# MITOCHONDRIAL INHERITANCE IN HAPLOID x NON-HAPLOID CROSSES IN *CRYPTOCOCCUS NEOFORMANS*

# MITOCHONDRIAL INHERITANCE IN HAPLOID x NON-HAPLOID CROSSES IN *CRYPTOCOCCUS NEOFORMANS*

By

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A Thesis

Submitted to the school of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2010) McMaster University
(Biology) Hamilton, Ontario

TITLE: Mitochondrial inheritance in haploid x non-haploid crosses in *Cryptococcus neoformans*

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SUPERVISOR: Professor Dr. Jianping Xu

NUMBER OF PAGES: viii, 111

## ABSTRACT

*Cryptococcus neoformans* is among the most common human fungal pathogen and the leading cause of meningoencephalitis among immunocompromised individuals. It consists of two divergent serotypes A and D as well as their hybrid serotype AD. Strains of serotype A and D are mostly haploid in the natural environment and among clinical isolates while AD hybrids are either aneuploid or diploid. *C. neoformans* is a bipolar heterothallic fungus in which completion of the sexual (meiotic) cycle is dependent upon interaction between cells of opposite mating type, *MAT***a** and *MAT*α. In crosses between haploid *MAT***a** and *MAT*α strains, most fusion products and meiotic segregants inherit mitochondrial DNA (mtDNA) from the *MAT***a** parent. Besides mating between haploid strains, haploid x aneuploid and haploid x diploid crosses, collectively referred to as haploid x non-haploid crosses, can also occur in *C. neoformans*. However, it was previously unknown whether the uniparental mitochondrial inheritance operates in *C. neoformans* haploid x non-haploid crosses as well. In my thesis, I analyzed mitochondrial inheritance in the fusant and meiotic segregant populations of the two types of opposite-sex haploid x non-haploid crosses, *MAT***a/a** and *MAT*α and *MAT***a** and *MAT*α/α. The study showed that: (1) unlike haploid x haploid crosses, mitochondrial inheritance in the progeny of haploid x non-haploid crosses was not dominantly uniparental although it was biased towards inheritance of *MAT***a**(/**a**) parent-specific mtDNA, and that (2) there was a significant variation within and between crosses of each type in the percent of progeny with other mtDNA types. The findings of my study expand our understanding of mitochondrial inheritance process in *C. neoformans* and, potentially, in other eukaryotes.

## ACKNOWLEDGEMENTS

The past two years' study in the Department of Biology at McMaster University was extremely enjoyable and rewarding in many respects. McMaster University provides excellent resources for learning and self-development which have greatly contributed to expanding my scientific knowledge of fungal genetics. The successful completion of my M. Sc. thesis, nevertheless, would not be possible without the help of people around me.

First of all, I would like to express gratitude to my supervisor Dr. Jianping Xu for his generous help with my research project both experimentally and knowledgably. Dr. Xu gave me the opportunity to experience the real science. His doors were open for me to answer questions and exchange ideas. For all three years I have known Dr. Xu, he has been my mentor, coach, and guide. His adherence to daily physical activities has positively impacted my life style as I have started going to a gym on a regular basis.

Next, I would like to express my gratitude to Sheng Sun for showing me experimental techniques. Without a doubt, exceptional work ethics I learnt from him will benefit me throughout my future career as a dentist. I would also like to thank Dr. Timothy Y. James for his constructive suggestions to my manuscript, assistance with data analysis as well as great ideas brought into the experimental designs. Moreover, I am very grateful to my committee member Dr. Brian Golding, who was always supportive and available for help. A Bioinformatics course Dr. Golding teaches has significantly improved my understanding of programming. In addition, I would like to give respect and credit to my family: my mother, grandmother, and sister—who supported and encouraged me throughout my study here. Lastly, my research success would not be possible without the help and care of my lab colleagues. I thank Mochan Li, Dr. Anuradha Chowdhary, Dr. Ping Zhang, Amanda Wilson, Rahul Nayak, David Bartfai, Sanjay Hiremath, and Hari Maganti (in no particular order).

This research is supported by National Sciences and Engineering Research Council (NSERC) Alexander Graham Bell Canada Graduate Scholarship to I. Skosireva and by an NSERC Discovery grant to Dr. J Xu.

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August 2010

## THESIS FORMAT

This thesis is organized in three chapters. The first chapter provides a general introduction that provides a general overview of this study. The second chapter presents my project that has been published in Current Genetics in April 2010, 56(2):163-176. Chapter 3 gives overall conclusions of the study as well as suggests future studies that will expand our understanding of mitochondrial inheritance in *Cryptococcus* species.

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 PATTERNS OF MITOCHONDRIAL INHERITANCE IN EUKARYOTES

Mitochondria are ubiquitous in eukaryotic cells. They play a vital role in cellular bioenergetics: mitochondria generate cellular energy in the form of adenosine triphosphate (ATP) by converting chemical energy through oxidative phosphorylation. Together with chloroplast DNA, mitochondria are unlike other eukaryotic organelles (e.g., ribosome, lysosome, Golgi apparatus, etc.) in that they contain their own genetic material, which is inherited independently of the nuclear genome. The inheritance of organelle DNA typically does not follow Mendelian principles. There are different ways to classify mitochondrial inheritance. Some reviews of different mtDNA inheritance modes start with the discussion of uniparental mtDNA inheritance mode and then provide variations and deviations from this mode observed in a particular species analyzed (Taylor 1986; Birky 2001; Xu 2005). Such a bottom-up approach is valuable as it emphasizes the prevalence of uniparental mitochondrial transmission mode among diverse species. In this thesis, however, I used a top-down approach to classify mtDNA inheritance—the classification that is based on the dependence of the organelle inheritance on a cell's sex (in higher eukaryotes) or mating type (in lower eukaryotes) (Figure 1.1). In contrast to the bottom-up approach, this categorization puts emphasis on the fact that any mtDNA inhertitance mode is dependent on a cell's sex. Looking at mitochondrial transmission from this perspective serves two main purposes: it better prepares the reader for and creates a smooth transition to Chapter 2, in which associations between mating type allele number in parental strains and a mtDNA inheritance pattern in progeny populations have been found.

MtDNA inheritance in eukaryotes could be categorized into two main types: (1) parent's sex/mating type-dependent and (2) progeny's sex/mating type-dependent (Figure 1.1). There are two subtypes of the parent's sex-dependent inheritance: (1) sample location-dependent and (2) sample location-independent. In the former, mitochondrial inheritance is affected by a progeny sampling location. This latter subtype could be further divided into hermaphroditic and yeast-like mtDNA inheritance modes. In species with a hermaphroditic mode of mitochondrial inheritance either parent can act as a mitochondrion donor irrespective of progeny'sex. Similar to the hermaphroditic mode, mitochondria inherited in a yeast-like mode could come from either mate, although the mechanism employed is quite different from that in the hermaphroditic inheritance mode. The mtDNA inheritance which depends on offspring's sex is also known as doubly-uniparental mitochondrial inheritance. The details of various mitochondrial inheritance modes and supporing evidence are provided below.

A uniparental mitochondrial transmission occurs in both main types of mtDNA inheritance, parent's and progeny's sex/mating type dependent. In this mode, all or the majority of sexual progeny inherit mtDNA haplotype from a parent of a higher dominan. Based on the proportion of progeny with a dominant parent-specific mtDNA, the uniparental inheritance could be either dominant or biased. In addition, there is also biparental mtDNA inheritance mode, in which progeny inherit mtDNA haplotypes from both mates (a.k.a. progeny heteroplasmic for mtDNA). Like uniparental inheritance, biparental inheritance has been detected in species exhibiting either one of mtDNA inheritance types. When discussing the biparental mode of organelle inheritance, it is important to consider such factors as the proportion of progeny with mtDNAs inherited from both parents over a total number of

progeny screened as well as a ratio of parental mtDNA haplotypes in a single progeny. If the ratio is significantly in favor of the mtDNA from one mate over the other then such a state would represent a biparental mtDNA transmission which is biased towards the propagation of mitochondria from a single mate. Mitochondrial leakage is a term commonly used in the discussion of mtDNA inheritance. The term refers to cases in which offspring are heteroplasmic for parental mtDNAs and/or inherit mtDNA entirely from a mate that normally does not contribute mitochondria to the next generation progeny (Barr et al 2005; Yan et al 2007; Skosireva et al 2010).

A detailed description of each mtDNA inheritance patterns presented above follows next. Importantly, a primary focus is given on the discussion of parental mtDNA haplotypes in progeny populations of intra-specific matings between species of the same ploidy level (i.e. same-ploidy crosses). Mitochondrial inheritance in offspring of matings between species of different ploidy (i.e. mixed ploidy crosses) is a focus of Subsection 1.2.

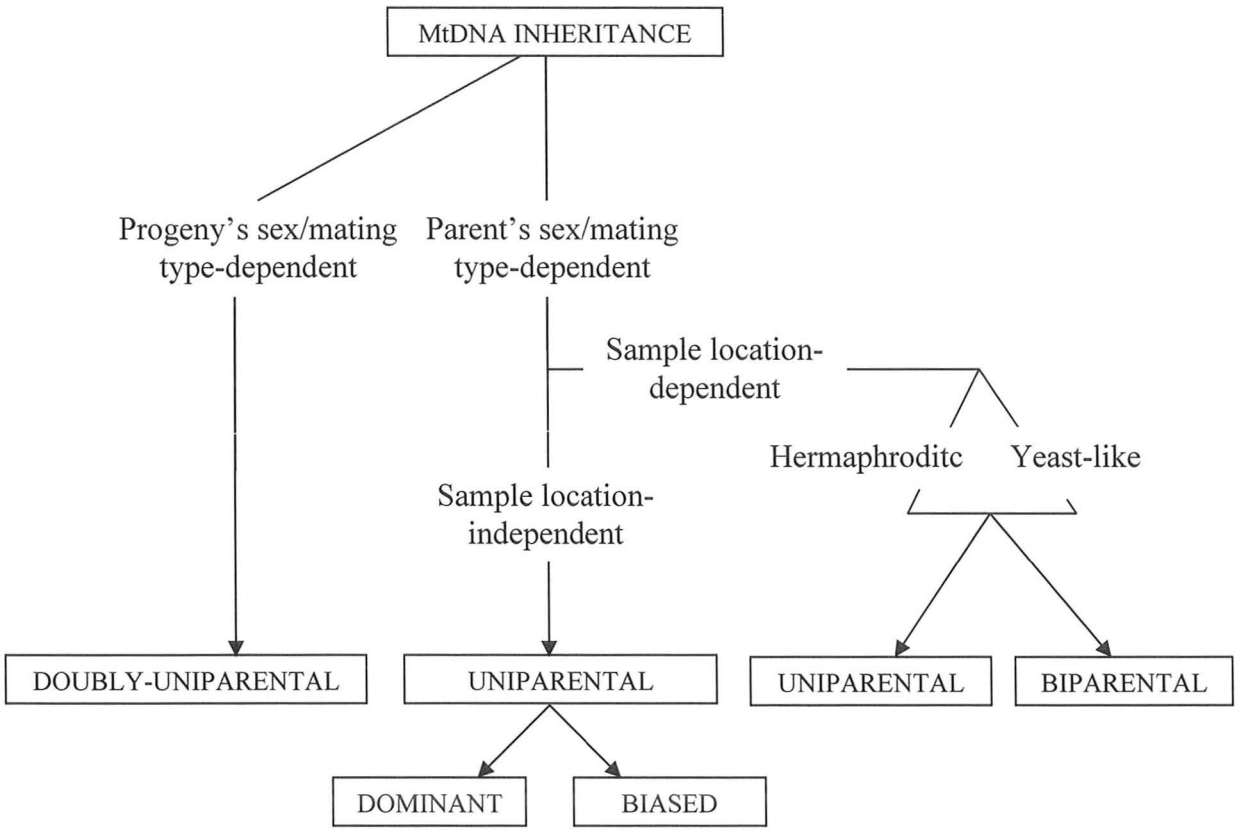


**Figure 1.1** Schematic representation of mitochondrial inheritance patterns in eukaryotes.

### 1.1.1 Uniparental mtDNA inheritance

Dominantly uniparental mtDNA inheritance is the most common pattern of mitochondrial inheritance in higher eukaryotes (e.g., animals, flowering plants, mosses, and ferns). In this mode, progeny mtDNA haplotype comes entirely from one of the two parents. In particular, offspring of intra-specific crosses in animals acquire predominantely maternal mitochondria, which establish themselves in all cell lines of the progeny (reviewed in Birky 2001; Xu 2005). Thus, a discussion of uniparental mtDNA transmission in almost all multicellular animals always implies the inheritance of maternal mitochondria. The first reported case of maternal mtDNA inheritance in animal offspring of an intra-specific mating was in rats of the Sprague-Dawley (Hayashi et al. 1978) and of the Wistar types (Kroon et al. 1978). Following these publications, numerous related studies reached the same conclusion in many species, including the pocket gopher *Geomys pinetis* (Avise et al. 1979), the fruit fly *Drosophila melanogaster* (Reilly and Thomas 1980), and humans (Giles et al. 1980).

Unlike eukaryotic microorganisms, higher eukaryotes are anisogamous, that is, their opposite-sex gametes are different in size. Specifically, a female gamete (egg) is usually larger than a male gamete (sperm). The "gamete size difference" hypothesis has been proposed to explain the maternal mtDNA inheritance in higher eukaryotes. According to the hypothesis, maternal inheritance is the result of a biased cytoplasmic contribution from the two gametes. Next, the evolution of anisogamy might have led to the mating success in higher eukaryotes for several reasons. First, a gamete's small size might contribute to the sperm's increased motility, leading to a greater mating success rate. The other advantage to small sperm size is the differences in the production of gametes: sperm are cheaper than eggs; thus, more sperm could be produced. In addition, a large egg serves as a better target

for tiny male gametes than a female gamete of a comparatively same size as sperm (Randerson and Hurst, 1999; 2001). Exceptions to the pattern of dominantly maternal mtDNA inheritance, nevertheless, have been documented. A case of mosaic (or mixed) mitochondrial inheritance was reported in a 28-year-old human male patient with a sporadic mitochondrial myopathy. Ninety percent of the patient's muscle tissue had paternal mtDNA while his blood, hair roots, and cultured fibroblasts inherited the maternal mtDNA haplotype (Schwartz and Vissing 2002). In addition, occasional sperm and/or biparental mtDNA inheritance has been also reported in intra-specific crosses of such animal species as the fruit fly *Drosophila simulans* (Kondo et al 1992) and cattle *Bos taurus* (Steinborn et al 1998). More evidence of parental mtDNA leakage comes from progeny of hybrid crosses in *Lepidopteran insects* (Lansman et al 1983), honeybee *Apis mellifera* L. (Meusel and Moritz, 1993), mouse *Mus musculus* (Gyllensten et al 1991; Shitara et al 1998), and sheep *Ovis aries* (Zhao et al. 2004). An exact proportion of each parental mtDNA molecules in a progeny cell is hard to obtain since most analytical techniques utilized today could not detect paternal mitochondria which are at levels less than about 5% of a cell's total mtDNA count. In addition, any frequencies of mtDNA haplotypes that were detected turned out to be highly unstable: egg mitochondria replaced sperm mitochondria in subsequent developmental stages (Meusel and Moritz 1993). The breakdown of mechanisms governing uniparental inheritance may result in the presence of mtDNA from the parent that usually does not contribute, leading to a parental mtDNA ratio variation.

The dominantly uniparental mtDNA transmission is also widespread in lower eukaryotes (e.g., slide molds, fungi, including yeast) that are capable of sexual reproduction. Unlike animals, most eukaryotic microorganisms are isogamous. Consequently, in these

species uniparental mtDNA inheritance manifests itself in the progeny's inheritance of mtDNA haplotype from a mating type parent of higher dominancy rather than from a larger gamete as in higher eukaryotes. Examples are numerous and include a basidiomycetous fungus *Cryptococcus neoformans*, and plasmodial slime molds *Physarum polycephalum* and *Didymium iridis*. In the fungus, most progeny which originated from a mating between haploid cells of opposite mating type inherited mitochondria strictly form a *MAT***a** parent rather than from a *MAT*α parent (Yan and Xu 2003). Unlike bipolar *C. neoformans* with two mating-type alleles, *P. polycephalum* has three multiallelic mating loci (Youngman et al. 1979; Kawano et al. 1987), which give rise to more than 700 different mating types (Moriyama and Kawano, 2010). For any pair of compatible mating types, mitochondria are inherited from a mating-type partner of higher dominance. Thus, in *Physarum*, almost all gametes can act as either donors or recipients of mtDNA as this completely depends on the mating type of the parent. This kind of unidirectional mtDNA inheritance constitutes a linear dominance hierarchy. Nevertheless, the phenomenon is not absolute in *P. polycephalum* since cells with mtDNA haplotypes from both parents were detected among the progeny populations from 21 out of 60 different mating combinations analyzed (Moriyama and Kawano, 2003). Therefore, depending on a specific cross examined, *P. polycephalum* plasmodia could exhibit either a dominant or a biased uniparental mtDNA inheritance pattern. Similarly, in a related slime mold, *D. iridis*, both dominant and biased uniparental inheritance patterns, including rare cases of biparental inheritance, were observed as well (Silliker and Collins 1988; Silliker et al 2002). As in case with *P. polycephalum*, the specific mtDNA inheritance pattern observed varied depending on the particular strains mated. Therefore, in lower eukaryotes a sex-dependent uniparental mtDNA inheritance in progeny

of intra-specific crosses heavily relies on a particular combination of mating partners analyzed.

### 1.1.2 Sample location-dependent mtDNA inheritance

In higher eukaryotes, the discussion of sample location-dependent mtDNA inheritance is primarily restricted to hermaphroditic plants (most flowering plants, or angiosperms), from which a hermaphroditic mode of mtDNA inheritance derives its name. Specifically, a unique feature of hermaphrodite plants, in which flowers bear both female and male reproductive organs, is reciprocal fertilization. Thus, reproduction is these species constitutes an example of intra-specific cross. To illustrate, take two plants of the same species: parents 1 and 2. When flowers of parent 1 are pollinated by pollen from plant 2, the seeds produced would contain the parent 1's mitochondria. In contrast, in a reciprocal cross (when pollen grains from plant 1 lands on the stigma of plant 2's flowers), the seeds produce would inherit mitochondria from parent 2. Thus, reciprocal fertilization in hermaphrodites gives rise to two progeny populations with either one of parental mtDNA haplotype depending on the sampling location of seeds. Importantly, the mtDNA inheritance mode in both progeny populations is dominantly uniparental. The examples include pedunculate oak *Quercus robur* L. (Dumolin et al 1995). In this species, the analysis of 143 progeny of five intra-specific crosses showed a dominantly maternal pattern of mitochondrial genome transmission as revealed by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) of mtDNA fragments amplified with universal primers. In plants, including angiosperms (flowering plants), reports of heteroplasmy caused by paternal leakage are rare (Mogensen 1996; Hattori et al 2002) and limited to the studies involving hybrid crosses between different strains or species of plants.

In lower eukaryotes, the hermaphroditic inheritance mode is widespread among fungal species of the phyla Ascomycota and Basidiomycota. For example, in the ascomycete *Neurospora crassa*, progeny's mtDNA haplotype depend on which parent is allowed to develop protoperithecia on crossing medium. The protoperithecial mate acts as maternal parent transmitting its mitochondria to the next generation while the other mate donates conidia, thus, acting as paternal parent. That a uniparental mtDNA inheritance pattern operates in. *N. crassa* has been demonstrated in a cross between a mitochondrial mutant "*poky*" strain and a wide-type strain. In crosses in which the "*poky*" strain was protoperithecial, the spores preferentially gave rise to "*poky*" strains, whereas the progeny of the reciprocal cross were mostly wild-type (Mitchell and Mitchell 1952). It has been speculated that uniparental organelle transmission observed in this species is due to an unequal cytoplasmic contribution of each parent with only nuclei from the paternal conidia migrating into the protoperithecium (Mitchell and Mitchell 1952). Nevertheless, rare cases of paternal cytoplasmic leakage were reported in *N. crassa* intra-specific crosses (Mitchell and Mitchell 1952). Hermaphroditic mtDNA inheritance mode also operates in such ascomycetes as *Podospora anserine* (Belcour and Begel 1977), *Ophiostoma ulmi* (Brasier and Kirk 1986), *Cryphonectria parasitica* (Milgroom and Lipari 1993), *Neurospora tetrasperma* (Lee and Taylor 1993), *Atkinsonella hypoxylon* (van Horn and Clay 1995), *Aspergillus nidulans* (Coenen et al 1996), *Epichloe typhina* (Chung et al 1996), and *Blumeria graminis* (Robinson et al 2002).

Crosses in basidiomycetous fungi are made by combining two monokaryotic (containing one haploid nucleus) strains of compatible mating types. These strains fuse to form a dikaryon, which is also a heterokaryon (i.e. contains two different haploid nuclei). At

the tip of dikaryon, meiosis takes place leading to the production of basidiospores. A classic example of the hermaphroditic mitochondrial transmission in basidiomycetous fungi is seen in *Coprinus cinereus*. In 1983, Baptista-Ferreira et al. reported that in *C. cinerues*, a mating between two compatible haploid, monokaryotic colonies led to the formation of dikaryons which segments contained two parental nuclei. Two discrete reciprocal dikaryons arose as a result of a reciprocal exchange of nuclei by mitosis and migration following an anastamosis between parental hyphae. Importantly, the migrations of parental nuclei through the cells of the recipient monokaryon were not accompanied with mitochondrial transmission. Consequently, progeny's mtDNA haplotype was always that of the resident mated monokaryon colony. The formation of colonies composed of sectors with different mtDNA haplotypes (i.e., mitochondrial mosaics) were later confirmed by May and Taylor (1988) in the analysis of dikaryons from 15 different monokaryon x monokaryon crosses in *C. cinereus*. May and Taylor (1988) also extended the findings by Baptista-Ferreira et al. (1983) by reporting cases of mating asymmetry in the form of unilateral nuclear migration observed in certain *C. cinereus* strain combinations. In the unilateral nuclear migration, only one parental monokaryon acted as nuclear donor but not nuclear acceptor. Nevertheless, as in the bilateral (reciprocal) nuclear migration, newly formed dikaryons contained mitochondria resident of the mated monokaryon and, thus, inherited mitochondria uniparentally (May and Taylor 1988). Interestingly, in *C. cinereus*, dikaryotic hyphae could be produced not only on both sides of a mating junction (a physical connection between the two monokaryons) but also at the junction itself. A dikaryon recovered at the junction contained mtDNA haplotypes of both mates which segregated rapidly during mitotic divisions subsequent to fusion (Baptista-Ferreira et al 1983; May and Taylor 1988).

Analogous to monokaryon x monokaryon matings in *C. cinereus*, bilateral nuclear migration takes place in a similar type of crosses in *Pleurotus ostreatus*, a commercially important edible mushroom (Matsumoto and Fukumasa-Nakai, 1996). Almost all of the dikaryons produced in these crosses had a mtDNA haplotype of the nuclear recipient monokaryon while dikaryons isolated from the junction zone commonly contained recombinant mitochondrial genomes. These results revealed that in *P. ostreatus*, mitochondria were inherited mostly in a hermaphroditic mode (uniparentally) although biparental inheritance via mtDNA recombination also occurred. It should be noted the phenomenon of bilateral nuclear migration described in *C. cinereus* may not be the case in other basidiomycetous fungi. In fact, biparental nuclear migration has not been observed in the field mushroom *Agaricus bitorquis* although a unilateral nuclear migration took place in a specific strain combination (Hintz et al. 1988). Like in *C. cinereus* matings, mitochondria remained immobile during the nuclear migration in *A. bitorquis* crosses. As a result, a single mtDNA type was present in the majority of the dikaryotic cells. Moreover, both parental mtDNA types were found only in dikaryons which arose from the junction zone of the two types of monokaryotic hyphae. However, similar to *C. cinereus*, dikaryons heteroplasmic for mtDNAs were transient: during vegetative growth, parental mitochondria segregated fast from the anastomosed cells giving rise to two dikaryons with the same nuclear components but different mtDNA. Hermaphroditic mode of mitochondrial inheritance was also reported in other higher basidiomycetes, such as *Coprinus congregatus* (Ross 1976), *Armillaria bulbosa* (Smith et al 1990), *Schizophillum commune* (Specht et al 1992), *Stereum hirsuitum X S. complicatum* (Ainsworth et al 1992), *Lentinula edodes* (Fukuda et al 1995), *Agrocybe aegerita* (Barroso et al 1997).

Another variation of the sample location-dependent mtDNA inheritance pattern, which also known as yeast-like (Yan 2006), was observed in three fungal species of the phylum Ascomycota: *Saccharomyces cerevisiae* (Thomas and Wilkie 1968), *S. castellii* (Petersen et al. 2002), and *Schizosaccharomyces pombe* (Thraikill and Birky 1980). In these species, no nuclear migration took place but rather parental nuclear genomes were transmitted to the next generation through replication. Different inheritance modes (e.g., uniparental or biparental) were detected in the species exhibiting a yeast-like mtDNA inheritance. The specific mode observed would vary depending on the location of progeny isolation relative to an intermediate or conjunction zone, which is basically a zygote or fusion product. Mating in these species begins with the fusion of two parental cells, resulting in a zygote formation. Importantly, in the zygote, the parental cells' cytoplasms likely do not mix completely. As a result, the parent-specific mtDNAs remain in distinct zones of the zygote as revealed by cytological observations in Nunnari et al. (1997). Depending on which place of the zygote a bud originates from, the progeny inherit mtDNA type from either one of the two parents or from both parents. Specifically, the buds arisen from the end of a zygote would give rise to the progeny with mtDNA inherited from one parent (a.k.a. being homoplasmic for mtDNA type). In contrast, buds originated from the middle or conjunction zone would acquire mitochondria from both parental cells (a.k.a. being heteroplasmic for parental mtDNA types) (Strausberg and Pelman 1978; Zinn et al. 1987). Generally, progeny heteroplasmic for mtDNA constitute a high proportion among the progeny of the fungal species with yeast-like mitochondrial inheritance mode. Thus, a yeast-like sample location-dependent mitochondrial inheritance mode could be viewed as a combination of uniparental and biparental mitochondrial inheritance modes. Importantly, a high frequency of

heteroplasmic progeny is what makes the outcome of this mode of mitochondrial inheritance different from that in a hermaphroditic mode.

The mitochondrial inheritance mode in the commercially important button mushroom *Agaricus bisporus* Lange (Imbach) (= *Agaricus brunnescens* Peck) is somewhat intermediate between hermaphroditic and yeast-like mtDNA modes. Unlike many other basidiomycetous species, hyphal sectors or compartments in *A. bisporus* are multinucleate. Nuclei of the same or different genotype(s) could be present within one hyphal compartment giving rise to homokaryotic and heterokaryotic mycelia, respectively. Although the ploidy and number of each nuclear types present in hyphal sectors have not been studied, the homokaryon is considered a genetically haploid mycelium while the heterokaryon is genetically diploid. As a result, two types of same ploidy crosses could take place in this species: (1) homokaryon x homokaryon and (2) heterokaryon x heterokaryon. The third possible pairing combination, heterokaryon x homokaryon, is an example of mixed ploidy crosses and this is discussed in Subchapter 1.2. No evidence of nuclear migration has been observed in 189 homokaryotic x homokaryotic pairings analyzed by Jin et al. (1992). Moreover, progeny of most homokaryon x homokaryon crosses showed a bias towards inheritance of only one of the two possible parental mitochondrial genotypes (Jin et al 1992). Importantly, in contrast to other higher basidiomycetes, the derived heterokaryons in this type of crosses originated *only* at the junction zone, a product of hyphal anastomosis of the parental homokaryons. Next, in both heterokaryon x heterokaryon and heterokaryon x homokaryon types of crosses, mtDNA type of each subculture was identical to that of the genetically more similar of the two original strains (hence, uniparental inheritance) without any evidence of mtDNA recombination taking place based on RFLP of two mitochondrial loci (Xu et al 1996). To summarize, the

absence of nuclear migration in *A. bisporus* L. crosses is what makes the mitochondrial inheritance in this species analogous to the yeast-like mode of mtDNA transmission. However, *A. bisporus* L.'s mtDNA inheritance pattern lacks a distinctive feature of the yeast-like mode—a high proportion of progeny heteroplasmic for mtDNA among the progeny population. Thus, a biased uniparental mitochondrial inheritance observed in this species is similar to the mtDNA inheritance patterns observed in basidiomycetous species exhibiting a hermaphroditic mode of inheritance.

### 1.1.3 Doubly uniparental mtDNA inheritance

The doubly uniparental mitochondrial inheritance, also known as a sex-limited mtDNA inheritance, was first documented in the marine blue mussel *Mytilus edulis*. The mtDNA inheritance in this species is very unique as it depends on the offspring sex. Particularly, in intra-specific crosses in *M. edulis*, the maternal mtDNA is passed to both male and female progeny but the paternal mtDNA is passed to only the male progeny (Skibinski et al 1994a; Skibinski et al 1994b; Zouros et al 1994). Among lower eukaryotes this mtDNA inheritance pattern has been identified in a basidiomycetous fungal plant pathogen *Ustilago maydis*. In opposite-sex crosses between yeast-like cells of mating types a1 and a2, progeny of mating type a1 with mtDNA haplotype inherited from either parent were present at equal frequencies. In contrast, 94 % of the mating type a2 progeny had the a2 parent-specific mtDNA (Wilch et al 1992). The mitochondrial genotyping of dikaryotic hyphae (progeny) was done by examination of the cells' mitochondrial RFLP phenotype.

## 1.2 THE BULLER PHENOMENON

### 1.2.1 Lower eukaryotes

In lower eukaryotes, intra-specific crosses between species of different ploidy occur commonly in higher basidiomycetes, which are mainly saprophytic fungi capable of generating dikaryotic mycelia. In these species, the mating between dikaryon and monokaryon mycelia (a.k.a. di-mon mating) can take place. In this type of mating, monokaryotic mycelia typically become dikaryotized through nuclear migration from dikaryotic mycelia (Figure 1.2). The nuclear exchange between the parental mycelia occurs according to compatibility as exerted by the incompatibility factors. This mode of dikaryotization was first reported in *Coprinus cinereus* (published as *C. lagopus*) by A. H. R. Buller in 1930 and 1931 and was later termed the Buller phenomenon by Quintanilha (1937). Following the initial discovery, more evidence of the Buller phenomenon came from the species *Coprinus* (Dickson 1934; Quintanilha 1939; Kimura 1958; Swiezynski and Day 1960; Prevost 1962 in Raper 1966; Traquair 1987; May and Taylor 1988), *Psilocybe coprophila* (Kimura 1958), *Schizophillum commune* (Ellingboe and Raper 1962; Crowe 1963), *Typhula incarnata* (Cavelier 1982), *T. idahoensis*, *T. ishikariensis* (Bruehl et al 1983), *Coriolus versicolor* (Aylmore and Todd 1984), *Stereum hirsutum* (Coates and Rayner 1985), *Laccaria bicolor* (Gardes et al 1990), *Echinodontium tinctorium* (Wilson 1991), *Armillaria* species (Rizzo and Harrington 1992; Carvahlo et al 1995), *Pleurotus ostreatus* (Vilgalys et al 1993; Tanesaka et al 1994), *Agaricus bisporus* (Xu et al 1996), *Pholiota nameko* (Nogami et al 2002), and recently from *Heterobasidion parviporum* (James et al 2009). Importantly, not all of the species listed have strictly uninucleate and binucleate hyphal compartments; some species, such as *S. hirsutum*, *H. parviporum*, and *A. bisporus*, have multinucleate hyphal

sectors. The Buller phenomenon that operates in the latter species is known as a cross between heterokaryotic and homokaryotic mycelia (hereafter referred to as he-ho pairings). A heterokaryon contains two types of nuclei while a homokaryon has a single type of nucleus. The homokaryon and the heterokaryon are considered genetically haploid and diploid mycelia, respectively. Moreover, based on these definitions, the terms dikaryon and heterokaryon could be used interchangeably as they both designate the presence of two nuclei of different genotypes in a cell. Lastly, the Buller phenomenon is used here as a general term as it encompasses both di-mon matings and he-ho matings.

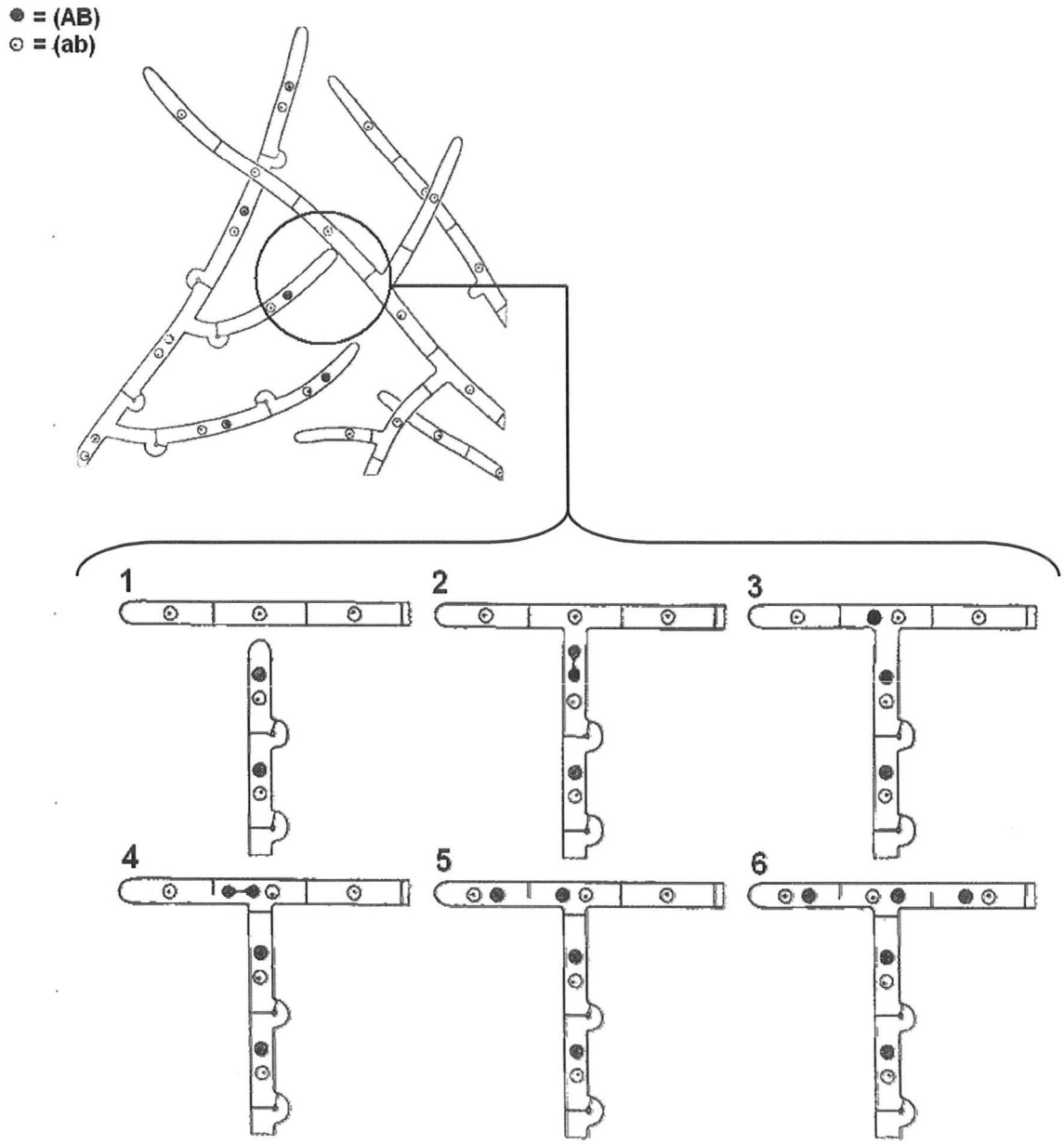


**Figure 1.2** Schematic representation of the diploidisation of a monokaryotic mycelium by a dikaryotic mycelium in a hemi-compatible di-mon mating in *Coprinus lagopus*. The diploid mycelium has a pair of nuclei (*AB* and *ab*) of opposite sex in each cell and is, thus, a heterokaryon. (1) The dikaryotic hypha grows toward the monokaryotic one, followed by (2) hyphal anastamosis and replication of the *AB* nucleus; (3) one of the daughter *AB* nuclei has migrated into the mated monokaryon, thus diploidising it; (4) the *AB* nucleus of the recently diploidised monokaryon divides again and (5) passes one daughter *AB* nucleus to monokaryotic cells which are adjacent to it. Adapted from Buller (1930).

Before we proceed with the illustration of the Buller phenomenon in different species, it is important to distinguish between two variations of di-mon/he-ho crosses: (1) fully compatible and (2) hemi-compatible. The first subtype, which could be represented as (A+B) x C (where A, B, and C are different nuclear types or different mating factors), is the cross between a mono/homokaryon which nuclear type is different from those of the parental heterokaryon. As a result, in a fully compatible mating, both nuclei (A and B) of the heterokaryon are mating type-compatible with the mono/homokaryon's nucleus (C) and either of them could migrate into the mono/homokaryon. In contrast, in a hemi-compatible mating (i.e., (A+B) x A), only one of the heterokaryon's nuclei is sexually compatible with a mono/homokaryon. Thus, only the heterokaryon's nucleus which is different from the mono/homokaryon's one would pass into the recipient haploid parent. Figure 1.2 demonstrates a diploidization process that would take place in this cross. A hemi-compatible mating could be a case when a parental mono/homokaryon of a di-mon/he-ho cross also happened to be one of the mono/homokaryons parental to the heterokaryon. In other words, strain of mating type A in cross (A+B) x A is also one of the parents in a monokaryotic x monokaryotic (or homokaryotic x homokaryotic) mating, which has led to the formation of heterokaryon (A+B), the diploid parent in the (A+B) x A cross. It is important to note that although a (A+A) x B cross could in theory take place, a heterokaryon with mating factors that are the same (A+A) would not normally arise since its production requires a mating between two mono/homokaryons of the same mating type (i.e., sexually incompatible). Thus, (A+A) x B cross is illegitimate. Furthermore, it is important to note that the notations used here to designate fully and hemi-compatible crosses [(A+B) x C and (A+B) x B] are simplistic notations which do not take into account a specific type of a fungus' mating

system. In fact, the majority of higher fungi have either a bipolar or a tetrapolar mating system, that is, a single mating type locus or two genetically unlinked loci, respectively.

Studies of fully and hemi-compatible crosses revealed the nuclear migration pattern in the progeny of these crosses. For instance, in the majority of heterokaryotization cases, nuclear selection generally favored the formation of derived heterokaryon with nuclei genetically less similar than the other permissible nuclear combinations (Kimura 1958; Coates and Rayner 1985). Several hypotheses have been proposed to explain the selective advantage of genetically dissimilar nuclei. According to one hypothesis, nuclear genetic heterogeneity may lead to a more effective override of the self-non-self rejection response that accompanies hyphal interactions (Coates and Rayner 1985). However, the nuclear bias could also be explained by interactions between nuclear and mitochondrial genomes. In the Buller phenomenon, it is nuclei but not mitochondria that migrate during mating into the recipient mycelium. The different modes of inheritance operating during di-mon and he-ho matings in basidiomycetes may help to avoid a genomic conflict between the nuclear and mitochondrial genomes in the newly formed heterokaryon. Therefore, the discussion of nuclear inheritance in these types of crosses is important for the understanding of mitochondrial inheritance in the progeny populations of these matings. Since some proteins localized in mitochondria are encoded by nuclear genes (e.g., cytochrome c, the α and β subunits of F1 adenosine triphosphotase, some subunits of cytochrome oxidase), proteins of mitochondria of a certain haplotype could be better expressed when they coexist with a nucleus or nuclei of compatible genotype(s). In return, the mitochondrial genotype could influence nuclear gene expression as was reported by Parikh et al. (1987) in *S. cerevisiae*. The relationship between the two genomes is thus not negligible. Compatibility between

nuclear and mitochondrial genotypes might be one of the factors which determine whether certain mating combinations would take place and which mate's mtDNA haplotype the progeny would preferentially inherit. Unfortunately, the inheritance of mitochondrial genome has received much less attention than the nuclear composition of the derived heterokaryon populations originated from di-mon/he-ho matings. Therefore, mitochondrial inheritance pattern is typically not presented.

In the basidiomycete *C. cinereus*, the mating system is remarkably flexible. Besides crosses between monokaryons (see 1.1.2 Sample location-dependent mtDNA inheritance), di-mon matings were also observed in this species (May 1988; May and Taylor 1988). Depending on a specific parental combination, the formation of nuclear mosaics was observed in *C. cinereus* di-mon crosses. For instance, genetic mosaics comprised of two possible nuclear combinations were detected in seven fully compatible di-mon matings tested (May 1988). Specifically, six crosses produced dikaryons all sectors of which contained the same nuclear genotype. The replicates of these three crosses confirmed the presence of dikaryon with the same nuclear combination. In the remaining three crosses, the nuclear combinations of all examined sectors of dikaryons were either the same as the one observed in the first trial or different. The derived dikaryons of the seventh fully compatible cross were unique as sectors with one nuclear combination were found to reside next to sectors with the other type of nuclear combination. Importantly, all novel dikaryotic mycelia generated in 13 fully compatible di-mon crosses, including the seven discussed above, contained a mtDNA resident in the mated monokaryon colonies (May and Taylor 1988). Besides *C. cinereus*, the di-mon matings were reported in many other species of the genus *Coprinus*, such as *C. sphaerosporus* (Dickson 1934), *C. fimetarius* (Quintanilha 1939), *C. lagopus* (Swiezynski

and Day 1960), *C. macrorhizus* f. *microsporus* (Kimura 1958), *C. radiatus* (Prevost 1962 in Raper 1966), and *C. psychromorbidus* (Traquair 1987).

Next, the nuclear migration was also extensively studied in fully compatible di-mon matings in *S. commune*. This fungus has a tetrapolar incompatibility system, thus a correct notation for a fully compatible di-mon cross is $(A^xB^x+A^yB^y)$ x $A^zB^z$, where x, y, and z refer to different alleles of the mating factors A and B. To set up crosses of this type, Ellingboe and Raper (1962) used highly isogenic strains. The nuclear genotypes of derived, newly established dikaryons were determined by screening for mating-type factors. The results revealed a highly preferential selection of one of the two parental dikaryon's nuclear genotypes by the monokaryon. Thus, contrary to the expectation, the derived dikaryons containing either of two alternative composite nuclear genotypes, $(A^xB^x+ A^zB^z)$ or $(A^yB^y+ A^zB^z)$, were not present in equal frequencies. These results were later confirmed by Crowe (1963). However, unlike Ellingboe and Raper (1962) who proposed that incompatibility factors were primarily responsible for the internuclear selection observed in these matings, Crowe (1963) came to a conclusion that the incompatibility barrier should be regarded as the sole mechanism determining the dikaryotising ability of a pair of nuclei in the establishment of the new dikaryon.

In addition, the Buller phenomenon was observed in the pine pathogen *A. gallica* (Carvahlo et al 1995). The subcultures of fully compatible di-mon pairings were examined with polymorphic nuclear and mitochondrial markers. Three patterns of nuclear inheritance were revealed in the derived heterokaryon populations in these crosses (in order of decreasing frequency): (1) replacement of resident haploid nuclei by nuclei of the parental

dikaryon, (2) recombinant nuclei, or (3) triploidy. In contrast, the progeny mtDNA genotype was always that of the resident monokaryotic parent.

*S. hirsutum*, *H. parviporum*, and *A. bisporus* are unlike all other species which have been discussed. The main distinction is a multinucleate state of these fungi's hyphal compartments or sectors. The Buller phenomenon which takes place in these species is known as a he-ho cross (see above). Unlike a dikaryotic parent in di-mon matings, a heterokaryotic mate in he-ho pairings has two types of nuclei in more than two copies per cell. In contrast, a homokaryotic parent contains multiple copies of a single type of nucleus. He-ho interactions may result in a number of outcomes, such as heterokaryotization of the parental homokaryon or displacement of the homokaryotic nuclei from their resident mycelium. Both types of crosses were analyzed in *S. hirsutum*, revealing different genetic compositions in the newly formed heterokaryons (Coates and Rayner 1985). In particular, the progeny's genotypes were classified into three types: (1) composite (recipient homokaryon's nucleus plus one of the heterokaryon's nuclei); (2) parental (nuclear genotype identical with that of the parental heterokaryon); and (3) novel (different from all composite and parental combinations). The frequencies of heterokaryons of each type were different in two types of crosses. Out of 135 fully compatible interactions, 73 % yielded only a single heterokaryon genotype, which in 82 % of cases was composite. The findings revealed a selective exclusion of one of the heterokaryon's nuclei. In 27 % of the cases, fully compatible pairings produced two heterokaryons, about a half of which had two possible composite genotypes and the other half had one composite and one parental nuclear genotype. The origin of newly formed heterokaryons with parental genotypes could have resulted from the replacement of the acceptor homokaryon's nuclei with the nuclei of the parental heterokaryon. Next, in the

hemi-compatible pairings, the proportions of derived heterokaryons with a single nuclear genotype (71 %) and two different genotypes (29 %) were similar to those obtained in the fully compatible interactions. Unfortunately, no data on mtDNA inheritance in the progeny of these crosses are available.

Similar to *S. hirsutum*, the life cycle of the root rot pathogen *H. parviporum* alternates between homokaryotic and heterokaryotic stages. Interestingly, he-ho pairings in this species resulted in a stable or transient formation of hyphae with three nuclei (trikaryons) in their compartments (James et al 2009). Trikaryons with certain nuclear combinations were favored. Specifically, 19 % of newly established trikaryons contained nuclei of three different mating types. Some of these trikaryons retained all three alleles while others lost one allele during sub-culturing steps.

Unlike studies on *S. hirsutum* and *H. parviporum*, he-ho pairings in the button mushroom *A. bisporus* were analyzed for both nuclear and mitochondrial inheritance (Xu et al 1996). Derived heterokaryons were screened for RFLPs at 18 nuclear loci and two regions of mtDNA. A recombination among nuclear loci but not among mitochondrial regions was observed. Particularly, five of the eight subcultures from the two he-ho crosses were recombinant for nuclear markers. The somatic recombination involved nuclear reassortment, exchange of genetic material between nuclei as well as crossing over between markers located on the same chromosome. The mtDNA type of each subculture was identical to that of the genetically more similar of the two original strains. Furthermore, no recombinant mtDNA haplotypes were observed.

Lastly, the di-mon and he-ho matings discussed above were set up in the laboratory conditions. However, the available evidence suggests that the Buller phenomenon is also

widespread in natural populations of basidiomyctes. For instance, an interaction similar to a di-mon mating occurs readily in *Heterobasidion annosum* (Fr.) Bref. (= *Fomes annosus* (Fr.) Karst.), an economically important fungal parasite of coniferous trees (Chase and Ullrich 1983). In fact, the spatial distribution of incompatibility alleles in *H. annosum* revealed genetic mosaics in some of the hyphae occupying individual or multiple hosts. A few incompatibility alleles were found shared in various paired combinations. In one instance, all basidiocarps on one host shared a single allele, which could be a case if a hemi-compatible di-mon mating had taken place.

### 1.2.2 Higher eukaryotes

The Buller phenomenon, described in higher fungi, does not take place in higher eukaryotes (e.g., mosses, ferns, flowering plants, and animals) due to two main reasons (Buller 1941). First, after the initiation of mating in higher fungi, the new dikaryotic cells are produced which terminate in the young basidia. In contrast, the only dikaryotic cells present in the higher eukaryotes are egg cells just after the entry of a sperm's nucleus. Importantly, karyogamy (i.e., nuclear fusion) in higher fungi takes place only in basidia, which are fungal cells distant in descent from those in which the dikaryotic state was originally established. Second, the dikaryotic fungal hyphae are long persistent. The new dikaryotic cells are formed via a conjugate nuclear division. On the contrary, the dikaryotic state of the fertilized ovum is transitory since it quickly turns into a synkaryotic (fused) diploid state in the process of karyogamy.

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# CHAPTER 2

# MITOCHONDRIAL INHERITANCE IN HAPLOID X NON-HAPLOID CROSSES IN *CRYPTOCOCCUS NEOFORMANS*

## PREFACE

### This chapter was published in a journal

Skosireva I, James, T.Y., Sun, S., and Xu, J. (2010) Mitochondrial inheritance in haploid x non-haploid crosses in *Cryptococcus neoformans*. *Curr Genet*. **56(2):**163-76.

### Authors' contribution

This study was conducted under the guidance of my supervisor, Dr. Jianping Xu. The laboratory portion of this study, writing the draft of the manuscript, and table and figure preparation were performed mainly by me. Dr. Timothy Y. James attempted experimental procedures pertaining to the differentiation of mechanisms controlling mtDNA inheritance with the use of vital dyes MitoTracker Green / Rhodamine B and fluorescent *in situ* hybridization analysis. Dr. James also described drawbacks of these experiments which appear in the paper's discussion section. Furthermore, Dr. James provided an excellent idea of how a $2^{-\Delta\Delta Ct}$ method of relative quantification by Livak and Schmittgen (2001) could be adapted for the determination of the *MAT* locus' diploid condition in non-haploid parents used in this study. Specifically, Dr. James pointed out that the use of an internal reference gene CNJ00540 in a quantitative real-time polymerase chain reaction (qRT-PCR) would be sufficient to reveal a *MAT* allele copy number in the parental strains. The results obtained with qRT-PCR were further supported based on the analysis of the parents' genotypes for 115 nuclear loci. This screening was performed by Sheng Sun. The five strains screened were among 163 strains that Sun and Xu analyzed and presented in their publication in Genetics (2007) 177(3): 1475–1486. Importantly, all seven R strains used in this study (non-haploid parents: R103, R104, R121, R149, R159; and reference strains for q-RT-PCR: R1-3 and R1-35) were AD hybrids obtained by Sun from a laboratory cross between strains CDC15 and JEC20 (Sun and Xu 2007). Finally, the manuscript was improved based on the suggestions and comments provided by Dr. Jianping Xu, Dr. Timothy Y. James, and Sheng Sun, who meticulously examined, edited and proofread it.

**Mitochondrial inheritance in haploid x non-haploid crosses in *Cryptococcus neoformans***

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**Running title**: MtDNA Inheritance in *Cryptococcus*

**ABSTRACT**

In the basidiomycetous yeast *Cryptococcus neoformans*, fusants and meiotic progeny from haploid-haploid (HH) crosses between strains of mating type **a** (*MAT***a**) and mating type alpha (*MAT*α) typically inherit mitochondrial DNA (mtDNA) from the *MAT***a** parent. In this study, we investigated the mtDNA inheritance pattern in haploid x non-haploid crosses. A total of 420 meiotic progeny and 173 fusants were obtained from five crosses and analyzed for two polymorphic mitochondrial markers. The percentage of meiotic progeny and fusants inheriting mtDNA from MATα or MATα/α parents ranged from 8% to 50%. The leakage was significantly greater than those observed in HH crosses, indicating that mtDNA inheritance is not uniparental in haploid x non-haploid crosses in *C. neoformans*. In addition, mtDNA leakage in the fusants, but not the meiotic progeny, of the *MAT*α/α x *MAT***a** cross was significantly higher than that in the *MAT***a**/**a** x *MAT*α cross, suggesting that the diploid parents with different mating types contribute differently in determining fusant mtDNA genotype in these crosses. Flow cytometry analysis revealed that meiotic progeny population of each cross was of mixed ploidy while the ploidy level of the selected fusants ranged from diploid to triploid.

**Key Words:** *Cryptococcus neoformans*, mating-type locus, mixed ploidy crosses, mitochondrial DNA inheritance

## INTRODUCTION

Unlike nuclear genes and genomes, the inheritance of mitochondrial genes and genomes does not obey Mendel's laws. In the majority of sexual eukaryotes, the mitochondrial DNA (mtDNA) is inherited from only one, usually the female, parent (Birky 2001; Xu 2005). However, the patterns of uniparental inheritance have been studied mostly in crosses between haploid gametes. In microorganisms, other types of mating are also possible. For example, in fungi, mating between diploid and haploid strains can occur (Korhonen 1978; Crandall and Caulton 1979; Niwa and Yanagida 1985); however, little is known about the mitochondrial inheritance in crosses between parents of different ploidy levels (mixed ploidy crosses).

*Cryptococcus neoformans* (*Filobasidiella neoformans*) is an encapsulated basidiomycetous yeast that can cause meningoencephalitis in immunocompromised patients. *C. neoformans* strains can be divided into two main serotypes, A and D, corresponding to var. *grubii* and var. *neoformans*, respectively. Serotypes A and D are found worldwide in both natural environments and in patients, with serotype A accounting for the majority of human infections (Franzot et al. 1999). Most natural serotype A and D strains of *C. neoformans* are haploid although diploid serotype A strains have also been found (Lin et al. 2009). Aside from the serotypes A and D strains in *C. neoformans*, there are also serotype AD strains, a group of recent hybrids between strains of serotypes A and D as demonstrated by gene genealogical analyses (Xu et al. 2002). Most serotype AD strains are either diploid or aneuploid and contain alleles typical of both serotypes A and D (Xu et al. 2000b; Lengeler et al. 2001; Chaturvedi et al. 2002; Xu et al. 2002; Xu and Mitchell 2003). Although less common than serotype A strains, serotype AD strains can be more prevalent than serotype D

strains among clinical isolates from certain geographic areas (e.g., San Francisco, CA) (Brandt et al. 1996).

*C. neoformans* has a bipolar mating system with one mating type (MAT) locus that has two alternative functional alleles, *MAT***a** and *MAT*α. The MAT locus spans ~100-120 kb and contains over 20 genes (Lengeler et al. 2002; Fraser et al. 2004). Under appropriate conditions, the haploid strains of opposite mating types, α and **a**, can fuse. Studies have shown that the MAT locus is involved in the control of mtDNA inheritance in *C. neoformans* (Xu et al. 2000a; Yan and Xu 2003). Specifically, in haploid-haploid (HH) crosses between *MAT***a** and *MAT*α parents most meiotic progeny (a.k.a. true progeny, basidiospores) inherit mitochondria from only the *MAT***a** parent (Yan and Xu 2003). This mitochondrial inheritance pattern was also observed in fusants (the fusion products of the parental strains which precede meiosis) from opposite-sex HH crosses in *C. neoformans* (Yan et al. 2004; 2007a; b). The precise mechanism by which mating type controls mtDNA inheritance has not been determined, but the master regulatory proteins Sxi1α and Sxi2a encoded by the *MAT* locus are involved (Yan et al. 2004; Yan et al. 2007a).

In many basidiomycete fungi, mating between dikaryons (two different haploid nuclei in the same cell) and monokaryons (one haploid nucleus in each cell), called di-mon mating, has been shown to occur (Buller 1930; 1931; Swiezynski and Day 1960; Aylmore and Todd 1984; Coates and Rayner 1985; Xu et al. 1996). The process that generates novel dikaryotic mycelia from di-mon mating is called the Buller phenomenon, named after the original discoverer A. H. R. Buller (1930). In these crosses, a variety of genetic exchange(s) and recombination(s) can occur. An analogous process can occur in those few basidiomycetes with a diploid mycelium; for example, in the pine pathogen *Armillaria gallica*, subcultures of

diploid-haploid pairings showed several patterns of inheritance for nuclear genes: replacement of resident haploid nuclei by diploid nuclei, recombinant nuclei, or triploidy. In contrast, the progeny mtDNA genotype is always that of the resident haploid parent (Carvahlo et al 1995). Similar genetic processes have also been observed in heterokaryon - homokaryon crosses in the button mushroom *Agaricus bisporus* (Xu et al. 1996).

In this study, we tested whether non-haploid and haploid mating occurs in *C. neoformans* and if so, what are the patterns of mitochondrial inheritance. Specifically, because both diploid and aneuploid cells have been found in *C. neoformans* in nature, two types of non-haploid and haploid matings are possible. The first is a cross between uninucleate diploid (2n) cells and haploid (n) cells (the HD crosses) and the second between haploid (n) and aneuploid ($2n\pm j$ where j is the haploid number of chromosomes, $1 \leq j < n$) cells (the HA crosses). Specifically, we tested whether the *MAT***a** parent-dependent mitochondrial inheritance identified in HH crosses operates in HD and HA crosses as well. We hypothesized that the progeny of opposite-sex HD and HA crosses, *MAT***a** x *MAT*α/α and *MAT*α x *MAT***a/a**, should inherit mtDNA from the parent carrying *MAT***a** allele(s), similar to those observed in HH crosses. To test this hypothesis, a total of 420 meiotic progeny from five crosses were analyzed for two mitochondrial loci. In addition, mtDNA genotypes of 173 fusants from both types of crosses were also obtained. Mitochondrial genotypes different from that of the *MAT***a** allele-bearing parent were observed in all crosses. The distinct mtDNA genotypes included *MAT*α-specific, recombinant, or heteroplasmic mtDNA types, collectively referred to as "mtDNA leakage," expressed in percentage over the total number of progeny. MtDNA leakage ranged from 8 to 40% of the total meiotic progeny populations.

Furthermore, mtDNA leakage in the fusant populations was in the range of 17.6-50%. We discussed the potential mechanisms for the different mitochondrial inheritance patterns.

## MATERIALS AND METHODS

**Strains and genetic crosses.** The strains used in this study are listed in Table 1. To determine mitochondrial inheritance patterns in the progeny of mixed ploidy crosses, we used two isogenic *C. neoformans* haploid strains YZX1 and YZX2. These two strains have the mitochondrial genotype typical of serotype A strains (mtA) and they differ from each other at the mating-type locus (Yan et al. 2004). Strain YZX1 has the *MAT*α mating type and is auxotrophic for adenine while YZX2 is its *MAT***a** counterpart (Yan et al. 2004). Two types of crosses, *MAT***a** x *MAT*α/α and *MAT*α x *MAT***a/a**, were performed. Specifically, *C. neoformans* YZX1 strain was crossed with two *MAT***a/a** diploid strains (R121 and R159), while the YZX2 strain was mated with three *C. neoformans MAT*α/α strains (R103, R104, and R149). Unlike strains YZX1 and YZX2, these five R strains had the mitochondrial genotype typical of serotype D strains (mtD) (Sun and Xu 2007). These R strains (including two others R1-3 and R1-35 used as reference strains in this study, see below) were the products of a cross between *C. neoformans* haploid strains of serotype D (JEC20) and serotype A (CDC15), and their genotypes were characterized for 115 nuclear loci using co-dominant markers (Sun and Xu 2007). The presence of two *MAT***a** or *MAT*α alleles in diploid and aneuploid parents was confirmed by real-time PCR (see below).

**Table 1.** *C. neoformans* strains used in this study and their genotypes [a]

| ***C. neoformans* stains** | **Serotype** | ***MAT*** | **Genotype** | **Reference/Source** |
|---|---|---|---|---|
| YZX1 | D | Dα | *ade2 mtA NEO* | Yan *et al.*, 2004 |
| YZX2 | D | D**a** | *ade2 mtA NEO* | Yan *et al.*, 2004 |
| JEC20 | D | D**a** | | Kwon-Chung *et al.*, 1992 |
| JEC21 | D | Dα | | Kwon-Chung *et al.*, 1992 |
| JEC50 | D | Dα | *ade2* | Sia *et al.*, 2000 |
| D14, D15 (RAS strains) | D | **a**DDα | | Sia *et al.*, 2000 |
| H99 | A | Aα | | John Perfect |
| CDC15 | A | Aα | | CDC[b] |
| R103, R104, R149 | AD | αAAα | *mtD* | Sun and Xu, 2007 |
| R121, R159 | AD | **a**DD**a** | *mtD* | Sun and Xu, 2007 |
| R1-3, R1-35, CDC92-5, CDC92-181 | AD | **a**DAα | | Sun and Xu, 2007; CDC[b] |

[a] mtA and mtD are the mitochondrial genotypes for serotypes A and D, respectively. The *neo* gene encodes resistance to the antibiotic G418. Strains with *ade2* auxotrophic marker require adenine for growth on the minimal medium SD. *ade2* mutants exhibit a pink colony phenotype due to accumulation of adenine precursor in cell cytoplasm. *MAT***a** mating type allele **a**, *MAT*α mating type allele α.

[b] Center for Disease Control and Prevention, 1600 Clifton Rd., Mailstop D-11, Atlanta, GA 30333.

Strains R1-3 and R1-35 were used as reference strains for real-time PCR assays to determine gene copy numbers within individual cells. These two strains were *MAT***a**/α AD hybrids from a laboratory cross between strains CDC15 and JEC20 (Sun and Xu 2007). Haploid *MAT*α strains of serotype A (H99 and CDC15) and haploid *MAT***a** strains of serotype D (YZX2 and JEC20) also served as additional reference strains for real-time PCR. D14 and D15 were synthesized diploid strains from Joe Heitman's lab at Duke University and their ploidy levels were confirmed by flow cytometry analyses (Sia et al. 2000). JEC20, JEC21, and JEC50 are confirmed haploid strains (Sia et al. 2000).

**Mating experiment.** Parental strains were pre-grown on YEPD agar (1% Bacto Yeast Extract, 2% Dextrose, 2% Bacto Peptone, 1.8% Difco Agar) and then a small amount of the cells was suspended in 0.3 ml sterile water. Mating mixtures were performed on V8 juice agar [4.5% V8 vegetable juice (Campbell Soup Co., Etobicoke, Ontario, Canada), 0.5 g/L $KH_2PO_4$, 4 % Difco Agar, pH ~ 7.0] by spotting 20 µl of each parental cell suspension. Single parental strains were spotted as negative controls in the vicinity of each other on the same V8 plates to test for self-filamentation behavior. The plates were incubated for 32 days in the dark at 25 °C. At the end of the incubation period, agar containing hyphal filaments and spores were cut from the hyphal area away from each mating spot, suspended in 0.5 ml sterile water, and then streaked to isolate pure meiotic progeny for analysis (Xu et al. 2000a). In addition, individual basidiospores from one of the crosses were isolated by micromanipulation with the aid of a manual micromanipulator (Singer MSM, Somerset, UK).

In addition to analyzing meiotic progeny, fusants of the two crosses, R103 x YZX2 and R159 x YZX1, were also obtained. The fusants are the fusion products of the parental strains. The crosses were set up on V8-juice agar and incubated in the dark at 25 °C for about 1-3

days before any signs of filamentation. Serial 2-fold dilutions of cell suspensions were prepared. Fusants were selected on SD minimal medium [2% dextrose, 2% Difco agar, 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% $(NH_4)_2SO_4$] supplemented with 220 ng/ml G418 disulfate salt (Sigma). Since the haploid YZX1 and YZX2 parents are *ade2 NEO* (G418 resistant, $G418^r$) while the non-haploid strains are prototrophic and G418-sensitive ($G418^s$) (Table 1), the fusants were expected to be wild-type and G418-resistant ($G418^r$). Thus, unlike the parental strains, the fusants can grow on the selective medium. The *ade2* genotype is a recessive mutation that abolishes the adenine biosynthetic pathway and leads to the accumulation of a cell-limited pink-red pigment in the cell cytoplasm only in haploid cells. In contrast, the *NEO* genotype results from a dominant marker that confers resistance to the antibiotic G418. Cells were incubated at 37°C for 3 days. Eighty-eight random fusants from each of the two crosses were picked off the plates, and screened for mitochondrial genotypes.

We also performed *MAT***a**/α x *MAT***a** and *MAT***a**/α x *MAT*α crosses by mixing diploid *MAT***a**/α strains R1-3, R1-35, CDC92-5 and CDC91-181 with YZX1 and YZX2 respectively (Table 1). However, we were unable to make them mate and produce recombinant progeny or fusants.

**Mitochondrial genotyping.** The mtDNA genotypes of the meiotic progeny and fusants were determined using the natural polymorphisms at the *ND2* and *ND5* loci between the mitochondrial genomes of serotype A and D strains (Supplemental Table 1). While there is no intron in the *ND5* gene of the mtA-type genome, there is one intron within the *ND5* coding sequence of the mtD-type genome (Litter et al. 2005; Toffaletti et al. 2004). At the *ND2* locus, the mtA and mtD – type genomes had different PvuII and ScaI restriction

digestion patterns that could be easily distinguished through PCR amplification, restriction enzyme digestions and gel electrophoresis. The conditions for PCR, agarose gel electrophoresis, and scoring followed those of Yan and Xu (2003) and Toffaletti et al. (2004). MtDNA genotypes may be different from either parent due to mtDNA recombination or heteroplasmy. Specifically, cells that inherited *ND2* gene from one parent and *ND5* from the other parent, or the opposite, were considered to have recombinant mtDNA genotypes. In contrast, cells heteroplasmic for mtDNA would contain one or both mtDNA genes from both parents (i.e., they would be heterozygous for *ND2* and/or *ND5* alleles). Heteroplasmic or recombinant mtDNA genotypes of the selected cells were reconfirmed by repeated PCR and PCR-RFLP analysis.

**Statistical analyses.** MtDNA leakages in fusants and meiotic progeny of each cross were compared with those previously determined for HH crosses. Statistical significance of the comparisons was determined using the Chi-square test on a two-by-two contingency table with 1 degree of freedom (d.f.). *P* values of $<0.05$ were considered statistically significant.

**Segregation analysis.** To confirm that the basidiospores isolated in this study were true meiotic segregants, a test for independence of the two nuclear markers, *ade2* and *NEO*, was conducted on a number of meiotic progeny from each cross. The cells analyzed included all meiotic segregants used in ploidy analysis (see below). Progeny cells stored in a –80 °C freezer were pre-grown on YEPD medium, washed with $H_2O$, and analyzed for the *ade2* auxotrophic marker by streaking on the minimal medium SD. The $Ade^-$ phenotype was confirmed by the formation of pink colonies when grown on SD medium supplemented with adenine (0.2 g/L). Sensitivity of basidiospores to G418 was confirmed by growth/no-growth

on SD medium supplemented with 220 ng/ml G418 disulfate salt. The parental strains were used as controls. Cells were incubated at room temperature for 3 days.

**Cell wall and nuclear staining.** Morphological features of *C. neoformans* haploid x non-haploid matings were examined using fluorescence microscopy. Crosses were set up on V8 medium (pH 5) as described above for mating reactions. The dikaryotic nuclear condition and clamp connections of the developing filaments were analyzed by staining with DAPI in SlowFade Gold from Molecular Probes (Invitrogen) and calcofluor white (Difco), respectively, as described in Lengeler et al. (2001) and Nielsen et al. (2003).

**Determination of gene copy number by Real Time – Polymerase Chain Reaction (RT-PCR) assays.** We used RT-PCR to confirm the diploid condition of the *MAT* chromosome in the parental strains. RT-PCR primers were designed for a locus on the *MAT* chromosome (*STE20***a** and *STE20*α), and a locus on one autosome (chromosome 10: CNJ00540) (Supplemental Table 1) using two internet-based interface programs, Primer-3 and Oligonucleotide Properties Calculator [http://www.basic.northwestern.edu/biotools/oligocalc.html]. Samples were amplified using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen™) in a 96-well plate (Applied Biosystems™). Individual real-time PCR reactions were carried out in 20 μL volumes containing 10 μL SuperMix, forward and reverse primers (each at a final concentration of 0.2 μM), 0.8 μL of ROX Reference Dye (1:10), sterile $H_2O$, and 4 μL of genomic DNA in four 5-fold serial dilutions. DNA extraction was done according to Xu et al. (2000a). RT-PCR was performed in the Stratagene MxP3000 Real Time System, using the same program for all genes: 2 minutes of hold at 50 °C (UDG incubation), 2 minutes of pre-incubation at 95 °C followed by 40 cycles for 15s at 95 °C and 30s at 62 °C. A no-template control (complete

amplification mixture without adding genomic DNA) was included in every RT-PCR experiment series to monitor for contaminants and primer-dimer formation. Melting curves were acquired and analyzed at the end of each reaction to control for specificity; a specific reaction should have a single melting peak corresponding to the PCR product being amplified.

To confirm that there were two copies of *MAT***a** and *MAT*α genes in the diploid or aneuploid parental strains, the $2^{-\Delta\Delta Ct}$ method of relative quantification (Livak and Schmittgen 2001) was adapted with some modifications. Specifically, two sets of primers specific for each of the target genes, serotype A *MAT*α (*STE20*αA) and serotype D *MAT***a** (*STE20***a**D), and one primer pair that could amplify both serotype A- and serotype D-specific alleles of an internal reference gene CNJ00540 were designed and analyzed (Supplemental Table 1). CNJ00540 encodes an orthologue of the well-characterized *S. cerevisiae* gene LYS2 encoding alpha-aminoadipate reductase. The serotype D allele at locus CNJ00540 can be recognized and cut with the restriction enzyme *Hae*III while the serotype A allele cannot be digested by this enzyme. The CNJ00540 locus was chosen since all the diploid and aneuploid parental strains were heterozygous at this locus (Sun and Xu 2007). The average cycle threshold (Ct) was calculated for each target gene and the internal control gene and the ΔCt (Ct, target gene – Ct, CNJ00540) was determined. We then calculated the mean ΔCt across all dilutions of the same sample. It was expected that the presence of both the target gene and the reference internal gene with the same copy number (1 or 2) in a sample would give ΔCt value not significantly different from zero (ΔCt = 0 in ideal situation). Here, genomic DNA extracted from *C. neoformans* H99, CDC15, JEC20, and YZX2 (all known to harbour a single copy of the STE20 and the CNJ00540 genes) were used as haploid calibrators. In

contrast, a 2-fold difference in copy number of CNJ00540 over *STE20* would generate ΔCt twice of that in the strains R1-3 and R1-35. This is because R1-3 and R1-35 have a single copy of both the *MAT***a** and the *MATα* alleles (Table 1) and both are heterozygous at CNJ00540. All other parental R strains were also heterozygous at CNJ00540, as confirmed by PCR-RFLP.

RT-PCR reaction efficiency and relative efficiency of amplification of *STE20* and CNJ00540 genes were calculated according to Ferreira et al. (2006).

**Determination of ploidy by fluorescence activated cell sorting (FACS).** The ploidy of the parents, selected fusants and meiotic progeny from each cross was determined by flow cytometry similar to the protocol used by Lin et al. (2008). Briefly, cells were harvested from YEPD medium, washed once in phosphate-buffered saline (PBS), and fixed in 1 ml of 70% ethanol overnight at 4 °C. Fixed cells were washed once with 1 ml of NS buffer (10 mM Tris-HCl [pH 7.6], 250 mM sucrose, 1 mM EDTA [pH 8.0], 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol), stained with propidium iodide (Fluka BioChemika) (0.5 mg/ml) in 0.2 ml of NS buffer containing 0.02 ml RNase A (1 mg/ml), and incubated with agitation at 4 °C overnight. Then 0.04 ml of stained cells was diluted into 1 ml of 50 mM Tris-HCl (pH 8.0) and sonicated for 13s using a Fisher Scientific* Model 100 Sonic Dismembrator. Flow cytometry was performed with 10,000 cells on a Becton-Dickinson LSR II. Strains JEC20, JEC21, and JEC50 were used as haploid controls, while D14 and D15 were used as diploid controls. Cells were first checked for proper nuclear straining by fluorescence microscopy and the stained cells were then analyzed by flow cytometry using the PE-A channel. The images of the samples were

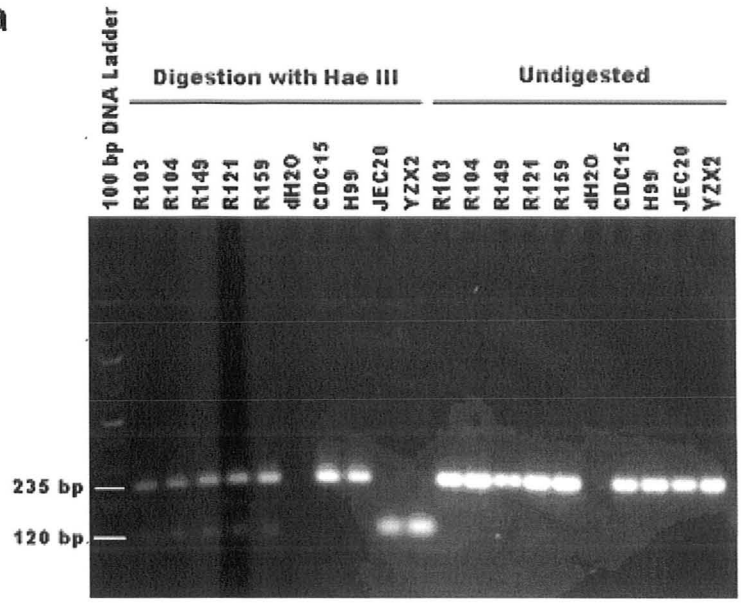
overlaid using WEASEL v2.6 software (http://www.wehi.edu.au/cytometry/WEASELDevelopment.html.)

## RESULTS

**Parental strains: sterility, ploidy and copy number of *MAT* locus.** We first performed a series of experiments to examine the ploidy of the strains (R103, R104, R121, R149, and R159) used in the haploid x non-haploid crosses that were generated from a previous serotype A x serotype D cross. After 32 days of incubation on V8 agar, none of these strains produced hyphal filaments. The presence of the opposite-mating type cells on the plate did not result in another parent exhibiting self-filamentous behaviour.

Fluorescence microscopy for nuclear straining revealed that the *MAT***a**/**a** and *MAT*α/α strains derived from the serotype A x serotype D cross were all uninucleate yeast cells. FACS analysis showed that all AD strains except R149 were diploid (FACS profiles of the control strains and the parental strains are shown in Fig. 1c and Supplemental Fig. 1, respectively). The FACS profiles of these strains overlapped with those of the diploid control strains D14 and D15. Strain R149 showed a scatter profile in between haploid and diploid strains and was likely an aneuploid (2n-j).

a




b

| Strain | ΔCt | | | |
|---|---|---|---|---|
| | Mean | Min | Max | SD |
| R103 | 0.44 | 0.15 | 0.76 | 0.31 |
| R104 | 0.50 | 0.45 | 0.64 | 0.09 |
| R149 | 0.47 | 0.33 | 0.68 | 0.15 |
| H99 | 0.51 | 0.08 | 0.81 | 0.31 |
| CDC15 | 0.42 | 0.24 | 0.75 | 0.24 |
| R1-3 | 1.30 | 1.11 | 1.42 | 0.14 |
| R1-35 | 1.62 | 1.49 | 1.82 | 0.15 |
| R121 | 0.22 | 0.14 | 0.28 | 0.06 |
| R159 | 0.52 | 0.40 | 0.59 | 0.08 |
| JEC20 | 0.43 | 0.31 | 0.53 | 0.10 |
| YZX2 | 0.45 | 0.38 | 0.55 | 0.07 |
| R1-3 | 0.97 | 0.89 | 1.05 | 0.07 |
| R1-35 | 1.17 | 1.09 | 1.29 | 0.09 |

c

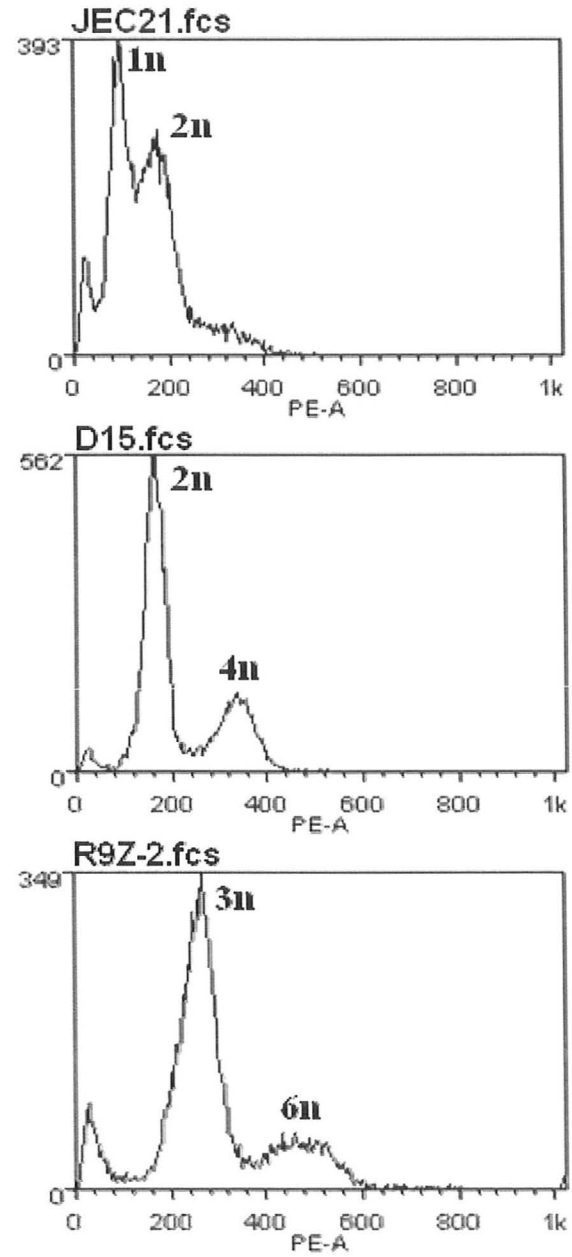



**Figure 2.1 Parental strains contain two MAT alleles and are either diploid or aneuploid.** (a) Serotype D allele-specific restriction enzyme digestion analysis of the control CNJ00540 locus with HaeIII. (b) Mean, minimum (Min), and maximum (Max) ΔCt (Ct, STE20 – Ct, CNJ00540) values from RT-PCR assay. (c) FACS profiles of the haploid control strain JEC21, diploid control strain D15, and fusant R9Z-2 from the R159 x YZX1 cross. 1n, 2n, 3n, 4n, and 6n indicate nuclear DNA content. The x-axis indicates fluorescence intensity reflecting the DNA content and the y-axis indicates cell count.

The diploid nature of the parents was also supported by the examination of the strains' genotypes for 115 genes on fourteen chromosomes determined previously by PCR-RFLP analysis (Sun and Xu 2007). Strains R103, R104, and R121 were heterozygous for a variable number of genes on all chromosomes, suggesting the presence of two complete sets of the chromosomes. Strains R159 and R149 were heterozygous for all genes on thirteen and twelve chromosomes, respectively, while they were homozygous for serotype A and D genes on chromosome 4. We demonstrated that these two strains, nonetheless, had two copies of chromosome 4 as well since the presence of two genome equivalents of the *MAT* encoded locus *STE20* was confirmed by RT-PCR (see below). Strain R149 likely contained a single copy of chromosome 13 (2n-1) since it contained alleles from only the serotype A parent on all six loci examined on this chromosome. However, we cannot exclude the possibility that part or all of chromosome 13 was present in two copies in strain R149 and all these markers were homozygous for the serotype A-specific alleles.

The presence of two (serotype A- and D-specific) CNJ00540 alleles in all diploid/aneuploid parents as well as in the *MAT***a**/α calibrator strains R1-3 and R1-35 was confirmed by PCR-RFLP analysis (digestion patterns for AD parents are shown in Fig. 1a). Therefore, the ratio of *STE20*αA (or *STE20***a**D) allele to CNJ00540 allele was expected to be 1:2 in the calibrators and 1:1 in control haploid strains. The ΔCt values of the haploid controls were similar to those of the diploid/aneuploid parents (Fig. 1b). In contrast, ΔCt of the calibrator strains R1-3 and R1-35 were more than twice of the latter values. This suggested the presence of two copies of MATα allele in strains R103, R104, and R149 and two copies of the *MAT***a** allele in R121 and R159 strains (Fig. 1b). The individual PCR efficiency for each gene was in a 90-110% range (data not shown). The relative efficiencies

of *STE20* and CNJ00540 genes of each strain were similar. In all cases, post-RT-PCR melting curve analysis demonstrated a consistent melting point of 84.4, 80.6, and 87.6 °C for *STE20*αA, *STE20***a**D and CNJ00540 genes, respectively (data not shown), suggesting that the selected primers were specific for the targeted genes.

The presence of two genome equivalents of the *MAT* locus in three diploid parents (R103, R104, and R121) was further supported by the examination of the strains' genotypes for other genes on chromosome 4 (Sun and Xu 2007). Out of eleven Chromosome 4 genes analyzed in a previous study, strains R103, R104, and R121were heterozygous for three, five, and one loci, respectively, consistent with the presence of two copies of chromosome 4 in these strains.

**MtDNA types of meiotic progeny and fusants.** A gel demonstrating the scoring of polymorphisms at the mitochondrial *ND5* and *ND2* genes for the parental and control strains are shown in Fig. 2. MtDNA leakage in the meiotic progeny populations of *MAT*α/α x *MAT***a** crosses ranged from 18.3-40% (Table 2, crosses No.1, 2, and 3). Despite the variation in mtDNA leakage, the majority of the meiotic progeny in each cross inherited mtDNA from the *MAT***a** parent. Progeny homoplasmic for *MAT*α/α mtDNA were the second most abundant, accounting for ~10-39% of all the progeny in each cross (Table 2, crosses No.1, 2, and 3). Recombinant mtDNA types were rare among the progeny and comprised ~1-8% of the population (Table 2, crosses No. 1, 2 and 3). No heteroplasmic progeny were identified (Table 2, crosses No. 1, 2, and 3). MtDNA leakage among the progeny from the cross R149 x YZX2 was significantly higher than that in the R104 x YZX2 cross (Chi-square test, d.f.=1, $P<0.01$) but it was not different from that of the other cross between R103 and YZX2. The fusants from the R103 x YZX2 cross showed 50% mtDNA leakage (Table 2, cross No. 1a),

**Figure 2.2 Parental and zygote mitochondrial genotypes at the *ND5* allele and PCR-RFLP at *ND2*.** (a) Mitochondrial genotypes of *C. neoformans* parental strains. Sterile $ddH_2O$ was used as a negative control and JEC21 and H99 as positive controls. Top, strain names in corresponding lanes are the same as those in Table 1. (b, c) Mitochondrial genotypes of selected zygotes from the representative R103 x YZX2 cross. Arrowheads designate heterozygosity for genes (zygotes No. 12, 21, 27). Zygotes No. 17, 28, have recombinant mtDNA types.

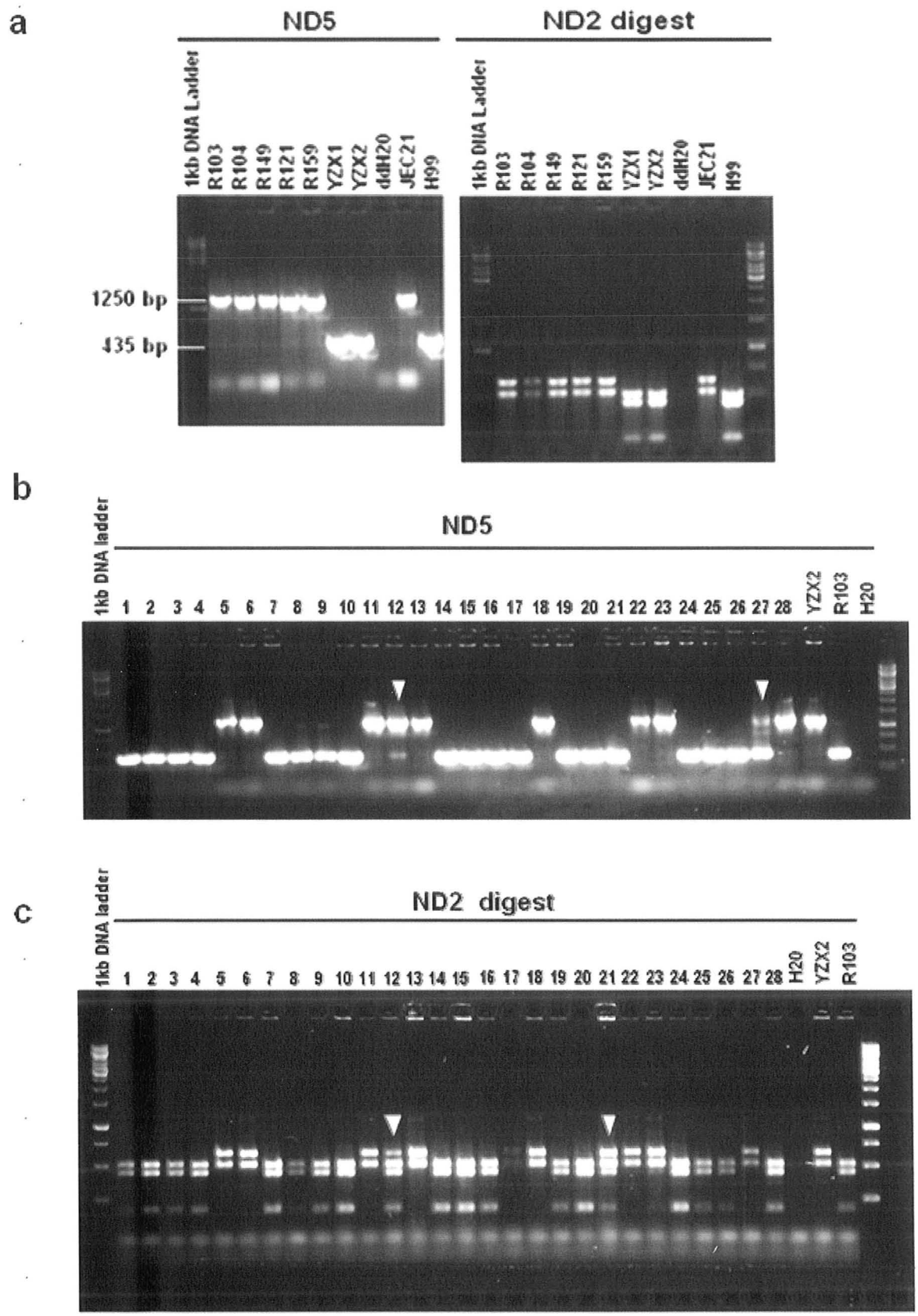

a
ND5
ND2 digest
1kb DNA Ladder
R103
R104
R149
R121
R159
YZX1
YZX2
ddH20
JEC21
H99
1250 bp
435 bp
b
ND5
1kb DNA ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
YZX2
R103
H20
c
ND2 digest
1kb DNA ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
H20
YZX2
R103

**Table 2.** Mitochondrial inheritance in mixed ploidy crosses

| Pattern of mating | Percent of meiotic progeny/fusants with mtDNA | | | | |
|---|---|---|---|---|---|
| | from the parent bearing *MAT***a** allele(s) | from the parent bearing *MAT*α allele(s) | Recomb-inant | Hetero-plasmic | Percent leakage |
| *MAT*α/α x *MAT***a** | | | | | |
| **1.** R103 x YZX2 | 70.9 | 23.3 | 5.8 | 0.0 | 29.1 |
| **1a.** R103 x YZX2 [a] | 50.0 | 37.5 | 5.7 | 6.8 | 50.0* |
| **2.** R104 x YZX2 | 81.7 | 10.3 | 8.0 | 0.0 | 18.3 |
| **3.** R149 x YZX2 | 60.0 | 38.8 | 1.2 | 0.0 | 40.0* |
| *MAT***a/a** x *MAT*α | | | | | |
| **4.** R121 x YZX1 | 92.0 | 8.0 | 0.0 | 0.0 | 8.0 |
| **5.** R159 x YZX1 | 64.6 | 33.0 | 0.0 | 2.4 | 35.0* |
| **5a.** R159 x YZX1 [a] | 82.4 | 11.8 | 1.1 | 4.7 | 17.6 |

[a] Crosses 1a and 5a show mitochondrial inheritance in fusants.

[b] "Percent leakage" refers to the proportion of progeny that are not homoplasmic for mtDNA from the *MAT***a** (or *MAT***a/a**) parent over the total number of progeny assayed in each cross.

* Percent leakage in the meiotic progeny of cross No. 3 is significantly higher than that in the cross No. 2, $P<0.01$ (chi-square test, d.f.=1). Percent leakage in the progeny of cross #5 is significantly higher than that in the cross No. 4, $P<0.01$ (chi-square test, d.f.=1). Percent leakage in the fusants and meiotic progeny of the same cross (crosses No.1 and 1a; 5 and 5a) was statistically different, $P\leq0.01$ (chi-square test, d.f.=1).

Similar to *MAT*α/α x *MAT***a** crosses, most of the meiotic progeny (64.6-92%) from the *MAT***a**/**a** x *MAT*α crosses inherited mtDNA from the *MAT***a** allele-carrying parent (Table 2, crosses No. 4 and 5). Eight percent mtDNA leakage in the progeny of the R121 x YZX1 cross was entirely due to the inheritance of the *MAT*α mtDNA (Table 2). This leakage was significantly lower than that in the progeny of the other cross of this type, R159 x YZX1 cross (Chi-square test, d.f.=1, $P<0.01$), as well as the lowest among the progeny populations of all the crosses analyzed. Nevertheless, the percentages of meiotic progeny homoplasmic for *MAT*α mtDNA in the *MAT***a**/**a** x *MAT*α type crosses were in a similar range (8-33%) as those in *MAT*α/α x *MAT***a** pattern of mating (Table 2). The two types of crosses, however, differed in the percentage of progeny with recombinant mtDNA type. Specifically, no recombinant mtDNA genotypes were identified among the meiotic progeny populations of the *MAT***a**/**a** x *MAT*α crosses. In addition, progeny heteroplasmic for mtDNA were detected in the R159 x YZX1 cross only, representing 2.4% of the population (Table 2, cross No. 5). In the latter cross, the mtDNA leakage in the progeny population was 35%, which is almost twice of that observed in the fusant population (17.6%) (Table 2, crosses No. 5 and 5a). This difference was statistically significant (Chi-square test, d.f.=1, $P<0.05$). The two populations also differed significantly in the percentage of cells homoplasmic for *MAT*α/α mtDNA (Chi-square test, d.f.=1, $P<0.01$). Nearly 12% of the fusants in the R159 x YZX1 cross inherited the *MAT*α mtDNA which is about a third of that in the meiotic progeny population of this cross. Similar to that of the R103 x YZX2 cross, cells heteroplasmic for mtDNA were present among the fusants from the R159 x YZX1 cross and comprised ~5% of the population (Table 2, cross No. 5a). For mtDNA genotypes of individual fusant and meiotic progeny at *ND2* and *ND5* loci, please refer to Supplemental Tables 3A to 3G.

The above-described fusants and basidiospores were obtained from those randomly germinated and formed colonies on our selective media. To examine whether our analyzed basidiospore population was representative of the original basidiospore population, we obtained and analyzed 49 basidiospores directly by micromanipulation from the hyphae produced by mating between R121 and YZX1 strains (Supplemental Figure 2, Supplemental Table 4). The majority of basidiospores (42/49, 85.7%) inherited mitochondria from the *MAT***a**/**a** parent, 8.2% had the *MAT*α parent-specific mtDNA type, and the rest (6.1%) were heteroplasmic with mtDNA from both parents. This leakage was not significantly different from the 8% leakage observed among the 88 meiotic progeny isolated by picking randomly germinated spores (chi-square test = 1.42, d.f. =1, $P>0.05$) (R121 x YZX1 cross in Table 2 and Supplementary Table 4). This result suggested that the randomly germinated spore population used in the initial screening was representative of the whole meiotic progeny population in these crosses.

**Segregation analysis of *ade2* and *NEO* in meiotic segregants.** As expected based on Mendelian segregation, cells with both parental ($Ade^-$ $G418^r$ and $Ade^+$ $G418^s$) and recombinant ($Ade^-$ $G418^s$ and $Ade^+$ $G418^r$) phenotypes were observed among the meiotic segregants of each cross (Table 3). Most of the progeny exhibiting a parental genotype had a mtDNA type and/or ploidy level different from that of the parental strain (Table 3, Supplemental Table 2). However, a 1:1:1:1 genotypic ratio was not observed. Our results are not un-expected. Although a result of equal segregation and independent assortment, the 1:1:1:1 ratio is characteristic of the F1 population of a cross in which the parents have the same copy number of gene/allele of interest. In the case of haploid x non-haploid cross e.g. *MAT*α/α x *MAT***a**, the haploid parent had one copy of mutant *ade2* gene whereas a non-

**Table 3.** Meiotic progeny phenotypes based on segregation of *ade2* and *neo* markers. [a]

| Cross | PARENTAL PHENOTYPES | | RECOMBINANT PHENOTYPES | | Total |
|---|---|---|---|---|---|
| | **$Ade^+$ $G418^s$ (non-haploid parents)** | **$Ade^-$ $G418^r$ (haploid parents)** | **$Ade^+G418^r$** | **$Ade^-$ $G418^s$** | |
| R103 x YZX2 | 47 (**10 alpha,** 37 a,) | 6 (**2 a**, 2 alpha, 2 rec) | 13 | 9 | 75 |
| R104 x YZX2 | 43 (**4 alpha**, 36 a, 3 rec) | 7 (**6 a**, 1 alpha) | 17 | 13 | 80 |
| R149 x YZX2 | 51 (**21 alpha**, 30 a) | 4 (**1 a**, 2 alpha, 1 rec) | 20 | 3 | 78 |
| R121 x YZX1 | **6 a** | 20 (**2 alpha**, 18 a) | 0 | 1 | 27 |
| R159 x YZX1 | 27 (**22 a**, 5 alpha) | 6 (**1 alpha**, 4 a, 1 het) | 14 | 1 | 48 |

[a] In brackets, number of meiotic segregants with different mtDNA type. In bold, number of meiotic progeny with mtDNA genotype and phenotype that are identical to the parental strain. Some of the meiotic progeny in bold had a ploidy level different from that of the parental strain (Supplemental Table 2 and Supplemental Fig. 1). "a" for the *MAT***a** (*MAT***a**/**a**) parent-specific mtDNA, "alpha" for the *MAT*α (*MAT*α/α) parent-specific mtDNA, "rec" for the recombinant mtDNA, "het" for heteroplasmic mtDNA.

**Ploidy level of meiotic progeny and fusants.** In total, 20 fusants and 76 meiotic progeny representing all crosses were tested for ploidy by FACS (Supplemental Table 2, Supplemental Figure 1). In both types of crosses, mating products of various ploidy level were found, including haploid, aneuploid (2n±j), diploid, and likely triploids. Since we did not have a positive triploid control, it was difficult to distinguish between aneuploids close to the triploid level and true triploid cells. Therefore, the ploidy of these cells were referred to as >2n. Nine out of 19 meiotic progeny from the *MAT***a/a** x *MAT*α crosses were >2n, whereas 4 out of 57 meiotic progeny from *MAT*α/α x *MAT***a** crosses were >2n. This difference was statistically significant $P<0.01$ (chi-square test, d.f. = 1). In contrast, 15 out of 20 fusants screened were >2n and the remaining 5 were diploid (2n) (Supplemental Table 2, Supplemental Fig. 1). There was no association of cell ploidy with mtDNA type of the meiotic progeny (or fusants).

**Morphological features of *C. neoformans* haploid x non-haploid mating.** All five matings produced filaments characteristic of mating. Staining with calcofluor white revealed the production of long hyphal filaments linked by fused clamp connections (Fig. 3a). The filaments contained paired nuclei (dikaryons) (Fig. 3c) and produced fertile basidia with four chains of basidiospores (Fig. 3b). The basidiospores from each cross remained viable after at least forty rounds of cell division as revealed by effective colony formation on YEPD agar. Successive re-streaking was part of cell preparation for mtDNA genotyping, FACS analysis, and fluorescent microscopy.

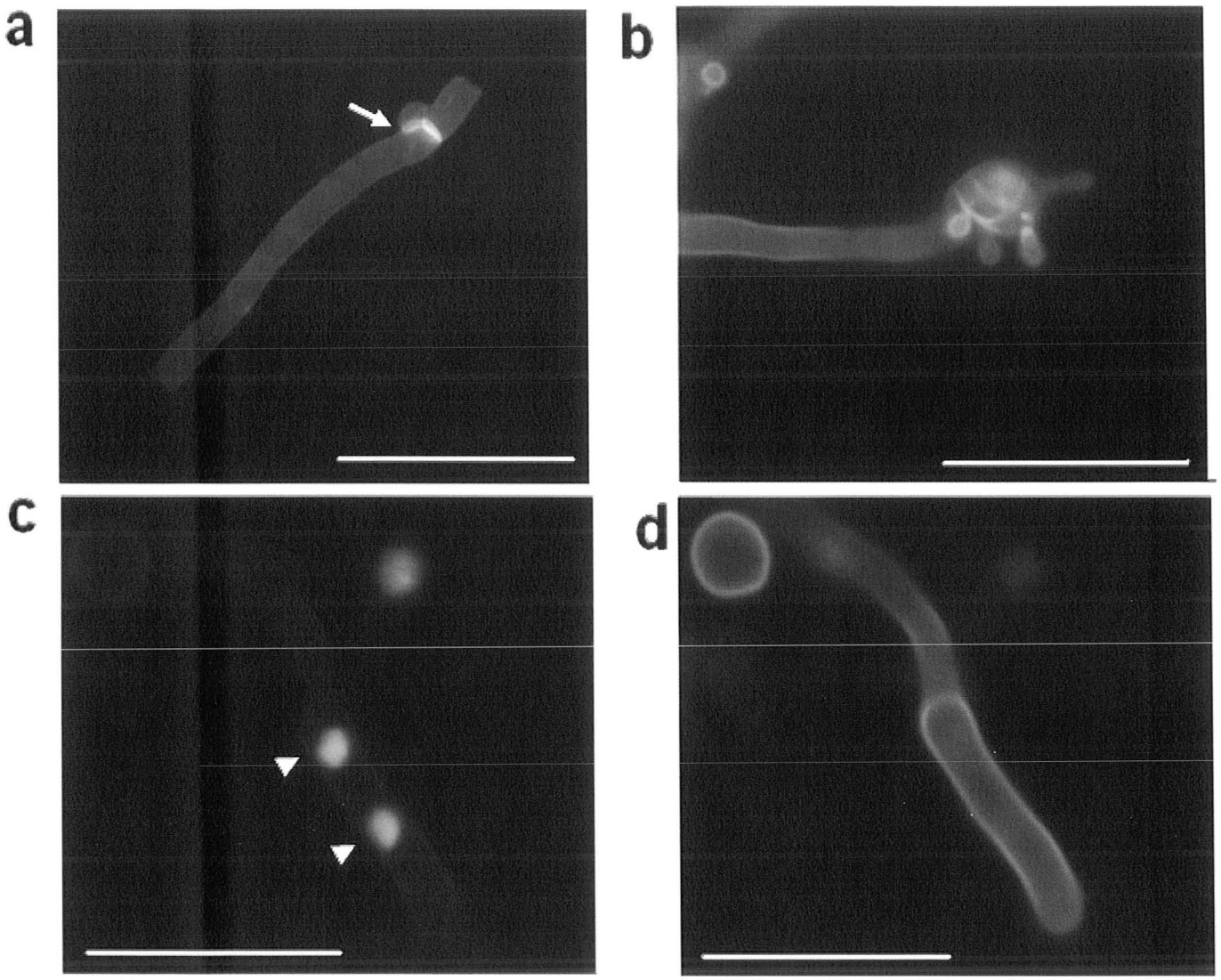



**Figure 2.3 Morphological features of *C. neoformans* haploid x non-haploid mating.** The non-haploid *MAT***a/a** R121 or R159 strains were mixed with the *MAT*α strain YZX1. (a) Calcofluor White staining of the septa of filaments produced in a R121 x YZX1 cross after 10 days of growth on V8 (pH 5.0) at 25°C in the dark. The arrow designates a fused clamp connection. Bar, 25 μm. (b) Example of the formation of four basidiospores on a basidium in a R159 x YZX1 cross produced after 10 days of growth on V8 (pH 5.0) at 25°C in the dark. Bar, 25 μm. (c) DAPI staining of nuclei in filaments produced in a R121 x YZX1 mating. Arrowheads designate paired nuclei in the dikaryons. Bar, 25 μm. (d) Light microscopy image of the filament seen in panel d.

## DISCUSSION

The patterns of mitochondrial inheritance have been of great interest to scientists ever since the discovery in the 1960s that mitochondria have their own DNA and their own patterns of inheritance. Mitochondria are the "energy factory" of the cells, play essential roles in cell metabolism and survival, and were derived from an ancient bacterial endosymbiosis event; therefore, the factors controlling mitochondrial inheritance are likely conserved. In this study, we analyzed progeny from haploid x diploid and haploid x aneuploid crosses and identified mtDNA inheritance patterns significantly different from those between haploid strains. Our study enhances the understanding of the diversity of mtDNA inheritance patterns in sexual eukaryotes.

In the study, we used sterile haploid and non-haploid (i.e. diploid and aneuploid) strains as parents in crosses. Parental sterility was confirmed by the absence of hyphal production after 32 days of incubation in the vicinity of each other on V8 plate. Although incapable of sexual reproduction on their own, the paired parental strains were sex compatible as revealed by the formation of hyphae. The latter had all the features characteristic of opposite-sex mating in serotype D *C. neoformans* such as dikaryotic hyphae with septa and fused clamp connections (Figure 3a,c,d), and club-shaped basidia bearing four spore chains (Figure 3b) (Kwon-Chung 1975). The sterility of the parental strains strongly suggests that the mycelium could not have originated from the haploid and/or diploid fruiting stimulated by pheromones produced by the strain with the opposite mating type. Haploid fruiting filaments are distinct for being monokaryotic and having unfused clamp connections (Wickes et al. 1996). That the isolated meiotic progeny were true meiotic progeny was further confirmed by the results of the segregation analysis. The presence of cells with the recombinant phenotypes ($Ade^+$

$G418^r$ and $Ade^-$ $G418^s$), the parental phenotypes ($Ade^-$ $G418^r$ and $Ade^+$ $G418^s$) but a non-parental mtDNA type and/or ploidy level (Supplemental Table 2) as well as the prevalence of $Ade^+$ phenotype among the meiotic progeny with ploidy level of at least 2n-j (including 2n, >2n) provide robust evidence that the cells analyzed were indeed meiotic segregants. The conclusion was further supported by similar marker segregational patterns among basidiospores isolated directly through micromanipulation (Table 3, Supplemental Table 4).

In *C. neoformans,* mtDNA is inherited almost exclusively from the *MAT***a** parent in opposite-sex HH crosses (Yan and Xu 2003). Three hypotheses have been proposed to explain the uniparental pattern of mtDNA inheritance in this species. The selective degradation model argues that *MAT*α mtDNA is selectively tagged and destroyed following cell fusion (Yan and Xu 2003). Evidence for the selective elimination of the mating type-specific mtDNA in controlling mtDNA inheritance in isogamous species has been shown in zygotes of the unicellular alga *Chlamydomonas reinhardtii* (Gillham 1994). Alternatively, the *MAT*α nucleus may migrate unidirectionally into the *MAT***a** cell while leaving its mitochondria behind. The fusion product, as a result, would preferentially contain *MAT***a** mitochondria (Yan et al. 2004). However, these two hypotheses are not mutually exclusive and may be complemented by the third mechanism, the non-random budding of zygotes after mating and cell fusion. According to the third mechanism, the parental cytoplasms might not be mixed completely after mating, and hyphal or bud formation occurs preferentially from the *MAT***a** parent side of the zygote (Yan and Xu 2003). Here, we discuss the relevance of the observations made in this study to these hypotheses.

A significantly greater mtDNA leakage was observed in all crosses in this study than in the earlier HH crosses reported by Yan and Xu (2003). Specifically, the overall mtDNA

leakage in the meiotic progeny of seven HH crosses between JEC20 and JEC21-mtA was 0.5% (Yan and Xu 2003). This leakage differed significantly from 8-40% mtDNA leakage observed in the meiotic progeny populations in this study (Table 2) (Chi-square tests, $P<0.01$ in all pairwise tests). In addition, mtDNA leakage in the basidiospores obtained by micromanipulation in this study (Supplemental Table 4) was significantly greater than in those of the HH crosses in Yan and Xu (2003); providing further evidence for greater mtDNA leakage in the progeny of haploid x non-haploid crosses than in the opposite-sex HH crosses in *C. neoformans*. Similarly, 17.6-50% mtDNA leakages in the fusants of the HD crosses (Table 2) were significantly higher than those identified in the various HH crosses (0-8%) (Yan et al. 2004; 2007a; b) (Chi-square test, $P\leq0.05$). Although the leakage frequency was higher than those found in HH crosses, mtDNA inheritance, except for the fusant population of the R103 x YZX2 cross (Table 2, cross No. 1a), was still biased towards the *MAT***a** (i.e. *MAT***a or** *MAT***a/a**) parent. This suggests that additional factors besides the *MAT* locus are involved in the control of mitochondrial inheritance in mixed ploidy crosses in *C. neoformans*. Ploidy of the parents could be one of the potential factors. An increase in ploidy level is often paralleled with an increase in cell size. FACS analysis revealed that this relationship holds true for *C. neoformans*: diploid cells of *C. neoformans* are larger in size than haploid cells as confirmed by flow cytometry and light microscopy in Lin et al. (2008, 2009). A positive correlation between ploidy level and cell size has been also observed in other fungal species such as *Saccharomyces cerevisiae* and *Candida albicans* (Galitski et al. 1999; Hubbard et al. 1985). Assuming that the cytoplasm of the two parental cells is completely mixed in haploid x non-haploid crosses, a greater proportion of the cytoplasm in the fusants would come from the parent with a higher ploidy level. The latter would thus also

contribute a greater amount of its mitochondria than a haploid parent. However, biased cytoplasmic inputs alone could not explain the higher level of mtDNA leakage observed in the *MAT***a**/**a** x *MAT*α (cross No.5) compared to those in *MAT*α/α x *MAT***a** crosses No. 1 and 2 (Table 2).

The parental cell size's effect on mitochondrial inheritance in fusants of HD crosses is also consistent with the model of selective degradation of the *MAT*α mtDNA. In a typical opposite-sex HH cross, the parental cells are isogamous, contributing the same amount of cytoplasm (and likely similar number of mitochondria) to the fusion product. Therefore, immediately after the fusion of the two parents, the ratio of *MAT***a**-to-*MAT*α mtDNA should be about 1:1. Later on during the course of mating as factors responsible for the selective degradation of *MAT*α mtDNA are activated, the initial mtDNA ratio would change dramatically in favor of the *MAT***a** mtDNA in HH crosses (0.5% leakage in Yan and Xu 2003). For still unknown reason(s), a dramatic decrease in the *MAT*α or *MAT*α/α mtDNA following the fusion of the parental strains might not have occurred in the opposite-sex HD crosses (Table 2). However, we did observe that the fusants from *MAT***a**/**a** x *MAT*α cross were more effective at eliminating mtDNA from the *MAT*α allele-bearing parent (17.6 % leakage) than those of *MAT*α/α x *MAT***a** cross (50% leakage) (Table 2, crosses No. 5a and 1a). In the *MAT*α/α x *MAT***a** cross, the cytoplasmic contribution of the *MAT*α/α diploid parent to the fusion products was probably higher than that of the *MAT***a** parent, resulting in the numerical dominance of *MAT*α mtDNA. Higher numbers of *MAT*α mtDNA might have interfered with the selective tagging and degradation process, which would explain a higher percentage of fusants with *MAT*α mtDNA (Table 2, cross No. 1a). In contrast, the *MAT*α mtDNA elimination process seems to operate more efficiently when *MAT***a** mtDNA is

present in numbers higher than *MAT*α mtDNA, as shown in the fusion products of *MAT***a/a** x *MAT*α cross (Table 2, cross No. 5a). Nevertheless, the proposed explanation alone does not account for the higher percent leakage observed in the fusants of *MAT***a/a** x *MAT*α than in a typical opposite-sex HH cross.

Aside from cell size effect, genetic factors other than those at the mating-type locus were likely involved in contributing to the differences in leakage between the *MAT***a/a** x *MAT*α and the *MAT*α/α x *MAT***a** crosses. It is also possible that interactions between nuclear and mitochondrial genes and genomes could contribute to the differences. For example, in the wheat pathogen *Phaeosphaeria nodorum,* there was evidence for significant cytonuclear disequilibrium that was correlated to differences in fitness among mtDNA haplotypes (Sommerhalder et al. 2007). In HH crosses in *C. neoformans*, the *SXI1*alpha and *SXI2*a genes located within the mating-type locus controlled mitochondrial inheritance (Yan et al. 2004; Yan et al. 2007a). Since these two genes are interacting transcription factors (Hull et al. 2002; 2005), they determine mtDNA inheritance likely through controlling the expression of other genes, including those not within the *MAT* locus (Yan et al. 2007a). The significant differences in mtDNA inheritance patterns between HD (or HA) and HH crosses may be due to ploidy-specific genetic factors in *C. neoformans*. Adenine auxotrophy of the haploid parents YZX1 and YZX2 is very unlikely to be responsible for the elevated mtDNA leakage of the progeny of the mixed ploidy crosses. These strains were also used in the studies of mitochondrial inheritance in *MAT***a** x *MAT*α HH crosses in *C. neoformans* described previously and were found to have no effect on mtDNA inheritance (Yan et al. 2004; Yan et al. 2007a, b). The highest mtDNA leakage observed in the latter was 8% (Yan et al. 2007b),

which is significantly lower than the maximum 50% mitochondrial leakage found in the haploid x non-haploid crosses ($x^2$, $P<0.01$).

An investigation was attempted to differentiate mechanisms controlling mtDNA inheritance with differential staining of mitochondria or mtDNA of the parental cells during mating on V8 agar. One method used the vital dyes MitoTracker Green and Rhodamine B (Invitrogen) that accumulate specifically in mitochondria. Although the mated and unmated cells remained viable and showed good fluorescence of the mitochondria, immediately after mating the dyes merged, i.e., all mitochondria of a zygote were stained with both dyes, likely due to the constant redistribution of the dyes between the cytoplasm and mitochondria (T. J. Collins, pers. comm.). A second method tested was fluorescent *in situ* hybridization (FISH) using oligonucleotide probes specific to the mtDNA of var. *grubii* and var. *neoformans* following the hybridization protocol of Röder et al. (2007) with minor modifications. A high level of autofluorescence, a common pattern in *Cryptococcus* species, also prohibited us from differentiating the parental mitochondria when using FISH. Future research will use GFP and RFP-tagged mitochondrial proteins to determine the localization of *MAT***a** and *MAT*α mitochondria during the early mating process.

The mtDNA leakage observed in the fusant populations of both types of mixed ploidy crosses differed significantly from those in the meiotic progeny (Table 2, crosses No. 1 and 1a; 5 and 5a). Specifically, the fusants typically have on average higher ploidy levels than meiotic progeny (Supplemental Table 2, Supplemental Figure 1), and they were prevented from filamentation and dikaryon formation after mating due to the short mating period on V8 agar and post-fusion selection at a high temperature (37°C) that was not conducive for filamentation. The observed extensive variation in mtDNA leakage in the five crosses (Table

2) thus seemed to favor the hypothesis of a random budding pattern of the newly formed zygotes in their production of daughter cells in our HA and HD crosses. The effect of zygote bud position on progeny mtDNA inheritance has been demonstrated in *S. cerevisiae*. In *S. cerevisiae* the cytoplasms from the two opposite mating type haploid parents do not fuse completely, and the positions where the daughter buds are formed have a significant effect on the daughter mitochondrial genotype (Stevens 1981). If random budding operates in *C. neoformans* mixed ploidy crosses, the *MAT*α (*MAT*α/α) mtDNA would end up in the mating product whenever the bud formation occurred from the side of the zygote containing this mtDNA. A greater number of *MAT*α mitochondria and mtDNA molecules in the zygote will likely increase the likelihood of obtaining heteroplasmic fusants with *MAT*α mtDNA (Table 2, cross No. 1a).

Isolation of mating products of different ploidy in both types of haploid x non-haploid crosses was consistent with the outcome of triploid meiosis. Aneuploids are common among the meiotic segregants of triploid cells, as has been shown in *S. cerevisiae* (Campbell et al. 1981) and the fission yeast *Schizosaccharomyces pombe* (Niwa and Yanagida 1985). In triploid meiosis the segregation of the homologous triad is expected to be two-by-one; that is, half of the meiotic products should be disomic (n+1) for any given chromosome while the other half should be haploid (n). Different homologous triads, however, segregate independently from each other; as a result, the meiotic product can be disomic for some chromosomes while being haploid for the other chromosomes (i.e., aneuploids). As expected, the majority of the fusants from the HD and HA crosses were >2n while some were not different from 2n. The isolation of diploid fusants suggests that triploid nuclei might not

be stable and can lose chromosomes quickly. The isolation of meiotic progeny with nuclear content >2n also indicates improper chromosome segregation during meiosis.

In this study, we used diploid and aneuploid strains as parents in crosses. The diploid strains also exist in natural *C. neoformans* populations, comprising about 8% of the *C. neoformans* serotype A isolates (Lin et al. 2009). It is therefore possible that HD and HA crosses could also occur in nature. If such crosses occur and if the mtDNA inheritance in these crosses is as leaky as they are identified here, we should expect to identify signatures of mtDNA recombination in natural populations. Signatures of recombination were recently found in natural populations of *Cryptococcus gattii*, a closely related species of *C. neoformans*. (Xu et al. 2009). At present, there is limited information about mitochondrial population structure in natural populations of *C. neoformans*.

## ACKNOWLEDGEMENTS

This work was supported by National Sciences and Engineering Research Council (NSERC) Alexander Graham Bell Canada Graduate Scholarship (IS) and by an NSERC Discovery grant (JX). We would like to acknowledge Hong Liang for technical assistance.

# APPENDIX

## SUPPLEMENTARY TABLES

**Supplemental Table 1.** PCR primers used in this study.

| DNA | Sequence (5′ → 3′) | Annealing temperature (°C) | Expected band size (bp) |
|---|---|---|---|
| *ND5* | F: ctattggtgttacaggagctcac [a]<br>R: gagccttcatacctgccttatttgc | 55 | 435[a] ; 1,250 [b] |
| *ND2* | F: tatgatggccgtagcgctatc [a]<br>R: tggtggtactcctgccattg [a] | 46 | 1,679[a,b] |
| STE20αA | F: agctgcgatccgttgtaagt<br>R: ttcctgatgccctgagaagt | 62 | 141[a] |
| STE20**a**D | F: cacgcgatcctcatcatcta<br>R: aagtgtcgggtcttgtacgc | 62 | 140[b] |
| CNJ00540 | F: gacttggcacgagacaaaca<br>R: ggcagaatggacctgagaag | 62 | 235 [a,b] |

[a] Serotype A
[b] Serotype D

**Supplemental Table 2.** Ploidy level of meiotic progeny and fusants.

| Strain | Ploidy[a] | $Ade2^+$ / $Ade^-$ | $G418^r$ / $G418^s$ |
|---|---|---|---|
| **Haploid controls** | | | |
| JEC21 | n | | |
| JEC20 | n | | |
| JEC50 | n | | |
| **Diploid controls** | | | |
| D15 | 2n | | |
| D14 | 2n | | |
| **Non-haploid parents** | | | |
| R103 | 2n | + | s |
| R104 | 2n | + | s |
| R149 | 2n-j | + | s |
| R121 | 2n | + | s |
| R159 | 2n | + | s |
| **Meiotic progeny of R103 x YZX2 cross** | | | |
| AB/R103-3 | 2n | + | s |
| AB/R103-10 | 2n | + | s |
| AB/R103-18 | 2n | + | s |
| AB/R103-39 | 2n-j | + | s |
| AB/R103-11 | 2n | + | s |
| AB/R103-26 | 2n-j | – | r |
| AB/R103-85 | 2n | – | r |
| AB/R103-57 | n | + | s |
| AB/R103-14 | 2n | + | s |
| AB/R103-37 | n | + | s |
| AB/R103-54 | 2n-j | – | s |
| AB/R103-74 | n | + | s |
| AB/R103-40 | >2n | + | r |
| AB/R103-36 | 2n-j | – | r |
| AB/R103-80 | 2n-j | – | s |
| AB/R103-48 | 2n-j | + | r |
| AB/R103-52 | n | + | r |
| AB/R103-29 | 2n-j | – | r |
| AB/R103-27 | 2n-j | + | s |
| AB/R103-88 | 2n-j | + | s |
| **Fusants of R103 x YZX2 cross** | | | |
| R3Z-19 | >2n | + | r |
| R3Z-20 | >2n | + | r |
| R3Z-25 | >2n | + | r |
| R3Z-18 | >2n | + | r |
| R3Z-16 | 2n | + | r |
| R3Z-22 | >2n | + | r |
| R3Z-10 | >2n | + | r |
| R3Z-24 | >2n | + | r |
| R3Z-17 | >2n | + | r |
| R3Z-21 | >2n | + | r |

**Supplemental Table 2** *continued*

| Strain | Ploidy[a] | $Ade2^+$ / $Ade^-$ | $G418^r$ / $G418^s$ |
|---|---|---|---|
| **Meiotic progeny of R104 x YZX2 cross** | | | |
| BC/R104-2 | n | + | r |
| BC/R104-15 | 2n-j | + | r |
| BC/R104-18 | n | + | s |
| BC/R104-19 | n | + | s |
| BC/R104-28 | 2n-j | – | s |
| BC/R104-34 | 2n-j | – | r |
| BC/R104-31 | n | + | r |
| BC/R104-20 | n | + | s |
| BC/R104-21 | 2n-j | – | s |
| BC/R104-3 | n | + | r |
| BC/R104-16 | 2n-j | – | r |
| BC/R104-84 | 2n | + | s |
| BC/R104-78 | n | + | s |
| BC/R104-73 | 2n | + | r |
| BC/R104-67 | 2n-j | – | s |
| BC/R104-64 | 2n-j | + | s |
| BC/R104-42 | >2n | + | s |
| BC/R104-43 | 2n-j | + | r |
| BC/R104-14 | >2n | + | r |
| BC/R104-57 | n | + | s |
| **Meiotic progeny of R149 x YZX2 cross** | | | |
| CD/R149-16 | n | + | r |
| CD/R149-20 | 2n-j | – | s |
| CD/R149-18 | >2n | + | s |
| CD/R149-28 | 2n | + | r |
| CD/R149-29 | 2n-j | + | s |
| CD/R149-30 | 2n-j | + | s |
| CD/R149-47 | n | + | s |
| CD/R149-21 | 2n-j | + | s |
| CD/R149-22 | n | + | r |
| CD/R149-19 | n | + | s |
| CD/R149-15 | 2n-j | + | r |
| CD/R149-39 | n | + | s |
| CD/R149-17 | n | + | s |
| CD/R149-80 | n | + | s |
| CD/R149-60 | n | + | r |
| CD/R149-61 | 2n-j | + | r |
| CD/R149-38 | n | + | s |
| **Meiotic progeny of R121 x YZX1 cross** | | | |
| EF/R121-13 | 2n-j | + | s |
| EF/R121-4 | >2n | – | r |
| EF/R121-6 | >2n | – | r |
| EF/R121-7 | >2n | – | s |
| EF/R121-8 | >2n | – | r |
| EF/R121-14 | 2n-j | + | s |

**Supplemental Table 2** *continued*

| Strain | Ploidy[a] | $Ade2^+$ / $Ade^-$ | $G418^r$ / $G418^s$ |
|---|---|---|---|
| **Meiotic progeny of R159 x YZX1 cross** | | | |
| FG/R159-62 | >2n | – | r |
| FG/R159-73 | >2n | + | s |
| FG/R159-68 | 2n | + | s |
| FG/R159-66 | 2n | + | s |
| FG/R159-47 | >2n | – | r |
| FG/159-2 | 2n-j | + | s |
| FG/R159-3 | >2n | + | s |
| FG/R159-27 | >2n | – | r |
| FG/159-69 | n | + | s |
| FG/159-82 | 2n | + | r |
| FG/159-83 | 2n | + | s |
| FG/R159-61 | 2n | + | s |
| FG/R159-70 | 2n | + | r |
| **Fusants of R159 x YZX1 cross** | | | |
| R9Z-23 | >2n | + | r |
| R9Z-28 | >2n | + | r |
| R9Z-45 | 2n | + | r |
| R9Z-5 | 2n | + | r |
| R9Z-1 | >2n | + | r |
| R9Z-3 | >2n | + | r |
| R9Z-4 | >2n | + | r |
| R9Z-2 | >2n | + | r |
| R9Z-6 | 2n | + | r |
| R9Z-8 | 2n | + | r |

[a] n: Haploid; 2n-j: aneuploid between haploid and diploid, 'j' is the haploid number of chromosomes, $1 \leq j < n$; 2n: Diploid, >2n: aneuploid between diploid and triploid, or triploid.

**Supplemental Table 3A.** Mitochondrial inheritance in meiotic progeny of R103 x YZX2 cross.[a]

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| AB-1 | 2 | 2 | | w |
| AB-2 | 2 | 2 | L | w |
| AB-3 | 2 | 2 | L | w |
| AB-4 | 2 | 2 | L | w |
| AB-5 | 2 | 2 | | w |
| AB-7 | 2 | 2 | | w |
| AB-8 | 2 | 2 | | w |
| AB-9 | 2 | 2 | L | w |
| AB-10 | 2 | 2 | L | w |
| AB-11 | 2 | 2 | L | w |
| AB-12 | 2 | 2 | L | P |
| AB-13 | 2 | 2 | L | w |
| AB-14 | 1 | 1 | L | w |
| AB-15 | 2 | 2 | L | w |
| AB-16 | 2 | 2 | L | w |
| AB-17 | 2 | 2 | L | w |
| AB-18 | 2 | 2 | L | w |
| AB-19 | 2 | 2 | L | P |
| AB-20 | 2 | 2 | L | w |
| AB-21 | 2 | 2 | L | w |
| AB-22 | 2 | 2 | L | P |
| AB-23 | 2 | 2 | L | w |
| AB-24 | 2 | 2 | L | w |
| AB-25 | 2 | 2 | L | P |
| AB-26 | 1 | 1 | L | P |
| AB-27 | 1 | 1 | L | w |
| AB-28 | 2 | 2 | L | P |
| AB-29 | 2 | 1 | L | P |
| AB-30 | 2 | 2 | L | w |
| AB-31 | 2 | 2 | L | w |
| AB-32 | 2 | 2 | L | w |
| AB-33 | 2 | 2 | L | w |
| AB-34 | 2 | 2 | L | P |
| AB-36 | 1 | 1 | L | P |
| AB-37 | 1 | 1 | s | w |
| AB-38 | 2 | 2 | s | w |
| AB-39 | 2 | 2 | s | w |
| AB-40 | 2 | 1 | s | w |
| AB-41 | 2 | 1 | s | P |
| AB-42 | 1 | 1 | s | w |
| AB-43 | 1 | 1 | s | P |
| AB-44 | 2 | 2 | s | w |
| AB-45 | 2 | 2 | s | w |
| AB-46 | 2 | 2 | s | w |
| AB-47 | 2 | 2 | s | w |
| AB-48 | 2 | 2 | s | w |
| AB-49 | 2 | 2 | s | w |
| AB-50 | 2 | 2 | s | w |
| AB-51 | 2 | 2 | s | w |
| AB-52 | 2 | 1 | s | w |

**Supplemental Table 3A** *continued*

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| AB-53 | 2 | 2 | s | w |
| AB-54 | 2 | 2 | s | P |
| AB-55 | 2 | 2 | s | w |
| AB-56 | 2 | 2 | s | w |
| AB-57 | 1 | 1 | s | w |
| AB-58 | 2 | 2 | s | w |
| AB-59 | 1 | 1 | s | w |
| AB-60 | 1 | 1 | s | P |
| AB-61 | 2 | 2 | s | w |
| AB-62 | 2 | 2 | s | w |
| AB-63 | 2 | 2 | s | P |
| AB-64 | 2 | 2 | s | w |
| AB-65 | 2 | 2 | s | w |
| AB-66 | 1 | 1 | s | w |
| AB-67 | 1 | 1 | s | w |
| AB-68 | 2 | 2 | s | w |
| AB-69 | 1 | 1 | s | w |
| AB-70 | 2 | 2 | s | w |
| AB-71 | 2 | 2 | s | w |
| AB-72 | 2 | 2 | s | w |
| AB-73 | 2 | 2 | s | w |
| AB-74 | 1 | 1 | s | w |
| AB-75 | 2 | 2 | s | P |
| AB-76 | 2 | 2 | s | w |
| AB-77 | 2 | 2 | s | w |
| AB-78 | 2 | 2 | s | w |
| AB-79 | 2 | 2 | s | w |
| AB-80 | 1 | 1 | s | P |
| AB-81 | 1 | 1 | L | w |
| AB-82 | 2 | 2 | L | w |
| AB-83 | 1 | 1 | L | w |
| AB-84 | 1 | 1 | L | P |
| AB-85 | 1 | 1 | s | w |
| AB-86 | 2 | 2 | s | w |
| AB-87 | 1 | 2 | s | w |
| AB-88 | 1 | 1 | s | w |

[a] "1" for homozygous for the R103 allele; "2" for homozygous for the YZX2 allele; and "3" for heterozygous for both parental alleles.

[b] "s" and "L" represent small and large colony size, respectively.

[c] "P" and "w" represent pink and white color of colony, respectively.

**Supplemental Table 3B.** Mitochondrial inheritance in meiotic progeny of R104 x YZX2 cross.[a]

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| BC-1 | 2 | 2 | S | W |
| BC-2 | 2 | 2 | S | W |
| BC-3 | 1 | 1 | S | W |
| BC-4 | 2 | 2 | S | P |
| BC-5 | 1 | 1 | S | W |
| BC-6 | 2 | 2 | S | W |
| BC-7 | 1 | 2 | S | W |
| BC-8 | 2 | 2 | S | W |
| BC-9 | 2 | 2 | S | W |
| BC-11 | 2 | 2 | S | W |
| BC-12 | 2 | 2 | S | W |
| BC-13 | 1 | 1 | S | W |
| BC-14 | 1 | 1 | S | W |
| BC-15 | 2 | 2 | S | W |
| BC-16 | 1 | 1 | S | P |
| BC-17 | 2 | 2 | L | P |
| BC-18 | 2 | 2 | L | W |
| BC-19 | 2 | 2 | L | W |
| BC-20 | 2 | 2 | L | W |
| BC-21 | 1 | 1 | L | P |
| BC-22 | 2 | 2 | L | W |
| BC-23 | 1 | 2 | L | P |
| BC-24 | 2 | 2 | L | W |
| BC-25 | 2 | 2 | S | W |
| BC-26 | 2 | 2 | S | W |
| BC-27 | 2 | 2 | S | W |
| BC-28 | 2 | 2 | S | P |
| BC-29 | 2 | 2 | S | W |
| BC-30 | 2 | 2 | S | W |
| BC-31 | 2 | 2 | S | W |
| BC-32 | 2 | 2 | S | W |
| BC-33 | 2 | 2 | S | W |
| BC-34 | 2 | 2 | S | P |
| BC-35 | 2 | 2 | S | W |
| BC-36 | 2 | 2 | S | W |
| BC-37 | 2 | 2 | S | W |
| BC-38 | 2 | 2 | S | W |
| BC-39 | 2 | 2 | S | W |
| BC-40 | 2 | 2 | S | P |
| BC-41 | 2 | 2 | S | W |
| BC-42 | 2 | 2 | S | W |
| BC-43 | 1 | 2 | S | W |
| BC-44 | 2 | 2 | S | W |
| BC-45 | 2 | 2 | S | W |
| BC-46 | 2 | 2 | S | W |
| BC-47 | 2 | 2 | S | W |
| BC-48 | 2 | 2 | S | P |
| BC-49 | 1 | 2 | S | W |
| BC-50 | 2 | 2 | S | P |
| BC-51 | 2 | 2 | S | P |

**Supplemental Table 3B** *continued*

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| BC-52 | 1 | 2 | s | w |
| BC-53 | 2 | 2 | s | P |
| BC-54 | 2 | 2 | s | w |
| BC-55 | 2 | 2 | s | w |
| BC-56 | 2 | 2 | s | w |
| BC-57 | 1 | 2 | s | w |
| BC-58 | 2 | 2 | s | w |
| BC-59 | 1 | 1 | s | P |
| BC-60 | 2 | 2 | s | w |
| BC-61 | 2 | 2 | s | w |
| BC-62 | 2 | 2 | s | w |
| BC-63 | 2 | 2 | s | P |
| BC-64 | 2 | 2 | s | w |
| BC-65 | 2 | 2 | s | w |
| BC-66 | 2 | 2 | s | w |
| BC-67 | 1 | 2 | s | P |
| BC-68 | 2 | 2 | s | P |
| BC-69 | 2 | 2 | s | P |
| BC-70 | 2 | 2 | s | w |
| BC-71 | 2 | 2 | s | w |
| BC-72 | 2 | 2 | s | P |
| BC-73 | 2 | 2 | s | w |
| BC-74 | 2 | 2 | s | w |
| BC-75 | 2 | 2 | s | P |
| BC-76 | 2 | 2 | s | P |
| BC-77 | 2 | 2 | s | w |
| BC-78 | 1 | 1 | s | w |
| BC-79 | 2 | 2 | s | P |
| BC-80 | 2 | 2 | s | w |
| BC-81 | 2 | 2 | L | w |
| BC-82 | 2 | 2 | L | w |
| BC-83 | 2 | 2 | L | P |
| BC-84 | 1 | 1 | L | w |
| BC-85 | 2 | 2 | L | w |
| BC-86 | 2 | 2 | L | w |
| BC-87 | 2 | 2 | L | w |
| BC-88 | 2 | 2 | L | P |

[a] "1" for homozygous for the R104 allele; "2" for homozygous for the YZX2 allele; and "3" for heterozygous for both parental alleles.

[b] "s" and "L" represent small and large colony size, respectively.

[c] "P" and "w" represent pink and white color of colony, respectively.

**Supplemental Table 3C.** Mitochondrial inheritance in meiotic progeny of R149 x YZX2 cross.[a]

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| CD-1 | 2 | 2 | L | P |
| CD-2 | 1 | 1 | L | W |
| CD-3 | 1 | 1 | L | W |
| CD-9 | 2 | 2 | L | W |
| CD-10 | 2 | 2 | L | W |
| CD-14 | 2 | 2 | L | W |
| CD-15 | 1 | 1 | L | W |
| CD-16 | 2 | 2 | L | P |
| CD-17 | 2 | 2 | S | P |
| CD-18 | 2 | 2 | S | W |
| CD-19 | 1 | 1 | S | W |
| CD-20 | 2 | 2 | S | W |
| CD-21 | 1 | 1 | S | P |
| CD-22 | 1 | 1 | S | W |
| CD-23 | 1 | 1 | S | P |
| CD-24 | 1 | 1 | S | W |
| CD-25 | 1 | 1 | S | W |
| CD-26 | 2 | 2 | S | W |
| CD-27 | 2 | 2 | S | W |
| CD-28 | 2 | 2 | S | P |
| CD-29 | 2 | 2 | S | W |
| CD-30 | 2 | 2 | S | W |
| CD-31 | 2 | 2 | S | W |
| CD-32 | 2 | 2 | S | W |
| CD-33 | 2 | 2 | S | W |
| CD-34 | 1 | 1 | S | P |
| CD-35 | 2 | 2 | S | W |
| CD-36 | 1 | 1 | S | W |
| CD-37 | 1 | 1 | S | W |
| CD-38 | 1 | 1 | S | W |
| CD-39 | 1 | 1 | S | W |
| CD-40 | 2 | 2 | S | P |
| CD-41 | 1 | 1 | S | W |
| CD-42 | 2 | 2 | S | W |
| CD-43 | 2 | 2 | S | W |
| CD-44 | 2 | 2 | S | W |
| CD-45 | 2 | 2 | S | W |
| CD-46 | 2 | 2 | S | W |
| CD-47 | 1 | 1 | S | W |
| CD-48 | 1 | 1 | S | P |
| CD-49 | 1 | 1 | S | W |
| CD-50 | 2 | 2 | S | W |
| CD-51 | 2 | 2 | S | P |
| CD-52 | 2 | 2 | S | W |
| CD-53 | 1 | 1 | S | P |
| CD-54 | 2 | 2 | S | W |
| CD-55 | 2 | 2 | S | W |
| CD-56 | 1 | 1 | S | W |
| CD-57 | 2 | 2 | S | W |
| CD-58 | 1 | 1 | S | W |

**Supplemental Table 3C** *continued*

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| CD-59 | 2 | 2 | s | P |
| CD-60 | 2 | 2 | s | w |
| CD-61 | 2 | 2 | s | w |
| CD-62 | 2 | 2 | s | w |
| CD-63 | 1 | 1 | s | P |
| CD-64 | 1 | 1 | s | P |
| CD-65 | 1 | 1 | s | w |
| CD-66 | 1 | 1 | s | P |
| CD-67 | 2 | 1 | s | P |
| CD-68 | 2 | 2 | s | P |
| CD-69 | 1 | 1 | s | P |
| CD-70 | 1 | 1 | s | w |
| CD-71 | 2 | 2 | s | P |
| CD-72 | 1 | 1 | s | P |
| CD-73 | 2 | 2 | s | w |
| CD-74 | 2 | 2 | s | w |
| CD-75 | 2 | 2 | s | P |
| CD-76 | 1 | 1 | s | P |
| CD-77 | 2 | 2 | s | w |
| CD-78 | 1 | 1 | s | w |
| CD-79 | 1 | 1 | s | P |
| CD-80 | 2 | 2 | s | w |
| CD-81 | 2 | 2 | s | w |
| CD-82 | 2 | 2 | s | w |
| CD-83 | 2 | 2 | s | P |
| CD-84 | 2 | 2 | s | w |
| CD-85 | 2 | 2 | s | w |
| CD-86 | 2 | 2 | s | w |
| CD-87 | 2 | 2 | s | w |
| CD-88 | 2 | 2 | s | P |

[a] "1" for homozygous for the R149 allele; "2" for homozygous for the YZX2 allele; and "3" for heterozygous for both parental alleles.

[b] "s" and "L" represent small and large colony size, respectively;

[c] "P" and "w" represent pink and white color of colony, respectively.

**Supplemental Table 3D.** Mitochondrial inheritance in meiotic progeny of R121 x YZX1 cross.[a]

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| EF-1 | 1 | 1 | S | P |
| EF-2 | 1 | 1 | S | W |
| EF-3 | 1 | 1 | S | W |
| EF-4 | 2 | 2 | S | P |
| EF-5 | 2 | 2 | S | P |
| EF-6 | 2 | 2 | S | P |
| EF-7 | 1 | 1 | S | P |
| EF-8 | 1 | 1 | S | P |
| EF-9 | 1 | 1 | L | W |
| EF-10 | 1 | 1 | L | W |
| EF-11 | 1 | 1 | L | W |
| EF-12 | 1 | 1 | L | W |
| EF-13 | 1 | 1 | L | W |
| EF-14 | 1 | 1 | L | W |
| EF-15 | 1 | 1 | L | W |
| EF-16 | 1 | 1 | L | W |
| EF-17 | 1 | 1 | L | W |
| EF-18 | 1 | 1 | L | W |
| EF-19 | 1 | 1 | L | W |
| EF-20 | 1 | 1 | L | W |
| EF-21 | 1 | 1 | L | W |
| EF-22 | 1 | 1 | L | W |
| EF-23 | 1 | 1 | L | W |
| EF-24 | 1 | 1 | L | W |
| EF-25 | 1 | 1 | L | W |
| EF-26 | 1 | 1 | L | W |
| EF-27 | 1 | 1 | L | W |
| EF-28 | 1 | 1 | L | W |
| EF-29 | 1 | 1 | L | W |
| EF-30 | 1 | 1 | L | W |
| EF-31 | 1 | 1 | L | W |
| EF-32 | 1 | 1 | L | W |
| EF-33 | 2 | 2 | S | P |
| EF-34 | 1 | 1 | S | P |
| EF-35 | 1 | 1 | S | P |
| EF-36 | 1 | 1 | S | P |
| EF-37 | 1 | 1 | S | P |
| EF-38 | 1 | 1 | S | P |
| EF-39 | 1 | 1 | S | W |
| EF-40 | 1 | 1 | S | W |
| EF-41 | 1 | 1 | L | W |
| EF-42 | 1 | 1 | L | W |
| EF-43 | 1 | 1 | L | W |
| EF-44 | 1 | 1 | L | W |
| EF-45 | 1 | 1 | L | W |
| EF-46 | 1 | 1 | L | W |
| EF-47 | 1 | 1 | L | W |
| EF-48 | 1 | 1 | L | W |
| EF-49 | 1 | 1 | S | P |
| EF-50 | 1 | 1 | S | P |

**Supplemental Table 3D** *continued*

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| EF-51 | 1 | 1 | s | w |
| EF-52 | 1 | 1 | s | P |
| EF-53 | 2 | 2 | s | P |
| EF-54 | 1 | 1 | s | P |
| EF-55 | 1 | 1 | s | P |
| EF-56 | 2 | 2 | s | w |
| EF-57 | 1 | 1 | s | P |
| EF-58 | 1 | 1 | s | P |
| EF-59 | 1 | 1 | s | w |
| EF-60 | 1 | 1 | s | P |
| EF-61 | 1 | 1 | s | P |
| EF-62 | 1 | 1 | s | P |
| EF-63 | 1 | 1 | s | w |
| EF-64 | 2 | 2 | s | P |
| EF-65 | 1 | 1 | L | w |
| EF-66 | 1 | 1 | L | w |
| EF-67 | 1 | 1 | L | w |
| EF-68 | 1 | 1 | L | w |
| EF-69 | 1 | 1 | L | w |
| EF-70 | 1 | 1 | L | w |
| EF-71 | 1 | 1 | L | w |
| EF-72 | 1 | 1 | L | w |
| EF-73 | 1 | 1 | L | w |
| EF-74 | 1 | 1 | L | w |
| EF-75 | 1 | 1 | L | w |
| EF-76 | 1 | 1 | L | w |
| EF-77 | 1 | 1 | L | w |
| EF-78 | 1 | 1 | L | w |
| EF-79 | 1 | 1 | L | w |
| EF-80 | 1 | 1 | L | w |
| EF-81 | 1 | 1 | L | w |
| EF-82 | 1 | 1 | L | w |
| EF-83 | 1 | 1 | L | w |
| EF-84 | 1 | 1 | L | w |
| EF-85 | 1 | 1 | L | w |
| EF-86 | 1 | 1 | L | w |
| EF-87 | 1 | 1 | L | w |
| EF-88 | 1 | 1 | L | w |

[a] "1" for homozygous for the R121 allele; "2" for homozygous for the YZX1 allele; and "3" for both parental alleles.

[b] "s" and "L" represent small and large colony size, respectively.

[c] "P" and "w" represent pink and white color of colony, respectively.

**Supplemental Table 3E.** Mitochondrial inheritance in meiotic progeny of R159 x YZX1 cross.[a]

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| FG-1 | 2 | 2 | S | W |
| FG-2 | 2 | 2 | L | W |
| FG-3 | 2 | 2 | S | W |
| FG-4 | 1 | 1 | S | W |
| FG-5 | 1 | 1 | L | W |
| FG-6 | 1 | 1 | L | W |
| FG-7 | 1 | 1 | S | P |
| FG-8 | 1 | 1 | S | W |
| FG-9 | 1 | 1 | L | W |
| FG-10 | 1 | 1 | L | W |
| FG-11 | 1 | 1 | L | P |
| FG-12 | 1 | 1 | L | W |
| FG-13 | 1 | 1 | L | W |
| FG-15 | 1 | 1 | L | W |
| FG-16 | 1 | 1 | L | W |
| FG-17 | 2 | 2 | S | W |
| FG-18 | 2 | 2 | S | W |
| FG-19 | 1 | 1 | S | W |
| FG-20 | 1 | 1 | S | W |
| FG-21 | 1 | 1 | S | W |
| FG-23 | 1 | 1 | S | W |
| FG-24 | 2 | 2 | S | W |
| FG-25 | 1 | 1 | S | W |
| FG-26 | 2 | 2 | S | W |
| FG-27 | 2 | 2 | S | P |
| FG-28 | 1 | 1 | S | W |
| FG-29 | 2 | 2 | S | W |
| FG-30 | 1 | 1 | S | W |
| FG-31 | 2 | 2 | S | W |
| FG-32 | 1 | 1 | S | W |
| FG-33 | 2 | 2 | S | W |
| FG-34 | 1 | 1 | S | P |
| FG-35 | 1 | 1 | S | W |
| FG-36 | 1 | 1 | S | W |
| FG-37 | 2 | 2 | S | W |
| FG-38 | 2 | 2 | S | W |
| FG-40 | 2 | 2 | S | W |
| FG-41 | 2 | 2 | S | W |
| FG-42 | 2 | 2 | S | W |
| FG-43 | 2 | 2 | S | W |
| FG-44 | 1 | 1 | S | W |
| FG-45 | 2 | 2 | S | W |
| FG-46 | 2 | 2 | S | W |
| FG-47 | 1 | 1 | S | P |
| FG-48 | 2 | 2 | S | P |
| FG-50 | 2 | 2 | S | W |
| FG-52 | 1 | 1 | L | W |
| FG-53 | 2 | 2 | L | P |
| FG-55 | 1 | 1 | L | W |
| FG-56 | 1 | 1 | L | P |

**Supplemental Table 3E** *continued*

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] |
|---|---|---|---|---|
| FG-57 | 2 | 2 | s | w |
| FG-59 | 2 | 2 | s | w |
| FG-60 | 3 | 1 | s | w |
| FG-61 | 1 | 1 | L | w |
| FG-62 | 1 | 1 | L | P |
| FG-63 | 2 | 2 | s | w |
| FG-66 | 1 | 1 | s | w |
| FG-67 | 1 | 1 | s | w |
| FG-68 | 1 | 1 | s | w |
| FG-69 | 1 | 1 | s | w |
| FG-70 | 1 | 1 | s | w |
| FG-71 | 1 | 1 | s | w |
| FG-72 | 1 | 1 | s | w |
| FG-73 | 1 | 1 | s | w |
| FG-74 | 1 | 1 | s | w |
| FG-75 | 3 | [c] | s | P |
| FG-76 | 1 | 1 | s | w |
| FG-77 | 1 | 1 | s | w |
| FG-78 | 1 | 1 | s | w |
| FG-79 | 1 | 1 | s | w |
| FG-80 | 1 | 1 | s | w |
| FG-81 | 2 | 2 | s | w |
| FG-82 | 1 | 1 | s | w |
| FG-83 | 1 | 1 | s | w |
| FG-84 | 1 | 1 | s | w |
| FG-85 | 1 | 1 | s | w |
| FG-86 | 1 | 1 | s | P |
| FG-87 | 1 | 1 | s | w |
| FG-88 | 1 | 1 | s | w |

[a] "1" for homozygous for the R159 allele; "2" for homozygous for the YZX1 allele; and "3" for heterozygous for both parental alleles.

[b] "s" and "L" represent small and large colony size, respectively.

[c] "P" and "w" represent pink and white color of colony, respectively.

**Supplemental Table 3F.** Mitochondrial inheritance in fusants of R103 x YZX2 cross.[a]

| Strain ID | NAD5 | ND2 digest |
|---|---|---|
| R3Z -1 | 2 | 2 |
| R3Z -2 | 2 | 2 |
| R3Z -3 | 2 | 2 |
| R3Z -4 | 2 | 2 |
| R3Z -5 | 1 | 1 |
| R3Z -6 | 1 | 1 |
| R3Z -7 | 2 | 2 |
| R3Z -8 | 2 | 2 |
| R3Z -9 | 2 | 2 |
| R3Z -10 | 2 | 2 |
| R3Z -11 | 1 | 1 |
| R3Z -12 | 3 | 3 |
| R3Z -13 | 1 | 1 |
| R3Z -14 | 2 | 2 |
| R3Z -15 | 2 | 2 |
| R3Z -16 | 2 | 2 |
| R3Z -17 | 2 | 1 |
| R3Z -18 | 1 | 1 |
| R3Z -19 | 2 | 2 |
| R3Z -20 | 2 | 2 |
| R3Z -21 | 2 | 3 |
| R3Z -22 | 1 | 1 |
| R3Z -23 | 1 | 1 |
| R3Z -24 | 2 | 2 |
| R3Z -25 | 2 | 2 |
| R3Z -26 | 2 | 2 |
| R3Z -27 | 3 | 1 |
| R3Z -28 | 1 | 2 |
| R3Z -29 | 1 | 1 |
| R3Z -30 | 1 | 1 |
| R3Z -31 | 1 | 1 |
| R3Z -32 | 2 | 2 |
| R3Z -33 | 2 | 2 |
| R3Z -34 | 3 | 2 |
| R3Z -35 | 2 | 2 |
| R3Z -36 | 1 | 1 |
| R3Z -37 | 1 | 1 |
| R3Z -38 | 2 | 2 |
| R3Z -39 | 2 | 2 |
| R3Z -40 | 1 | 1 |
| R3Z -41 | 2 | 2 |
| R3Z -42 | 2 | 2 |
| R3Z -43 | 1 | 1 |
| R3Z -44 | 1 | 1 |
| R3Z -45 | 2 | 2 |
| R3Z -46 | 1 | 1 |
| R3Z -47 | 1 | 1 |
| R3Z -48 | 2 | 2 |
| R3Z -49 | 2 | 2 |

**Supplemental Table 3F** *continued*

| Strain ID | NAD5 | ND2 digest |
|---|---|---|
| R3Z-50 | 1 | 1 |
| R3Z-51 | 2 | 2 |
| R3Z-52 | 1 | 1 |
| R3Z-53 | 1 | 1 |
| R3Z-54 | 1 | 1 |
| R3Z-55 | 1 | 1 |
| R3Z-56 | 2 | 2 |
| R3Z-57 | 1 | 1 |
| R3Z-58 | 2 | 2 |
| R3Z-59 | 2 | 2 |
| R3Z-60 | 2 | 2 |
| R3Z-61 | 2 | 2 |
| R3Z-62 | 3 | 1 |
| R3Z-63 | 2 | 2 |
| R3Z-64 | 2 | 2 |
| R3Z-65 | 2 | 2 |
| R3Z-66 | 2 | 2 |
| R3Z-67 | 1 | 1 |
| R3Z-68 | 1 | 1 |
| R3Z-69 | 1 | 1 |
| R3Z-70 | 2 | 2 |
| R3Z-71 | 2 | 1 |
| R3Z-72 | 1 | 2 |
| R3Z-73 | 2 | 2 |
| R3Z-74 | 1 | 1 |
| R3Z-75 | 2 | 2 |
| R3Z-76 | 2 | 2 |
| R3Z-77 | 2 | 2 |
| R3Z-78 | 1 | 1 |
| R3Z-79 | 3 | 2 |
| R3Z-80 | 1 | 1 |
| R3Z-81 | 2 | 2 |
| R3Z-82 | 2 | 2 |
| R3Z-83 | 2 | 1 |
| R3Z-84 | 1 | 1 |
| R3Z-85 | 1 | 1 |
| R3Z-86 | 1 | 1 |
| R3Z-87 | 1 | 1 |
| R3Z-88 | 2 | 2 |

[a] "1" for homozygous for the R103 allele; "2" for homozygous for the YZX2 allele; and "3" for heterozygous for both parental alleles. All fusants were wild-type G418-resistant and produced white colonies.

**Supplemental Table 3G.** Mitochondrial inheritance in fusants of R159 x YZX1 cross.[a]

| Strain ID | NAD5 | ND2 digest |
|---|---|---|
| R9Z-1 | 1 | 1 |
| R9Z-2 | 2 | 2 |
| R9Z-3 | 1 | 1 |
| R9Z-4 | 1 | 1 |
| R9Z--5 | 1 | 1 |
| R9Z-7 | 1 | 1 |
| R9Z-8 | 1 | 1 |
| R9Z-9 | 2 | 2 |
| R9Z-11 | 1 | 1 |
| R9Z-12 | 3 | 1 |
| R9Z-13 | 1 | 1 |
| R9Z-14 | 1 | 1 |
| R9Z-15 | 3 | 1 |
| R9Z-16 | 2 | 2 |
| R9Z-17 | 1 | 1 |
| R9Z-18 | 1 | 1 |
| R9Z-19 | 1 | 1 |
| R9Z-21 | 1 | 1 |
| R9Z-22 | 2 | 2 |
| R9Z-23 | 3 | 1 |
| R9Z-24 | 1 | 1 |
| R9Z-25 | 2 | 2 |
| R9Z-26 | 1 | 1 |
| R9Z-27 | 1 | 1 |
| R9Z-28 | 1 | 1 |
| R9Z-29 | 1 | 2 |
| R9Z-30 | 1 | 1 |
| R9Z-31 | 1 | 1 |
| R9Z-32 | 1 | 1 |
| R9Z-33 | 1 | 1 |
| R9Z-34 | 1 | 1 |
| R9Z-35 | 1 | 1 |
| R9Z-36 | 1 | 1 |
| R9Z-37 | 1 | 1 |
| R9Z-38 | 1 | 1 |
| R9Z-39 | 1 | 1 |
| R9Z-40 | 1 | 1 |
| R9Z-41 | 1 | 1 |
| R9Z-42 | 1 | 1 |
| R9Z-43 | 1 | 1 |
| R9Z-44 | 1 | 1 |
| R9Z-45 | 2 | 2 |
| R9Z-46 | 1 | 1 |
| R9Z-47 | 1 | 1 |
| R9Z-48 | 1 | 1 |
| R9Z-49 | 1 | 1 |
| R9Z-50 | 1 | 1 |
| R9Z-51 | 1 | 1 |
| R9Z-52 | 1 | 1 |

**Supplemental Table 3G** *continued*

| Strain ID | NAD5 | ND2 digest |
|---|---|---|
| R9Z-53 | 1 | 1 |
| R9Z-54 | 1 | 1 |
| R9Z-55 | 1 | 1 |
| R9Z-56 | 1 | 1 |
| R9Z-57 | 1 | 1 |
| R9Z-58 | 1 | 1 |
| R9Z-59 | 3 | 1 |
| R9Z-60 | 1 | 1 |
| R9Z-61 | 1 | 1 |
| R9Z-62 | 1 | 1 |
| R9Z-63 | 1 | 1 |
| R9Z-64 | 1 | 1 |
| R9Z-65 | 1 | 1 |
| R9Z-66 | 1 | 1 |
| R9Z-67 | 1 | 1 |
| R9Z-68 | 1 | 1 |
| R9Z-69 | 1 | 1 |
| R9Z-70 | 1 | 1 |
| R9Z-71 | 1 | 1 |
| R9Z-72 | 1 | 1 |
| R9Z-73 | 1 | 1 |
| R9Z-74 | 1 | 1 |
| R9Z-75 | 1 | 1 |
| R9Z-76 | 2 | 2 |
| R9Z-77 | 1 | 1 |
| R9Z-78 | 1 | 1 |
| R9Z-79 | 1 | 1 |
| R9Z-80 | 1 | 1 |
| R9Z-81 | 1 | 1 |
| R9Z-82 | 2 | 2 |
| R9Z-83 | 1 | 1 |
| R9Z-84 | 1 | 1 |
| R9Z-85 | 2 | 2 |
| R9Z-86 | 2 | 2 |
| R9Z-87 | 1 | 1 |
| R9Z-88 | 1 | 1 |

[a] "1" for homozygous for the R159 allele; "2" for homozygous for the YZX1 allele; and "3" for heterozygous for both parental alleles. All fusants were wild-type G418-resistant and produced white colonies.

**Supplemental Table 4.** Mitochondrial genotype of the basidiospores derived by micromanipuation from the hyphae of the R121 x YZX1 cross.[a]

| Strain ID | NAD5 | ND2 digest | Colony size[b] | Colony color[c] | Ade[d] | G418[e] |
|---|---|---|---|---|---|---|
| M-R121-1 | 1 | 1 | s | w | + | s |
| M-R121-2 | 2 | 2 | s | w | + | s |
| M-R121-3 | 3 | 1 | s | w | + | s |
| M-R121-4 | 1 | 1 | s | w | + | s |
| M-R121-5 | 3 | 1 | s | w | + | s |
| M-R121-6 | 1 | 1 | s | w | + | s |
| M-R121-7 | 1 | 1 | s | w | + | s |
| M-R121-8 | 1 | 1 | s | w | + | s |
| M-R121-9 | 1 | 1 | s | w | + | s |
| M-R121-10 | 1 | 1 | s | w | + | R |
| M-R121-11 | 1 | 1 | s | w | + | R |
| M-R121-12 | 2 | 2 | s | w | + | s |
| M-R121-13 | 1 | 1 | s | w | + | s |
| M-R121-14 | 1 | 1 | s | w | + | s |
| M-R121-15 | 3 | 1 | s | w | + | s |
| M-R121-16 | 1 | 1 | s | P | – | R |
| M-R121-17 | 1 | 1 | s | w | + | s |
| M-R121-18 | 1 | 1 | s | w | + | s |
| M-R121-19 | 1 | ? | s | w | + | R |
| M-R121-20 | 1 | 1 | s | w | + | s |
| M-R121-21 | 1 | 1 | s | w | + | R |
| M-R121-22 | 1 | 1 | L | w | + | s |
| M-R121-23 | 1 | 1 | L | w | + | s |
| M-R121-24 | 1 | 1 | s | P | – | R |
| M-R121-25 | 1 | 1 | s | w | + | s |
| M-R121-26 | 1 | 1 | L | w | + | s |
| M-R121-27 | 1 | 1 | L | w | + | s |
| M-R121-28 | 1 | 1 | s | w | + | s |
| M-R121-29 | 1 | 1 | s | w | + | s |
| M-R121-30 | 1 | 1 | L | w | + | s |
| M-R121-31 | 1 | 1 | s | w | + | R |
| M-R121-32 | 1 | 1 | L | w | + | s |
| M-R121-33 | 1 | 1 | s | w | + | s |
| M-R121-34 | 1 | 1 | L | w | + | s |
| M-R121-35 | 1 | 1 | s | w | + | s |
| M-R121-36 | 1 | 1 | s | P | – | s |
| M-R121-37 | 2 | 2 | s | w | + | s |
| M-R121-38 | 1 | 1 | s | w | + | s |
| M-R121-39 | 1 | 1 | s | P | – | s |
| M-R121-40 | 1 | 1 | L | w | + | s |
| M-R121-41 | 1 | 1 | s | w | + | s |
| M-R121-42 | ? | 1 | s | w | + | s |
| M-R121-43 | 1 | 1 | s | w | + | s |
| M-R121-44 | 1 | 1 | s | P | – | s |
| M-R121-45 | 1 | ? | s | P | – | R |
| M-R121-46 | 1 | 1 | s | w | + | R |
| M-R121-47 | 1 | 1 | L | w | + | R |
| M-R121-48 | 1 | 1 | s | w | + | s |
| M-R121-49 | 2 | 2 | s | w | + | R |

**Supplemental Table 4** *continued*

[a] "1" for homozygous for the R121 allele; "2" for homozygous for the YZX1 allele; and "3" for heterozygous for both parental alleles; "?" missing data.

[b] "s" and "L" represent small and large colony size, respectively.

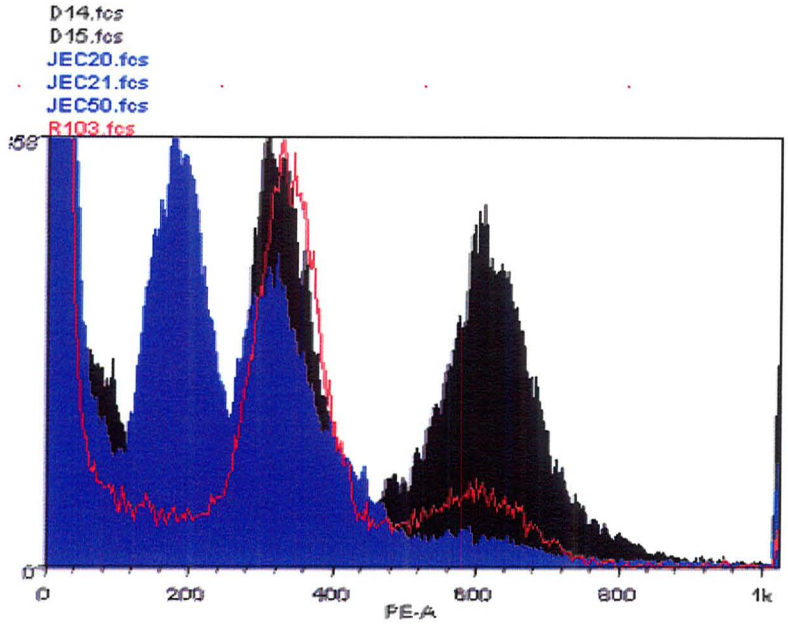
[c] "P" and "w" represent pink and white color of colony, respectively.

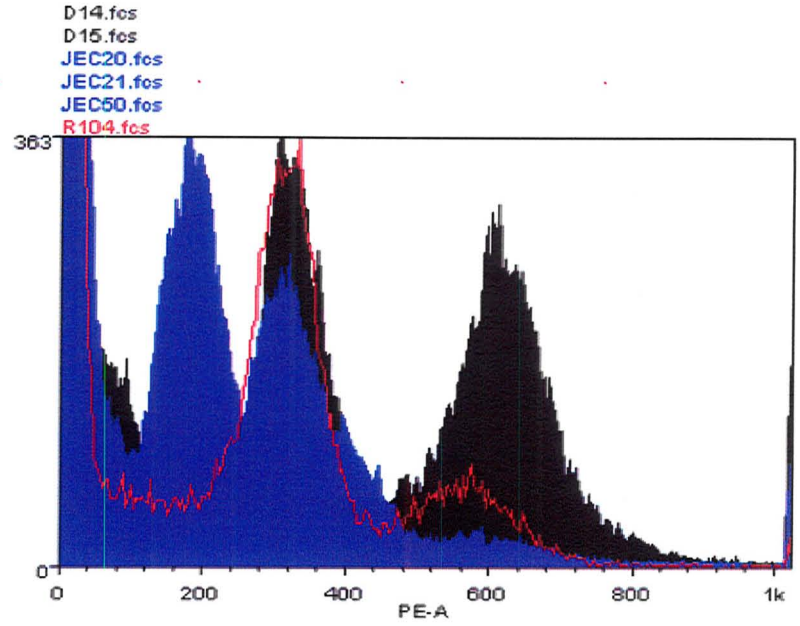
[d] "+" and "-" represent autotrophic and auxotrophic for adenine, respectively.

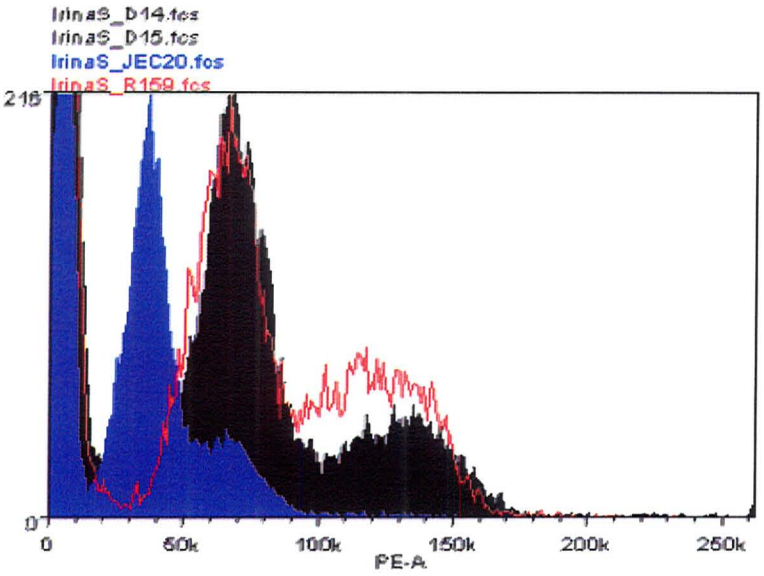
[e] "R" and "s" represent resistant and susceptible to the antibiotic G418, respectively.

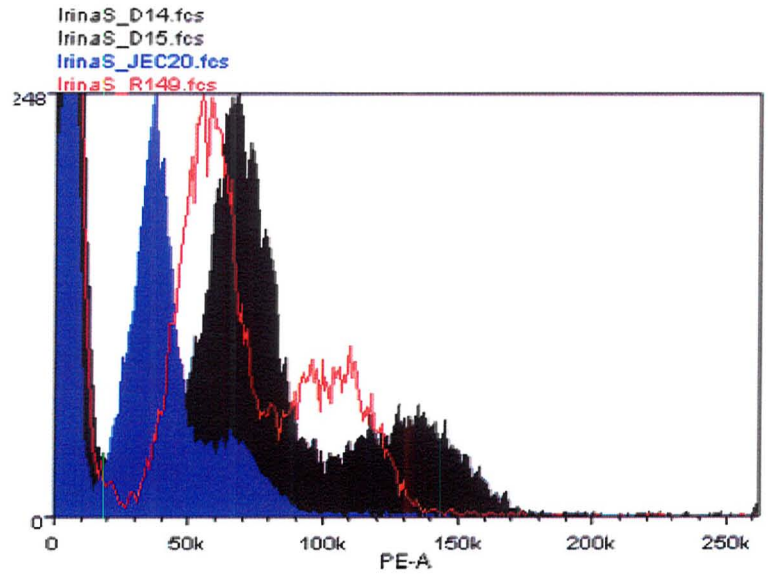
## SUPPLEMENTARY FIGURES

**Supplemental Figure 2.1** Overlapping FACS histograms. The black filled histograms are of the control diploid strains D14 and D15. The blue filled histograms are of the control haploid strains JEC20, JEC21, and JEC50. The red line histogram is of a sample strain.

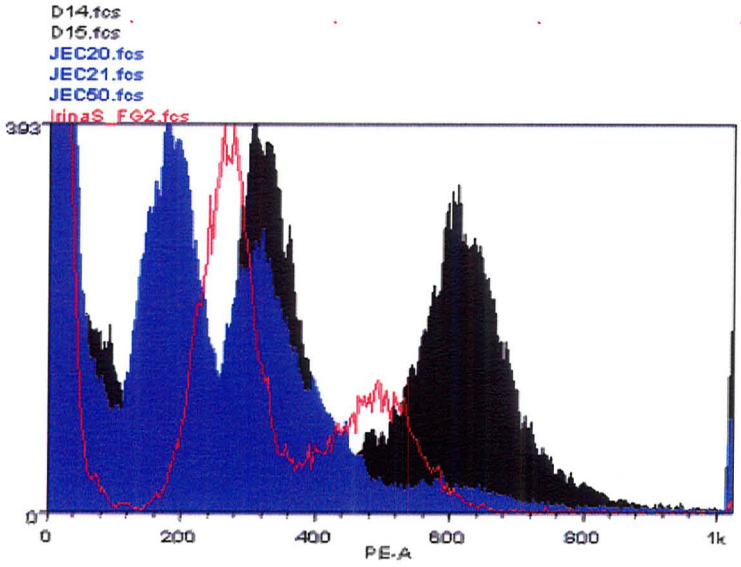

D14.fcs
D15.fcs
JEC20.fcs
JEC21.fcs
JEC50.fcs
R103.fcs
0
0
200
400
600
800
1k
PE-A

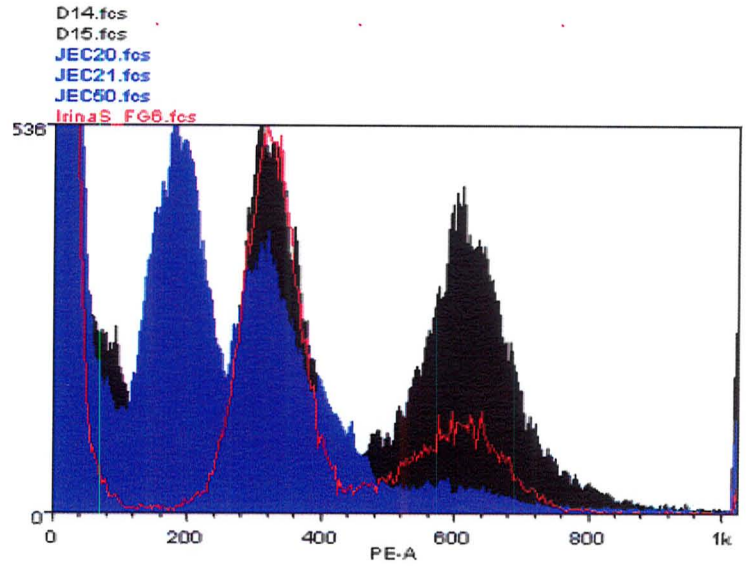

D14.fcs
D15.fcs
JEC20.fcs
JEC21.fcs
JEC50.fcs
R104.fcs
363
0
0
200
400
600
800
1k
PE-A

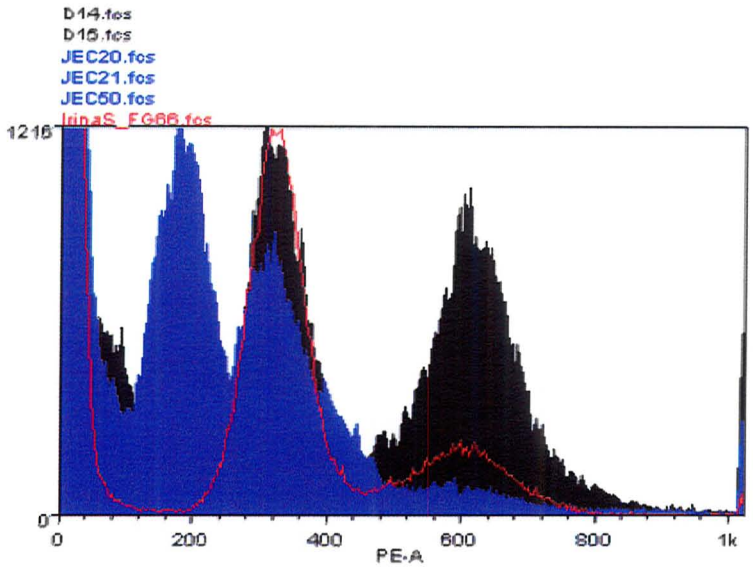

IrinaS_D14.fcs
IrinaS_D15.fcs
IrinaS_JEC20.fcs
IrinaS_R159.fcs
0
0
50k
100k
150k
200k
250k
PE-A

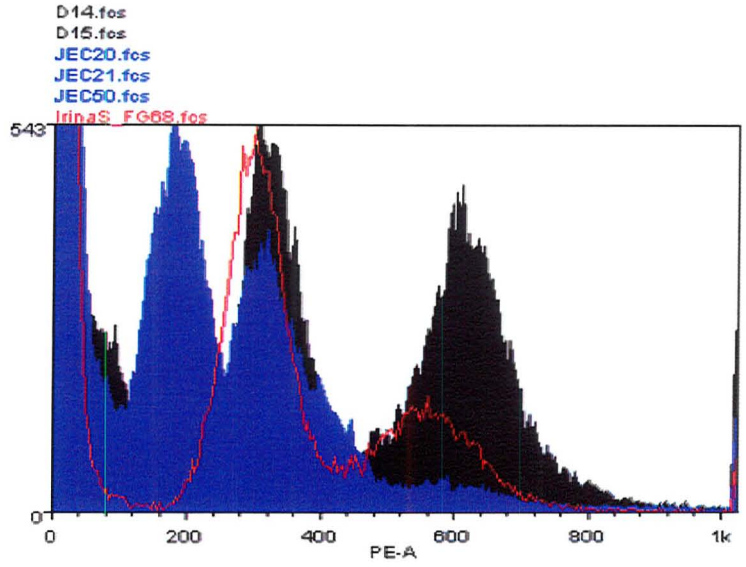

IrinaS_D14.fcs
IrinaS_D15.fcs
IrinaS_JEC20.fcs
IrinaS_R149.fcs
248
0
0
50k
100k
150k
200k
250k
PE-A

**Supplemental Figure 2.1** *continued*

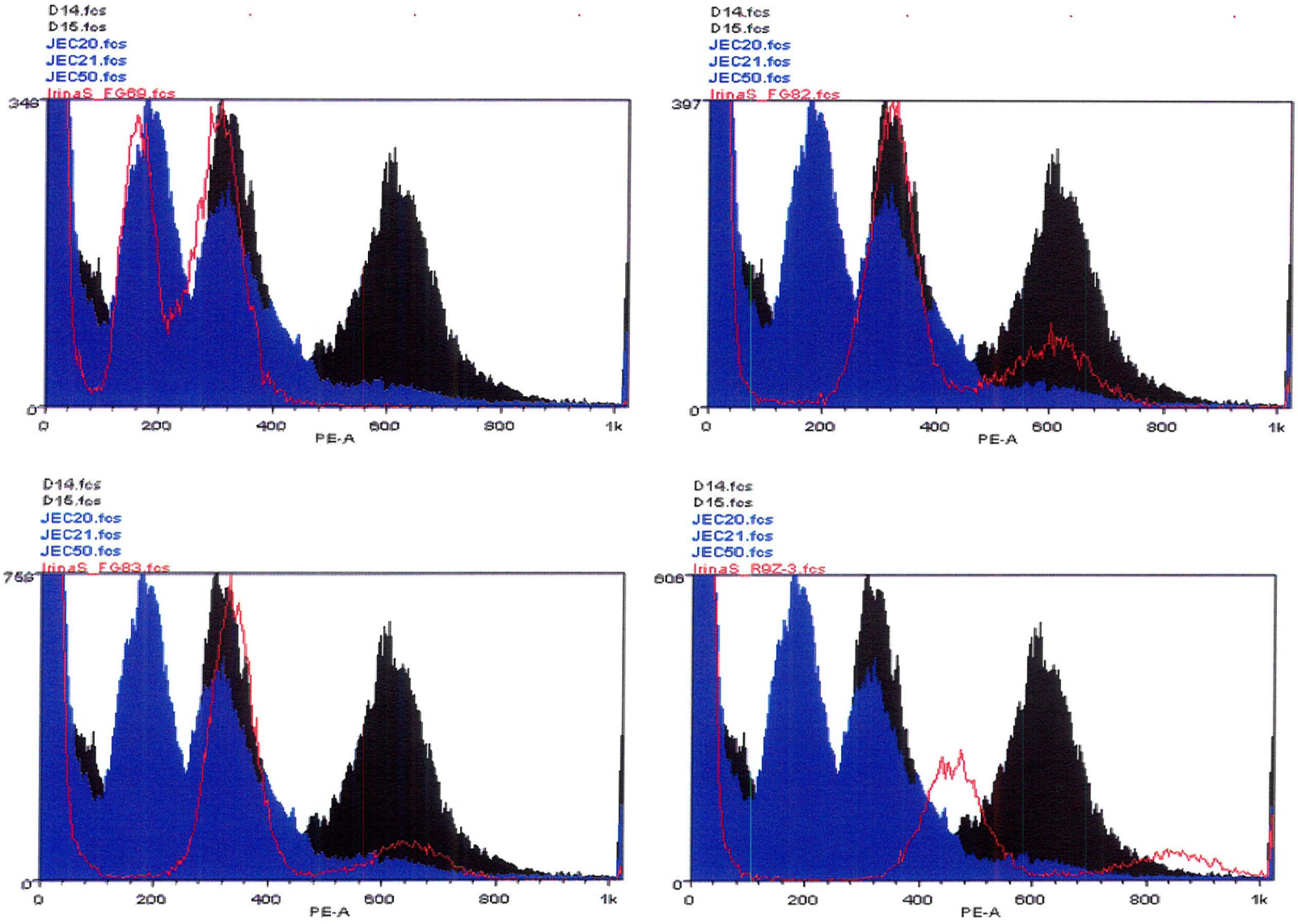


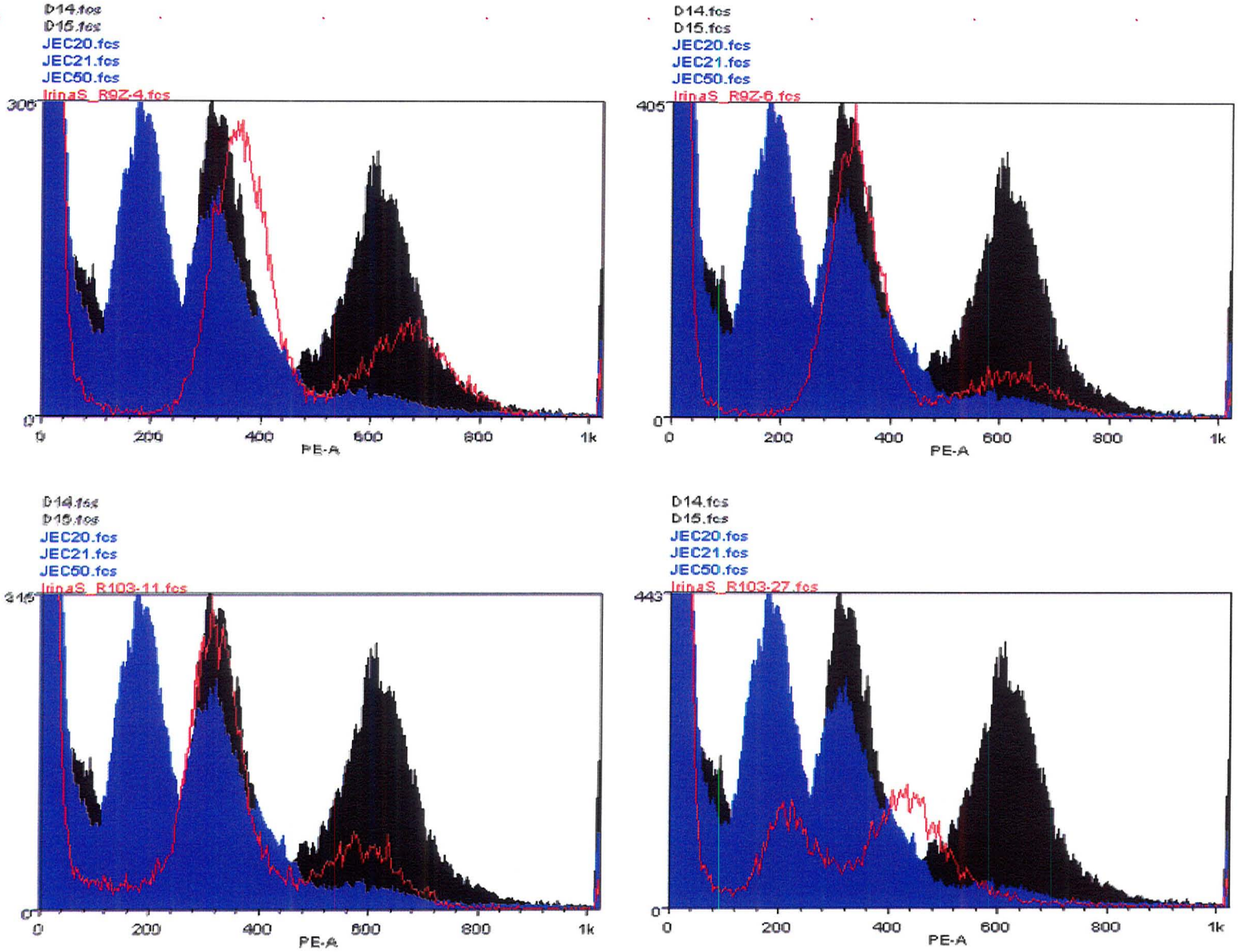



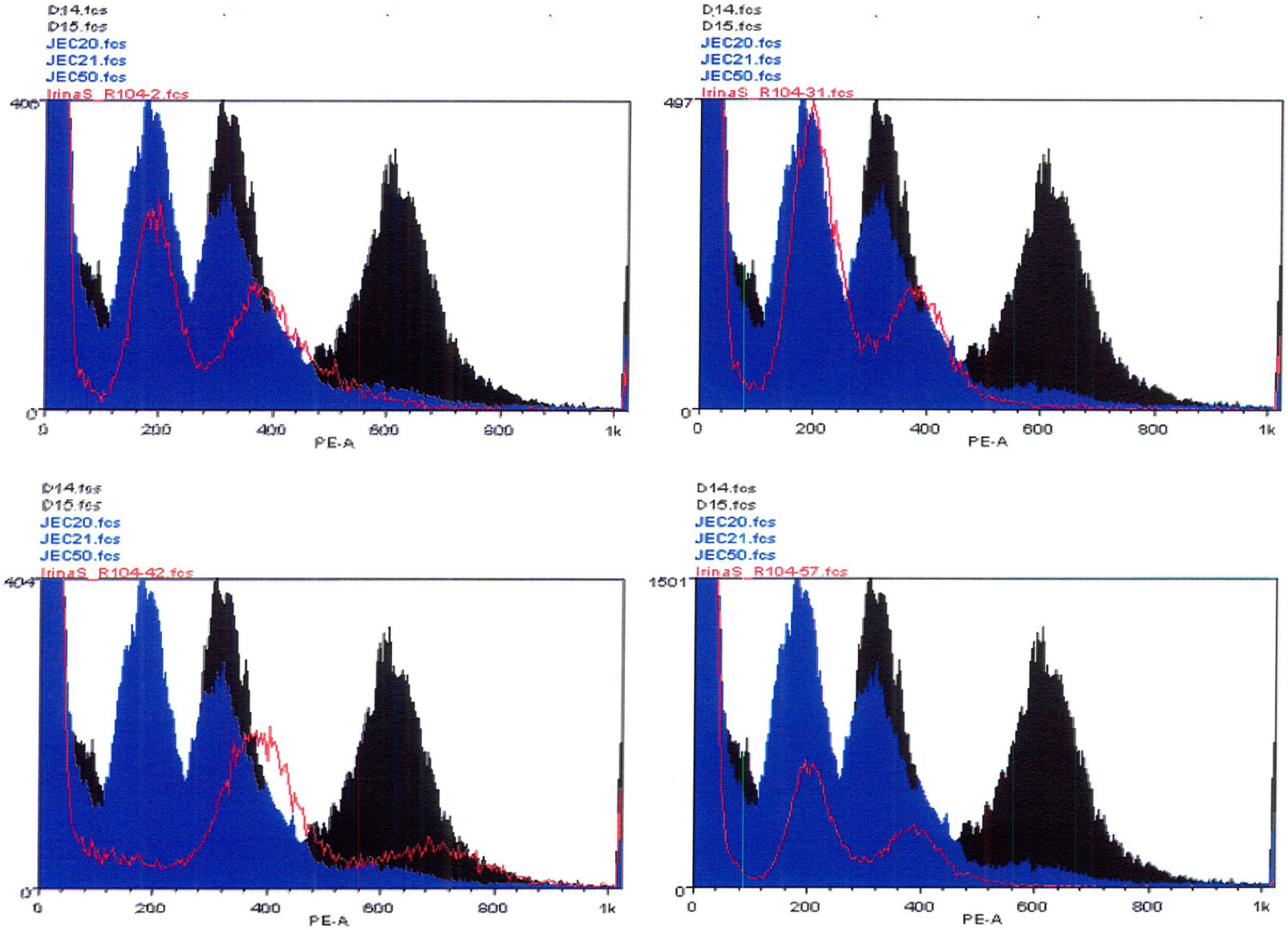
**Supplemental Figure 2.1** *continued*



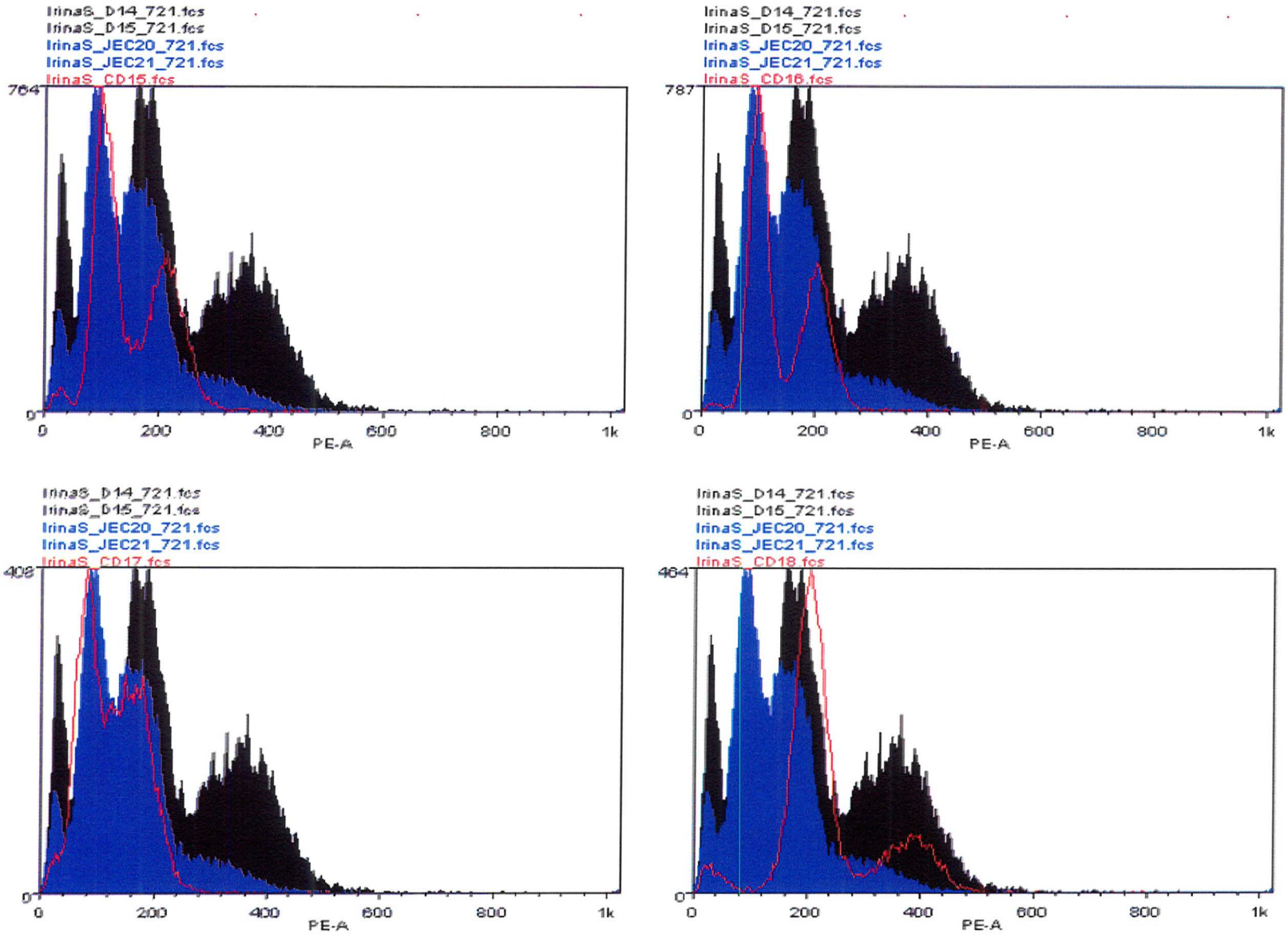
**Supplemental Figure 2.1** *continued*



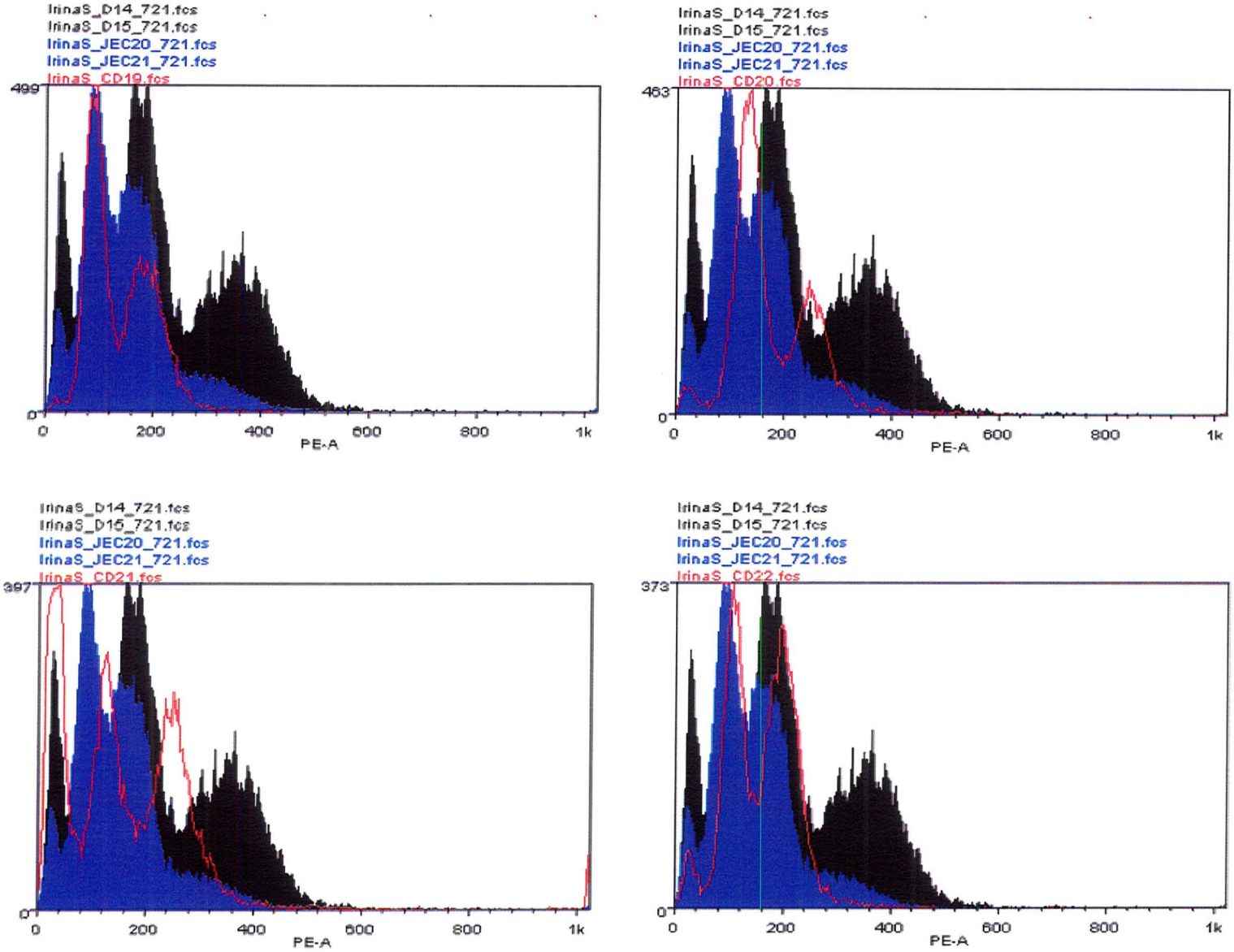
**Supplemental Figure 2.1** *continued*



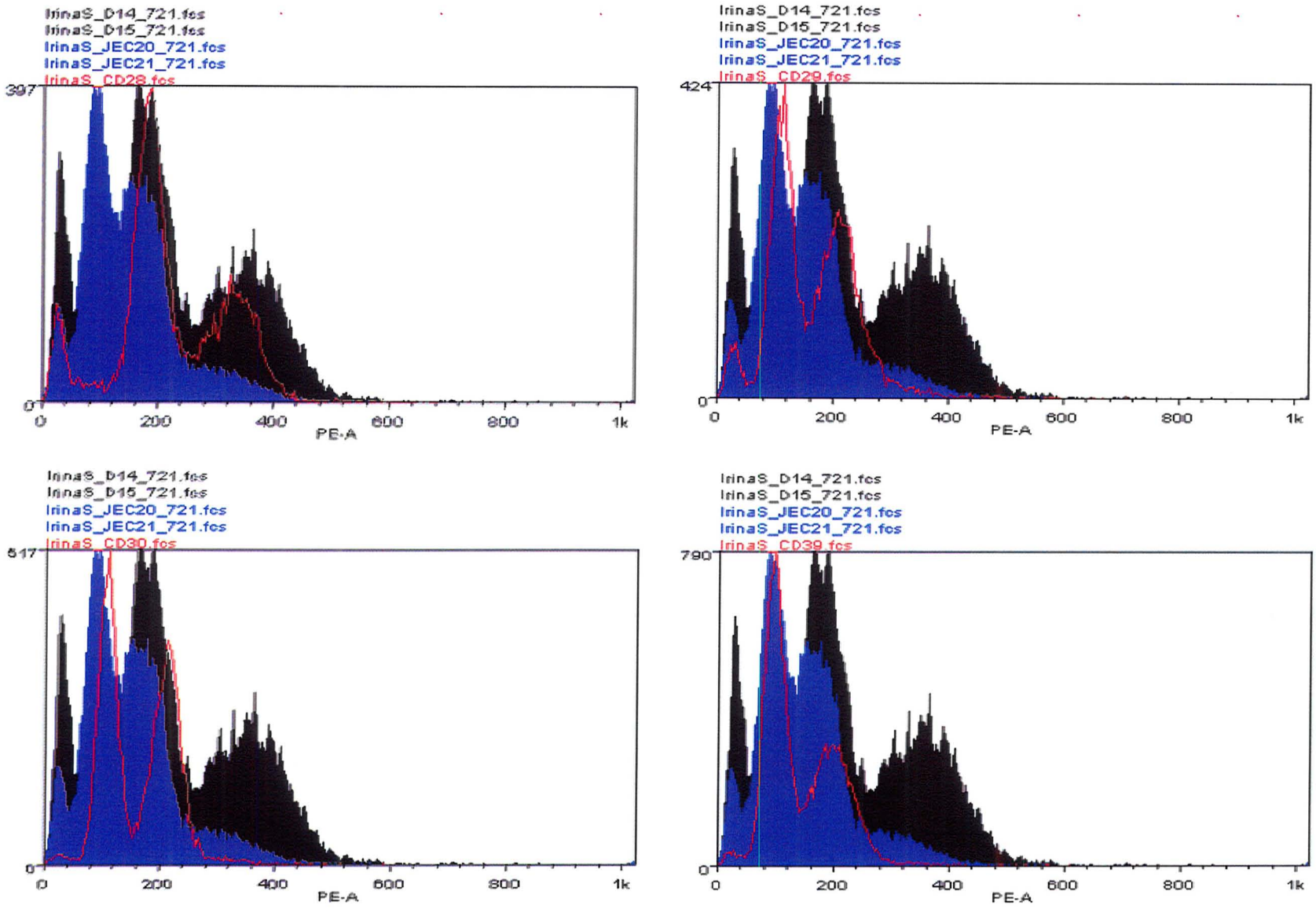
**Supplemental Figure 2.1** *continued*



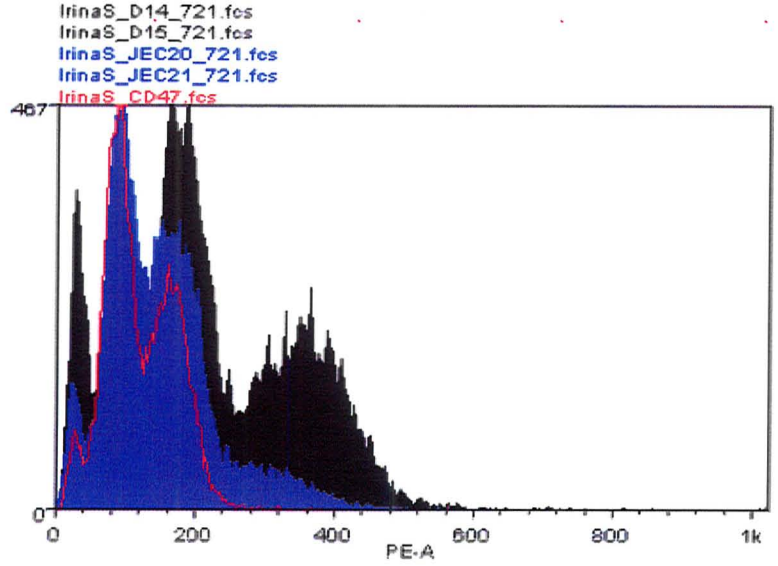
**Supplemental Figure 2.1** *continued*

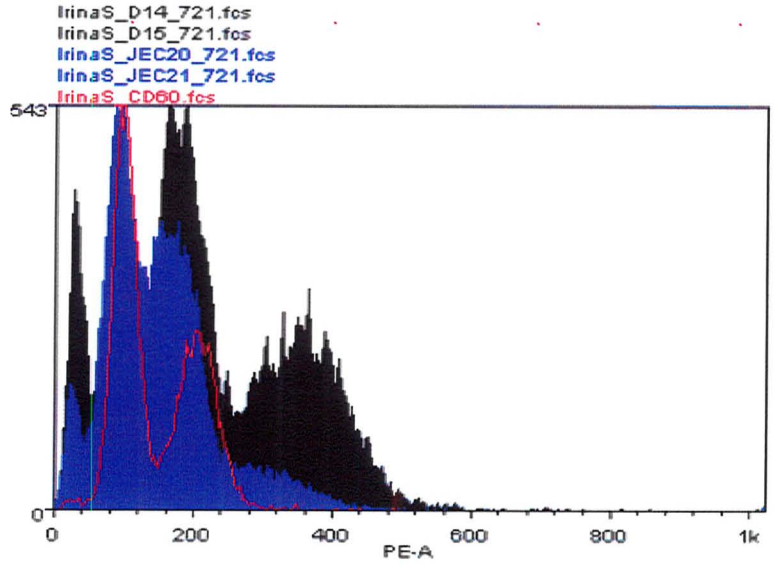


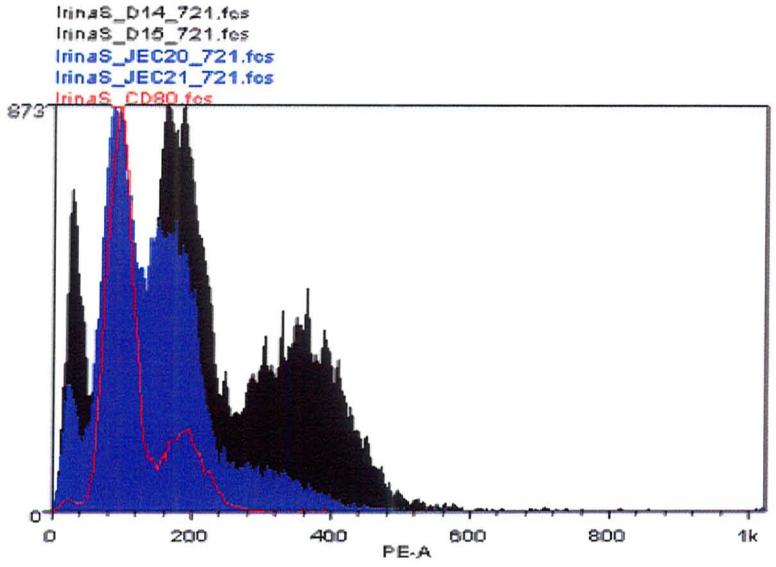
**Supplemental Figure 2.1** *continued*

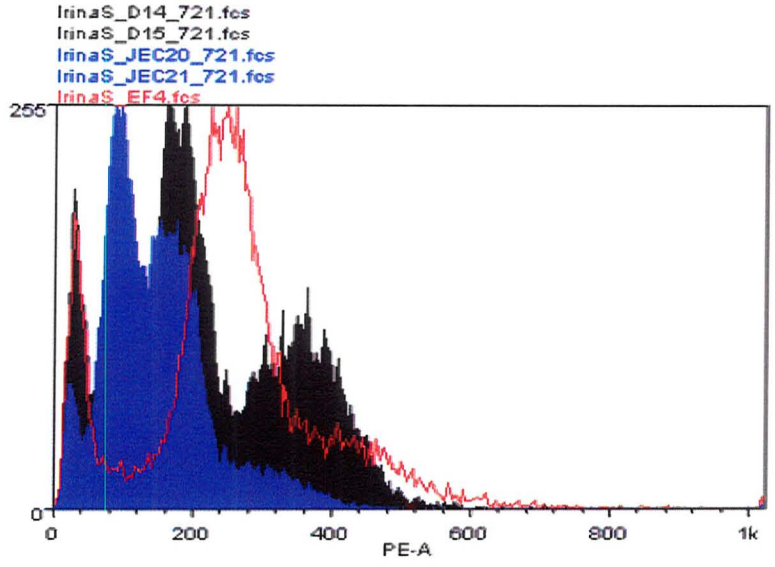


**Supplemental Figure 2.1** *continued*

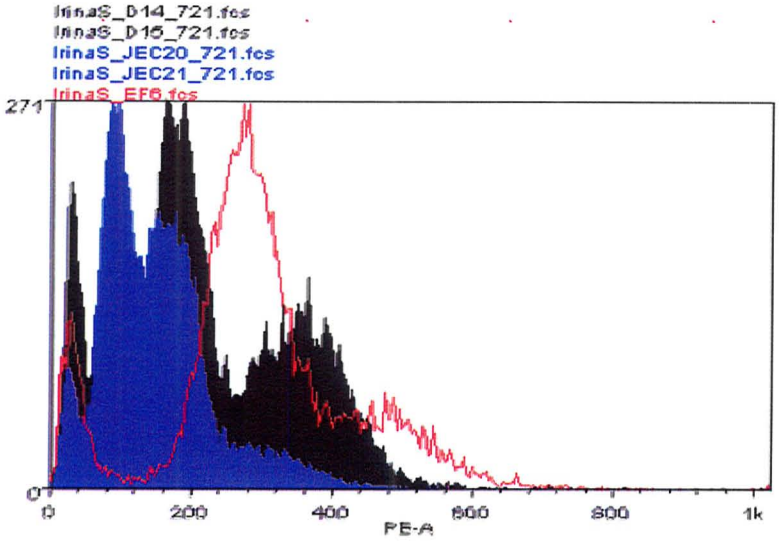


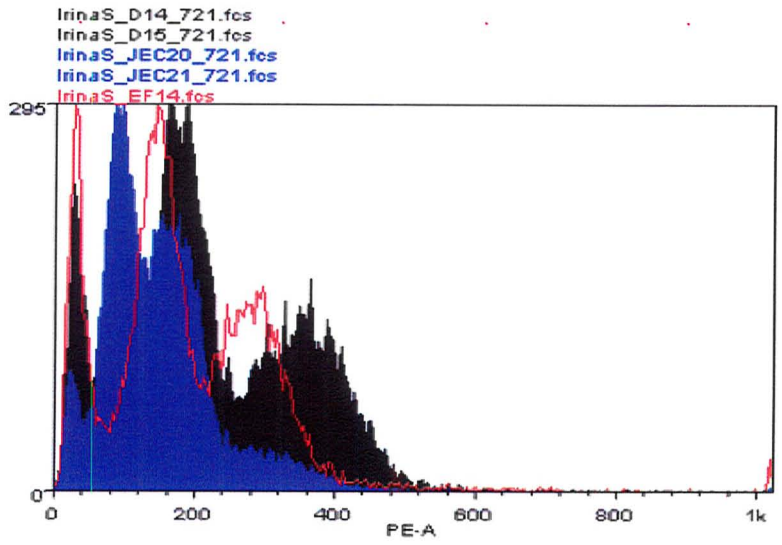



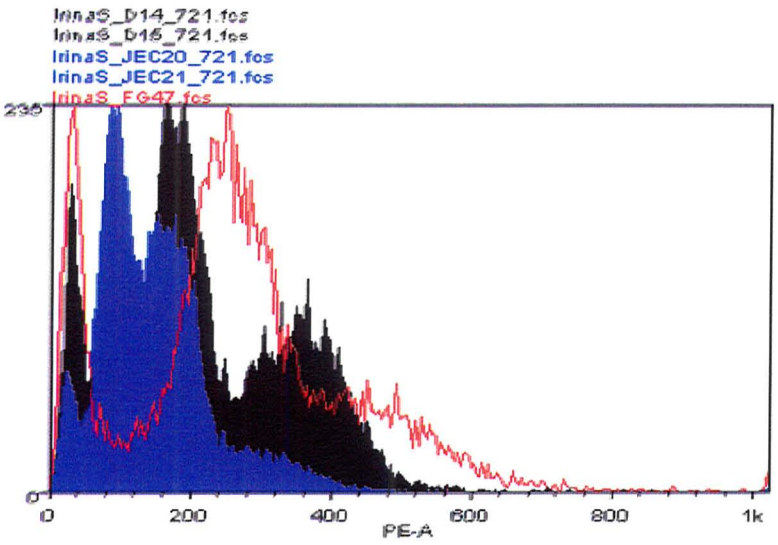



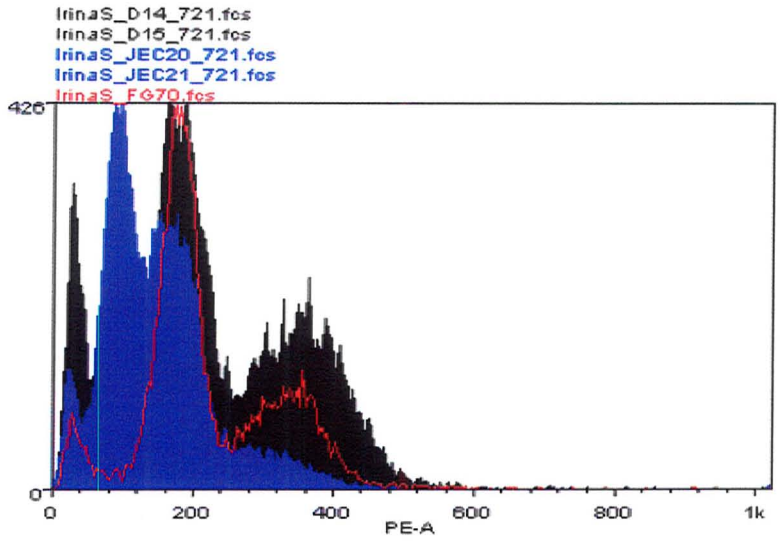



**Supplemental Figure 2.1** *continued*

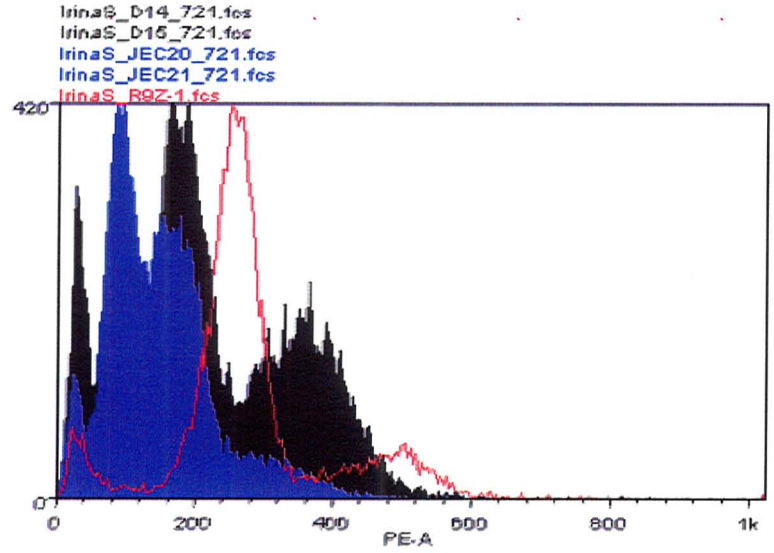


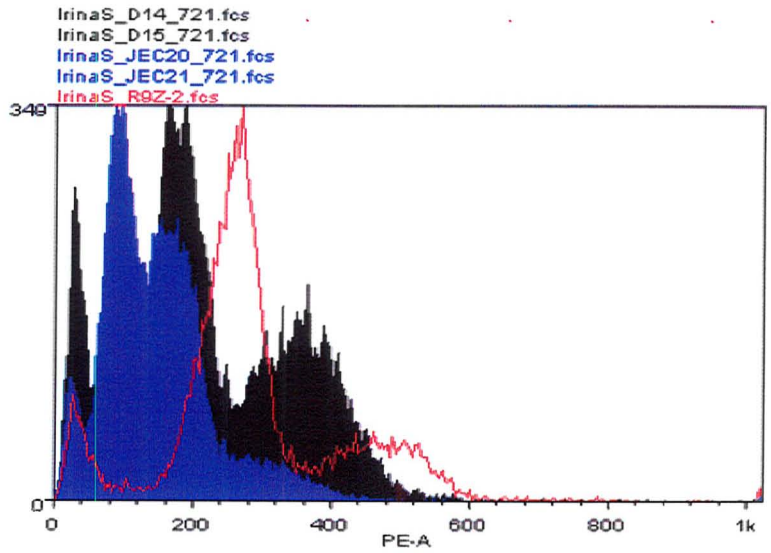



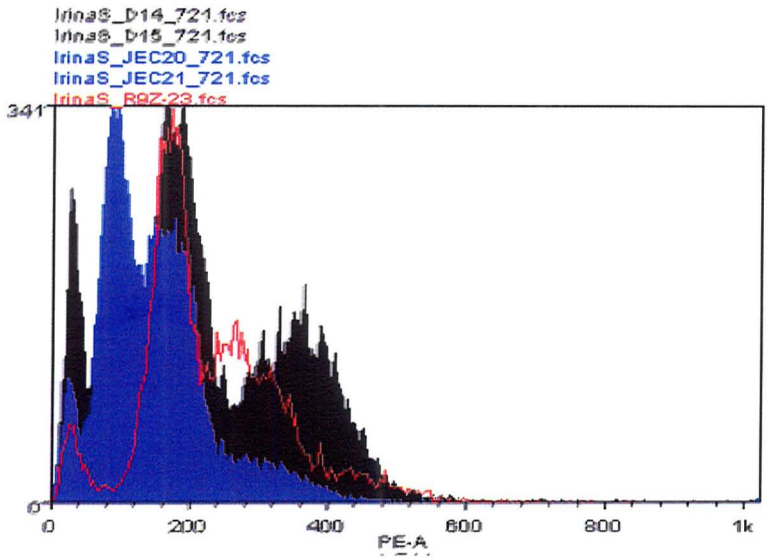



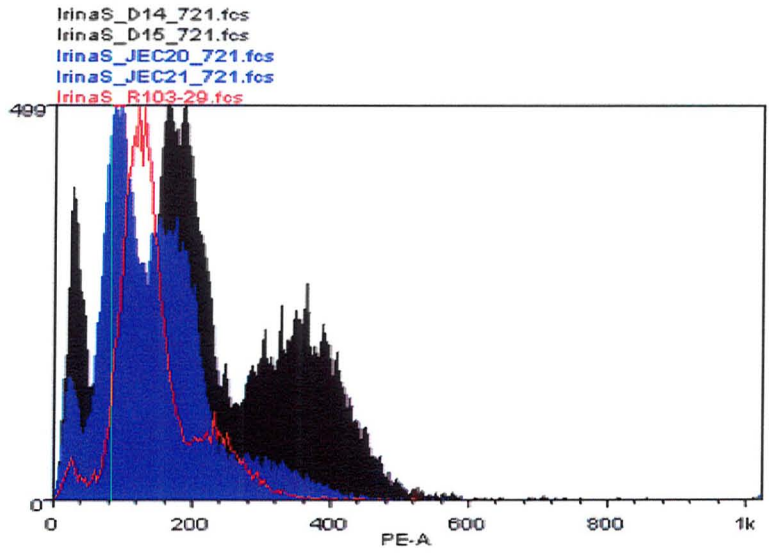



**Supplemental Figure 2.1** *continued*



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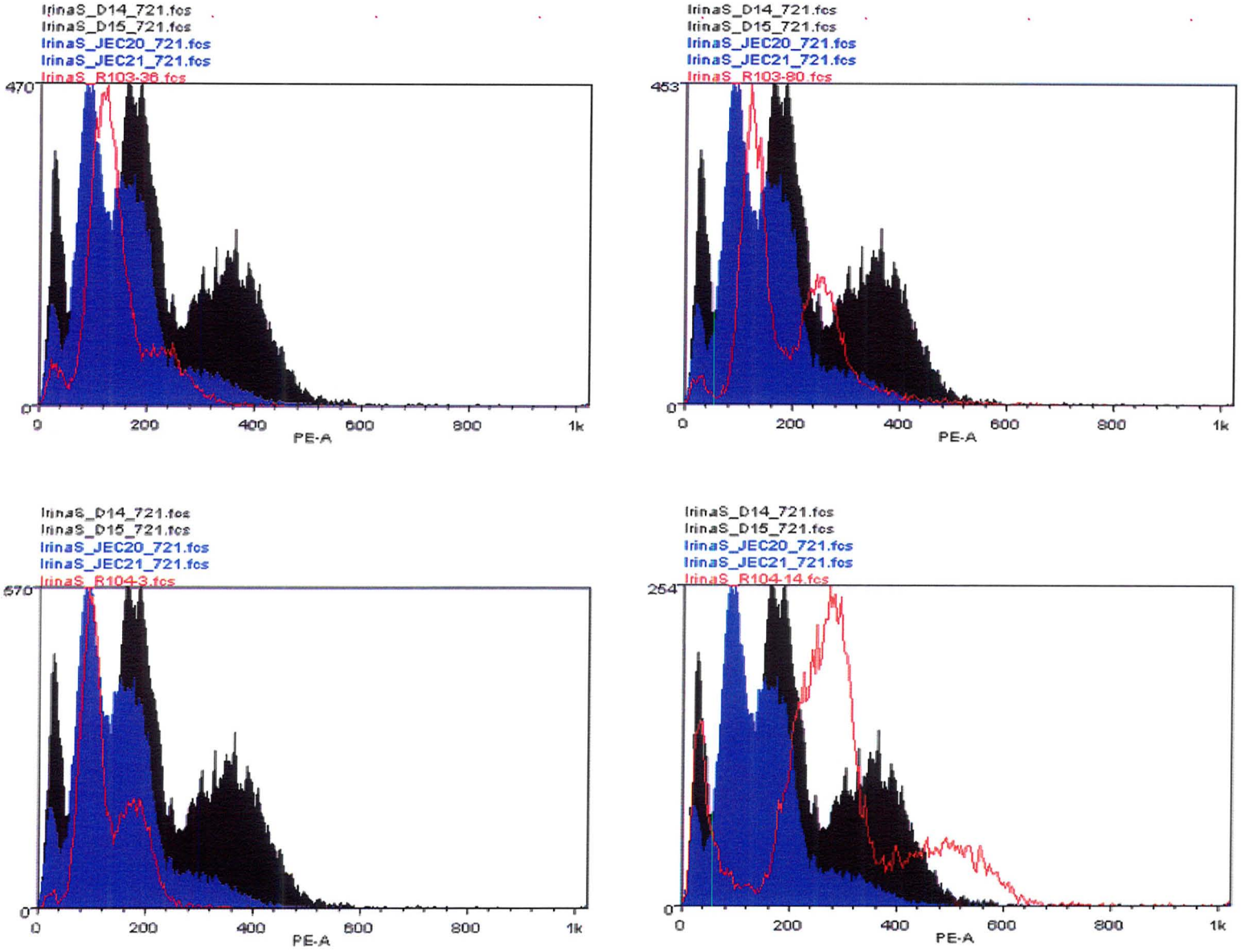

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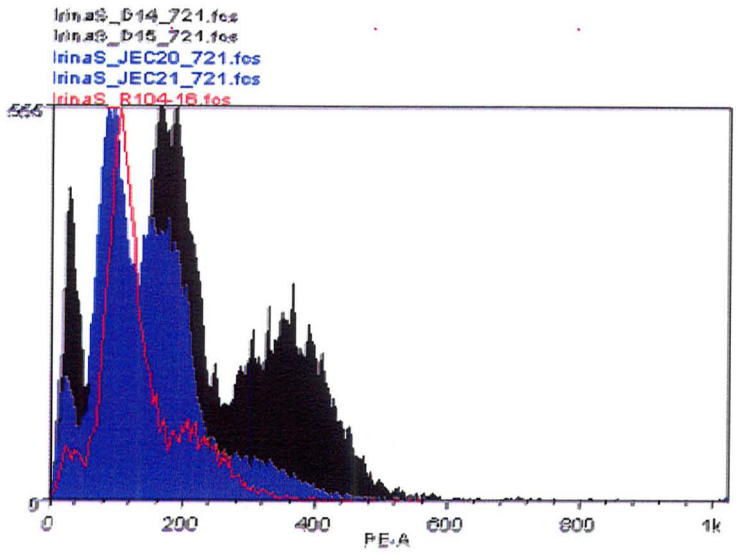


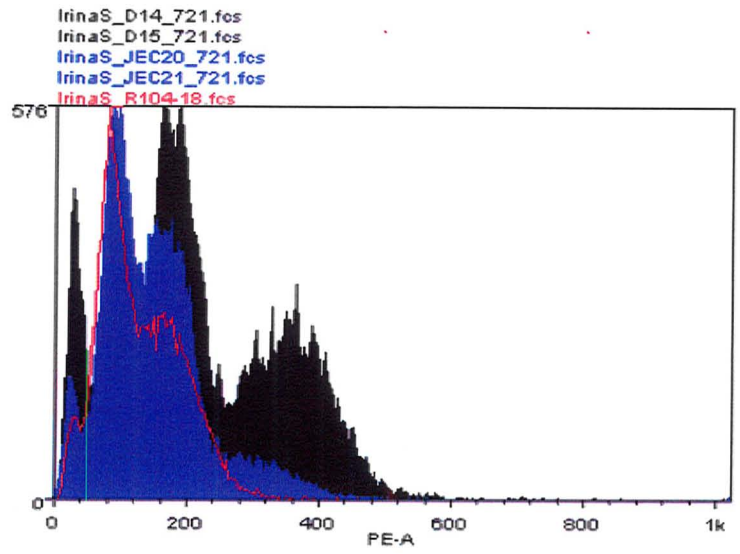


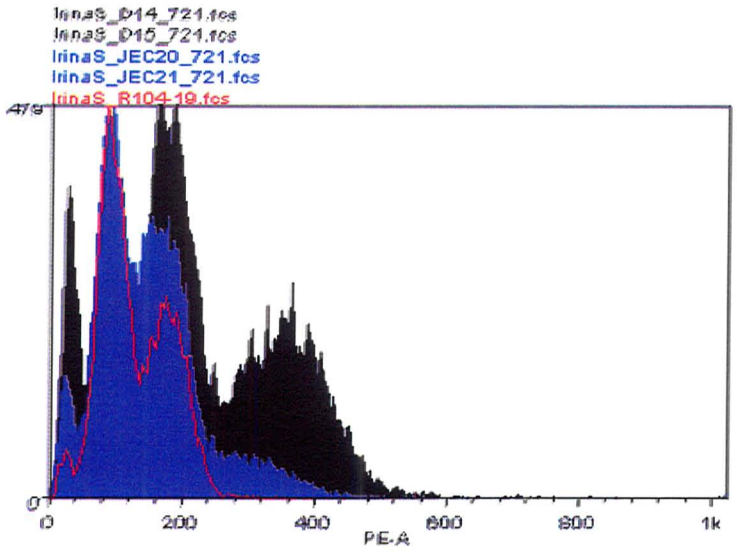


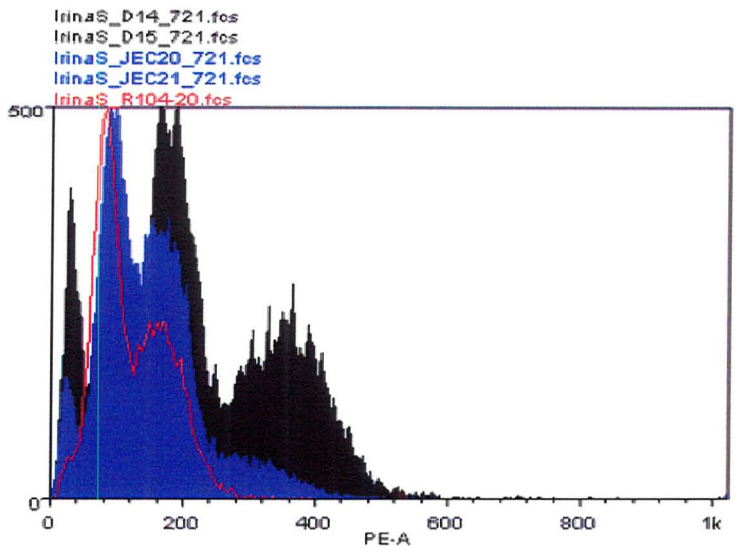

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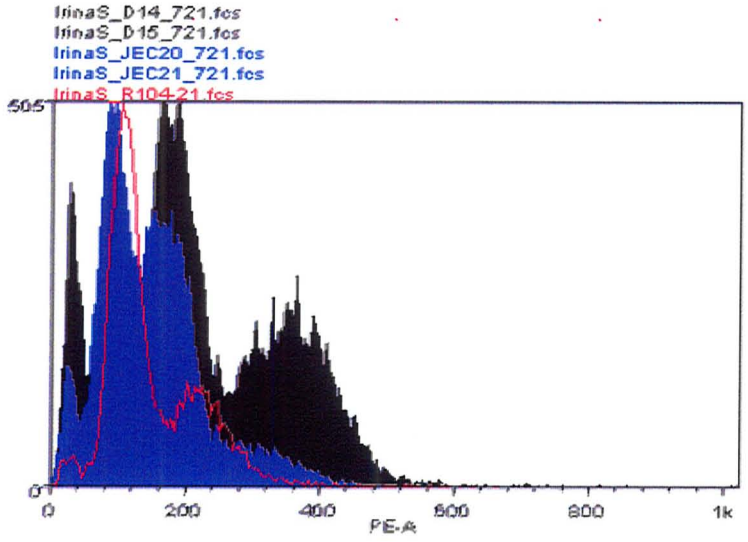


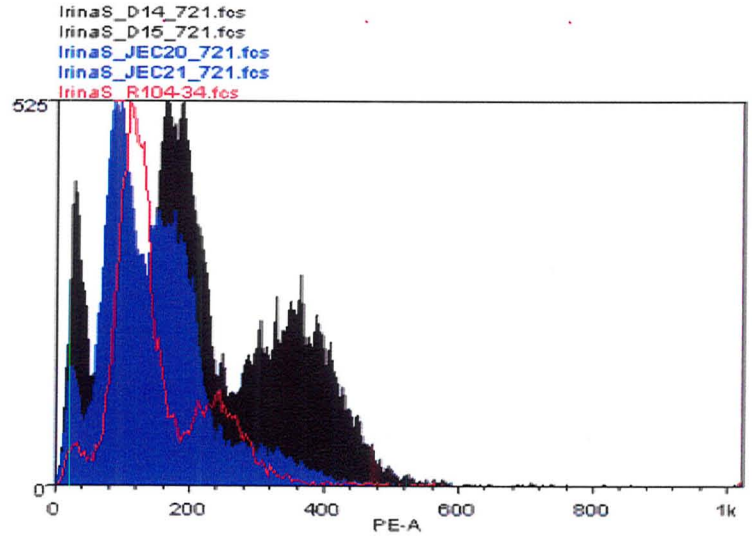


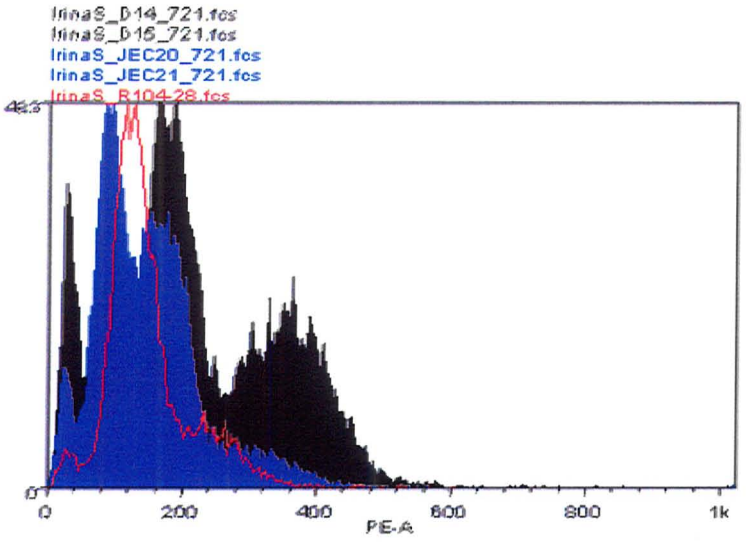


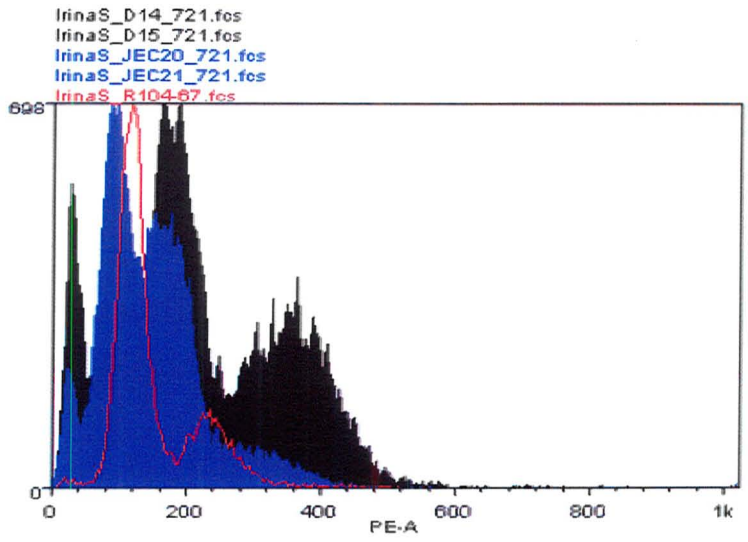

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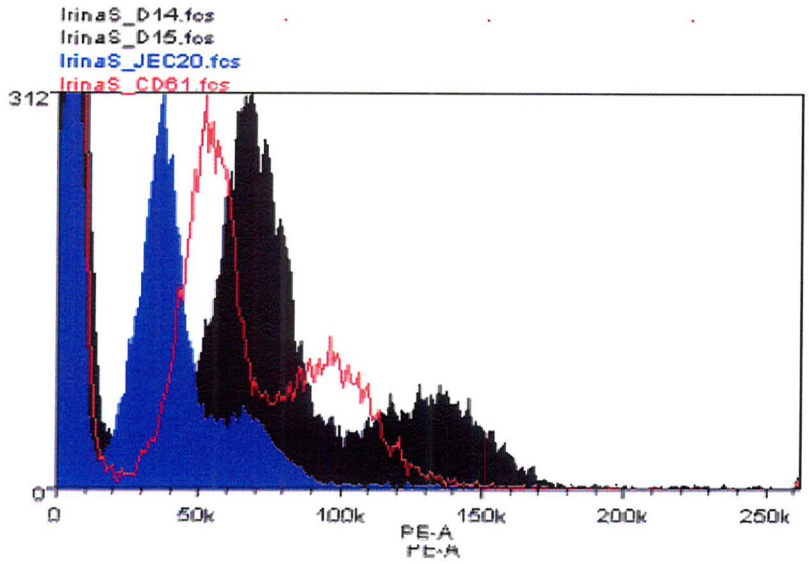


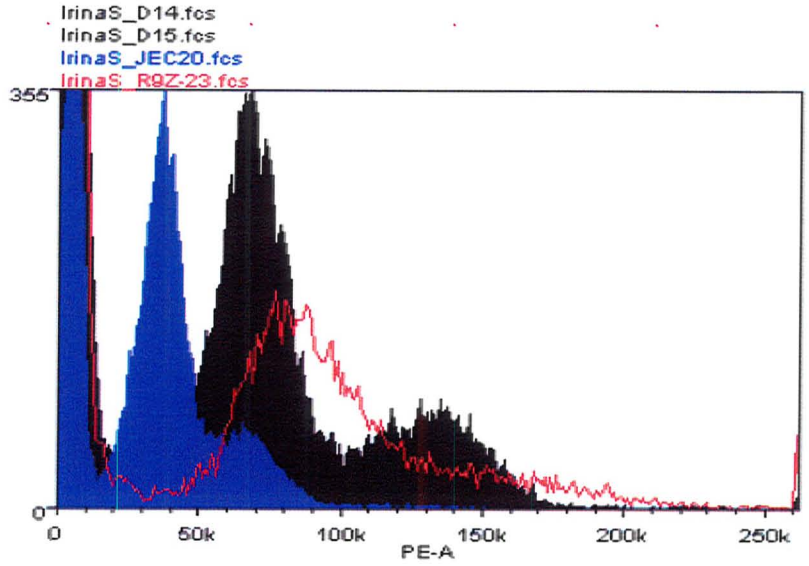


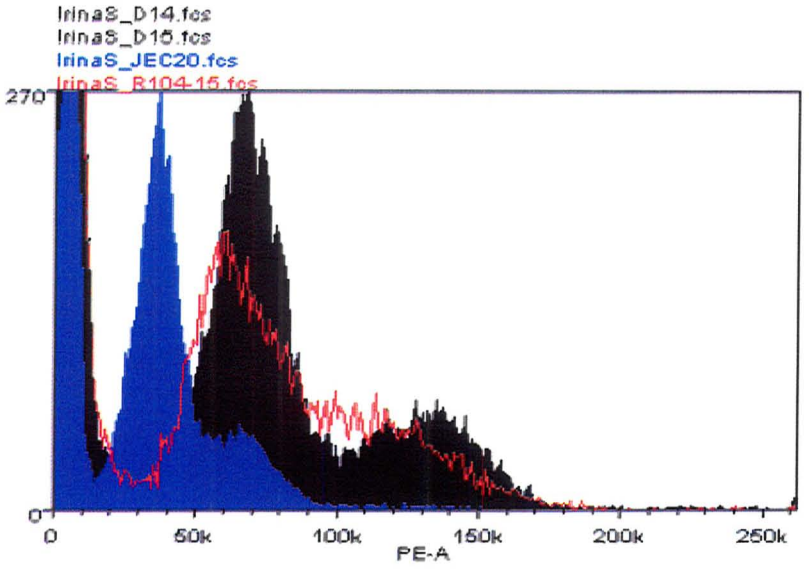


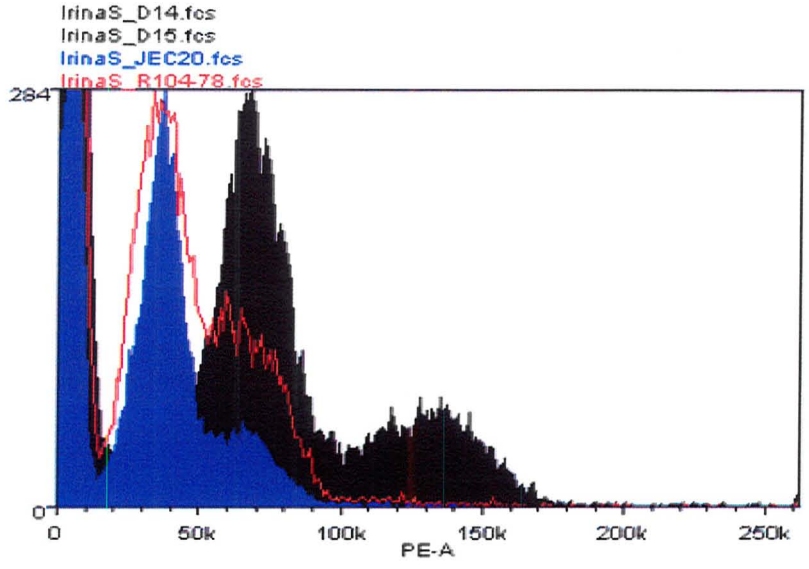

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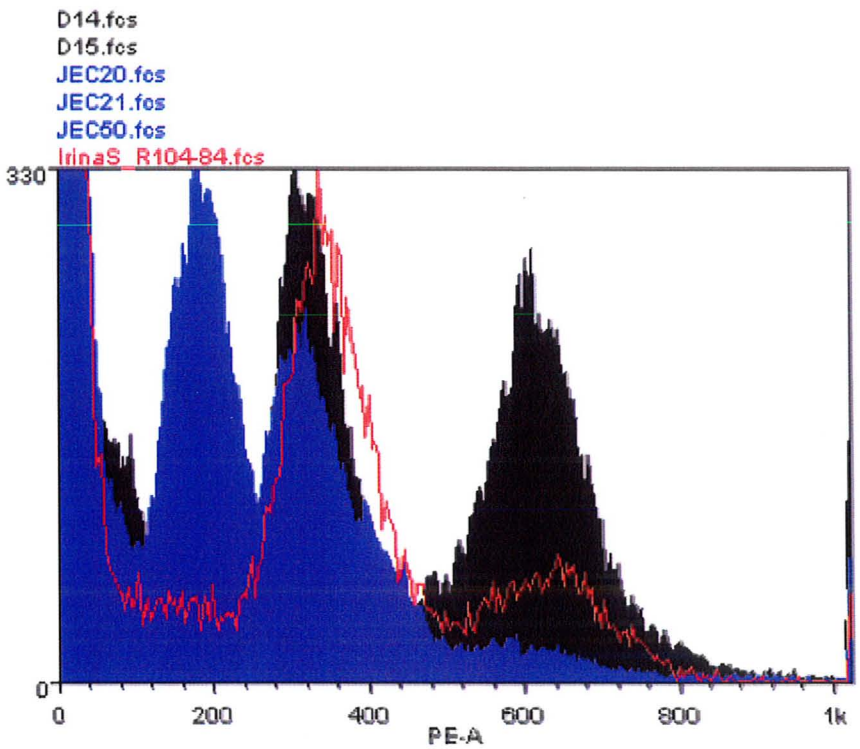

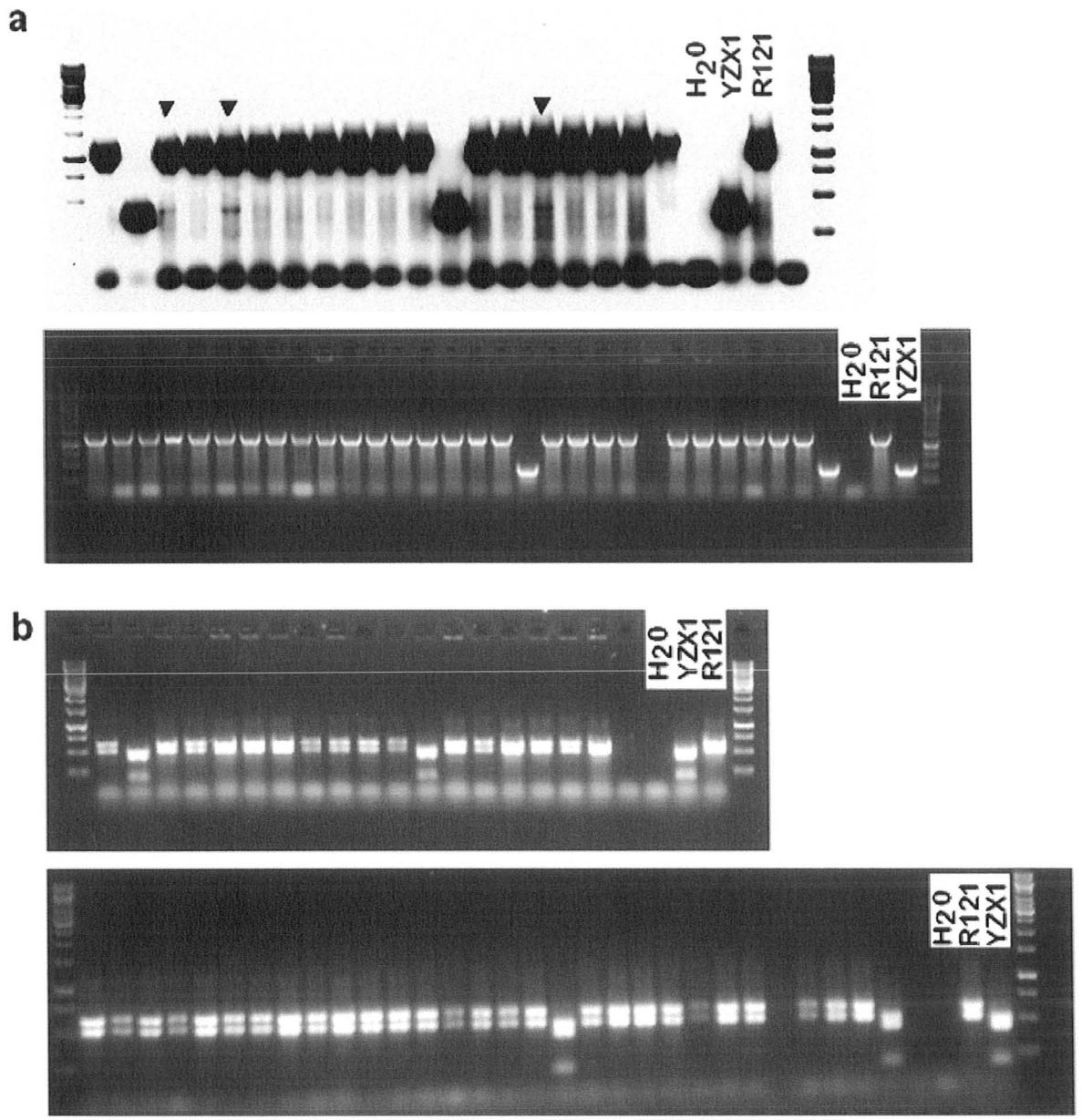


**Supplemental Figure 2.2** Mitochondrial genotypes of 49 basidiospores isolated by micromanipuation from the hyphae of the representative R121 x YZX1 cross (see Supplemental Table 4 for tabulated results). (a) Mitochondrial genotypes at the *ND5* locus. (b) PCR-RFLP at the *ND2* locus. Arrowheads designate heterozygousity at the specific locus. Sterile ddH2O was used as a negative control while R121 and YZX1 parental strains as positive controls. At the two ends of each gel is the 1kb DNA ladder.

# CHAPTER 3

## GENERAL CONCLUSIONS AND PERSPECTIVES

In this thesis, I introduced an overview of mitochondrial inheritance patterns in the sexual crosses of a variety of species, including matings between species of different ploidy (Chapter 1). In Chapter 2, I presented the study that revealed that: (1) mitochondrial leakages in the fusant and meiotic progeny populations of the two types of haploid x non-haploid crosses were significantly different from those in opposite-sex haploid x haploid crosses in *C. neoformans* (17.6-50% versus 0-8% (Yan et al. 2004; 2007a; b) in fusant populations, respectively; 8-40 versus 0.5% (Yan & Xu, 2003) in basidiospores, respectively); (2) there was a significant variation in mtDNA leakage among the basidiospore populations of the same cross (Table 2); as well as (3) mtDNA leakage was significantly different in fusant populations of different types of cross (Table 2). *C. neoformans* haploid parents, YZX1 and YZX2, used in the two types of crosses in this study, were isogenic (except for *MAT* locus). These strains were derivatives of strains JEC21 and JEC20, respectively (Yan et al 2004). Importantly, both YZX1/YZX2 and JEC20/JEC21, including the derivatives of the latter, were employed in the studies of mitochondrial inheritance in haploid x haploid crosses in which a uniparental pattern of the organelle inheritance was observed (Yan & Xu, 2003; Yan et al. 2004). Unlike the haploid x haploid matings, the opposite-sex parent of YZX1/YZX2 strains in the mixed ploidy crosses were non-haploid and, thus, larger in cell size. A larger cell size should positively translate in higher copy number of mitochondria in the cell which is important for that cell's unimpeded growth. Based on these findings and assumptions, we proposed that the mtDNA leakages observed in the fusant and basidiospore populations of haploid x non-haploid crosses were likely a result of difference in mitochondrial number of two parents and ploidy-specific effects of genes' products involved in the control of mitochondrial inheritance in *C. neoformans*.

Future studies should focus on investigating mitochondrial inheritance mechanism(s) in haploid x haploid and mixed ploidy crosses in *C. neoformans*. A suppression of a high level of autofluorescence in *Cryptococcus* species would be important for a success of these studies, especially if FISH analysis would be employed. Next, ploidy-specific effects of genetic factors, such as *Sxi1α* and *Sxi2***a**, on mitochondrial leakage in haploid x non-haploid crosses could potentially reveal whether a copy number of these essential regulators of mtDNA inheritance (as Yan et al (2007a) research in haploid x haploid matings revealed) governs mtDNA inheritance in these crosses. Furthermore, it would be interesting to analyze mtDNA inheritance patterns in sexual progeny of intra- and intervarietal crosses in *Cryptococcus gattii* as well. MtDNA inheritance patterns that would be observed in these crosses could provide a further support for the selective degradation hypothesis as a primary mechanism governing mitochondrial inheritance in *Cryptococcus* species. Finally, the assessment of cytonuclear disequilibrium in *C. neoformans* would allow a deeper insight into mechanism of mitochondrial inheritance in this species. Cytonuclear disequilibrium could be revealed by comparing patterns of mitochondrial restriction fragment length polymorphism (RFLP) diversity with patterns of nuclear RFLP diversity. Such a study would also disclose effects of gene flow and sexual reproduction on the population genetic structure and evolutionary history of the human fungal pathogen *C. neoformans*.

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