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A TAXONOMIC STUDY OF THE FAMILY DICTYOSTELIACEAE BASED ON RIBOSOMAL DNA SEQUENCES

KYOUNG-MI KANG AND NAM-KEE CHANG

Department of Biology Education,
Seoul National University,
Shillim-dong, Kwanak-gu, Seoul 151-742, Korea

Abstract

The sequences of the 5.8S nuclear ribosomal DNA and the internal transcribed spacer (ITS1 and ITS2) regions from 12 species of Dictyosteliaceae were analyzed. These regions were extremely divergent in what we consider to be a single family. In the ITS1 and ITS2 regions there were high nucleotide length variations between two genera, *Dictyostelium* and *Polysphondylium*. The results of sequence alignments revealed that the sequences of the 5.8S rDNA had sufficient divergences to be used for a taxonomic study of these taxa and the ITS regions were useful to discriminate between closely related species.

Introduction

Dictyostelid cellular slime molds or Dictyostelids are most abundant in the surface humus layers of forest soils and live as unicellular bacteria-feeding myxamoeba which, under the conditions of food depletion, aggregate to form a fungus-like multicellular structure (Bonner, 1967; Raper, 1984). Since Oskar Brefeld isolated and described *Dictyostelium mucoroides* in 1869, the taxonomy of dictyostelids have been studied by Bonner (1967), Olive (1975), Cavender *et al.*, (1981), Raper (1984), Hagiwara (1989), etc. Dictyostelids are taxonomically comprised in the order Dictyosteliales (Raper, 1984). The order Dictyosteliales contains two families, Dictyosteliaceae and Acytosteliaceae. The family Dictyosteliaceae contains three genera, *Dictystelium, Polysphondylium* and *Coenonia* (Raper, 1984). The last genus was founded on *C. denticulata* by van Tieghem in 1884. However, this monotypic genus has not been rediscovered anywhere (Bonner, 1967; Hagiwara, 1989). So far, about 70 species of dictyostelids have been discovered in soils of various forests (Swanson *et al.*, 1999; Cavender & Vadell, 2000).

The traditional and current classification of dictyostelids has been based on morphological and developmental characteristics (Bonner, 1967; Olive, 1975; Raper, 1984; Hagiwara, 1989; Vadell & Cavender, 1998). These characteristics are sometimes too ambiguous to differentiate one species from other morphologically similar species and may vary under different culture conditions. In recent years, several alternative taxonomic approaches have been proposed. These include biochemical tests, sequence analysis of ribosomal genes and spacers, and isozyme electrophoresis. The rDNA ITS regions (ITS1, ITS2, and the 5.8S gene) have been widely used to discriminate various taxa at the family, generic and subgeneric levels (Carbone & Kohn, 1993; Driver *et al.*, 2000). In addition, variations in these regions have been used to identify species or strains, as markers in population genetic studies (Lanfranco *et al.*, 1999). However, the molecular taxonomy of dictyostelids has not previously been addressed at the intra-family level. In the present study, we compared the sequences of the rDNA ITS regions from 12 species of dictyostelids, assessed variations between two genera and among 12 species and constructed the phylogenetic tree among them.

E-mail: kang73@snu.ac.kr(corresponding author)

Materials and Methods

The species used in this study are as listed in Table 1. Eleven species were isolated from the soils collected at Mt. Nam in Seoul and Mt. Halla in Cheju island, Korea (Kang *et al.*, 1998), identified by the morphological characteristics based on the reports of Hagiwara (1989) and Raper (1984), and deposited in the Korean Collection for Type Cultures. One species, *D. discoideum* (KCTC 16360, ATCC 11735) was purchased from the Korean Collection for Type Cultures.

Table 1. List and Gene Bank accession number of Dictyostelids used in this study.

Species	Voucher	Gene Bank accession number
D. aureo-stipes	KCTC 16859[a]	AF219106
D. discoideum	KCTC 16360, ATCC 11735	V00192[b]
D. giganteum	KCTC 16858	AF219102
D. minutum	KCTC 16855	AF219105
D. mucoroides	KCTC	AF351197
D. polycephalum	KCTC 16856	AF219104
D. purpureum	KCTC 16857	AF219103
D. sphaerocephalum	KCTC	AF351199
P. candidum	KCTC 16861	AF219110
P. pallidum	KCTC 16863	AF219108
P. tenuissimum	KCTC 16862	AF219109
P. violaceum	KCTC 16860	AF219107
Physarum polycephalum		V01159
Didymium iridis		X60210

[a] KCTC=deposited at the Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology (KRIBB)

[b] Sequence from Olsen & Sogin (1982) which is identical with our result.

Genomic DNA was extracted from the amoeba stage of 12 species 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 & Roth, 1970). DNA was prepared according to Nellen *et al.*, (1987).

The polymerase chain reaction (PCR) was applied to amplify the ribosomal DNA including 140bps of 18S rDNA, full length of ITS1, 5.8S rDNA and ITS2, and 40bps of 28S rDNA. The primers were designed based on the sequence of *Dictyostelium discoideum* (Ozaki *et al.*, 1984) and the primer binding sites were at the end of the 18S rDNA and at the beginning of the 28S rDNA. The primer sequences are: 18S Forward=CACACCGCCCGTCGCTCCTACCGATCG, 28S Reverse=TCCTCCGCTTAC TGATATGC. 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 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 5 min at 94°C was followed by 35 cycles of 1 min at 95°C for denaturation, 1 min at 55°C for annealing and 1min at 72°C for extension. A final extension at 72°C for 7 min was conducted after the thermal cycling.

To clone the PCR products, pT7Blue(R) vector (Novagen, Madison, USA) was used. The blunt-ended vector and the cleaned PCR products were ligated and transformed to an

E. coli strain, JM109 (Promega, Madison, USA). The electroporation method using Electroporator || (Invitrogen, San Diego, USA) was applied to the transformation of *E.coli*. The cloned plasmids 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. The second sequencing was directly performed with the PCR products.

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 & 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). Phylogenetic tree was constructed by maximum parsimony method using PAUP.

Results and Discussion

Complete sequences of the rDNA ITS regions including parts of 28S and 18S rDNA were obtained for 12 dictyostelids and these sequences were submitted to the GenBank Nucleotide Database (Table 1). In case of *D. discoideum,* our sequences were almost identical to the sequences that were reported by Olsen & Sogin (1982) except a few base changes at the ITS regions. The length and GC contents of ITS1, ITS2 and 5.8S rDNA are presented in Table 2. The sequences and the length of the ITS1 and ITS2 regions varied among different species. The length of ITS1 ranged from 177 bps (*P. pallidum*) to 329 bps (*D. discoideum*). The difference of ITS1 between two genera was about 120 bps in length. The lengths of ITS2 of *Polysphondylia* except *P. violaceum* were longer than those of *Dictyostelia*. The length of ITS2 ranged from 384 bps (*P. violaceum*) to 639 bps (*P. tenuissimum*). The length of 5.8S rDNA was 162 or 163 bps in all taxa except *D. aureostipes* (172bps). The GC contents of all regions were lower than 50 % and showed a great variance in the ITS regions, ranging from 21.1 % (*P. tenuissimum*) to 40.3 % (*D. polycephalum*) in ITS1, from 17.4% (*P. violaceum*) to 43.4% (*D. polycephalum*) in ITS2.

At the beginning of this study, we focused on the ITS1 and ITS2 regions rather than the 5.8S rDNA, because the ITS1 and ITS2 regions are widely used to study systematics of most organisms (Yuan & Kupfer, 1995; Saenz & Taylor, 1999). In general, as ribosomal RNA genes evolve slowly, they have been used to compare very distant taxa and are less useful at finer taxonomic levels (Wainwright *et al.*, 1993). There is little divergence in 5.8S rDNA, but there is a sufficient divergence in ITS1 and ITS2 at the level of family and genus. However, the rDNA ITS regions of isolates used in this study were extremely divergent even in what we consider to be a single family. 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 and 5.8S rDNA regions. According to the multiple sequence alignment by CLUSTAL X, the sequences of ITS1 and ITS2 had too many ambiguous and gap sites to be used for the further analysis (data not shown). There are also extremely divergent ITS sequences at the genus/species level in some fungi (Redecker *et al.*, 1999; Dodd *et al.*, 2000).

Table 2. The length and GC contents of ITS1, 5.8S rDNA and

Species	ITS1		5.8S rDNA		ITS2		Total	
	Length (bps)	GC contents (%)	Length (bps)	GC contents (%)	Length (bps)	GC contents (%)	Length (bps)	GC contents (%)
D. aureo-stipes	318	32.7	172	50.0	604	30.6	1094	34.3
D. discoideum	329	27.7	162	42.0	575	43.1	1066	38.2
D. giganteum	296	38.8	162	43.8	451	37.7	909	39.2
D. minutum	310	28.7	163	41.7	485	25.8	958	29.4
D. mucoroides	293	30.4	162	42.6	421	35.9	876	35.3
D. polycephalum	325	40.3	162	44.4	548	43.4	1035	42.6
D. purpureum	308	22.7	162	42.6	400	27.3	870	28.5
D. sphaerocephalum	283	34.3	162	43.2	425	38.8	870	38.2
P. candidum	254	31.1	165	42.4	815	37.1	1234	36.5
P. pallidum	177	30.2	162	45.1	635	25.2	974	27.6
P. tenuissimum	180	21.1	162	45.1	639	24.6	981	27.3
P. violaceum	204	24.0	162	42.6	384	17.4	750	24.7

We analyzed relationships among species using the sequence alignments of the 5.8S rDNA and compared the sequences of ITS regions to discriminate between closely related species that had higher similarity in the 5.8S rDNA. We aligned the sequences of the 5.8S rDNA of 14 taxa including *Physarum polycephalum* and *Didymium iridis* as outgroup (Fig. 1). *Physarum polycephalum* and *Didymium iridis* are acellular slime molds (Plylum *Myxomycota*) which have something in common with cellular slime molds (Phylum *Acrasiomycota*, Class *Acrasiomycetes*, Order *Dictyosteliales*); they both lead double lives (myxamoeba and spore). Of the aligned 175 sites, 56 characters were constant and 88 characters were parsimony-informative for all the sequences surveyed. The nucleotide distances among all pairwise comparisons for 5.8S rDNA are summarized in Table 3. Mean character differences within dictyostelids ranged from 0.000 (*P. pallidum* and *P. tenuissimum*) to 0.311 (*D. aureostipes* and *D. minutum*). Mean character differences between dictyostelids and outgroup ranged from 0.425 (*D. aureostipes* and *P. polycephalum*) to 0.500 (*D. polycephalum* and *P. polycephalum*). Using heuristic search (TBR swapping algorithm), only one most parsimonious tree was obtained. Bootstrapping with 100 replicates was conducted. The result of phylogenetic analysis of the 5.8S rDNA sequences of the 14 taxa is shown in Fig. 2. It was unexpected that the clade was not formed according to genera, *Dictyostelium* and *Polysphondylium*.

In general, molecular data had a tendency to be consistent with morphology (Dodd *et al.*, 2000; Gottlieb *et al.*, 2000). The values of total character differences were low between morphologically similar species (Table 3). The most similar species, *P. pallidum* and *P. tenuissimum* had identical nucleotide sequences of the 5.8S rDNA. A comparison of sequences in the ITS regions revealed that two species shared 95% similarity in the ITS1 regions and 97.7% in the ITS2 regions (data not shown). *D. mucoroides* and *D. sphaerocephalum* which have been treated as the same species because of their morphological similarity by Hagiwara (1989) were closely related in 5.8S rDNA sequence data. Of 162 characters, only two nucleotides differed. But, a sequence similarity of the ITS regions was lower than 70%. Therefore, the data from the ITS and 5.8S rDNA sequences strongly suggest that *D. mucoroides* and *D. sphaerocephalum* may not be the same biological species as stated in Raper (1984).

Table 3. Pairwise distances of the 5.8S rDNA sequences among taxa. Mean character differences (adjusted for missing data) are shown above

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 Ddis	-	0.288	0.209	0.188	0.188	0.231	0.062	0.062	0.055	0.056	0.062	0.154	0.494	0.490
2 Daur	46	-	0.224	0.250	0.250	0.311	0.281	0.281	0.281	0.288	0.288	0.306	0.461	0.425
3 Pcan	33	37	-	0.122	0.122	0.238	0.184	0.190	0.177	0.196	0.215	0.228	0.461	0.444
4 Ppal	30	40	19	-	0.000	0.234	0.150	0.144	0.163	0.175	0.188	0.200	0.494	0.484
5 Pten	30	40	19	0	-	0.234	0.150	0.144	0.163	0.175	0.188	0.200	0.494	0.484
6 Dmin	37	50	38	37	37	-	0.238	0.250	0.244	0.231	0.250	0.263	0.487	0.477
7 Dmuc	10	45	29	24	24	38	-	0.012	0.019	0.043	0.074	0.160	0.494	0.490
8 Dsph	10	45	30	23	23	40	2	-	0.019	0.043	0.062	0.160	0.494	0.490
9 Dgig	9	45	28	26	26	39	3	3	-	0.037	0.068	0.167	0.481	0.477
10 Dpur	9	46	31	28	28	37	7	7	6	-	0.056	0.154	0.487	0.484
11 Pvio	10	46	34	30	30	40	12	10	11	9	-	0.136	0.487	0.477
12 Dpol	25	49	36	32	32	42	26	26	27	25	22	-	0.500	0.497
13 Ppol	76	71	70	76	76	74	76	76	74	75	75	77	-	0.097
14 Diri	75	65	67	74	74	72	75	75	73	74	73	76	15	-

(Ddis = *D. discoideum*, Daur = *D. aureo-stipes*, Pcan = *P. candidum*, Ppal = *P. pallidum*, Dmin = *D. minutum*, Dmuc = *D. mucoroides*, Dsph = *D. sphaerocephalum*, Dgig = *D. giganteum*, Dpur = *D. purpureum*, Pvio = *P. violaceum*, Dpol = *D. polycephalum*, Ppol = *Physarum polycephalum*, Diri = *Didymium iridis*)

The presence of polar granules (PG) in the spore is a very important characteristic as a taxonomic criterion above the species rank, and it was used to divide the genus *Dictyostelium* into two groups, PG-positive and PG-negative *Dictyostelia* (Raper, 1984; Hagiwara, 1989). Among eight *Dictyostelia* used in the present study, *D. aureo-stipes, D. minutum* and *D. polycephalum* were PG-positive *Dictyostelia.* The highest distance values were those obtained between isolates belonging to PG-positive *Dictyostelia.* Sequence homologies among PG-negative *Dictyostelia* were significantly greater than among PG-positive *Dictyostelia.* It was unexpected that *P. violaceum* was closer to PG-negative *Dictyostelia* such as *D. purpureum, D. discoideum* and *D. sphaerocephalum* than other white *Polysphondylia. P. violaceum* is distinguished from the other *Polysphondylia* by its violet color, the pattern of aggregation, the tips of sorophores and consolidated polar granules in spores and seems to be closer to some PG-positive *Dictyostelia* such as *D. aureo-stipes* (Hagiwara, 1989).

In the present study, we showed that dictyostelids have a high level of sequence variation in 5.8S rDNA as well as in the ITS regions and that the sequences of 5.8S rDNA could be used as an aid to the taxonomy of the family Dictyosteliacea. Nevertheless, sequences of the ITS regions could be more useful than those of 5.8S rDNA to discriminate between very closely related species.

Our knowledge of the rDNA variation of dictystelids is still limited. Clearly, more sequence data and taxonomic sampling of two genera are needed to enhance our confidence in these preliminary findings. A detailed analysis of morphological data compared with molecular data would also help to establish new taxonomic criteria for these taxa.

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D.discoideum                TTAAGCATAA ACGGTGAATA CCTCGACTCC TAAATCGATG AAGACCGTAG CAAAGTGCGA
D.aureo-stipes              A..G...C.T TT...CG... .....G...T CG......C. .......... ..........
D.giganteum                 .......... ......G... .......... .......... .......... ..........
D.minutum                   .......... .......... .........T .......... .......... ..........
D.mucoroides                .......... ......G... .......... .......... .......... ..........
D.polycephalum              .......... .......... .....G.... ...T...... .......... ..........
D.purpureum                 .......... .......... .......... .......... .......... ..........
D.sphaerocephalum           .......... ......G... .......... .......... .......... ..........
P.candidum                  G......... .T....G... .....G.... .......... .......... ..........
P.pallidum                  G......... .T....G... .....G.... .......... .......... ..........
P.tenuissimum               G......... .T....G... .....G.... .......... .......... ..........
P.violaceum                 .A........ .......... .......... .......... .......... ..........
Physarum polycephalum       --.CCGT.GG G..A..G..T G..T.GTG.. .GCT....C. ....G..C.. TG..AC....
Didymium iridis             --.CCGT..G G..A..G..T G..G.GTA.. CGCT....C. ....G..C.. TG..AC....

D.discoideum                TAATTCACTT GAATTGCA-- GCCTACTGGG ATATTTGAAA TGTTTAACGC ACATGATGAC
D.aureo-stipes              .......... ......T.GC .TT...C.C. ...G.C...C ....G..... ..........
D.giganteum                 .......... ........-- .......... ...G...... C...G..... ..........
D.minutum                   .......T.. .T......T- .A....ATTC .A.A.C..G. ....G..... .T..CGCA.G
D.mucoroides                .......... ........-- .......... ...G...... ....G..... ..........
D.polycephalum              .........A ........-- .A....C... .C.G.C.... C...G..... ........GT
D.purpureum                 .......... ........-- .......... ...G...... ....G..... .......AG.
D.sphaerocephalum           .......... ........-- .......... ...G...... ....G..... ..........
P.candidum                  .......... ........-- .A......T. .A.G.C.... C...G..... ..........
P.pallidum                  ...G.....C ........-- .A......T. .A.G.C.... ....G..... ..........
P.tenuissimum               ...G.....C ........-- .A......T. .A.G.C.... ....G..... ..........
P.violaceum                 .......... ........-- .......... ...G...... ....G..... ........G.
Physarum polycephalum       ...C.TTTG. ..C.C...-- -..CT...T. ..CAAC.TCT CC..G...AT TAG..-C.G.
Didymium iridis             ...CG.TTG. ..T.C...-- -..CT...T. ..CAAC.TC. CC.CG...AT CG...-C.G.

D.discoideum                --------AT CGGTCCTTTC GGAT--TAGG TGTTATACTT GGGTGA-GAG TGGTC
D.aureo-stipes              CGACTGCCCC TT.CAAAAGG ..CAGC.... CC--.CT..C .TT...-... ACC.T
D.giganteum                 --------.. T......... ....--..A. .........C. ......-... ...CT
D.minutum                   ------GCTC TA.A-..CA. .TCA---... CC..TC.... ..C...-... CA..T
D.mucoroides                --------.. T.......A. ....--..AA .......... ......-... ...CT
D.polycephalum              --------.. .A....GCAA ....--...A ..CC...... ..A...-T.. C.A.A
D.purpureum                 --------.. T......... ....--..A. ..C....... ......-... ...CT
D.sphaerocephalum           --------.. T......... ....--..AA ...C...C. ......-... ...CT
P.candidum                  --ATTGGCTC TTT.A.AGAG TT.A---.T. CC--.C.... .AT...-... .C.CA
P.pallidum                  ---------A TT.-A..CC. CCGAGT..AA ...C.C.... ..T...-... .C.CA
P.tenuissimum               ---------A TT.-A..CC. CCGAGT..AA ...C.C.... ..T...-... .C.CA
P.violaceum                 --------.. T........T ....--..A. ...C...... ......-... C.A..
Physarum polycephalum       ---------C TT.-....CG ..CA--CT.. CC--CCCT.G ..A..CCTT. .CC.T
Didymium iridis             ---------C TT.-....CG ..CA--CT.. CC--CCC..G .TT..CCTT. -CC.T
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Fig. 1. Aligned sequences of the 5.8S rDNA of 12 dictyostelids and 2 outgroup by Clustal X. Alignment gaps are indicated by dashes and conserved bases are indicated by dots. The DNA sequence from left to right reads from 5' to 3' ends.

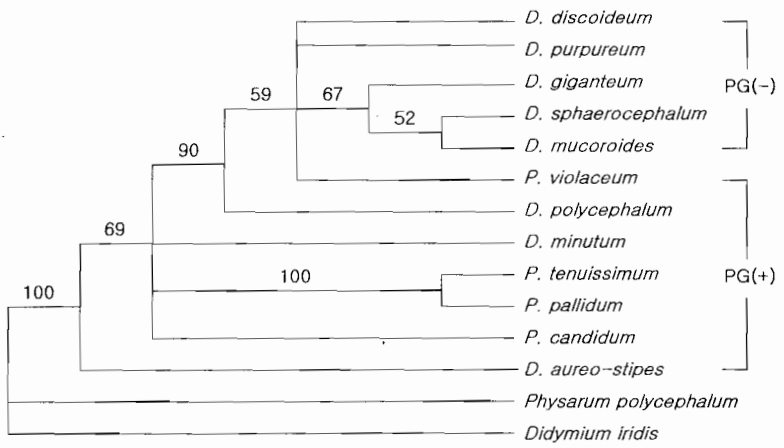

Fig. 2. Consensus phylogenetic tree obtained by the maximum parsimony method with 100 bootstrap replicates. Bootstrap percentages are shown on the tree.

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