Breaking the "one nucleus, one whole genome" rule: *Neurospora crassa* separates its haploid chromosomes into different nuclei

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Abstract

Neurospora crassa has long served as a key fungal genetics model, facilitating discoveries like the "one gene, one enzyme" hypothesis. It produces many conidia, mostly with two or three nuclei and each nucleus was once thought to contain a full haploid genome. Using flow cytometry and FISH, we found that *N. crassa* haploid chromosomes are divided among nuclei, each conidium is haploid – challenging the "one nucleus, one whole genome" rule.

22 **Main**

23 Nuclei define eukaryotes. They assist the separation of genetic materials and gene expressions. It
24 has been established in molecular genetics and cell biology that each nucleus contains at least
25 one complete set of chromosomes necessary for proper genomic function and cell development,
26 the "one nucleus, one whole genome" rule. However, our recent study challenges this long-
27 standing principle. In two plant pathogenic fungi belonging to the Sclerotiniaceae family,
28 soilborne *Sclerotinia sclerotiorum* and airborne *Botrytis cinerea*, we uncovered that their haploid
29 chromosomes are separated among different nuclei (1). Such finding prompted the next question
30 on how widespread such phenomenon is. As both *S. sclerotiorum* and *B. cinerea* have
31 multinuclear spores but also produce uninuclear spermatia that are not viable likely due to
32 chromosome losses, we searched the literature to identify fungi with bi- or multinuclear spores
33 and frequent nonviable uninuclear cells, which are potential candidates that may also be breaking
34 the "one nucleus, one whole genome" rule. *Neurospora crassa* came out of our literature
35 analysis.

36

37 *N. crassa* is a non-pathogenic ascomycete commonly known as bread mold. It has been serving
38 as a classical model organism in fungal genetics and molecular biology (2,3). It was the first
39 filamentous fungus fully sequenced, making it a preferred model for genome-wide experimental
40 approaches (4,5). Its genome is approximately 40 Mb in size and comprises 7 chromosomes (4–
41 7).

42

43 *N. crassa* can be easily recognized by their abundant orange conidial spores, which are typically
44 multinucleate, majority of which have 2 to 3 nuclei (8,9) (Figure 1A). Although it does produce

uninuclear conidia as well, less than 15% of them are viable likely due to incomplete chromosome sets (10,11). To quantify the DNA amount in each nucleus of *N. crassa*, we employed flow cytometry through fluorescence-activated cell sorting (FACS) using purified nuclei from mycelia. Haploid and diploid *Saccharomyces cerevisiae* (baker's yeast) strains BY4741 and BY4743 were used as controls. Based on the relative fluorescence intensity (RFI) of nuclei (Figure 1B and C), each *N. crassa* mycelial nucleus contained about a quarter of its total genome, suggesting that each nucleus within *N. crassa* mycelia harbors only a subset of its full genome (Figure 1C).

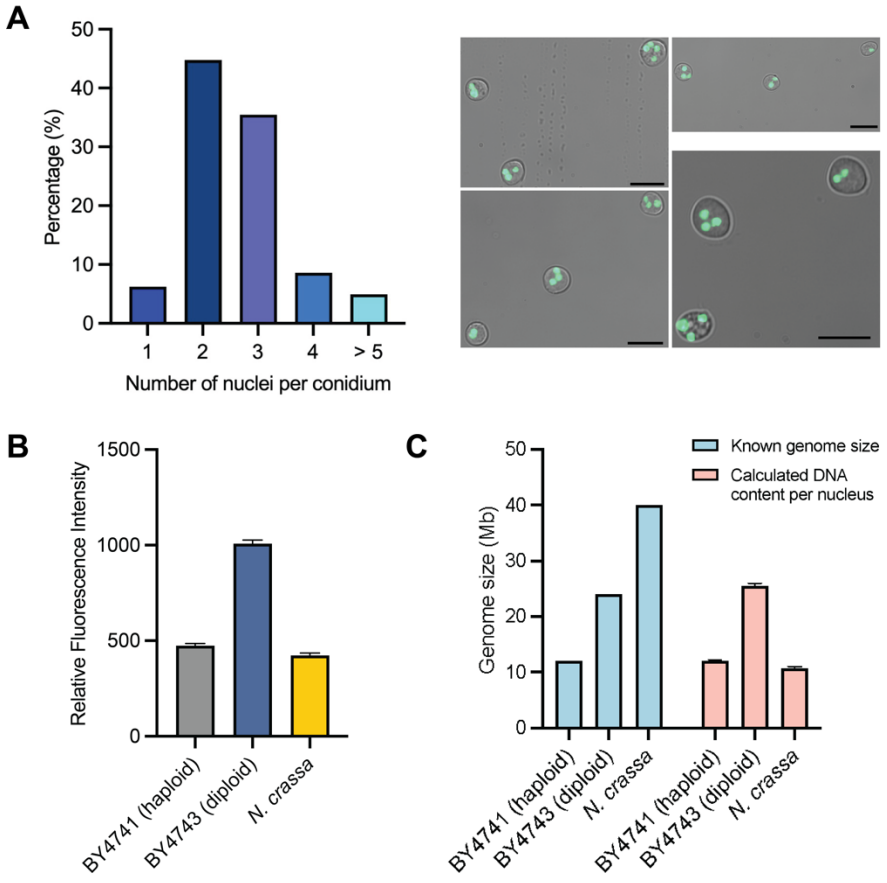
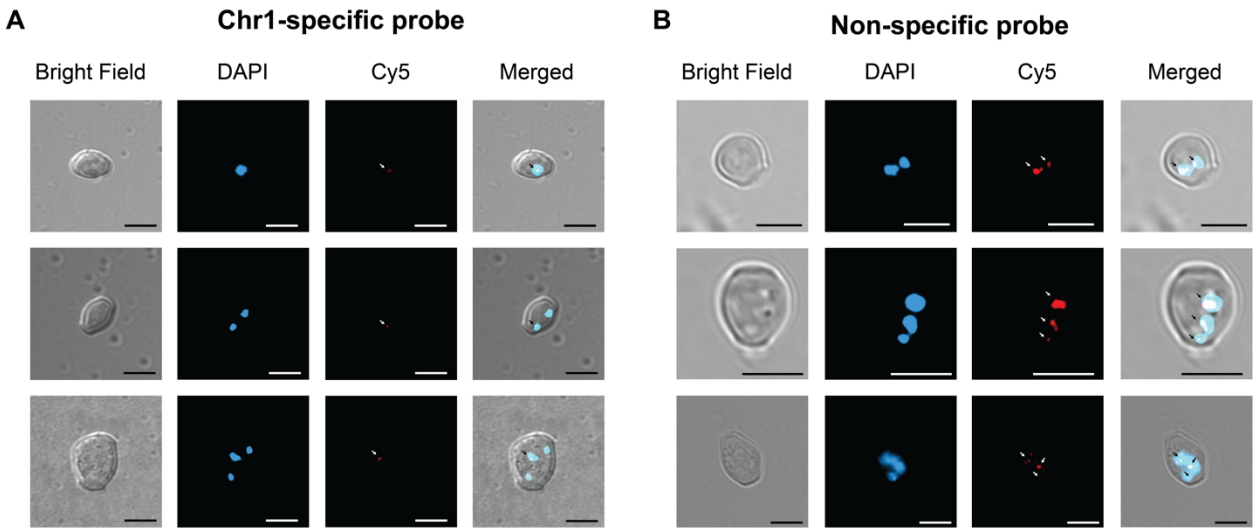


Figure 1. *N. crassa* conidia harbor multiple nuclei and each nucleus only contain parts of the total haploid genome.

(A) Quantification of nuclei number in the conidium of *N. crassa* (left). The fluorescence microscopy images show the nuclei in conidia of the *N. crassa* histone H1-eGFP strain (right). Scale bar, 10 µm.
(B) Propidium iodide (PI) fluorescence intensity of sorted purified nuclei from *Saccharomyces cerevisiae* BY4741 (haploid yeast), BY4743 (diploid yeast), mycelia of *N. crassa*. One hundred nuclei from each strain were sorted into

each well of a 96-well plate by a Sony MA900 cell sorter. Data from 15 wells were collected for each strain. The PI fluorescence intensity values were calculated based on the mean fluorescence intensity of each individual well. Error bars indicate the standard deviation of the values from 15 wells.
(C) Calculated DNA content per nucleus in *S. cerevisiae* BY4741 (haploid), BY4743 (diploid) and *N. crassa*. The known genome sizes of these strains are 12, 24, and 40 Mb, respectively. The DNA content in each nucleus was calculated by multiplying the genome size of the haploid yeast strain BY4741 by the fold-change values derived from data in Fig B. Error bars indicate the standard deviation (n = 15).

To further test whether each nucleus contains a full set of the haploid chromosomes in *N. crassa* conidia, we performed fluorescence in situ hybridization (FISH) using a chromosome 1 (Chr1)-specific probe (Supplementary figure 1A). As shown in Figure 2, hybridization signals were consistently detected in only one nucleus per conidium. Notably, Chr1-specific signals were never detected in multiple nuclei within the same conidium (Figure 2A and C). As a control, a non-specific probe that is present in all chromosomes (Supplementary figure 1B) yielded signals in all nuclei with positive hybridizations, confirming that probe accessibility was not limiting (Figure 2B and C). These FISH results support that the chromosome inside the nuclei of the bi- or tri- nuclear conidium are different. Together with the flow cytometry results, each *N. crassa* conidium seems to carry a complete set of haploid chromosomes distributed among one or multiple nuclei. The conidia are thus haploid regardless of their nuclei number. This feature will offer a major practical advantage. For gene replacement experiments to knock out or modify target genes, protoplasts made from conidia can be used, skipping the step of uninucleate microconidia purification in current methods. The same with mutagenesis for forward genetic analyses. Just as with *S. sclerotiorum*, binucleate ascospores or multinuclear conidia can be used with UV or chemical mutagens to generate pure haploid mutants (12).



C

Probe	No. of conidia without probe signal	No. of conidia with probe signal in one nucleus	No. of conidia with probe signals in two or more nuclei	Total numbers of conidia
Chr1-specific probe	20	122	0	144

Probe	No. of conidia without probe signal	No. of conidia with probe signals in over half of nucleus	No. of conidia with probe signals in all nuclei	Total numbers of conidia
Non-specific probe	15	29	79	123

Figure 2. A *N. crassa* conidium can separate its haploid chromosomes into one, two, or three nuclei.

(A) FISH signals of Chr1-specific (left) and non-specific (right) probes in representative conidia. Blue and red signals are from DAPI-stained nuclei and hybridized probe, respectively. The hybridized Cy5 probe signals are pointed out by arrows. Scale bars, 5 μm.
(B) Summary of FISH results for Chr1-specific (top) and non-specific (bottom) probes in one or multiple nuclei of examined conidia.

Interestingly, although uninucleate *N. crassa* conidia (microconidia) are viable, they exhibit a very low germination rate (10,11), whereas uninucleate spermatia cells in *S. sclerotiorum* and *B. cinerea* are nonviable. This led to the improvement of *N. crassa* mutagenesis protocols where uninucleate spores were firstly purified before mutagenesis, reinforcing the "one nucleus, one whole genome" dogma (11,13). We believe the reason why a small fraction of the uninucleate

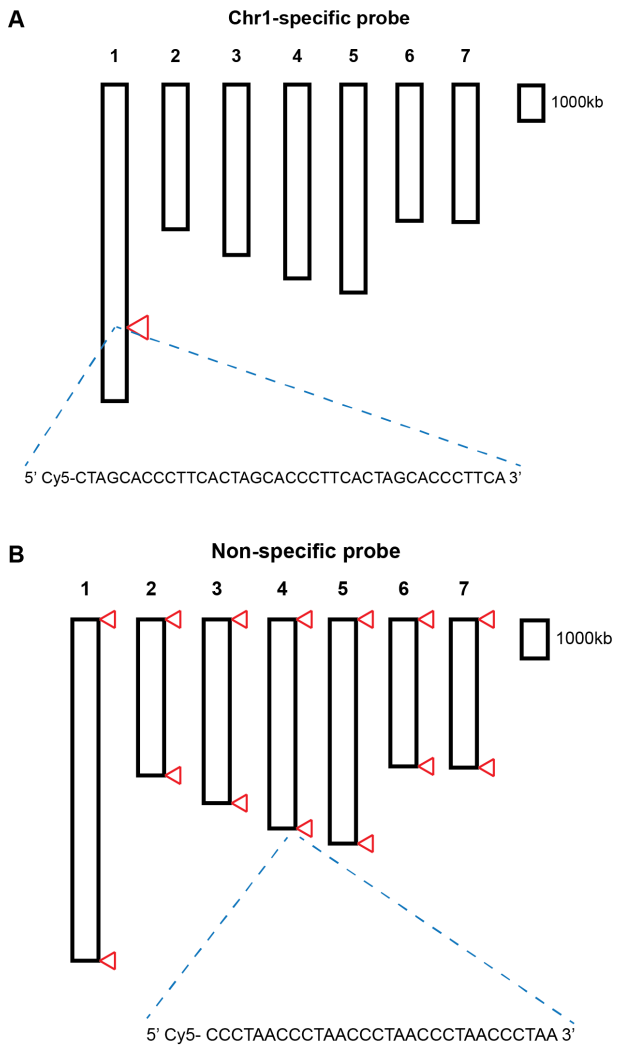
cells is viable in *N. crassa* is probably due to its low haploid chromosome number. Its 7 chromosomes may occasionally be packed into just one nucleus, allowing the spore to be viable. As for *S. sclerotiorum* and *B. cinerea*, 16 or 18 chromosomes are seemingly almost impossible to be packed simultaneously into a single nucleus during their nuclear division.

In summary, our findings in *N. crassa* suggest that dividing haploid chromosomes across multiple nuclei may be more widespread than previously recognized. It is not restricted to sclerotia-forming plant fungal pathogens of the Sclerotiniaceae family. Other Ascomycetes and Basidiomycetes with known bi- or multi-nucleate ascospores and basidiospores, such as *Morchella, Poronia, Oudemansiella* and *Cortinarius*, also warrant further investigation (14–18). Studying these organisms could provide important insights into the spread of this phenomenon and advance our understanding of chromosome biology and genome evolution. Moreover, the precise molecular mechanisms underlying such chromosome splitting phenomenon remain puzzling and represent an intriguing area for future research. For instance, how do different nuclei housing the full complement of chromosomes communicate and coordinate with each other? What are the key genes and proteins involved in nuclear coordination? Are there special checkpoints to ensure proper chromosomal distribution? Advanced live-cell imaging and single-nuclei sequencing could be particularly valuable for tracking chromosomal segregation and assessing genetic variation or stability across multiple nuclei.

Methods

Relative fluorescence intensity (RFI) determination by flow cytometry

120 Flow cytometry was performed following the previous study using nuclei from *Saccharomyces*
121 *cerevisiae* and mycelia of *N. crassa* (1).
122
123 **FISH analyses**
124 Through BLAST searches of the *N. crassa* genome, a chromosome 1 (Chr1)-specific probe was
125 identified, which is located in a non-coding region with a single successive hybridization site
126 (Supplementary figure 1A). Additionally, a non-specific repetitive probe was identified that can
127 target all chromosomes (Supplementary figure 1B).
128
129 Conidia fixation and FISH were performed following the previous study with minor changes (1).
130 100 ng of probe was used. The hybridization was performed overnight at 37 to 42℃ for non-
131 specific probe, and at 45℃ for 22 hours for the Chr1-specific probe. Nuclei were stained with 1
132 μg/ml DAPI before imaging.
133
134 **Microscopy observation**
135 Images of the Histone-GFP strain and FISH samples were performed with the ZEISS AXIO
136 Imager M2 microscope at 200× or 1000× magnification. Scale bars are shown in each image and
137 specified in the figure legends.



Supplementary figure 1. Chromosomal locations of the specific and non-specific FISH probes.

(A)-(B) The chromosomal regions in *N. crass* where the Chr1-specific (A), and non-specific (B) probes can hybridize to are indicated by red triangles. The Chr1-specific probe occurs once, while the non-specific probe is repeated from seven to eleven times across different chromosomes. The probe sequences are shown at the bottom of each panel. Scale bars showing chromosome lengths are shown on the right.

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Author Contributions

X.L. conceived the idea; J.T. and X.L. wrote the manuscript draft; J.T. and Y.X. conducted the experiments. L.T. and Y.Z. revised the original manuscript. All authors edited and approved the final manuscript.

Competing financial interests

The authors declare no competing interests.

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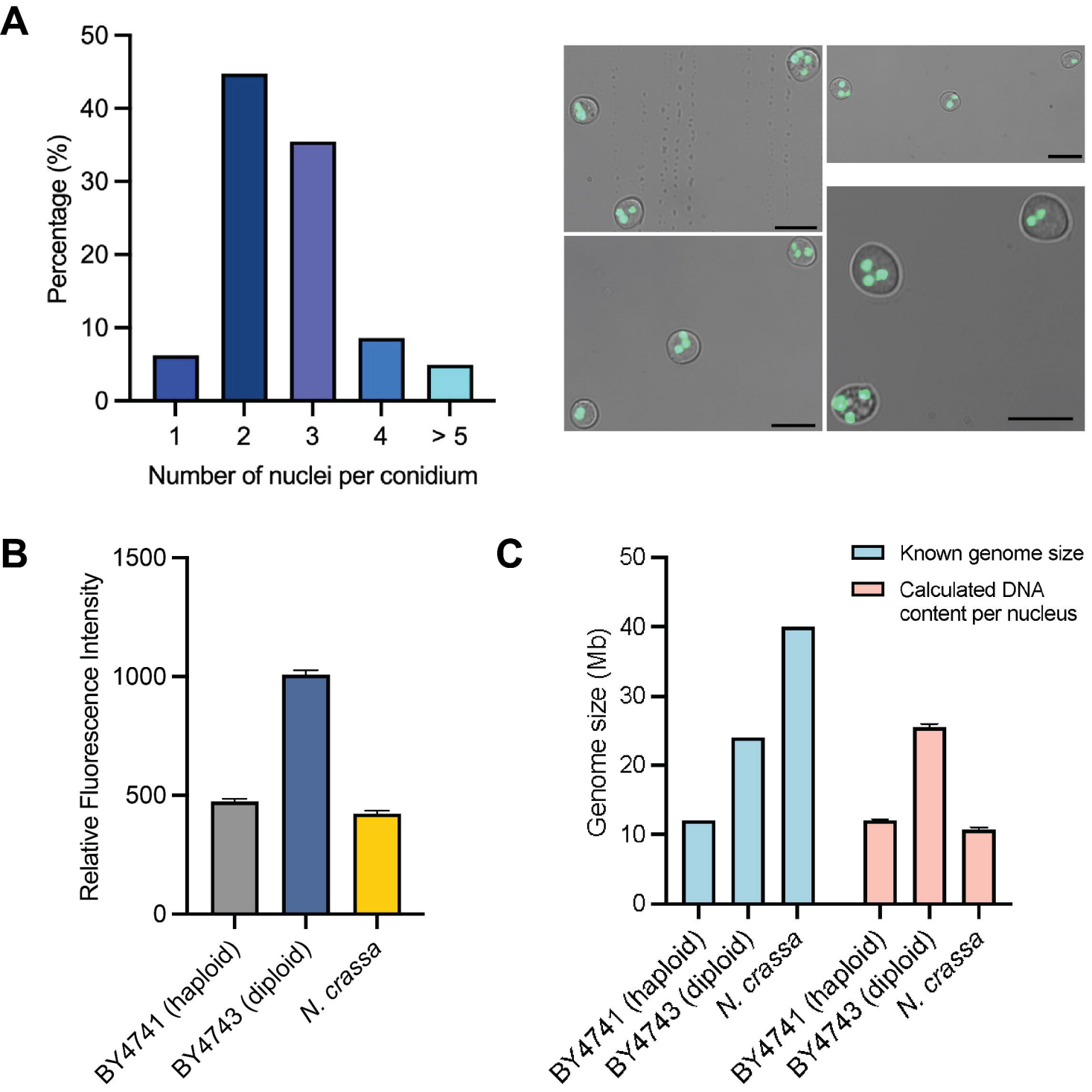
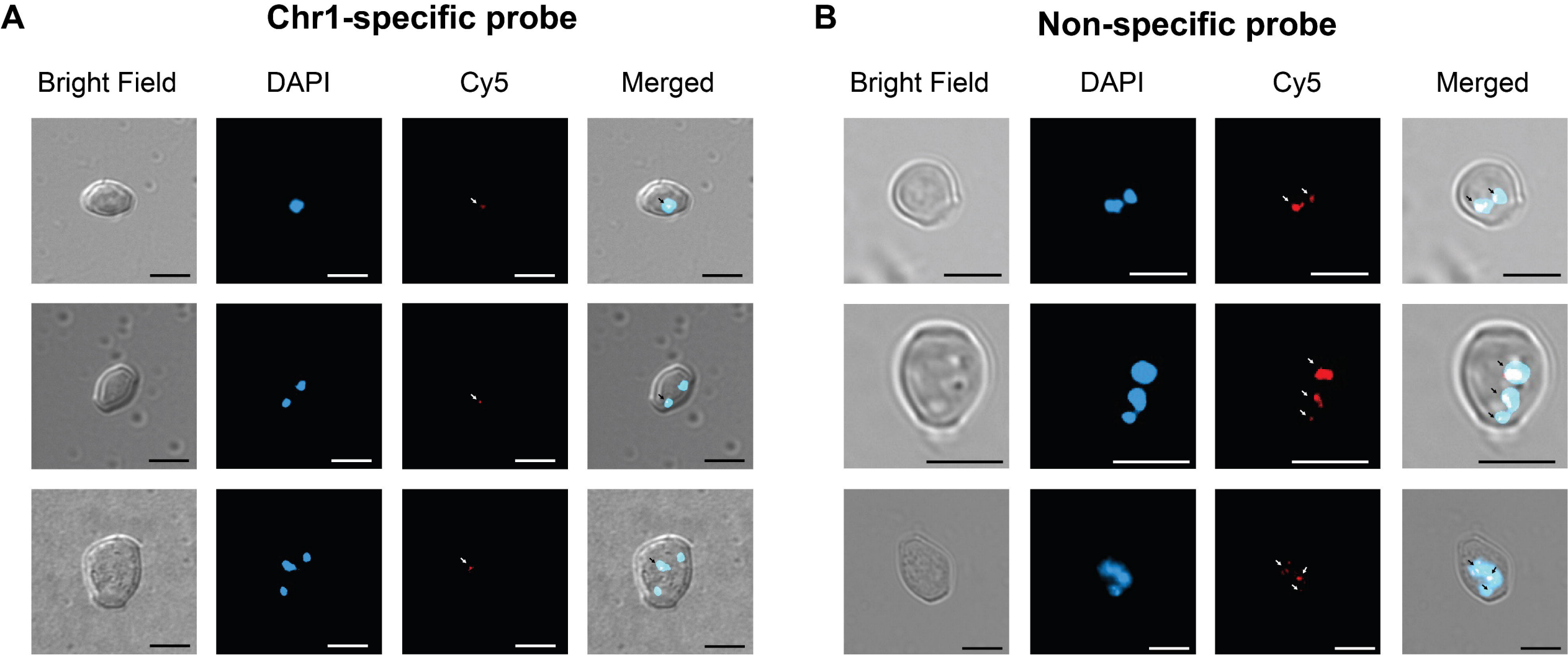

A
Percentage (%)
50
40
30
20
10
0
1
2
3
4
> 5
Number of nuclei per conidium
B
Relative Fluorescence Intensity
1500
1000
500
0
BY4741 (haploid)
BY4743 (diploid)
N. crassa
C
Genome size (Mb)
50
40
30
20
10
0
Known genome size
Calculated DNA content per nucleus
BY4741 (haploid)
BY4743 (diploid)
N. crassa
BY4741 (haploid)
BY4743 (diploid)
N. crassa



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