Intraspecific variation in response to spore-to-spore cultivation in the myxomycete, *Didymium squamulosum*

Winsett, KE

Department of Biological Sciences, Science and Engineering 632, University of Arkansas, Arkansas, USA

Winsett, KE 2011 – Intraspecific variation in response to spore-to-spore cultivation in the myxomycete, *Didymium squamulosum*. Mycosphere 2 (5), 555–564, Doi 10.5943/mycosphere/2/2/5

Spore-to-spore cultures were attempted for 94 specimens of *Didymium squamulosum* using common methods of laboratory cultivation. Specimens varied in age, morphology, method of collection, geographic origin and substratum. Spores from 35 specimens germinated in culture, only nine of which successfully went from spore-to-spore in laboratory culture. No patterns were obvious regarding germination success, plasmodium production or fruiting body formation from the varying factors among the specimens, although no spores from any specimen older than six years germinated.

Key words – laboratory cultivation – myxogastrid – phenotypic plasticity – slime mold

Article information
Received 10 October 2011
Accepted 20 October 2011
Published online 29 October 2011
Corresponding author: Katherine E. Winsett – e-mail – katherine.winsett@gmail.com

Introduction

Since the description of spore-to-spore culture of *Didymium difforme* and *Badhamia utricularis* (Berk.) by Lister (1901), an effort has been made to do the same for as many species of myxomycetes (Myxogastrids or plasmodial slime molds) as possible. Haskins & Wrigley de Basanta (2008) reported that, spore-to-spore culture had been achieved for approximately 10% of all known species of myxomycetes. Members of the Physarales are the most common species in culture and are assumed to be the easiest to cultivate under laboratory conditions (Haskins & Wrigley de Basanta 2008). It has been known for a long time that species of myxomycetes display variation in germination when laboratory conditions are altered to include differences in abiotic factors such as pH associated with natural extracts in culture media (Smart 1937).

In light of the differences noted in the environmental conditions under which particular species are found in nature (Stephenson et al. 2008), it may be that this ecological variation would translate to laboratory conditions as well.

Little data is available regarding intraspecific variation in cultivation success. Clark et al. (2002) obtained only 13 viable cultures that would go spore-to-spore for the myxomycete *Arcyria cinerea* from 55 field collections of the species. It is assumed that unequal cultivation success is a common occurrence, although little such information is found in the literature. The results of recent intraspecific molecular studies using DNA sequencing suggest that significant within-species variation is found at the molecular level (Winsett & Stephenson 2008, 2011). How this relates to the recognized phenotypic variation in cosmopolitan species such as *Didymium squamulosum* is unknown.

Didymium squamulosum (Alb. & Schwein.) Fr. (Fig. 1) is a common morphological species found worldwide (Martin & Alexopoulos 1969). Myxomycetes

can produce two vegetative forms, the amoeboflagellate, which multiply by mitosis and can coalesce to form a multinucleate plasmodium. Classification and species concepts are based upon the development of fruiting bodies from plasmodia in nature or laboratory culture. Complex reproductive strategies including heterothallic and non-heterothallic forms are known to occur in myxomycetes. *D. squamulosum* is known to include both types, and is thus considered to be a species complex of sibling biological species (El Hage et al. 2000). Significant morphological variation in *D. squamulosum* is recognized within the taxonomic description though it is, as yet, unclear how such variation is related to the known species complex (Martin & Alexopoulos 1969, El Hage et al. 2000, Clark & Haskins 2010, 2011).

In order to examine the the phenotypic variation of *D. squamulosum* under controlled laboratory conditions, and thus separate from any effect of variation in ecological condition, an attempt was made to cultivate 96 isolates of *D. squamulosum* from spore-to-spore, using standard culturing methods (e.g. Clark 1995, Haskins & Wrigley de Basanta 2008, Haskins, personal communication; Wrigley de Basanta, personal communication). The specimens cultured originated from moist chamber cultures or field collections and represented a diversity of geographic localities, habitats and ages (Table 1). All attempts to culture these specimens were carried out during the summer and autumn of 2010. An initial method for germination and culture was attempted, followed by adaptations to the method in an effort to promote germination in those isolates that did not germinate during the first attempt.

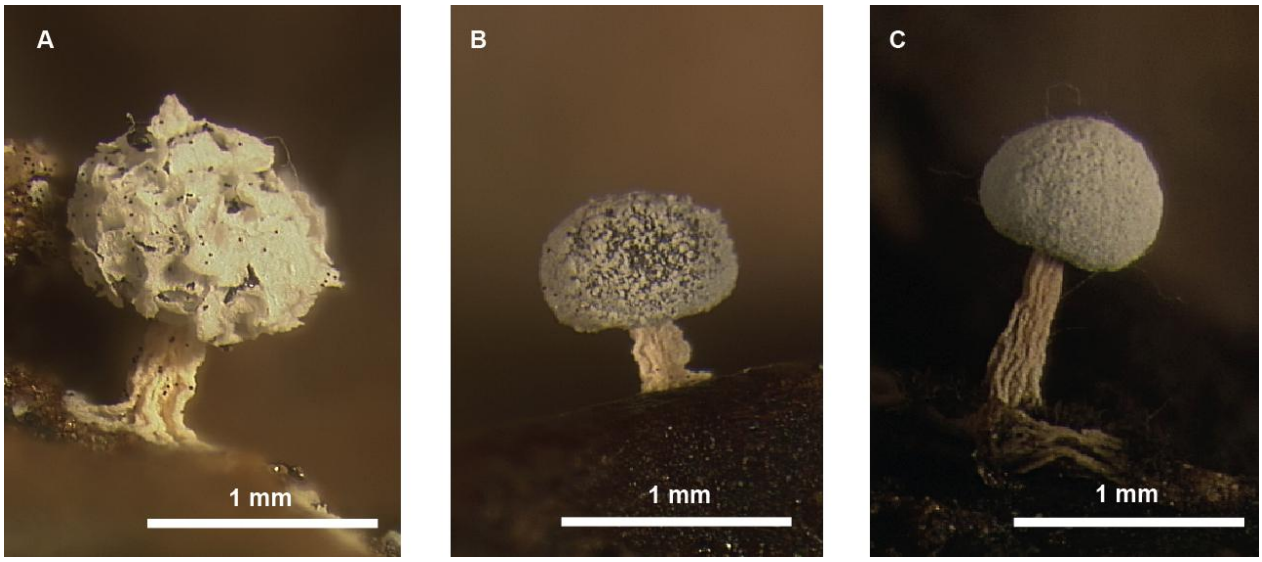

Fig 1 (A-C) – Three common morphological forms of *Didymium squamulosum.* Image A shows the typical flaky aspect to the peridium. Stalk variation common to the species is also shown, with images A and B showing the short stout stalk and image C indicating the longer stalk possible in this species. All three images show the fluted stalk typical of *D. squamulosum*. The stalk is commonly found with lime deposits present, most notably apparent in image A.

Methods

Spores were removed from 94 herbarium specimens of *Didymium squamulosum* (Table 1). The specimens, collected between 1998 and 2006, were housed in the University of Arkansas myxomycete herbarium (UARKM). Initially, spore germination was attempted by placing most of the spores from a single sporangium on sterile 1.5% water agar plates (15 g Bacto agar, 1 L distilled water) with a surface film of sterile deionized water. Germination plates were stored at room temperature in indirect light. When germination occurred and strong populations of amoebae were found, a block of agar with amoebae present was placed upside down on the surface of sterile half-strength cornmeal agar plates (CM/2) (8.5 g Bacto corn

meal agar, 12.5 g Bacto agar, 1 L distilled water). *Escherichia coli* cells from liquid culture were streaked onto the plate to serve as food organisms. Following plasmodium formation, an agar block of plasmodium was transferred to a clean CM/2 plate, and crushed sterile oat flakes were added. Plasmodia were allowed to grow and fruit in natural light. Using these methods, spores from 31 specimens of *D. squamulosum* germinated.

An additional four specimens germinated following the use of an adaptation of the general method in which approximately 0.5 mL of a "natural substrate solution" was added to the plate of water agar on which spores were sown in mass. This solution was prepared by soaking a small piece of substrate material (approximately 1 g) in 10 mL of sterile deionized water. The substrate material was taken from the herbarium specimen that served as the original source of the spores used for culturing. This technique was developed as a result of collaboration and discussions with Ms. Diana Wrigley de Basanta (see Wrigley de Basanta et al. 2010) and is based on the assumption that the mélange of biotic and abiotic factors present on this substrate favored fruiting, and likely germination, for the original isolate that fruited in nature or in a moist chamber culture.

For the cultures in which fruiting body production was observed, specimens were prepared for herbarium storage. The agar block substrate along with the fruiting bodies was cut out and glued to acid-free cardstock paper trays cut to fit into cardboard pill boxes. The specimens were accessioned with the same accession number as the original specimen and deposited in UARKM.

Results

Of the 94 spore-to-spore cultures attempted, less than 1% (nine) were successful. In 35 (37.2%) cultures, spore germination occurred. Of these, only 13 formed plasmodia, nine of which resulted in fruiting body formation. No characteristic of the original herbarium specimen was common to all of the successful spore-to-spore cultures. Though most spores that successfully germinated in culture came from moist chamber, successful cultures were also started with spores from collections originating from field collections, aerial litter and ground litter, and from different geographic localities. Of the collections that only germinated in culture, variation in fruiting body source, substrate and locality was found. The only commonality that can be inferred relates to the age of the specimen.

No specimens that germinated were older than 6 years at time of agar culture. The oldest specimen that germinated was collected from a moist chamber culture in 2004, and the most recent were collected in October 2009 (less than one year since collection at the time the work was done). However, age of the collection could not predict whether germination would occur. Of the most recently collected specimens (2009), only four of 15 specimens germinated on agar.

Discussion

The absence of a pattern in the germination and growth success in spore-to-spore culture of a single species of myxomycete suggests the existence of intraspecific variation that reflects varying responses to ecological conditions and requirements to complete the life cycle. *Didymium squamulosum* is a cosmopolitan morphological species found in a number of habitats and on various substrates. The variation in germination and growth described herein can be added to the significant morphological variation that is also recognized within this species (El Hage et al. 2000, Winsett, unpublished data). There is not yet any mechanism describing phenotypic plasticity in this or any other species of myxomycete, nor is there a complete understanding of intraspecific molecular groups or types. Recent data suggest that intraspecific groupings do exist within species, but these have not been connected to morphological variation (El Hage et al. 2000, Winsett & Stephenson 2008, 2011, Winsett, unpublished data).

While no clear pattern could be detected regarding cultivation success, it must be noted that most of the successful isolates were obtained from moist chamber culture. Seven of the nine isolates that successfully went spore-to-spore in culture and nine of the 13 isolates that produced plasmodia originated

Table 1 Collection data and culture results for each specimen included in the study. Accession numbers marked with an asterisk (*) are 14 collections that did not germinate using initial methods and were further studied using a mélange solution to promote germination.

Accession number	Geographic origin	Substrate	From moist chamber (MC) or field collected (FC)	Year collected	Germination	Plasmodium formation	Fruiting body formation
24034	Hawaii, USA	Aerial litter	MC	2004	X		
26253	North Dakota, USA	Aerial litter	FC	2006	X	X	
26282	North Dakota, USA	Aerial litter	FC	2006	X	X	X
26283	Colorado, USA	Aerial litter	FC	2006	X	X	
26293	Kansas, USA	Aerial litter	FC	2006	X		
26301	Oklahoma, USA	Aerial litter	FC	2006	X		
26537	North Dakota, USA	Aerial litter	FC	2006	X		
26573	North Dakota, USA	Aerial litter	FC	2006	X	X	X
31858	Guatemala	Aerial litter	MC	2006	X		
31885	Guatemala	Aerial litter	MC	2007	X		
31886	Guatemala	Aerial litter	MC	2007	X		
31890	Guatemala	Aerial litter	MC	2007	X		
31905	Guatemala	Ground litter	MC	2007	X		
31907	Guatemala	Aerial litter	MC	2007	X		
31910	Guatemala	Aerial litter	MC	2007	X		
31922	Guatemala	Aerial litter	MC	2007	X		
34688	Guatemala	Aerial litter	MC	2008	X	X	X
34689	Guatemala	Aerial litter	MC	2007	X	X	
34691	Guatemala	Aerial litter	MC	2007	X	X	X

Table 1 continued. Collection data and culture results for each specimen included in the study. Accession numbers marked with an asterisk (*) are 14 collections that did not germinate using initial methods and were further studied using a mélange solution to promote germination.

Accession number	Geographic origin	Substrate	From moist chamber (MC) or field collected (FC)	Year collected	Germination	Plasmodium formation	Fruiting body formation
34692	Guatemala	Aerial litter	MC	2007	X		
34697	Guatemala	Aerial litter	MC	2007	X	X	X
34711	Guatemala	Aerial litter	MC	2007	X		
34729	Guatemala	Aerial litter	MC	2007	X	X	X
34900	South Africa	Ground litter	MC	2006	X		
35278	South Africa	Ground litter	MC	2006	X	X	
38107	South Africa	Ground litter	MC	2006	X		
38162	South Africa	Aerial litter	MC	2006	X		
38173	South Africa	Coarse woody debris	MC	2006	X		
38855	South Africa	Aerial litter	MC	2006	X	X	X
38860	South Africa	Aerial litter	MC	2006	X	X	X
38864	South Africa	Ground litter	MC	2006	X	X	X
*43763	New Zealand	Palm frond	FC	2009	X		
*43785	New Zealand	Palm frond	FC	2009	X		
*43786	New Zealand	Palm frond	FC	2009	X		
*43790	New Zealand	Palm frond	FC	2009	X		
9727	New Zealand	Palm frond	FC	1998			
24034	Hawaii, USA	Aerial litter	FC	2004			
26038	North Dakota, USA	Aerial litter	FC	2006			
26039	North Dakota, USA	Aerial litter	FC	2006			

Table 1 continued. Collection data and culture results for each specimen included in the study. Accession numbers marked with an asterisk (*) are 14 collections that did not germinate using initial methods and were further studied using a mélange solution to promote germination.

Accession number	Geographic origin	Substrate	From moist chamber (MC) or field collected (FC)	Year collected	Germination	Plasmodium formation	Fruiting body formation
26260	Oklahoma, USA	Aerial litter	FC	2006			
26263	North Dakota, USA	Aerial litter	FC	2006			
26266	Wyoming, USA	Aerial litter	FC	2006			
26285	Kansas, USA	Ground litter	FC	2006			
26309	Kansas, USA	Aerial litter	FC	2006			
26558	New Mexico, USA	Aerial litter	FC	2006			
26592	Colorado, USA	Aerial litter	FC	2006			
26607	North Dakota, USA	Aerial litter	FC	2006			
26644	Kansas, USA	Aerial litter	FC	2006			
26689	Kansas, USA	Aerial litter	FC	2006			
26848	New Zealand	Palm petiole	FC	2002			
27869	New Zealand	Palm frond	FC	1998			
28032	New Zealand	Palm frond	FC	1998			
28035	New Zealand	Palm frond	FC	1998			
28046	New Zealand	Palm frond	FC	1998			
28062	New Zealand	Palm frond	FC	1998			
28074	New Zealand	Palm frond	FC	1998			
28079	New Zealand	Palm frond	FC	1998			
28100	New Zealand	Palm frond	FC	1998			

Table 1 continued. Collection data and culture results for each specimen included in the study. Accession numbers marked with an asterisk (*) are 14 collections that did not germinate using initial methods and were further studied using a mélange solution to promote germination.

Accession number	Geographic origin	Substrate	From moist chamber (MC) or field collected (FC)	Year collected	Germination	Plasmodium formation	Fruiting body formation
28105	New Zealand	Palm frond	FC	1998			
28126	New Zealand	Aerial litter	MC	1998			
28151	New Zealand	Palm frond	FC	1998			
28239	New Zealand	Palm frond	FC	1998			
31864	Guatemala	Aerial litter	MC	2007			
31884	Guatemala	Ground litter	MC	2007			
31898	Guatemala	Ground litter	MC	2007			
31903	Guatemala	Coarse woody debris	MC	2007			
34581	South Africa	Ground litter	MC	2006			
34583	South Africa	Ground litter	MC	2006			
34661	Guatemala	Aerial litter	MC	2007			
34693	Guatemala	Aerial litter	MC	2007			
34857	South Africa	Aerial litter	MC	2006			
*43742	New Zealand	Palm frond	FC	2009			
*43745	New Zealand	Palm frond	FC	2009			
*43746	New Zealand	Palm frond	FC	2009			
*43761	New Zealand	Palm frond	FC	2009			
*43762	New Zealand	Palm frond	FC	2009			
*43773	New Zealand	Palm frond	FC	2009			
*43777	New Zealand	Palm frond	FC	2009			
*43787	New Zealand	Palm frond	FC	2009			

Table 1 continued. Collection data and culture results for each specimen included in the study. Accession numbers marked with an asterisk (*) are 14 collections that did not germinate using initial methods and were further studied using a mélange solution to promote germination.

Accession number	Geographic origin	Substrate	From moist chamber (MC) or field collected (FC)	Year collected	Germination	Plasmodium formation	Fruiting body formation
*43791	New Zealand	Palm frond	FC	2009			
*43792	New Zealand	Palm frond	FC	2009			
44647	New Zealand	Ground litter	FC	2001			
44674	New Zealand	Palm petiole	FC	2005			
44690	New Zealand	Palm petiole	FC	2006			
44973	New Zealand	Palm petiole	FC	2005			
44974	New Zealand	Palm petiole	FC	2006			
44975	New Zealand	Palm petiole	FC	2006			
44976	New Zealand	Palm petiole	FC	2006			
44977	New Zealand	Palm frond	FC	2009			
44979	New Zealand	Palm frond	FC	2005			
44981	Costa Rica	Flower spike	FC	1999			
CSAK013	New Zealand	Palm petiole	FC	2001			
CSAK049	New Zealand	Palm petiole	FC	2002			
OTA58428	New Zealand	Ground litter	FC	2004			

from fruiting bodies that developed in moist chamber culture of substrate material. Perhaps growth in moist chamber culture provides opportunity for full maturation of the sporocarp. However, instances have been personally observed when spores of fruiting bodies in moist chamber culture developed abnormally suggesting care must be taken to allow fruiting bodies to fully mature in these conditions as well as examination of spores to determine if normal development occurred.

The technique of using a mélange solution from soaking substrate material was attempted for 14 isolates, all of which were collected at the same locality in October 2009 (Table 1). These isolates all fruited over the same few days with very similar morphology and identical sequences for a mitochondrial marker (Winsett, unpublished data). Because *D. squamulosum* is known to occur as non-heterothallic (presumably apomictic) strains (El Hage et al. 2000, Clark & Stephenson 2003), it was assumed that these field collections could

be different isolates of the same genetic strain. Furthermore, the assumption was made that these isolates would behave in a similar fashion in culture, with similar germination rates and growth success. Interestingly, despite their recent collection, none of the isolates germinated on water agar alone, and only four of the 16 isolates germinated using the substrate mélange technique. If these specimens were members of the same clone, it would be reasonable to assume they would behave similarly in culture, responding the same way to any set of culture conditions.

Along with the biological conclusions that can be drawn from these data, it should also be noted that the interpretation of data from laboratory cultivation, meant to investigate if phenotypic variation holds up in common culture conditions, could be made more difficult by the unequal success of germination within a particular species. This series of spore-to-spore culture attempts in *D. squamulosum* was undertaken to enhance an intraspecific molecular study of the same species. The expectation was to use the fruiting bodies formed in culture to determine if the extensive morphological variation seen in these herbarium specimens would be seen also when cultivated in common conditions. *D. squamulosum* is known to occur, perhaps commonly, as non-heterothallic strains (El Hage et al. 2000, Clark & Stephenson 2003). Such genetic isolation would serve as a mechanism for the accumulation of phenotypic variation within morphological species. Understanding whether the source of morphological variation could be ecological or genetic using evidence from whether or not variation was sustained in common culture would be important for investigating myxomycete species. The value of the information that can be drawn from the laboratory cultivation of myxomycete isolates has been significantly reviewed (Clark & Haskins 2010, 2011). Uneven germination rates and the inability to predict which specimens will be successful in spore-to-spore culture is a limitation in the study of the morphological species concept.

Acknowledgements

This project was supported by National Science Foundation Planetary Biodiversity Inventory (PBI) grant (DEB-0316284; PI Steven L. Stephenson) and NSF Partnerships for Enhancing Expertise in Taxonomy (PEET) grant (DEB-0329102; PI Frederick W. Spiegel) at the University of Arkansas. George Ndiritu, Carlos Rojas, Adam W. Rollins, Clive Shirley and Steven L. Stephenson are gratefully acknowledged for providing many of the specimens used in this study.

References

Clark JD. 1995 – Plasmodial incompatibility in the myxomycete *Didymium squamulosum*. Mycologia 95, 24-26.

Clark JD, Haskins EF. 2010 – Reproductive systems in the myxomycetes: a review. Mycosphere 1, 337-353.

Clark JD, Haskins EF. 2011 – Principles and protocols for genetical study of myxomycete reproductive systems and plasmodial coalescence. Mycosphere 2, 487-496.

Clark JD, Stephenson, SL. 2003 – Biosystematics of the myxomycetes *Didymium squamulosum*, *Physarum compressum* and *Physarum melleum*: additional isolates. Mycotaxon 85, 85-89.

Clark JD, Schnittler M, Stephenson SL. 2002 – Biosystematics of the myxomycete *Arcyria cinerea*. Mycotaxon 82, 343-346.

El Hage, N, Little, C Clark, JD, Stephenson SL. 2000 – Biosystematics of the *Didymium squamulosum* complex. Mycologia 92, 54-64.

Haskins EF, Wrigley de Basanta D. 2008 – Methods of agar culture of myxomycetes: an overview. Revista Mexicana de Micología 27, 1-7.

Lister A. 1901 – On the cultivation of Mycetozoa from spores. Journal of Botany 39, 5-8.

Martin GW, Alexopoulos CJ. 1969 – The Myxomycetes. University of Iowa Press, Iowa City.

Smart RF. 1937 – Influence of certain external factors on spore germination in the myxomycetes. American Journal of Botany 24, 145-159.

Stephenson SL, Schnittler, M, Novozhilov, YK. 2008 – Myxomycete diversity and distribution from the fossil record to the present. Biodiversity and Conservation 17, 285-301.

Winsett KE, Stephenson SL. 2008 – Using ITS sequences to assess intraspecific genetic relationships among geographically separated collections of the myxomycete *Didymium squamulosum*. Revista Mexicana de Micología 27, 59-65.

Winsett KE, Stephenson SL. 2011 – Global distribution and molecular diversity of *Didymium difforme*. Mycosphere 2, 135-146.

Wrigley de Basanta D, Lado C, Estrada-Torres A. 2010 – *Licea eremophila*, a new myxomycete from arid areas of South America. Mycologia 102, 1185-1192.