GENETIC VARIABILITY AMONG *BLASTOSCHIZOMYCES CAPITATUS* ISOLATES FROM DIFFERENT CLINICAL SOURCES

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Sixteen clinical isolates and nine ATCC reference strains of *Blastoschizomyces capitatus* were analysed genetically, examined for the cellobiose, arbutin and salicin assimilation and tested for the aspartyl-proteinase secretion. The restriction endonuclease analysis (REA) with *Hpa*II and *Hinf*I enzymes and the electrophoretic karyotype (EK) were investigated. Both the restriction enzymes revealed two groups (I, II) constituted by the same isolates: 17 isolates (68%) in group I and 8 (32%) in group II. The EK analysis revealed sixteen groups. These data prompts for a genetic variability of the isolates of *Blastoschizomyces capitatus* and their account in two distinct genetic groups as suggested by REA. This grouping was confirmed by examing the utilisation of cellobiose, arbutin and salicin. The tests for secretory aspartyl proteinase (Sap) were positive only for three isolates, suggesting a marginal role of this specific enzyme in pathogenesis for these isolates.

Disseminated fungal infections (*Candida* or *Aspergillus* species) are causes of morbidity and mortality in neutropenic patients with haematological or oncologic disorders. Recently, among fungal microorganisms, *Blastoschizomyces capitatus* has been recognised as an uncommon yet increasing, opportunistic pathogen (1-3).

Blastoschizomyces capitatus (4-6) can be distinguished from other morphologically similar organisms, i.e., *Geotrichum candidum* and *Trichosporon beigelii*, by its (i) formation of annelloconidia at the tip of a percurrently proliferating conidiogenous cell, (ii) growth at 45°C on standard nutrient media such as SGA, (iii) resistance to cycloeximide, and (iv) inability to utilise urea (6). Formerly it was also known as *Geotrichum capitatum* (7-11) and/or *Trichosporon capitatum* (12-14). All these names are considered as synonyms (7-9). Moreover, several authors have considered *B. capitatus* as a different species with respect to *T. capitatum* by morphogenetic characteristics (6) creating the specific genus *Blastoschizomyces*. Published cases have already reported several *B. capitatus* infections including disseminated infection, osteomyelitis and intervertebral discitis, onychomycosis, respiratory tract infection, septicaemia, meningitis, kidney, liver involvement and endocarditis (2-3, 6, 12-20). More of these infections were discovered in Europe (85% of the reported cases) than in North America (10% of the reported cases) (6).

Key words: blastoschizomyces capitatus, REA (Restriction endonucleases Analysis), EK (electrophoretic karyotype), carbon compounds utilisation, Sap (secretory aspartyl proteinase)

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This fungus is commonly present in the soil (21), often isolated in stools of children, on the human skin, and in the digestive and respiratory tracts (13-14).

Despite the information on the biology and taxonomy of this fungus, little is known about its epidemiology and/or virulence. Adhesion to human tissues, proteinase production, ability to form biofilms (slime) in glucose-containing solutions remain to be defined. Moreover at this time, a complex genetic analysis of isolates of this fungus is lacking. In this work, we investigated the genetic characteristics and the aspartyl-proteinase secretion of nine ATCC reference strains of *B. capitatus* and sixteen clinical strains from different sources, mostly blood, detected as *B. capitatus* by morphological and biochemical properties.

MATERIALS AND METHODS

Microorganisms and culture conditions

Twenty-five isolates of *Blastoschizomyces capitatus* were utilized in this work (Tab. I). The yeast-like isolates were identified by examination of well established morphological and biochemical characteristics. The cycloheximide resistance of these organisms was examined on BBL Mycosel (Becton Dickinson Microbiology System, Cockeysville, Md) slants incubated at 30°C. Conidial morphology and ontogeny were observed by use of 7- to 10-day-old potato dextrose agar (Unipath S.p.a., Garbagnate Milanese, Milan, Italy) plates and cornmeal agar (Unipath) slide cultures. Potassium nitrate assimilation was determined with a nitrate test medium as reported by others (22). Biochemical tests were performed by using ID32 C yeast identification system strips with an ATB reader (BioMérieux Italy S.p.A., Roma, Italy) (Tab. I). All the isolates were grown in YPD, 1% yeast extract (w/v) (Biolife Italiana, Milano, Italy), 2% (w/v) peptone (Biolife Italiana) 2% (w/v) D-glucose (Carlo Erba Reagenti, Milano, Italy) at 30°C in a horizontal shaking incubator (Sanyo Gallenkamp PLC, Belton Park, Loughborough, UK). The strains were maintained in Cryo vials (Delchimica Scientific Glassware, Napoli, Italy) from overnight liquid culture supplemented with 30% (v/v) glycerol (Sigma, Sigma Chemical Co., St. Louis, USA) and stored at –80°.

Carbon source growth test

The ability to grow on cellobiose, arbutin and salicin as carbon sources was tested in liquid broth in according to van der Walt & Yarrow (23). The isolates were pre-grown in Sabouraud for 24 h at 30°C. The cultures were diluted first 1:50 and successively 1:20 with 0.67% (w/v) yeast nitrogen base (YNB, Difco) without carbohydrate. Finally, 100 µl of the last diluted cultures were added to 5 ml of YNB supplemented of 1% (w/v) of carbohydrate to test. The growing was scored after 1 week at room temperature in horizontal shaker incubator (Sanyo Gallenkamp PLC, Belton Park, Loughborough, UK).

DNA preparation

Whole-cell DNA was prepared as in the previously described yeast method (24). Briefly, three colonies of each isolate were grown separately to stationary phase, as assessed by measurements of optical density of cultures, in 10 ml of YPD medium at 30°C in 50-ml sterile disposable polypropylene tubes (Delchimica Scientific Glassware). For spheroplast formation, the harvested cells were washed in SE buffer 1.2 M D-sorbitol, 0.1 M EDTA pH 7.5, and 14 mM β-mercaptoethanol (Sigma), with 25 µl of 5 mg/ml (w/v) Lyticase (4000 U/mg; Sigma) solution and incubated at 37°C for 1 h. The spheroplasts were centrifuged and incubated in lysis buffer, 50 mM Tris-HCl, 20 mM EDTA pH 7.5 and 1% (w/v) sodium dodecyl sulphate (Sigma) for 30 min at 65°C. 1.5 ml of 5 M potassium acetate (Sigma) was added and the mixture was kept on ice for 1 h and centrifuged. The pellet was discarded and the supernatant was treated for DNA purification (25).

Restriction endonuclease analysis (REA)

The following restriction endonucleases were utilised *Hpa*II and *Hinf*I (New England Biolabs, Hitchin, Hertfordshire, England, UK) in accordance with the manufacturer. Whole cell DNA 7 µg were incubated with 50-60 units of each enzyme. Samples were electrophoresed in 1% (w/v) agarose (BioRad Laboratories S.r.l., Segrate-Milano, Italy) in 1 X TBE buffer, 90 mM Tris base, 90 mM boric acid and 2 mM EDTA pH 8 (Sigma) at 30 V overnight. Gels were stained in 0.5 µg/ml (w/v) ethidium bromide for 30 mins and destained in distilled water. Images of the gels were obtained by Gel-Doc 2000 (BioRad). The sizes of the bands were calculated by Gels software programme by Gel Doc 2000 apparatus (26).

Agarose plug preparation and electrophoretic karyotyping

Agarose plugs were prepared from all the yeast isolates (ATCC and clinical strains) as reported previously (27). Samples were examined for electrophoretic karyotype by pulsed-field electrophoresis with CHEF (Contour-clamped Homogeneous Electric Field) Mapper apparatus (BioRad) (28). The electrophoretic running conditions were: a pulse-time of 35 min at 65 V constant voltage for 74 h in 1% (w/v) agarose gel, running buffer 0.5 X TAE, 45 mM Tris-acetate and 1 mM EDTA pH 8 (Sigma) at a constant temperature of 14°C. Gels were stained in 0.5 µg/ml (w/v) ethidium

bromide for 30 min. and de-stained in distilled water. Images of the gels were obtained by Gel-Doc 2000.

Proteinase production

The ability to secrete aspartyl proteinase was assessed in a solid medium as previously described (29-30). Briefly, the yeasts were pre-grown in YPD medium and induced to proteinase secretion in BSA agar, 1.17% (w/v) yeast carbon base, 0.01% (w/v) yeast extract and 0.2% (w/v) BSA; (Sigma) at pH 5.0. Enzyme activity was measured as the diameter of a lytic area on BSA and scored as – to ++ as previously described (29-30). The determinations were confirmed by testing the hydrolysis of bovine haemoglobin (BH) by a spectrophotometer (30).

RESULTS

Carbon source growth test

The test revealed two groups of isolates. The isolates ATCC 62963, GER A, GER B, RC 1, RC 2, RM 1, RM 2, RM 5 were able to grow in salicin, arbutin and cellobiose. All the other isolates were unable of growing on the same carbon compounds.

RFLPs analysis

Twenty-five isolates of *B. capitatus* were examined for restriction fragment length polymorphisms (RFLPs) of whole cell DNA using *Hpa*II and *Hinf*I restriction enzymes. The *Hpa*II digestion constituted two groups of isolates named I and II (Fig. 1, A and B). Among the isolates of group I the strains RM4 and Urine116 showed a slightly different pattern, thus, group I was subdivided into three subgroups named a, b and c (Table II A). Also the electrophoretic pattern generated by *Hinf*I digestion (Fig. 2, A and B) constituted two groups of isolates, I and II. Surprisingly, *Hinf*I groups were constituted by the same isolates as for *Hpa*II groups (Table II B). Also in this case, group I was subgrouped (Table II B). However, with both the tested enzymes, seventeen isolates (68%) were in group I and eight isolates (32%) were in group II.

Karyotype analysis

According to the karyotype analysis (Fig. 3, A and B) sixteen different electrophoretic patterns were identified based on the number and/or the size of the presumptive chromosomal bands. For all the isolates, with the previously described electrophoretic conditions, we identified three presumptive

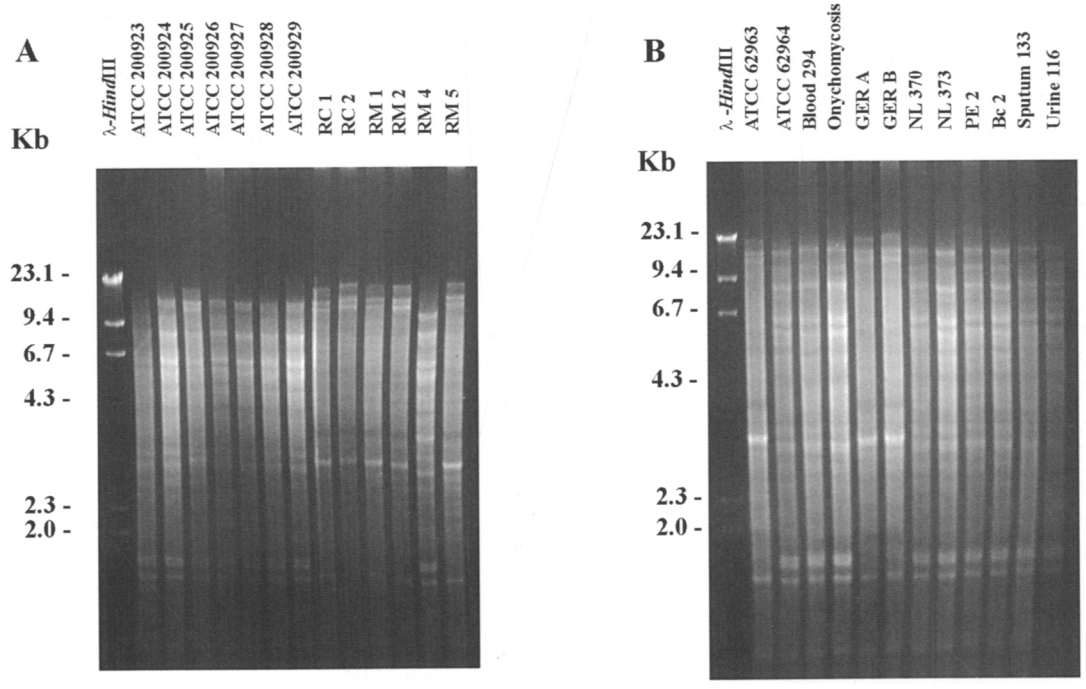

Fig. 1. *Figures 1A and 1B show the REA HpaII profiles of all the isolates of B. capitatus tested in this work. λ DNA digested HindIII was the DNA size marker, the sizes are showed on the left. Codes of the isolates are on the top of the figures.*

Codes	Source	Pathology	Geographic Source	ID32 C code
ATCC 200923*	Urine	Onchologic	Pescara [Italy]	2000010001
ATCC 200924*	Blood	Hemopathy	Pescara [Italy]	3200010003
ATCC 200925*	Blood	Hemopathy	Pescara [Italy]	3200010003
ATCC 200926*	Blood	Hemopathy	Pescara [Italy]	2200010001
ATCC 200927*	Blood	Hemopathy	Pescara [Italy]	2000010001
ATCC 200928*	Blood	Hemopathy	Pescara [Italy]	2200010001
ATCC 200929*	Blood	Hemopathy	Pescara [Italy]	2200010001
RC1	Blood	Hemopathy	Reggio Calabria [Italy]	2200010001
RC2	Blood	Hemopathy	Reggio Calabria [Italy]	2200010001
RM1	Blood	Hemopathy	Rome [Italy]	2200010001
RM2	Blood	Hemopathy	Roma [Italy]	2000010001
RM4	Blood	Hemopathy	Roma [Italy]	2000010001
RM5	Blood	Hemopathy	Roma [Italy]	2000010001
ATCC 62963**	Blood	Hemopathy	Great Britain	3000010001
ATCC 62964**	Blood	Hemopathy	Great Britain	2000010001
Blood 294	Blood	Hemopathy	Pescara [Italy]	2200010001
Onychomycosis	Nail	Healthy subject	Pescara [Italy]	2000010001
Ger. A	Blood	Hemopathy	Germany	3010010001
Ger. B	Blood	Hemopathy	Germany	3210010003
NL 370	Blood	Hemopathy	Netherlands	2200010001
NL 373	Blood	Hemopathy	Netherlands	2200010001
Pe 2	Blood	Hemopathy	Pescara [Italy]	2000010021
Bc 2	Blood	Hemopathy	Pescara [Italy]	2000010001
Sputum 133	Mucosite	Hemopathy	Pescara [Italy]	2000010003
Urine 116	Urine	Hemopathy	Pescara [Italy]	3010010001

Table I. *The table shows the Laboratory codes of all the isolates utilized in this work, the source of isolation and the associated pathology, the geographic sources and the ID 32C codes found. Note the number 1 in the third code position for Ger A, Ger B and Urine 116 that means the cellobiose assimilation.*

A

Group	Sub-group	Isolates
I	a	ATCC 200923, ATCC 200924, ATCC 200925, ATCC 200926, ATCC 200927, ATCC 200928, ATCC 200929, ATCC 62964, Blood 294, Onychomycosis, NL 370, NL 373, PE 2, BC 2, Sputum 133
	b	RM4
	c	Urine 116
II		ATCC 62963, GER A, GER B, RC 1, RC 2, RM 1, RM 2, RM 5

B

Group	Sub-group	Isolates
I	a	ATCC 200923, ATCC 200924, ATCC 200926, ATCC 200927, ATCC 200928, ATCC 200929, ATCC 62964, Blood 294, Onychomycosis, NL 370, NL 373, PE 2, BC 2, Sputum 133, RM4, Urine 116
	b	ATCC 200925
II	a	GER A, GER B, RC 1, RC 2, RM 1, RM 2, RM 5,
	b	ATCC 62963

Table II. *In panel **A** groups and sub-groups found after digestion with HpaII are listed; in panel **B** the grouping after digestion with HinfI is shown. It is interesting to note that in both the panel the same isolates constitute group I or group II.*

chromosomal bands with the unique exception of the strain RC 1 that showed four well separated bands. All the bands had a size ranging from 2.6 up to 6 Mbps (Table III, A and B). *Schizosaccharomyces pombe* was used as reference CHEF DNA marker having three well separated chromosomes sizing 3.5, 4.6 and 5.7 Mbps (31). The biggest band of our isolates, however, did not seemed to be a unique band in several isolates. A number of four presumptive chromosomes for our isolates could be suggested for a whole size of approximately 18 Mbps.

Proteinase production

Proteolytic activity was observed only for isolates Bc 2 and NL 370, respectively ++ and + for group I, and for RC 2 scored as ++ for group II.

DISCUSSION

Blastoschizomyces capitatus is a new emerging fungal pathogen able to cause invasive disease especially in immunocompromised hosts (1-3, 15-16, 18-19, 32-35). Recently this fungus was the causative agent of onychomycosis in a healthy subject (17). The morphological characteristics of *B. capitatus* have been described (4-6), but the virulence properties of this microorganism are, at this time, not well known and very few descriptions of its molecular characteristics are available. Two REA typing are reported in literature (17, 32).

In this study, the polymorphism of the restriction endonuclease fragments generated by *Hpa*II and *Hinf*I and electrophoretic karyotype were performed as genetic approach. The REA profiles (Fig. 1A, 1B, 2A, 2B) distinguished twenty-five isolates in two distinct genetic groups constituted by the same isolates for both the tested enzymes (Table II A, II B). Among the ATCC strains, only ATCC 62963 belonged to group II; apparently ATCC 62964 and ATCC 62963 were epidemiologically related (21). A similar result, for example, has been reported in literature for *C. parapsilosis* distinguished by the REA in three genetic groups (36), assessed as three different species by DNA-DNA re-association and DNA relatedness (37). A similar finding seems possible for *B. capitatus*. Groups I and II, confirmed by both endonuclease used, could be two different species. Several authors reported DNA relatedness experiments on a pool of isolates identified as *G. capitatum*, synonym of *B. capitatus*, detecting the presence of two species: *G. capitatum* and *G. clavatum* (10-11). A particular biochemical property can identify these two species: negative or positive, respectively, cellobiose assimilation. Moreover, *G. clavatum* is also positive to arbutin and salicin assimilation (10-11). Our biochemical tests for identification with ID32 C revealed three isolates positive to cellobiose utilization: Urine 116, Ger A and Ger B; number 1 in

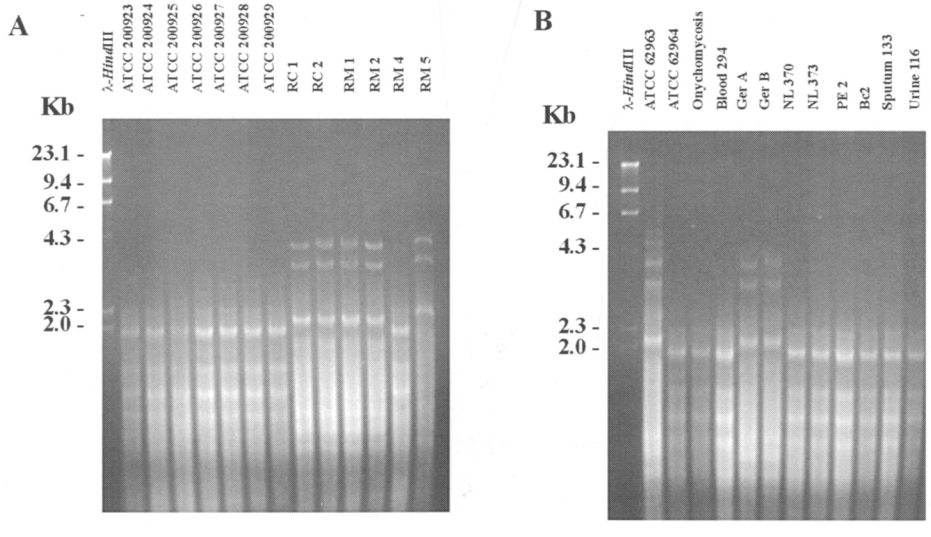

Fig. 2. *Figures 2A and 2B show the REA HinfI profiles of all the isolates of B. capitatus tested. λ DNA digested HindIII was the DNA size marker, the sizes are shown on the left. Codes of the isolates were written on the top of the figures.*

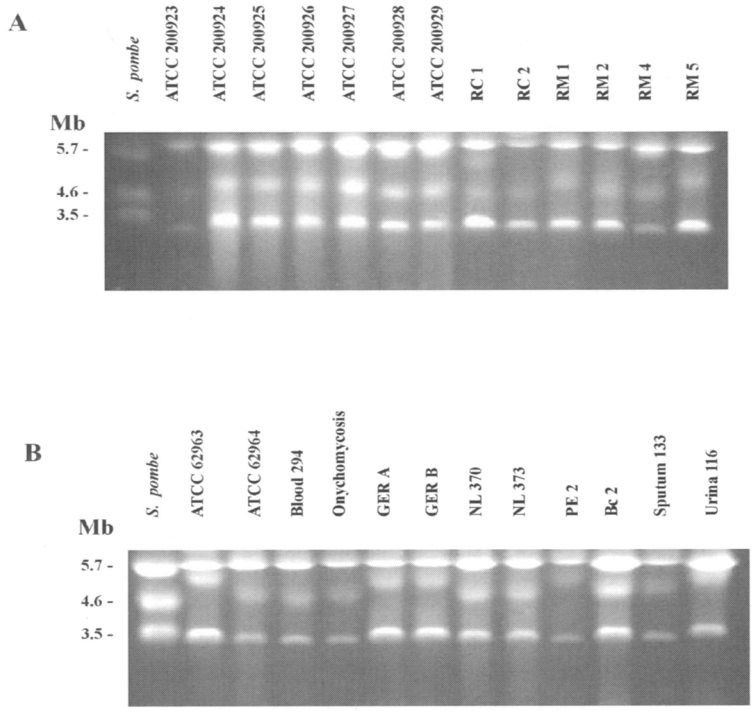

Fig. 3. *The figures 3A and 3B show the EK profiles of all the isolates of B. capitatus tested. Schizosaccharomyces pombe (BioRad) was utilised as a chromosomal DNA size marker. The sizes of the chromosomal bands of the fission yeast have been written on the left of the panel. Codes of the isolates were written on the top of the figures.*

third position in eight-number code of ID 32 C. We expected an identical REA profile, but in this case, they were different: Urine 116 was in group I, Ger A and B in group II (Tab. II, A and B). This data was also confirmed in Southern hybridisation experiments (26) with *B. capitatus* specific DNA probes derived from two ATCC strains (utilized in this work) specific for a single REA group (data not shown). On the contrary, performing the carbon assimilation test, we confirmed the REA distribution: all the isolates in group II utilised the cellobiose, the isolates in group I were unable to. The isolates of group II were also able to utilise arbutin and salicin.

According to the electrophoretic karyotype analysis, *B. capitatus* showed three or four chromosomal bands (Fig. 3, A and B). This pattern, for the number and/or the size of these bands is similar to the electrophoretic karyotype of the fission yeast *Schizosaccharomyces pombe* that we utilised as a marker in EK (31). Based on the putative number of the chromosomal bands, we suggest a genome size ranging from 13.2 Mb to 19 Mb. This major genomic size was established for RC 1 which clearly showed four bands, ATCC 200923 showed the smallest genomic size. The information of the size of *B. capitatus* was quite close to *C. albicans* (16-17 Mb) (38-39) and *S. pombe* (13.8 Mb) (31, 38). The EK analysis distinguished the isolates in sixteen groups based on number and/or size of the chromosomal bands, with higher discrimination with respect to the REA analysis as reported in the literature (7). Only in two EK classes we found isolates of both the REA groups. No information was obtained by this method about the correct species attribution.

We conclude that for a correct attribution of the species name, *B. capitatus* or *G. clavatum*, instead of a routine identification test, it is necessary to perform the assimilation test or, as shown in this work, a restriction endonuclease analysis utilising ATCC 62964 and ATCC 62963 as group marker I and II, respectively. Our results confirm that *B. capitatus* and *G. capitatum* are synonymous. To settle this controversial genetic aspect definitively, we suggest the finding of a species-specific genomic DNA probe derived from ATCC group marker strain and addressing a PCR method for clarifing the species attribution.

The capability of fungi to secrete or to express lytic

A

Group	Chromosome size [Mb]	Isolates Group I	Isolates Group II
I	6 - 4.6 - 2.6	ATCC 200923	
II	6 - 4.8 - 3	ATCC 200924, ATCC 200925, ATCC 200926, ATCC 200927	RM 1
III	6 - 4.7 - 2.8	ATCC 200928, ATCC 200929	
IV	6 - 5.4 - 4.6 - 3		RC 1
V	6 - 4.6 - 2.8		RC 2
VI	6 - 4.7 - 3		RM 2
VII	5.9 - 4.6 - 2.6	RM 4	
VIII	5.9 - 4.8 - 3		RM 5

Table III. *The table reports the sizes of the putative chromosomal bands of all the isolates found in the EK analysis distinguished also by the REA grouping. In **A** are described the isolates shown in Fig. 3A, in **B** are described the isolates showed in Fig. 3B. Composing all the sizes and the number of the chromosomal bands we constituted sixteen groups.*

B

Group	Chromosome size [Mb]	Isolates Group I	Isolates Group II
I	5.8 - 5.2 - 3.4	PE2	ATCC 62963
II	5.8 - 4.7 - 3.3	ATCC 62964, Onychomycosis	
III	5.8 - 4.6 - 3.3	Blood 294	
IV	5.8 – 5 - 3.4		GER A, GER B
V	5.8 - 4.7 - 3.4	NL 370, NL 373	
VI	5.8 - 4.9 - 3.5	BC 2	
VII	5.8 - 4.9 - 3.3	Sputum 133	
VIII	5.8 - 5.2 - 3.5	Urine 116	

enzymes is considered an important factor of virulence, directly involved in fungal invasion and destruction of host tissues. In particular, the production of secretory aspartyl proteinase (Sap) has been underlined as a most relevant virulence factor in *Candida* (30). The enzymes of the Sap family can be involved in the degradation of important number of proteins, directly engaged in the host defence, such as immunoglobulins, cytokines and complement (29). Only Bc 2, NL 370 and RC 2 were aspartyl proteinase producers. Therefore, this property seems not to be relevant in *B. capitatus*, but other proteases could be involved in penetrating tissues or degrading the host defense as happened in the lesions of vertebral bodies observed in a patient (16).

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