

## First Report of Plasmodiophorid Slime Molds and Olpidium Species in a Member of the Cucurbitaceae

Jonathan D Hulse\*

Clemson University, Poole Agricultural Center, Jersey Ln, Clemson, Miami University, Center for Advanced Microscopy and Imaging, Oxford, USA

\*Corresponding Author: Jonathan D, Clemson University, Poole Agricultural Center, Jersey Ln, Clemson, Miami University, Center for Advanced Microscopy and Imaging, Oxford, USA. E-mail: ectorhizo@gmail.com

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### Abstract

*Cucurbita maxima* is an incredibly diverse species, and it is suggested to have more cultivated forms than any other crop. It has many medicinal uses including anti-diabetic, anti-oxidant, anticancer, and anti-inflammatory properties. It is also a major food source for wide variety of world cultures, due to its fiber content, carbohydrates,  $\beta$ -carotene, vitamins, alkaloids, minerals, fatty acids, flavonoids, and polysaccharides. Detection of root-associated microbes in *C. maxima* has not been well documented in the scientific literature. A multi-phase approach was implemented to first verify fungal associations in *C. maxima*, and then second, to document the colonization of fungi in *C. maxima* grown in a conventional agricultural system. *Cucurbita maxima* grown in southwest Ohio showed relationships with members of the Zygomycota and Plasmodiophorid Slime Molds. This study provides a first report of a relationship between *Olpidium* spp. and Plasmodiophorid Slime Molds in *C. maxima* in the United States. The images provided in this manuscript are the first photographic documentation of these organisms in *C. maxima* to date. This is also a first report of Plasmodiophorid Slime Molds in any member of the family Cucurbitaceae.

**Keywords:** Mycorrhizae; Fungi; *Cucurbita Maxima*; Cucurbitaceae; Plasmodiophorid Slime Mold; Zygomycota; Olpidium; Slime Mold; Microbiology; Microbe; Microscopy; Laser Scanning Confocal Microscopy

### Introduction

Members of the Cucurbitaceae have traditionally been used as model organisms for botanical experiments. Cucurbits such as *Cucumis sativus* Linnaeus, *Cucumis melo* Linnaeus, *Citrullus lanatus* (Thunb.) Matsun. and Nakai, *Cucurbita moschata* Duchesne ex Poir., *Cucurbita maxima* Duchesne ex Poir., *Cucurbita ficifolia* Bouche, and *Cucurbita pepo* Linnaeus are all economically important members of this family [1-10]. Cucurbits have been used extensively to study fungal/host relationships. These research topics range from host induced resistance to pathogens, to research that reports on cucurbit nutrient acquisition by beneficial mycorrhizas, as well as projects that study the effects of fungal infection in cucurbits on the tolerance of the host plant to adapt to abiotic stresses [9,11].

Members of the genus *Cucurbita* are used by different human cultures across the globe and are a notable source for nutrition and medicinal treatments. *Cucurbita maxima* produces the largest number of different fruit shapes of any species in this economically important clad, and misidentification of the species can lead to confusion when using this cucurbit for botanical research [12]. The species epithet of *Cucurbita maxima* suggests vigorous growth of fruit, which is the most massive of any cucurbit. *Cucurbita maxima* originated in South America and were domesticated around 4000 years ago [13]. Various forms of *C. maxima* were disseminated to Europe in the 16<sup>th</sup> century, and subsequently were taken by European explorers to the Indian sub-continent, and Southeast-Asia [13]. *Cucurbita maxima* is used medicinally, and is reported to have anti-diabetic, anti-oxidant, anti-cancer, and anti-inflammatory properties [14,10]. It is also a major food source for wide variety of world cultures, due to its fiber content, carbohydrates,  $\beta$ -carotene, vitamins, alkaloids, minerals, fatty acids, flavonoids, and polysaccharides [10].

The diverse Kingdom Fungi can be found in overlapping ecosystems, habitats, niches, and geographic locations, forming diverse communities. 'Fungal-like organisms' is a broad term used to characterize microorganisms classified in multiple domains, which contain morphological similarities to 'true fungi', but are genetically dissimilar based upon current molecular characterization tools. Fungi and fungal-like organisms inhabit root systems of many plants, forming many different types of mycorrhizal associations, ranging from endo-, ecto-, ectendo-, and orchidaceous- mycorrhizae. Fungi and fungi-like organisms also live in the rhizosphere that surrounds plant roots, forming complex communities. These organisms may interact with a host plant in a spectrum of ways, from beneficial to pathogenic. The types of organisms that form mycorrhizal associations with herbaceous plants are not entirely known and could be important links to understanding agricultural ecosystems. The following research on host/microorganism interactions has been described in the literature for fungi and fungal-like organisms.

"Fungal-like organisms" include Plasmodiophorid Slime Molds, which are an asexual protozoan clade within the Rhizaria supergroup [15]. No sexual stage of the *Plasmodiophora* group has been elucidated, so the biological species concept is very difficult to implement in this small, but economically significant, clade [15]. Plasmodiophorids were first characterized by a cruciform morphology of the nucleus in 1899 [15]. Since the discovery of this clade, morphological and molecular analyses have revealed 10 genera (*Ligniera* Maire and A. Tison, *Membranosorus* Ostenfeld and H.E. Petersen, *Octomyxa* Couch, J. Leitn. and Whiffen, *Plasmodiophora* Woronin, *Polymyxa* Ledingham, *Sorodiscus* Lagerh. and Winge, *Sorosphaera* Brunch., *Spongospora* Wallr., *Tetramyxa* Goebel) and 35 species [15]. Classic morphological characteriza-

tion was determined by the shape and number of associated sporosori, but new characterization and classification are based upon ribosomal 18S sequences [15]. Ecologically, Plasmodiophorids are obligate parasites of hosts that range from angiosperms to algae and Oomycota [15]. To date, no Plasmodiophorid has been successfully cultured outside of its host [15]. Plasmodiophorids can be found in terrestrial, freshwater, and marine habitats, since water is needed for the movement of primary and secondary zoospores. Plasmodiophorids are of economic importance to humans, notably as plant pathogens in agricultural and aquatic ecosystems [15]. The most economically important terrestrial diseases caused by Plasmodiophorids are club root disease of brassicas, caused by *Plasmodiophora brassicae* Woronin, and powdery scab of potatoes, caused by *Spongospora subterranea* (Wallr) Lagerh., [15]. Neuhauser, *et al.* (2009) described a new Plasmodiophorid pathogen of *Vitis vinifera* Linnaeus, *Sorosphaera viticola* Neuhauser.

Zygomycota is an old term used to describe a paraphyletic group that is currently not recognized as a distinct phylum and is being molecularly characterized into distinct phylogenetic divisions [16] states that *Olpidium* sp. are known to transmit viruses within cucurbit hosts and are thus an economically and ecologically important obligate parasite of members of the *Cucurbitaceae*. *Olpidium* sp are also stated to transmit viruses within hosts, other than *Cucurbitaceae* [17]. While Campbell, *et al.* [16] obtained stellate resting spores from *Cucumis sativus*, it should be surveyed for in other members of the *Cucurbitaceae*. Mochizuki T., *et al.* [18] stated that they used *C. maxima* as host plants to maintain the zygomycete fungi *Olpidium* sp in culture but have never shown *Olpidium* sp. in agriculturally cultivated *C. maxima*. Sekimoto, *et al.* [19] published a multi-gene phylogeny placing *Olpidium* in the phylum Zygomycota, while Mochizuki, *et al.* [18] published a manuscript stating that *Olpidium* is in the phylum Chytridiomycota. The publication by Sekimoto, *et al.* [19] precedes Mochizuki, *et al.* [18] and the molecular phylogeny should take precedence in this circumstance, placing *Olpidium* sp. into the paraphyletic phylum Zygomycota.

This research project is designed to verify a presence of Plasmodiophorid Slime Molds in the family of *Cucurbitaceae*, and to furthermore survey *C. maxima* an infection with Plasmodiophorid Slime Molds and the Zygomycete *Olpidium* sp. Preliminary and secondary surveying of agricultural fields in the Eastern United States will be the primary methods for determining such infections.

## Methods

### Phase I: Survey

The first phase was a survey of *Cucurbita maxima* roots collected from farms in the eastern United States, and the use of traditional staining techniques to discern the presence or absence of fungal and 'fungal-like' organism associations in the host. Various microscopic techniques are commonly utilized to image these fungi, and the use of differential stains is a critical step in the imaging process. Differential stains are used to provide contrast between the fungal tissues and the host plant cells. The first phase was conducted in order to establish a justification for further exploration into the colonization mechanisms of fungus and fungal-like organisms in *C. maxima*.

Root samples were collected from 3 farms in southeastern Ohio, 1 farm in West Virginia, and 2 locations in Maryland. Farms were selected based upon their different agricultural practices, ranging from certified USDA Organic, uncertified organic, unsprayed, and conventional treatment. Root samples were collected randomly from multiple plants of each cultivar, and then roots were chosen at random for staining and microscopic analysis. According to the growers at each farm, the cultivars included Blue Hubbard, Burgess Buttercup, Dills Atlantic Giant, Rouge Vif d'Etampes, Red Kuri, Sweet Meat, and Turk's Turban.

### Seed Germination

Seeds of *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard were purchased from Seed Savers Exchange®. Ten seeds of each cultivar were placed in filter paper lined Petri-plates, moistened with de-ionized water. This was replicated 10 times, for a total of 100 seeds of each cultivar. Seeds were incubated at 22°C under 24 hours of florescent lights. Seedlings were transferred from filter paper to 3" peat pots, filled with moistened Farfard® 3B potting soil. Plants were grown on light carts or in light boxes under fluorescent lights at 22°C with a regime of 18 hours of light, and 6 hours of dark.

### Field Cultivation

Research was conducted at Miami University's Ecology Research Center (ERC) on Somerville Road, north of Oxford, Butler County, Ohio. The field is approximately 1/2 hectare in size and is adjacent to the ERC access road. The field is bounded by a gravel access road on the east, and abandoned fields on the other 3 sides. Across the access road is a secondary/tertiary growth deciduous forest. The bordering fields are comprised of *Poaceae*, and invasive forbs, such as *Cirsium arvense* (L.) Scop., and *Taraxacum officinale* F.H. Wigg, and other native herbaceous plants, including *Solidago* sp. The field was previously planted with a *Glycine max* (L.) Merr. - *Zea mays* L. rotation on alternating years. The field was left fallow for a year prior to this study.

The field was disked twice and tilled before planting. No chemical treatments were applied to the field pre- or post-planting. The field was relatively level, except for a slight depression toward the southwest. The field is partially shaded in early morning by the shadows created from the forest on the east side of the field. This shadow rapidly decreases, allowing for more than 12 hours of direct sunlight on the field.

Four-week-old seedlings of *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard were transplanted in a non-randomized pattern that contained rows of 26 individuals, with 2.4m spacing between the plants, and between the rows. Two rows of the same cultivar were planted adjacent to each other. Plants were irrigated by hand for the first 10 days post-planting.

### Root Sampling

During each sampling event, 10 plants of each cultivar were randomly destructively sampled, and five roots from each plant were sub-sampled from the total roots collected. Sampling took place during three evenly spaced times during the growing season. The sampling took place on July 2<sup>nd</sup>, August 2<sup>nd</sup>, and September 2<sup>nd</sup>.

3<sup>rd</sup>, 2015. Roots were stored in plastic bags at 4°C until processing within 48 hours post-harvest. Only one, 1 cm segment was used from each root, and the rest of the sample was frozen at -80°C for future analysis.

### Staining Techniques

Root staining was a modification of the Brundrett, *et al.* [20] methods. Samples were heated in 10% (w/v) aqueous potassium hydroxide (KOH) solution for 50 minutes at 95°C. KOH was decanted off of the samples, and 5% hydrochloric acid (HCL) (v/v) was added to neutralize the pH. The samples were kept in 5% HCL for 5 minutes at 20°C. Then, 1% Trypan blue (w/v) was added to the storage tubes. The samples were incubated at 100°C for 5 minutes, and immediately washed with 20°C distilled water. Samples were cut into 1 cm sections and mounted on glass slides within 50% lactic acid-glycerol (v/v) and stored at 4°C.

### Light Microscopy

Root segments were examined for distinct morphological features using bright field light microscopy and laser scanning confocal microscopy and imaged with an Olympus AX - 70 light microscope, and a Zeiss 710 laser scanning confocal microscope. Presence of Plasmodiophorid Slime Mold infection was determined by the presence of sporosori, and presence of Zygomycota was determined by the presence resting spores.

### Results

Preliminary sampling of *C. maxima* in the Eastern United States showed a presence of both sporosori and resting spores, suggesting that a Plasmodiophorid Slime Mold infection and Zygomycota infection was present in *C. maxima*. Plasmodiophorid Slime Molds were observed with Bright Field Light Microscopy after sampling events from Phase I in *C. maxima* cv. Rouge Vif d'Etampes collected from Mountain Valley Orchards (Figure 1A). Plasmodiophorid sporosori were observed with Laser Scanning Confocal Microscopy in *C. maxima* cv. Rouge Vif d'Etampes collected from phase I sampling at Mountain Valley Orchards (Figure 2A-2D). Members of the Zygomycota were observed during Phase I sampling. Stellate resting spores produced by the genus *Olpidium* sp. are indicated with an arrow, showing the constricted apical morphology typical to members of the Chytridomycota, but are molecularly identified to be in the Zygomycota (Figure 3A-3D) [17].

Secondary sampling at the Ecology Research Station in Butler County Ohio also yielded positive results, showing a presence of both Plasmodiophorid Slime Molds and the Zygomycete *Olpidium* sp in *C. maxima*. Sporosori of unknown plasmodiophorid species in *C. maxima* cv. Golden Hubbard collected from phase II sampling at Miami University's ERC are shown with an arrow and imaged with Bright Field Light Microscopy (Figure 1B-1H).

**Figure 1:** Plasmodiophorid slime molds found during Phase I and II. A. Sporosori of unknown plasmodiophorid species in *C. maxima* cv. Rouge Vif d'Etampes collected from Mountain Valley Orchards. B-H. Sporosori of unknown plasmodiophorid species in *C. maxima* cv. Golden Hubbard collected from Miami University's ERC. Mag. bar is 50 µm.

**Figure 2:** Plasmodiophorid slime molds sampled during Phase I and II in *C. maxima*. A-D. Sporpsori of unknown plasmodiophorid species in *C. maxima* cv. Rouge Vif d'Etampes collected from phase I sampling at Mountain Valley Orchards, labeled with arrows, imaged with Laser Scanning Confocal Microscopy.

**Figure 3:** Zygomycota found in Phase I. A - D. Stellate resting spores produced by the genus *Olpidium* sp. indicated with an arrow, showing the constricted apical morphology typical to members of the Chytridiomycota, but are molecularly identified as Zygomycota. Mag. bar = 50  $\mu$ m.

## Discussion

Cucurbits are cultivated worldwide for nutrition, medical, and cultural uses, and the species *Cucurbita maxima* was used as a model organism for this research project due to a void in information about microbial communities in the scientific literature. *Cucurbita maxima* is an economically important crop and its microbial communities should be studied in more detail because they can either be beneficial to the host plant or can harbor parasites and pathogens that may potentially kill or weaken the host organism and reduce agricultural yields.

Plasmodiophorids are of economic importance to humans, notably as plant pathogens in agricultural and aquatic ecosystems [15]. This is the first time that Plasmodiophorid Slime Molds have been documented in any member of the Cucurbitaceae, and more narrowly, the first time being documented in *Cucurbita maxima*. Plasmodiophorid Slime Molds are known to vector plant viruses to host organisms, and this report provides justification to further study the role of this organism in members of the Cucurbitaceae. The host organism as well as the number and distribution of divisions/units of the sporosori were traditionally used to identify Plasmodiophorid Slime molds. Since Plasmodiophorid Slime Molds have never been found in a member of the Cucurbitaceae, the Plasmodiophorid Slime Mold found in this study will be considered a new species, or multiple new species. Morphologically, the sporosori found in *C. maxima* most likely resemble members of the genus *Ligniera*, *Polymyxa* and *Sorosphaera*. Molecular identification should be used to correctly identify this organism. Funding and time constraints did not allow for the molecular identification to be completed during this study, but host root tissues have been stored for further molecular analysis. This is also a first report of Plasmodiophorid Slime Molds being imaged with Laser Scanning Confocal Microscopy. This form of microscopy is a useful tool to visualize sporosori within the host cells. Since Plasmodiophorid Slime Molds have never been detected with a member of the Cucurbitaceae, these images provided are useful in morphologically characterizing these organisms. Molecular identification needs to be conducted in order to identify these unknown species. Morphological characteristics suggest

these are members of the genera *Ligniera*, *Polymyxa*, *Sorosphaera*, but morphology alone cannot be used to determine the species. There are potentially 3 novel species of Plasmodiophorid Slime Molds discovered during this study. Since Plasmodiophorid Slime molds have never been described in Cucurbitaceae, these could potentially belong to a novel genus as well. Further molecular identification of these organisms is required to correctly place these organisms within a clade.

This is a first report of the fungal genus *Olpidium* (A. Braun) J. Schrot. being documented from field sampling in the host *Cucurbita maxima* Mochizuki, et al. [18] stated that they used *C. maxima* as host plants to maintain inoculum in culture but have never shown *Olpidium* sp. in agriculturally cultivated *C. maxima*. To date, *Olpidium* sp. has not been shown in the literature to have any agricultural interaction with *C. maxima* to my knowledge. Sekimoto, et al. [19] published a multi-gene phylogeny placing *Olpidium* in the paraphyletic phylum Zygomycota, while Mochizuki, et al. [18] published a manuscript stating that *Olpidium* is in the phylum Chytridiomycota, without citing Sekimoto, et al. [19]. The publication by Sekimoto, et al. [19] precedes Mochizuki, et al. [18] and the molecular phylogeny should take precedence in this circumstance, placing *Olpidium* sp. into a phylum Zygomycota. It is recommended that a research group conduct Evo-Devo studies to elucidate what genes are involved in producing the iconic flask shaped sporophore of the Chytridiomycota and see if these genes are conserved in the Zygomycota. Since *Olpidium* has never before been documented from field sampled *C. maxima*, this could be representative of a new species. Molecular identification of the ITS1 and ITS2 regions, as well as SSU and LSU genes will need to be conducted in the future, in order to officially name this species.

## Conclusion

Plasmodiophorids are of economic importance to humans, notably as plant pathogens in agricultural and aquatic ecosystems [15]. This is the first time that Plasmodiophorid Slime Molds have been documented in any member of the Cucurbitaceae, and more narrowly, the first time being documented in *Cucurbita maxima*. Since these Plasmodiophorid slime molds have never been discovered in this family of plants, they very well could be new species. Plasmodiophorid Slime Molds are known to vector plant viruses to host organisms, and this report provides justification to further study the role of this organism in members of the Cucurbitaceae. This is a first report of the fungal genus *Olpidium* (A. Braun) J. Schrot. being documented from field sampling in the host *Cucurbita maxima*. Since *Olpidium* has never before been documented from field sampled *C. maxima*, this could be representative of a new species. Molecular identification of the ITS1 and ITS2 regions, as well as SSU and LSU genes will need to be conducted in the future, in order to officially name this species.

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