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Ploidy and chromosomal number in *Tuber aestivum*

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Abstract

Chromosome number is generally considered to be the most accurate and direct measure of ploidy. In fungi, however, counting of chromosomes is difficult and inaccurate because of their small size. To date truffles have been characterized using molecular approaches (analysis of DNA and PCR-based techniques) in order to find out differences among species, but not by a cytogenetical approach. Although the small size of truffle chromosomes is of some hindrance for a cytogenetic study, in the present work *Tuber aestivum* chromosome counts were determined in metaphase configurations from haploid nuclei of ascospores by aceto-orcein staining. Nuclear chromosome number and topography were also evaluated by propidium iodide DNA staining using confocal microscopy. *Tuber aestivum* was found to possess a basic number of 5 or 6 chromosomes, medium length ≤0.95 μm. The karyology of ascospores during their developmental stages was also investigated.

Keywords: Truffle; Chromosome; Ascomycetes karyotype; Ascomycotina; Pezizales

1. Introduction

Some truffles (Ascomycotina) of the genus *Tuber* Micheli ex F.H. Wigg. are economically important due to the organoleptic properties of some species (*Tuber magnatum* Pico, *T. melanosporum* Vittad., *T. aestivum* Vittad., *T. borchii* Vittad.). Truffles are also biologically interesting since they are hypogeous fungi living under microaerobic conditions and possess abnormal mitochondria [1,2] when grown under anaerobic conditions similarly to *Saccharomyces cerevisiae* and other yeasts in the Ascomycotina. Both basic and applied concerns suggest this work will provide a better understanding of the reproductive cycle of truffles of the genus *Tuber,* first by detecting chromosome number both in spores and hyphae.

Karyotyping has often been used in the classification of animals and plants; moreover, the reproductive cycle of some species can be assessed only if the ploidy of somatic and reproductive cells is known. Chromosome number is considered to be the most accurate and direct measure of ploidy, but counting chromosomes of fungi is difficult and inaccurate because of their small size [3–5]. Electrophoretic karyotypes of several Ascomycetes are also known [6,7]. To date several truffles have only been characterized molecularly through the analysis of DNA, rRNA and other PCR-based techniques in order to discern

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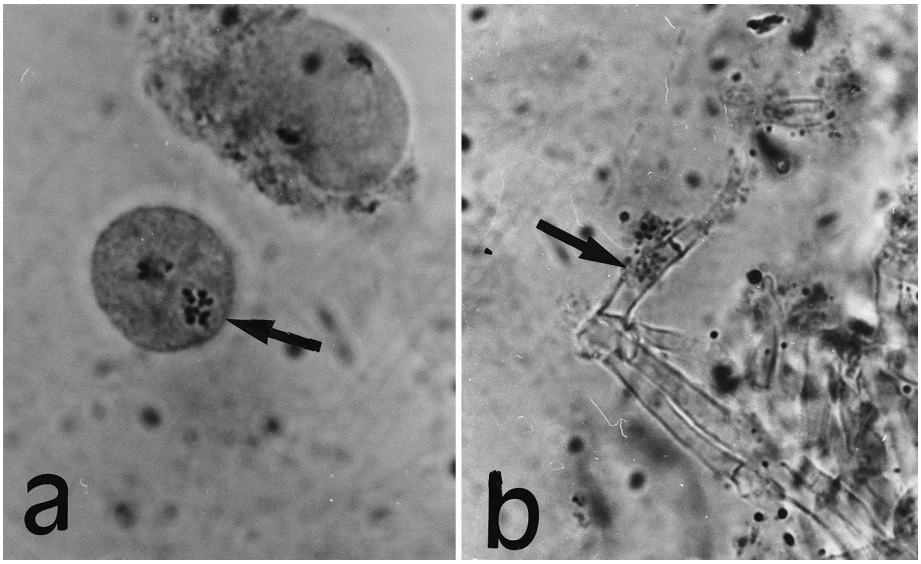

Fig. 1. Light microscopy of aceto-orcein stained *Tuber aestivum* chromosomes (arrowed). a: Sporal chromosomes; b: hyphal chromosomes. Magnification 1200×. Seven sporal and twenty hyphal metaphases were scored.

differences among species [8–10]; however, none of these species has been investigated using a cytogenetical approach. In the present work *T. aestivum* chromosomes were observed and measured in metaphases of haploid ascospores and dikaryotic or diploid hyphal metaphases by aceto-orcein staining, permitting counting of chromosomes. Nuclear chromosome number was also evaluated by propidium iodide DNA staining for confocal microscopy. We also investigated the karyology of ascospores during their developmental stages to better understand sporal karyological changes.

2. Materials and methods

Immature *T. aestivum* specimens were collected close to L'Aquila or Teramo (Abruzzo, Italy) and used for study soon after collection or else stored at −25°C for 2–4 days prior to karyotypical analysis.

2.1. Staining of truffle chromosomes and nuclei for light microscopy

Thin slices of the truffles (1–2 mm) were treated with asci wall lytic enzymes cellulase, protease and chitinase L2265 (Sigma) with 0.01% (w/v) colchicine overnight, fixed in ethanol/glacial acetic acid (3:1 (v/v), overnight), hydrolyzed in 1 M HCl for 30 min at R.T. and squashed in 45% (v/v) acetic acid and orcein solution. The cover slides were removed by dipping in liquid nitrogen and samples dehydrated in graded alcohol series, dipped in xylene; finally the slides were mounted in Canadian balsam.

2.2. DNA staining by propidium iodide for confocal microscopy

DNA staining was performed using a method [11] modified for *T. aestivum* specimens. Thin truffle slices were treated with 0.01% (w/v) colchicine overnight at 20°C, fixed in ethanol/acetic acid 20 (3:1 (v/v) overnight), dipped in 1 mg ml^{-1} $NaBH_4$ solution for 10 min. Lipids were removed using acetone treatment for 2 h. Slices were treated with block/permeabilization buffer (0.2% BSA, 0.1% Triton X-100, PBS) for 5 min, stained with 1 μg ml^{-1} propidium iodide (Sigma) for 15 min, rinsed 2 times with block/permeabilization buffer for 5 min and mounted with Vectashield™ (Vector Laboratories, Burlingame, CA, USA) to prevent photobleaching. Slides were stored

in the dark at 4°C before observation by confocal microscopy using a Sarastro 2000 microscope (Molecular Dynamics, CA, USA).

2.3. Reagents

The reagents used were of the purest grade available and generally obtained from Sigma or Merck.

3. Results

3.1. Light microscopy

The orcein-staining procedure was sufficient to make the chromosomes of both spores and hyphae visible. Treatment including asci wall lytic enzymes prior to squashing and staining was found to improve the results. Chromosomes and chromatin were strongly stained showing very little or no background. Five chromosomes (Fig. 1a and Table 1) could be observed in cells at metaphase during mitoses of sporal haploid nuclei. A chromosomal medium size of approximately 0.95 μm (roundish in shape) was estimated. We have not observed differently shaped chromosomes. Hyphal chromosomes are very similar to spore chromosomes in shape and size (Fig. 1b) and as reported in Table 1 and Fig. 1b, 10 chromosomes/metaphase were scored.

Table 1
Chromosome number of *Tuber aestivum*

Source	Hyphal metaphases	Sporal metaphases
Monti della Laga, Teramo, Italy	9	5
	9	5
	9/10	6
	9/10	6
	10	
	10	
	10	
	10	
	11	
	11	
	12	
San Marco, L'Aquila, Italy	8/9	5
	9/10	5/6
	9/10	5/6
	10	
	10	
	10	
	10/11	
	10/11	
	10/11	

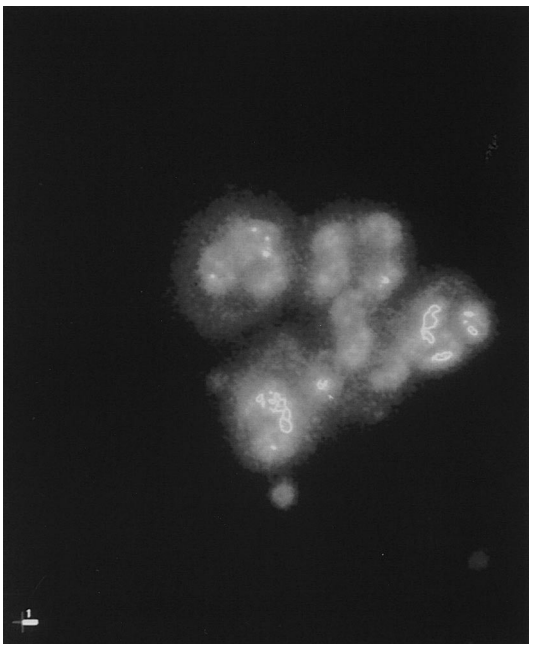

Fig. 2. *Tuber aestivum* sporal nuclei stained with propidium iodide. Scale bar = 1 μm. More than 500 sporal nuclei were examined.

3.2. Confocal microscopy

The nuclear chromosome number and topography were evaluated by propidium iodide DNA staining for confocal microscopy of sporal metaphases. Fig. 2 shows chromosomes constituting a butterfly-shaped body. Autofluorescence did not allow chromosome counting as clearly as by light microscopy.

3.3. Karyology

During ascospore formation, the nuclei in the sporigenous area divide, originating first two and later 4–8 daughter nuclei (Fig. 3a,b,c). In the ripening spore, the wall is formed first, while reticulation is formed later (Fig. 3d). The dividing nuclei migrate close to the wall previous to occupying the center of the forming spores while the cytoplasm becomes vacuolate.

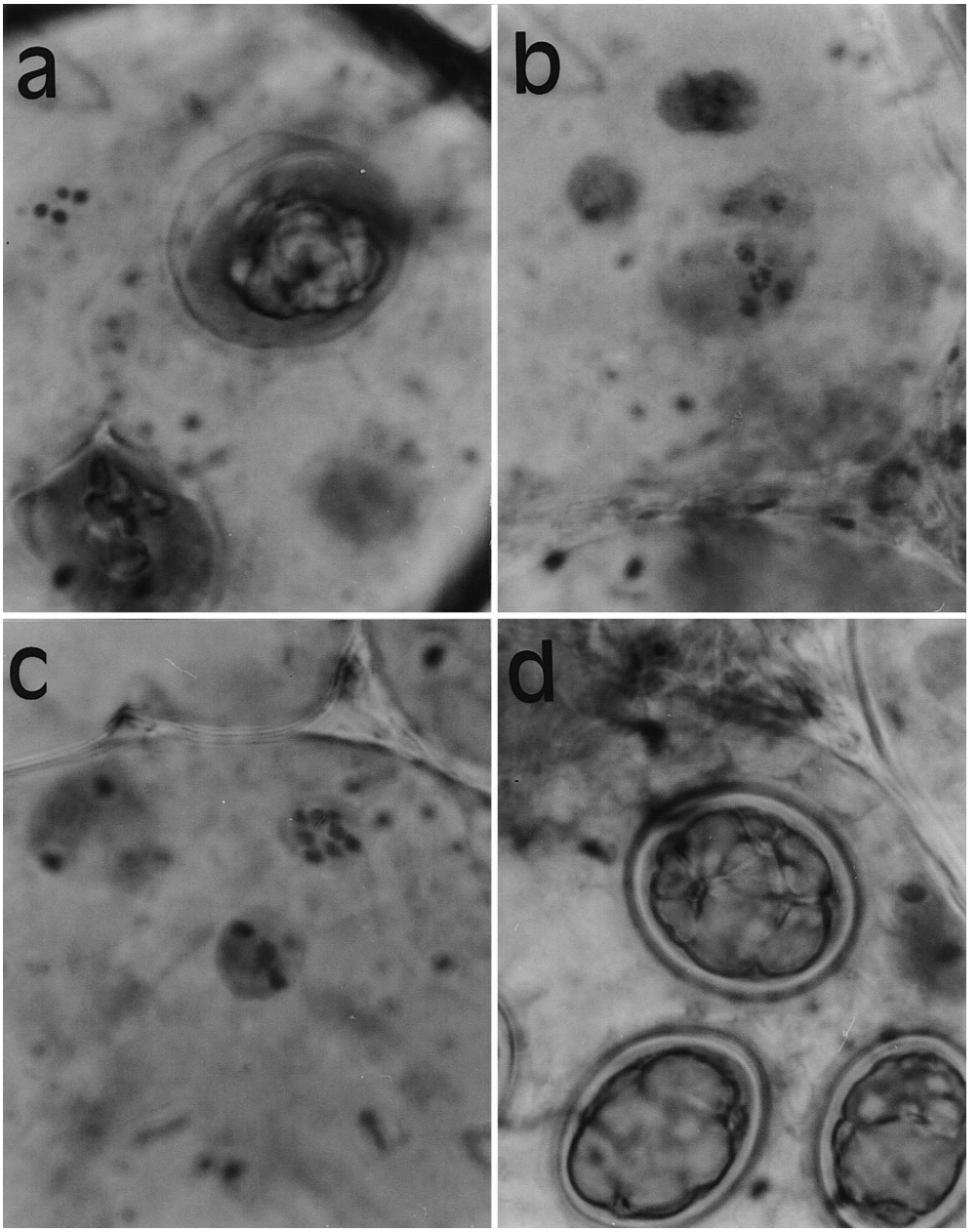

Fig. 3. *Tuber aestivum* ascospores. a–c: Karyological variations; d: ripe ascospores. More than 500 ascospores were examined.

4. Discussion

The numbers of spore and hyphal chromosomes, 5.4 ± 0.1 (n) and 10 ± 0 ($2n$) respectively, strongly suggest a haploid chromosome number of 5 for *T. aestivum*; this value of n is in the range of those of several Ascomycetes, for example *Neurospora crassa* ($n = 7$) [6] and also the mean bp content calculated from the mean metaphase chromosomal size. In fact by assuming that the metaphase chromosomes of *T. aestivum* undergo a contraction of the same order of magnitude of those of other metaphasic chromo-

somes (about 5000×) [12] then 0.95 μm (chromosomal length)×5000 would give 4.75 mm of linear DNA, equivalent to about 14 Mbp/chromosome, that is about in the range of some *N. crassa* chromosomes (4–12.6 Mbp), as estimated by CHEF [13]. This is in line with truffles as well as *N. crassa* being Ascomycotina. Our findings show that truffles share chromosomal dimensions with other fungi which are considerably smaller than those of plants and animals. This is consistent with fungi having smaller genomes compared to plants. However, a more direct measure of DNA content/genome is required to exactly assess *T. aestivum* genomic size.

On the basis of the karyological observations in parallel to chromosome counts, the reconstruction of the 'sporal stage' (when spores are produced within the asci) may be attempted from a cytogenetical point of view. From the diploid presporigenous hyphae ($2n=10$) premeiotic (sporogonial cells) would arise, which after meiosis should give rise to haploid ($n=5$) spores. This would suggest that hyphal dikaryotic cells [14] might give rise to diploid synkaryotic presporal hyphae. In situ hybridization to a nucleolar organizer probe [15,16] should mark haploid, dikaryotic and synkaryotic nuclei and may help to further clarify the nuclear cycle of *T. aestivum*.

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