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Regional Variations of Cellular Slime Molds Referred to Ribosomal DNA

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ABSTRACT: Regional variations of Dictyostelid cellular slime molds were examined using molecular data. The intertranscribed spacer regions including the 5.8S ribosomal DNA of 2 species(*D. purpureum, P. violaceum*) of Cellular Slime Molds were sequenced and analyzed. Among 13 strains of *D. purpureum* and 12 strains of *P. violaceum* analyzed, each two strains were obtained from ATCC and the others were isolated from the forest soils in Korea. The sequences of the 5.8S ribosomal DNA were conserved among the strains of the same species, but unexpectedly highly variable among species. A high level of genetic diversity was found which was best resolved at the genus/species level as well as the family level by sequence data from the ITS 1 and ITS 2 regions. According to the sequence alignments by CLUSTAL X and the phylogeographic analyses by PAUP, 12 strains of *P. violaceum* were divided into three groups among which there were no difference of the morphological characteristics. Among 13 strains of *D. purpureum*, genetic variations were related to two morphological types, the temperate and subtropical type. There was no variation pattern according to geography in Korea, but there were some variations between Korea and other countries.

Key words: Dictyostelid, Intertranscribed spacer region, Regional variation.

INTRODUCTION

Dictyostelid cellular slime molds or dictyostelids are normal components of soil microbial communities. Feeding on soil bacteria, the dictyostelids help control and modify soil bacterial populations, consequently stimulate decomposition and mineralization of soil nutrients. Dictyostelids also appear to serve as excellent bioindicators of general soil microbial activity.

Since Oskar Brefeld isolated *Dictyostelium mucoroides* and described it in 1869, the ecological studies of the dictyostelids have been performed by Singh (1947), Cavender and Raper (1965), Bonner (1967), Olive (1975), Traub and Hohl (1976), Cavender *et al.* (1981), Raper (1984), Hagiwara (1989), Vadell *et al.* (1995).

The dictyostelids have been recovered from soils of temperate deciduous forest, tropical deciduous and seasonal evergreen rainforest, boreal coniferous forest, and tundra by various investigators around the world. And in Korea, they have been recovered mainly from soils of forest (Shim 1998).

Worldwide, eighty species of dictyostelids were found to occupy various forest soils. These species distributions fall into one of four categories: cosmopolitan, disjunct, restricted, and pantropical (Swanson *et al.* 1999). *P. violaceum* and *D. purpureum* used in this study fall just short of cosmopolitan, *D. purpureum* being somewhat limited to forest soils of lower latitudes, in world. In Korea, *D. purpureum* and *P. violaceum* are widely distributed and have high occurrence frequency (Shim 1998).

In previous studies, they are focused on the distributions of species simply based on morphological characteristics. In fact, all the ecological researches on dictyostelids were related to the morphology and physiology. However, the development of molecular biological techniques introduced molecular geography into species' distributions. These include biochemical tests, sequence analysis of ribosomal genes and isozyme electrophoresis (Lanfranco *et al.* 1999).

In dictyostelids, the molecular data of ribosomal DNA have been used in phylogeny and comparing the species (Kang and Chang 2000, 2001).

We propose here to make use of the molecular geographic approach to try to reveal the degree of intraspecific variations, relations between genetic variations and morphology and distribution patterns with the intraspecific phylogeny based on variation in sequences of ITS regions.

MATERIALS AND METHODS

25 strains of 2 species that were identified by the morphological characteristics based on the reports of Hagiwara (1989) and Raper (1984) used in this study are as represented in Fig. 1. 4

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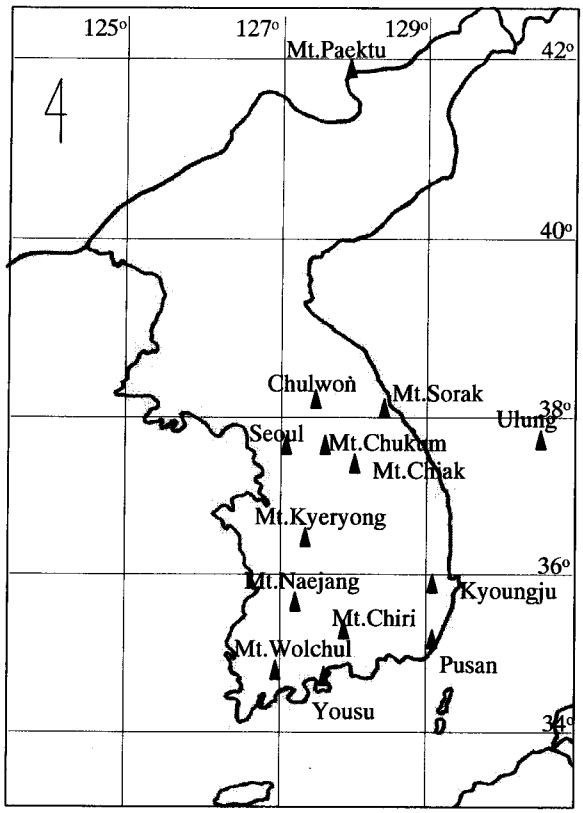

Fig. 1. Collection locations of *D. purpureum* and *P. violaceum*.

strains were purchased from the American Type culture Collection (ATCC). Genomic DNAs were extracted from the amoeba stages that were grown for 12-15 hours from spores on LP agar. Amoebae were collected by washing the surface of the agar media with modified HL-5 media (Schwalb and Roth, 1970). DNAs were prepared according to Nellen *et al.* (1987).

The polymerase chain reaction (PCR) was applied to amplify the 18S rDNA. The primers were designed based on the sequence of *Dictyostelium discoideum* (Ozaki *et al.*, 1984) and the primer binding sites were at the beginning of the 18S rDNA and at the beginning of the 5.8S rDNA. The primer sequences are : 18S Forward=CACACCGCCCGTCGCTCCTACCGATCG, 28S Reverse=TCCTCCGCTTACTGATATGC. The 50 μl of PCR reaction mixture consisted of 34.5 μl sterile distilled water, 5 μl of 10 × Taq polymerase buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$), 2 μl of 5 mM dNTP, 5 unit of Taq DNA polymerase (TaKaRa Biochemicals, Japan), 2 μl each of 10 mM primer 18S Forward and 28S Reverse and approximately 5-10 ng genomic DNA. The thermal cycling was performed on a Perkin-Elmer Thermal Cycler with the following condition: 1 cycle of 5min at 94℃ was followed by 35 cycles of 1min at 95℃ for denaturation, 1min at 55℃ for annealing and 1min at 72℃ for extension. A final extension at 72℃ for 5 min was conducted after the thermal cycling. The amplified products were purified by QIAprep spin miniprep kit (QIAGEN Inc., Chatsworth, USA). Then they were reacted with Thermosequenase kit (Amersham Life Science, Cleveland, USA). The samples were separated on a 4 % Long Ranger gel (FMC BioProducts, Rockland, USA) and sequenced with the Automatic DNA Sequencer, Long ReadIR 4200 (LI-COR Inc, Lincoln, USA). To verify the accuracy of sequences, all the samples were sequenced twice.

Sequences were aligned by multiple alignment method of the computer software CLUSTAL X (Thompson *et al.* 1997). The determination of ITS1, 5.8S rDNA and ITS2 was based on the genomic sequence of D. discoideum (Olsen and Sogin 1982, Ozaki *et al.* 1984). The length and GC contents of each region were analyzed. Pairwise comparisons were carried out for the 5.8S rDNA using the heuristic search of PAUP (ver. 4.0b; Swofford 1998). Aligned sequences were analysed using both

Table 1. The lengths and GC contents of ITS 1, 5.8S rDNA and ITS 2 regions of *D. purpureum*

	ITS 1		5.8S rDNA		ITS 2		Total	
	Length	GC contents	Length	GC contents	Length	GC contents	Length	GC contents
PAm	309	23.3	162	43.8	469	33.3	941	31.8
PCa	306	22.9	162	42.0	400	27.8	868	28.7
PCk	306	23.2	162	42.6	400	28.0	868	29.0
PCr	306	22.9	162	43.3	400	28.8	868	29.5
PCw	306	22.5	162	42.6	400	27.5	868	28.6
PJp	306	23.2	162	44.1	400	27.8	868	29.1
PKj	306	22.9	162	42.6	400	28.0	868	28.9
PKr	306	22.9	162	42.9	400	28.3	868	29.0
PPs	306	23.2	162	42.6	400	28.5	868	29.3
PPt	306	22.5	162	42.6	400	28.0	868	28.8
PSe	306	22.2	162	42.6	400	28.0	868	28.7
PUl	306	23.2	162	42.6	400	28.0	868	29.0
PWc	306	22.9	162	42.6	400	28.0	868	28.9

Abbreviations : PAm (America), PCa (Mt. Chiak), PCk (Chukum), PCr (Mt.Chiri), PCw (Chulwon), PJp (Japan), PKj (Kyoungju), PKr (Mt. Kyeryong), PPs (Pusan), PPt (Mt. Paektu), PSe (Seoul), PUl (Ulung), PWc (Mt. Wolchul).

Table 2. The lengths and GC contents of ITS 1, 5.8S rDNA and ITS 2 regions of *P. violaceum*

	ITS 1		5.8S rDNA		ITS 2		Total	
	Length	GC contents	Length	GC contents	Length	GC contents	Length	GC contents
VCk	204	24.0	162	42.6	385	17.4	751	24.6
VCr	244	25.4	160	44.4	450	29.6	854	31.1
VCw	238	25.6	161	44.1	450	30.0	849	31.3
VFr	205	24.4	162	42.0	386	17.1	753	24.4
VJp	216	21.8	161	42.9	417	18.2	794	24.2
VKr	238	25.6	161	44.1	451	29.0	850	30.9
VNj	207	21.7	161	43.8	416	20.0	784	25.4
VSe	244	25.4	161	44.1	450	28.9	855	30.8
VSk	220	22.7	162	42.6	387	17.1	769	24.1
VSr	238	25.6	161	44.1	450	30.0	849	31.2
VWc	220	22.7	162	42.6	387	17.1	769	24.1
VYs	205	22.0	161	42.9	416	20.2	782	25.3

Abbreviations : VCk(Chukum), VCr(Mt.Chiri), VCw(Chulwon), VFr(France), VJp(Japan), VKr(Mt. Kyeryong), VNj(Mt. Naejang), VCa(Mt. Chiak), VSe(Seoul), VSk(Mt. Nam, Seoul), VSr(Mt.Sorak), VWc(Mt. Wolchul), VYs(Yousu).

Table 3. Sequence divergence of *D. purpureum*. Above diagonal is mean character differences and below diagonal is total character differences

	PPs	PUl	PKr	PCw	PCa	PCr	PKj	PWc	PCk	PPt	PSe	PJp	PAm
PPs		0.0035	0.0046	0.0115	0.0115	0.0081	0.0046	0.0069	0.0104	0.0150	0.0161	0.0173	0.0936
Pul	3		0.0035	0.0104	0.0104	0.0115	0.0058	0.0081	0.0115	0.0161	0.0173	0.0184	0.0948
PKr	4	3		0.0069	0.0069	0.0104	0.0046	0.0069	0.0104	0.0150	0.0162	0.0173	0.0938
PCw	10	9	6		0.0115	0.0150	0.0092	0.0115	0.0127	0.0173	0.0184	0.0196	0.0934
PCa	10	9	6	10		0.0127	0.0069	0.0069	0.0104	0.0104	0.0115	0.0173	0.0913
PCr	7	10	9	13	11		0.0081	0.0081	0.0115	0.0161	0.0173	0.0184	0.0948
PKj	4	5	4	8	6	7		0.0023	0.0058	0.0104	0.0115	0.0127	0.0890
PWc	6	7	6	10	6	7	2		0.0035	0.0081	0.0092	0.0104	0.0867
PCk	9	10	9	11	9	10	5	3		0.0069	0.0104	0.0138	0.0879
PPt	13	14	13	15	9	14	9	7	6		0.0035	0.0138	0.0879
Pse	14	15	14	16	10	15	10	8	9	3		0.0150	0.0890
PJp	15	16	15	17	15	16	11	9	12	12	13		0.0925
PAm	81	82	81	81	79	82	77	75	76	76	77	80	

parsimony and distance methods using PAUP (ver. 4.0b; Swofford 1998).

RESULTS AND DISCUSSION

25 strains of cellular slime molds were analyzed. ITS regions from these cellular slime molds were amplified with 18S Forward and 28S Reverse primers. The PCR products were directly sequenced with PCR primers and *E. coli* genome was not amplified during PCR.

The length and GC contents of ITS 1, ITS 2 and 5.8S rDNA are represented in Table1 and Table 2. Total lengths of ITS 1, 5.8S rDNA and ITS 2 regions of *Dictyostelium purpureum* ranged from 868 to 941(PAm) bases, with an ITS1 of 306 - 309 (PAm) bases, a 5.8S rDNA of 162 bases and an ITS2 of 400 to 469 (PAm) bases (Table 1). In *P. violaceum*, total lengths ranged from 751 (VCk) to 855 (VCr) bases, with an ITS 1 of 204 (VCk and VSe) to 244 (VCr) bases, a 5.8S rDNA of 160 (VCr) - 162 bases and an ITS 2 of 385 (VCk) to 451 (VKr) bases (Table 2). According to Kang and Chang (2001), total lenghs of ITS regions are highly variable among species, especially in *P. violaceum*, it is very short. All the strains of *P. violaceum* have short sequences of ITS regions.

The GC contents of the ITS regions were lower than 50% and were variable among strains, from 28.6 (PCw) to 318 (PAm) in *D. purpureum* (Table 1) and 24.1 to 31.3 (VCw) in *P. violaceum*. (Table 2) The GC contents of ITS 1 and ITS 2 (17.1 to 33.3) are lower than 5.8S rDNA(42.0 to 44.4) in both species. GC contents of cellular slime molds are relatively lower than other eukaryotes. GC contents are similar to that of *Saccharomyces cerevisiae*, but quite different from those of *Xenopus lavis, Physarum poly-*

Table 4. Sequence divergence of *P. violaceum*. Above diagonal is mean character differences and below diagonal is total character differences

	VCw	VSr	VKr	VSe	VCr	VSk	VWc	VCk	VFr	VNj	VYs	VJp
VCw		0.0012	0.0024	0.0059	0.0059	0.1921	0.1921	0.1801	0.1850	0.2147	0.2205	0.2218
VSr	1		0.0012	0.0047	0.0047	0.1908	0.1908	0.1788	0.1837	0.2147	0.2205	0.2205
VKr	2	1		0.0047	0.0047	0.1882	0.1882	0.1761	0.1810	0.2131	0.2189	0.2189
VSe	5	4	4		0.0117	0.1895	0.1895	0.1774	0.1823	0.2186	0.2244	0.2179
VCr	5	4	4	10		0.1950	0.1950	0.1817	0.1879	0.2176	0.2234	0.2221
VSk	146	145	143	144	148		0.0000	0.0067	0.0134	0.1797	0.1854	0.1794
VWc	146	145	143	144	148	0		0.0067	0.0134	0.1797	0.1854	0.1794
VCk	134	133	131	132	135	5	5		0.0093	0.1801	0.1878	0.1804
VFr	138	137	135	136	140	10	10	7		0.1833	0.1903	0.1829
VNj	164	164	163	167	166	131	131	130	132		0.0090	0.0191
VYs	168	168	167	171	170	135	135	135	137	7		0.0230
VJp	171	170	169	168	171	132	132	131	133	15	18	

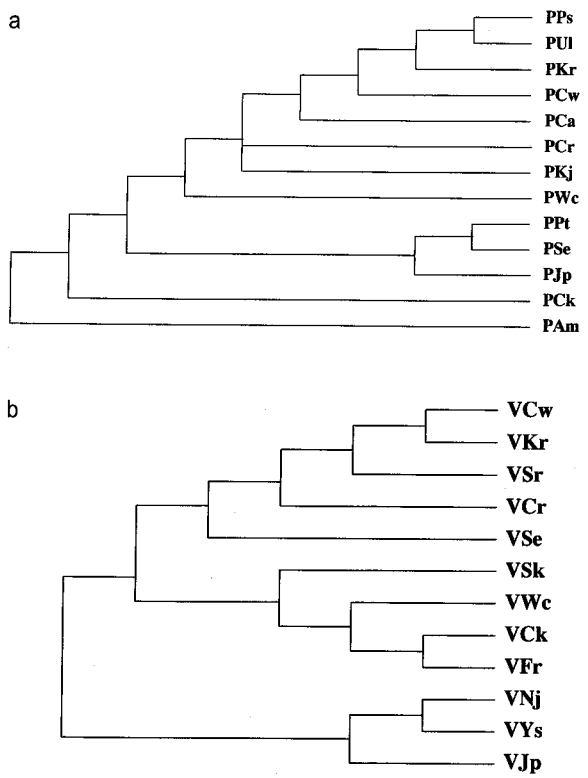

Fig. 2. One of the 26 most parsimonious tree of *D. purpureum* (a) and one of the 36 most parsimonious tree of *P. violaceum* (b).

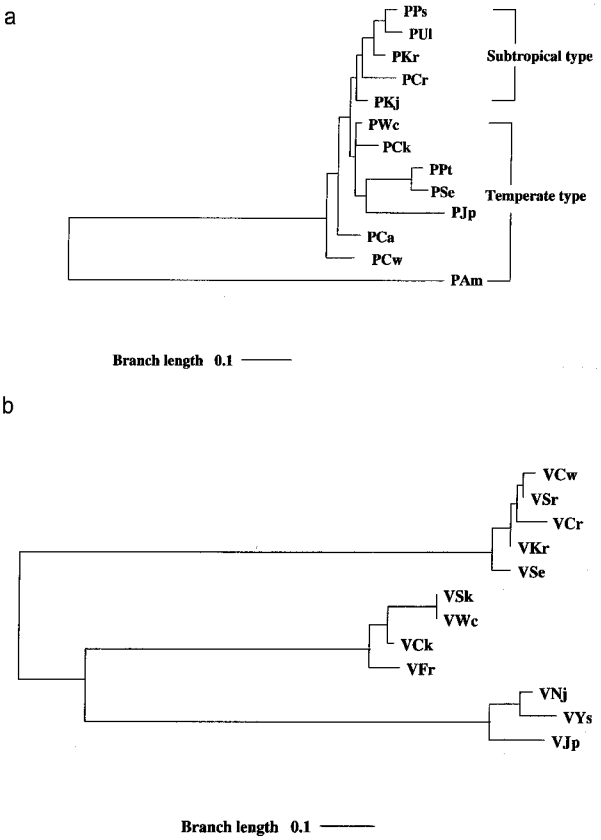

Fig. 3. Neighbor Joining tree of *D. purpureum* (a) and *P. violaceum* (b).

cephalum and rat (Ozaki *et al.* 1984). In general there is the tendency that the GC contents of rDNA of the lower eukaryotes such as yeast, dictyostelids is lower than that of higher eukaryotes, and the ITS 1 regions is extremely AT rich.

According to the multiple sequence alignments by CLUSTAL X, sequence alignments of ITS 1 and ITS 2 have many ambiguous and gap sites in *P. violaceum*. However there is a no great variation in *D. purpureum*. Pairwise comparisons between all possible combinations were carried out for ITS regions (Tables 3 and 4). Sequence divergences are ranged from 0.0023 (PKj and PWc) to 0.0948 (PAm and PUl, PCr) in *D. purpureum* (Table 3) and from 0.0012 (VSr and VCw, Vkr) to 0.2221 (VCr and VJp) in *P. violaceum* (Table 4). The sequence divergences of 5.8S rDNA are high among the species (Kang and Chang 2001). Moreover,

there are too great divergence to align the sequences of ITS 1 and ITS 2. But in this result, ITS regions have enough divergence to study the intraspecific variations.

Using only the potentially informative data with gaps coded as missing, we obtained the phylogenetic tree of *D. purpureum* (Fig 2a). Of the 953 aligned positions from the ITS 1, 5.8S rDNA and ITS 2 regions of the 12 strains, 845 characters were constant, 80 characters were variable, 28 characters of which were phylogenetically informative. The parsimony analyses generated 26 most parsimonious trees with the entire ITS and 5.8S rDNA sequences(Fig. 2a). They had a length of 184 steps, a consistancy index(CI) of 0.832, a retention index (RI) of 0.846 and a rescaled consistancy index (RC) of 0.732. In *P. violaceum*, of the 883 total characters, 622 characters were constant, 26 characters were variable, 235 characters of which were phylogenetically informative. The parsimony analyses generated 36 most parsimonious trees with the entire ITS and the 5.8S rDNA sequences (Fig. 2b). They had a length of 184 steps, a consistency index(CI) of 0.832, a retention index (RI) of 0.846 and a rescaled consistency index (RC) of 0.732.

Neighbor Joining trees were generated using PAUP (ver.4.0b; Swofford 1998) (Fig. 3a and 3b). According to Hagiwara (1989), *D. purpureum* have two subtypes. One is named temperate form which is often makes large sorocarps, 6 mm in height and produces comparatively elongated spores (2.1< length/width < 3.0). The other is named subtropical form that makes smaller sorocarps, darker or rather blackish sori and thicker spore. 5 strains, PPs, PUl, PKr PKj and PCr are subtropical forms according to Hagiwara (1989) and the others are temperate form. In NJ tree, 5 strains of subtropical form are grouped into one clade (Fig. 3a).

In *P. violaceum*, there are three distictive clades: clade 1 includes VCw, VKr, VSr, VCr and Vse, clade 2 includes VSk, VWc, VCk, VFr, and clade 3 includes VNj, Vys, VJp (Fig. 3b). However, there is no distinctive differences in morphological characteristics which divide the *P. violaceum* into 3 clades, and there is no regional patterns which explains the genetic divergences. In fact, *P. violaceum* have very various morphology, which includes symmetry, branch length and number of branch. This is because that all the Polysphondylia which have violet spore were generally classified into *P. violaceum*. Recently, Vadell and Cavender (1998) and Kang and Chang (2001) have identified 2 new violet Polysphondylia. However, all strains used in this research are *P. violaceum*. Therefore this study shows that *P. violaceum* have 3 genetic subtypes according to ITS regions, however this is not able to be expalined by morphological characteristcs. Moreover other information of gene can divide *P. violaceum* into other patterns.

Both species shows no distinctive regional variations in sequences of ITS regions. However, PJp and PAm have the highest divergence within the temperate types in *D. purpureum*, and VFr and VJp have the highest divergence within each clade in *P. violaceum* (Table 4).

In general the occurrence frequency per site is low, and in some species, it is difficult to culture and harvest the amoeba (*D. minutum* and *D. polycephalum*). Some species which have high frequency are not be able to used to study because of the ambiguity of species determination (*P. tenuissimum* and *P. pallidum*). Therefore the more species and the larger regions studied may reveal the more about regional variations of dictyostelids.

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