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Patterns of mitochondrial inheritance in the myxogastrid *Didymium iridis*

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Abstract: Seven strains of the Central American A1 mating series of *Didymium iridis* were crossed in all possible combinations. Individual plasmodia were isolated and grown to a stage where total DNA could be isolated for DNA-DNA hybridization with cloned mitochondrial DNA probes to determine the pattern of mitochondrial inheritance. Random, biased, and dominant patterns of uniparental mitochondrial inheritance were observed, as well as rare cases of biparental inheritance, depending on the particular parental strains mated. The diverse patterns suggest that the factors controlling mitochondrial inheritance in *D. iridis* are complex. Differences between trials of the same matings suggest that non-genetic factors may also influence mitochondrial inheritance.

Key Words: biparental inheritance, isogamous mating, mitochondrial transmission genetics, myxomycetes, uniparental inheritance

INTRODUCTION

Uniparental inheritance appears to be a common feature of organelle inheritance, though exceptions do occur (Sears 1980, Birky 1994, 1995, 2001, Gillham 1994, Ohno 1997). In oogamous or anisogamous organisms, uniparental organelle inheritance could be explained by a lack of transmittance of one parent's organelles. However, the assumption of organelle exclusion in higher eukaryotes, specifically mammals, has been challenged by recent studies (Ankel-Simons and Cummins 1996, Sutovsky et al 1999). The reason for uniparental organelle inheritance in isogamous organisms has been more difficult to discern. Nuclear-cytoplasmic and intra-organelle competition have been proposed as driving forces in the evolution of uniparental inheritance (Eberhard 1980, Partridge and Hurst 1998). Godelle and Reboud (1995) proposed that specialization of organelles to cell lines has lead to uniparental inheritance. Birky (2001), however, has argued that uniparental inheritance should not be regarded as a general feature of organelle inheritance given its varying extent and the diversity of underlying mechanisms responsible for this inheritance pattern.

Perhaps no group of organisms provides a greater challenge to uniparental inheritance than the plasmodial slime molds or Myxogastria (Olive 1975). In these organisms mating occurs by complete fusion of isogametes that presumably contribute equally to the initial mitochondrial population in the zygote (Silliker and Collins 1988, Alexopoulos et al 1996). The zygote develops into a multinucleate cell, the plasmodium, by mitosis without cell division (Alexopoulos et al 1996). This precludes mitochondrial segregation by means of cell division as a mechanism for establishing homoplasty, a homogeneous organelle population. Further, the multiple allelic mating systems common in heterothallic myxogastrids (Betterley and Collins 1983) means that mitochondrial donor and recipient strains cannot be simply designated by mating type as would be possible in a binary mating system (Hurst 1992, Hurst and Hamilton 1992). Yet despite these challenges, uniparental inheritance occurs in the Myxogastria (Kawano et al 1987, Silliker and Collins 1988, Kawano and Kuroiwa 1989, Meland et al 1991). A mating-type hierarchy was shown to govern uniparental mitochondrial inheritance in *Physarum polycephalum* (Kawano and Kuroiwa 1989). Interestingly, the control could be overridden by the presence of a mitochondrial plasmid (Kawano et al 1991, 1993).

Uniparental inheritance was also found in *Didymium iridis*, though either parent seemed equally likely to be the mitochondrial donor in the cross studied (Silliker and Collins 1988). A low incidence of biparental inheritance was also observed in *D. iridis* (Silliker and Collins 1988). It was possible that the lack of hierarchy seen in *D. iridis*, as opposed to *P. polycephalum*, was due to a peculiarity of the particular

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cross studied. Alternatively, a novel pattern of inheritance could be operating in *D. iridis*. In this study seven strains of the A1 mating series of *D. iridis* were crossed in all possible combinations and the mitochondrial inheritance of the progeny was determined by DNA-DNA hybridization, in an attempt to understand the rules that govern mitochondrial inheritance in *D. iridis*.

MATERIAL AND METHODS

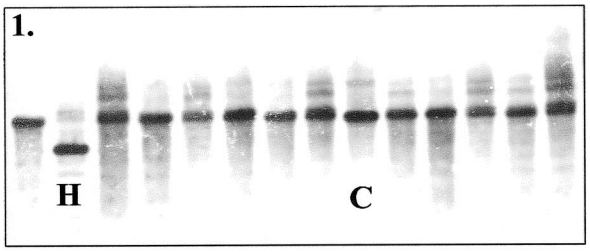
Strains and cultivation.—All *D. iridis* strains used in this study were members of the A1 Central American mating series, which consists of 12 mating types capable of interbreeding with each other but reproductively isolated from the other heterothallic mating series in this morpho-species (Clark et al 1991). Mating types within a series are designated by superscript numbers. Dr. Jim Clark kindly provided the following strains: Hon1–7 (mating type A^2), Pan2–44 (A^8), and CR2–26 (A^6) from the Collins mating type testers collection (Collins and Betterley 1982) and Gua2–2 (A^{13}) which was isolated by Clark and Landolt (1993). Strains Hon1–2 (A^1), CR5–5 (A^2), and Pan2–16 (A^7) were obtained directly from Collins' collections. Haploid strains were maintained in liquid axenic culture in peptone-glucose-yeast (PGY) media supplemented with heat killed bacteria (HKB) as previously described (Silliker et al 1988). Matings were initiated by combining 0.5 mL volumes of stationary phase cultures ($>10^6$ amoebae/mL) in a 14 mL disposable culture tube and incubating for 1 h at 23 C. A concentrated suspension of live *Escherichia coli* was prepared by suspending the bacteria grown on a Nutrient Agar (Difco) slant tube (15 × 150 mm) in 5.0 mL of sterile distilled water. The concentrated bacterial suspension, 0.5 mL, was added to the mating mixture after incubation and prior to dividing the mixture onto four buffered half-strength corn meal agar (CMA/2: 10 mM Na_2HPO_4; 10 mM KH_2PO_4; 8 g Difco Corn Meal Agar and 12 g agar) plates (100 × 15 mm). The plates were observed daily with a dissecting microscope (250×) until small plasmodia were visible. Individual plasmodia were excised onto a block of agar with a sterile needle and transferred to a fresh CMA/2 plate (60 × 15 mm). Within a day the plasmodium migrates away from the agar block and any remaining amoebae. The isolated plasmodium was then transferred to a CMA/2 plate (100 × 15 mm) coated with concentrated bacterial suspension. Once the plasmodium covered the plate, or had appeared to consume all the bacteria, it was harvested for DNA isolation (usually five to seven days after the plasmodium is excised from the cross plate). The possibility of fusions between very small plasmodia, prior to isolation, cannot be ruled out, however, the density of plasmodial formation makes this unlikely.

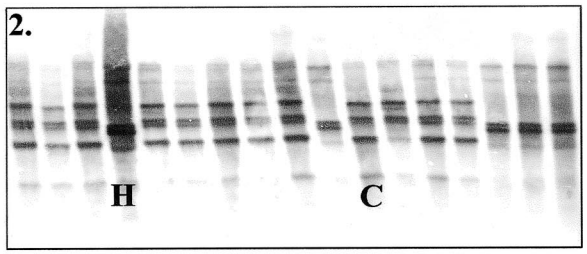
DNA isolation.—The procedure for isolating DNA from amoebae and plasmodia are identical except where noted. Cultures of amoebae 50 mL (10^6 amoebae/mL) were concentrated by centrifugation (1650 *g*, 10 min). Plasmodia were flooded with 3.0 mL ice-cold sterile distilled water and dislodged from the plate with a sterile glass rod. Centrifugation at 1650 *g* for 10 s pellets the plasmodia and an overlying slime layer. The water and slime were removed by aspiration. Either the amoebal or plasmodial pellets were suspended in 500 μL Lysis Buffer (10 mM Tris pH 8.0, 100 mM EDTA pH 8.0, and 0.5% SDS). Proteinase K was added to a final concentration of 10 μg/mL; then the lysate was incubated at 50 C. A 30 min incubation was sufficient for amoebal DNA isolation but plasmodial lysates were incubated for two days to assist the breakdown of slime which is inhibitory to subsequent enzymatic manipulations. Proteins were removed by an equal volume chloroform:isoamyl (24:1) extraction. Nucleic acids were precipitated by addition of 2/3 vol isopropanol and centrifugation (16 000 *g*, 10 min). RNA was degraded by suspending the nucleic acid pellet in 500 μL RNAse A (100 μg/mL in10 mM Tris, 1 mM EDTA pH 8) at 37 C for 30 min. After precipitation the DNA was suspended in TE Buffer (10 mM Tris, 1 mM EDTA pH 8), typically 200 μL for the amoebal isolations and 25 μL for the plasmodial isolations.

Probes and hybridization.—The mtDNA probes were obtained by digesting Pan2–16 mtDNA purified on CsCl gradients (Silliker and Collins 1988) with *Eco* RI or *Kpn* I according to the manufacturer's instructions. Restriction fragments were cloned into the pBluscript SKII+ vector (Stratagene, La Jolla, California) using a Fast-Link DNA Ligation Kit (Epicentre Technologies, Madison, Wisconsin). Hybridization confirmed the clones' mitochondrial origin. The following probes were chosen for this study because they highlight easily identifiable polymorphisms between the parental strains: *Eco* RI-2 (6.5 kb), *Eco* RI-4 (3.5 kb), *Kpn* I-3 (7.3 kb), and *Kpn* I-5 (4.5 kb). With the exception of a 2.5 kb overlap between *Eco* RI-2 and *Kpn* I-3, unique restriction maps indicate that these probes are from different regions of the genome. Standard procedures were used for gel electrophoresis (Sambrook et al 1989). Bi-directional transfers of DNA from gels were according to the method of Smith and Summers (1980). Probes were labeled by the random-primed method with digoxigenin and detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase that cleaves the chromogenic substrates, nitro-blue tetrazolium and 5-bromo-4-chloro-indoyl-phosphate (these procedures were performed as described in the Roche Molecular Biochemicals DIG Application Manual, Indianapolis, Indiana).

RESULTS

Eighteen crosses were made representing all possible combinations of seven *D. iridis* strains, not including compatible matings between strains with like mitochondrial types (e.g., Hon1–2 and Hon1–7 or Pan2–16 and Pan2–44). Blots of *Eco* RI digested total DNA were hybridized with cloned mtDNA fragments that highlighted distinctive strain polymorphisms; total DNA from haploid parental strains was included for comparison. Representative blots are pictured in FIGS. 1–3. FIGURE 1 depicts a trial where all the prog-





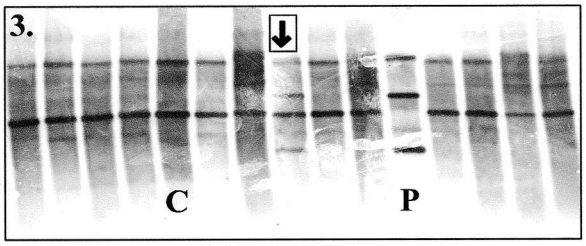


FIG. 1–3. Hybridization of mtDNA probes to haploid parents and diploid progeny. 1. Cross Hon1–2 × CR5–5 probed with *Eco* RI-2 showing uniparental inheritance of the CR5–5 mitochondrial type. 2. Cross Hon1–2 × CR2–26 probed with *Eco* RI-4 showing uniparental inheritance of either parental mitochondrial type. 3. Cross CR2–26 × Pan2–44 probed with *Kpn* I-3 showing uniparental inheritance of the CR2–26 parental mitochondrial type and biparental inheritance in the lane marked with an arrow. Note that in the biparental individual the mitochondrial DNA of the Costa Rican parent (CR2–26) appears to be in higher concentration. The lanes containing total DNA from the haploid parental strains are marked with one-letter abbreviations.

eny showed uniparental inheritance in favor of one parent. FIGURE 2 depicts a trial where all the progeny show uniparental inheritance but either parental mitochondrial type could serve as the donor. In FIG. 3 most of the progeny show uniparental inheritance in favor of one parent, however, one individual shows biparental inheritance (lane indicated by the arrow).

The inheritance data for all crosses are summarized in FIG. 4 and TABLE I. The relationships between strains with respect to mitochondrial inheritance are complex. Three overall patterns have been identified. In several crosses, one parent is always the mitochondrial donor, for example, of the thirty-two individuals analyzed from the cross between Hon1–7 and Pan2–44, all thirty-two progeny inherited the Pan2–44 mitochondrial type. Therefore, we call this pattern dominant inheritance. At the other extreme is the random pattern where either parent seems equally likely to be the mitochondrial donor. The cross between Hon1–7 and CR2–26 demonstrates this pattern. We call the third and most common pattern biased. In this case, one parent is the more frequent mitochondrial donor. A χ^2 test for each cross was calculated to distinguish between random and biased inheritance. If the calculated χ^2 value for a cross, based on the hypothesis of 50:50 inheritance, had a probability of less that 5%, the inheritance pattern was considered biased, not random (see TABLE I for probabilities). The three patterns discussed, dominant, biased, and random, describe ratios of uniparental progeny. Biparental inheritance occurred rarely, in 8 of the 403 progeny studied (~2%). These are indicated in FIG. 4 by the letter B, for biparental, since the probe detected both parental mitochondrial types.

Further complexities can be noted in these inheritance patterns (FIG. 4). Sibling strains that have the same mitochondrial type do not show similar inheritance patterns when mated to the other strains. For example, Hon1–2 and Hon1–7 are haploid strains that were derived through sporulation of the Hon1 diploid plasmodium. They have the same mitochondrial type but differ in mating type. If one compares the behavior of Hon1–2 and Hon1–7 when mated with Pan2–16, the Hon1–2 mitochondrial type is favored over Pan2–16, but the Pan2–16 mitochondrial type is favored over Hon1–7. Pan2–44 has the same mitochondrial type as Pan2–16. When Pan2–44 is mated with Hon1–7 it is not merely favored, as it is with Pan2–16, instead its inheritance is completely dominant. However, the Pan2–44 mitochondrial type is favored in crosses with Hon1–2. In contrast, the bias was reversed with Pan2–16. These results show that mitochondrial type is not a good predictor of the mitochondrial donor in a cross since strains with identical mitochondrial types do not show similar inheritance patterns when mated to a third strain.

Strains with the same mating type do not show similar inheritance patterns when mated with other strains. Hon1–7 and CR5–5 both have the A^2 mating type. When Hon1–7 is crossed with CR2–26, either parent is likely to be the mitochondrial donor. However, when CR5–5 is crossed with CR2–26, inheritance is strongly biased towards the CR2–26 parent. The CR5–5 parent also shows biparental inheritance in some crosses, which has not been observed with the Hon1–7 strain. These results show that mating type is not a good predictor of the mitochondrial

	Hon1-2	Hon1-7	CR5-5	CR2-26	Pan2-16	Pan2-44	Gua2-2
Hon1-2 (A^1)	—	—	1:28	6:19	13:4	3:17	9:0
Hon1-7 (A^2)		—	—	6:8	8:19	0:32	22:0
CR5-5 (A^2)			—	2:20	3:16:B3	0: 29:B2	16:0
CR2-26 (A^6)				—	18:2	29:0:B1	11:0
Pan2-16 (A^7)					—	—	5:22
Pan2-44 (A^8)						—	25:1:B2
Gua2-2 (A^{13})							—

FIG. 4. Results of mitochondrial inheritance in 18 crosses of strains from the A1 mating series of *Didymium iridis*. Strain mating types are in parentheses. The first number in each box indicates the number of individuals inheriting the mitochondrial type of the parent listed to the left, the second number indicates the number of individuals inheriting the mitochondrial type of the parent listed at the top of the column. Individuals showing biparental inheritance are indicated with the letter B, for biparental. Haploid strains derived from the same diploid plasmodia (Hon1–2 and Hon1–7 or Pan2–16 and Pan2–44) were not crossed because the sibling strains have the same mitochondrial type. Two strains, CR5–5 and Hon1–7, have the same mating types and are therefore incompatible mates. The ratios indicate a range of inheritance patterns from random, to biased, to completely dominant inheritance, with rare cases of biparental inheritance.

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Further, there is no evidence for a mating type hierarchy of inheritance as is seen in *P. polycephalum* (Kawano and Kuroiwa 1989). Reading across the top line of FIG. 4 one can see that CR5–5 and CR2–26 are the likely mitochondrial donors when mated with Hon1–2; however, Hon1–2 is the likely donor when mated with Pan2–16. But Pan2–16 is the likely donor when mated with CR5–5. Similarly, CR2–26 is the preferred donor when mated with every strain except Hon1–7, where either parent is equally likely. Yet, Hon1–7 can be the recipient when mated with many of the other strains that only receive from CR2–26. Despite the strong bias observed in individual crosses, there is no discernable pattern of hierarchy between strains.

An interesting observation can be made when one looks at the data broken into actual experimental trials (TABLE I). In cross c. Hon1–2 × Pan2–16, the Hon1–2 parent is the dominant donor in the first trial and the Pan2–16 parent is the dominant donor in the second trial despite the fact that the cumulative data indicate a bias toward the Hon1–2 parent. Cross g represents another trial where the bias shifts from one trial to the next. These data suggest that environmental conditions or stochastic events at the

TABLE I. Mitochondrial inheritance in crosses between seven strains of *Didymium iridis*

Cross	Trial	Results[a]	Inheritance type[b]	χ^2 probability[c]
a. Hon1-2 × CR5-5	1	0:16	Biased	$P < 0.001$
	2	1:12		
b. Hon1-2 × CR2-26	1	5:13	Biased	$P < 0.01$
	2	1:6		
c. Hon1-2 × Pan2-16	1	13:0	Biased	$P < 0.05$
	2	0:4		
d. Hon1-2 × Pan2-44	1	2:10	Biased	$P < 0.01$
	2	1:7		
e. Hon1-2 × Gua2-2	1	3:0	Not done[d]	
	2	6:0		
f. Hon1-7 × CR2-26	1	3:1	Random	$P > 0.05$
	2	3:2		
	3	0:5		
g. Hon1-7 × Pan2-16	1	0:10	Biased	$P < 0.05$
	2	8:6		
	3	0:3		
h. Hon1-7 × Pan2-44	1	0:10	Dominant	$P < 0.001$
	2	0:22		
i. Hon1-7 × Gua2-2	1	13:0	Dominant	$P < 0.001$
	2	9:0		
j. CR5-5 × CR2-26	1	0:3:B1	Biased	$P < 0.001$
	2	0:3		
	3	0:6		
	4	2:8		
k. CR5-5 × Pan2-16	1	0:2	Biased	$P < 0.01$
	2	0:2:B1		
	3	3:12:B2		
1. CR5-5 × Pan2-44	1	0:12:B1	Biased	$P < 0.001$
	2	0:12		
	3	0:5:B1		
m. CR5-5 × Gua2-2	1	4:0	Dominant	$P < 0.001$
	2	12:0		
n. CR2-26 × Pan2-16	1	3:0	Biased	$P < 0.01$
	2	12:2		
	3	3:0		
o. CR2-26 × Pan2-44	1	6:0	Biased	$P < 0.001$
	2	23:0:B1		
p. CR2-26 × Gua2-2	1	2:0	Dominant	$P < 0.001$
	2	9:0		
q. Pan2-16 × Gua2-2	1	1:4	Biased	$P < 0.01$
	2	2:4		
	3	2:14		
r. Pan2-44 × Gua2-2	1	1:1:B2	Biased	$P < 0.001$
	2	7:0		
	3	17:0		

[a] The numbers indicate the number of individuals inheriting the mitochondrial type of the first and second parent listed, respectively.

[b] The hypothesis of random (50:50) inheritance was tested for the accumulated data for each cross by χ^2 analysis. Crosses with a χ^2 value $P > 0.05$ were considered to be showing random inheritance. Crosses with χ^2 values $P < 0.05$ were considered to be showing biased inheritance unless one parent was never the donor; in those cases, the mode of inheritance was considered dominant. Biparental individuals were ignored for purposes of the χ^2 analysis. However, if any biparental individuals were observed in an otherwise dominant cross the mode of inheritance was considered biased.

[c] Probability of calculated χ^2 value based on an hypothesis of random inheritance.

[d] The χ^2 test cannot be performed on data sets less than ten.

time of mating may influence the pattern of inheritance in at least some of the crosses.

DISCUSSION

In an earlier study with *D. iridis* (Silliker and Collins 1988), plasmodia were harvested 42–60 d after mating so that a sufficient cell mass could be obtained for DNA isolation and purification on CsCl gradients. Alternatively, plasmodia were sporulated at about 20 d after mating; amoebae and plasmodia produced from the germinating spores were harvested for DNA analysis. In this study, plasmodia were harvested and the DNA isolated at about 8–12 d after mating. Despite the substantial decrease in time between mating and DNA isolation the data still support uniparental inheritance as the typical mode of mitochondrial inheritance in *D. iridis*.

However, a significant number of individuals showing biparental inheritance were also detected (~2%). It is not clear whether these individuals are en route to becoming homoplastic or whether the mixtures represent a persistent association. In this experimental plan, all of the plasmodium was used for DNA isolation and analysis to maximize the chance of detecting biparental inheritance, but future experiments could be designed to follow the mitochondrial population over time. An interesting possibility is that the individuals showing biparental inheritance are the result of unusual multiple cell and nuclear fusions that have been described in *D. iridis* (Ross and Cummings 1970). Unequal numbers of parental nuclear types might upset typical inheritance patterns. It can be noted that when mixtures are found, the two parental types are rarely present at equal frequency. It is also highly likely that through the use of the polymerase chain reaction, a higher number of biparental individuals would have been detected. These data support Birky's (1995, 2001) contention that uniparental inheritance should be considered a quantitative trait where the frequency of parental alleles inherited is considered rather than trying to impose the discrete categories of uni- or bi-parental inheritance on organisms.

Though in any particular cross only a small portion of the genome was detected by probes (3.5–7.3 kb), other regions of the genome were tested in other crosses. The particular probe chosen for a mating was based solely on its ability to unambiguously differentiate between the haploid parental strains; this varied depending on the strains being mated. Collectively, the probes used cover 19.3 kb of the mitochondrial genome. It is possible that through recombination other regions of the other parent's genome could have been transmitted, though we have no evidence to support this. Alternatively, recombinant genomes might have been transmitted at frequencies too low to detect by hybridization. Kawano et al (1991, 1993) have shown that in *P. polycephalum* a mitochondrial plasmid promotes mitochondrial fusion and recombination. A mitochondrial plasmid has been detected in *D. iridis* (Silliker and Speitzer 1997) that has no homology to the first five open reading frames of the *P. polycephalum* mF plasmid (Nakagawa et al 1998). Interestingly, all of the strains in this study (and all A1 series strains tested–nine independently isolated strains) show homology to the plasmid sequence cloned from the Pan2–16 strain (Silliker and Speitzer 1997).

The different patterns of uniparental inheritance observed, dominant, biased, and random, suggest that genetic compatibility factors may be involved in determining which mitochondrial type is inherited. However, the differences observed between trials also suggest that environmental or stochastic factors may also influence inheritance. In this study, equal volumes of stationary phase cells were combined to initiate mating. Previous researchers (Ross 1979, Shipley and Holt 1982) demonstrated that mating is highly influenced by cell fusion competency, which is induced by a pheromone that accumulates at high haploid cell densities. Perhaps subtle differences in the parent cells' physiological mating competency influence the mitochondrial donor in a particular mating.

Though *D. iridis* and *P. polycephalum* share most life history features, including multiple mating types, isogamy, and somatic fusion, the mechanism of mitochondrial inheritance appears to be different. In *P. polycephalum*, a mitochondrial donor hierarchy was observed which was determined by the alleles at the *matA* mating type locus (Kawano and Kuroiwa 1989). Our data do not support a mitochondrial donor hierarchy in *D. iridis* and the mating type alleles do not appear to be directly involved in determining mitochondrial inheritance. It should be noted, that Clark (1983) has demonstrated other differences in the mating types of *D. iridis* and *P. polycephalum*. Because these organisms share unusual characteristics it is tempting to expect them to behave similarly, however, based on LSU rRNA sequences, Johansen et al (1992) estimated a divergence time of 400–500 million years for these two genera. Since uniparental inheritance is believed to have evolved independently numerous times (Birky 1995, Gillham 1994) the differences between *D. iridis* and *P. polycephalum* may represent different evolutionary paths to uniparental inheritance. The degree to which *D. iridis* and *P. polycephalum* are exemplars for the Myxogastria as a whole will depend upon future studies of other members of this group.

Mirfakhrai et al (1990) showed that mitochondrial inheritance in the cellular slime mold *Polysphondylium pallidum* was uniparental. Further, they showed that the mitochondria associated with the *mat2* parent were always inherited in crosses between closely related strains. However, when more distantly related strains were mated the *mat1* parent occasionally served as the mitochondrial donor. Shitara et al (1998) found paternal mitochondrial leakage in interspecific mouse crosses, but no leakage when hybrid females were backcrossed to the paternal strain. They concluded that the stringent controls that prevent paternal mitochondrial inheritance within species were only partly effective in interspecific crosses. This raises the question of whether the disorderly inheritance patterns seen in *D. iridis* are due to mating strains that are only distantly related. Betterley and Collins (1983) found nearly uniform isozyme patterns within the A1 mating series (their analysis included all the strains used in this study with the exception of Gua2–2). They interpreted this uniformity as evidence of interbreeding in this mating series, however, little is actually known about the degree of gene flow in these Central American strains. It is possible that locally fewer mating types exist, essentially collapsing into a binary mating system with well-defined donor and recipient strains, as Hurst and Hamilton (1992) would predict. Only extensive sampling of mating and mitochondrial types in localized populations will resolve this question.

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