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Phylogeny of the genus *Kluyveromyces* inferred from the mitochondrial cytochrome-*c* oxidase II gene

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A phylogenetic analysis of 17 species belonging to the genus *Kluyveromyces* and 12 reference and outgroup species was performed using mitochondrial cytochrome-*c* oxidase II gene sequences. The genus *Kluyveromyces* appears as a polyphyletic taxon formed by species included within the following four main groups. The *Kluyveromyces phaffii* group encompasses the species *Kluyveromyces blattae*, *K. phaffii* and *Kluyveromyces yarrowii*. The *Kluyveromyces marxianus* group is a monophyletic group consisting of the species *Kluyveromyces aestuarii*, *Kluyveromyces dobzhanskii*, *Kluyveromyces lactis*, *K. marxianus* and *Kluyveromyces wickerhamii*. The monophyletic *Kluyveromyces thermotolerans* group is formed by *K. thermotolerans*, *Kluyveromyces waltii* and *Saccharomyces kluyveri* (which appears in the mitochondrial tree as the sister clade of the *K. marxianus* group). Finally, the *Saccharomyces cerevisiae* group contains the remaining *Kluyveromyces* species, as well as the reference *Saccharomyces* species (*sensu lato* and *sensu stricto*) and *Candida glabrata* (the phylogenetic relationships within this group are unclear according to the bootstrap test). The phylogenetic relationships obtained for this mitochondrial gene are, for the most part, congruent with previous trees based on nuclear rRNA sequences, except for the position of *K. yarrowii* and the close relationship between the *K. marxianus* and *K. thermotolerans* groups. These differences, as well as the existence of these groups, are discussed in the context of previous studies based on phenotypic, genetic and molecular data. Although additional studies are required to decipher the phylogenetic relationships between the genus *Kluyveromyces* and the closely related genera *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*, future changes to their taxonomic status should take account of the existence of these four groups of *Kluyveromyces* species.

Keywords: mitochondrial DNA, cytochrome-*c* oxidase II, *coII*, *Kluyveromyces*, molecular phylogeny

INTRODUCTION

The genus *Kluyveromyces* was originally established by van der Walt (1956a) on the basis of *Kluyveromyces polysporus*, a newly isolated yeast that formed large multispored asci containing reniform spores. In the same year, an additional species, *Kluyveromyces africanus*, was assigned to the genus (van der Walt, 1956b). The ascospore shape was considered to be a generic characteristic and thus the diagnosis of the genus *Kluyveromyces* was emended by van der Walt (1965) to include yeasts producing fewer reniform spores (generally four per ascus). Consequently, many former *Saccharomyces* species and the genera *Fabospora*, *Zygofabospora*, *Dekkeromyces* and *Guilliermondella* were transferred to the genus *Kluyveromyces*. However, the variability of ascospore shape in

Abbreviations: BV, bootstrap value; NJ, neighbour-joining.

The EMBL accession numbers for the sequences in this paper are AJ235911–AJ235928.

Kluyveromyces lactis or *Kluyveromyces marxianus* did not support the view that ascospore shape underlies meaningful lines of development; later studies redefined the concept of the genus as more features were taken into account. Publications on *Kluyveromyces* taxonomy based on DNA base composition and DNA–DNA hybridization (Fuson *et al.*, 1987; Vaughan-Martini & Martini, 1987) and more recent reviews (Lachance, 1993, 1998) have established the current basis for the classification of the species of the genus *Kluyveromyces*.

The genus *Kluyveromyces* currently consists of 18 species, including the new ones, i.e. *Kluyveromyces sinensis* (Li *et al.*, 1990), *Kluyveromyces hubeiensis* (Li *et al.*, 1992), *Kluyveromyces piceae* (Weber *et al.*, 1992) and *Kluyveromyces bacillisporus* (Lachance *et al.*, 1993), some of which have been the subject of extensive investigations concerning their genetics, ecology and evolution.

The reconstruction of evolutionary trees by using 18S rRNA gene sequences (Cai *et al.*, 1996; James *et al.*, 1997, 1998), partial 26S rRNA gene sequences (Kurtzman & Robnett, 1998) and restriction analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers (Molina *et al.*, 1992; Shen *et al.*, 1994; Belloch *et al.*, 1998a) presents *Kluyveromyces* as a polyphyletic genus. These studies suggest the existence of three groups: one, which is monophyletic, is constituted by the species *K. lactis*, *K. marxianus*, *Kluyveromyces aestuarii*, *Kluyveromyces dobzhanskii* and *Kluyveromyces wickerhamii*. The members of this significant group are highly related and appear clearly separated from the other *Kluyveromyces* species. This is consistent with the ability of these strains to mate in the laboratory (Johannsen, 1980) and with the results of DNA base composition and relatedness (Fuson *et al.*, 1987; Vaughan-Martini & Martini, 1987), isoenzyme analysis (Sidenberg & Lachance, 1983, 1986) and karyotyping (Sor & Fukuhara, 1989; Viljoen *et al.*, 1989), although there is evidence of heterogeneity in these species at the intraspecific level (Cottrell *et al.*, 1987; Belloch *et al.*, 1997; Lehmann *et al.*, 1992; Molnar *et al.*, 1996). A second group includes the species *K. africanus*, *Kluyveromyces delphensis*, *Kluyveromyces lodderae*, *K. polysporus* and *Kluyveromyces yarrowii*, which appear intermixed with species belonging to the genera *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces* in a polyphyletic group. The species *Kluyveromyces blattae* and *Kluyveromyces phaffii* form distinct lines quite separate from each other and from the other two groups.

Comparisons of rRNA genes are presently used to assess phylogenetic relationships among yeasts pertaining to the *Saccharomycetaceae* family, in which all the above genera are included (Kurtzman & Robnett, 1991, 1998; Hendriks *et al.*, 1992; Wilmotte *et al.*, 1993; Boekhout *et al.*, 1994; Cai *et al.*, 1996). However, a few studies involving other genes (Hoeben *et al.*, 1993) corroborate the conclusions obtained from the rRNA phylogenetic analyses.

Mitochondrial DNA is widely employed in evolutionary studies of higher eukaryotes because it is a small molecule, consists mostly of coding sequences and lacks the genetic complexity that complicates the interpretation of nuclear data. However, yeasts show a paucity of mitochondrial genes, which in some species is compensated for by a structural complexity in the intergenic regions, resulting from the distribution of optional introns (Wilson & Fukuhara, 1991; Hardy & Clark-Walker, 1991; Clark-Walker & Weiller, 1994), rearrangements and/or insertions/deletions that encompass entire genes (Hardy *et al.*, 1989; Wilson *et al.*, 1989; Hoeben *et al.*, 1993; Piškur *et al.*, 1998), together with a complex pattern of changes from the universal code of codon usage (Brunner & Coria, 1989; Clark-Walker & Weiller, 1994).

In the present study, we have sequenced the mitochondrial gene *COII* (also *COX2*), which encodes subunit II of the cytochrome-*c* oxidase complex, of 17 species of the genus *Kluyveromyces*. These *COII* gene sequences have been used to determine the phylogenetic relationships among the species of the genus *Kluyveromyces* and other ascomycetous yeasts.

METHODS

Yeast strains. The DNA sequences of the mitochondrial cytochrome-*c* oxidase II gene were obtained from 17 type strains of species of the genus *Kluyveromyces*. The accession numbers of these sequences in the EMBL database, the strain references for the Spanish Type Culture Collection (CECT) and their origins are given in Table 1.

Of the ascomycetous yeast *COII* sequences available in the EMBL sequence library, those corresponding to species of the genus *Saccharomyces* and the species *Candida glabrata* were included in the ingroup and those from species of the genus *Dekkera* (anamorph *Brettanomyces*) were chosen as the closest outgroup to *Kluyveromyces* according to previous phylogenetic analyses based on 18S rRNA genes (Cai *et al.*, 1996; James *et al.*, 1997, 1998).

DNA extraction, amplification and sequencing. Total DNA was prepared according to the method described by Querol *et al.* (1992). The *coII* gene region was amplified by PCR using the high-fidelity conditions described by Kwiatowski *et al.* (1991). The following primers, designed by comparing available sequences from other yeast species, were used to amplify *coII*: COII-3 (5′-ATTTATTGTTCRTTTAATCA-3′) and COII-5 (5′-GGTATTTTAGAATTACATGA-3′).

PCR products were cleaned with the GeneClean Purification Kit (Bio101) and directly sequenced using the *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer), according to the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer (model 373A). The following two additional internal primers were used to obtain the whole sequence of both strands: COII-i3 (5′-TCTTAATTGACCATCTTCTAA-3′) and COII-i5 (5′-TTATTATATTTATGTGATGAAGT-3′).

Phylogenetic inference. The *coII* gene sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). For phylogenetic inference, we used the neighbour-joining (NJ) method (Saitou & Nei, 1987). The number of taxa precluded the use of maximum-likelihood analysis for phylogenetic

Table 1. Yeast strains analysed in the present study, isolation sources and EMBL accession numbers for their *COII* sequences

Culture-collection abbreviations: CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; CECT, Colección Española de Cultivos Tipo, Spanish Type Culture Collection, University of València, València, Spain. Accession numbers AJ235911 to AJ235928 correspond to sequences obtained in our laboratory; the other sequences from reference and outgroup species were retrieved from the EMBL sequence database.

Species	Strain designation	Isolation source	Accession no.
Kluyveromyces aestuarii	CECT 1949^T (= CBS 4438)	Estuarine mud	AJ235911
Kluyveromyces africanus	CECT 1963^T (= CBS 2517)	Soil	AJ235922
Kluyveromyces bacillisporus	CECT 1979^T (= CBS 7720)	*Quercus* exudate	AJ235923
Kluyveromyces blattae	CECT 1964^T (= CBS 6284)	*Blatta orientalis*	AJ235928
Kluyveromyces delphensis	CECT 1954^T (= CBS 2170)	Dried figs	AJ235921
Kluyveromyces dobzhanskii	CECT 1952^T (= CBS 2104)	*Drosophila pseudoobscura*	AJ235915
Kluyveromyces lactis	CECT 1961^T (= CBS 683)	Gassy cheese	AJ235914
Kluyveromyces lodderae	CECT 1126^T (= CBS 2757)	Soil	AJ235925
Kluyveromyces marxianus	CECT 10585^T (= CBS 712)	–	AJ235916
Kluyveromyces phaffii	CECT 10646^T (= CBS 4417)	Soil	AJ235927
Kluyveromyces piceae	CECT 11327 (= CBS 7738)	Rhizosphere	AJ235920
Kluyveromyces polysporus	CECT 1960^T (= CBS 2163)	Soil	AJ235924
Kluyveromyces sinensis	CECT 11332^T (= CBS 7660)	Dead bird	AJ235919
Kluyveromyces thermotolerans	CECT 1951^T (= CBS 6340)	–	AJ235912
Kluyveromyces waltii	CECT 1965^T (= CBS 6430)	Exudate of *Ilex integra*	AJ235913
Kluyveromyces wickerhamii	CECT 1966^T (= CBS 2745)	*Drosophila montana*	AJ235917
Kluyveromyces yarrowii	CECT 1958^T (= CBS 8242)	Tanning fluid	AJ235926
Candida glabrata	CBS 138^T	Human faeces	X69430
Dekkera anomala	CBS 77	Stout beer	X64822
Dekkera bruxellensis	CBS 74^T	Lambic beer	X64823
Dekkera bruxellensis	CBS 5512 (neotype of *Brettanomyces custersii*)	Bantu-beer brewery	X64824
Dekkera custersiana	CBS 4805^T	Bantu-beer brewery	X64826
Dekkera naardenensis	CBS 6042^T	Lemonade	X64821
Eeniella nana	CBS 1945^T	Bottled beer	X64825
Saccharomyces kluyveri	CECT 10677^T (= CBS3082)	*Drosophila pinicola*	AJ235918
Saccharomyces bayanus	CBS 375		AJ002019
Saccharomyces cerevisiae	Unknown		V00685
Saccharomyces paradoxus	SDI-2 (*Saccharomyces douglasii*)		X95975
Saccharomyces exiguus	CBS 379^{NT}		X69429

reconstruction, although it has been used for evolutionary model testing and parameter estimation.

NJ trees and the statistical confidence of a particular group of sequences in the NJ trees, evaluated by the bootstrap test (1000 pseudoreplicates), were performed using the computer program MEGA version 1.0 (Kumar *et al*., 1993). Among-site rate variation was also incorporated into the analyses by using different gamma-corrected distances. To estimate the alpha parameter of the gamma distribution, we used the method of moments and the maximum-likelihood methods of Sullivan *et al*. (1995) and Yang & Kumar (1996), all of which were implemented in the PAMP program of the PAML version 1.3 package for Windows (Yang, 1997).

RESULTS

The yeast mitochondrial *COII* gene (in those species for which complete sequences are available) ranges in size from 744 bp (*K. lactis*) to 756 bp (*C. glabrata*, *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*) and contains no introns. The region sequenced in the present study encompasses a $\approx$ 600 bp segment of the gene (except the 5′ and 3′ ends), corresponding to positions 124–708 in the *Saccharomyces cerevisiae COII* coding sequence (Fox, 1979).

The 29 *COII* partial sequences yielded 600 nucleotide positions aligned for all species (585 positions if gaps are not considered). *K. blattae* was the only species showing two insertions, corresponding to one TCT codon (encoding Ser) and a tandem repeat of four AAT codons (Asn).

In all of the species in the alignment, 268 variable nucleotide sites (45 %) were observed, of which 222 are

phylogenetically informative (37%). Of the variable sites, 75 correspond to first-codon positions (58 are informative), 50 correspond to second-codon positions (37 are informative) and 143 correspond to third-codon positions (127 are informative). If the outgroup species of the genus *Dekkera* are excluded and only the closely related species of the genera *Kluyveromyces* and *Saccharomyces* – and also *C. glabrata* – are considered as ingroup species [as indicated by Cai *et al.* (1996) and James *et al.* (1997)], the number of variable nucleotide positions is 212 (36·2%), of which 157 are informative (26·8%). These variable sites correspond to 51 first-codon positions, 39 second-codon positions and 122 third-codon positions (33, 24 and 100 of them are informative, respectively). The inferred amino acid sequences are also very variable. There are 90 variable amino acid positions (out of 200) among the whole set of species (68 are informative), of them 23 are fixed in the ingroup (67 are variable and 46 informative).

The *COII* fragment from the ascomycetous yeasts under study have a high proportion of A + T (a mean of 73·8%, ranging from 72·1% in *K. piceae* to 76·2% in *K. blattae*), especially in third-codon positions (ranging from 90·8% in *K. sinensis* to 97·4% in *K. bacillisporus*), as has been previously reported for mtDNA from different fungi (Sueoka, 1988).

The mean ratio of transitions to transversions for all pairwise comparisons is $0{\cdot}45 \pm 0{\cdot}16$; if comparisons involving the outgroups are excluded, the mean ratio is $0{\cdot}49 \pm 0{\cdot}19$, with values ranging from 0·091 (between the outgroup species *Dekkera anomala* and *Dekkera custersiana*) to 2·33 (between the sibling species *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*). In general, the highest ratios are among the more related species and the lowest ratios are for comparisons between more distant ones. A strong bias for transitional substitutions between closely related species with a loss of this bias between more distantly related species has been previously demonstrated for *Drosophila* mtDNA (and insect mtDNA in general), which also shows a very high A + T content. This trend has been explained as a fast saturation of transitional substitutions due to strong biases in both base composition and substitution patterns (DeSalle *et al.*, 1987; Liu & Beckenbach, 1992; Barrio *et al.*, 1994). Moreover, very low transition/transversion ratios also indicate that divergence times among species are quite old (they are similar to those observed among the comparisons between pairs of *COII* sequences from representatives of different insect orders; see Liu & Beckenbach, 1992). Consequently, not only have transitions reached saturation, but saturation could also be affecting transversional substitutions.

As the compositional bias can lead to underestimation of the true amount of divergence and hence affect phylogenetic reconstructions (Saccone *et al.*, 1993; Collins *et al.*, 1994), this factor should be taken into account in the models used to estimate nucleotide divergence. Three distance procedures have been developed to correct compositional bias. One of these, the Tamura & Nei (1993) distance procedure, was developed to compensate for overall base composition bias (and also transition/transversion bias) on the assumption that all taxa have approximately the same nucleotide composition. The other distance measures, the LogDet (Lockhart *et al.*, 1994) and the procedure of Galtier & Gouy (1995), were designed to infer phylogenies from DNA sequences of unequal base composition. However, the three distance estimates produce the same tree topology with the NJ tree-building method, regardless of the model used.

Fig. 1 shows the NJ tree generated by using Tamura–Nei distances obtained from all substitutions. We can conclude from this tree that the genus *Kluyveromyces* is not a monophyletic group and that its species can be divided into four 'groups', two of which are well-supported monophyletic groups according to the bootstrap test (bootstrap values > 70% are considered to indicate well-established groups according to Hillis & Bull, 1993). The first branch corresponds to the first group of three 'oldest' species (in this order): *K. phaffii*, *K. yarrowii* and *K. blattae* (to be designated as the *K. phaffii* group, named after the first species from this group to be described), whose phylogenetic relationships are unclear according to their bootstrap values (BVs). The second group of species, referred to as the *K. thermotolerans* group, includes the species pair *K. thermotolerans*/*Kluyveromyces waltii* and the species *Saccharomyces kluyveri*. They form a well-supported monophyletic group (BV 80%) that clusters, in turn, with the third group of species with high bootstrap support (BV 98%). This third group, the *K. marxianus* group, is also monophyletic (BV 96%) and contains the species *K. aestuarii*, *K. dobzhanskii*, *K. lactis*, *K. marxianus* and *K. wickerhamii*, whose relationships are well established except for the positions of the last two species. The fourth group (hereafter referred to as the *Saccharomyces cerevisiae* group) comprises a series of clusters of species belonging to the genera *Kluyveromyces* and *Saccharomyces* and which are, in most cases, not well established according to the confidence limits of the bootstrap test. This group includes the following: (i) the highly related species pair *K. delphensis*/*C. glabrata* (BV 91%), which are distantly related to the species *K. bacillisporus* (BV 33%); (ii) the four-species cluster *K. lodderae*/*K. piceae*/*K. sinensis*/*Saccharomyces exiguus* (BV 65%); (iii) the monophyletic *Saccharomyces sensu stricto* group (BV 84%); and (iv) the species pair *K. africanus*/*K. polysporus* (with a low BV of 47%).

Another potential difficulty with distance estimation is whether the data conform with the assumptions of the model. In this instance, these data do not appear to meet the expectations from the Poisson distribution, which is the method underlying most of the commonly used distance estimation procedures (Swofford *et al.*, 1996). This can be shown by testing whether the distribution of character changes fits a Poisson dis-

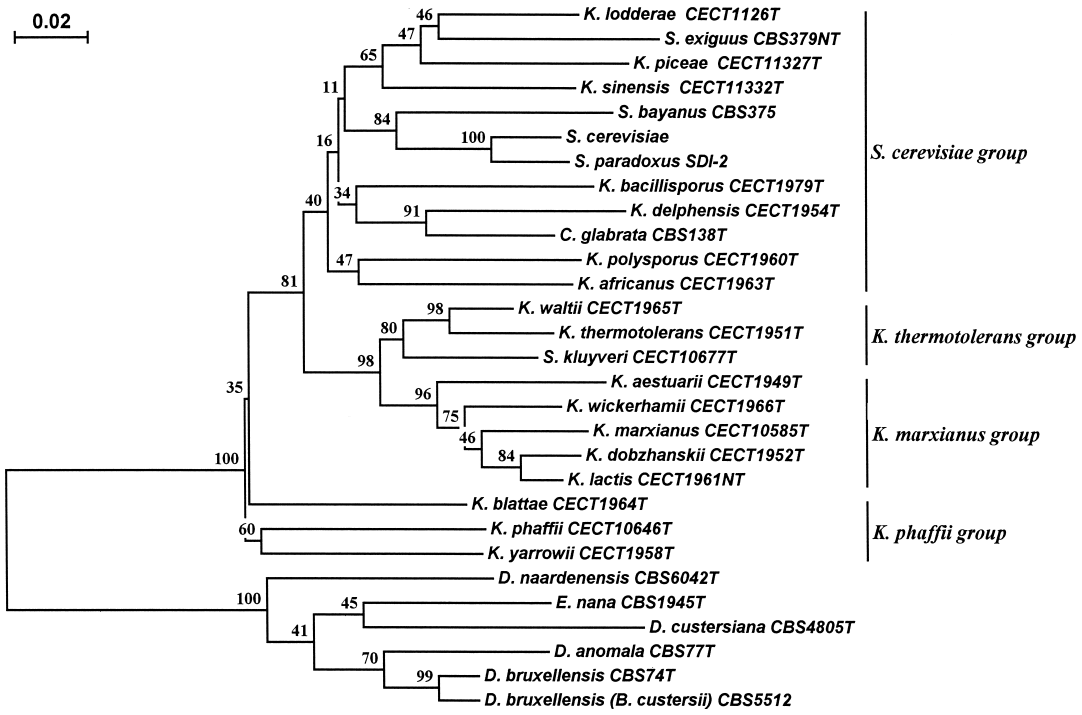

Fig. 1. Neighbour-joining tree based on total nucleotide divergence between pairs of *COII* sequences corrected according to the method of Tamura & Nei (1993). Percentage bootstrap values (based on 1000 pseudoreplicates) are given on the nodes. Bar, 0·02 substitutions per nucleotide.

tribution or is instead more appropriate to a Γ-distributed rates model. To test this, the tree topology depicted in Fig. 1 was evaluated by using PAMP programs (Yang, 1997), which estimate the shape parameter, α, of the Γ-distribution, by using the method of moments, and by using the methods of Sullivan *et al.* (1995) and Yang & Kumar (1996). The estimates for the α parameter were as follows: 0·58, according to the method of moments; 0·40, according to the method of Sullivan *et al.* (1995); and 0·37, according to the maximum-likelihood method of Yang & Kumar (1996). Moreover, a likelihood ratio test, performed with the results of the BASEML program of the PAML package (Yang, 1997), indicates that the among-site distribution of nucleotide substitution rates fits better into a Γ-distributed rates model than a Poisson distribution ($\chi^2 = 981$, d.f. $= 1$, $P < 0{\cdot}0001$).

When the phylogeny is estimated by using the Tamura–Nei distance model modified for the Γ-distribution (Rzhetsky & Nei, 1994), the answer depends on the value used for the α parameter. Thus, for $\alpha = 0{\cdot}58$, the same topology as that of the tree depicted in Fig. 1 is obtained with the same significant groups and clusters. When α values of 0·37 or 0·40 are used, a slightly different branching order is observed (not shown) in which *Saccharomyces kluyveri* appears as a sister lineage of the cluster formed by the *K. thermotolerans* and *K. marxianus* groups but the other significant groups or clusters do not differ.

As mentioned before, transitional substitutions can become saturated in very A+T-rich mtDNAs, which will introduce a bias into the phylogenetic reconstructions. One escape from this bias is to rely exclusively on conservative changes, i.e. transversions in our case, which accumulate at slower rates than transitions. The NJ tree derived from nucleotide distances based on transversions, according to the Tamura–Nei model, is depicted in Fig. 2(a). This tree shows the same groups and clusters as the NJ tree based on total substitutions, but the bootstrap values are, in several cases, smaller, e.g. the *Saccharomyces sensu stricto* group is not significant in this tree (BV 51%). This could be due to the smaller number of informative substitutions (i.e. only transversions) and to the fact that transversions can also be saturated. In this tree, the *K. marxianus* and *K. thermotolerans* groups again appear as two closely related (BV 92%) monophyletic groups (BV 96% and 73%, respectively). It is also worth noting that the *K. phaffii* group appears in this tree as a cluster, but with low bootstrap support (BV 48%).

Constrained positions and conservative changes are thought to be particularly reliable in the phylogenetic analysis of DNA sequences because their phylogenetic signals are less likely to be erased by parallel- and back mutations and saturation. We have relied only on transversions to overcome a potential problem of saturation of transitions, but nonsynonymous substi-

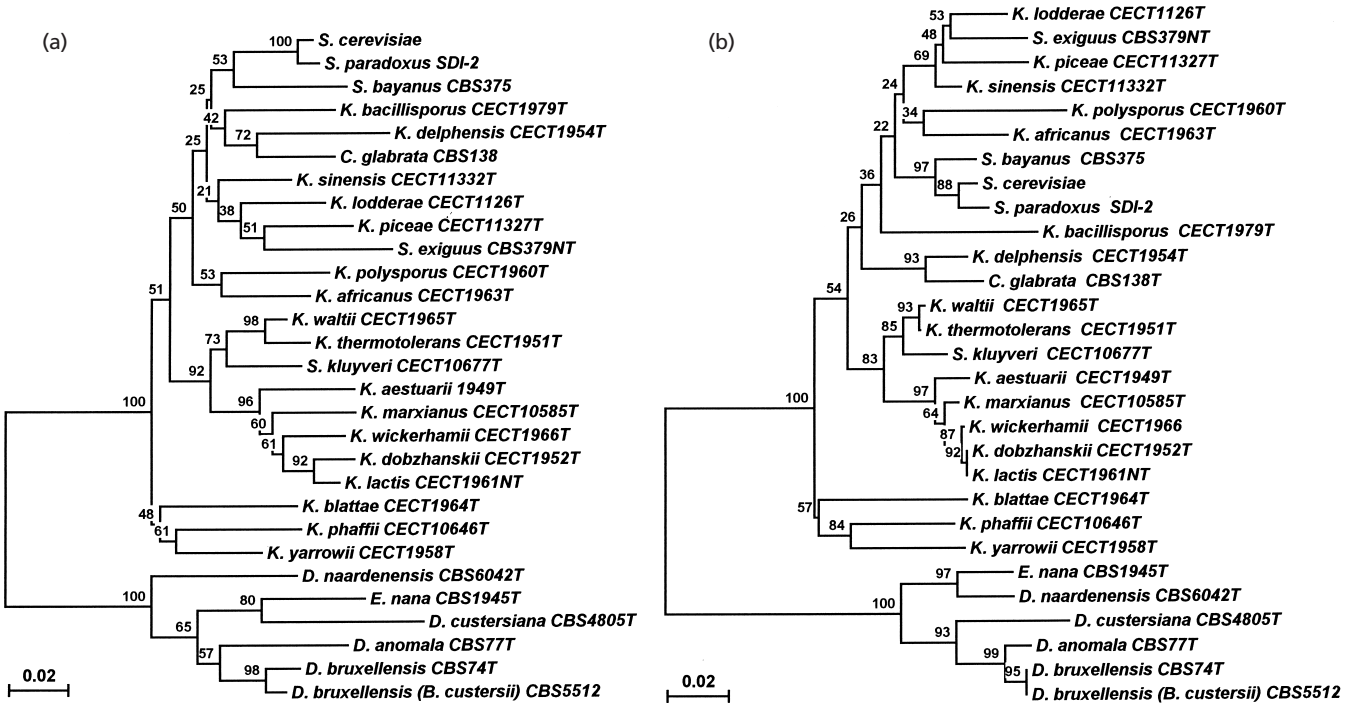

Fig. 2. Neighbour-joining trees based on *COII* gene sequences, using transversions only (a) or nonsynonymous substitutions only (b). Transversions were corrected according to the method of Tamura & Nei (1993) and nonsynonymous substitutions were obtained using the unweighted method of Nei & Gojobori (1986). Percentage bootstrap values (based on 1000 pseudoreplicates) are given on the nodes. Bar, 0·02 transversion substitutions per site (a) or nonsynonymous substitutions per nonsynonymous site (b).

tutions also accumulate at a slower rate than synonymous substitutions. Fig. 2(b) shows the NJ tree based on distances derived from nonsynonymous substitutions by the unweighted method of Nei & Gojobori (1986). This nonsynonymous NJ tree yields monophyly for the same groups and clusters, but with higher bootstrap support for some of them. Thus, the *K. marxianus* and *K. thermotolerans* groups are again monophyletic and well-resolved groups (BV 97% and 85%, respectively) that appear as sister clades with bootstrap support of 83%. The *K. lodderae*/*K. piceae*/*K. sinensis*/*Saccharomyces exiguus* cluster is at the limit of support in the bootstrap test (BV 69%) and the *K. phaffii*/*K. yarrowii* species pair appears well supported (BV 84%).

Phylogenetic reconstructions derived from the analysis of the mitochondrial *COII* gene sequences are, for the most part, congruent with those previously obtained from the nuclear 18S rRNA gene sequences (Cai *et al.*, 1996; James *et al.*, 1997, 1998; Kurtzman & Robnett, 1998) and partial 26S rRNA gene sequences (Kurtzman & Robnett, 1998). Nonetheless, there are notable differences in the positions of some species: *K. yarrowii* appears as part of a clear species pair with *K. polysporus* within the *Saccharomyces cerevisiae* group in the 18S rRNA and 26S rRNA trees and as the sister taxon of *K. phaffii* within the *K. phaffii* group in the *COII* gene tree.

Another important difference is the position of the monophyletic *K. thermotolerans* group in the trees. Thus, this group is included within the heterogeneous *Saccharomyces cerevisiae* group in the 18S rRNA gene trees from Cai *et al.* (1996) and James *et al.* (1997) or appears as the first group to diverge (basal clade) in the 26S rRNA gene tree from Kurtzman & Robnett (1998), but it is the sister clade of the *K. marxianus* group according to the *COII* gene tree. The species *K. thermotolerans* and *K. waltii* (and also *Zygosaccharomyces cidri* and *Zygosaccharomyces fermentati*; *Saccharomyces kluyveri* was not included) also appear as the sister clade of the *K. marxianus* group in the 18S rRNA NJ tree obtained by Kurtzman & Robnett (1998, see their Fig. 1), although this relationship is not supported by the bootstrap test (BV <50%). The different topologies obtained by different authors with the same 18S rRNA sequences coupled with the low bootstrap support for the nodes are indicative of a lack of resolution of the relationships between groups of *Kluyveromyces* species.

In the case of the 26S rRNA tree obtained by Kurtzman & Robnett (1998, see also their Fig. 1), the basal position of the *K. marxianus* group and the short branches connecting these species compared to the branches connecting the other species could be considered as indicative of an artifactual rooting due to the outgroup species, the Archiascomycete *Schizo-*

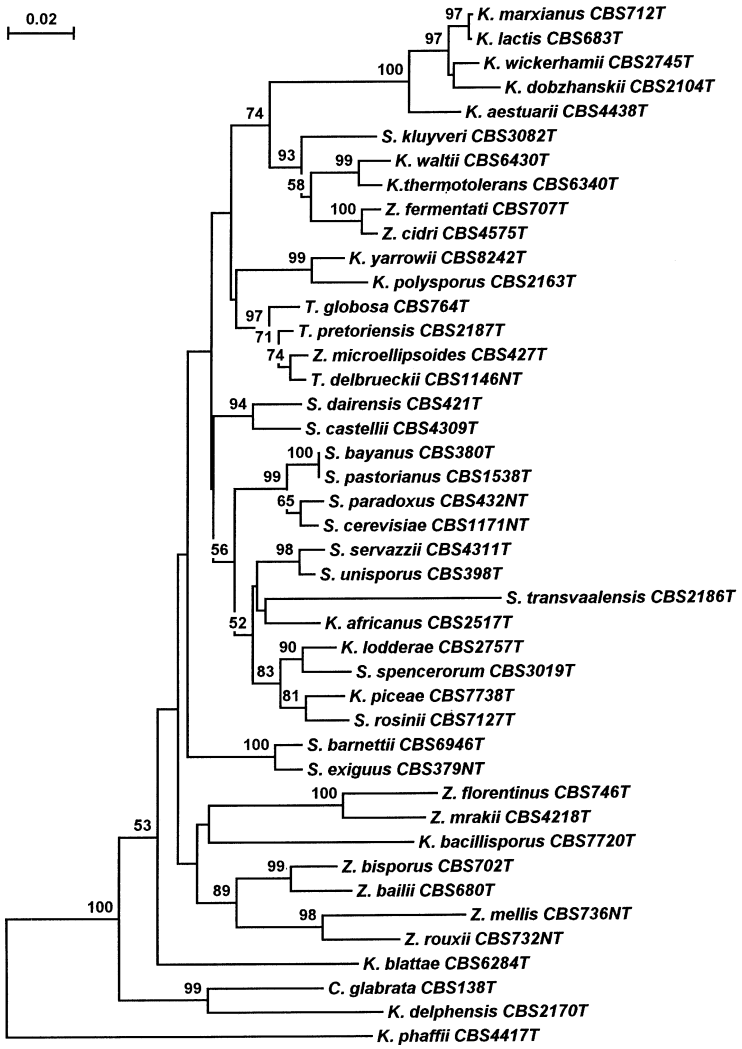

Fig. 3. Neighbour-joining tree obtained from the reanalysis of the same 26S rRNA sequences used by Kurtzman & Robnett (1998) to obtain the tree depicted in their Fig. 1, but excluding from the analysis the outgroup *Schizosaccharomyces pombe* and including *Saccharomyces kluyveri*, *Kluyveromyces bacillisporus*, *Kluyveromyces piceae* and *Candida glabrata*. The unrooted tree is shown rooted at the midpoint. Nucleotide divergences were obtained according to the Jukes & Cantor (1969) method, as in the original analysis. Percentage bootstrap values are also based on 1000 pseudoreplicates; only those higher than 50 % are indicated on the nodes.

saccharomyces pombe, which is too distant with respect to the ingroup. Connecting a distant outgroup to a tree can be very problematic, as there may be so many changes along the branch connecting the ingroup to the outgroup that the sequences effectively become randomized (Swofford *et al.*, 1996, p. 478). This can lead to 'long-branch attraction' effects with artifactual rooting along longer ingroup branches, or to wrong rooting due to convergence. For this reason, it is often preferable to obtain an unrooted tree rather than to include a highly divergent outgroup taxon in the analysis. Fig. 3 is the unrooted tree obtained from reanalysis (under the same conditions, i.e. using the NJ method and Jukes & Cantor distance measures) of the same 26S rRNA sequences used by Kurtzman & Robnett (1998) to obtain the NJ tree depicted in their Fig. 1, but excluding from the analysis the outgroup *Schizosaccharomyces pombe* and including *Saccharomyces kluyveri*, *K. bacillisporus*, *K. piceae* and *C. glabrata*. The most interesting change in the topology of the new tree with respect to the original one is the fact that the monophyletic *K. marxianus* and *K. thermotolerans* groups (also including *Z. cidri* and *Z. fermentati*) are supported as sister groups according to the bootstrap test (BV 73 %), as in the *COII* tree. From the comparison between this tree and the *COII* trees, we can conclude that the groups supported by bootstrap values higher than 70 % in the *COII* tree are also well supported in this 26S rRNA gene tree, indicating a high congruence between both gene trees.

DISCUSSION

The phylogenetic relationships among the species of the genus *Kluyveromyces* obtained from the sequence analysis of the mitochondrial gene *COII* are, for the most part, congruent with the trees obtained from the reanalyses of the nuclear 18S and 26S rRNA gene sequences obtained by Cai *et al.* (1996), James *et al.* (1997) and Kurtzman & Robnett (1998). In both nuclear and mitochondrial phylogenies, the genus *Kluyveromyces* appears as a polyphyletic taxon formed by species that are included within the following four main groups. The '*Saccharomyces cerevisiae*' group is a large group of species belonging to the present genera

Kluyveromyces, *Saccharomyces* (*sensu lato* and *sensu stricto*), *Torulaspora* and *Zygosaccharomyces* and whose phylogenetic relationships are unsolved in many cases. The *K. marxianus* group is clearly monophyletic and comprises the species *K. aestuarii*, *K. dobzhanskii*, *K. lactis*, *K. marxianus* and *K. wickerhamii*. The monophyletic *K. thermotolerans* group is formed by the species *K. thermotolerans*, *K. waltii* and *Saccharomyces kluyveri*, according to the *COII* gene tree, but also includes *Z. cidri* and *Z. fermentati* according to the ribosomal trees; this group appears as a sister clade of the monophyletic *K. thermotolerans* group in both nuclear and mitochondrial trees, although this relationship is supported by bootstrap analysis in the 26S and *COII* trees but not in the 18S tree. Finally, the *K. phaffii* group includes the species *K. blattae* and *K. phaffii*, according to 18S rRNA gene and *COII* gene trees (and also *K. yarrowii*, on the basis of the mitochondrial gene), but does not appear as such a group in the 26S tree.

The recalcitrant nature of the phylogenetic relationships among the different lineages within the *Saccharomyces cerevisiae* group of species might seem to be simply the result of two factors: (i) the low levels of divergence in the case of the phylogenetic reconstructions based on the conserved 18S or 26S rRNA genes and, simultaneously, (ii) a saturation effect in the case of the phylogeny based on the highly variable mitochondrial gene. Alternatively, it may be that the lack of resolution of phylogenetic relationships within the *Saccharomyces cerevisiae* group is actually evidence of a rapid phyletic radiation of the *Saccharomyces cerevisiae* group. Short internode lengths and similar levels of sequence divergence among taxa have been suggested as being consistent with hypotheses of rapid origin and radiation of lineages (Kraus & Miyamoto, 1991). This rapid radiation could be a consequence of the adaptation and colonization of new habitats, as has been already proposed to explain the appearance of *Saccharomyces cerevisiae* mediated by the duplication of its genome (Wolfe & Shields, 1997).

Unlike the *K. phaffii* group, the existence of the other three groups is well supported by other phenotypic, genetic and molecular data. The species of *Kluyveromyces* included in the *Saccharomyces cerevisiae* group (but also the *K. phaffii* group species) exhibit poor nutritional capabilities, form asci with either few or many spores and are isolated from restricted habitats (almost exclusively soils). The species included in the monophyletic and related *K. marxianus* and *K. thermotolerans* groups show greater nutritional versatility, form few spores per ascus and are isolated from very diverse habitats (van der Walt, 1970; van der Walt & Johannsen, 1984; Barnett *et al.*, 1990).

This classification according to their general characteristics was confirmed by phenetic analyses based on morphology, isolation sources, physiology, etc. Thus, Campbell (1972), Poncet (1973), and also Lachance (1993) in a recent review, proposed the division of the members of the *Kluyveromyces* genus into the following three main groups: (i) the nutritionally restricted species, which are those included within the *Saccharomyces cerevisiae* group in the *COII* tree; (ii) the nutritionally versatile species group, which correspond to the monophyletic *K. marxianus* group; and (iii) the *K. thermotolerans*/*K. waltii* species pair, which form, with *Saccharomyces kluyveri*, a monophyletic group related to the *K. marxianus* group in the *COII* tree. The close phenetic relationship between the *K. thermotolerans*/*K. waltii* pair and *Saccharomyces kluyveri* (and also some species of *Zygosaccharomyces*) was also suggested by Lachance (1993).

The analysis of genetic interbreeding among species of the genus *Kluyveromyces* (Johannsen & van der Walt, 1978; Johannsen, 1980) also showed two groups of species. The first one, which includes the species from the *Saccharomyces cerevisiae* group, is characterized by the absence of interfertility. The second one, corresponding to the *K. marxianus* group, is characterized by the ability to hybridize under laboratory conditions (although in most cases hybrids are allopolyploids or revert to one of the parental species) between strains of the present species *K. aestuarii*, *K. dobzhanskii*, *K. lactis*, *K. marxianus* and *K. wickerhamii* (the *K. marxianus* group) and, at a lower frequency, between *K. marxianus* and *K. thermotolerans* and between *K. lactis* and *K. waltii* (the *K. thermotolerans* group). Although it was demonstrated that the *K. thermotolerans* strain used by Johannsen & van der Walt (1978) to construct the hybrid represents a rare variety of *K. thermotolerans* or a distinct but related species and that the hybrid reverted to *K. marxianus* (Vaughan-Martini *et al.*, 1987), the formation of hybrids between some species of the *K. marxianus* and *K. thermotolerans* groups could be considered indicative of a relationship between these two monophyletic groups.

The comparative analysis of electrophoretic chromosome patterns (Belloch *et al.*, 1998b) revealed the existence of two groups of *Kluyveromyces* species distinguished by their karyotypes. The species of the first group (which correspond to the *Saccharomyces cerevisiae* group species and *K. phaffii* and *K. yarrowii*) exhibit chromosomal patterns of many small chromosomes, similar to that of *Saccharomyces cerevisiae*. The species of the second group (which corresponds to the *K. marxianus* and *K. thermotolerans* groups and the species *K. blattae*) exhibit patterns with fewer but larger chromosomes. Phenotypic characters together with electrophoretic karyotypes were used by Kock *et al.* (1988) to propose that the genus *Kluyveromyces* evolved from mycelioid, hybridizing 'primitive' ancestors associated with diverse habitats towards the unicellular, non-hybridizing 'evolved' taxa restricted to specific habitats. During this process, a reduction in phenotypic and genetic characters occurred, while an increase in the number of small

chromosomes was observed in their karyotypes. This evolutionary scenario is compatible with the phylogeny, presented here, based on the *COII* gene.

As already mentioned, the mitochondrial gene tree and the rRNA gene trees are generally congruent in the phylogenetic relationships among species of the genus *Kluyveromyces*, although there are interesting differences in the positions of some species. The first remarkable difference corresponds to the position of *K. yarrowii* in the trees. This species is a sister taxon to *K. polysporus* in the 18S and 26S rRNA gene trees, but forms a species pair with *K. phaffii* within the *K. phaffii* group in the *COII* gene tree. *K. polysporus* and *K. yarrowii* also appeared as a species pair (although this was not significant according to bootstrap tests) in phylogenetic reconstructions based on the restriction-pattern analysis of the 18S rRNA (Shen *et al.*, 1994) and the restriction-map analysis of the ribosomal region encompassing the 5.8S rRNA gene and the two internal transcribed spacers (Belloch *et al.*, 1998a). However, the first analysis was performed with the same gene sequenced by Cai *et al.* (1996) and the second was performed with an adjacent region of the same transcription unit.

Van der Walt *et al.* (1986) indicated in the original description of *K. yarrowii* that this species shows some physiological agreement with *K. blattae* and *K. phaffii* (but also with *K. africanus*). This was confirmed by Lachance (1993), who pointed out that *K. phaffii* and *K. yarrowii* are probably related because they are almost indistinguishable physiologically. The question of whether *K. yarrowii* should be included in the *K. phaffii* group, with *K. blattae* and *K. phaffii*, or in the *Saccharomyces cerevisiae* group remains unanswered and requires further study.

The second difference between the previous ribosomal trees and the mitochondrial trees corresponds to the position of the monophyletic cluster of species, *K. thermotolerans*/*K. waltii*/*Saccharomyces kluyveri* (the *K. thermotolerans* group), which is included within the heterogeneous *Saccharomyces cerevisiae* group in the 18S trees obtained by Cai *et al.* (1996) and James *et al.* (1997) as well as the 26S tree obtained by Kurtzman & Robnett (1998), but which appears as a sister group of the *K. marxianus* group, according to the *COII* gene tree.

However, this difference is in fact spurious. The different topologies obtained by different authors with the same 18S rRNA sequences coupled with the low bootstrap support for the nodes are indicative of a lack of resolution of the relationships between groups of *Kluyveromyces* species. One of these topologies is the one obtained by Kurtzman & Robnett (1998), which shows both *K. marxianus* and *K. thermotolerans* as sister clades, although this relationship is not supported by the bootstrap analysis. In the case of the 26S rRNA sequences such a difference is due to the use of a very distant outgroup in the tree obtained by Kurtzman & Robnett (1998). However, after the exclusion of this outgroup from the analysis, both the *K. marxianus* and *K. thermotolerans* groups also appear as sister clades in the reanalysis of the 26S rRNA sequences, which agrees with the *COII* tree.

In addition, several observations support the close relationship between the *K. marxianus* and *K. thermotolerans* groups. As seen before, the *K. thermotolerans*/*K. waltii* species pair has been related to the *K. marxianus* group with respect to interfertility assays (Johannsen, 1980) and electrophoretic karyotyping (Belloch *et al.*, 1998b). The electrophoretic isozyme patterns of Sidenberg & Lachance (1983) also indicated that *K. thermotolerans* shares with *K. wickerhamii* and *K. dobzhanskii* a more or less intermediate status between *K. marxianus* and *K. lactis*. Moreover, these workers also found a singular and unexpectedly high level of electrophoretic similarity between *K. waltii* and *Kluyveromyces bulgaricus* (now included in the taxon *K. marxianus*). In a subsequent study, Sidenberg & Lachance (1986) also showed that *K. waltii* shares a number of characteristics with either *K. thermotolerans* or *K. marxianus*, indicating that *K. waltii* may occupy an intermediate position in the genus, between the latter two species.

In this way, Kock *et al.* (1988) also included *K. thermotolerans* and *K. waltii* in the same group as *K. marxianus* and its relatives in accordance with their morphology, physiology, interfertility, long-chain fatty acid composition and electrophoretic karyotypes.

In a very recent paper, Piškur *et al.* (1998) analysed the structure and genetic stability of mitochondrial genomes from *Saccharomyces* species (both *sensu lato* and *sensu stricto*) and concluded that *Saccharomyces kluyveri* is a clearly separated species according to its mtDNA characteristics (*Saccharomyces kluyveri* is a petite-negative yeast and the other *Saccharomyces* species are petite-positive). These workers postulate that the petite-positive character evolved specifically in the ancestor of the *sensu lato* and *sensu stricto* yeasts (the so-called *Saccharomyces cerevisiae* group) after separation from *Saccharomyces kluyveri* and other related petite-negative species of the genera *Kluyveromyces* (only some species from the *K. marxianus* group were tested; personal communication), *Zygosaccharomyces* and *Torulaspora*. With respect to *Saccharomyces* species, this scenario is also compatible with the mitochondrial tree.

Recently, Wolfe & Shields (1997) presented molecular evidence for an ancient whole-genome duplication in *Saccharomyces cerevisiae*. In a subsequent study, Keogh *et al.* (1998) postulated from the analysis of gene-order arrangements, chromosome numbers and 18S rRNA gene phylogenetic reconstructions of ascomycetous yeast species that the genome duplication occurred before the divergence of the *Saccharomyces sensu stricto* species and after the divergence of this lineage from *Saccharomyces kluyveri* (i.e. within the *Saccharomyces cerevisiae* group according to the ribosomal trees). This conclusion was based on the

observations that *Saccharomyces kluyveri* has a smaller number of chromosomes and a lower percentage of linkage conservation of adjacent genes compared to the chromosome pattern and gene order in *Saccharomyces cerevisiae*. However, on the basis of the mitochondrial tree, the genome duplication could have occurred after the divergence of the *Saccharomyces cerevisiae* and *K. marxianus* groups (i.e. at the time of the possible rapid radiation of the *Saccharomyces cerevisiae* group) and before the divergence of the *Saccharomyces sensu stricto* species. This is more congruent with the similar levels of linkage conservation showed by *K. marxianus*, *K. lactis* and *Saccharomyces kluyveri* (83, 74 and 75 %, respectively) and the similar characteristics of their chromosomal patterns.

There has long been debate as to how the biological classification should reflect the evolutionary relationships of the organisms. Lachance (1993), in a revision of the genus *Kluyveromyces*, pointed out that 'nomenclatural changes should be avoided at all costs, unless the existing usage clearly violates an important principle or causes the persistence of erroneous ideas'. As a follower of the principles of the 'evolutionary systematics school', he considered the rearrangement of paraphyletic taxa to be undesirable but the reorganization of polyphyletic taxa unquestionable. Although further studies including additional species are required to confirm the phylogenetic relationships of the current genera (*Kluyveromyces*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*), future nomenclatural changes should take account of the existence of these three groups of *Kluyveromyces* species.

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