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Clonality structure in *Candida dubliniensis*

C. Badoc [a], T. De Meeûs [b], S. Bertout [a], F.C. Odds [c], M. Mallié [a], J.-M. Bastide [a,*]

[a] *Laboratoire d'Immunologie et de Parasitologie MENRT EA 2413, Université Montpellier I, Faculté de Pharmacie, 15, Av. Charles Flahault, P.O. Box 14491, 34093 Montpellier Cedex 5, France*

[b] *Centre d'étude sur le Polymorphisme des micro-organismes, Equipe ESS, UMR CNRS/IRD 9926, IRD, 911 Avenue d'Agropolis, P.O. Box 5045, 34032 Montpellier Cedex 1, France*

[c] *Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, UK*

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Abstract

Multilocus enzyme electrophoresis was performed on 76 European strains of *Candida dubliniensis*. Ten of the 20 enzyme-encoding loci were polymorphic, giving rise to 10 electrophoretic types within the sample studied. Investigation of the population genetics of a subset of 36 strains from HIV-infected patients in London showed the existence of strong heterozygote deficits and excesses associated with significant linkage disequilibria between pairs of loci. These findings, together with the predominance of multilocus genotypes, strongly suggest that *C. dubliniensis* is mainly (if not totally) clonal. Analysis of genotypes of a larger number of strains should confirm this conclusion and improve our understanding of the epidemiology of this pathogen. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Molecular typing; Population genetics; Reproduction mode

1. Introduction

Candida dubliniensis is a *Candida* species recently described by Sullivan et al. [1,2]. This fungus is an agent of oropharyngeal and systemic candidiasis in immunocompromised patients [1,2]. Until 1995, *C. dubliniensis* was misidentified as *Candida albicans* since the two species are phenotypically similar in their production of chlamydospores on rice–agar–Tween medium, and germ tube formation in serum [2–4].

C. dubliniensis is now recognized as a separate species, with distinctive phenotypic characteristics such as a lack of β-glucosidase, a dark green colony color on CHROMagar Candida, failure to grow at 45°C and specific carbohydrate assimilation profiles. It also has a very distinct genomic organization [2]. The species is considered a minor general constituent of the normal human flora; nevertheless, most known isolates of *C. dubliniensis* have been recovered from the oral cavities of HIV-infected patients with oropharyngeal candidiasis [3]. However, even with appropriate tests of species identification, clinical strains of *C. dubliniensis* remain rare [3].

C. dubliniensis possesses 9–10 chromosome-sized bands in electrophoretic gels [2], and since the conventional method does not allow direct visualization of chromosomes, the ploidy status of *C. dubliniensis* has not yet been determined. No sexual cycle is known for *C. dubliniensis*, nor has any process resembling meiosis or spore formation been detected [2]. The only mode of reproduction known for *C. dubliniensis* is asexual. But the question of this yeast's population structure is far from settled. Some low level of genetic exchange may occur but there is still no conclusive evidence for recent sexual recombination as no population study of this yeast has been published so far. The apparent absence of sexual reproduction in *C. dubliniensis* is very unusual because most of its relatives among the budding yeasts (e.g. *Saccharomyces cerevisiae*) have retained sexual cycles [5]. A notable exception is *C. albicans*, which seems to lack a natural sexual cycle [6–8] although it possesses homologues of mating

* Corresponding author. Tel.: +33 (4) 67 63 52 02; Fax: +33 (4) 67 41 16 17.

E-mail address: jean-marie.bastide@pharma.univ-montpl.fr (J.-M. Bastide).

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loci in *S. cerevisiae* and has recently been shown to be capable of mating when genes at the mating loci are altered appropriately [9,10].

Although mating and meiosis have not been observed for *C. dubliniensis*, the prevailing mode of reproduction of this organism in nature can be investigated by several population genetics methods. The two fundamental genetic consequences of sexual reproduction are segregation of alleles at a given locus and recombination of genotypes between loci. If both processes can be shown to be lacking, it suggests that sexual reproduction is absent or restricted [8].

In the present study, the genotype of 76 European strains of *C. dubliniensis*, including the type strain CBS 7987, was investigated by multilocus enzyme electrophoresis (MLEE) for 20 enzyme loci. These data were computer-analyzed by several population genetics software packages to determine the degree of intraspecies polymorphism and the prevailing mode of natural reproduction of this recently described species.

2. Materials and methods

2.1. Yeast strains and clinical isolates

We studied 76 strains of *C. dubliniensis* (Table 1), including the type strain of the species CBS-7987 (IVP 2710). All the isolates were identified as *C. dubliniensis* by DNA fingerprinting with the moderately repetitive *C. albicans*-specific oligonucleotide sequence Ca3 and standard yeast phenotypic techniques [1]. Isolates were grown on Sabouraud chloramphenicol agar medium (BioMérieux, Marcy l'Etoile, France) at 37°C. The 36 strains used for the linkage disequilibrium and F_{is} analysis were isolated from 15 patients from London (Table 1).

2.2. Multilocus enzyme electrophoresis (MLEE)

MLEE was performed as previously described [8,11]. In this study, 20 enzyme systems were tested: peptidase A (PEP.A; substrate, Val-Leu), peptidase B (PEP.B; substrate, Leu-Gly-Gly), peptidase C (PEP.C; substrate, Phe-Pro), peptidase D (PEP.D, substrate Leu-Ala), peptidase E (PEP.E, substrate Lys-Leu), glucose phosphate isomerase (GPI), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6PD), phosphoglucomutase (PGM), aspartate aminotransferase (AAT), alcohol dehydrogenase (ADH), fructokinase (FK), fumarase (FUM), malico enzyme (ME), mannose phosphate isomerase (MPI), purine nucleoside phosphorylase (NP), 6-phosphogluconate dehydrogenase (6PG), hexokinase (HK), sorbitol dehydrogenase (SDH), and isocitrate dehydrogenase (IDH).

Alleles were numbered in increasing order of mobility. Each isolate was characterized for the various polymorphic enzyme-encoding loci. Distinct multilocus variants were designated electrophoretic type (ET).

2.3. Statistical analysis

2.3.1. Linkage disequilibrium analysis

Linkage disequilibria expected to be found in a clonally reproducing population [12] were tested by the exact probability test for genotypic linkage disequilibrium performed with the GENEPOP version 1.2 program [12,13]. This test computed unbiased estimates, by the Markov chain method, of the exact probabilities of random association for all contingency tables corresponding to all possible pairs of loci in each population.

2.3.2. F_{is} analysis

F-statistics are a set of tools devised by Wright [14] to partition heterozygote deficiency into within- and between-population components. They are widely used by population biologists to assess levels of genetic structuring of natural populations [15]. In particular, F_{is} measured the heterozygote deficit within a population. F_{is} was estimated by the Weir–Cockerham unbiased estimators (f for F_{is}) [16] and computed by the program F-STAT version 1.2.

The presence of both deficit and excess of heterozygotes (respectively negative and positive F_{is}) from one locus to another and from one population to another is generally associated with clonality [17]. The deviation from zero of these indices was tested using permutation procedures (performed by F-STAT). Absence of heterozygote deficit or excess (i.e. $F_{is} = 0$) was determined by permutating alleles within sites. The observed value was compared with that for the corresponding randomly generated distribution. Providing a sufficiently large number of permutations was analyzed (10 000 in our case), an unbiased estimate of the exact probability of type I error (the probability of getting by chance values more extreme than the one observed) was obtained. This test could be carried out over all loci and all populations [14,16].

2.3.3. Multiple testing

Because multiple testing enhances type I error, the sequential Bonferroni procedure [18] was used when necessary; this involves dividing the desired significance level (i.e. $\alpha = 0.05$) by the number of remaining comparisons.

3. Results

Ten loci (50%) were polymorphic among the 20 enzyme loci studied (Table 2). We identified 22 alleles giving 10 different ETs for the 76 *C. dubliniensis* strains (Table 1). Of these multilocus patterns, ET1 was represented by 46 strains, ET4 by six strains, ET5, ET6 and ET7 by five strains each, ET2 and ET3 by three strains, and ET8, ET9 and ET10 by one strain each. This first analysis

Table 1
Number, origin, and characteristics of the *C. dubliniensis* strains within ET

ET[a]	Strain (IVP)	Origin, patient	Sample date	Source
ET 1	2776	**London** (UK), **BO**	1985	Feces
	2777	**London** (UK), **93577**	1986	Feces
	2778	**London** (UK), **BO**	1985	Feces
	2780	**London** (UK), **LB**	1986	Oropharynx
	2781	**London** (UK), **LB**	1986	Feces
	2782	**London** (UK), **LB**	1986	Oropharynx
	2783	**London** (UK), **LB**	1986	Oropharynx
	2784	**London** (UK), **LB**	1986	Oropharynx
	2788	**London** (UK), **LB**	1986	Feces
	2790	**London** (UK), **LB**	1986	Feces
	2796	**London** (UK), **LB**	1986	Feces
	2797	**London** (UK), **LB**	1986	Oropharynx
	2818	**London** (UK), **IC50**	?	Feces
	2820	**London** (UK), **TT**	?	Feces
	2821	**London** (UK), **JB**	?	Feces
	2822	**London** (UK), **JB**	?	Oropharynx
	2824	**London** (UK), **JB**	?	Oropharynx
	2835	**London** (UK), **093C**	1993	Oropharynx
	2836	**London** (UK), **236C**	1993	Oropharynx
	2709	CBS-7988, Melbourne (Australia)	1992	Oropharynx
	2710	CBS-7987, Dublin (Ireland)	1988	Oropharynx
	2775	Bangor (Wales, UK)	1985	Oropharynx
	2798	Madrid (Spain)	1986	Oropharynx
	2809	Houston (TX, USA)	1981	Oropharynx
	2806	Leeds (UK)	1975	Oropharynx
	2807	Leicester (UK)	1978	Oropharynx
	2808	Leicester (UK)	1981	Oropharynx
	2810	Sheffield (UK)	1982	Oropharynx
	2811	Leicester (UK)	1988	Oropharynx
	2813	Leicester (UK)	1989	Oropharynx
	2815	Leicester (UK)	1990	Oropharynx
	2817	Leicester (UK)	1990	Oropharynx
	2828	Quebec (Canada)	1992	Oropharynx
	2759	Belgium	?	Oropharynx
	2772	Montpellier (France)	1990	Oropharynx
	2831	San Antonio (TX, USA)	1993	Oropharynx
	2832	Frankfurt (Germany)	1993	Oropharynx
	2833	Frankfurt (Germany)	1993	Oropharynx
	2837	Turnhout (Belgium)	1994	Oropharynx
	2838	Antwerp (Belgium)	1994	Oropharynx
	2839	Netherlands	1994	Oropharynx
	2829	Quebec (Canada)	1992	Oropharynx
	2830	Brussels (Belgium)	1993	Oropharynx
	2773.1	Montpellier (France)	1991	Oropharynx
	2773.2	Montpellier (France)	1991	Oropharynx
	2774	Montpellier (France)	1990	Oropharynx
ET 2	2791	**London** (UK), **LB**	1986	Feces
	2792	**London** (UK), **LB**	1986	Feces
	2793	**London** (UK), **LB**	1986	Oropharynx
ET 3	2840	**London** (UK), **EK**	?	Feces
	2779	Bangor (Wales, UK)	1985	Vagina
	2726	Montpellier (France)	1999	Oropharynx
ET 4	2794	**London** (UK), **BB**	1986	Sputum
	2795	**London** (UK), **BB**	1986	Sputum
	2785	**London** (UK), **BB**	1986	Feces
	2786	**London** (UK), **BB**	1986	Feces
	2787	**London** (UK), **BB**	1986	Feces
	2789	**London** (UK), **BB**	1986	Oropharynx

[a]Electrophoretic types, ET: see Table 2.

Table 1
Number, origin, and characteristics of the *C. dubliniensis* strains within ET

ET[a]	Strain (IVP)	Origin, patient	Sample date	Source
ET 5	2812	**London** (UK), **NE**	1988	Oropharynx
	2799	Madrid (Spain)	1986	Oropharynx
	2800	Valencia (Spain)	1986	Oropharynx
	2801	Valencia (Spain)	1986	Oropharynx
	2802	Valencia (Spain)	1986	Oropharynx
ET 6	2805	**London** (UK), **OD003**	1975	Oropharynx
	2803	Valencia (Spain)	1986	Oropharynx
	2804	Leeds (UK)	1973	Oropharynx
	2760	Belgium	?	Oropharynx
	2761	Belgium	?	Oropharynx
ET 7	2814	**London** (UK), **NE**	1990	Oropharynx
	2816	**London** (UK), **JB**	1990	Oropharynx
	2825	Paris (France)	1992	Oropharynx
	2826	Dublin (Ireland)	1992	Oropharynx
	2827	Brussels (Belgium)	1992	Oropharynx >
ET 8	2819	**London** (UK), **OD187**	?	Feces
ET 9	2823	**London** (UK), **TC**	?	Feces
ET 10	2834	**London** (UK), **TUNJ**	1993	?

therefore showed poor intraspecific polymorphism (Table 2). As the ploidy status of the *C. dubliniensis* species is still undetermined, we had to consider the possibility of diploidy and haploidy.

To ascertain the prevailing mode of reproduction of *C. dubliniensis*, we had to work on samples of reasonable size, and the 36 London strains fitted this requirement. Nineteen strains belonged to ET 1, six to ET 4, three to ET 2, two to ET 7, and one to ET 3, 5, 6, 8, 9 and 10, respectively. All these 10 ETs were also encountered in the other sampling locations (40 strains, Table 1). Analysis of the London strain population with the GENEPOP program revealed strong linkage disequilibria between pairs of loci (Table 3). This result did not depend on the ploidy status of a species because the test used is genotypic (i.e. not allelic).

To undertake a F_{is} analysis, we had to consider the hypothesis of diploidy for the *C. dubliniensis* species. The F_{is} analysis showed a strong heterozygote deficit for eight loci (NP, AAT, FUM, PEP.B, G6PD, ADH, GPI and 6PG) with P values ranging from 10^{-4} to 10^{-2} (Table 4), and two excesses of heterozygotes for the PEP.E and HK loci ($P < 10^{-4}$, Table 4). The analysis was not possible for the IDH locus (no significant polymorphism).

We also undertook the same analysis with the London strains, excluding replicated ETs from each patient, to check for possible pseudo-replication biases. With the remaining 18 strains, the results led to similar conclusions, as all F_{is} stayed significantly different from 0 (being negative or positive) and 18% of pairs of loci (out of 45 testable pairs) remained significantly linked.

4. Discussion

Fixed heterozygosity at loci PEP.E and HK might be explained by gene duplications and/or aneuploidy, providing *C. dubliniensis* was haploid. However, *C. dubliniensis* was more closely related to diploid species (e.g. *C. albicans*, *C. tropicalis*) than to haploid ones (e.g. *C. glabrata*) [2]. The diploid hypothesis was thus more likely for this species and the F_{is} analysis we undertook appeared relevant to us.

On the basis of these preliminary results, *C. dubliniensis*

Table 2
Isoenzyme pattern obtained for each ET observed

ET	NP	AAT	FUM	PEP.B	PEP.E	HK	G6PD	ADH	GPI	6PG	IDH
ET 1	1/1	1/1	1/1	1/1	1/3	1/2	1/1	1/1	1/1	1/1	1/1
ET 2	1/1	1/1	1/1	1/1	1/3	1/2	1/1	2/2	1/1	1/1	1/1
ET 3	1/1	2/2	1/1	2/2	1/2	1/2	1/1	2/2	1/1	1/1	1/1
ET 4	3/3	1/1	2/2	1/1	1/2	1/2	2/2	1/1	2/2	1/1	1/1
ET 5	1/1	1/1	1/1	1/1	1/3	1/2	1/1	1/1	1/1	2/2	1/1
ET 6	1/1	2/2	1/1	2/2	1/2	1/2	1/1	1/1	1/1	1/1	2/2
ET 7	1/1	2/2	1/1	2/2	1/2	1/2	1/1	1/1	1/1	1/1	1/1
ET 8	1/1	1/1	1/1	1/1	1/3	1/2	1/1	1/1	1/1	3/3	1/1
ET 9	3/3	1/1	1/1	2/2	1/2	1/2	1/1	1/1	2/2	1/1	1/1
ET 10	3/3	1/1	1/1	1/1	1/2	1/2	2/2	1/1	2/2	1/1	1/1

Table 3
Significant linkage disequilibria and sequential Bonferroni corrections

Loci pair	P value	Nb tests	α' (0.05/Nb tests)
NP-GPI	**0.00000***	36	**0.001388**
NP-PEP.E	**0.00000***	35	**0.001428**
NP-G6PD	**0.00000***	34	**0.00147**
PEP.E-G6PD	**0.00000***	33	**0.001515**
PEP.E-GPI	**0.00000***	32	**0.001562**
G6PD-GPI	**0.00000***	31	**0.001613**
FUM-G6PD	**0.00019***	30	**0.001666**
NP-FUM	**0.00172***	29	**0.001724**
FUM-GPI	**0.00176***	28	**0.001786**
PEP.B-PEP.E	0.00246†	27	0.001852
FUM-PEP.E	0.00297†	26	0.001923
PEP.B-GPI	0.02522†	25	0.002000
NP-PEP.B	0.02585†	24	0.002083

Nb tests: number of remaining tests. *Value significant at the Bonferroni level; †value significant at the type I error level (5%).

seemed to have a prevailingly clonal mode of reproduction (at least in the sample investigated in this work), but other potential factors might generate deviations from the patterns expected in a panmictic population. We had to consider each of these separately: physical linkage of the 10 polymorphic loci, natural selection, Wahlund effect (the London strains might come from different populations), and haploidy with duplicated loci.

If the loci were physically proximal on the same chromosome, linkage disequilibrium might occur in panmictic populations [19]. The loci in our study were chosen without knowledge of their chromosome location. There are 9–10 chromosomes in *C. dubliniensis* [2]. In considering the existence of 10 haploid or five diploid chromosomes, the probability that all 10 polymorphic loci were located on the same chromosome was small (2.7×10^{-9} and 5.12×10^{-7}, respectively). Even if some loci were on the same chromosome, crossing over made it unlikely that multilocus linkage disequilibrium would be sustained in natural population, unless the loci were all situated within a small DNA segment [8].

If natural selection favored certain allelic combinations, or certain multilocus associations, it would generate departures from both Hardy–Weinberg expectations and linkage equilibrium. If two common multilocus genotypes were strongly favored by natural selection within a sexual population, the loci positively selected might not necessarily be the ones evidenced by our enzyme assays but might be others tightly linked to them ('hitchhiking'). In any case, natural selection could sustain linkage disequilibrium only by eliminating the genotypes that had arisen by recombination. However, as the number of loci in disequilibrium increased, the fraction of the population that needs to be eliminated (the 'genetic load' of the population) soon becomes unbearable [20].

In a large geographical area, different allelic frequencies might be found in different populations, even if each separate population was panmictic. The result of the combination of samples from different local populations would be linkage disequilibrium and apparent heterozygote deficits (the Wahlund effect). Geographical subdivision did not account for our results; the strains studied were all collected in a restricted area (London, UK), which reduced this possible source of error. Furthermore, the same ETs were found in very distant locations (e.g. ET 1 in Spain, Belgium and France, ET 5 in Spain), suggesting low levels of population structure, and such a hypothesis required all loci to display heterozygote deficits, which was not our case, providing *C. dubliniensis* is diploid.

Heterozygote deficit might be explained by the presence of null alleles, but in a population with a sexual cycle homozygotes for the null alleles would be present at each generation. In our study no strains showed a null phenotype for the enzyme loci tested. As a result, null alleles could hardly explain our positive F_{is} (Table 4). Indeed, Brookfield's method [20] to estimate null allele frequencies necessary to explain all positive F_{is} did not provide convincing results. The goodness of fit of such estimates was globally tested with an exact binomial test. The absence of observed blanks against the expected ones (4) is too low a value to be explained by chance ($P=0.013$).

Table 4
F_{is} estimators within samples when testing absence of heterozygote deficit or excess

Locus	F_{is} values	Proportion of randomizations that show a deficit of heterozygote	Proportion of randomizations that show an excess of heterozygote
NP	1	0.0001	1.0000
AAT	1	0.0107	1.0000
FUM	1	0.0001	1.0000
PEP.B	1	0.0001	1.0000
PEP.E	**−1**	**1.0000**	**0.0001**
G6PD	1	0.0001	1.0000
ADH	1	0.0001	1.0000
GPI	1	0.0001	1.0000
6PG	1	0.0001	1.0000
HK	**−1**	**1.0000**	**0.0001**

Indicative adjusted nominal level (5%) for one table is: 0.00250.
Data not available for the IDH locus.

Self-fertilization results in heterozygote deficiencies and linkage disequilibria. It might account for some of our results, but the presence of heterozygosity excesses (Table 4) suggested that departures from panmixia in the present study were not conveniently explained by self-fertilization, providing *C. dubliniensis* is diploid.

In tests for linkage disequilibrium, the percentage of significant *P* values at the type I error level ($\alpha = 0.05$) was generally higher than expected: 13 tests out of 36, i.e. 36.1% of significant *P* values (Table 3), and eight tests out of 45 (18%) without repeated ETs within patients, instead of the expected 5% under the null hypothesis. Moreover, after Bonferroni correction, nine *P* values remained significant (i.e. 25%) (Table 3). With only 36 strains, these highly significant results strongly suggested that recombination was absent or at least very rare in this yeast. Moreover, the presence of multilocus genotypes (ET) worldwide confirmed our interpretation.

Our conclusion is that clonal reproduction is the most parsimonious overall explanation for the results. A clonal population structure does not imply that sex is totally absent, but only that it is too rare an event to break a prevalent pattern of clonality [21]. This study contributed to a better understanding of the diversity, the genome dynamics and the mode of reproduction of the yeast *C. dubliniensis*. To confirm this clonal mode of reproduction, further experiments should be performed to determine if homologues of genes that function in both mating (e.g. *GPA*1, *STE*20, and *STE*6) and meiosis (e.g. *DMC*1) in *S. cerevisiae* [5] could be identified in *C. dubliniensis* as has been done for *C. albicans* [9,10]. Indeed, *C. albicans* expresses a mating-type-like (*MTL*) locus that resembles the mating-type (*MAT*) locus of the sexually reproducing yeast *S. cerevisiae*. Such data obtained for *C. dubliniensis* would raise the possibility that this species has a complete sexual cycle under particular growth conditions which remain to be determined.

It is important to keep in mind that some clones may have a widespread distribution. Genetically differentiated clones should be separately investigated for relevant medical features such as pathogenicity, virulence and resistance to antifungal agents. Association between particular genetic makeups and medical characteristics would facilitate selective effective methods for control of *C. dubliniensis*, an opportunistic emergent pathogen.

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