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Phylogenetic relationships among non-pathogenic isolates of dark septate endophytes from *Ericaceae* plants

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Abstract

A total of 91 isolates of dark septate endophytes（DSE）were isolated from the family Ericaceae plants. Internal Transcribed Spacer（ITS）and 5.8S rDNA sequence were determined in 10 non-pathogenic isolates of DSE, including a *Heteroconium chaetospira*. The determined sequences were subjected to Neighbor-joining analysis of MEGA4. We identified *Heteroconium chaetospira*（syn. *Cladophialophora chaetospira*）on the first time in Japan from Ericaceae plant. Three isolates had a close relationship with *Leptodontidium orchidicola*. Identities of non DSE isolates like Pj029 and Ro034 were still unknown and remain so until isolates sporulate or until more taxa will be sequenced. Strong bootstrap support within the phylogenetic tree between the *Pleosporales* isolates and Pj022 confirmed their congeneric status, while Ro24 and Ro012 were exhibited close affinity to *Cyphellophora* sp. All DSE isolates had the ability to form intercellular structures within the epidermal cells of blueberry（*Vaccinium corymbosum* L.）.

Key words：dark septate endophyte, Ericaceae, phylogenetic analysis, rDNA ITS

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Introduction

Dark septate endophytes（DSE）are broadly classified as conidial and sterile septate fungal endophytes that form melanized structures like inter- and intracellular hyphae and microsclerotia in plant roots and are known to have affinities for ascomycetes［1］. Based on these morphological characteristics, numerous taxa have been classified as DSE, including *Chloridium paucisporum, Leptodontidium orchidicola, Heteroconium chaetospira, Phialocephala dimorphosphora, Phialocephala fortinii* and *Phialophora finlandia*［12］. The interactions of DSE and their hosts are controversial, having been suggested to be pathogenic, neutral or beneficial［9, 12, 19, 25, 32, 34］. Such large number and wide taxonomic range of hosts have confirmed their ecological importance［12］, particularly the unique interface of the Ericaceae where the DSE occurrence overlaps the ecological niches of ericoid mycorrhizae（ERM）. The nutrient acquisition and transfer of ERM to its Ericaceae host in strongly acidic soils has been well studied in contrast to DSE［3, 20］. The range and relative importance in Ericaceae are still unclear［9］therefore it is critical to investigate the functional aspects of the interaction between the two organisms involved in the association.

Leptodontidium orchidicola Sigler & Currah sp. nov. is relatively common, but poorly known species in the DSE complex. The taxa isolated and identified by Currah et al.［5］from healthy root and tuber segments of some boreal terrestrial orchids native to Canada and has been isolated from plants of alpine and subalpine habitats in Western Canada［9］, from *Salix* shrubs of Northern-Alberta, Canada［20］, more recently from a temperate terrestrial orchid of two distinct sites in Germany, Central Europe［23］. More intensive research shed a light on the distribution and ecological function of *Heteroconium chaetospira*（Grove）M.B. Ellis. It has been isolated from wood pulp［14］, millipede droppings［8］, arable soil［7］, Chinese cabbage roots［16］and more recently from Western Canadian forest communities［16］. Symbiotic association between *H. chaetospira* and Chinese cabbage was reported by Usuki et al.［28］and was also confirmed as a potential biocontrol agent against clubroot［17］.

The identification of DSE in culture often difficult, because the conidiogenesis and sporulation are very rare and their cultural and vegetative characteristics are similar［1, 19］. Particularly the species of *Cadophora, Leptodontidium* and *Phialocephala* genus often mask the slow growing species of *Heteroconium* genus. It often makes difficult to transfer them into pure culture. Molecular analysis is a reliable way to differentiate certain taxa of DSE complex［1］. The rDNA internal transcribed spacer（ITS）sequences have proved to be useful in the identification of taxa of DSE［24, 30, 31］. The aim of the present work was to determine the phylogeny of selected DSE of three Ericaceae plants native to Japan by partial sequence of the rDNA-ITS region.

Materials and methods

Root sampling

The experimental site is located in the Azalea garden of the Graduate

School of Horticulture of Chiba University, Matsudo, Japan (35° 46'N, 139° 54'E). Root samples were randomly collected from the outer periphery of the root system from 3 ericaceous species of *Rhododendron obtusum* (Lindl.) Planch., *Rhododendron pulchrum* Sweet and *Pieris japonica* (Thunb.) D. Don ex G. Don. For each species, three plants were randomly chosen to collect root sub-samples. The *R. obtusum* plants were 3 meters apart from each other, while the sampled plants of *R. pulchrum* and *P. japonica* having a smaller canopy were 1.5 meters from each other. Nine soil core samples were obtained from three randomly chosen plant of each species, 27 soil cores from the three Ericaceae species in total. Soil particles attached to roots were carefully removed using a forceps under dissecting microscope followed by washing roots with tap water for 2 hours before sterilization. Root samples were cut into 2-3 mm segments followed by sterilization in ethanol (70% w/v) for 30 s and in sodium hypochlorite (1% available chlorine) for 90 s. Finally sterilized root samples were rinsed 3 times for 60 s using sterile distilled water. Sterilized root segments - 300 from each species - were placed on malt extract agar (MEA, 2% contained 15 mg streptomycin and 15 mg gentamycin per L^{-1} media) in 90×15 mm Petri dishes and incubated at 18℃ for 1 month. After the appearance of the first isolates from the host epidermal cells, primary selection was done to eliminate those isolates without DSE and ERM characteristics. Only the dark, slow growing and septate fungi were selected and subcultured to MEA media.

Inoculation test

An inoculation experiment was conducted using highbush blueberry (*Vaccinium corymbosum* L.) seedlings *in vitro* for the rapid assessment of colonization ability. Due to the high germination index of blueberry seeds and the close phylogenetic relation with the sampled Ericaceae shrubs (all belong to Ericaceae) made it as a useful plant material for inoculation test [6]. Moreover, the function of ERM and DSE reported to be the same in Ericaceae plant family [25, 28]. Blueberry seeds were extracted from a commercial available frozen blueberry fruits. Surface sterilization of seeds in ethanol (70% w/v) for 30 s followed by sodium hypochlorite (1% available chlorine) for 120 s was done. Sterilized seeds on PDA media (90×15 mm Petri dishes) were then incubated to germinate at 21℃ for 16 hr day and 8 hr night light conditions. Seed germination was observed after 23 days. After appearance of hypocotyls, seedlings were transferred to 20 mL test tubes, containing 10 mL of modified Mitchell and Read media (NH_4Cl, 32 mg; $CaCl_2 \cdot 7H_2O$, 43.5 mg; $MgSO_4 \cdot 7H_2O$, 10 mg; KCl, 5.5 mg; $FeCl_3$, 3.75 mg; sucrose, 2 g; KH_2PO_4, 210 mg; pyridoxine, 100μg; thiamine, 100g, agar (Difco) 10 g; distilled water, 1,000 mL). Final pH before autoclaving was adjusted to 6.0 [6]. Lower part of each test tube was covered with aluminum foil to prevent light penetration into the root zone. Seedlings were then inoculated with each of the 91 isolates in 3 replicates. Control (seedlings with no inoculation) was also included. After putting a sterilized sponge plug in each tube to avoid cross contamination, tubes were incubated at 18℃ for 16 hr day and 8 hr night light conditions. Ten weeks after inoculation, the seedlings were destructively harvested. Roots were stained with trypan blue (0.1% w/v) to determine root colonization by fungal endophytes. Colonization intensity was measured using the slide method described by Trouvelot et al. [27] with a slight modification on counting the infected cortical cells rather than length of the infected root portions. Thirty root segments per treatments were prepared and mounted between two slide glasses in lactoglycerol (lactic acid, glycerol, distilled water 1:1:1) and a dissecting microscope was used under 30x magnification.

DNA extraction and ITS- RFLP analysis

The genomic DNA of each isolates was extracted by Fast DNA® Kit (MP Biomedicals, USA). The ribosomal DNA ITS region was amplified with 25 pmol of ITS1F and ITS4 primers [30]. The amplification was carried out in an automated PCR Thermal Cycler TP-600 (Takara Bio Inc.) with 35 cycles of 94℃ for 60 s, 50℃ for 60 s and 72℃ for 60 s, followed by 72℃ for 10 min. Electrophoresis was carried out with 1.5% (w/v) agarose gels stained with ethidium bromide and visualized under UV light (ATTO printgraph Type GX, ATTO Corporation, Japan) to check the presence of amplified ribosomal DNA ITS regions. The amplified ITS regions of each samples were digested for 3 hrs at 37℃ with restriction endonucleases *Afa*I, *Hae*III, *Hinf* I, *Msp*I, (Takara Bio Inc., Shiga, Japan) and *Cfo*I (Roche Diagnostics GmbH.), respectively. The DNA fragments were separated in a 3% (w/v) agarose gel under 50V for 75 min, stained and viewed as described earlier. One STEP ladder 50 (Wako Nippon Gene) base pair ladder (0.05-2.0 kbp) was used as a reference point for base pair length. The base pair length of each restriction fragment length polymorphism (RFLP) fragments was calculated using CPAtlas 2.0 software (www.lazarsoftware.com).

Sequencing and phylogenetic analysis

Prior to preparation of sequencing reaction mixture, the PCR product was purified with SUPREC™-PCR (Takara Bio Inc.) purification column. The sequencing reaction mixture was prepared with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing product was purified with Centri-Sep™ Columns (Princeton Separations Inc., Adelphia, NJ., USA) and analyzed on an ABI PRISM® 3100 Genetic Analyzer. The sequencing data of isolates variable groups were subjected to homology search using the BLAST (blastn) program through the GenBank database to match the closest sequence. Phylogenetic relation between the isolates of the present

Table 1 Colony characteristics of subcultured isolates derived from ericaceous roots after three weeks of incubation at 18℃ on MEA (2%).

Isolates †	Color of colony	Texture (aerial mycelium)	Margin color of the colony	Growth rate (diam., mm d^{-1})
Rp005	Dark grey	Felt like	—	0.07
Rp011	Olive / dark grey	Dense, flat	Cream	0.21
Rp022	Grey	Fluffy	—	0.16
Ro012	Olive / dark grey	Fluffy	Submerged, black	0.14
Ro024	Brown / grey	Felt like	Brown	0.21
Ro034	Dark grey	Felt like	Brown	0.04
Pj022	Grey	Dense, flat	—	0.08
Pj023	Light grey	Fluffy	Submerged, black	0.02
Pj029	Grey	Fluffy	—	0.29
H. chaetospira	Light grey	Felt like	Submerged, brown	0.41

† Rp, Ro and Pj means that each isolate was respectively isolated from *Rhododendron pulchrum*, *R. obtusum and Pieris japonica*

study and the closest sequences from isolates derived from the homology search (BLAST) were combined to create a neighbor-joining tree by using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. [26]. The distances were calculated using p-distance model. Branch lengths were proportional to base pair differences. The confidence levels were calculated from 500 replicate bootstrap samplings. Colonization intensity data of seedlings were analyzed using general linear model (GLM) (SAS, 1995). Tukey test at $P<0.05$ was used for comparison of treatment mean.

Results and discussion

The number of fungal isolates derived from three Ericaceae host plants varied and were 26, 34 and 31 for *Rhododendron pulchrum*, *Rhododendron obtusum* and *Pieris japonica* respectively (data not shown). After 14 days of inoculation, seedlings appearance exhibited responses. Among the total of 91 isolates, 82 isolates caused pathogenic symptoms. The symptoms were manifested mainly in stunted growth, discoloration and necrotic blight of the blueberry leaves. Based on this response of host plant, a primary selection was carried out among the isolates. Out of the 91 isolates, only 9 did not show any pathogenic symptoms and were then subjected to molecular characterization. One additional DSE (*Heteroconium chaetospira*) isolated by Narisawa et al. [18] was also included in the experiment; while the remaining 82 isolates were excluded, due to their pathogenic effects. The morphological characteristics of colony of each isolates were presented in Table 1. The microscopic differentiation of isolates did not show any significant morphological character and reproductive structures to identify them. The isolates remained sterile under culture, therefore microscopic observation did not provide any distinct taxonomic character. Owing to the absence of morphological clues for sorting the studied isolates, RFLP digestion of the ITS region of each isolates were aimed to distinguish them based on their restriction fragments. Variable fragment sizes (data not shown) of the studied isolates were not provided opportunity for further grouping; consequently all of the 10 isolates were subjected for sequencing. The neighbor-joining tree clustered the isolates into four distinct clades (Fig. 1.).

Compiling the results of homology search (blastn) and phylogenetic analysis, four isolates had DSE affinities. The phylogenetic positions of Rp011, Rp022 and Pj023 were set to an individual clade (clade I) having strong bootstrap support. Within the clade I numerous isolates were represented the *Leptodontidium* genus. Our Rp011 isolate has a 98% sequence similarity with a *L. orchidicola* (AF486133) (Table 2) which was isolated from a boreal terrestrial orchid (*Platanthera hyperborea* L.) in Alberta, Canada [10], although within the phylogenetic tree it was more closely set to other *L. orchidicola* (AY606312) their sequence similarity was also 98% and was isolated from healthy root tips of Norway spruce in Lithuania [15]. Similarly to a DSE fungus (AF168783) which exhibited 98% sequence similarity that was isolated from a perennial *Ranunculus* sp. located in an alpine tundra site of Niwot Ridge, Colorado, USA [22]. Other *Leptodontidium* like isolates of our study, Rp022 and Pj023 were exhibited 99% sequence similarity with the previously mentioned strain of *L. orchidicola* (AY606312). Moreover, significant homology (98%) was found between Rp022, Pj023 and *Leptodontidium* sp. (FN393420) recovered from *Holcus lanatus* a characteristic grass species of nutrient poor fen-meadows in Spain. All of our *L. orchidicola* - like isolates (Rp022, Pj023 and Rp011) were able to form intracellular structure in the epidermal cells of blueberry seedlings without damaging the integrity of host cells (Fig. 2.). This supported the findings of Fernando and Currah [9], on the colonization ability of alpine plants *in-vitro* by *L. orchidicola*, while the effects of those *L. orchidicola* isolates were strongly host-specific.

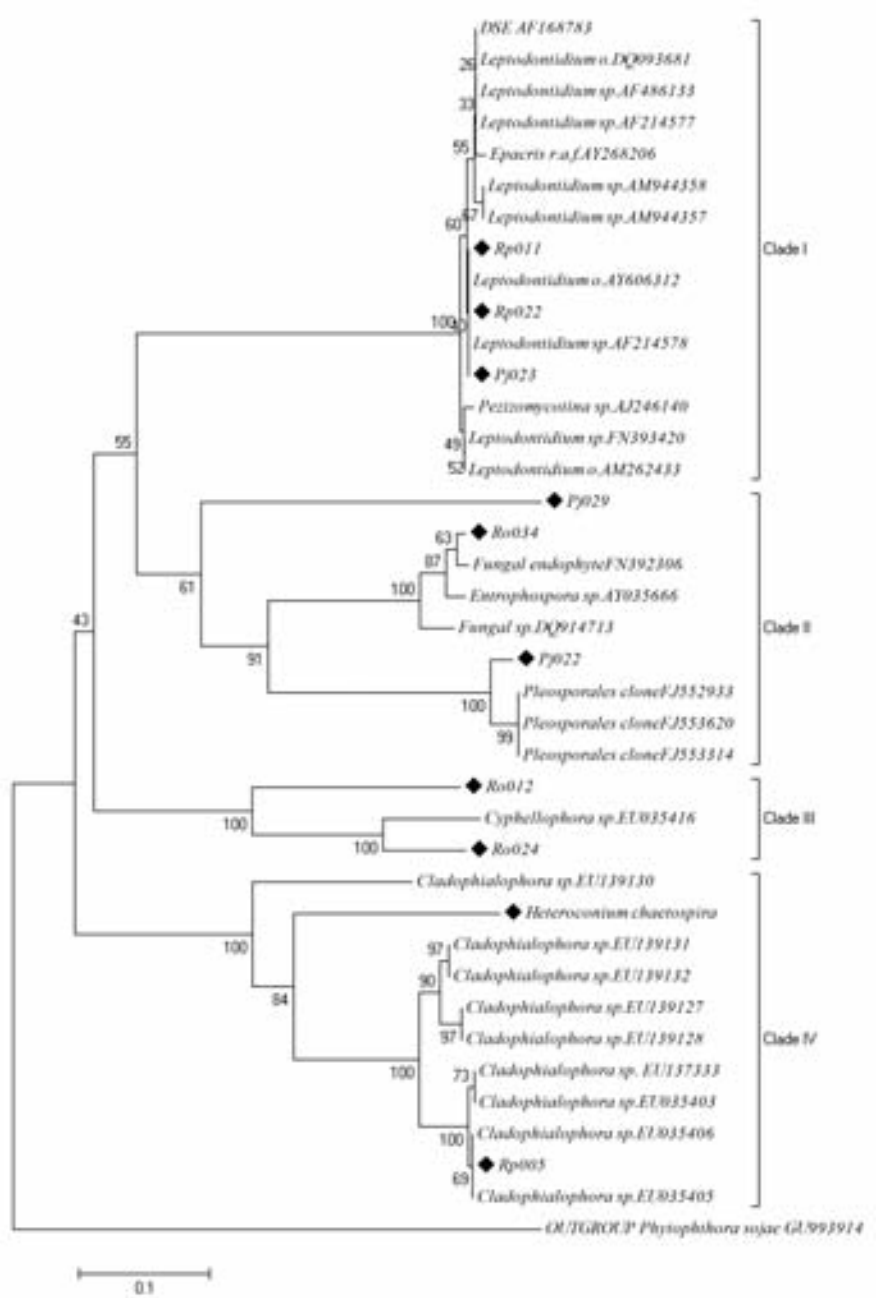

Fig. 1. Neighbor-joining tree of dark septate endophytes obtained from GenBank database combined with the isolates of current study (◆). Distances were calculated using the p-distance method based on the alignment of 5.8 S and ITS1 rDNA regions. Numerical values above the branches indicate the confidence levels for grouping from 500 replicates. Branch lengths are proportional to base pair differences. Outgroup was *Phytophthora sojae* (GU993914). (*Leptodontidium o.* = *Leptodontidium orchidicola, Epacris* r.a.f. = *Epacris* root associated fungi).

Isolates Pj029 and Ro034 had weak homology % (Table 2) and moderate bootstrap support within the clade II. Identities of isolates in this clade are still unknown and remain so until isolates sporulate or until more taxa will be sequenced. The monophyletic origin of clade II still questionable, although the bootstrap support was strong (90) among the subclades, but the *Entrophospora* sp. (AY035666) represented internal branch belongs to Glomeromycota. In contrast with this *Pleosporales* (FJ553314, FJ553620, FJ552933) dominated monophyletic internal branch that was belong to Ascomycota. The strong bootstrap support between the *Pleosporales* isolates and Pj022 confirmed their congeneric status. Clade III represented by *Cyphellophora* sp. (EU035416) and a strong bootsrtrap support (100) may closely dis-

Table 2 GenBank accession codes of closest matches derived from BLAST (blastn) search

Isolate	GenBank accession no.	Species	Host / Source	Location	Similarity % over the 5.8S and rDNA ITS1 region	Overlaping number of basepairs (isolate / species)
Rp005	EU035406	*Cladophialophora chaetospira*	Wheat field / soil	Germany	100%	267 / 267
Rp011	AF486133	*Leptodontidium orchidicola*	*Platanthera hyperborea*	Switzerland	98%	297 / 301
Rp022	AF214578	*Leptodontidium orchidicola*	Boreal forest	Canada, Alberta	99%	216 / 218
Ro012	EU035416	*Cyphellophora laciniata*	Human	Switzerland	86%	232 / 267
Ro024	EU035416	*Cyphellophora laciniata*	Human	Switzreland	86%	232 / 267
Ro034	AY035666	*Entrophospora sp.*	Arable soil	Switzerland	94%	318 / 336
Pj022	DQ914713	Fungal *sp.*	*Elaeocarpus dentatus*	New Zealand	92%	158 / 170
Pj023	AF214578	*Leptodontidium orchidicola*	Boreal forest	Canada, Alberta	99%	216 / 218
Pj029	GU062276	*Strumella sp.*	*Alnus incana* / branch	Latvia	88%	174 / 196

posed with Ro024 and Ro012 isolates, but their homology % over the sequenced region was rather weak (Table 2).

The Rp005 isolate had a high sequence similarity with *Cladophialophora chaetospira* (EU035406) (Table 2) and clustered in a strong bootstrap supported monophyletic clade (clade IV) with other *Heteroconium chaetospira* (syn. *Cladophialophora chaetospira*) strains [3]. *H. chaetospira* isolated by Narisawa et al. [16] was also clustered in clade IV with Rp005. High sequence similarity (100%) along with close phylogeny position, confirmed the possible conspecificity of Rp005 and *Cladophialophora chaetospira* (EU035406) isolates. Narisawa et al. [18] found close phylogenetic relation between two *Heteroconium chaetospira* isolates with a *Cladophialophora devriessi* and *Cladophialophora bantiana*. Internal branches among the two genus (i.e *Cladophialophora* and *Heteroconium*) exhibited strong bootstrap support (74%) in their study. Both *H. chaetospira* strains (i.e Rp005 and *H. chaetospira* isolated by Narisawa) [17] in our study had the ability to form intracellular structure in blueberry host that resembled ERM coil. Darkly pigmented chains of Rp005 (*H. chaetospira* obligate synonym *C. chaetospira*) were the undoubted evidence of intercellular colonization, while the intracellular colonization was manifested in the presence of darkly colored conglomerates of *H. chaetospira* hyphae in the epidermal cells (Fig. 2.). The formation of intracellular structure in Ericaceae by *H. chaetospira* was previously reported in axenic *R. obtusum* var. *kaempferi* [29] inoculated with a *H. chaetospira* (MAFF 238955) derived from the roots of Chinese cabbage (*Brassica campestris* L.). However, the recovery of our Rp005 isolate from *R. pulchrum* indicates the first isolation of *H. chaetospira* (obligate synonym as *C. chaetospira*) from an Ericaceae in Japan. Previously, there was no report about the presence of any DSE species in a complete endophytic survey of *R. obtusum* var. *kaempferi* at approximately 30 km NE from the sample site of the current study [28]. Phylogenetic analysis based on ITS1 and ITS2 sequences is not considered adequate to study relationships among members of higher taxa by some authors [2]. However, if there is any series of taxa that link other distant taxa, phylogenetic analyses using ITS1 [13] or the entire ITS region is appropriate and useful [10]. It is necessary to use both morphological characteristics and molecular techniques on that case where the later not convinced the proper taxonomy of the isolate.

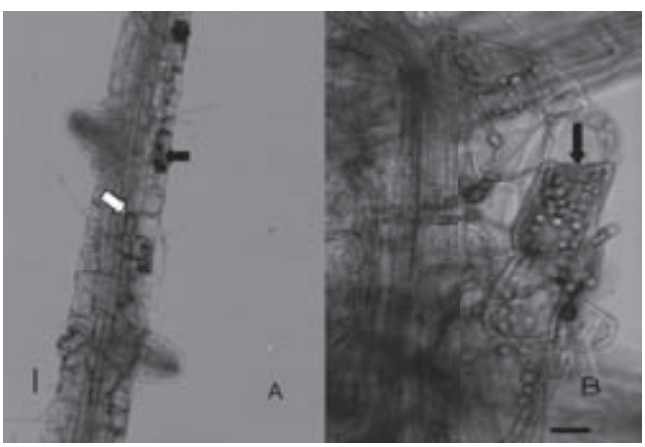

Fig. 2. (A) Chain-like hypha of Rp005 form intercellular (white arrowhead) and intracellular (black arrowhead) structures in the epidermal cells of blueberry (*Vaccinium corymbosum* L) seedling. Bar is 100μm. (B) Epidermal cells of blueberry (*Vaccinium corymbosum* L.) seedling heavily colonized (arrowhead) by hyphal complexes of Rp011. Bar is 30μm.

Our result suggests that the Rp005 isolate appropriately disposed in *H. chaetospira*, while Rp022, Pj023 and Rp011 had close phylogenetic affinity with *L. orchidicola*. To differenciate closely related species the fragment of beta-tubulin gene sequence can provide rapid and reliable identification in species level, due to the higher sequence variability than rDNA ITS [35]. Future direction of the research could be to test the ability of organic nitrogen uptake of each DSE isolates and their

transfer to their Ericaceae host in pure quartz sand culture.

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