commercial strains were also found to carry 11.1 and/or 12.1 kb elements. Because *L. edodes* is known to contain some double-stranded RNA viruses (Ushiyama et al., 1977), RNase and DNase digestion tests were perfomed on these six elements. They were found to be digested by DNase, but not by RNase (data not shown). They were also found to have their own distinct properties as described hereafter. The known pLLE1 plasmid (about 11.0 kb) of *L. edodes* (Katayose et al., 1990) was estimated to be 11.1 kb in molecular size in the present electrophoretic analysis. These results suggested that the six low-molecular-weight DNA elements are not the result of random fragmentation of the high-molecular-weight nuclear or mitochondrial genome DNA but probably extrachromosomal DNA molecules replicating separately from the genomes, namely, DNA plasmids, and are mitotically stable.

To examine whether the plasmids of the same or different molecular sizes found in the wild isolates from geographically various regions share sequence homology with each other, they were subjected to Southern hybridization analysis under stringent assay conditions using the entire molecule of each plasmid purified from representative isolates as a hybridization probe. As the results in Table 2 and Fig. 2 indicate, plasmid molecules of the same size shared strong sequence homology with each other regardless of geographical origin. It was also shown that 11.1 kb plasmids detected in this study were homologous to the pLLE1 plasmid found in the commercial strain Me1610 of *L. edodes* by Katayose et al. (1990) and that both plasmids were similar in restriction pattern, as described hereafter. In contrast, the plasmids of 9.0 and 11.1 kb showed no sequence homology with each other or with the plasmids of 9.8, 10.8, 12.1, and 12.3 kb, while the latter four plasmids were found, unexpectedly, to share significant sequence homology with each other. Cross-hybridization signals of high intensity were generally observed among restriction fragments containing one side terminal or the near-central part of the four plasmid molecules (Fig. 3). No sequence homol-

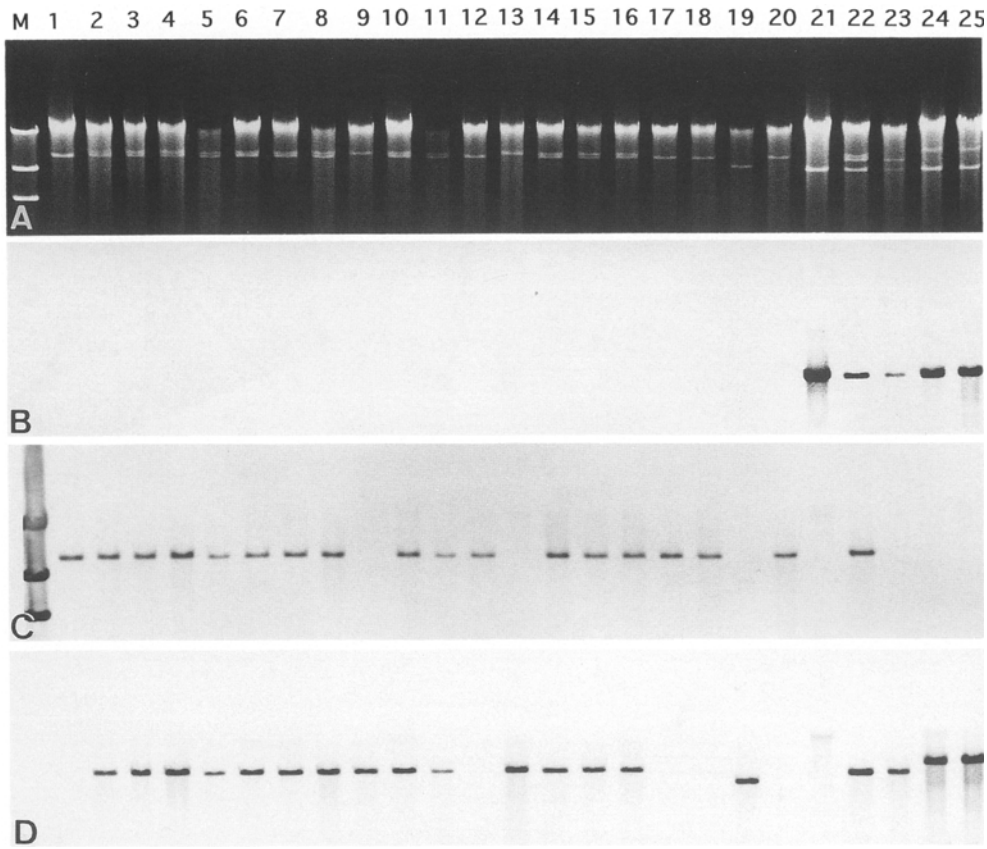

Fig. 2. Southern hybridization analysis of sequence homology among six kinds of plasmids of different sizes from 25 representative isolates of *Lentinula edodes*.
A: ethidium bromide staining of 1% agarose gel. B, C and D: hybridization patterns probed with Digoxigenin-labelled 9.0 kb, 11.1 kb and 9.8 kb plasmids, respectively. Lane M, *Hin*d III-digested λDNA size markers. The isolates of lane 1 to 25 are presented in the same order in Table 2.

ogy was observed between any of the six plasmids and nuclear or mitochondrial DNAs of their hosts and non-hosts. These hybridization data suggested that the plasmids of the same sizes were of identical types and that those of 9.0 and 11.1 kb were distinct homologous types of plasmids. It was also reasonable to consider that the remaining four plasmids were related types, namely, variants belonging to a different homology group from the

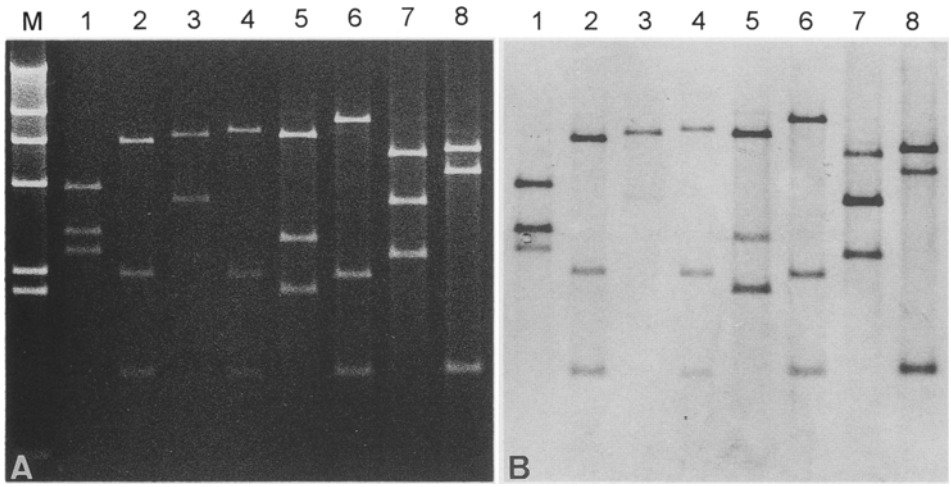

Fig. 3. Southern hybridization analysis of sequence homology among the four variant plasmids of the same homology group from *Lentinula edodes*.
A: ethidium bromide staining of 1% agarose gel. B: hybridization pattern probed with *Eco*RI and *Hae*III double-digested Digoxigenin-labelled pLE3A plasmids. Lane M, *Hin*d III-digested λDNA size markers; lane 1, *Bam*HI-digested pLE3A; lane 2, *Hae*III-digested pLE3A; lane 3, *Bam*HI-digested pLE3B; lane 4, *Hae*III-digested pLE3B; lane 5, *Bam*HI-digested pLE3C; lane 6, *Hae*III-digested pLE3C; lane 7, *Bam*HI-digested pLE3D; lane 8, *Hae*III-digested pLE3D.

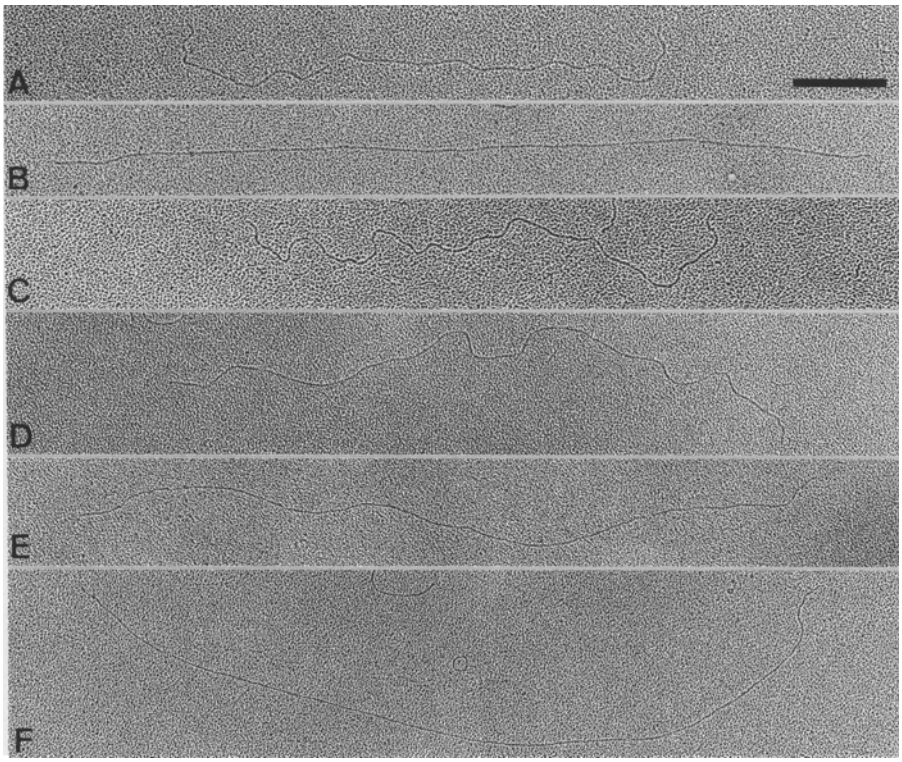

Fig. 4. Electronmicrographs of the six plasmids from *Lentinula edodes*.
A, pLE1; B, pLE2; C, pLE3A; D, pLE3B; E, pLE3C; F, pLE3D. Bar=0.5 μm.

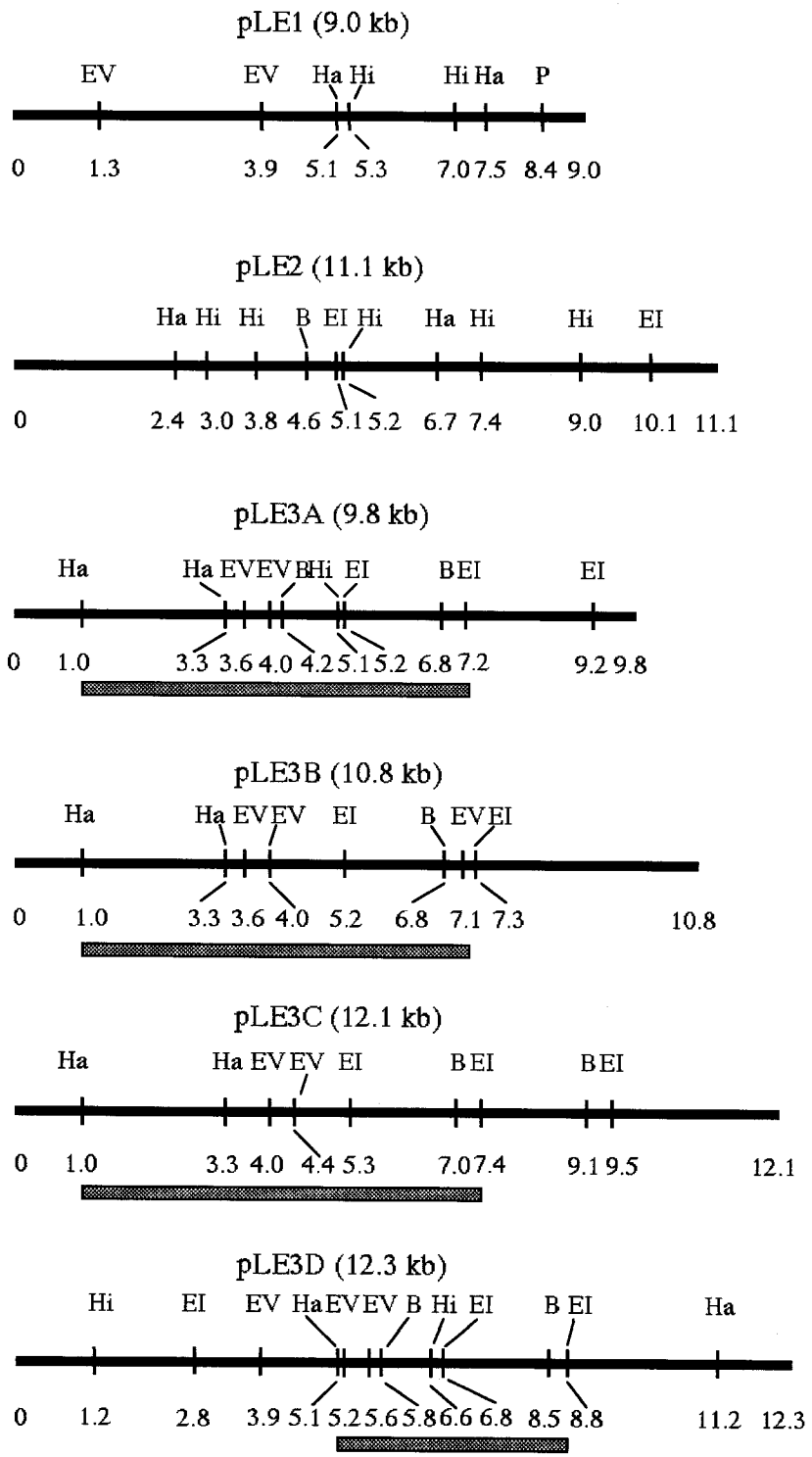

Fig. 5. Restriction site maps of the six plasmids carried by *Lentinula edodes*.
EI, *Eco*RI; EV, *Eco*RV; B, *Bam*HI; Ha, *Hae*III; Hi, *Hin*dIII; P, *Pvu*II. On each map, endonucleases not marked have no cleavage site on that plasmid. The shadowed boxes show regions in which the relative orientation of certain restriction sites is similar among the four variant plasmids of the same homology group.

former two homology groups. Hence, the six kinds of plasmids from *L. edodes*, those of 9.0, 11.1, 9.8, 10.8, 12.1 and 12.3 kb, were designated as pLE1, pLE2 (=pLLE1), pLE3A, pLE3B, pLE3C and pLE3D, respectively and were assigned to three different homology groups.

Characterization of plasmids To examine some of their properties, the six plasmids isolated from representative wild isolates were analyzed by digestion with *Bam*HI, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, and *Pvu*II restriction endonucleases. The result is shown in Fig. 5. Each of the six plasmids was found to have a unique restriction pattern. Moreover, the restriction pattern of pLE2 from Japanese wild isolates matched that of pLLE1 reported previously (Katayose et al., 1990). For each plasmid, the total molecular weight of fragments produced by single or double endonuclease digestion coincided with that before digestion, but the number of fragments derived from the double digestion was always one less than the total number derived from single digestions with the same two endonucleases. The results indicated that the six *L. edodes* plasmids might all be linear molecules. A linear structure with two distinct ends was confirmed by electron microscopy (Fig. 4). Based on these restriction analysis data and determination of two terminal restriction fragments from each plasmid by labeling the 3′ ends of the whole genome with terminal transferase, restriction site maps for the six linear plasmids were constructed (Fig. 5).

Comparison of the site maps of pLE3A, pLE3B, pLE3C and pLE3D revealed that, although variations in restriction sites occurred over the whole molecule, the relative orientation of certain restriction sites in the near-central part (shadowed boxes in Fig. 5) was similar. This similarity appeared to correlate with the occurrence of cross-hybridization among them. Minor restriction site variations were also recognized among DNA molecules of pLE1 and pLE2 plasmids from geographically distant wild isolates, but these do not alter the total molecular sizes.

When the proteinase K treatment step was omitted before phenol extraction during the process of total DNA isolation, it was difficult to detect the four pLE3 plasmids by agarose gel electrophoresis, and pLE1 and pLE2 also appeared as much less intensive bands. The fact that the proteinase K treatment is essential for positive detection of plasmids suggested the association of proteins with the plasmid DNA molecules. Further characterization of physicochemical profiles of the six plasmids will

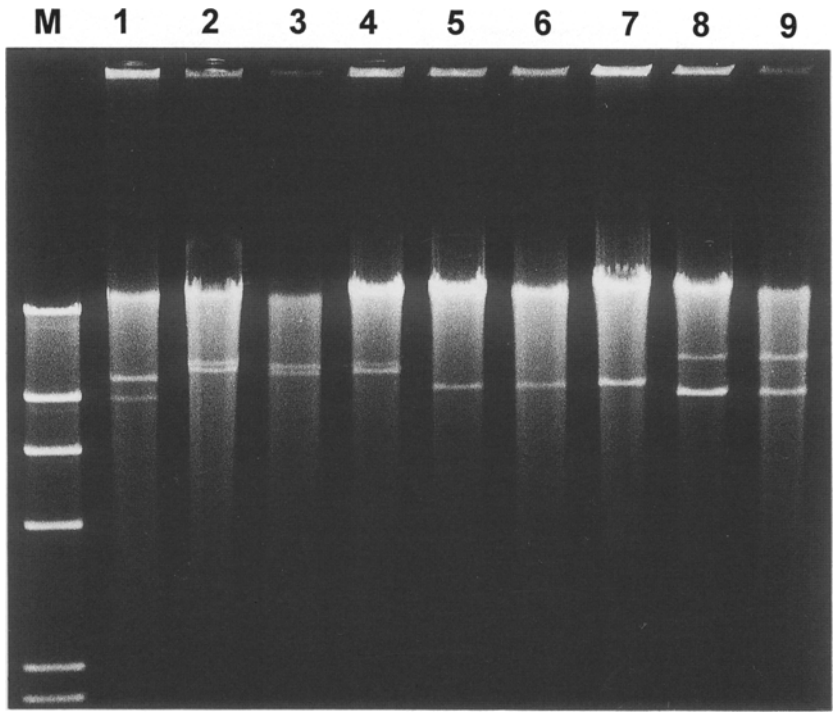

Fig. 6. Electrophoretic analysis of total DNA samples of newly established dikaryons derived from reciprocal crossing among compatible monokaryons having different kinds of plasmids.
Lane M, *Hin*d III-digested λDNA size markers; lane 1, PN1502a monokaryon with pLE3B and pLE1; lane 2, newly established dikaryon (PN1502a × J1158a) from J1158a monokaryon side retaining pLE3C and pLE2; lane 3, J1158a monokaryon with pLE3C and pLE2; lane 4, newly established dikaryon (J1158a × BO689a) from J1158a monokaryon side retaining pLE3C and pLE2; lane 5, newly established dikaryon (J1158a × BO689a) from BO689a monokaryon side retaining pLE3A; lane 6, BO689a monokaryon with pLE3A; lane 7, newly established dikaryon (BO689a × NZ1569a) from BO689a monokaryon side retaining pLE3A; lane 8, newly established dikaryon (BO689a × NZ1569a) from NZ1569a monokaryon side retaining pLE3D and pLE1; lane 9, NZ1569a monokaryon with pLE3D and pLE1.

be reported elsewhere.

Transmission pattern of plasmids in sexual crosses and their intracellular location To study the transmission of the six plasmids in sexual crosses, four representative dikaryotic isolates, namely, PN1502 with pLE1 and pLE3B, BO689 with pLE3A, J1158 with pLE2 and pLE3C, and NZ1569 with pLE1 and pLE3D, were dedikaryotized into their two component monokaryons with different nuclei according to the protoplast isolation and regeneration method of Fukumasa-Nakai et al. (1994). These monokaryons were examined for the presence of plasmids by electrophoretic analysis of their total DNAs and found to retain a set of all plasmids harbored by the original dikaryons. Next, reciprocal crossings were carried out between PN1502a monokaryon (with pLE1 and pLE3B), J1158a monokaryon (with pLE2 and pLE3C), BO689a monokaryon (with pLE3A) and NZ1569a monokaryon (with pLE1 and pLE3D). All newly established dikaryons carried the same set of plasmids as the nuclear recipient monokaryons (Fig. 6), indicating that the plasmids are uniparentally (maternally) transmitted in sexual crosses, as is the case for cytoplasmic genetic elements, that is, mitochondrial genome DNAs (Fukuda et al., 1995). Moreover, the two new dikaryons from each reciprocal cross, which have the same nuclei but different plasmid types, were shown to be similar in their morphological properties. The plasmids retained by the dikaryons were also effectively

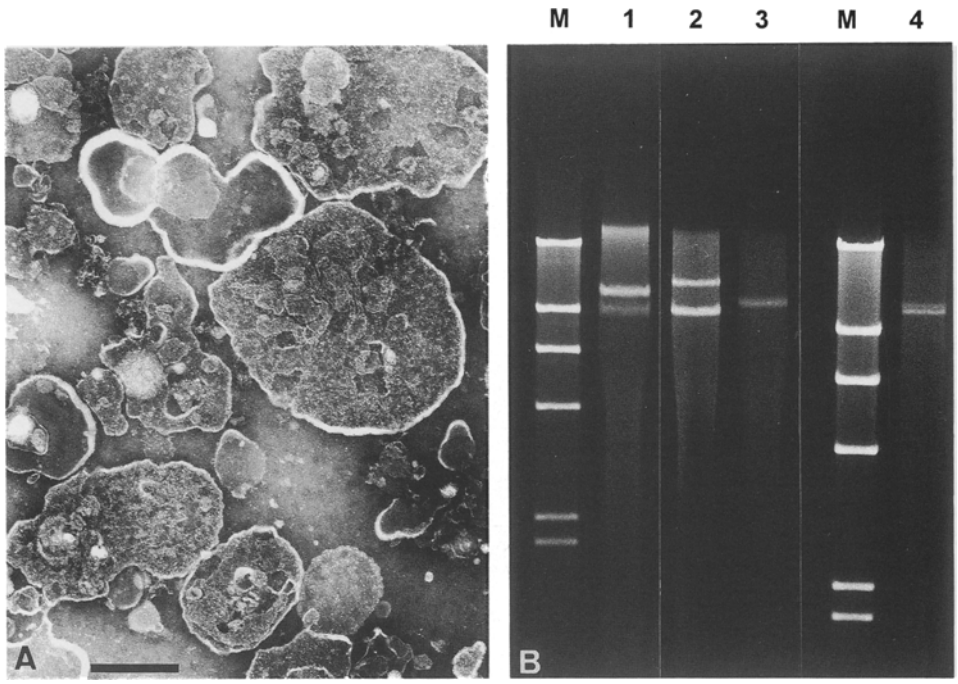

Fig. 7. Electronmicrograph (A) of mitochondria fractionated from fresh mycelium of *Lentinula edodes*, and electrophoretic analysis (B) of DNA preparations from the mitochondrial samples.
A: 1% phosphotungstic acid negative staining of mitochondria. Bar=0.5 μm. B: ethidium bromide staining of 0.7% agarose gel. Lane M, *Hind* III- digested λDNA size markers; lanes 1 to 4, mitochondrial DNA preparations from PN1502 isolate with pLE3B and pLE1, NZ1569 isolate with pLE3D and pLE1, BO689 isolate with pLE3A, and J1158 isolate with pLE3C and pLE2, respectively.

Table 3. Distribution of six plasmid types in geographically distant wild isolates of *Lentinula edodes*.

Wild isolates from	Plasmid types detected[a]					
	pLE1	pLE2	pLE3A[b]	pLE3B[b]	pLE3C[b]	pLE3D[b]
Japan	−	+	−	−	+	−
Nepal	−	+	−	−	−	−
Thailand	−	+	−	−	−	−
Borneo	−	−	+	−	−	−
Papua New Guinea	+	+	−	+	−	−
New Zealand	+	−	−	−	−	+

a) +, detected; −, not detected.
b) Plasmid type unique to each geographically distinct region.

transferred and maintained in the individual basidiospore progeny, indicating that the plasmids were stable through the cell cycle of sexual reproduction. To further examine the intracellular location of these plasmids, mitochondrial fractions were purified from fresh mycelial cultures of the same four representative isolates (Fig. 7A). Electrophoretic analysis of DNAs from these mitochondrial fractions revealed that all plasmids were associated consistently with the mitochondrial DNA preparations (Fig. 7B). This result, together with the finding of uniparental transmission of the six *L. edodes* plasmids, suggested that these plasmids are all located in mitochondria.

Geographical distribution of plasmids pLE1 and pLE2 were widely distributed in wild isolates from Papua New Guinea and New Zealand, and from Papua New Guinea and northern hemispherical regions including Japan, Nepal, and Thailand, respectively (Table 3). The four other plasmids, pLE3A, pLE3B, pLE3C, and pLE3D, were unique to wild isolates from Borneo, Papua New Guinea, Japan, and New Zealand, respectively. pLE3B was found only in the four isolates (PN1499, PN1500, PN1502, and PN1503) collected only from the highlands of Papua New Guinea at about 3,000 m elevation, in which it was present in combination with pLE1 alone or pLE1 and pLE2. In the Japanese isolates, pLE3C was frequently found in combination with pLE2. All four New Zealand isolates used contained both pLE1 and pLE3D. These findings indicated that the multiple plasmids present in the same isolates were all from mutually different homology groups.

Discussion

The present study revealed the presence of six kinds of linear mitochondrial DNA plasmids in the shiitake mushroom, *L. edodes*. Five of them, pLE1, pLE3A, pLE3B, pLE3C and pLE3D, were detected for the first time in this study, and pLE2 was identical to the pLLE1 reported previously in a commercial strain of this fungus (Katayose et al., 1990). Southern hybridization analysis revealed that the six plasmids belonged to three different homology groups, and pLE3A, pLE3B, pLE3C and pLE3D were variant types constituting a common homology group. Presumably, the four variant plasmids differentiated from a common ancestral plasmid by re-arrangement of DNA sequences in mainly large-scale insertions or deletions during their evolution. In contrast, pLE1 and pLE2 have each maintained an identical molecular size without any major restriction site variations, although they both have wider geographical distributions more than the four variant plasmids. However, at present we have no sufficient evidence to prove the plasmid diversification in *L. edodes* in natural populations. More detailed information on their physicochemical properties including sequence comparison is needed.

The six plasmids of *L. edodes* were present singly or in combinations of two or three different plasmid types in about 80% of wild isolates examined. Multiple plasmids present in the same isolate were always from different homology groups. This suggests that plasmids of the same homology group can not occupy the same host, and that the four variant plasmids may be incompatible with each other. The coexistence of different plasmid families in the same isolates is a general phenomenon observed in many other fungi (Meinhardt et al., 1990). On the other hand, each of the *L. edodes* plasmids was mitotically and meiotically stable, whether present singly or with another type. This suggests the independent replication of these plasmids, and relates to their very high frequency of distribution in natural populations. The widespread incidence of *L. edodes* plasmids suggests that they may be neutral genetic elements for their hosts, in contrast to the exceptional case of the kalilo plasmid of *Neurospora* species, which reduces its incidence in populations through its destructive effect on hosts (Arganoza et al., 1994; Debets et al., 1995; Yuewang et al., 1996). The finding that two *L. edodes* reciprocal dikaryons with the same nuclei but different plasmid types showed similar morphological properties also supports this idea. In this regard, Robison and Horgen (1994) reported that for *A. bitorquis*, the presence or absence of one or both of pEM and pMPJ plasmids is not correlated to any obvious phenotype.

Interesting patterns were noticed in the geographical distribution of the *L. edodes* plasmids. pLE1 was found in wild strains distributed widely in the southern hemisphere from the South Pacific to Australasia, while pLE2 was commonly associated with the northern hemisphere strains of South Pacific-Asian distribution. This plasmid distribution pattern suggests that the ancestral strains of *L. edodes* with pLE1 and/or pLE2 originally inhabited the South Pacific area and subsequently they came to retain only one type of plasmid as they spread their geographical distribution to the north or south. This speculation conforms with the hypothesis, based on nuclear ribosomal DNA sequence analysis, that the origin of Asia-Australasian-distributed *L. edodes* is in the South Pacific (Hibbett et al., 1995). In addition, our findings provide better understanding of the phylogenetic relation of Asia-Australasian-distributed natural populations of *L. edodes* with significantly macromorphological variations (Shimomura et al., 1992). It is also interesting to note that the delimited natural populations harboring one of the four variant plasmids (pLE3A, pLE3B, pLE3C and pLE3D) of the same homology group correspond to the four phylogenetically divergent lineages deduced from recent molecular genetic studies using mitochondrial DNA restriction fragment length polymorphism (RFLP) analysis (Fukuda et al., 1994) and nuclear ribosome DNA sequence and RFLP analysis (Hibbett et al., 1995; Nicholson et al., 1995).

To incorporate the plasmid properties in the elucidation of *L. edodes* phylogeny, more extensive studies are necessary. However, the present results suggest that the plasmid distribution patterns allow estimation of the geographical source of *L. edodes* wild isolates.

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