

# The D1/D2 domain of the large-subunit rDNA of the yeast species *Clavispora lusitaniae* is unusually polymorphic

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## Abstract

Ten different versions of the D1/D2 divergent domain of the large-subunit ribosomal DNA were identified among interbreeding members of the yeast species *Clavispora lusitaniae*. One major polymorphism, located in a 90-bp structural motif of the D2 domain, exists in two versions that differ by 32 base substitutions. Three other polymorphisms consist of a two-base substitution, a two-base deletion, and a single-base deletion, respectively. The polymorphisms are independent of one another and of the two mating types, indicating that the strains studied belong to a single, sexually active Mendelian population. Several strains were heterogeneous for one or more of the polymorphisms, and one strain was found to be automictic and capable of producing asci on its own by isogamous conjugation or by bud–parent autogamy. These observations suggest circumspection in the use of sequence divergence as the principal criterion for delimiting yeast species.

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**Keywords:** *Clavispora lusitaniae*; D1/D2 large-subunit rDNA polymorphism; Sequence heterogeneity; Species concept

## 1. Introduction

The genus *Clavispora* Rodrigues de Miranda (1979) [1] comprises two species, *Clavispora lusitaniae* and *Clavispora opuntiae* [2]. The latter has been recovered repeatedly in hundreds of samples of necrotic cacti world-wide and seems to be confined to that habitat. *C. lusitaniae* occurs

in a broad array of substrates of plant and animal origin, as well as industrial wastes and clinical specimens. Although the two species are fairly easy to distinguish on the basis of three growth tests, both exhibit a certain amount of intraspecific variability in their morphological and physiological characteristics and in their mating behavior [3,4], as well as in various genetic markers [5,6]. Mating reactions among strains of *Clavispora* species are generally reproducible [7,8], but some exceptions have been noted [4]. Mating does not occur between the two species.

Five strains (CBS 4413, KCTC 7268, MTCC 1001, UCDFST69-106, UWO92-308.1) were independently found to differ from the type culture of *C. lusitaniae* by 32 substitutions in the D2 variable domain of the large-subunit ribosomal DNA. In the absence of interfertility data, this would serve as strong grounds to treat them as members of a distinct species. In the case of strain

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CBS 4413, however, this would have mandated a re-interpretation of some criteria used to delineate species, as the latter was chosen to be the isotype of the species on the basis of its ability to mate and form asci with strain CBS 6936<sup>T</sup>, the holotype of the species [1]. In addition, the nuclear DNAs of the two strains form 94% heteroduplex DNA [3]. Here, we present evidence that *C. lusitanae* as a whole is unusually polymorphic with respect to rDNA sequences.

## 2. Materials and methods

### 2.1. Microorganisms

The origins of the strains are given in Table 1. The strains were chosen to represent isolates from a variety of origins and mating types. Mating and ascus formation were assessed by mixing strains in pairs on yeast carbon base agar (Difco) supplemented with 0.01% ammonium

Table 1  
Selected properties of *C. lusitanae* considered in this study

Strain number	Sexuality		Position and type of D1/D2 sequence polymorphisms				Isolation data
	Mating type	Fertility	177	197	390	406–496 <sup>a</sup>	
CBS 6936 <sup>T</sup>	h <sup>+</sup>	++	AT	–	T	H1	Citrus, Israel
CBS 5901	h <sup>–</sup>	++	AT	–	T	H1	Crow droppings
WM 1037	h <sup>+</sup>	Rare asci	AT	–	T	H1	Clinical, Australia
WM 1038	h <sup>+</sup>	++	AT	–	T	H1	Clinical, Australia
WM 02.92	h <sup>–</sup>	++	AT	–	T	H1	Clinical, Australia
UWO-S86-287	h <sup>–</sup>	++	AT	–	–	H1	Columnar cactus necrosis, Venezuela
UWO-S79-257.1	h <sup>+</sup>	++	AT	–	T	H2	Boojum tree, Mexico
UWO-S79-267.12	h <sup>+</sup>	++	AT	–	T	H2	Agria cactus necrosis, Mexico
UWO94-423.2	h <sup>+</sup>	+	CC	–	–	H3	Pulque, Mexico
CBS 4413 <sup>I</sup>	h <sup>–</sup>	Rare asci	AT	–	T	I1	Pig digestive tract, Portugal
CBS 4415	h <sup>–</sup>	++	AT	–	T	I1	Pig digestive tract, Portugal
CBS 4870	h <sup>–</sup>	+	AT	–	T	I1	Clinical, Finland
MTCC 1001	automictic	++	AT	–	T	I1	Citrus, India
UWO85-286.1	h <sup>–</sup>	++	AT	–	T	I1	<i>Drosophila</i> sp., USA
CBS 4414	h <sup>–</sup>	++	AT	TA	T	I1	Pig digestive tract, Portugal
CBS 5030	h <sup>–</sup>	++	AT	TA	T	I1	South Africa
CBS 5094	h <sup>–</sup>	++	AT	TA	T	I1	Clinical, Germany
KCTC 7268	?	?	AT	TA	T	I1	Beer, Korea
UWO92-308.1	h <sup>+</sup>	+	AT	TA	–	I1	Crushed agave, Mexico
CBS 1944	h <sup>+</sup>	++	AT	–/TA	T	I1	Clinical, Norway
CBS 5299	h <sup>–</sup>	++	AT	–/TA	T	I1	Milk of mastitic cow, Greece
UCDFST69-106	h <sup>+</sup>	++	AT	–/TA	T	I1	Wet dates
UWO-WN9.20	h <sup>–</sup>	++	AT	TA/–	T	I1	Chocolate factory waste, Canada
WM 02.196	h <sup>–</sup>	++	AT/CC	–	T	I1	Clinical, Australia
WM 02.348	h <sup>+</sup>	++	AT/CC	–/TA	T	I1	Clinical, Australia
UWO92-291.1	h <sup>+</sup>	++	CC	–	–	I2	Agave rot, Mexico
UWO94-217.3	h <sup>+</sup>	++	CC	–	–	I2	<i>Drosophila</i> , Mexico
UWO94-252.1	h <sup>–</sup>	Rare zygotes	CC	–	–	I2	Agave, Mexico
CBS 2866	h <sup>+</sup>	++	CC	–	T	I3	Clinical, The Netherlands
CBS 7270	h <sup>+</sup>	++	CC	–	T	I3	Clinical
UWO-G90-207.5	h <sup>+</sup>	++	CC	–	–	I4	Columnar cactus necrosis, Antigua
UWO-G90-244.1	h <sup>–</sup>	++ (ovoid)	CC	–	–	H/I	Prickly pear cactus necrosis, Antigua
UWO-S80.29	h <sup>+</sup>	++	CC	–	T	H/I	Agave rot, Arizona
UWO83-1068.1	h <sup>–</sup>	++	CC	–	T	I/H	Prickly pear fruit, Cayman Islands
WM 1036	h <sup>+</sup>	++	AT	–/TA	T	H1/I1	Clinical, Australia
WM 02.446	h <sup>–</sup>	++	AT/CC	–	T	H1/I1	Clinical, Australia
UWO93-323.1	h <sup>–</sup>	++	AT	–	T/–	I1/I2	Agave rot, Bahamas
CBS 7068 <sup>T</sup> <i>C. opuntiae</i>	h <sup>+</sup>	(++)	CC	TA	C	O	Prickly pear necrosis, Australia

Data for the type strain of *C. opuntiae* are included for comparison. Polymorphic sites for which strains were heterogeneous are given in order of decreasing relative abundance.

<sup>T</sup>Type culture, <sup>I</sup>isotype.

Culture collection abbreviations: CBS=Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. KCTC=Korean Type Culture Collection, Daejeon, South Korea. MTCC=Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. UCDFST=University of California, Davis, CA, USA. UWO=University of Western Ontario, London, ON, Canada; UWO-G=received from P.F. Ganter, Tennessee State University, Nashville, TN, USA; UWO-S=received from W.T. Starmer, Syracuse University, Syracuse, NY, USA; UWO-W=received from J. Fein, formerly of Weston Food Research, Toronto, ON, Canada. WM=Centre for Infectious Diseases and Microbiology, Westmead Hospital, Westmead, Australia.

<sup>a</sup>H1, H2, and H3 are minor variations on the D2 sequence found in the holotype of *C. lusitanae*, CBS 6936 (Fig. 1). I1 to I4 are variations on the sequence determined for the isotype, CBS 4413. The version found in *C. opuntiae* is designated 'O'.

sulfate. After 2 days at 25°C, the mixtures were examined in the light microscope. The strains were characterized morphologically and physiologically by standard methods [9].

## 2.2. DNA sequencing

Sequencing of the D1/D2 variable domains of the large rDNA subunit was performed in five different laboratories, using various procedures for sequencing polymerase chain reaction (PCR) products. The sequencing reactions used primers NL1 and NL4 [10] or LR0R and LR16 [11]. In a few instances (strains CBS 6936, CBS 4413, and UWO92-308.1), the same sequences were determined in two or more laboratories and were found to be perfectly reproducible. The sequencing templates were prepared either by direct PCR amplification on whole cells [12] or on purified DNA [13]. Some of the heterogeneous PCR products were cloned using the pGEM<sup>®</sup>-T Easy Vector System (Promega, Madison, WI, USA). Purified PCR products (30 ng, GFX<sup>™</sup> PCR DNA gel band purification kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA)) were ligated according to the manufacturer's instructions. The plasmid was grown in *Escherichia coli* JM109 High Efficiency Competent Cells (Promega), and a single colony suspended in sterile double distilled water was used as template for the amplification of cloned large-subunit fragments. Internal transcribed spacer (ITS)/5.8S sequences were determined using the primers IT1 and IT2 [14] or those described by White et al. [15]. Actin gene sequences were determined as described elsewhere [16]. The sequences were aligned and the tree constructed with the program DNAMAN (Vaudreuil, Quebec, QC, Canada). The RNA secondary structure was determined with O. Matzura's program RNAdraw, version 1.0 (<http://rnadraw.base8.se/>).

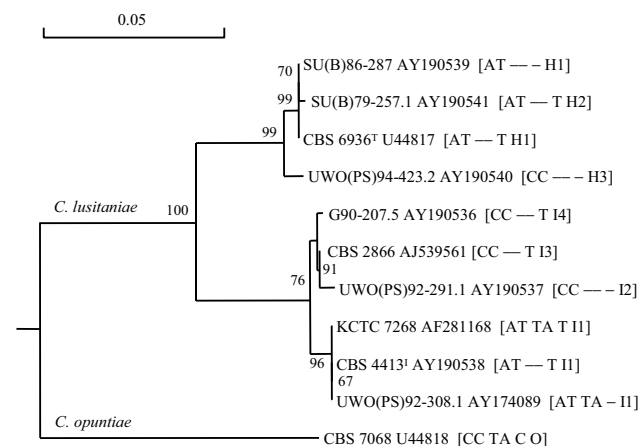


Fig. 1. Neighbor-joining phylogram of the various polymorphic sequences of the D1/D2 domains of the large-subunit rDNA of *C. lusitaniae*. Bootstrap values were determined from 1000 iterations. The scale shows 5% divergence in base substitutions. The four sequence polymorphisms are described symbolically in brackets (see Table 1).

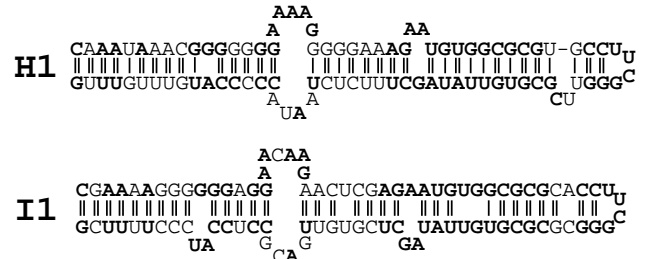


Fig. 2. Secondary structure of the H1 and I1 polymorphic regions of the D2 domain of the large-subunit ribosomal RNA in *C. lusitaniae*. Double lines show Watson-Crick pairing, and single lines show GU pairs. Conserved bases are in boldface.

## 3. Results and discussion

### 3.1. D2 polymorphism and heterogeneity

The sequences of the D1/D2 regions of *C. lusitaniae* strains could be assigned to 10 categories (Fig. 1). A major polymorphism was found in a 90-bp region of the D2 domain. The version found in strain CBS 6936<sup>T</sup> (holotype of the species) differed by 32 base substitutions from that found in strain CBS 4413<sup>I</sup> (isotype of *C. lusitaniae* and former type of *Candida lusitaniae*). In Table 1 and Figs. 1 and 2, the two versions have been labeled H1 and I1, respectively. Within the 90-base region, minor variations (individual substitutions) have been detected and the relevant sequences are designated H2, H3, I2, I3, and I4 in Table 1 and Fig. 1. Five strains were heterogeneous, containing a mixture of both the H and I versions. In those cases, the sequence electropherograms showed mixed peaks indicative of the two bases found in the different versions. The PCR products for two of these strains were cloned and the individual sequences were determined to be H1 and I1. One strain was heterogeneous for the I1 and I2 versions. The H/I polymorphism is located in a structural motif in the ribosomal RNA product, designated the C1\_2 subhelix in *Saccharomyces cerevisiae* [17]. The two versions found in *C. lusitaniae* are generally similar in their predicted secondary structure (Fig. 2).

Other minor variations were also identified. One is an AT/CC polymorphism at position 177 (D1 domain, counting from the 5' end of the sequencing primer NL1). Another is the frequent deletion of a TA doublet, at position 197. The doublet was found in five strains of *C. lusitaniae* and in its sister species, *C. opuntiae*. Additionally, the rDNA was heterogeneous for the presence or absence of this doublet in six other strains, causing the sequencing electropherograms to become illegible beyond that point. The whole sequence could be reconstructed by comparing the reactions obtained separately with the forward and reverse primers. A single-base (T) deletion at position 390 in the D2 domain was found in some strains, and one strain was heterogeneous for the presence or absence of that base.

The outgroup principle sustains that versions of the

polymorphic sequences that are shared with *C. opuntiae* can be considered plesiomorphic (ancestral). The five strains of *C. opuntiae* whose D1/D2 sequences are available contained the CC version of the AT/CC polymorphism and the TA doublet, indicating that they are plesiomorphic and that the TA doublet was lost by deletion during the phyletic development of *C. lusitaniae*. The polymorphic T at position 390 corresponded to a C in *C. opuntiae*, but given the adjacent bases, it is also likely that the variation found in *C. lusitaniae* is due to loss, and not gain, of a base. As to the 90-base polymorphic region of the D2, the version found in *C. opuntiae* is more or less equidistant from the H and I versions, making it difficult to draw a conclusion about character polarity in this case.

### 3.2. Other sequences

The question arises as to whether other gene sequences exhibit comparably high degrees of variation. The sequence of a 979-bp segment of the actin gene has been reported for strains CBS 6936<sup>T</sup> and CBS 4413 [16] (GenBank AJ389065 and AJ389066). The two strains differ by a single substitution in that actin segment. This portion of the actin gene may vary by as much as 11 substitutions among strains of some yeast species, and the divergence hitherto observed between species is of 17 substitutions or more (H.M. Daniel, unpublished observations). On that basis, one would conclude that actin gene sequences support the notion of conspecificity for these two strains, in spite of their considerable variation in the D1/D2 domains.

Strains MTCC 1001 (AY174102) and UWO92-308.1 (AY174089), which share the I1 version of the polymorphic D2, differ by 18 substitutions in their ITS rDNA sequences, and these in turn differ by 3–21 substitutions from various other *C. lusitaniae* ITS sequences available in the GenBank database.

Kato et al. [18] examined sequence divergence in the topoisomerase II gene for several pathogenic *Candida* species. Although *C. lusitaniae* was represented by only one strain in their study, the species apparently has accumulated more substitutions than comparable species, suggesting that it may have evolved more rapidly. The authors pointed out that other sequences, including the D1/D2 domains of the large-subunit rDNA of *Clavisporea*-related genera [19], also have a higher substitution rate. These genera correspond to the family Metschnikowiaceae and its anamorphs.

### 3.3. Physiological variation

A small amount of variation exists in the nutritional characteristics of various strains of *C. lusitaniae* [2]. Most noteworthy in this study were two strains. CBS 4413 grows weakly on sorbose, ribitol, xylitol, and lysine,

whereas most other strains exhibit strong responses for those tests. The strain is also unusual in that it assimilates D-glucosamine. The most pronounced difference was seen in strain UWO92-308.1, which fails to grow on sucrose, raffinose, maltose, salicin, ribitol, and N-acetyl-glucosamine, all of which are normally assimilated. These differences may account in part for the reduced mating vigor of the two strains. It is interesting to note that the two strains have very similar D1/D2 sequences (Fig. 1).

### 3.4. Variation in sexual reactivity and species status

With the exception of strains KCTC 7268 (not available for testing), MTCC 1001, and CBS 7068<sup>T</sup> (the type of *C. opuntiae*), all strains listed in Table 1 produced a mating reaction when mixed in compatible pairs. The mating type h<sup>+</sup> had been assigned arbitrarily to the type strain of *C. lusitaniae*, CBS 6936<sup>T</sup> [3], and all strains that mate with it are designated h<sup>-</sup>. In Table 1, the intensity of the mating reactions is reported as ‘++’ when mature asci and liberated ascospores were abundant after 2 days, and ‘+’ when ascus formation was less intense. Strain CBS 4413 formed asci only very rarely, but some asci contained ascospores (Fig. 3a). All matings involving strain UWO(PS)-94-252.1 were characterized by an abundance of cells with conjugation tubes (Fig. 3b), but with only a few zygotes (Fig. 3c), and no ascospores. Matings involving strain G90-244.1 were unusual in that ovoid, as opposed to clavate, ascospores were produced (Fig. 3d). The vast majority of strains of *C. opuntiae* (exemplified here by strain CBS 7068<sup>T</sup>) form mature asci in various amounts when mated appropriately [7], but sexual reactivity between the two species is absent.

Interestingly, strain MTCC 1001 forms abundant asci in a single, apparently haploid culture, suggesting that it is

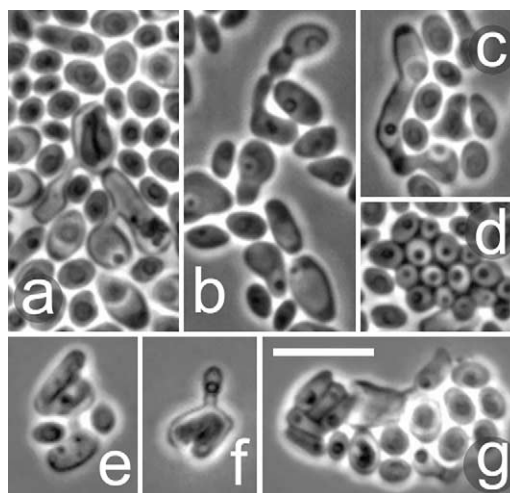


Fig. 3. Asci and ascospores of *C. lusitaniae*. a: Asci formed by strains S79-257.1 and CBS 4413. Conjugation tubes (b) and zygote (c) formed by strains CBS 6936 and UWO94-252.1. d: Ovoid ascospores formed by strains G90-244.1 and G90-207.5. e–g: Asci formed by the autotictic strain MTCC 1001. Scale bar = 5  $\mu$ m.

homothallic. Some of the asci arise out of isogamous conjugation (Fig. 3e), and others from bud–parent cell conjugation (Fig. 3f). This is the only report, to our knowledge, of an automictic strain in the genus *Clavispora*.

The widespread formation of mature asci in most mixtures of haploid mating types of *C. lusitaniae*, albeit with some variation in intensity, indicates that the strains in question are conspecific. In all cases, the intensity of mating was dependent on the individual mating partners, and not on the genetic distance of the two members of a pair, at least as inferred by their D1/D2 sequences. This suggests that the poor mating was the result of individual deficiencies, and not due to genetic divergence between the strains. As to the self-fertility encountered in strain MTCC 1001, in the absence of other data, the description of a separate species might have been warranted. However, the sequencing data provide no basis for separation, as the D2 version observed in that strain is similar to that of several other strains studied (Table 1). The physiological profile of the strain is perfectly typical of the species. The presence of both heterothallic and homothallic strains in a single species is not completely unknown, as for example in *Kluyveromyces lactis* [20]. Considerable evidence supports the view that homothallism is a derived trait that arises repeatedly in descendants of heterothallic ancestors [21]. The present example supports this idea.

In the absence of mating compatibility data, DNA/DNA reassociation has been used with considerable success to assess species boundaries [22]. In the present case, it is particularly fortunate that the amount of heteroduplex formation between strains CBS 6936<sup>T</sup> and CBS 4413<sup>I</sup> is known [3], not only because they are the holotype and the isotype, respectively, of *C. lusitaniae*, but also because the latter strain exhibits rather poor mating characteristics, which could, on its own, be interpreted as evidence for treating them as separate species. The high level of DNA reassociation (94%) confirms conspecificity.

### 3.5. Sequence variation and species boundaries

The joint distributions of mating types and sequence polymorphisms (Table 1) was analyzed in 2×2 contingency tables to see if significant associations might exist between any of these variations. A strong correlation between any of them would have indicated that the evolutionary history of *C. lusitaniae* has been relatively free of sexual recombination. Moreover, a perfect correspondence between the mating types and the large D2 polymorphism (H versus I) would have represented strong evidence that these strains are sexually inactive and have in fact speciated along the lines of the mating types. However, all calculated  $\chi^2$  values were non-significant at the 0.05 level, suggesting that the four sequence markers are distributed randomly with respect to the mating type, and that the strains in question indeed share a common gene pool. Visual evaluation of the data in Table 1 might suggest a

linkage disequilibrium involving the TA insertion at position 197 (D1 domain) on the one hand, and the AT doublet at position 177 or the II version of the D2 polymorphism on the other. However, that conclusion is not supported statistically.

The presence of heterogeneities in some strains provides further evidence that homogenization of rDNA repeats has not been sufficient to compensate for frequent sexual recombination in the species. Heterogeneity within strains further confirms that the polymorphisms are present in a single species, but confounds any attempt to speculate on the origin of the polymorphisms.

The emerging conclusion is that strains hitherto designated members of *C. lusitaniae* are part of one interbreeding population and constitute a single evolutionary unit, a single biological species. This may appear to contradict the increasingly popular view that yeasts that differ by more than 1% in their D1/D2 region should be regarded as separate species. This view is in fact a serious misinterpretation of the generalizations made by Kurtzman and Robnett [19] in their seminal paper. These authors very clearly stated that sequence divergence can be used as a guideline or a predictor of species boundaries. The guideline was empirically based on the sequence divergence values obtained with pairs of strains that were previously known to be or not to be conspecific by other criteria, the most fundamental of which is the formation of ascospores following mating. Implicit in Kurtzman and Robnett's generalization was the proviso that the 1% guideline must be interpreted in the context of the whole organism and its life history. The study of gene sequence divergence has revolutionized the way we approach yeast systematics, but should not cause one to lose sight of the biological evidence. One extremely complex set of genes takes precedence over any other source of information used in delineating species. These are the genes that control zygote formation and meiosis. These genes, at least in heterothallic yeasts, determine whether or not strains collectively follow a common evolutionary path, and in so doing tell us in the clearest possible terms whether or not they are conspecific.

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