Principles and Protocols for Genetical Study of Myxomycete Reproductive Systems and Plasmodial Coalescence

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The diplo-haplontic life cycle of myxomycetes produces a genetic system with clonal gametes, which provide a number of opportunities and difficulties that those unfamiliar with the group might find confusing. Therefore, we provide a guide to the Mendelian genetics of the myxomycetes for those who are interested in expanding their taxonomic and environmental research in this group. The ability of the haploid unicellular myxamoeba, which reproduces by mitotic division to produce a clonal population, and to convert directly into a gamete, is the basis of the special characteristics of myxomycete genetics. Since the same clonal lines can be used in multiple different specific crosses, genetic information, which can be used in future crosses, can be built up for those lines. This allows the researcher to study multi-gene traits such as the plasmodial coalescence system.

Key words – fusion – heterothallism – homothallism – self-recognition – sibling-species

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Introduction

Several species of myxomycetes have been used to study the genetics and cell biology of a number of basic biological systems (Collins 1979). However, cultural and genetic information can also be very useful in the study of myxomycete taxonomy, population biology and ecology (Clark & Haskins 2010). Therefore, since not everyone working in these areas is familiar with the advantages, difficulties, and peculiarities of the slime mold lifecycle and associated genetic system, we decided to provide a primer to assist them in adding these useful procedures to their studies.

The myxomycetes have two vegetative stages, the multinucleate coenocytic plasmodium and the small uninucleate myxamoeba (Collins 1979, Stephenson & Stempen 1991). They both feed on microorganisms and other organic matter which they can ingest. The plasmodium grows larger and may fragment to form new plasmodia, form a resistant sclerotium under adverse condition, or sporulate when it runs out of food. The spores produced by plasmodial sporulation then germinate to produce myxamoebae. The myxamoebae grow and divide mitotically to form a clonal population of genetically identical cells, which encyst under adverse conditions, or produce a plasmodium when they reach high population densities. The formation of plasmodia by the myxamoebae occurs either by means of heterothallic sexual fusion, or by some non-heterothallic mechanism (Collins 1979, Clark & Haskins 2010). The myxomycetes with non-heterothallic reproduction are either homothallic (sexual

fusion occurs between any two myxamoebae) or apogamic (no sexual fusion occurs). It is generally believed that homothallism is either very rare, or does not occur in the myxomycetes, and thus most, if not all non-heterothallic isolates are apogamic (Clark & Haskins 2010). DNA ploidal level studies on a number of apogamic isolates found that myxamoebae could convert directly into diploid or polyploidy plasmodia without the need for sexual fusion (Therrien et al. 1977). Therefore, Mendelian genetical studies are limited to the species and isolates of slime molds which exhibit heterothallic sexual reproduction.

The heterothallic myxomycetes have a diplo-haplontic life cycle, with two vegetative stages; the diploid coenocytic plasmodium that contains many nuclei, and the haploid uninucleate myxamoeba. This type of life cycle, which is found in only a few organism (some yeasts) besides the myxomycetes produces a somewhat unique system of genetic analysis. Since the myxamoebae divide mitotically to produce a clonal population of identical cells that can also covert directly into gametes you have, in effect, a genetic system with clonal gametes. An isolated spore, or myxamoeba, will grow and form a large population that can be repeatedly used in a number of different crosses. It can also be stored for fairly long periods since it produces resistant cysts, and the population is potentially immortal (Clark 1992). Thus, the exact identical cross can be repeated many times at different dates, and the genetic information for each clonal population can be accumulated from many different crosses. This allows the genetic analysis of multigene traits such as the plasmodial incompatibility system. While this system has a number of advantages, it is also fairly labor intensive and requires considerable cultural manipulation.

Culture and myamoebal clones

To accomplish genetic studies in the myxomycetes, the organism must not only be in culture, but it must also grow well and be emendable to cultural manipulations. Thus, the first priority for anyone considering genetic studies in the myxomycetes, is to establish the organism in a useable cultural system (Spiegel et al. 2005, Haskins & Wrigley de Basanta 2008). Someone with no working background in the culture of myxomycetes, could gain experience by obtaining a *Physarum polycephalum* culture to try out the different techniques. This species is easy to work with and generally available (can be obtained from a biological supply company that provides teaching materials). Food organisms such as *Kiebsiella*, *Escherichia*, and yeasts are also available from these sources.

Once a myxomycete isolate is in sustained culture, clonal populations, derived from single spores or amoeba, are isolated for genetic analysis. It is of the utmost importance that sterile technique be used during all aspects of culture work. All materials and utensils must be properly sterilized, and the work area cleaned of dust and other sources of contaminants. Also, if possible, the culture area should be distant from any area where field material is examined, since this is a major source of contamination and mites (these can become a major problem, since they can carry contaminants between Petri dishes). Thus, careless technique can lead to unwanted fungal and bacterial contaminants that could quickly over run the culture and destroy your work.

One method of cloning is the manual isolation of a single spore or amoeba using a dissecting microscope with 60× magnification and substage lighting. This method requires a certain level of dexterity and patience, in addition to a good microscope. A normal looking fairly fresh sporangium is selected and spores extracted from it with a sterilized jeweler's forceps (the tip is dipped into 90% alcohol which is burned off in a flame and then allowed to cool before use). The extracted spores are spread in a long streak on the surface of an agar plate, or alternatively they may be deposited on the plate in a small drop of sterilized water (water can be sterilized and stored for later use in small screw cap vials) and then spread over the plate with a sterilized loop. A third method of spreading the spores uses a slender cut wedge of sterilized Millipore filter held by forceps to pick up spores from the sporangium (this is especially useful for the more delicate sporangial species). Once the spores are spread on the agar, those which are distantly separated from one another are good candidates for isolation. One method of isolation

uses a flame sterilized needle (very thin and small) in a holder, to cut out a tiny agar block, upon which the spore rest, and to pick it up with the needle tip (requires a steady hand and practice). Alternatively, sterile Millipore wedges can be used to pick up the spore (sticks to the filter). The spores are then transferred to a fresh agar plate (rubbed off of the needle or filter paper). When a spore has been successfully isolated (can be checked with the dissecting microscope), an appropriate food organism is added. A growing myxamoebal population may be diluted and spread on agar using the same methods and single myxamoeba isolated.

A second method of cloning is by means of spore dilution. A small number of spores (20–40) are removed from the sporangium with sterile forceps, placed in a small drop of sterile water, and spread on the surface of an agar plate with a glass right angle spreader. Then 0.3–0.5 mL of a food micro-organism suspension is dispensed on the plate and spread with the spreader. The food organism will form a lawn on the agar surface and after several days the germinating spore will grow and divide to form a clear plaque in this lawn. Well separated, circular amoebal plaques can then be cut out and transferred to fresh agar plates. Alternatively, spores from a sporangium can be placed in sterile water with a food organism, and the mixture spread on the plate. In this case samples can be taken (sterilely) and examined with a microscope to judge the dilution factor, prior to spreading onto the plate. When working with a species that has a high level of non-heterothallic isolates, it may be easier to spot a number of loopfulls of the diluted mixture onto the plates. If all of the spots which produce myxamoebae also produce plasmodia then the isolate is non-heterothallic and further isolations are usually unnecessary. Single myxamoeba can also be isolated from growing myxamoebal populations by this method.

When an appropriate number of clones have been established, It is essential to determine the appropriate culture conditions for long term maintenance of these stocks and to establish an appropriate transfer schedule. This may vary from weekly transfer, to long term (a year or so) stasis conditions (Haskins & Wrigley de Basanta 2008). The myxamoebal clones of many species will survive for up to a year in screw cap slant tubes partially filled with water.

Reproductive systems

Once you have an isolate in culture, the first step, in genetic analysis, is the determination of its reproductive system. It can be heterothallic, and therefore subject to genetic studies, or non-heterothallic, and therefore not amenable to genetic manipulations. This determination can be quite straightforward. Non-heterothallic isolates produce plasmodia from clones derived from single spore or myxamoebal isolations, while the clones of heterothallic isolates must be crossed in order to produce plasmodia. Fifteen to twenty clones should be established for the new isolate, and grown on the appropriate medium in Petri dishes. If the majority of clones produce plasmodia, the isolate is likely to be non-heterothallic. In this case one of the resulting plasmodia should be induced to sporulate, and a second generation of clones derived from it; if they also produce plasmodia by themselves, the isolate is non-heterothallic. The non plasmodia forming clones, from a non-heterothallic isolate may be due to a number of different causes; they may just be sterile, they may not be myxomycetes (amoeboid contaminants), or they could be revertants (Collins 1980) to heterohallism (some apomictic isolates occasionally produce haploid myxamoebae which can undergo sexual crossing).

Crossing

If the clones derived from an isolate do not produce plasmodia by themselves, then they should be crossed to test for heterothallism. Crossing should be done with growing clones and is usually done on a dilute agar medium such as half strength corn meal agar (or other agar media that has been found to benefit a particular species). Clones which have reached growth stasis and have a high level of resistant cysts present; should be transferred to fresh medium before crossing, so that fresh growing cultures are used in the cross. The actual cross, making sure to use sterile techniques, is made by streaking a loopful of myxamoebae from one clone onto the plate, and then streaking a loopful of another clone across it in an × pattern (or the two loopfuls can be used to

make a mixed puddle). The cross is then set aside and monitored for plasmodial formation over a period of time. Most species which produce aphanoplasmodia require a water film (sterile distilled water) on the agar surface of the cross plate, in order to grow and produce plasmodia.

Mating Types

Heterothallic isolates of a myxomycete species are expected to produce myxamoebal clones that have a one to one ratio of the two mating types present in an isolate. However, a skewed ratio may be present in some isolates, where one mating type is rare or absent (Mc Guinness & Haskins 1985). These abnormal ratios are probably due to a number of different causes such as meiotic drive, aneuploidy or selfing (see Clark & Haskins 2010). In any case, the usual procedure is to cross four to six clones in all possible pair-wise combinations (Punnett square, Fig. 1); these four to six clones will generally contain at least one of each of the two mating types. Since, plasmodia will occur only when the cross consists of two different mating types, the clones can be assigned to the two types, based upon the crossing results (clones having the same mating type will not produce plasmodia when crossed, while clones with different mating types will produce plasmodia). Occasionally, an expected plasmodium is not produced in a particular cross. This can be due to a number of reasons: poor myxamoebal growth, homozygousity for a lethal mutant, or simple error (the cross was not really made). Once you have determined the mating types from these initial crosses, two vigorous clones of opposite mating type can be selected to serve as tester clones (clones 1 and 3). These tester clones can then be used to determine the mating type of any other clones from that isolate (Fig. 2).

A plasmodium from the original series of crosses should then be selected (the 1 × 3 cross or another which produces vigorous plasmodial growth) and induced to sporulate. A second series of clones isolated from it can then be tested for mating type, to insure that the system is stable. Mating types are usually designated with the capital letter A and a numerical superscript (clone 1 = A^1, clone 3 = A^2).

Multiple Alleles

Every myxomycete species that has been extensively investigated has displayed a system of multiple alleles at the mating locus (Clark & Haskins 2010). Therefore, when the tester clones of two different isolates of the same species are crossed against each other in all possible combination, three different patterns may occur (Fig. 3). If the two isolates have the same two mating types, they will produce plasmodia in half of the crosses (the two sets of similar mating types will not produce plasmodia); if they have one mating type in common, they will produce plasmodia in three-quarters of the crosses (the two similar mating types will not produce plasmodia); and if they have no mating types in common, they will produce plasmodia in all of the crosses. The new mating types can then be given a designation.

In a like manner, the mating type testers of any additional isolates can then be crossed to the known mating type tester, to determine their mating types.

Biological species

Some isolates, which are morphologically identical, are genetically isolated from each other and are thus biological sibling species. Thus, many myxomycete morphological species are in reality a complex of related biological species that cannot inbreed with each other. This situation is detected by crossing tester clones of the different isolates against each other, as in the test for multiple alleles. However, in this case no plasmodia are formed in the crosses of clones from the different isolates (Fig. 4).

Plasmodial coalescence

The fusion or non-fusion of plasmodia was once considered to indicate the occurrence of physiological races within a species (Gray 1945), but is now known to be due to a complex genetic self-recognition system. The plasmodia of most myxomycete species is a large multinuclear cell covered with a slime sheath (lacking in the aphaneroplasmodia of Stemonitales species). These plasmodia are motile and are constantly forming and reforming a reticulate structure by means of fusion between different areas. Since there are no physical

Clones	1	2	3	4	5	6
1	-	PP	PP	00	00	00
2		-	00	PP	PP	PP
3			-	PP	PP	P0
4				-	00	00
5					-	00

Clones 1, 4, 5, and 6 have one mating type, and clones 2 and 3 have a different mating type. The 3 × 6 cross produced plasmodia in only one of the two replicate crosses, but the overall pattern indicates that plasmodia should be expected in this cross.

Fig. 1 – Primary crossing matrix to determine mating types: six clones tested in all possible pair-wise combinations with two separate replications of each cross (P indicate that a plasmodium formed in that cross and a 0 indicates no plasmodium).

Clones	7	8	9	10	11	12	13	14	15
Tester: 1	PP	PP	00	0P	00	PP	PP	00	PP
3	00	00	PP	00	PP	00	00	PP	00

Clones 9, 11, and 14 have the same mating type as clone 1. Clones 7, 8, 10, 12, 13, and 15 have the same mating type as clone 3. The 1 × 10 cross produces plasmodia in only one of the two replicate crosses, but the pattern supports the mating type determination (like clone 3)

Fig. 2 – Secondary crossing matrix to determine mating types of the remaining clones: two replications of each cross are indicated.

If both isolate have the same two mating types: then clone Y–1 = A^2 and clone Y–2 = A^1

X isolate clones:		X–1 = A^1	X–3 = A^2
Y isolate clones:	Y–1	PP	00
	Y–2	00	PP

If the two isolates have only one mating type in common: then clone Y–1 = A^2 and clone Y–2 = A^3 (a new type).

X isolate clones:		X–1 = A^1	X–3 = A^2
Y isolate clones:	Y–1	PP	00
	Y–2	PP	PP

If the two isolates have no mating types in common: then clone Y–1 = A^3 and clone Y–2 = A^4 (both are new mating types)

X isolate clones:		X–1 = A^1	X–3 = A^2
B isolate clones:	Y–1	PP	PP
	Y–2	PP	PP

Fig. 3 – Crossing matrix to determine multiple alleles. The designated tester clones from isolate X (X–1 and X–3) are crossed to the mating type un-designated tester clones of isolate Y (Y–1 and Y–2).

X isolate clones:		X–1 = A^1	X–3 = A^2
Y isolate clones:	Y–1	00	00
	Y–2	00	00

Since the Y isolate clones do not mate with the X isolate clones, they belong to a different mating group and therefore the Y isolate clones are generally designated as a new mating series: clone Y–1 = $A2^1$ and clone Y–2 = $A2^2$.

Fig. 4 – Crossing matrix to determine sibling species.

barriers to fusion between the different regions of a single plasmodium, there can also be no physical barriers to the coalescence of two different plasmodia. However, while two genetically identical plasmodia will always undergo coalescence, most plasmodia of the same species do not fuse and retain their individuality. This non-coalescence has been shown (Ling & Clark 1981) to be due to a complex genetic system consisting of a number of different genes, each having a dominant (F) and a recessive (f) allele. Thus, each gene locus in the diploid plasmodium can have one of three different genotypes: homozygous dominant (FF), heterozygous (Ff) or homozygous recessive (ff). However, each locus has only two genotypes: dominant (F- = FF and Ff) and recessive (ff). For two plasmodia to fuse they must be phenotypically identical at all of the different fusion gene loci. Since there may be ten or more fusion gene loci in a species, the number of different possible fusion phenotypes can be very large (10 loci would produce 1,024 phenotypes, which would not fuse with each other). However, most isolates from nature are only heterozygous for 2–4 loci (the rest are homozygous) and, therefore, segregate to produce 4, 8, or 16 different phenotypes. Since the F_2 phenotypic ratio of a three gene polygenic system is 27:9:9:9:3:3:3:1, a three gene polygenic system is on the outer limits of normal Mendelian analysis. However, the occurrence of clonal gametic lines (myxamoebal) in the myxomycetes allows for a modified analysis that makes it possible to examine larger polygenic systems.

Fusion tests

The study of coalescence requires a rapid method to determine if two different plasmodia undergo fusion or not. This determination can be made by a simple visual inspection in most cases, since the two plasmodial will form a single functional plasmodium if they fuse. However, this determination can be made more rapidly and easier with a dissecting microscope with substage lighting, since one can observe the flow of cytoplasm between the two plasmodia after the formation of common veins at the contact point (illustrated in Collins & Haskins 1972, Clark & Collins 1973, Carlile 1973). Dissecting microscopes also allow one to observe the minute reactions that can occur during a non-fusion contact between the two plasmodia.

Vigorous plasmodia growing on an agar plates can be cut out and positioned on another plate to produce a fusion test. A block of agar (approximately 5 × 25 millimeters), on which the plasmodium is present, is cut out with a spatula and placed on it edge on a new a agar plate. A second block, from a different plasmodium, is then placed about an 25 mm away from the first block. The two blocks should have the surfaces upon which the plasmodia occur facing each other, so that when the plasmodia come off of the blocks they will move toward each other. When the two plasmadia meet they will push up against each other and, if they are compatible, they should fuse within a few minutes of contact. However, for unknown reasons, some compatible combinations may require a longer period (up to 30 min) of time before they undergo fusion.

Back crosses

Since a normal F_2 analysis requires a large number of progeny in order to detect the rarer classes in a polygenic system, a backcross system using clonal gametes, which requires fewer progeny, is a better method of studying coalescence genetics. A vigorous parent (P) generation plasmodium, such as the 1×3 (= 1.3) cross, is chosen and induced to sporulate. Approximately 25 progeny clones (can be designated 1.3–1, 1.3–2, 1.3–3, etc.) are then isolated from this fruiting and crossed back to the two parent clones (1 and 3). Approximately half of the new clones should mate with each of the two parent clones. The resulting plasmodia can be designated 1.3–1 × 1, 1.3–2 × 1, 1.3–3 × 3, etc. Each of the backcross plasmodia is then fusion tested against the parent (1.3) plasmodium; all of the backcrosses that fuse with the 1.3 plasmodium belong to a fusion phenotype. Then a plasmodium which does not fuse with the parent (1.3) plasmodium is chosen as a new tester and all of the remaining backcross plasmodia are fusion tested against it; again any plasmodia that fuse with it belong to a second fusion phenotype. This process is repeated, with the selection of new testers, until all of the backcross plasmodia can be placed into a distinct fusion phenotype. Each set of backcrosses (the backcrosses to the two parent

clones), will produce 1, 2, 4, 8, 16, etc. fusion phenotype classes, dependent upon the number of recessives alleles in the particular parent clone (one class = no recessive, two classes = 1 recessive, four classes = 2 recessives, eight classes = 3 recessives, etc.). Figs 5–6 are examples of backcross results.

The backcrosses to parent clone 1 indicate segregation for one gene, and the backcrosses to parent clone 3 indicate segregation at two loci. The parent 1.3 plasmodium is therefore heterozygous at three loci, and its genotype can be arbitrarily designated as Aa, Bb, Cc and its phenotype as A-, B-, C- (for convenience these will be given as AaBbCc and A-B-C-). Since the backcrosses to parent clone 1 only produces two different fusion classes, parent clone 1 must have dominant alleles at two of the three loci, so it can be arbitrarily assigned the genotype ABc, and therefore parent clone 3 would have an abC genotype (parent plasmodium 1.3 must be heterozygous at all three loci = Abc × abC = AaBbCc). Since the backcrosses to parent clone 1 only produces two fusion classes, and one of them (the 1.3 class) is all dominant (A-B-C-), the other class (1.3–6 × 1) must have an A-B-cc phenotype, since parent clone 1 carries dominant alleles at the A and B loci, and thus the only way it can be different from the 1.3 class (A-B-C-) is to be recessive (cc) at the C locus.

The backcrosses to parent clone 3 produce four fusion phenotypes, with one of them being A-B-C-, since it fuses with the parental 1.3 plasmodium. The other three classes, therefore, have one of the following three genotypes/phenotypes: AabbC-, aaBbC- or aabbC- (they all must have a dominant C phenotype derived from parent clone 3 and recessive a and b alleles (clone 3 has the abC genotype). In order to correlate the classes with their phenoltypes, a new series of crosses must be undertaken. A vigorous plasmodium from one of the three classes is chosen (such as 1.3–7 × 3) and induced to sporulate. A new series of clones are isolated from it and then crossed to the original parent generation clone 3, and then tested to place them in a fusion class. For convenience the clones derived from the 1.3–7 × 3 plasmodium will be designated as X–1, X–2, X–3 etc. (Fig. 7). The second generation backcrosses are only shown for crosses to the parent clone 3, since the crosses to parent clone 1 would not provide any new information.

Two fusion classes indicates that the 1.3–7 × 3 plasmodium is heterozygous for either the A or B locus. You can then arbitrarily designate the 1.3–7 × 3 plasmodium (and its fusion class cohorts) as having an aaBbC- genotype/phenotype and, therefore, the 1.3–3 × 3 plasmodium (and its fusion class cohorts) must be aabbC- (if the 1.3–7 × 3 plasmodium is heterozygous only for Bb, then this class must receive a b allele in order to be different). This just leaves the AabbC- genotype/phenotype undesignated, so it must correlate with the 1.3–10 × 3 plasmodium.

If the 1.3–7 × 3 plasmodium was homozygous recessive for A and B (aabbC-), then the backcrosses would produce only a single fusion class. The other two classes could then be arbitrarily assigned the AabbC- and aaBbC- genotypes/phenotypes.

Further analysis

At this point testers for five of the eight possible fusion phenotypes (1.3 = A-B-C-, 1.3–6 × 1 = A-B-cc, 1.3–7 × 3 = aaB-C-, 1.3–3 × 3 = aabbC-, 1.3–10 × 3 = A-bbC-) have been designated, leaving only three classes to be determined (aaB-cc, A-bbcc, and aabbcc). The best way to proceed is to construct a clone that is recessive (abc) for all three loci. The 1.3–3 × 3 plasmodium is homozygous recessive at loci a and b and heterozygous (Cc) for locus c (phenotypically dominant, but it must carry a recessive c allele from its 3 parent). Thus, if plasmodium 1.3–3 × 3 is sporulated and clones isolated from it, they will have either the abc or abC genotype. If these clones are crossed amongst themselves they will produce plasmodia of two fusion phenotypes, aabbC- and aabbcc. The aabbC- class will fuse with the 1.3–3 × 3 tester and, therefore, the other class will have the aaabbcc phenotype (sixth fusion phenotype). The clones that produced this class will have the all recessive genotype abc.

Crossing an all recessive (abc) genotype clone (designated Y–1) of the appropriate mating type (A^2), to the original parent clone 1 (Abc), will produce an A-bbcc phenotype plasmadium (seventh fusion phenotype). The final fusion phenotype (aaB-cc) requires another cross and progeny analysis. Another all.

Classes	1.3	1.3–6 × 1
	1.3–1 × 1	1.3–6 × 1
	1.3–2 × 1	1.3–8 × 1
	1.3–9 × 1	1.3–14 × 1
	1.3–11 × 1	1.3–19 × 1
	1.3–18 × 1	1.3–22 × 1
	1.3–21 × 1	

Fig. 5 – Fusion phenotype classes from the backcrosses to parent clone 1 (A^1 mating type).

Classes	1.3	1.3–7 × 3	1.3–3 × 3	1.3–10 × 3
	1.3–5 × 3	1.3–7 × 3	1.3–3 × 3	1.3–10 × 3
	1.3–12 × 3	1.3–13 × 3	1.3–4 × 3	1.3–16 × 3
	1.3–20 × 3	1.3–23 × 3	1.3–15 × 3	1.3–17 × 3
		1.3–24 × 3		

Fig. 6 – Fusion phenotype classes from the backcrosses to parent clone 3 (A^2 mating type).

Classes	1.3–7 × 3	1.3–3 × 3	1.3–10 × 3
	X–7 × 3	X–5 × 3	0
	X–2 × 3	X–10 × 3	
	X–9 × 3	X–17 × 3	
	X–16 × 3		

If heterozygous for Aa or Bb.

Fig. 7 – Second generation backcrosses to parent generation clone 3 (A^2 mating type).

recessive (abc) clone (designated Y–2), of the appropriate mating type (A^1), crossed to original parent clone 3 (aBC) will produce a plasmodium (Y–2 × 3) having the aaBbCc genotype Progeny clones from this plasmodium will occur in four equal classes-aBC, aBc, abC, or abc- and when backcrossed to the Y–1 (abc) clone will produce plasmodia having a aaB-C-, aaB-cc, aabbC-, or aabbcc phenotype. These plasmodia can then be fusion tested against the known fusion class testers for three of the possibilities: 1.3–7 × 3 (aaB-C-), 1.3–3 × 3 (aabbC-), Y–2 × 3 (aabbcc). Therefore, the plasmdia that do not fuse with any of these testers must have the aaB-cc phenotype (the eighth fusion phenotype).

Clone genotypes

Once you have testers for all of the fusion phenotypes, and have identified all recessive (abc) myxamoebal clones, the genotype of any other clone can be identified by crossing it to the all recessive clone of the appropriate mating type to produce a plasmodium. This plasmodium can then be fusion tested against the known fusion testers to determine its phenotype, which will then indicate the genotype of the unknown clone. For example, a clone with an unknown (aBC) genotype crossed to the abc clone will produce a plasmodium with the aaB-C- phenotype (to produce the aaB-C- phenotype the unknown has to be aBC). Using this procedure, a set of clones having all of the possible genotypes can be identified.

Linkage

In some case, different coalescence gene loci may be located close together on the same chromosome and will, therefore, tend to maintain their original association unless recombination takes place. This linkage (Ling & Clark 1981) can be detected in the backcrosses by the occurrence of unequal fusion phenotype classes. In fig. 6, four classes were recovered, indicating segregation of two heterozygous loci. In this case, all four types were recovered in reasonably equal numbers (typical of the free recombination of un-linked loci). However, if

the parental configurations of the two segregating loci (AB from parent clone 1and ab from parent clone 3) occur significantly more frequently than the recombinant configuration (Ab and aB), then the two genes are linked. The parental configurations occur in the 1.3 class (A-B-C-) and the 1.3–3 × 3 class (aabbC-). So, if these two classes had occurred more frequently than the other two classes (1.3–7 × 3 and 1.3–10 × 3). the A and B loci would have been linked.

Variations and additional loci

Since the analysis of any isolate will depend upon the number of heterozygous genes present, there can be no set procedure that covers all situations. However, the use of backcrosses, and the accumulation of genetic information for specific clones and fusion testers, will provide a set of procedures that can handle considerable variation.

An extensive investigation of plasmodial coalescence in a number of isolates of the same species will likely reveal additional loci involved in determining plasmodial fusion. However, this basis procedure can still be utilized, to produce detailed information concerning the clonal genotypes and fusion tester phenotypes for each isolate. Once this information is determined, the testers of the different isolates can be tested against each other to determine the amount and kind of overlap between the different isolates. Analysis of these results could then be used to suggest specific crosses that might elucidate the overall genetic system controlling coalescence. This kind of analysis can become quite involved (Ling & Clark 1981), and could require considerable time and effort, therefore, unless the information is specifically needed for a research project, it is probably unwise to attempt.

Cytotoxic loci

Studies (Ling & Clark 1981) have shown that there are basically two types of genes controlling plasmodial coalescence, with the genes acting either pre- or post-fusion. The pre-fusion loci prevent the fusion of membranes which would allow coalescence, and the post-fusion loci terminate the events after membrane fusion has allowed cytoplasmic mixing. These later loci are generally called clear zone loci since the mixed cytoplasm (usually a small area) undergoes a cytotoxic reaction, which causes the cytoplasm to coagulate (forming a clear area) and die. The different genes that control coalscence can be characterized as non-fusion or clear zone loci by careful observation (Clark & Collins 1973) of the contact zone between two plasmodium, which different phenotypically at a single compatibility loci using a 60× dissecting microscope with substage lighting. This is usually necessary since the fused region of a clear zone may be quite small, however, in certain cases (Carlile 1973) the gene is so weak that the reaction takes several hours to occur and the clear zone affects a large proportion of the fused plasmodium. These fusion tests need to be conducted between a plasmodium with all recessive phenotype (aabbcc) and another plasmodium having a single dominant locus phenotype (such as A-bbcc). This will show whether or not the A loci controls a pre- or post-fusion reaction. If a post-fusion clear zone is produced, it will also provide a guide to the strength of the reaction (weak genes cause a slow reaction and therefore a larger affected area). The B and C genes would be examined using the same procedure.

Non-heterothallic isolates

Although the non-heterothallic isolates do not have mating types and, therefore, crossing and genetic analysis is impossible, they still display a coalescence phenotype. Since it is likely that they are apomictic, and thus every spore is genetically identical, all of the progeny from a sporangium have the same coalescence phenotype and will fuse with each other. However, if homothallism does occur, it would be possible for a homothallic isolate to be heterozygous for the coalescence genes and thus produce a number of different fusion classes by segregation; but since sexual fusion takes place only after a certain density of myxamoebae occurs, almost all fusion will be between genetically identical sibling myxamoebae, to produce homozygous plasmodia having a single fusion class. This is because sexual fusion would occur between adjacent myxamoebae, which due to mitotic division, would almost always be clonal siblings.

Since there is a large number of possible phenotypes in the species, the fusion of the

plasmodia of two isolates from nature is a strong indication of relatedness. Fusion test can thus be used to determine the occurrence, abundance, and extent of a clonal non-heterothallic population (Stephenson et al. 2004).

Conclusions

Culture has become an increasingly important aspect of myxomycete taxonomy and ecological studies. However, if one takes the trouble to culture the organism, there is an opportunity to then undertake genetic studies that will enhance the original project. While myxomycetes are relatively easy to work with, their diplo-haploid life cycle with clonal gametes has some unique aspects, which provide both genetic problems and opportunities. Therefore, we have provided what we hope is a simple and understandable set of principles and protocols for the genetic study of myxomycete reproduction and plasmodial coalescence.

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To all myxomycete workers, past, present, and future, we hope that this work will help insure the continued research in this interesting group of organisms.

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