Phylogenetic analysis of the psychrophobic yeast *Arxiozyma telluris* and the reinstatement of *Candida pintolopesii* (van Uden) Meyer et Yarrow and *Candida slooffii* van Uden et do Carmo Sousa

Stephen A. James,1 Matthew D. Collins2 and Ian N. Roberts1

Author for correspondence: Stephen A. James. Tel: +44 1603 255000. Fax: +44 1603 458414.
e-mail: Steve.James@bbsrc.ac.uk

A phylogenetic analysis was conducted upon ten strains of the psychrophobic yeast *Arxiozyma telluris* using nuclear rDNA (18S and 26S) and mitochondrial cytochrome-c oxidase subunit II (COX2) gene sequences. Strains examined included those described originally as *Candida slooffii*, *Torulopsis bovina* (= *Candida bovina*) and *Torulopsis pintolopesii* (= *Candida pintolopesii*), which are all currently accepted as synonyms of *Arxiozyma telluris*. Comparative 18S rDNA sequence analysis showed that these strains formed a genealogically highly related group, which was phylogenetically distinct from any other ascomycetous species studied. The results showed that *A. telluris*, as currently described, appears to be composed of a complex of closely related but nevertheless separate taxa, which was phylogenetically distinct from any other ascomycetous species studied. The results showed that *A. telluris*, as currently described, appears to be composed of a complex of closely related but nevertheless separate taxa. rDNA and COX2 gene sequence data revealed that CBS 1787T, the type strain of *C. pintolopesii*, the currently recognized asexual form (anamorph) of *A. telluris*, along with strains CBS 2676 and CBS 2985 formed a distinct taxon that is phylogenetically separate from *A. telluris*. Similarly, the sequence data also showed that *C. slooffii* is a distinct taxon and support the reinstatement of this species. However, with regard to the relationship between the type strains of *A. telluris* (CBS 2685T) and *C. bovina* (CBS 2760T), discrepancies were observed between the rDNA and COX2 sequence datasets, and these results are discussed in more detail.

**Keywords:** yeast, *Arxiozyma telluris*, rDNA, COX2

**INTRODUCTION**

As a result of taxonomic revisions in the mid-1970s (van der Walt & Johannsen, 1975; von Arx et al., 1977), the genus *Saccharomyces* became restricted to diploid species that formed unornamented, glabrous ascospores and were characterized by the coenzyme Q6 system (Yarrow, 1984). *Saccharomyces telluris* (originally described as *Saccharomyces tellustris*; van der Walt, 1957) remained distinct from all other *Saccharomyces* species, as it produced warty ascospores. In this respect, *S. telluris* resembled species of the genus *Issatchenka*, but the absence of the coenzyme Q7 system led to its exclusion from this genus (Kurtzman et al., 1980). Therefore, van der Walt & Yarrow (1984) introduced the new genus *Arxiozyma* to accommodate *S. telluris*, and renamed the species *Arxiozyma telluris*. The type strain of the species was originally isolated from soil in South Africa (van der Walt, 1957), but the majority of *A. telluris* strains recorded subsequently have been recovered from the alimentary canals of warm-blooded animals such as cows, horses, pigs and rodents (Kreger-van Rij, 1958; van der Walt, 1970; van der Walt & Yarrow, 1984; Barnett et al., 2000). One noteworthy feature of the species is that many of its strains are characterized by their high minimum growth temperature, which ranges from 20 to 30 °C (Mendonça-Hagler & Phaff, 1975; van Uden & Buckley, 1970; van Uden & Vidal-Leira, 1970; Watson et al., 1981). As a result, *A. telluris* is
often referred to as psychrophobic (Mendonça-Hagler & Phaff, 1975). This is a very distinctive feature, rarely observed in yeast species, and possibly reflects adapt-ation to a specific ecological niche.

At present, the species *A. telluris* includes strains classified originally as *Candida slooffii*, *Torulopsis bovina* (= *Candida bovina*) and *Torulopsis pintolopesii* (= *Candida pintolopesii*) (Kurtzman, 1998a; Barnett et al., 2000). Morphologically and physiologically, these species were found to be extremely similar to one another and to *S. telluris*, with species differentiation being based mainly on the minimum temperature of growth and the ability to form ascospores or pseudo-myceilia (van Uden & Buckley, 1970; van Uden & Vidal-Leira, 1970; Mendonça-Hagler & Phaff, 1975). These species were later reduced to synonymy with *S. telluris* following an nuclear (n) DNA–nDNA hybridization study by Mendonça-Hagler & Phaff (1975), in which the nDNAs of all four species were shown to display high degrees of reassociation (80 % or greater) with one another. As a consequence, *C. pintolopesii* is currently recognized as the asexual state (anamorph) of *A. telluris* (Mendonça-Hagler & Phaff, 1975; Kurtzman, 1998a; Barnett et al., 2000).

In a biochemical study, Watson et al. (1981) discovered that the most distinctive difference between these four yeast taxa was their respiratory characteristics. While *S. telluris* and *T. bovina* were both shown to be respiration-competent (containing typical respiratory chain enzymes and cytochromes), *C. slooffii* and *T. pintolopesii* on the other hand were found to be respiration-deficient and lacked cytochrome aa₃ and cytochrome-c oxidase. Watson et al. (1981) highlighted the fact that the temperature limits of growth (20–44 °C) for *S. telluris* and *T. bovina* were different from those of *C. slooffii* (27–43 °C) and *T. pintolopesii* (24–43 °C). Although not contradicting the earlier findings of Mendonça-Hagler & Phaff (1975), these authors raised the possibility that the various isolates belonged to more than one species.

In order to understand better the species inter-relationships of these yeasts, a phylogenetic study based on 18S rRNA gene (rDNA) and 26S rDNA D1/D2 sequences was carried out upon representative strains of the original four species. The sequence of the mitochondrial COX2 gene (also known as COII), which encodes subunit II of the cytochrome-c oxidase complex, was also determined for each strain and the resulting phylogenetic tree was compared with those generated from the two rDNA sequence datasets.

**METHODS**

**Yeast strains and cultivation.** The ten strains of *A. telluris* examined in this study were obtained from the National Collection of Yeast Cultures (NCYC), Norwich, UK, and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Strain CBS 17877 (= IGC 24057) was isolated from the liver of a white mouse (*Mus musculus*) and is the type strain of *C. pintolopesii* (formerly *T. pintolopesii*).

Strain CBS 24197 was isolated from the caecum of a horse and is the type strain of *C. slooffii* (van Uden & do Carmo Sousa, 1957). Strain CBS 2675 was recovered from rodent droppings in New Zealand. Strain CBS 2676 was recovered from the udder of a mouse in The Netherlands. Strain CBS 2678 was isolated from the crop of a turkey from the UK. Strain CBS 2685 (= IGC 26637) was isolated from soil in South Africa and is the type strain of *A. telluris* (originally described as *S. tellustris*; van der Walt, 1957). Strain CBS 2670 was recovered from the caecum of a cow and is the type strain of *C. bovina* (van Uden & do Carmo Sousa, 1957). Strain CBS 2778 was isolated from the caecum of a rat. Strain CBS 2985 was recovered from the peritoneal fluid of a dead guinea pig in Indonesia. StrainNCYC 2475 was isolated from the beak of a white-crowned pigeon in the USA. All strains were grown on YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar; pH 5.5) at 30 °C.

**PCR amplification of 18S and 26S rDNA.** Amplification of the 18S rRNA gene was performed as described by James et al. (1994). The variable D1 and D2 regions of the 26S rDNA were amplified using the PCR protocol described by James et al. (1994) and the conserved fungal oligonucleotide primers NL1 and NL4 (O'Donnell, 1993). The mitochondrial COX2 gene was amplified using the oligonucleotide primers COII-5 and COII-3 (Belloch et al., 2000). The cycling parameters used to amplify the COX2 gene were: 94 °C for 2.5 min followed by two cycles of 94 °C for 2 min, 45 °C for 1.5 min and 72 °C for 1.5 min, followed by 33 cycles of 92 °C for 1.5 min, 45 °C for 1.5 min and 72 °C for 1.5 min, with a final extension step for 5 min at 72 °C. All amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen) following the manufacturer’s instructions.

**DNA sequencing and sequence analysis.** Direct sequencing of the rDNA (18S and 26S) and COX2 PCR products was performed using a Taq DyeDeoxy cycle sequencing kit (PE Biosystems) and an Oligosense thermal cycler (Hybaid) according to the manufacturers’ recommendations. Near-complete 18S rDNA sequences (approximately 1760 nucleotides) were determined using the primers detailed by James et al. (1994), while the 26S rDNA D1/D2 sequences were determined using primers NL1 and NL4 (O’Donnell, 1993). In the case of COX2, these sequences were determined using the amplification primers COII-5 and COII-3 (Belloch et al., 2000). Purified sequence reaction mixtures were electrophoresed with a PE Biosystems model 373A automated DNA sequencer.

The rDNA and COX2 sequences were aligned using the multiple-sequence alignment program PILEUP (Feng & Doolittle, 1987) contained within the GCG software package version 10 (Genetics Computer Group, 1991). Sequence identity values were calculated using the program GAP (data not shown). Phylogenetic analyses were performed using PHYLIP version 3.572 (Felsenstein, 1993). Distance matrices were generated using the DNADIST program with the Jukes–Cantor distance measure and rooted phylogenetic trees were constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987) and the NEIGHBOR program. *Kluyveromyces lactis* was used as the outgroup to root the 18S rDNA tree, while *Saccharomyces cerevisiae* was used to root both the 26S rDNA and COX2 trees. The stability of the individual branches of each tree was assessed using the bootstrap method (Felsenstein, 1985) with the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE.
RESULTS

18S rRNA gene sequence analysis

Comparative analysis of the 18S rRNA gene sequence data revealed the ten strains of *A. telluris* to be highly related to one another, exhibiting sequence identity values that ranged from 99.5 to 100% (corresponding to nine to zero base differences). Fig. 1 shows the NJ tree constructed from the 18S rRNA gene sequence data and depicts the genealogical relationship between the ten *A. telluris* strains and representative members of the genera *Kluyveromyces*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*, 18S rRNA gene sequences of which were determined in previous studies (Cai et al., 1996; James et al., 1994, 1996, 1997). As can be seen from the phylogenetic tree in Fig. 1, the *A. telluris* strains collectively formed a distinct lineage (bootstrap value 100%) which, apart from displaying a loose association with *Saccharomyces servazzii*, showed little phylogenetic affinity to any other species examined. Indeed, a similar result was observed (data not shown) when the phylogenetic analysis was expanded to include all currently accepted members of the genus *Saccharomyces* (Barnett et al., 2000), to which genus this species was originally assigned (as *S. telluris*; van der Walt, 1957, 1970).

Despite forming a distinct lineage (bootstrap value 100%) and possessing 18S rRNA gene sequences that were extremely similar to one another, it is evident from the phylogenetic tree shown in Fig. 1 that the ten *A. telluris* strains can be divided into five subgroups. The five subgroups and their phylogenetic relationships to one another are discussed below.

*A. telluris* CBS 1787T (*C. pintolopesii* type strain), along with strains CBS 2676 (originally identified as *T. pintolopesii*; Kreger-van Rij, 1958) and CBS 2985, formed the largest subgroup, which was quite separate from the other *A. telluris* strains examined. These three strains were found to possess identical 18S rRNA gene sequences, which differed from that of the *A. telluris* type strain (CBS 2685T) by a total of six base substitutions (99.6% sequence identity).

In the second subgroup,CBS 2760T (*C. bovina* type strain) paired with CBS 2678, a strain isolated originally from the crop of a turkey and also identified as *C. bovina* (Kreger-van Rij, 1958). Together, these two strains were found to have 18S rRNA gene sequences

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**Fig. 1.** Dendrogram showing the phylogenetic relationship of *A. telluris* strains to the type strains of other species of the genera *Candida*, *Kluyveromyces*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*, based on 18S rRNA gene sequences. The tree was constructed by using the NJ method (Saitou & Nei, 1987). Bootstrap values from 100 replications, expressed as percentages, are given at branch-points (only values > 50% are shown). Bar, 2 estimated base substitutions per 1000 nucleotide positions.
that differed by a single base substitution. Currently, the species name *C. bovina* is considered a synonym of *C. pintolopesii* (Kurtzman, 1998a; Barnett et al., 2000), following the nDNA–nDNA hybridization study of Mendonça-Hagler & Phaff (1975). However, as can be seen from Fig. 1, the type strains of *C. bovina* (CBS 2760T) and *C. pintolopesii* (CBS 1787T) formed separate lineages, displaying only 99.5% sequence identity (corresponding to a total of nine base substitutions) to one another.

In the third subgroup, NCYC 2475 paired with CBS 2685T (*A. telluris* type strain), with the two strains possessing 18S rRNA gene sequences that differed by a single base substitution (99.9% sequence identity). Together, these two strains formed a close association with the two strains of the fourth subgroup, which was composed of CBS 2675 and CBS 2778. Despite originally being identified as strains of *T. pintolopesii* (CBS 2675) and *C. bovina* (CBS 2778), these two strains were nevertheless found to possess identical 18S rRNA gene sequences and to be most closely related to NCYC 2475 (displaying 99.9% sequence identity), itself classified originally as *C. bovina*.

The remaining strain, CBS 2419T, formed a separate phylogenetic lineage (Fig. 1) and appeared to represent an outlier to the other *A. telluris* strains examined. This strain was isolated originally from the caecal contents of a horse and was described by van Uden & do Carmo Sousa (1957) as the type strain of a new species of *Candida*, *C. slooffii*. However, a detailed comparison of the 18S rRNA gene sequence of CBS 2419T with that of the *A. telluris* type strain (CBS 2685T) revealed that the two sequences differed by a total of seven base substitutions (99.6% sequence identity).

### 26S rDNA D1/D2 sequence analysis

Although analysis of the 18S rRNA gene sequences revealed that the ten *A. telluris* strains could be differentiated into five subgroups, resolution of the interrelationships between some of the subgroups was statistically poorly supported (i.e. low bootstrap values). Such a lack of statistical support is no doubt due to the overall high level of sequence conservation of the 18S rRNA gene. In an attempt to address this problem and also to study these strains in greater phylogenetic detail, attention was focused on the sequence analysis of the D1/D2 variable domains of the 26S rRNA gene. This region has proved particularly useful for the differentiation of sibling species (see Kurtzman & Blanz, 1998), especially for species that cannot be distinguished on the basis of 18S rRNA gene sequences (e.g. *S. cerevisiae* and *Saccharomyces paradoxus*; James et al., 1997). Typically, conspecific strains display 1% or less sequence divergence in this region of rDNA, while sequence divergence between strains of separate biological species generally (though not always) ranges from greater than 1% to as much as 47% (Peterson & Kurtzman, 1991; Kurtzman, 1998b; Kurtzman & Blanz, 1998).

As with the 18S rRNA gene sequence data, comparative analysis of the *A. telluris* 26S rDNA D1/D2 sequences revealed these yeasts to be closely related to one another. However, in contrast to the 18S rRNA gene, the levels of sequence divergence observed between the 26S rDNA D1/D2 sequences were notably higher, with sequence identity values ranging from 94.8 to 100% (corresponding to 27 to 0 base differences). Fig. 2 shows the NJ tree constructed from the 26S rDNA D1/D2 sequence data and, although the overall topology of this tree is not identical to that of the 18S rRNA gene tree (Fig. 1), the ten *A. telluris* strains can nevertheless be divided into the same five subgroups.

Within the individual subgroups, *A. telluris* strains have 26S rDNA D1/D2 sequences that are either identical (i.e. CBS 2678 and CBS 2760T, the *C. bovina* type strain; CBS 1787T, the *C. pintolopesii* type strain, and CBS 2676) or that differ from one another by a single base substitution (i.e. CBS 2985 and CBS 1787T; CBS 2675 and CBS 2778; CBS 2685T, the *A. telluris* type strain, and NCYC 2475). Such sequence similarity would indicate that the strains within each subgroup are conspecific, as they display less than 1% sequence divergence in this region of the 26S rDNA (Kurtzman & Blanz, 1998). However, when the 26S rDNA D1/D2 sequences of strains belonging to separate subgroups were compared, levels of sequence divergence were notably higher, ranging from 2.3 to 5.2%.

Such levels of sequence divergence would suggest that the five subgroups of *A. telluris* identified in this study are distinct taxa, possibly all worthy of separate species status (Kurtzman & Blanz, 1998).

### COX2 gene sequence analysis

Using primers COII-5 and COII-3 (Belloch et al., 2000), approximately 600 nucleotides of the COX2 gene were determined for each of the ten *A. telluris* strains. Based on the complete sequences that have been determined to date, the yeast mitochondrial COX2 gene appears to range from 744 bp (*K. lactis*; Hardy & Clark-Walker, 1991) to 756 bp (*Candida glabrata* [Clark-Walker & Weiller, 1994], *Kluyveromyces thermotolerans* [Clark-Walker & Weiller, 1994] and *S. cerevisiae* [Fox, 1979]) and to contain no introns. Consequently, using this primer set, approximately 80–85% of the COX2 gene can be determined (i.e. both strands sequenced).

As with the rDNA (18S and 26S) sequence data, comparative analysis of the COX2 sequences revealed the ten *A. telluris* strains to be closely related to one another, exhibiting sequence identity values that ranged from 89.5 to 100% (corresponding to 58 to 0 base substitutions). The largest sequence variation was
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Fig. 2. Dendrogram showing the phylogenetic relationship between strains of the five subgroups of *A. telluris* identified in this study (see text for a detailed discussion), based on 26S rDNA D1/D2 sequences. The tree was constructed by using the NJ method (Saitou & Nei, 1987). Bootstrap values from 100 replications, expressed as percentages, are given at branch-points (only values > 50% are shown). Bar, 4 estimated base substitutions per 1000 nucleotide positions.

seen between the COX2 sequence of CBS 2419T (*C. slooffii* type strain) and those of strains CBS 2678 and CBS 2760T (*C. bovina* type strain), which were identical. CBS 2419T was the only *A. telluris* strain analysed that was found to contain insertions (two single bases) in its COX2 sequence. Both insertions were located toward the 3' end of the gene, at positions 672 and 675 (based on *S. cerevisiae* COX2 numbering; Fox, 1979). Besides CBS 2678 and CBS 2760T, strains CBS 1787T (*C. pintolopesii* type strain) and CBS 2676 were also found to possess identical COX2 sequences, as did the pair of strains CBS 2675 and CBS 2778.

Fig. 3 shows the NJ tree generated using Jukes–Cantor distances and is based on a COX2 sequence alignment of 551 nucleotides (including gaps). As with the 26S rDNA D1/D2 sequence data (Fig. 2), the ten *A. telluris* strains can be subdivided into five distinct subgroups on the basis of their COX2 gene sequences. Three of these subgroups are identical to those delimited on the basis of 26S rDNA D1/D2 sequences (Fig. 2). These correspond to 26S rDNA subgroups 1 (CBS 1787T, the *C. pintolopesii* type strain, CBS 2676 and CBS 2985), 2 (CBS 2675 and CBS 2778) and 5 (CBS 2419T, the *C. slooffii* type strain). However, in contrast to the 26S rDNA D1/D2 sequence data (Fig. 2), where CBS 2685T (*A. telluris* type strain) andNCYC 2475 formed a distinct group (supported by a bootstrap value of 98%), this was not the case on the basis of the COX2 gene sequence data. As Fig. 3 shows, NCYC 2475 formed a separate lineage, which showed no close association to any other *A. telluris* strain, while CBS 2685T, along with strains CBS 2678 and CBS 2760T (*C. bovina* type strain), formed a distinct group that was statistically well supported (bootstrap value 100%). Indeed, the COX2 sequences of CBS 2685T and CBS 2760T revealed that the *A. telluris* and *C. bovina* type strains differed from one another by only six base substitutions. This contrasted with their 26S rDNA D1/D2 sequences, which were found to differ from one another by a total of 16 base substitutions, and suggested that the two strains belonged to separate taxa (Kurtzman, 1998b).

Within the individual subgroups, the *A. telluris* strains had COX2 sequences that were either identical (i.e. CBS 1787T and CBS 2676; CBS 2675 and CBS 2778; CBS 2678 and CBS 2760T) or differed by a single base substitution (i.e. CBS 1787T and CBS 2985) or, in the case of strains CBS 2685T and CBS 2760T, by six base substitutions. However, when the COX2 sequences of strains belonging to separate subgroups were compared, the levels of sequence variation were much higher and ranged from 21 (between CBS 2675 and NCYC 2475) to 58 (between CBS 2419T and CBS 2760T) base substitutions. In the majority of pairwise strain comparisons, with the notable exception of strains CBS 2685T and CBS 2760T, the level of observed COX2 sequence variation was at least twice that seen between the corresponding 26S rDNA D1/D2 sequences.

One further point to note regarding the COX2 gene sequences determined in this study related to that of NCYC 505T, the type strain of *S. cerevisiae*. When this sequence was compared with the *S. cerevisiae* COX2
sequence held in the EMBL database (accession no. V00685), the two sequences were found to differ by a total of 18 base substitutions (representing 3.0% sequence divergence). This discrepancy in sequences raised concerns regarding the identity of the S. cerevisiae strain used by Fox (1979), for which no strain number was given.

DISCUSSION

At present, the species A. telluris includes strains originally classified as C. slooffii (van Uden & do Carmo Sousa, 1957), T. bovina (= C. bovina) and T. pintolopesii (= C. pintolopesii) (Kurtzman, 1998a; Barnett et al., 2000). Both morphologically and physiologically, these species were found to be extremely similar to one another and to S. telluris (= A. telluris; van der Walt & Yarrow, 1984) and, as few carbon sources are utilized by these yeasts (Kurtzman, 1998a; Barnett et al., 2000), species differentiation was limited mainly to differences in minimum temperature for growth and the ability to form ascospores or pseudomycelium (van Uden & Buckley, 1970; van Uden & Vidal-Leira, 1970; Mendonça-Hagler & Phaff, 1975). The species names C. slooffii, T. bovina and T. pintolopesii were later reduced to synonymy with S. telluris, following the nDNA–nDNA hybridization study of Mendonça-Hagler & Phaff (1975).

In the present study, 18S rDNA sequence analysis of ten strains of A. telluris, including strains originally classified as C. bovina, C. pintolopesii and C. slooffii, revealed this species to form a distinct lineage that, apart from displaying an extremely loose association with S. servazzii, showed little phylogenetic affinity to any other species examined (which included representative members of the genera Kluyveromyces, Saccharomyces, Torulaspora and Zygossaccharomyces) (Fig. 1). Indeed, a similar result was observed when the phylogenetic analysis was expanded to include all currently accepted members of the genus Saccharomyces (Barnett et al., 2000).

This finding is perhaps not unexpected, particularly if the morphology and physiology of A. telluris are considered. Unlike members of Saccharomyces, which produce one to four smooth, spheroidal to short ellipsoidal ascospores per ascus (Vaughan-Martini & Martini, 1998; Barnett et al., 2000), A. telluris produces one to two warty (i.e. verrucose), spheroidal to ovoidal ascospores per ascus (van der Walt, 1957; van der Walt & Yarrow, 1984; Kurtzman, 1998a). In this respect, A. telluris resembles species of the genus Issatchenkia (Barnett et al., 2000). However, as Kurtzman et al. (1980) pointed out, S. telluris possesses the coenzyme Q6 system, whereas members of Issatchenkia possess the coenzyme Q7.

The results of the 18S rDNA sequence analysis (Fig. 1) clearly support the reclassification of S. telluris by van der Walt & Yarrow (1984) as A. telluris. Nevertheless, it is also evident from the phylogenetic tree shown in Fig. 1 that this species as currently described (Kurtzman, 1998a; Barnett et al., 2000) is, in fact,
composed of a number of subgroups, all possibly worthy of separate species status. However, accurate resolution of these subgroups on the basis of 18S rDNA sequences was severely hampered by the overall sequence conservation of the 18S rRNA gene, with the *A. telluris* strains examined in this study displaying at least 99-5% sequence identity to one another. In order to improve the phylogenetic resolution of these subgroups and to determine their taxonomic status, the sequence of the more variable D1/D2 domain of the 26S rRNA gene was determined for each individual strain.

As Fig. 2 shows, comparative analysis of the 26S rDNA D1/D2 sequence data readily resolved the ten *A. telluris* strains into the same five subgroups (labelled 1 to 5; Fig. 2) that were revealed initially on the basis of 18S rDNA sequences (Fig. 1). Although the two rDNA regions differentiated the *A. telluris* strains into the same subgroups, differences were observed in the overall topologies of the two trees (Figs 1 and 2). These differences are possibly due to the fact that the 18S rRNA gene is more conserved than the D1/D2 domain of the 26S rRNA gene, with the latter displaying far greater levels of sequence divergence and consequently being far more useful for studying close genealogical relationships (e.g. those seen between sibling species; Kurtzman, 1998b; Kurtzman & Blanz, 1998).

Detailed analysis of the 26S rDNA D1/D2 sequences revealed that the *A. telluris* strains within each subgroup possessed sequences that were either identical or differed by a single base substitution (i.e. displayed < 10% sequence divergence). In contrast, *A. telluris* strains belonging to separate subgroups were found to have 26S rDNA D1/D2 sequences that displayed far greater sequence variation, ranging from 12 to 27 base substitutions (i.e. 2-3-5.2% sequence divergence). Thus, on the basis of 26S rDNA D1/D2 sequence divergence, the results indicated that the *A. telluris* strains within each subgroup were conspecific, while each individual subgroup of *A. telluris* strains appeared worthy of separate species status (Kurtzman, 1998b; Kurtzman & Blanz, 1998; Kurtzman & Robnett, 1998). Interestingly, the type strains of *A. telluris* (CBS 2685T), *C. bovina* (CBS 2760T), *C. pintolopesii* (CBS 1787T) and *C. slooffii* (CBS 2419T) were found to segregate into separate subgroups (subgroups 3, 4, 1 and 5, respectively; Fig. 2), indicating that they belonged to separate species. However, these findings directly contradict those of Mendonca-Hagler & Phaff (1975), who considered these four species to be conspecific, based on the fact that representative strains of the four taxa were found to display high levels of nDNA homology (> 80%) with one another.

In an effort to address (and possibly to resolve) the taxonomic confusion arising from the conflicting rDNA sequence and nDNA-nDNA homology datasets, sequence analysis was also carried out on the mitochondrial COX2 gene. In a recent study, Belloch et al. (2000) used COX2 to study the phylogeny of the genus *Kluveromyces* and demonstrated its use as a possible alternative to rDNA sequences for inferring the genealogical relationships of yeasts.

Fig. 3 shows the resulting phylogenetic tree derived from the COX2 gene sequences. As in both the 18S and 26S rDNA-derived trees (Figs 1 and 2), the ten *A. telluris* strains could be subdivided into five subgroups on the basis of their COX2 sequences. A comparison of Figs 1 and 2 revealed that three of the COX2-derived subgroups were identical (i.e. same strain content) to those delimited on the basis of their 26S rDNA D1/D2 sequences (namely subgroups 1, 2 and 4; Fig. 2). However, in contrast to the 26S rDNA data (Fig. 2), where CBS 2685T (*A. telluris* type strain) paired with NCYC 2475 (to form subgroup 3; Fig. 2), on the basis of the COX2 sequence data, this strain (CBS 2685T) was found to be closely related to CBS 2678 and CBS 2760T (*C. bovina* type strain) and the three strains together formed a statistically significant group (bootstrap value 100%) that was quite separate from NCYC 2475.

Overall, the COX2 gene sequence data were in good agreement with the 18S and 26S rDNA sequence data and indicated that, contrary to the earlier findings of Mendonca-Hagler & Phaff (1975), both *C. pintolopesii* and *C. slooffii* should be reinstated as separate species on the basis of their unique rDNA and COX2 sequences (see below). However, with regard to CBS 2685T (*A. telluris* type strain), the rDNA and COX2 sequence data were not in agreement and failed to establish the taxonomic position of this yeast with regard to the other strains studied. One possible explanation for the discrepancy between the rDNA and COX2 sequence datasets is that CBS 2685T could represent a hybrid strain. Such a finding was recently reported by Groth et al. (1999), who studied a novel *Saccharomyces* isolate, C1D1 (= CBS 8614), recovered from cider and showed it to be a natural hybrid between three different *Saccharomyces sensu stricto* species. The combined sequence data of the present study suggest that CBS 2685T could represent a natural hybrid that arose following the mating of two strains similar to NCYC 2475 and CBS 2678 (or CBS 2760T). This possibility is supported by the fact that the nuclear-encoded 18S rDNA and 26S rDNA of CBS 2685T are similar in sequence to those of NCYC 2475 (Figs 1 and 2), while its mitochondrial COX2 gene sequence is similar to those of CBS 2678 and CBS 2760T (which possess identical COX2 gene sequences) (Fig. 3). This could suggest that a strain related to NCYC 2475 contributed part or all of the nuclear genome of CBS 2685T, while a strain closely related to CBS 2678 and CBS 2760T contributed the mitochondrial DNA molecule. However, further work on these strains (beyond the scope of the present study) needs to be carried out to explore in more detail the possibility that CBS 2685T might represent a hybrid yeast strain and, if so, to establish the origins of its nuclear and mitochondrial genomes. Such work will
undoubtedly help to resolve the genealogical relationships between these yeast strains and particularly those between CBS 2685T (A. telluris type strain), CBS 2678, CBS 2760T (C. bovina type strain) and NCYC 2475.

**Reinstatement of species**


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