Chromosome length polymorphism in *Cryptococcus neoformans* clinical and environmental isolates

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Summary

A protocol for intact DNA preparation from the basidiomycetous yeast *Cryptococcus neoformans* has been developed and applied to karyotyping *C. neoformans* isolates displaying different degrees of capsule formation. A total of 46 strains have been analyzed: 23 (50%) isolated from environmental samples (pigeon droppings), all of them belonging to *C. neoformans* var. *neoformans*; and 23 (50%) from clinical samples (human and veterinarian) including 10 isolates of *C. neoformans* var. *neoformans* and 13 isolates of *C. neoformans* var. *gattii*. Our results showed a global genome size ranging from 14.2 to 20.9 Mb for variety *neoformans* and from 7.9 to 16.8 Mb for variety *gattii*. The karyotype diversity was very high for variety *neoformans* (29 different patterns for the 33 analyzed strains) and lower for variety *gattii* (six different patterns for 13 strains). No grouping among variety *neoformans* strains from the same origin was found indicating very high genome diversity for this variety, irrespectively of the origin of the strains.

Key words *Cryptococcus neoformans*, Karyotype, Environmental isolates, PFGE

Polimorfismo de tamaño cromosómico en aislamientos clínicos y ambientales de *Cryptococcus neoformans*

Resumen

Se describe un nuevo método de extracción de DNA intacto de la levadura *Cryprococcus neoformans* y su aplicación para la obtención del cariotipo de la misma mediante electroforesis en gel de campo pulsado. El método discrimina entre cepas con diferente grado de formación de cápsula y ha sido ensayado en un total de 46 aislamientos: 23 (50%) procedentes de medio ambiente (heces de paloma), todos ellos pertenecientes a la variedad *neoformans*; y 23 (50%) procedentes de muestras clínicas (humanas y veterinarias) que incluyeron 10 aislamientos de *C. neoformans* var. *neoformans* y 13 de *C. neoformans* var. *gattii*. Los resultados muestran un tamaño global del genoma de 14,2 a 20,9 Mb para la variedad *neoformans* y de 7,9 a 16,8 Mb para la variedad *gattii*. La variabilidad de cariotipo es muy elevada en la variedad *neoformans* (29 patrones diferentes para las 33 cepas analizadas) y menor para la variedad *gattii* (séis patrones diferentes para 13 cepas). Los aislamientos de la variedad *neoformans* no mostraron agrupamiento incluso entre cepas del mismo origen, lo que indica una muy elevada diversidad genómica para esta variedad, e independiente del origen de las cepas.

Palabras clave *Cryptococcus neoformans*, Cariotipo, Aislamientos ambientales, Electroforesis de campo pulsado

Cryptococcus neoformans is encapsulated yeast causing life-threatening infection in inmunocompromised hosts, such as those receiving corticosteroids or infected with the human inmunodeficiency virus. This yeast has been classified into two varieties: *C. neoformans* var. *neoformans* and *C. neoformans*. var *gattii* [1]. In addition, each variety presents two possible capsular antigens, thus, there are two serotypes for variety *neoformans* (A, and D) and two for var. *gattii* (B, and C). The introduction of Pulsed Field Gel Electrophoresis (PFGE) allowed a new approach to the study of fungal genetics. The ability to separate intact fungal chromosomes has led to the disco-

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very that most species exhibit Chromosome Length Polymorphism (CLP) and a relationship between CLPs and adaptation to different environmental conditions has been shown [2]. Since 1989, some PFGE studies have been carried out trying to find significative relationships between karyotype and phenotype for *Cryptococcus neoformans* [3-6]. All these studies have shown high karyotype variability in *C. neoformans*, and even demonstrated the capability of this yeast to rearrange its genome during an infection process [7]. For other pathogenic fungi a relationship between karyotype variability and environmental adaptation has been demonstrated [2]. In view of these results, our goal was to study strains of *Cryptococcus neoformans* isolated from two different environments, pigeon droppings and clinical samples (human and veterinarian). Since it has been documented that *C. neoformans* strains causing infection are of environmental origin [8] the comparison of karyotypes from environmental and clinical samples could allow us to ascertain whether *C. neoformans* undergoes stable chromosome rearrangements during the infection process. The goal of this work is to present such a comparison.

MATERIALS AND METHODS

Strains. A total of 46 strains of *Cryptococcus neoformans* were studied. Eleven were obtained from human clinical samples (Hospital de San Juan, Alicante, Spain, and Hospital de Montevideo, Uruguay); 12 from veterinarian samples (goat) (Facultad de Veterinaria. Cáceres. Spain), and 23 isolated from pigeon droppings in Alicante and Santa Pola, Spain [9]. Thirty-three (71.7%) of them belonged to the variety *neoformans*, and 13 (28,3%) to *C. neoformans* var *gattii* (Table 1). The identification was carried out by carbon compound assimilation (Auxacolor, Sanofi-Pasteur. Paris. France), urease and phenoloxidase production; growth at 37 °C and sensitivity to cycloheximide. The variety was determined both by growth on CGB agar [1] and D-proline assimilation [10]. Strains were maintained at 4 °C in sterile distilled water.

DNA extraction. Although there are several protocols described for *C. neoformans* intact DNA preparation [5, 11, 12], we had to optimize a new one for reproducible preparation of intact DNA from *C. neoformans* var. *gattii* strains. A modification of the method described by Wickes et al [12] was used. Cells were grown on YED broth (1% yeast extract; 2% dextrose) for 18 hours at 30 °C with gently agitation (150 rpm). Then they were washed three times in 50 mM EDTA pH 7.5. A second set of washes (x2) was then carried out in 125 mM EDTA pH 7.5 for strains belonging to variety *neoformans* and in 125 mM EDTA pH 7.5 with 1 M sorbitol for those of variety *gattii*. To obtain protoplasts, cells were treated with 10 mg/ml lysing enzymes from *Trichoderma harzianum* (Sigma) in CEPES (40 mM citric acid, 120 mM Na_2HPO_4, 1,2 M sorbitol, 20 mM EDTA pH 7,5, 5 mM DTT) at 37 °C for 30 or 60 min for variety *neoformans* and *gattii* respectively. Protoplasts were embedded in 1.6% low melting point agarose with 10 mg/ml lysing enzymes from *Trichoderma harzianum* (Sigma) and lysed overnight by incubating at 50 °C with ESP (0,5 M EDTA pH 9.0; 1% Na-Laurylsarcosine and 2 mg/ml Proteinase K). Agarose blocks were washed in 0.5 M EDTA pH 9,0 three times (or until the brown color disappeared) and kept in this solution at 4 °C until electrophoresis was preformed.

Electrophoresis conditions. Different electrophoresis conditions (voltage, temperature, pulse time and buffer solution), were assayed depending on the size range of DNA to be separated (see figure legends). Two PFGE systems were used: CHEF DRIII variable-angle (Bio-Rad), and LKB Navigator (Pharmacia). Gels were made with 1% Low EEO agarose (Hispanlab). The buffer was TBE 1X (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) for electrophoresis at high voltage (6 V/cm) and TAE 1X (40 mM Tris Acetate, 2 mM EDTA) for electrophoresis at low voltage (3 V/cm).

Data analysis. Pairwise comparisons of the band patterns were manually performed, and a matrix (presence-absence for each character) constructed. The data was computed using the NTSYS program version in an IBM PC [13].

Table 1. Origin and variety of the isolates studied.

Isolate	Origin	Variety
1	Environmental. Alicante	*neoformans*
2	Environmental. Alicante	*neoformans*
3	Environmental. Alicante	*neoformans*
4	Environmental. Alicante	*neoformans*
5	Environmental. Alicante	*neoformans*
6	Environmental. Alicante	*neoformans*
7	Environmental. Alicante	*neoformans*
8	Environmental. Alicante	*neoformans*
9	Environmental. Santa Pola	*neoformans*
10	Environmental. Santa Pola	*neoformans*
11	Environmental. Santa Pola	*neoformans*
12	Environmental. Santa Pola	*neoformans*
14	Environmental. Alicante	*neoformans*
15	Environmental. Alicante	*neoformans*
16	Environmental. Alicante	*neoformans*
17	Environmental. Alicante	*neoformans*
18	Environmental. Alicante	*neoformans*
19	Environmental. Alicante	*neoformans*
20	Environmental. Alicante	*neoformans*
21	Environmental. Santa Pola	*neoformans*
22	Environmental. Santa Pola	*neoformans*
23	Environmental. Santa Pola	*neoformans*
24	Environmental. Santa Pola	*neoformans*
13	Human/CSF/Alicante	*neoformans*
Cl2	Human/CSF/Alicante	*neoformans*
1795	Human/Montevideo	*gattii*
1796	Human/Montevideo	*neoformans*
2042	Human/Montevideo	*neoformans*
2043	Human/Montevideo	*neoformans*
2044	Human/Montevideo	*neoformans*
2045	Human/Montevideo	*neoformans*
2046	Human/Montevideo	*neoformans*
2047	Human/Montevideo	*neoformans*
2048	Human/Montevideo	*neoformans*
48	Goat/Lung/Cáceres	*gattii*
49	Goat/Lung/Cáceres	*gattii*
50	Goat/Lung/Cáceres	*gattii*
51	Goat/Liver/Cáceres	*gattii*
52	Goat/Brain/Cáceres	*gattii*
53	Goat/Cáceres	*gattii*
54	Goat/Lung/Cáceres	*gattii*
55	Goat/Lung/Cáceres	*gattii*
56	Goat/Intestine/Cáceres	*gattii*
58	Goat/Lung/Cáceres	*gattii*
59	Goat/Lung/Cáceres	*gattii*
60	Goat/Lung/Cáceres	*gattii*

RESULTS AND DISCUSSION

PFGE has been widely applied to the study of fungal genomic organization, both for yeast and filamentous fungi [2]. Prior to electrophoretic chromosome separation, the cell wall has to be removed and the protoplast thus formed have to be embedded in agarose and lysed in order to allow the obtention of intact DNA. However, many fungi have cell walls very resistant to physical, chemical and enzymatic treatments, which renders the protoplast obtention very difficult. This is especially true for fungi

such as *Cryptococcus neoformans* belonging to Basydiomycetes for which several intact DNA preparation protocols have been described [5,11,12]. Only one of these methods [11] considers the different varieties or the amount of capsule formed. However, in our hands, the strains with thicker capsules (very frequent in variety *gattii*) need both more washes and longer incubations in spheroplasting solution than the strains that present thinner capsules. However, irrespectively the amount of capsules formed, it is necessary to use a high concentration (10 mg/ml) of lysing enzymes in the spheroplasting solution in order to obtain enough amount of DNA suitable for PFGE, as it was concluded from the optimization experiments we carried out varying lysing enzymes concentration, temperature and incubation time (data not shown).

More than 50 gels corresponding to different separation conditions were analyzed. Chromosome patterns for a given strain was stable *in vitro*. Our results showed that *C. neoformans* var. *neoformans* harbored from nine to 13 chromosomes with a global genome size about 17 Mb, with a range between 14.2 and 20.9 Mb (Figures 1, 2 &3). For *C. neoformans* var *gattii*, we found from seven to 12 chromosomes with a global genome size of 10.0 Mb (range: 7.9-16.8 Mb). However, as discussed below, these numbers are likely to be minimal estimates because of the difficulty of separating the largest chromosomes.

Chromosome sizes vary from 420 to >3000 Kb. Twenty-two (96%) of *C. neoformans* strains from clinical origin showed bands under 700 Kb while only six (26%) of the environmental isolates (Figure 3). On the contrary, for environmental strains, larger fragments were most frequently represented, specially the 1800 Kb band that was obtained in 52% (9) of these strains and was absent in isolates of clinical origin (Figure 3). In order to calculate accurately the genome size it is necessary to separate all the chromosomes. In our case, this could only be accomplished running at least two different electrophoresis (with different pulse times and voltages, see Figures 1 and 2) for each strain. This is due to the fact that larger chromosomes remain non resolved in the compression region (see Figure 1) when conditions appropriate for separating smaller chromosomes are used, rendering the size estimation for larger chromosomes very inaccurate. This could somehow explain the differences in genome size reported in the literature. The global genome sizes reported for *C. neoformans* varies from the 15-17 Mb [11] to 24,5 Mb [5, 12]. In fact, most of the published results estimated the size of largest chromosomes as "larger than 2.200 Kb" [3,4,12]. There were no common fragments for all strains studied. The most conserved bands were those of 1050 Kb (63% of the strains); 850 Kb (54%); 1400 Kb (48%) and 1150 Kb (46%). None of these fragments were specially represented attending the variety and/or the origin of the isolates. For *C. neoformans* var. *neoformans*, the smallest chromosome varied among strains but in most cases was larger that 650 kb (Figure 3). However, almost all the *C. neoformans* var. *gattii* strains analyzed presented chromosomes of 420 and 580 kb (Figure 3). Wickes *et al.* [12] suggested that the presence of the small chromosome in the 500 kb range, could be used for identifying strains belonging to the variety *gattii*. Although we found a high presence of these bands in strains belonging to variety *gattii* (85% of the strains showed bands <750kb), we also found three (9%) var. *neoformans* strains that harbor such small chromosomes. Therefore we consider that the use of this criterion for identification of var. *gattii* strains could be misleading.

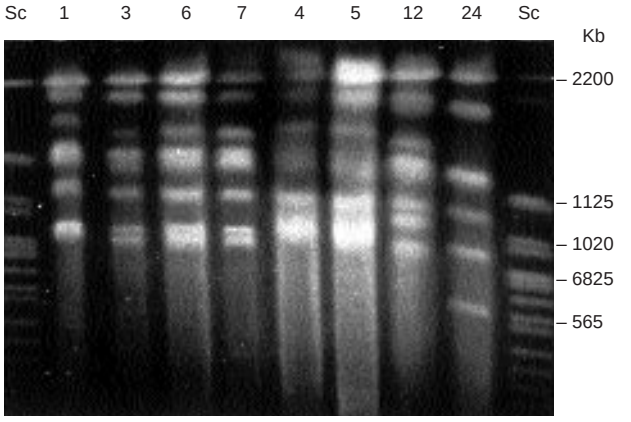

Figure 1. PFGE of *Cryptococcus neoformans* var. *neoformans* environmental isolates. Electrophoresis conditions: voltage: 6 V/cm; pulse time: 60-120 seconds; temperature: 14 °C; buffer: 1x TBE. Time: 24 hours. Sc: Total genomic DNA from *Saccharomyces cerevisiae* as DNA size marker (BioRad).

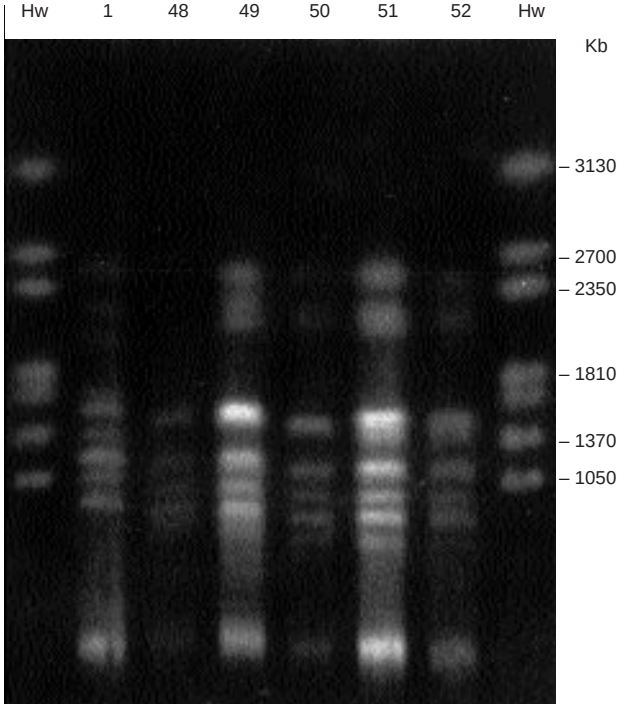

Figure 2. PFGE of *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii* strains. Electrophoresis conditions: voltage: 3V/cm; pulse time: 250-900 s; temperature: 14 °C; buffer: TAE x1. Time: 50 h. Hw: Total genomic DNA from *Hansenula wingei* as DNA size marker (Bio Rad). Strains: 1: *C. neoformans* var. *neoformans* from pigeon droppings. 48-52: *C. neoformans* var. *gattii* from goat tissues.

The chromosome pattern varied among strains for both origins and varieties (Figure 3), although this variation was somehow smaller for the variety *gattii* since we have obtained 29 different chromosome patterns for the 33 var. *neoformans* strains analyzed and six different patterns for 13 variety *gattii* strains (Figure 3). Our results agree with that of other authors, finding a higher variability in *C. neoformans* var. *neoformans* than in var. *gattii* strains. The karyotype variability (see figures 3 and 4) for clinical isolates was very high, since 15 chromosome pat-

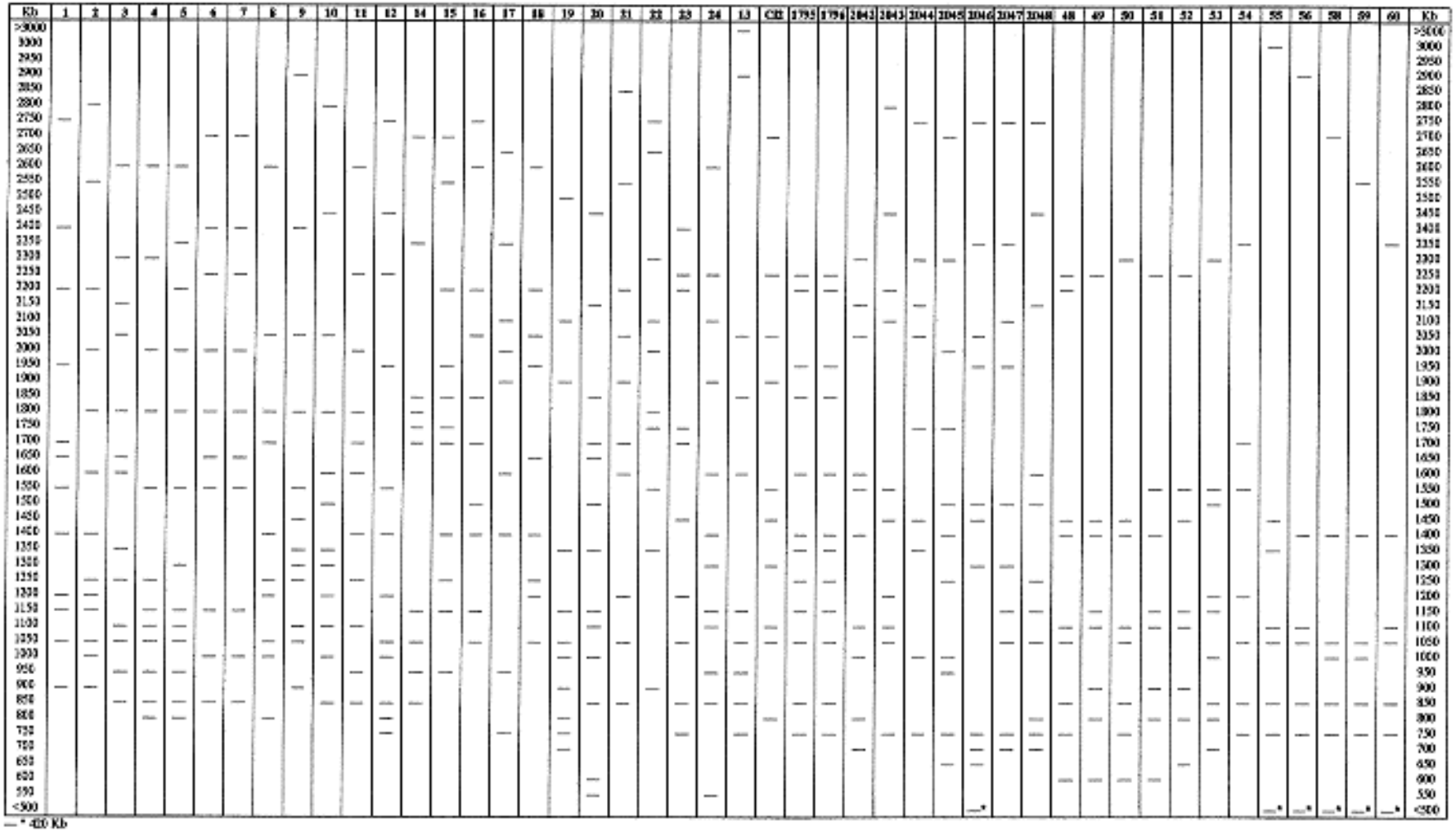

Figure 3. Representation of PFGE patterns of *Cryptococcus neoformans* isolates. Strains from 1-24: *C. neoformans* var. *neoformans* environmental strains. Strains from 13 to 2048: *C. neoformans* var. *neoformans* clinical strains. Strains from 48 to 60: *C. neoformans* var. *gattii* strains. Numbers indicate molecular size in kilobasepairs.

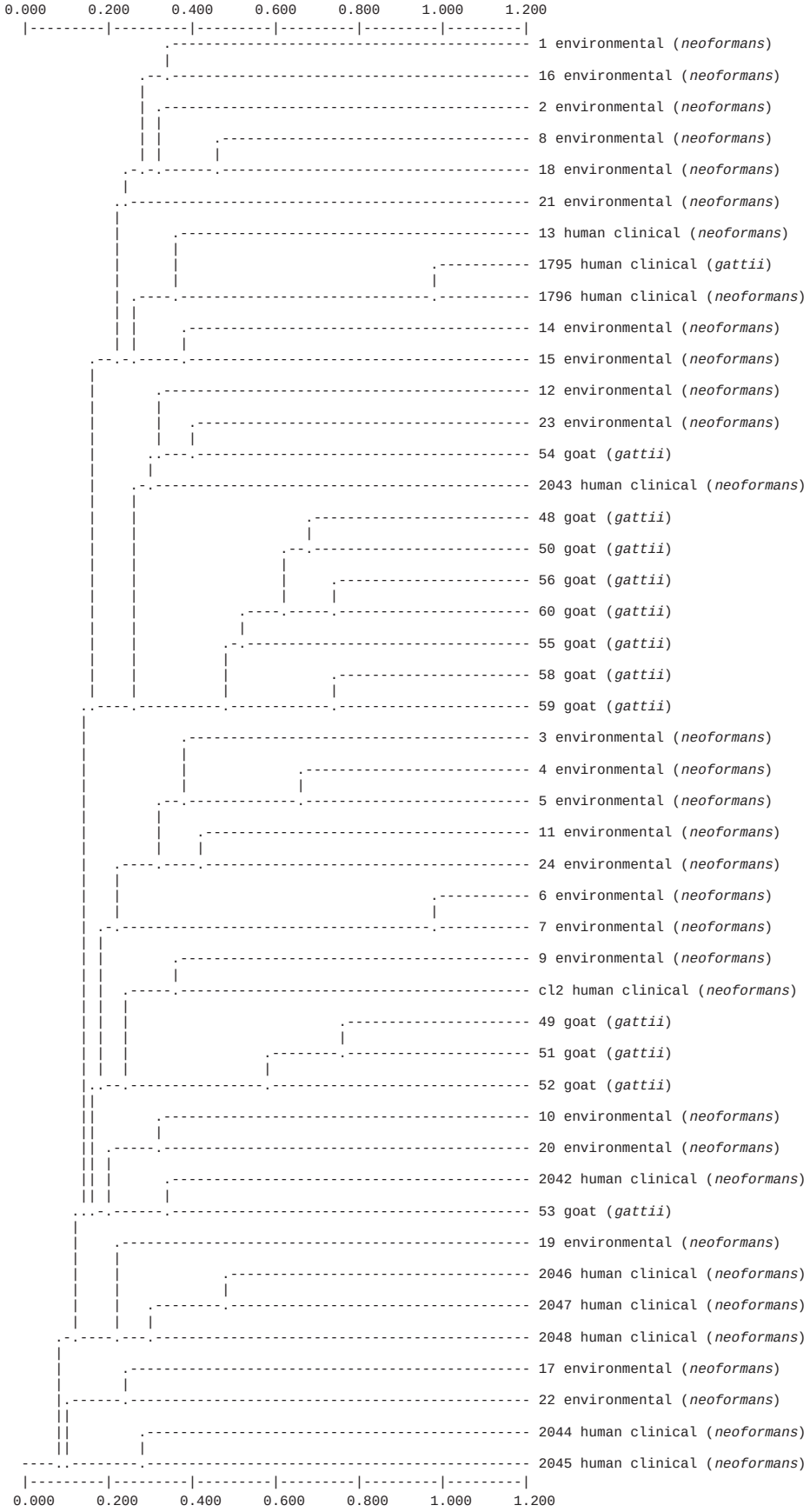

Figure 4. Dendrogram obtained by pairwise comparisons of the PFGE patterns (data computed with NTSYS program). Strains: 1-12 and 14-24: *C. neoformans* var. *neoformans* environmental strains; 13, Cl2, and 1796-2048: *C. neoformans* var. *neoformans* human clinical strains; 1795: *C. neoformans* var. *gattii* human clinical strain. 48-60: *C. neoformans* var. *gattii* from goat.

terns were found for the 23 strains analyzed. However, this high diversity of karyotypes was also found in the strains from environmental origin isolated from pigeon droppings. For the 23 isolates of this origin, 20 different chromosome patterns were obtained. In fact, these strains were not grouped (see figure 4) according to their karyotypes but dispersed in the dendrogram with those of clinical origin. Even the strains isolated from pigeon droppings from the same cage or sampling point (Table 1) display a high karyotype diversity (Figure 1, strains 12 and 24). This environmental diversity of *C. neoformans* karyotypes seems higher than expected making any correlation between karyotype and environmental for var. *neoformans* strains very difficult. However, the strains belonging to var. *gattii* were less diverse regarding their karyotype, as shown in figure 4, where they are grouped together and apart from var. *neoformans* strains.

The origin of this high karyotype variability has been addressed for different fungi such as *Candida albicans* and *Coprinus cinereus* [14,15]. These studies suggest that karyotype variability is due to mobility of genome fragments that include either "non sense" sequences (B or dispensable chromosomes) or multiple copies or a given sequence, such rDNAs. The mobility of these elements would result phenotypically neutral although, in some case, a relationship between CLP and adaptation to environmental conditions has been reported [2,16]. However, more studies are needed in order to clarify the role that this high CLP plays in *C. neoformans* adaptation to the environment and its putative relationship with its pathogenicity.

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