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Determining Mating Type and Species of a Natural Isolate of *Dictyostelium* using Molecular Techniques and Sexual Crosses

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Running Title: Mating type Identification in *Dictyostelium*

Abstract

Cellular slime molds, *Dictyostelium*, are bactivorous, soil-dwelling amoebae. When food is available, cells reproduce asexually by binary fission. Under certain environmental conditions, such as darkness and humidity, *Dictyostelium* reproduces sexually. The sexual cycle is uniquely social and has unusual features. First, sexual reproduction in most eukaryotes usually involves fusion of two gametes. But, in *Dictyostelium,* triparental inheritance is observed (Bloomfield *et al.* 2019), in which gamete fusion involves more than two gametes. Second, most eukaryotic species have two sexes or mating types, but *Dictyostelium* species are known to have more than two mating types. Even though *Dictyostelium* species show variations in sexual interactions, they look morphologically very similar. New natural isolates of *Dictyostelium* are generally identified and classified based on morphology (Hagiwara *et al.* 2004). Because variation in sexual interactions has genetic basis, many researchers agree that morphological species identification is not accurate, and that mating type identification be used to identify new isolates (Kawakami and Hagiwara 1999; Hagiwara *et al*. 2004). The goals of this project were 1) to identify the mating type of OZK11A (a natural isolate from the Ozark region of Arkansas) by gene presence/absence assay; and 2) to study macrocyst production by crossing cells of OZK11A strain with strains of known mating types. From the results of PCR, DNA sequencing and sexual crosses, we conclude that OZK11A is a mating type III, and it belongs to *Dictyostelium discoideum* species. We have also developed a novel, simple and cheap protocol to produce macrocyst.

Introduction

Cellular slime molds, *Dictyostelium,* are bactivorous, soil- dwelling amoebae found in moist soil all over the world. It transits back and forth between unicellular and multicellular stages depending upon the availability of food and other environmental conditions. When food is available, the haploid unicellular amoebae go through vegetative cycle in which they live a solitary life and reproduce asexually by binary fission. Depletion of food causes starving amoebae to undergo asexual developmental cycle to form fruiting bodies. Each fruiting body has a basal disc, stalk and sorus containing haploid spore cells at the top (Kessin 2001). Under certain environmental conditions, such as darkness, humidity and at a temperature on the higher side of tolerance, *Dictyostelium* reproduces sexually by macrocyst formation (Raper 1984; Kessin 2001). Thus, *Dictyostelium* uses three different reproduction strategies depending upon environmental conditions (Urushihara and Muramoto 2006). The sexual reproduction in *Dictyostelium* is uniquely social and has the following unusual features. 1) Usually, sexual reproduction in eukaryotes involves fusion of two gametes and the fusion process is tightly controlled to prevent polypoidy. But, in *Dictyostelium,* multiple gametes fuse to form syncytia. 2) Unlike other eukaryotes, gametes are morphologically indistinguishable from parental cells (Douglas *et al.* 2016). 3) Usually, two gametes contribute toward nuclear DNA and mitochondrial DNA is inherited from one of the gametes. In *Dictyostelium*, due to fusion of multiple gametes and hence mixing of cytoplasms, triparental inheritance is observed (Bloomfield et al. 2019) where a progeny inherits nuclear DNA from two gametes (two parents) and mitochondrial DNA from a third gamete (parent). 4) Most eukaryotic species have two sexes, but in dictyostelids, homothallic and heterothallic mating systems are found where some species of *Dictyostelium* are known to have more than two sexes (here after referred as mating types) (Bloomfield *et al*. 2010). For example, *Dictyostelium*

purpreum Olive has seven mating types and *D. giganteum* has four *and Dictyostelium discoideum* has three mating types and a few self-fertile homothallic strains (Hiromitsu 2004).

Sexual Reproduction

The hallmark of sexual reproduction in *Dictyostelium* is formation of macrocyst (Hagiwara *et al.* 2004). Macrocyst is formed when two sexually mature or fusion-competent cells of compatible mating types fuse to form a diploid zygote, which then attracts and ingests surrounding cells to form a multilayered structure called macrocyst (Bloomfield *et al.* 2010). Environmental conditions such as darkness and humidity, temperature, presence of calcium and absence of phosphate leads to the production of fusion specific proteins necessary for sexual maturation and cell fusion (Nickerson and Raper 1973; Erdos *et al.* 1976). The fusion-competent cells keep proliferating by binary fission.

More than two fusion-competent cells or gametes of compatible mating type fuse to form a large multinucleated giant cell (Saga *et al.* 1983; Urushihara *et al.* ,1990). Thus, there is mixing of cytoplasms from more than two gametes (Ishida *et al.* 2005) leading to triparental inheritance as mentioned above. Following gametic fusion, several hours later, the syncytia undergo cytokinesis and result in binucleate cells. The nuclei of binucleate cells fuse to form diploid zygotes (Ishida et al. 2005). Following zygote formation, zygote releases chemoattractant, cyclic adenosine monophosphate (cAMP), as a result neighboring haploid amoebae get attracted and form large cell aggregates around it (O'Day 1979). The aggregated cells lay down layers of cellulose and are also cannibalized by the zygote for nutrition (O,Day and Lewis 1975; Raper 1984; Kessin 2001). This stage is called precyst which then eventually matures into a macrocyst. A macrocyst is a single celled structure with three surrounding walls. It undergoes meiosis followed by repeated mitoses to form haploid amoebae (Okada *et al.* 1986). After a period of dormancy, macrocyst germinates to release haploid amoebae in the environment. It is not certain what triggers macrocyst to germinate but light, temperature or release of certain molecules from nearby bacteria could serve as triggering factors (Nickerson and Raper 1973). Depending on the local conditions, these amoebae can start vegetative, sexual or asexual development cycle.

The Mating System in D. discoideum

Both homothallic and heterothallic mating systems are known to exist in *Dictyostelium discoideum* (Robson and Williams 1980). Cells of homothallic strains can fuse with themselves (clonal fusion) to undergo sexual cycle, while for sexual reproduction to occur in heterothallic strains, cells of two different strains of compatible mating types must live together or find each other (Urushihara 1992). Heterothallic strains of *Dictyostelium discoideum* belong to three mating types (I, II and III). These mating types are determined by presence of sex specific genes at a genetic locus, called mat locus, on chromosome 5 (Bloomfield *et al.* 2010). Three forms of the mat locus specify mating types of this species. Mating type I and III are determined by presence of *matA* and *matS* genes respectively. Both genes have unique gene sequence and hence code for proteins that are not only different from each other but are also not similar to known protein families. Mating type II has *matB* and *matC* genes, which are homologs of *matA* and *matS* respectively, and therefore, can mate with Type I and III but for reasons not known, not with itself (Bloomfield *et al.* 2010). It is a mere speculation that mating type II may have arisen as a result of fusion of sex determining loci of types I and III. The three mating types can mate with each of the other two but not with itself. Thus, following sexual crosses are found to be successful: type I/type II, type I/type III, and type II/type III (Bloomfield *et al.* 2010).

The mating type locus in *D. discoideum* is completely sequenced and the key sex- determining genes (*matA* and *matS*) at this locus lack homology to other known proteins, therefore, this knowledge can be successfully used as a tool to identify mating type and species identity of natural isolates of *Dictyostelium.* New natural isolates of *Dictyostelium* are generally identified and classified based on morphology of fruiting bodies (color, size and shape) (Hagiwara *et al.* 2004). Because many new isolates show similar morphology, and identification of species based on morphology alone could be biased and hence inaccurate, it is important that mating type identification based on gene sequence be used as a tool to assign species to newly found isolates of *Dictyostelium*.

OZK11A is a natural isolate from the Ozark region of Arkansas. It was collected by J.C. Landolt and S. Stephenson and deposited by J.C. Landolt to the Dicty Stock Center, Chicago, IL. We identified the mating type of OZK11A by gene presence/absence assay and investigated its mating behavior by arranging sexual crosses with two test strains, AX2-GFP (mating type I) and HM1558 (mating type III).

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Materials and Methods

To ascertain the mating type and species of a natural isolate of *Dictyostelium*, OZK11A, we obtained OZK11A and two strains of known mating type, AX2-GFP and HM1558 from the Dicty Stock Center, Chicago, IL. These two mating type strains were used as test strains in the gene presence/absence assays and sexual crosses.

AX2-GFP strain is a mating type I and has expression vector, pDdA15gfp, to express GFP (green fluorescent protein) from actin 15 promoter and neomycin resistant gene. HM1558 strain has blasticidin S resistance cassette that expresses RFP (red fluorescent protein) and it has *matS* and *matT* genes introduced at the mating type locus making it mating type III.

AX2-GFP and HM1558 cells were grown in HL-5 medium (10g dextrose, 10g proteose peptone, 5g yeast extract, 0.97g Na_2HPO_4, 0.48g KH_2PO_4, and 0.03g streptomycin, volume to 1 liter and autoclaved) supplemented with G418-sulphate (APExBIO Technology, Houston, TX) and Blasticidin S HCL (APExBIO Technology, Houston, TX) respectively at a final concentration of 5µg/mL and maintained at 22 ºC. Since OZK11A is a wild type natural isolate, it was grown in heat-killed *Klebsiella aerogenes* broth (*K. aerogenes* culture grown overnight at 37°C in a sterile culture flask to stationary phase in SM broth (10 g glucose, 10 g proteose peptone,1 g yeast extract,1 g MgSO4∗7H2O, 1.9 g KH2PO4,0.6 g K2HPO4 volume to 1 liter and autoclaved) then autoclaved and cooled to room temperature).

Gene Presence/Absence Assay:

Genomic DNA Extraction: Genomic DNA was extracted from the above three strains of *Dictyostelium* using Promega Wizard genomic DNA purification kit (Promega Inc. Madison, WI) following manufacturer's instructions. To check the presence and integrity of the extracted genomic DNA samples, 5 µL of each sample was electrophoresed on 1% agarose gel and stained with GelRed.

Gene Sequences and Primer Design: Three pairs of polymerase chain reaction primers were designed (see Table 1) for each of the mating type genes, *matA, matC* and *matS*, from DNA sequences (GeneBank accession numbers FN543123.1, FN543124.1, FN543122.1, respectively). OligoAnalyzer program (IDT, Coralville, Iowa, USA) was used to design the primers. The primers were obtained from Integrated DNA Technologies (Coralville, IA) at 25mM concentration each.

PCR Reaction Composition and Conditions: Genomic DNA of the three strains were used as template to perform gene presence /absence assay using polymerase chain reaction (PCR) technique. PCR reactions were performed in a thermal cycler (Mastercycler® nexus gradient, Eppendorf Inc. USA). All reaction volumes were 50 µL and had the following reagents from TakaRa Bio USA, Inc. (San Jose, CA): 4 µL of dNTP mixture (2.5mM each), 1unit (0.25 µL) of

Table 1. Primer sequences used for amplifying mating type genes *matA, matS and matC.*

Primer Name	PCR Primers Nucleotide Sequence 5'-3'	Target	Sequence Length	Mating Type
matAFor	CACACTAAACATGGACCCAC	GenBank: FN543123.1 base (1008-1027)	343 base pairs	Type I
matARev	CCTAAATCTTTACCAAGTCA	GenBank: FN543123.1 base (1331-1350)		
matCFor	AGAGGTTTGACTTGGGTTGG	GenBank: FN543124.1 base (1167-1186)	401 base pairs	Type II
matCRev	CCATTTCAATCACTCCTACCA GC	GenBank: FN543124.1 base (1545 – 1568)		
matSF	GTATAAACCACTCACAGCTGA TA	GenBank: FR666792.1, Strain ZA3A base (309 - 331)	310 bp	Type III
matSR	CTAAAGGCAGGCACCTTC	GenBank: FR666792.1, Strain ZA3A, base (601-618)		
NmatSF	CGATCAGTTGGAAAACATTAC	GenBank: FN543122.1, Strain WS2162 base (743- 772)	664 base pairs	Type III
NmatSR	GGATAGCCAAAAAACTAGTTT	GenBank: FN543122.1, Strain WS2162, base (1383-1407)		

TakaRa ExTaq polymerase, 5 µL of 10X Ex Taq buffer. 1 µL of forward and reverse primers were added at a final concentration 2.5 µM each and 2 µL of acetylated BSA was added at a concentration of 10mg/mL (Promega Inc. Madison, WI). Nuclease-free water was added to obtain the total reaction volume. The cycling conditions for all reactions had denaturation at 94°C for 1 minute, extension of 72°C for 1 minute and a final extension at 72°C for 10 minutes. Each reaction went through 30 cycles and annealing conditions varied depending on the pair of primers used.

Sequencing Amplified Gene Fragments: PCR amplified products were electrophoresed on 2 % gel, stained with GelRed and visualized using gel documentation system. After confirming size and integrity of the PCR products on the gel, they were purified using Promega Wizard SV Gel and PCR-Clean-Up-System (Promega Inc. Madison, WI) following manufacturer's instructions. Purified samples were sequenced using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the DNA Sequencing Core Facility, UAMS, Little Rock, AR. Sequenced data was analyzed using FinchTV (version 1.5.0, Geospiza Inc., USA) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) software programs.

Sexual Crosses and Macrocyst Formation

Prior to setting up sexual crosses, amoebae of the two strains, AX2-GFP and HM1558 were grown axenically in HL-5 medium in presence of selective antibiotics G418 and Blasticidin respectively as these strains contain relevant expression vectors. The antibiotics were omitted in all sexual crosses because OZK11A is a natural isolate lacking selectable marker.

To study sexual reproduction, three pairwise two-way sexual crosses were set up in triplicate between two test strains (AX2-GFP (type I), HM1558 (type III)) and OZK11A (unknown type). All sexual crosses were maintained in heat-killed *K. aerogenes* broth. For each strain, $2x10^6$ cells were harvested, pelleted by centrifugation (Avanti JXN-26,Beckman Coulter Inc. USA) at 2000 rpm for 2 minutes at 4ºC and washed with phosphate development buffer (5mM Na_2HPO_4, 5mM KH_2PO_4, 1mM $CaCl_2$, 2mM $MgCl_2$, pH 6.5, store at 4ºC) twice and suspended in 50 µL of Hl-5 medium. For each of the two- way crosses, 2 X 10^6 cells of each strain were plated in 65 mm X 35 mm culture dish in 5 mL of heat- killed *K. aerogenes.* Each of the three control plates had 4 X 10^6 cells of one strain in heat-killed *K. aerogenes*. The plates were kept in a plastic box and maintained at 22^0C in a low temperature incubator. Wrapping the plastic box with black paper from all sides and keeping moist paper towels in the box surrounding the plates created dark and humid conditions necessary for macrocyst formation. Plates were observed after a week for presence of macrocysts. Pictures were taken on days 7, 9 and 12 using an inverted microscope (Zeiss Primovert, Jena, Germany) using 40 X objective lens.

Results

Gene Presence/Absence Assay:

To establish mating type of the natural isolate, OZK11A, PCR method was used to determine the presence of mating type genes (*matA, matC* and *matS*) from its genomic DNA sample as described in the materials and methods section. *Dictyostelium* strains AX2 -GFP and HM1558 of known mating types (mating type I and III) were used as test strains. To further ascertain the presence of mating type gene, PCR amplified products were sequenced and then analyzed using NCBI 's BLAST tool.

When PCR was performed using a pair of primers (Table 1) for *matA* gene (target 343 bp), amplified product of the expected size, 343 bp, was seen on the gel for AX2-GFP strain (Figure 1 (a)) conforming that AX2-GFP is mating type I. No desired amplification product was visible on the gel for the other two strains, HM1558 and OZK11A, even after altering primer concentrations and PCR conditions numerous times. PCR reactions using primers for *matC* gene (target size 401 bp) did not result in amplified product for all the three strains even after altering reactions and reaction conditions several times (results not shown). This result was expected for AX2-GFP and HM1558 because these strains are mating type I and III respectively. When two different pairs of primers for *matS* gene were used to amplify the targets (310 bp and 664 bp) using genomic DNA of all the three strains, amplified products of expected size were visible on the gel for OZK11A (Figure 1 (b) and (c)).

The PCR amplified products were cleaned and sequenced to confirm definitively the specificity of amplification. The sequenced data was observed and trimmed using FinchTV. The quality of the data appeared clean for all the three samples, as the chromatograms had evenly-spaced peaks of similar heights and baseline peaks ("noise") was not there at all. Each one of these sequenced nucleotide sequences was compared to standard databases of all known genes using BLASTn. BLASTn results for 310 bp gene product amplified using *matS* gene primers and genomic

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DNA of OZK11A strain showed 99.19 percent identity (figure 2(a)) to *Dictyostelium discoideum*, *matS* gene, strain ZA3A (GenBank: FR666792.1). BLASTn comparison for 664 bp gene product amplified using a different pair of primers for *matS* gene and genomic DNA of OZK11A resulted in three sequences with significant alignments. One of these alignments showed 98.8 percent identity to *Dictyostelium discoideum*, mating type locus, strain WS2162 (GenBank: FN543122.1) (Figure 2(b)).The 343 bp gene product amplified from genomic DNA of AX2-GFP strain using *matA* gene primers, showed 98.94 percent identity to *Dictyostelium discoideum*, mating type locus, strain WS205 (GenBank: FN543123.1).

Sexual Crosses and Macrocyst Formation

Sexual crosses were set up as described in the materials and methods section. Sexual cross between OZK11A and HM1558 (type III) did not result in macrocysts in all three test plates even after 10 days (Figure 3 (c)). As expected, sexual cross between the two test strains, AX2-GFP (type I) and HM1558 (type III) resulted in macrocysts on day 7 (Figure 3(a)). The macrocysts were big and numerous. Sexual cross between OZK11A and the test strain, AX2-GFP (type I), resulted in delayed mating as macrocysts were seen on day 9. The macrocysts resulted from this cross were small and few in number (Figure 3(b)).

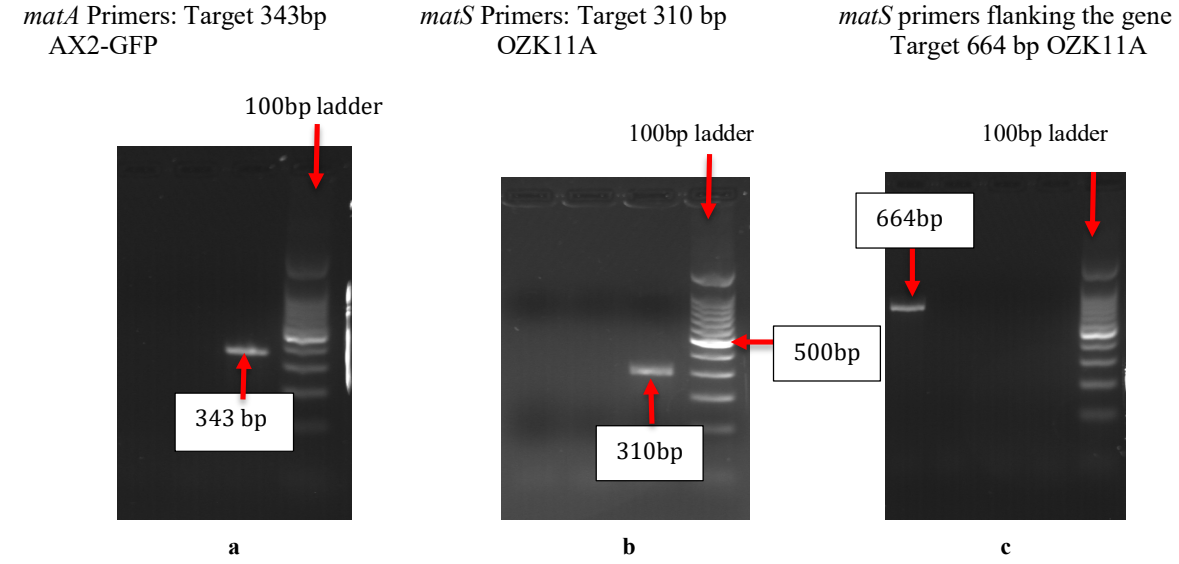

Figure 1. PCR amplification of *matA* and *matS* gene fragments using genomic DNA of AX2-GFP, HM1558 and OZK11A. (a) Shows amplification of 343 bp *matA* gene fragment for AX2-GFP. (b) 310 bp gene fragment was amplified for OZK11A strain when *matS* primers were used. (c) Primer pair flanking the *matS* gene amplified 664 bp product for OZK11A.

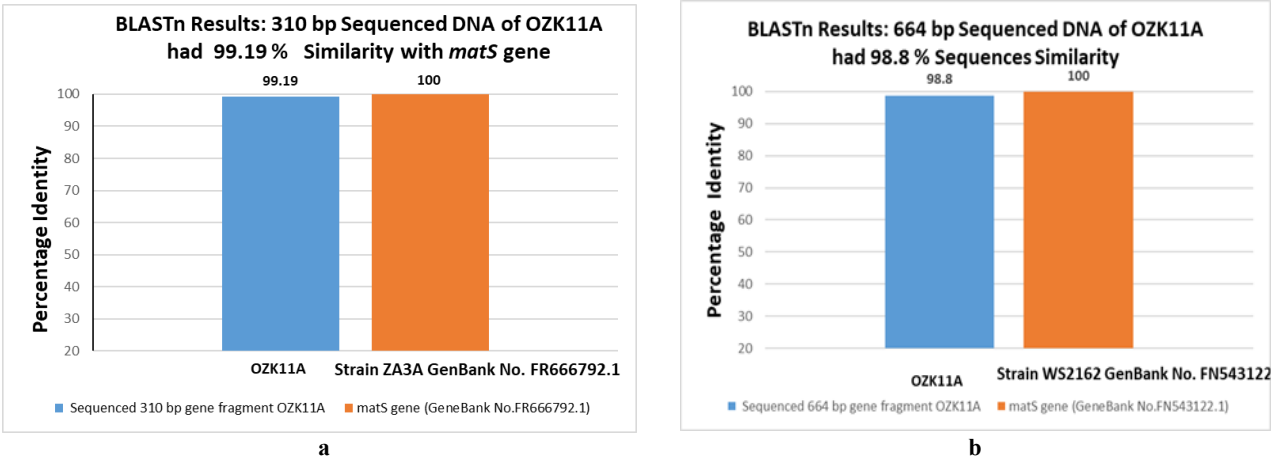

Figure 2. (a) 310 bp gene product amplified using *matS* gene primers and genomic DNA of OZK11A strain showed 99.19 percent identity to *Dictyostelium discoideum, matS* gene, strain ZA3A (GenBank: FR666792.1). (b) 664 bp gene product amplified using *matS* gene primers and genomic DNA of OZK11A strain showed 98.8 percent identity to *Dictyostelium discoideum,* mating type locus, strain WS2162, mating type III (GenBank: FN543122.1).

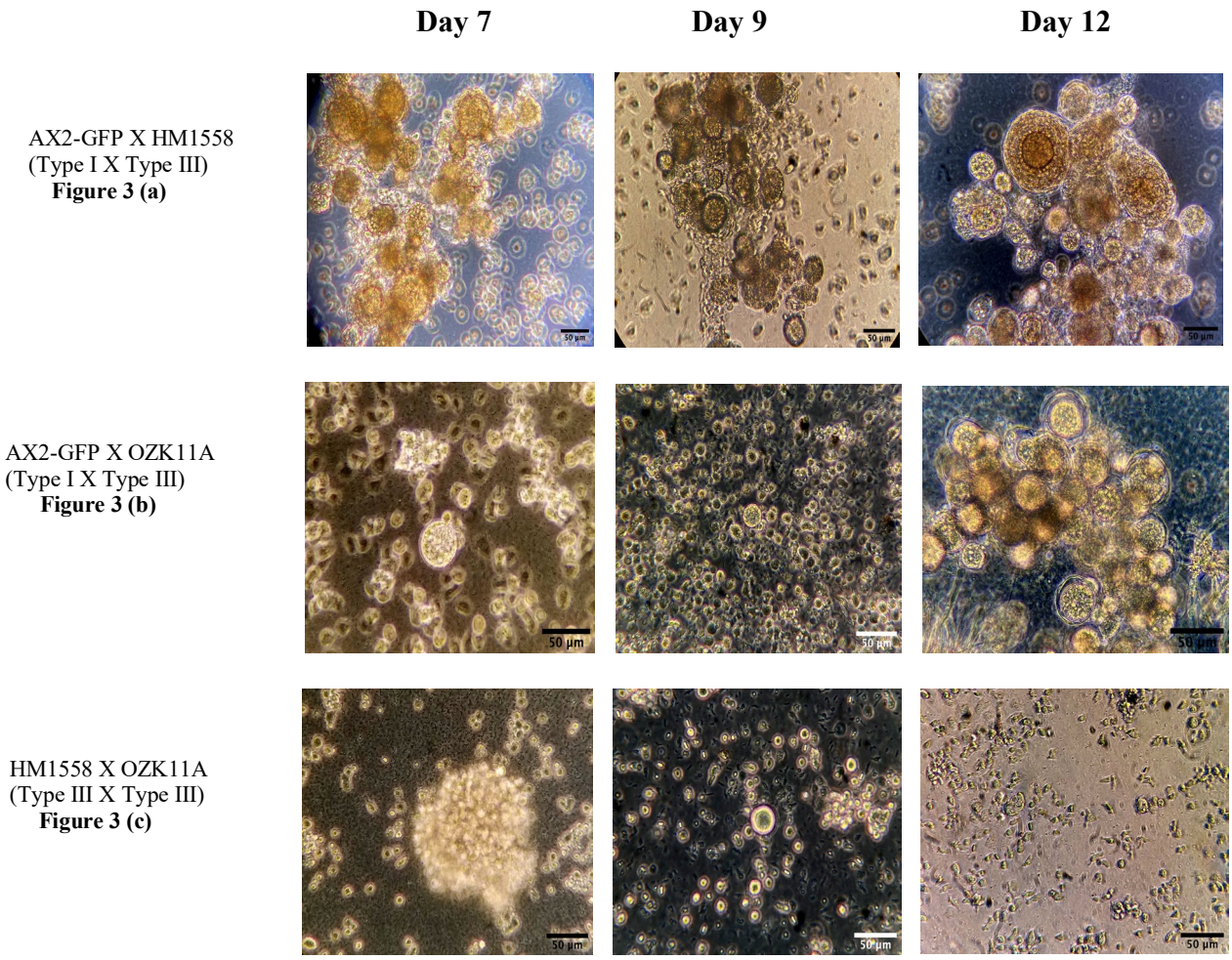

Figure 3**.** Results of the three pairwise two-way sexual crosses. (a) Sexual cross between the two test strains, AX2-GFP (type I) and HM1558 (type III) resulted in macrocysts on day 7. The macrocysts were big and numerous. (b) Sexual cross between OZK11A and the test strain, AX2-GFP (type I), resulted in delayed mating as macrocysts were seen on day 9. The macrocysts resulted from this cross were small and few in number. (c) Sexual cross between HM1558 and OZK11A did not result in macrocyst indicating they both belong to the same mating type (type III). Scale bar, 50µm.

Discussion

The examined strain, OZK11A, is a natural isolate from Ozark region of Arkansas. Morphologically, OZK11A amoebae look more elongated, tubular and branched than the test strains. It is difficult to predict the species type of OZK11A just by observing its morphology and comparing it with the known strains of *Dictyostelium discoideum*. When three different PCR reactions were performed using genomic DNA of OZK11A and primers for the genes, *matA*, *matC* and *matS* , which are sex specific genes of mating types I, II and III respectively, amplified products of expected size for *matS* gene were visible on the gel but not for the other two genes. Further, when these PCR amplified DNA samples were sequenced, the sequencing data and BLAST analysis indicated that OZK11A had more than 99% match with other known mating type III strains at the *matS* locus. So, we conclude that OZK11A has sex –specific *matS* gene in its sex-determining locus and hence it is mating type III strain of *Dictyostelium discoideum*.

Three pairwise sexual crosses of OZK11A with the test strains of mating type I and mating type III resulted in macrocyst formation with the type I but not with the type III. These results indicate that OZK11A is mating type III since (heterothallic) strains of the same mating type do not mate with each other. When OZK11A was crossed with itself, we did not get macrocysts even after two weeks, indicating that OZK11A gametes are not autogamous and therefore, OZK11A is not a homothallic strain of *Dictyostelium discoideum*.

Thus, from the results of PCR, DNA sequencing and sexual crosses, we conclude that OZK11A belongs to mating type III of *Dictyostelium discoideum*.

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Conclusion

We have ascertained the mating type and species of a natural isolate, OZK11A, using easy and widely available molecular techniques such as PCR and sequencing. The methods and set of primers for sex specific genes described here would make mating type and species identification of new natural isolates of *Dictyostelium discoideum* fast and accurate. This approach can also be used for identifying new isolates that belong to different species of *Dictyostelium* .

We have also developed a novel, simple and cheap protocol for macrocyst production. There are several protocols for macrocyst production in the published literature that requires use of expensive chemicals and materials (Bloomfield *et al.* 2019). To create phosphate free environment and dark and humid conditions necessary for macrocyst production, we used inexpensive, non-hazardous and readily available materials and obtained macrocyst in seven days like the other protocols.

Since we have identified the mating type of OZK11A (type III), researchers can utilize this strain to further understand mating behavior of *D. discoideum.* It can also be used as a test strain to identify mating type of unknown strains.

We believe that species identification of natural isolates of *Dictyostelium* is not only important for taxonomic purposes but also help in exploring and understanding how these bactivorous natural isolates of *Dictyostelium* interact with soil microbes and impact soil microbial composition and dynamics.

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