Identification of *Diachea leucopodia* on Strawberry from Greenhouse in Korea

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We have detected the slime mold, *Diachea leucopodia* (GNU06-10) in a strawberry greenhouse located in Sancheong-gun, Gyeongnam. Typical fruiting bodies had developed gregariously on the strawberry leaves, petioles, and plant debris on ground soil habitat, and also surprisingly on plastic pipes and a vinyl covering. Field samples were examined via stereomicroscopy, light microscopy, and SEM for the determination of morphological characteristics. Dark-brown to black spores formed gregariously within the stipitate cylindrical sporangium, and were covered by an iridescent peridium, which may be intact at maturity, or may have disintegrated. The upper portion of the peridium generally breaks up to expose the spores, whereas the lower portion was usually persistent. The results of energy dispersive X-ray spectrometer (EDS) analysis showed that lime was present in the stalk and columella but absent from the spores, capillitium, and peridium. The above characteristics confirm its taxonomic position in the genus *Diachea*. However, this genus is intermediate in character between the Physarales and Stemonitales of the Myxogastromycetidae. Hence, this genus had been classified as a member of the Stemonitales until the mid-1970's, on the basis of its iridescent peridium and noncalcareous capillitial system, similar to *Comatricha* of the Stemonitaceae. By way of contrast, emphasis on morphological characteristics, most notably the calcareous stalk and typical columella, places *Diachea* within the order Physarales. The presence of a phaneroplasmodium during the trophic stage and lime deposition in its sporophores, as was confirmed in this work, supported the inclusion of *Diachea* in the Physarales, and the noncalcareous capillitial system verified its identification as a member of the Didymiaceae. Further characteristics of the species *D. leucopodia* include the following: phaneroplasmodium, spore globose 7.5 μm in diameter, very minutely roughened; sporangia 500 $\mu\text{m} \times 1 \text{ mm}$, more or less cylindrical, gregarious, stalked 1.2 mm; stalk and columella white.

KEYWORDS : *Diachea leucopodia*, Digital mapping, Energy dispersive X-ray spectrometry, Slime mold, Strawberry

The strawberry (*Fragaria × ananassa*) is cultivated commonly in greenhouses in Korea, between September and April. In late October, 2006 in Sancheong-gun, Gyeongnam, unusual fruiting bodies were observed to have developed extensively in patches throughout the strawberry phyllosphere, e.g. on the leaves, petioles, and plant debris on soil habitat, and even on plastic pipes and the interior surfaces of vinyl coverings, where the overnight condensation of moisture into free water is inevitable--this must have established favorable conditions for the gregarious and extensive fruiting observed. These rarely observed fungal fruiting bodies were detected only in one greenhouse. To date, this species has been collected only once from that particular locality. Here, we describe the morphological characteristics that are essential to the identification of *Diachea leucopodia*, which is found uncommonly on the strawberry plant.

Materials and Methods

***Diachea leucopodia* sample (GNU06-10) collection and in vitro culture.** Slime mold fruiting bodies were

sampled from strawberry plants, debris on soil, plastic pipes, and vinyl coverings in a strawberry greenhouse, which was quite humid and located near high mountains in the Sancheong-gun, Gyeongnam area of Korea in October, 2006. The samples were examined with a dissecting microscope to assess sporophore development prior to being cultured. The moist chambers consisted of sterile petri plates fitted with a sheet of filter paper on the dish bottom. Fresh extracts of strawberry leaves were filtered through a bacteria-proof filter, then laid on the filter paper. Black spore masses were transferred to the petri plates, which were incubated in a slanted position at room temperature (25~28°C). *In vitro* spore germination to generate myxoamebae and the plasmodial network was monitored microscopically.

Morphological observation of field collection and energy dispersive X-ray spectrometry (EDS) analysis for lime deposition. The sporophore morphology and the surface structures of the sporangia, columella, capillitium, peridium, spores, stalk, and hypothallus of the field samples were assessed via stereomicroscopy and light microscopy. Field emission scanning electron microscopy (FESEM) was conducted with a Philips XL30 S FEG

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(Netherland) at a voltage of 20 kV. Sporophores of the slime molds were adsorbed on a double-face carbon ribbon on the specimen mount, which was then gold-coated and subjected to Energy Dispersive X-ray Spectrometry analysis and mapping (EDS, OXFORD Link-ISIS) for lime deposition.

Results

Sporophore morphology and *in vitro* culture. The morphology of fruiting bodies that developed on the strawberry plants and ground habitat is described in Fig. 1A and B. The FESEM micrograph in Fig. 1C shows the cylindrical sporangia; globose, very minutely echinulated (Fig. 2) spores, capillitium, columella, single peridium, stalk and hypothallus. Further relevant characteristics, in

detail, are as follows. Sporangia: stipitate, closely gregarious, cylindric, ochre to grayish black, apex obtuse, base slightly umbilicate or navel-shaped, total height 2.2 mm, up to 0.5 mm broad; peridium single, membranous, iridescent distinct in paler peridium, the upper portion dehisces to expose the spores while the lower portion remains persistent; stalk long, up to 1.2 mm in height, chalk white, looks very prominent against the black sporangium, broader at the base, tapering towards the apex, striated; hypothallus white, not very well developed. Columella: an elongation of the stipe, thick, blunt, white, calcareous, tapering, reaching two-thirds to the apex. Capillitium: profuse, anastomosing, flexuous threads, white. Spores: dark-brown to black, medium-brown in shade in transmitted light, globose, very minutely roughened, 7.5 μ m in diameter.

The spores (Fig. 1Da) germinated and produced a hap-

Fig. 1. Fruiting bodies and other morphological features of *Diachea leucopodia* associated with the strawberry plant. A, mature sporophores pedicellate sporangia, which is elongated, cylindrical on strawberry plant and also on plastic pipes; B, weed, leaf lesions affected by colonized sporophores and also on debris on ground soil, and interior surface of vinyl coverings; C, Scanning electron micrographs of partly dehiscent membranous peridium showing portion of columella and capillitia of *Diachea leucopodia*; D, *In vitro* cultivation of *Diachea leucopodia*. a, spore germination (light microscopy); b, phaneroplasmodium (digital camera); c, phaneroplasmodium (stereomicroscopy); d, Primordia with expanded apices of sporocarps (stereomicroscopy).

Fig. 2. EDS spectra of element deposition for spore against stalk or spore against carbon ribbon. Portion of SEM view shown as a tetragon was subjected to EDS analysis (SPs) and EDS digital Ca mapping (SPm). SPs-1 shows the EDS spectrum for spores adsorbed on a carbon ribbon. LE, Longitudinal extrusion of membrane wrinkled and folded up due to desiccation.

loid myxamoeba, which further developed into a phanero-plasmodium (Fig. 1Db and c) and up to differentiation into primordia of sporocarps and apex expansion on filter-paper supplied with fresh extracts of strawberry leaves at ambient temperature.

EDS analysis and digital mapping for lime deposition.

Spores adsorbed on carbon ribbon and on/against the stalk were subjected to dual-purpose EDS analysis, as shown in Fig. 2. Calcium content for spores on the carbon ribbon detected at 3.7 KeV were null at 3.1 cps (SPs-1), as compared to 18 cps for spores against the stalk (SPs). This controversy was attributed to the fact that the penetration limits of the probes into the specimens ranged up to 8 μ m (Bozzola and Kussell, 1999), which is beyond the dimension of spores that happened to lie on the stalk.

Therefore, the SEM images were mapped for lime deposition, as shown in SPm of Fig. 2, which demonstrates excellent comparison. The intensity of lime deposition was higher for the stalk (Fig. 3ST and STs), such that the shady area corresponding to spore should exhibit a lower cps as compared to the stalks, which are typically described as being conspicuously calcareous or limy.

The somewhat persistent portion of the peridium (Fig. 3PE) was contrasted against the columella (above) and the stalk (below) (Fig. 3PEm) for Ca content with 11 cps, a value lower than that measured in the columella and stalk.

The capillitial thread was smooth and flexible; Ca content was null at 2.2 cps, which is well illustrated as less intensive networks against columella on the mapping (Fig. 3COM). Columella, an extension of the stalk, was higher

in Ca content at 18 cps, and the stalk is traditionally described as calcareous (Fig. 3CO, COs and COM).

The stalk and hypothallus was surely calcareous with cps units of 38 and 50, respectively (Fig. 3STs and HYs). Those low-intensity linear patterns on the stalk corresponded to the longitudinal extrusion of the membrane (LE), which was wrinkled and linearly folded (Fig. 3ST and STm). This result indicated that, on the surface of the stalk, a membranous tissue which was distinct from the stalk tissue by virtue of its low Ca content, developed throughout the sporophore.

Discussion

The traditional classification of Myxogastria distinguishes five orders--the Echinosteliales, Liceales, Trichiales, Physarales, and Stemonitales--which are distinguished by spore color and sporophore morphology. This includes the presence or absence of a capillitium and its ornamentation, as well as the presence or absence of lime deposits. Recently, the phylogenetic analysis of separate and combined elongation factor 1-alpha (EF1 α) and small-subunit ribosomal RNA (ssu rRNA) data sets resulted in the division of the five traditionally recognized orders of Myxogastria into three distinct groups (Fiore-Donno *et al.*, 2005): The orders Physarales and Stemonitales, both of which possess dark spores, were recognized as the third group, i.e., the dark spored clade. This supports the notion that the two orders are quite closely related to one another.

The spores are always dark, and the capillitium is well developed in both the Physarales and Stemonitales. However, lime deposits have been detected in one or several parts of the fructification in the Physarales. The Stemonitales can be readily distinguished from the Physarales by the lack of lime deposits in all parts of the fruiting body, but with few exceptions--most notably *Leptoderma iridescent*--lime crystals are generally present in the hypothallus and sporangial base (Farr, 1981). The genus *Diachea*, which exhibits mixed characteristics (i.e. a Stemonitoid capillitium and a physaraceous lime-bearing stalk), further suggests the presence of a link between the two orders. Accordingly, at higher orders, the taxon of this genus has been a point of controversy. It is puzzling that this genus is included as a member of the Stemonitidae in the NCBI taxonomy database, even with the disclaimer that the database is not an authoritative taxonomical source. Thus, we attempted to rectify the issue.

The genus *Diachea*, with its glittering gold, bronze or bluish iridescent peridium, along with the color contrast of the calcareous white or colored stalk, possesses a beauty seldom matched by other myxomycetes (Keller *et al.*, 2004). The taxonomic history of the genus *Diachea* emphasizes the iridescent peridium and noncalcareous capillitial system in a fashion similar to that of *Comatricha* or

Fig. 3. EDS spectra of element deposition for peridium (PE), capillitial thread (CA), columella (CO), stalk (ST) and hypothallus (HY) (left column). Portions of SEM view shown as tetragons were subjected to EDS analysis (middle column) and the whole view was X-ray mapped for Ca deposition correspondingly (PEm, CAm, COm, and HYm) with the exception of STm, for which only the tetragon portion was mapped (right column). LE, refer to the footnote of Fig. 2.

Lamproderma, the common genera in the order Stemonitales (Martin and Alexopoulos, 1969). Another morphological trait of this genus is the typical columella, a sterile columnar supporting structure which is an extension of the stalk located within the sporangium, and is similar to that of *Stemonitis* (Stemonitales). However, a pseudocolumella in the center of the sporangium occurs in some Physarum species (Ulloa and Hanlin, 2000). Accordingly, Ghosh and Dutta (1962) classified *Diachea leucopodia* as a member of the family Stemonitidaceae of the order Stemonitales, even though they detected lime in the sporangia and columella. By way of contrast, when the emphasis

is placed on morphological characteristics, such as the calcareous stalk and columella composed of either granules or crystals, *Diachea* is shown to belong to the order Physarales.

The localization of lime deposits is a pivotal characteristic for the definition of families: the Physaraceae has the limy capillitium, whereas the Didymiaceae does not. Thus, this genus was transferred to the Didymiaceae of the order Physarales from Stemonitales (Alexopoulos and Saenz, 1975). Via EDS analysis, we have provided evidence for lime deposition in stalk, columella and hypothallus—this result is in concord with previously reported results (Farr,

1981). Keller *et al.* (2004) previously elucidated the taxonomic hierarchy of the genus *Diachea* in the order Physariales and family Didymiaceae, and provided a key to the species of *Diachea*, including *leucopodia*. Recently, Cavalcanti *et al.* (2006) identified *Diachea leucopodia* as a member of the family Didymiaceae of order Physariales in Brazil. Further research to support this new system should include a study of phaneroplasmodium trophic stages and the subhypothallic pattern of sporophore development, which support the inclusion of *Diachea* in the Physariales and Didymiaceae. A redundant exterior membrane that is folded up and longitudinally extruded along the stalk through dehydration are suggestive of the continuity of the peridium with the pedicel and with the hypothallus formed from the calcareous layer of the phaneroplasmodium. This characteristic is distinctive for subhypothallic sporocarp development, which again reinforces its inclusion in the Physariales. Thus, the NCBI taxonomy database should provide the most relevant nomenclature and classification information as well.

Diachea leucopodia fruiting bodies have reportedly been detected on ground habitats such as leaf litter, mixed litter of decomposing twigs, wood fragments, leaves, and occasionally on the stems of herbaceous plants. This species has been collected from leaf litters and also from living leaves of weeds growing on Mt. Man-roe san, Jincheon, Choongbuk, Korea (Cho, 2003). In this study, we have identified *Diachea leucopodia* growing on a strawberry plant and plant debris in soil, and also grow-

ing, unexpectedly, on inert materials such as vinyl coverings and plastic pipes.

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