NUCLEAR DNA CONTENT, LIFE CYCLE AND PLOIDY IN TWO NEOTTIELLA SPECIES (PEZIZALES, ASCOMYCOTA)

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Genome size, life cycle, and ploidy were determined for Neottiella vivida (Nyl.) Dennis and N. rutilans (Fr.) Dennis. The relative DNA content was measured from fruit-bodies using cytofluorometry; genome size was obtained by comparison with two standards: conidia of Trichopheea hemisphaerioides (23.3 Mb) and a spore-print from Pleurotus ostreatus (25 Mb). Neottiella vivida and N. rutilans were found to have genomes of approximately 750 Mb and 530 Mb, respectively. The ploidy level of N. rutilans is 50, that of N. vivida was calculated to be 70. In N. vivida, meiotic division occurs in the ascus apex where giant mitochondria with a DNA content of 60 Mb, 54 Mb, and 30 Mb were found. The two species are morphologically very similar and can be distinguished only by their ascospore ornamentation, which is reticulated in N. rutilans and warted in N. vivida. Due to endoreduplication in the uninucleate ascospores of N. vivida, the value of their nuclear DNA content is 2C. In N. rutilans, endoreduplication is not arrested at the 2C value but may proceed at a different rate in spores. Thus N. rutilans reveals heterogeneity in ploidy levels of sporal nuclei. In N. vivida and N. rutilans, differences in spore ornamentation may result from different patterns of gene expression regulated by the ploidy-dependent gene.

Quantification of changes in nuclear DNA has significantly contributed to a better understanding of the life cycle of several fungi (Olive, 1953; Bryant & Howard, 1969; Collins, 1979; Franklin et al., 1983; Anderson, 1982; Collins et al. 1983; Whisler et al., 1983; Horgen et al., 1985; Bresinsky et al., 1987; Wittmann-Meixner, 1989; Bayman & Collins, 1990; Weber, 1992).

Changes of nuclear DNA content occur at DNA replication and cell division, in the evolution of species, and during differentiation of cells in an organism. Ascomycetes with a sexual cycle double their ploidy upon fertilization in the ascus and reduce their ploidy by half at meiosis, producing ascospores. In the vegetative mycelium, the nuclear DNA content varies during the mitotic (G1 versus G2 phases) cycle of cell division. In the development of the organism, specialized polyploid cell types may arise through endocycles, i.e. cell cycles lacking cell division. Cells differing only by their ploidy are identical in terms of DNA sequence information, but are often quite different in terms of developmental, morphological, and physiological characteristics (Galitski et al., 1999; Hieter & Griffiths, 1999).

The term genome denotes the DNA of the haploid chromosome complement in terms of quality and quantity. The DNA content of the unreplicated haploid nuclear genome is known as its 1C-value (Swift, 1950). Dividing cells pass through a regular, repeated sequence of events known as the cell cycle. The cell cycle is divided into interphase and mitosis. Interphase is a period of chromosome duplication. Interphase can be divid-
ed into three phases, which are designed G1, S, and G2. Mitosis and cytokinesis together are referred to as the M phase of the cell cycle. The specialized resting, or dormant, state is called the G0 phase (G-zero phase). In the cell cycle, progression is mainly controlled at two crucial transition points, called checkpoints – one at the end of G1 and another at the end of G2. It is at the G1 checkpoint that the control system either arrests the cycle or triggers a process that will initiate the S phase (synthesis phase). At the G2 checkpoint, the control system again either arrests the cycle or initiates mitosis (Raven et al., 1999).

Species in Sclerotiniaceae and Leotiaceae (Helotiales) often have uninucleate spores with the 2C-value (Weber, 1992). The undivided nuclei of the large-budded fraction of *Saccharomyces cerevisiae* (Meyen ex Reese) Hansen are arrested in the anaphase or metaphase, i.e. in the G2 phase of the cell cycle (Hanna et al., 1995). However, the nuclei of spores of most species in Helotiales (Weber, 1992), as well as *Glomus versiforme* (P. Karst.) S.M. Berch (Biancotti et al., 1995) and species in the genera *Pleurotus* (Fr.) P. Kumm. and *Phellinus* Quél. (Kullman, 2000) are arrested in the G0/G1 phase (at the 1C-value).

Hence, although measurements *per se* cannot reveal whether each individual nucleus actually progresses through the cell cycle or not, kinetic information can be inferred from DNA content (position of the cell cycle). Progression through the S-phase and mitosis is expressed by changes in nuclear DNA content. The position of the nuclei in the cell cycle can therefore be estimated on the basis of measurement of DNA content. When measuring nuclei in the haplophase (nuclei with a 'single set' of chromosomes), a distribution curve is obtained whose first maximum (basic DNA content), indicating nuclei in the cell cycle G0/G1 phase, corresponds to genome size (1C-value – the DNA content of the unreplicated haploid nuclear genome, unit) (see Kullman, 2000).

In studies of polyploidy in fungi, variation of nuclear relative DNA content due to the mitotic cycle was tested by Bresinsky et al. (1987), Wittman-Meixner & Bresinsky (1989), Weber (1992), and Weber & Bresinsky (1992). It was shown that basic nuclear DNA content is the same in young and old mycelia (irrespective of the medium and the age of the culture), as well as in fruit-bodies, sclerotia or conidia. In young mycelia, DNA values are comparable to those of the fruit-body. Most nuclei are in the G0/G1 phase of the cell cycle. In haplonts nuclear DNA content in this cell cycle phase corresponds to their genome size.

In the dikaryophase, two nuclei combine to form a zygote (nuclei in diplophase with a 'double set' of chromosomes). In fungi the zygote is the only diploid (2n) cell. In the ascus, the nucleus is divided immediately by meiosis (zygotic meiosis), thus restoring the haploid (n) condition in the life cycle (n – the haploid chromosome set, unit). The cell nuclei may undergo endoreduplication (DNA replication in absence of mitotic cell division) and endopolyploidy ('many set' of chromosomes) can be assumed to occur (x – the basic chromosome set, the basic DNA content in germ-line polyploids) (Nagel, 1978).

Since in some cases the content of nuclear DNA in the fruit-body (often in tips of paraphyses but also in all other cell types) appeared to be larger, endopolyploidy was assumed to occur in ascomycetes (Weber, 1992) and in basidiomycetes (*Paxillus*, *Serpula*, and *Leucogyrophana*) (Meixner & Bresinsky, 1988; Wittman-Meixner & Bresinsky, 1989).
In this study the nuclear behaviour of two moss parasites, the ascomycetes *Neottiella vivida* and *N. rutilans*, is examined. The main aim was to establish whether there occur changes in ploidy during fungal growth and morphogenesis, as well as to determine absolute genome sizes and ploidy levels in these species.

**MATERIALS AND METHODS**

Fruit-bodies of *Neottiella vivida* (Nyl.) Dennis, Norway, Tromsö, Brennfjell, 26 Aug. 1998, A. Jakobson (TAA 135733) and *N. rutilans* (Fr.) Dennis, Finland, Kilpisjärvi, Sana, 25 Aug. 1998, A. Jakobson (TAA 135730), were fixed in Carnoy (Romeis, 1948) and kept at 4°C until needed.

The slides used for measuring relative nuclear DNA content were also used for measuring spore dimensions with an ‘AMPLIVAL’ microscope equipped with an HI 100 immersion objective. The length (l) and width (w) of spores are presented in the following form: \( l_{\text{mean}} \times w_{\text{mean}} \ \mu \text{m} \), where \( l_{\text{mean}} \) and \( w_{\text{mean}} \) denote the mean values of the length and width of 20 spores from a specimen. The variation coefficient is equivalent to the standard deviation as a percentage divided by the mean value.

For staining nuclei the material was squeezed between the slide and the cover-slip and subjected to the DAPI-staining procedure described in Bresinsky et al. (1987), Wittmann-Meixner (1989), Weber (1992), and Büttner (1999). The relative DNA content in nuclei and mitochondria was measured by cytofluorometry at the Institute of Botany, Regensburg University, using a Zeiss UNIVERSAL photomicroscope, equipped with an III RS epifluorescence illuminator, and an 03 Zeiss microscope photometer. The measured fluorescence intensity (in arbitrary units = a.u.) is proportional to DNA content in the nucleus. When measuring nuclei in the haplophase, a distribution curve is obtained whose first maximum, indicating nuclei in the cell cycle G0/G1 phase, corresponds to the genome size.

The resulting fluorescence histograms can be analyzed for calculating the difference in nuclear DNA content between the specimens. By including an internal standard, relative DNA content is converted to the absolute amount. The genome size of an unknown specimen is obtained by dividing the mean relative DNA content of the unknown G0/G1 population of nuclei by the mean of the standard G0/G1 population of nuclei and by multiplying the result by the genome size of the standard. Usually DNA content is expressed in picograms (pg) or in base pairs (bp), kilobases (kb, a stretch of 1000 nucleotide pairs in DNA) and megabase pairs of nucleotides (Mb) (NB 1pg = 965 Mb, see Bennet & Leitch, 1995).

The genome size of *N. vivida* and *N. rutilans* was estimated by comparison with two standards: conidia from a pure culture of the ascomycete *Trichophaea hemisphaerioides* (Mounton) Graddon (TFC 97-71 from TAA 147708) and a spore-print from the oyster mushroom *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. (TAA 142824). The genome size of *T. hemisphaerioides* was 23.3 Mb, and that of *P. ostreatus* 25 Mb (Kullman, 2000).

**RESULTS AND DISCUSSION**

From the standpoint of nuclear cytology, *N. vivida* and *N. rutilans* have a life cycle typical of ascomycetes (Rossen & Westergaard, 1966; Weber, 1992; Weber & Bresins-
The cycle includes a monokaryotic thallus (with nuclei in the haplophase), a dikaryophase, confined upon fructification, and asci which represent zygotes. Here the nuclei are in the diplophase of the life cycle. When the ascus nucleus divides, division is meiotic and meiospores are formed (Figs. 1, 3, 4a-h).

**Somatic nuclear behaviour**

The presence of up to double DNA content in the nuclei of hyphae and paraphyses indicates mitotic cell cycle phases G0/G1 and G2/M (Figs. 2, 6d, e). During the growth of a fruit-body nuclei divide within the hyphae but remain stable within the paraphyses at maturity (except for the nuclei of their apical cells).
In *N. vivida*, the mean DNA content in the nuclei of the paraphyses (DNA content of the nuclei at the tip was not measured in this case) was 750 Mb ± 9% and in the nuclei of the hyphae 770 Mb ± 36%. In the first case the measured nuclei were only in the G0/G1 phase, while in the second case, most nuclei were in the G0/G1 phase and some were in the G2/M phase. The mean DNA content of the hyphae is larger and more variable compared with the mean DNA content of the paraphyses due to the mitotic division of the first. Hence the nuclei of the paraphyses are more suitable for measurement of the C-value than the nuclei of the subhymenium hyphae.

When measuring fluorescence on nuclei, noise (additional light including the auto-fluorescence of cells) accounts for 6% when paraphyses are used, 12% when hyphae are used, and 12% when spores are used from total measured fluorescence (fluorescence of nuclear DNA-DAPI complex + noise). For this reason too, paraphyses should be preferred in determination of relative DNA content and genome size. In this case the 1C-value, i.e. genome size, was determined as 750 Mb for *N. vivida* and as 530 Mb for *N. rutilans* (see Materials and Methods).

**Development of the ascus**

The asci of *N. vivida* develop from croziers according to the *Neottiella*-type pattern of karyogamy (Read & Beckett, 1996; Chiu & Moore, 1999) (Fig. 1). Two nuclei in the hooked cell undergo conjugate mitoses (Mi) after which two septa are formed creating three cells (Fig. 1a–c). The three cells of the crozier are termed the terminal cell, the penultimate cell, and the stalk cell, representing respectively the first, the second, and the third cell of the crozier. The penultimate cell is binucleate, whereas the two other cells are uninucleate. At first, two prefusion nuclei with the basic DNA content are located in the terminal cell and in the stalk cell, respectively. The nucleus of the terminal cell then migrates into the stalk cell (Figs. 4a, 5). This binucleate cell, containing non-sister nuclei, may become the ascus mother cell, in which karyogamy (K) takes place (Fig. 5). In the young ascus, the first meiotic division (Me1) (Figs. 1f, 4c) and the second meiotic division (Me2) (Figs. 1g, 4d) give rise to four daughter nuclei with the basic DNA content, each of which divides by mitosis (Mi) to form eight ascospore nuclei (Figs. 1h, 4e). Formation of ascospores results from the infolding of the membranes around the nuclei (Figs. 1i, 4f). In a maturing spore, the nucleus with condensing chromatin remains between two vacuoles (Figs. 1j, 4h).

**Changes in ploidy level during DNA replication and cell division at the time of sporulation — Fig. 6**

After karyogamy the DNA content of the nucleus has the 4C-value. The mean DNA content was determined as 3060 Mb (Fig. 6a). The C-value can be measured exactly after the first meiotic division (Me1) when the formed nuclei have a stable DNA content (2C) until the next division (Figs. 1f, 4c). In such nuclei, the mean DNA content was 1500 Mb (then 1C = 1500 : 2 = 750 Mb, the same 1C-value like in paraphyses).

After the second meiotic division (Me2; Figs. 1g, 6b) and mitosis (Mi; Fig. 1h) the following DNA synthesis may occur asynchronously (Fig. 3d). After Me2, mean nuclear DNA content was 770 Mb and after Mi 810 Mb. Thereafter, eight uninucleate ascospores with the 2C-value are formed. In such nuclei, mean DNA content was measured at 1350 Mb for *N. vivida* (Fig. 6c).
Fig. 4. Photomicrographs of fruit-body of *N. vivida* (a, b, d, f: fluorescence micrographs; c, e, g – l: normal micrographs). a. nuclei in croziers and young asci; b. zygote nucleus; c. nuclei in the course of the first meiotic division; d. nuclei in the course of the second meiotic division; e. nuclei after postmeiotic mitosis; f. young nuclei of ascospores; g. endoreduplication in spore; h. fertilizing spore with condensing chromatin, nucleus between vacuoli; i. giant mitochondria in the apex of an ascus; j. nuclei in a hypha; k. nuclei in a hair; l. nuclei in a paraphysis. Bar = 10 µm.
The spore nuclei of *Neottiella* species studied here undergo endoreduplication (DNA replication in absence of mitotic cell division). In *N. rutilans*, endoreduplication is not arrested at the 2C-value as in *N. vivida*, but may proceed at a different rate in spores. In *N. rutilans* spores with DNA values of 2C, 3C, 5C, and 6C have been measured and endopolyploidy can be assumed to occur at different levels. These uninucleate spores are heteroploid. No differences have been found in spore sizes between the two specimens studied: spore sizes in *N. rutilans* have been measured at 23.1 μm ± 5% × 12.8 μm ± 5% and in *N. vivida* at 22.8 μm ± 4% × 13.0 μm ± 3%.

**Ascogenous hyphal differentiation**

There is evidence that species may reveal considerable variation in the pattern of ascogenous hyphal differentiation; significant variation can even be found within a single genus (*Thelebolus* Tode: Fr., Kimbrough, 1981). The precise ontogeny of ascogenous hyphae is usually difficult to study. *Neottiella vivida* with extremely large nuclei is the most appropriate species for this kind of research.

In *N. vivida*, ascogenous hyphae may branch repeatedly. In this case both cells, the penultimate cell and the stalk cell (containing also the migrate nucleus from the terminal cell), elongate to form a new crozier (Figs. 4a, 5). However, in the case of the *Neottiella* type pattern of karyogamy, asci can be formed one after another from the terminal and the stalk cells, while the penultimate cell initiates formation of new croziers continuously (Fig. 5). All nuclei of ascogenous hyphae, except for nuclei in the initial penultimate cell, can be potentially used in karyogamy for formation of zygotes.

**Time of premeiotic DNA synthesis**

Premeiotic DNA replication in fungi is known to occur usually before and sometimes after karyogamy. In *N. vivida*, premeiotic DNA replication occurs before karyogamy, as in *N. rutilans* (Rossen & Westergaard, 1966) and *Neurospora crassa* Shear & B.O. Dodge (Iyengar et al., 1977), and synchronously in all four nuclei of the crozier cells (Figs. 3a, b, terminal and stalk cells are not shown) (Figs. 4a, 5). It is possible that delayed premeiotic DNA synthesis is a general condition in fungi with a homokaryotic fruit-body (Bayman & Collins, 1990).

**DNA content of giant mitochondria**

Giant mitochondria are visible in the ascus apex. The DNA content of giant mitochondria was determined to be 60 Mb, 54 Mb, and 30 Mb (Fig. 4i).

The mitochondrial genome size of eight ascomycetes was found to be from 18.9 b.k. (*Torulopsis glabrata* (H.W. Anderson) Lodder & N.F. de Vries) to 108 b.k. (*Bretanomyces custersii* Florenz.) (Weber, 1993). Obviously, the mitochondrion of *N. vivida* with high DNA content contains multiple strands of DNA. Different DNA contents of mitochondria reveal the existence of a different number of strands in their nucleoids. Kuroiwa et al. (1996) examined the development of giant mitochondria of the plant *Pelargonium zonale* Ait after during megalasporogenesis and megalagametogenesis. They found that DNA content within the stacked mitochondrion increased up to 40 times compared with that at the megaspore mother cell stage; a single stack of mitochondria contained 340–1700 Mb DNA.
Nuclear genome size

In this study the genome size of *N. rutilans* was determined to be 530 Mb and that of *N. vivida* 750 Mb. Genome sizes for Pezizales ranged from 12 Mb for *Pulvinula* sp. (author’s unpublished data) to 750 Mb for *N. vivida*. The majority of genome sizes reported earlier for other fungi fall in this range too (Durán & Gray, 1989; Wittman-Meixner, 1989; Zolan, 1995).

The overall genome size estimated in this study for *Neottiella* species was significantly larger than that of the ascomycetous yeast *Saccharomyces cerevisiae* (13 Mb, data are available on internet: ftp.ebi.ac.uk) and other filamentous fungi. For example, the following genome sizes have been determined: for *P. ostreatus* 21 Mb (Sagawa & Nagata, 1992) and 24 to 30 Mb (two subpopulations in one spore-print differing in DNA content by 4.9 Mb (20%), Kullman, 2000), 31 Mb (Peberdy et al., 1993) and 35 Mb (Larraya et al., 1999), for *Trichophaea hemisphaeroides* (Mouton) Graddon 23 Mb (Kullman, 2000), for *Penicillum Paxilli* Bainier 23 Mb (Itoh et al., 1994), for *Histoplasma capsulatum* Darling 23 to 32 Mb (Carr & Shearer, 1998), for *Emericella (Aspergillus) nidulans* (Eidam) Vuill. 26 Mb (Timberlake, 1978) to 31 Mb (Brody & Carbon, 1989), for *Podospora anserina* (Rabenh.) Niessl 34 Mb (Javerzat et al., 1993), for *Neurospora crassa* Shear & Dodge 39 Mb (Orbach, 1992) and 43 to 45 Mb (Radford
Ploidy levels and speciation

When among ascomycetes the ploidy level of *N. rutilans* is approximately 50 (Weber, 1992), then, using genome sizes, the ploidy level of *N. vivida* was calculated...
to be 70. The two species are very similar and can be distinguished only by their spores, which possess an incomplete reticulum in *N. rutilans* and delicate regular warts in *N. vivida*. Heteroploid spores of *N. rutilans* have a high variability of spore ornamentation. Some spores have more restricted warts which are not completely connected to form a reticulate ornament. According to Galitski et al. (1999) (see also Hieter & Griffiths, 1999), cells of the ascomycetous yeast *Saccharomyces cerevisiae*, differing only in their ploidy, are identical in terms of DNA sequence information and relative gene dosage, but show different patterns of gene expression. In *N. vivida* and *N. rutilans*, the differences in spore ornamentation may be the result of different gene expressions regulated by a ploidy-dependent gene.

As the spores of *N. rutilans* are heteroploid, some spores of *N. rutilans* and *N. vivida* have the same DNA content. There is as yet no evidence of haploidization of these spore nuclei at germination. It is also known that two closely related species of Leotiales, *'Hymenoscyphus' equisetinus* (Velen.) Dennis and *'H. rhodoleucus* (Fr.) Z.S. Bi, having the same genome size, differ only in the relative DNA content of their uni-nucleate spores (1C and 2C, respectively) and in spore width (Weber, 1992). It can be speculated that in such cases polyploidy will repeatedly confirm taxonomically described speciation, as suggested by Bresinsky & Wittmann-Bresinsky (1995) for Boletales.

**CONCLUSIONS**

Within the true fungi, species of *Neottiella* serve as excellent objects for cytogenetic investigations due to their extraordinarily large nuclear DNA content and low cell autofluorescence. *Neottiella vivida* and *N. rutilans* were found to have genomes of approximately 750 Mb and 530 Mb, respectively.

*Neottiella rutilans* is the first ascomycete in which heteroploidy of spores has been discovered. Probably different levels of endopolyploidy in its spores account for the large variability of spore ornamentation within one specimen.

Giant mitochondria were found in the ascus, indicating intensive aerobic respiration and energy generation for metabolic activity associated with meiospore formation. It is demonstrated in flowering plants that the number of plastids ultimately produced per cell is a function of the level of endopolyploidy (Butterfass, 1967, 1973). The converse idea is that nuclear DNA replication is regulated by plastids, as appears to be the case with *Chlamydomonas* Ehrenberg (Blamire et al., 1974). This problem needs further research in fungi.

The *Neottiella*-type pattern of karyogamy is in principle maximally economical. All nuclei of ascogenous hyphae, except for the nuclei in the initial, i.e. penultimate cells, have the potential to be used in karyogamy for production of zygotes. In this case, all replicated DNA in ascogenous hyphae can eventually be used for spore production. However, there are two alternatives allowing regulation of spore production: formation of asci one after another, or formation of new croziers, i.e. new branches (instead of asci), for simultaneous production of a large number of asci. It can be speculated that the first alternative is more frequent in species with small primitive ascomata, while the second alternative occurs in species with large, more differentiated ascomata. However, this argument requires further study.
ACKNOWLEDGEMENTS

The research was supported partly by the DFG (Deutsche Forschungsgemeinschaft) and by grants Nos. 3580 and 4989 of the Estonian Science Foundation. I thank Prof. A. Bresinsky for useful consultations and all-round help. I thank Dr. M. Rahi and Dr. A. Raitviir for critical comments on the manuscript. Sincere thanks are due to Mrs. E. Jaigma for revising the English text of the manuscript.

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