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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGIAS
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OCORRÊNCIA DE LEVEDURAS EM ESPÉCIES VEGETAIS NATIVAS DA MATA ATLÂNTICA, PARQUE ESTADUAL DA SERRA DO MAR – NÚCLEO PICINGUABA, SÃO PAULO.

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Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Área de Concentração: Microbiologia Aplicada).

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RESUMO

No período de março de 1999 a fevereiro de 2002, foram coletadas 311 flores e 72 frutos de 19 espécies vegetais nativas de Mata Atlântica na região de Ubatuba, SP – núcleo Picinguaba, com o objetivo de descrever as espécies de leveduras presentes. Os locais de coleta abrangeram uma grande extensão de áreas de mata de encosta, planície, restinga e beira mar. Ainda, 75 amostras da água acumulada nos tanques de outras duas espécies vegetais foram analisadas com o mesmo objetivo. Trezentas e vinte e seis linhagens de leveduras foram isoladas, das quais, 75,8% apresentaram afinidade ascomicética e 24,2% basideomicética. O gênero *Candida* foi predominante, seguido por *Metschnikowia*, *Hanseniaspora*, *Bullera* e *Cryptococcus*. Entre os ascomicetos e seus anamorfos, 37 espécies foram identificadas, sendo que a espécie mais freqüentemente isolada em flores e frutos foi *Hanseniaspora uvarum*, com 22 e 20 isolados, respectivamente. Outras espécies que também foram isoladas com freqüência em flores foram *Bullera unica* e *Metschnikowia koreensis*. Como esperado, muitas das linhagens isoladas não se enquadraram dentro dos padrões descritos na literatura e, nesses casos, as mais freqüentes tiveram a região do domínio D1/D2 do rDNA sequenciada. Todos os oito isolados da nova espécie *Candida leandrae* foram obtidos a partir de frutos de *Leandra reversa* (Melastomataceae), sugerindo forte associação entre ambos. Das amostras da água de tanque foram isoladas cinco novas espécies. De *Canistropsis seidelii* (Bromeliaceae) foram descritas duas novas espécies: *Candida bromeliacearum* e *Candida ubatubensis*. A partir de *Heliconia velloziana* (Heliconiaceae) foram descritas *Candida heliconeae*, *Candida picinguabensis* e *Candida sanpauloensis*. Alguns isolados designados como *Candida* sp. A, B, C, D, E, F, G, H, I e *Debaryomyces* sp. A, B, C e outros, também não corresponderam às características das espécies-padrão descritas na literatura e ainda não foram seqüenciados.

ABSTRACT

From March of 1999 to February of 2002, 311 flowers and 72 fruits of 19 native plant species of the Atlantic Forest in the region of Ubatuba, SP Picinguaba area were sampled for the presence of yeasts. The sites of collection included a great extension of coastal areas like hillsides, plains, restinga and seashores. Seventy five samples of the water accumulated in the tank of two other plant species were also examined. Three hundred and twenty six yeast strains were isolated, with 75.8% being ascomycetes and 24.2% basideomycetes. The genus *Candida* was predominant, followed by *Metschnikowia*, *Hanseniaspora*, *Bullera* and *Cryptococcus*. Regarding the ascomycetous and their anamorphs, 37 species were identified, and *Hanseniaspora uvarum* was the prevalent in flowers and fruits, with 22 and 20 isolated respectively. *Bullera unica* and *Metschnikowia koreensis* were also frequently isolated from flowers. As expected, many of the strains did not fit the standard found in literature and most of them had their D1/D2 domain of rDNA sequenced. All the eight strains of new species, *Candida leandrae* were isolated from fruits of *Leandra reversa* (Melastomataceae), suggesting a strong association between them. Another five new species were isolated from tank water as follows: *Candida ubatubensis* and *Candida bromeliacearum* from *Canistropsis seidelii* (Bromeliaceae) and *Candida heliconeae*, *Candida sanpauloensis* and *Candida picinguabensis* from *Heliconia velloziana* (Heliconiaceae). Some strains previously identified as *Candida* sp. A, B, C, D, E, F, G, H, I and *Debaryomyces* sp. A, B, C and others, could not be identified as well but they were not sequenced to date.

1. INTRODUÇÃO

1.1. Leveduras: Características gerais.

O termo levedura descende do latim “levere” que significa levantar, referindo-se ao comportamento do dióxido de carbono durante a fermentação, o qual parece levantar a superfície líquida como uma espuma. Mesmo as leveduras estando intimamente ligadas aos processos fermentativos, as pessoas ignoravam a existência destes microrganismos (PHAFF et al., 1978).

Presume-se que os microrganismos façam parte da alimentação do homem desde os tempos que estes viviam apenas da coleta de frutos. O uso de leveduras pelo homem data desde A. C., como a fabricação de cerveja na Suméria, no Egito antigo onde foi encontrado um cardápio com dezesseis tipos diferentes de pães e seis tipos de vinhos assim como a fermentação de vinhos na Assíria e de pão em Roma (DAVENPORT, 1980; KURTZMAN, 1998).

O conceito de levedura foi estabelecido em 1680 com Antonie van Leeuwenhoek, o qual observou em uma gota de cerveja, numerosas células descritas como corpos globulares geralmente esféricos ou ovais. Posteriormente, Pasteur em 1835 demonstrou que as leveduras possuem habilidade respiratória e fermentativa (PHAFF et al., 1978).

O primeiro sistema de identificação foi desenvolvido por Emil C. Hansen em 1896, mas somente em 1931 Stelling-Dekker criaram o primeiro sistema de classificação completo e acessível para leveduras esporuladas. Diddens e Lodder em 1941 publicaram estudos sobre leveduras não esporuladas (PHAFF et al., 1978).

Atualmente as mais completas obras para a identificação de leveduras são as publicações de KURTZMAN e FELL (1998) que apresenta 689 espécies descritas e BARNETT, PAYNE e YARROW (2000), com 706 espécies descritas, ao lado de outros, como Yeasts of the World em 2002 e até mesmo on-line, como disponibilizado em “http://www.cbs.knaw.nl”

1.2. Distribuição e ocorrência na Natureza

As leveduras são particularmente notáveis entre os microrganismos pela sua diversidade morfológica e bioquímica. São definidas como fungos, cujo estado sexual não apresenta corpos de frutificação e o crescimento vegetativo ocorre por brotamento ou fissão. São microrganismos predominantemente unicelulares, não móveis, sendo a maioria saprófita e alguns parasitas oportunistas (MILLER, 1979; LACHANCE e STARMER, 1998).

A maioria das espécies de leveduras vive como saprófitas, mas em certas ocasiões, podem ser parasitas oportunistas (MILLER, 1979). Historicamente, as leveduras estão

associadas a processos fermentativos e substratos que contenham açúcares (hexoses). Entretanto, a habilidade das leveduras em assimilar grande número de compostos orgânicos, expande a sua capacidade de dispersão e de ocupação dos nichos ecológicos que contenham estes compostos (PHAFF e STARMER, 1980). Assim, as plantas mostram-se como excelentes habitats para uma grande quantidade de leveduras, pois, através da atividade fotossintética açúcares simples são produzidos, bem como polissacarídeos e outros compostos de carbono, incluindo algumas vitaminas, o que oferece uma gama de substratos para o crescimento destas, o que tem sido observado em vários ambientes (PHAFF e STARMER, 1980; 1987; BANNO e MIKATA, 1981; ROBBS et al., 1989; PHAFF, 1990a, b.).

Muitos dados sobre leveduras de regiões temperadas estão disponíveis na literatura, havendo importantes linhagens resultantes destes estudos. Contudo, apenas recentemente, estudos sobre leveduras em ambientes tropicais têm se tornando mais freqüentes. Alguns estudos sobre diversidade de leveduras realizados no Brasil, demonstraram que diferentes habitats como insetos, flores e frutos, apresentam comunidades de leveduras características, com biótipos diferentes, e até mesmo novas espécies (HAGLER et al., 1993; MORAIS et al., 1995; SANTOS et al., 1996).

Alguns estudos ilustram bem esta afirmativa. Assim como MORAIS (1991), em um estudo sobre a ocorrência de espécies de leveduras associadas a moscas do gênero *Drosophila* em ambientes florestais do Rio de Janeiro, observou que predominaram as leveduras apiculadas do gênero *Kloeckera* e ainda *Candida, Kluyveromyces, Debaryomyces e Hansenula.* Em ambiente degradado pode-se notar que os frutos de manga parecem ter sido o alimento mais abundante e fonte das leveduras isoladas das moscas do grupo *Drosophila melanogaster*.

ROSA et al. (1994) observaram que a levedura *Clavispora opuntia* está intimamente associada ao sítio alimentar da larva da mariposa *Silgelgaita* sp. no cacto *Pilosocereus arrabidae*, no qual esta espécie é dominante, confirmando observações de PHAFF e STARMER (1987), os quais verificaram que espécies de *Drosophila* são vetores importantes de leveduras, onde o inseto adulto e a larva necessitam de leveduras como suplemento nutritivo, acabando por disseminá-las no ambiente. ROSA et al. (2003), ainda verificou que a espécie *Starmerella meliponinorum* está fortemente associada a duas espécies de abelhas: *Tetragonisca angustula* e *Frieseomelitta varia.* A espécie *Melipona quadrifasciata* apresentou isolados relacionados com *Candida apicola*, que pertence a mesma "clade" de *Starmerella*, o que pode significar uma relação mutualistica entre as espécies estudadas.

Outros trabalhos sobre leveduras no Brasil também foram significativos como a comunidade de leveduras em goiabas (ABRANCHES et al., 2000), leveduras associadas com flores e frutos de regiões semi-áridas do nordeste brasileiro (SANTOS et al., 1996) e a sucessão de leveduras em frutos de *Parahancornia amapa* (MORAIS et al., 1995).

1.3. Ocorrência de leveduras em flores e frutos

A química dos hospedeiros, insetos vetores e interações entre microrganismos representam um importante papel no estabelecimento e distribuição das espécies de leveduras na natureza (STARMER et al., 1987; LACHANCE e STARMER, 1998). Flores e frutos são tradicionalmente considerados excelentes habitats para leveduras, representando uma sucessão efêmera de microhabitats durante o desenvolvimento e deterioração (SANTOS et al., 1996). A maioria das plantas que florescem produzem

néctar na base da corola de suas flores. Neste local a concentração de carboidratos costuma ser elevada e portanto podemos presumir a ocorrência de leveduras veiculadas aos insetos visitadores.

Os vetores, devido suas preferências por nichos específicos, promovem uma distribuição desigual das leveduras e criam barreiras para a troca genética entre espécies de diferentes substratos e microhabitats, são, no entanto, os principais responsáveis pela disseminação das leveduras de flor em flor. Os principais insetos visitadores são as abelhas, vespas, borboletas e besouros. As estimativas feitas pelos autores LAST e PRICE (1969), foram de que a ocorrência de leveduras chega a atingir índices de 10^6 células por flor ou inflorescência. Entretanto, algumas flores apresentaram baixos números de células. As leveduras podem até mesmo não ocorrer, no caso de flores que não possuem glândulas nectaríferas ou não são visitadas por insetos polinizadores.

A microbiota de flores é geralmete constituída por um grupo bem definido, formado pelos gêneros *Auerobasidium, Cryptococcus, Rhodotorula, Sporobolomyces* e *Bullera* dentre os basideomicetos e *Metschnikowia* e *Candida* dentre os ascosmicetos (PHAFF, 1990a).

Recentemente uma gama de novas espécies de leveduras tem sido descobertas em flores coletadas em diversos ambientes e climas (LACHANCE et al., 1998, 1999; ROSA e LACHANCE 1998; ROSA et al., 1999b; HONG et al., 2001), em flores e insetos associados (KURTZMAN e ROBNETT, 1998b; LACHANCE et al., 1998b; LACHANCE e BOWLES, 2002;), isoladas ainda de solo, insetos como abelhas, exudatos, vasos condutores de plantas e polpa de frutas (PÉTER et al., 1997; ROSA, et al., 1999a, 2003; MIDDELHOVEN et al., 1999, 2001; LACHANCE et al., 2001;

STARMER, et al., 2001; KURTZMAN, 2001; ZAO et al., 2002; MORAIS et al. 2004, TRINDADE et al. 2004).

Uma flor para se transformar em fruto sofre muitas mudanças fisiológicas que acabam influindo na presente população de leveduras. Por esse motivo, nem sempre as populações encontradas nas flores são as mesmas dos frutos. Segundo SKINNER et al., (1980), a microbiota natural de leveduras dos frutos é geralmente composta pelos gêneros *Rhodotorula, Sporobolomyces, Cryptococcus, Torulopsis, Candida, Pichia, Hansenula, Kloeckera, Hanseniaspora* e mais raramente por *Saccharomyces e Schizosaccharomyces.*

As espécies que primeiramente colonizam os frutos maduros ou em início de deterioração são caracterizadas pelo rápido crescimento e baixo perfil de assimilação, são geralmente apiculadas fermentadoras como *Kloeckera, Hanseniaspora* e *Saccharomycodes* (LAST e PRICE, 1969; PHAFF, 1990a, b). O processo de amadurecimento do fruto não altera somente a população de leveduras, mas também outras comunidades de microrganismos. A exaustão de nutrientes e a intolerância ao etanol pode limitar a variedade de espécies, as quais podem ser substituídas por outras mais generalistas, como é o caso das espécies apiculadas, que possuem baixa tolerância ao etanol produzido durante a fermentação (SPENCER et al., 1992; MORAIS et al., 1995; SANTOS et al., 1996; ABRANCHES et al., 2000; 2001).

PRADA e PAGNOCCA (1997) realizaram um levantamento das leveduras existentes em frutos nativos da Mata Atlântica no litoral sul paulista, especificamente na Reserva Florestal Juréia-Itatins. O trabalho mostrou que alguns gêneros predominaram, especialmente *Candida* e *Kloeckera,* os quais ocorreram em uma grande variedade de substratos. Neste trabalho também foram isoladas leveduras pretas e outras com

afinidade basidiomicética, sendo que alguns desses isolados não foram identificados segundo a metodologia disponível e utilizada naquele momento.

HAGLER et al., (1993), isolaram leveduras de água acumulada em duas espécies de bromélias, *Neoregelia cruenta* de dunas a beira mar e Q*uesnelia quesneliana* de região de mangue, próximo a cidade do Rio de Janeiro. As leveduras isoladas de *N. cruenta* foram típicas de superfície vegetal, sendo a maioria basidiomicetos ou leveduras pretas, como *Aureobasidium pullulans*, a mais freqüente. No entanto, em *Q. quesneliana* foram mais comuns os ascomicetos e seus anamorfos. Os resultados sugeriram que as comunidades microbianas em bromélias são tipicamente autóctones. Neste trabalho os autores também notaram um número considerável de linhagens não identificadas, as quais foram consideradas como prováveis novos biotipos.

Estudos como os descritos anteriormente, mostraram a evidente necessidade da continuação deste modelo de pesquisa, pelos possíveis desdobramentos representados pela descoberta de microrganismos ainda não descritos devido ao grande número de linhagens que não puderam ser identificadas pelos métodos tradicionais.

1.4. Uso de biologia molecular na taxonomia de leveduras

O uso do rDNA tem resultado em sérias mudanças na taxonomia de leveduras durante os últimos anos, conduzindo a uma classificação mais precisa. Além de facilitar a descoberta, classificação e a identificação de muitas novas espécies.

A ferramenta molecular para a sistemática de leveduras, foi inicialmente descrito por BICKNELL e DOUGLAS (1970), os quais observavam a diferença entre espécies pelo nível de reassociação na subunidade 25 do rRNA e no DNA nuclear. Duas leveduras eram consideradas pertencentes à mesma espécie quando apresentavam mais

de 80% de reassociação no DNA (PRICE et al., 1978). Este método fornece informações limitadas, adequadas apenas para discriminação entre espécies irmãs e os resultados não podem ser diretamente usados em comparações com outros taxa em estudos posteriores (KURTZMAN e PHAFF, 1987; KURTZMAN, 1992, 1994). Outros métodos, como a porcentagem de guanina e citosina (G + C % mol) presente em todo o DNA genômico e o RAPD (random amplified polymorphic DNA) também são utilizados para a identificação de leveduras, embora cada um destes métodos tenha um nível de resolução limitado (VALENTE et al, 1999).

O seqüenciamento de rDNA/rRNA está sendo amplamente utilizado por possibilitar a análise de distância e proximidade entre muitos tipos de organismos. Uma vez que está presente em todos os organismos vivos, um grande número de informações está contido em uma simples seqüência, sendo possível medir a proximidade e a distância das relações evolucionarias. Algumas regiões no DNA/RNA ribossomal são bem conservadas em todos os microrganismos, que servem como base para a análise das relações evolucionárias (KURTZMAN e BLANZ, 1998; VALENTE et al, 1999). A escolha de uma região para seqüenciamento é uma das questões mais difíceis na taxonomia molecular de leveduras, pois, é importante saber qual região do DNA escolher para resolver cada nível de relação entre os organismos. Segundo SOGIN et al. (1986), há uma preferência pelo uso do rDNA para inferências filogenéticas pois este é universal, o que permite a princípio, comparações entre qualquer organismo.

O seqüenciamento da região 25S do rDNA foi feito ao mesmo tempo do 18S, através da procura de uma região que contivesse informações suficientes para chegar mais perto das relações entre as espécies. O comprimento da porção seqüenciada da região 25S-635, é de aproximadamente 600 nucleotídeos, estabelecendo o uso da região

D1/D2 para a análise entre espécies (KURTZMAN e ROBNETT, 1997). Um extenso banco de dados sobre D1/D2 existe para leveduras ascomicéticas e basidiomicéticas, inclusive as patogênicas para humanos (BOEKHOUT et al., 1994; KURTZMAN e ROBNETT 1995, 1998a; FELL et al., 1998, 2000).

PETERSON e KURTZMAN (1991) e KURTZMAN e ROBENETT (1991, 1997, 1998a) demonstraram que a seqüência nucleotídica do domínio D1/D2 da subunidade maior do DNA ribossomal (rDNA), é suficientemente variável para identificar todas as espécies conhecidas de leveduras ascomicéticas. Em muitos casos, espécies que apresentaram mais de 1% de substituição neste domínio provaram ser espécies distintas. As seqüências do domínio D1/D2 estão disponíveis para todas as espécies conhecidas de leveduras ascomicéticas (KURTZMAN e ROBENETT 1995, 1997, 1998a) e estes dados estão disponíveis também para leveduras basidiomicéticas, embora em alguns casos seja necessário seqüênciar mais de uma região do DNA para se obter uma identificação precisa (FELL et al. 2000). Neste caso, as regiões escolhidas tem sido a ITS (Internal Transcribed Spacer) do rDNA e a região IGS (Intergenic spacer), (FELL e BLATT, 1999; SCORZETTI et al., 2002; NGUYEN et al., 2004).

1.5. Objetivos do trabalho

a) Isolar e identificar leveduras em ambiente de floresta tropical úmida na região Norte do Estado de São Paulo;

b) Descrever novas espécies de leveduras;

c) Formação de uma coleção a partir destes isolados, os quais estão sendo conservados em laboratório, viabilizando estudos posteriores quanto ao potencial de utilização em processos industriais ou biotecnológicos.

Os resultados obtidos estão apresentados sob forma de artigos preparados para publicação, cada um no formato exigido pela revista ao qual foram ou serão submetidos, denominados “Capítulos”.

No item Apêndice estão descritas outras informações relevantes que não puderam ser incluídas no corpo da tese, tais como, códigos de estocagem de todas as linhagens isoladas, listagem dos principais banco de culturas, fotos das espécies vegetais das quais foram isoladas as novas espécies e dos principais locais de coleta.

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2. CAPÍTULO 1:

Candida leandrae sp. nov., an asexual ascomycetous yeast species isolated from tropical plants.

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Candida leandrae sp. nov., an asexual ascomycetous yeast species isolated from tropical plants

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The novel yeast species *Candida leandrae* is described based on eight isolates from decaying fruits of *Leandra reversa* Cogn. (Melastomataceae) in an Atlantic rainforest site in Brazil, one from a Convolvulaceae flower in Costa Rica and one from a drosophilid in Hawai'i. The strains differed in their colony morphology, one being butyrous and smooth and the other being filamentous and rugose. Sequences of the D1/D2 domains of the large-subunit rRNA gene from both morphotypes were identical. *C. leandrae* belongs to the *Kodamaea* clade and is closely related to *Candida restingae*. The two species can be separated on the basis of growth at 37 °C and the assimilation of melezitose, negative in the novel species. The type culture of *C. leandrae* is strain UNESP 00-64R^T (=CBS 9735^T=NRRL Y-27757^T).

The *Kodamaea* clade (Rosa *et al.*, 1999; Lachance *et al.*, 1999) contains a growing number of interesting yeast species associated with plants (Lachance *et al.*, 2001), mushrooms (Nakase *et al.*, 1999) and associated insects. During a screening of yeasts associated with Atlantic rainforest fruits, an asporogenous species physiologically similar to *Candida restingae* was isolated from fruits of *Leandra reversa* Cogn. (Melastomataceae). The yeast presented two different colonial morphotypes, one being butyrous and smooth and the other being filamentous and rugose. Sequencing of the D1/D2 domains of the large-subunit rRNA gene indicated that the morphotypes are conspecific and confirmed a close relationship with *C. restingae*. In an independent study of the yeast community of Convolvulaceae flowers and associated insects in Hawai'i and Costa Rica, two additional strains with similar growth characteristics and D1/D2 sequences were recovered. We now describe the novel species as *Candida leandrae*.

Yeast isolation and characterization

C. leandrae strains were isolated independently from three localities. Eight strains were recovered from fruits of *L. reversa* (Melastomataceae). Ten fruits were collected in Picinguaba area, an Atlantic Rain Forest site at the 'Serra do Mar' State Park in São Paulo state, Brazil, during the summer of 2000. The fruits were individually blended in 2 ml sterilized distilled water and 0·1 ml of the suspension was spread on YM agar (1 % glucose, 0·5 % peptone, 0·3 % malt extract, 2 % agar) containing 100 mg chloramphenicol l^{-1} (Trindade *et al.*, 2002). Plates were incubated at 25 °C for 3–5 days. Strain UWOPS 00-612b2 was recovered from *Drosophila floricola* collected in a flower of *Ipomoea indica* (Convolvulaceae) on the Island of Oahu, Hawai'i. The fly was captured aseptically and allowed to deposit yeast cells on the agar medium. Strain UWOPS 01-664c3 was isolated from a flower of *Merremia tuberosa* (Convolvulaceae), near Dos Rios, Guanacaste Province, Costa Rica. Flower scrapings were streak-inoculated on the agar medium. The plates were kept at room temperature until colonies were sufficiently differentiated. Representative yeast colonies were purified and maintained in YM slants or in liquid nitrogen. Yeasts were characterized by standard methods (Yarrow, 1998) and their identification was attempted using the keys of Kurtzman & Fell (1998) and the CD-ROM *Yeasts of the World* (Boekhout *et al.*, 2002).

DNA sequence analysis

Yeast DNA was extracted and purified according to a protocol recommended for the Genomic Prep. Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech). The divergent D1/D2 domain (nucleotides 63–642 for *Saccharomyces cerevisiae*) at the 5′ end of the large-subunit rRNA gene was symmetrically amplified with primers

The GenBank/EMBL/DDBJ accession number for the large-subunit rRNA sequence of strain UNESP 00-64R^T is AY449659.

NL-1 and NL-4 (O'Donnell, 1993). Each PCR was performed with the Ready-To-Go kit (Amersham Pharmacia Biotech) adding 1·5 µl solution containing approximately 100 ng DNA; 1·6 µl NL-1 primer; 1·1 µl NL-4 primer (6 pmol each) and 17·8 µl Milli-Q water. The sequence products were resolved in an ABI Prism 377 DNA sequencer (Applied Biosystems) at the Center for the Study of Social Insects – UNESP, Rio Claro, São Paulo, Brazil. Alternatively, the DNA was amplified directly from whole cells and sequenced as described by Lachance *et al.* (1999). The sequences were edited and aligned using CLUSTAL W 1.4 (Thompson *et al.*, 1994) provided in the program DNAMAN 4.1 (Lynnon Biosoft, Vaudreuil, Québec, Canada), which was used also for construction of the neighbour-joining tree.

Species delineation, classification and ecology

The novel species *C. leandrae* formed two distinct colony morphotypes (Fig. 1). Six strains (UNESP 00-63L, 00-64L, 00-65L and 00-66L; UWOPS 00-612b2 and 01-664c3) formed butyrous and smooth colonies and four (UNESP 00-63R, 00-64R^T, 00-65R and 00-66R) formed rugose and membranous colonies. The colony phenotypes were stable within each strain. The strains had nearly identical physiological profiles and the sequences of their D1/D2 large-subunit rRNA gene regions were identical. Sequence analysis further demonstrated that the species is related to *C. restingae* and belongs to the *Kodamaea* clade (Fig. 2). Extensive variability in colony morphology has been noted for *Kodamaea kakaduensis* (Lachance *et al.*, 1999), *Kodamaea anthophila* and *Kodamaea nitidulidarum*, but not for *C. restingae* (Rosa *et al.*, 1999). The D1/D2 sequences of *C. leandrae* and *C. restingae* differed by nine substitutions and three gaps in 467 bases, indicating that the two taxa represent phylogenetically distinct species. The isolates were examined after growth on most common sporulation media (5 % malt extract agar, cornmeal agar, Fowell acetate agar and dilute V8 agar), but asci were not formed. Mixed pairs showed no signs of conjugation, suggesting that *C. leandrae* occurs in nature in the asexual form.

Species in the *Kodamaea* clade have been isolated mostly from flowers and associated insects (Lachance *et al.*, 2001). *Kodamaea ohmeri* has been recovered sporadically from a variety of substrates including flowers, insects or clinical specimens (Kurtzman, 1998; Rosa *et al.*, 2003). Other species in the clade are associated with flowers and insects in tropical ecosystems (Rosa *et al.*, 1999; Lachance *et al.*, 1999, 2001), or with mushrooms (Nakase *et al.*, 1999), where they are presumably vectored by fungivorous insects. *C. restingae* was isolated from cactus flowers in Brazil and from insects collected in a cactus flower in Costa Rica (Lachance *et al.*, 2001). One strain was isolated from an unidentified fruit in an Atlantic Rain Forest site in Minas Gerais, Brazil. *C. leandrae* is probably associated with insects that visit decaying fruits and flowers in tropical ecosystems. Among several fruits collected in Picinguaba area, only decaying fruits of *L. reversa* yielded *C. leandrae*, suggesting a possible association with insects that exhibit a preference for that tree species. The very low frequency of recovery in hundreds of Convolvulaceae flowers or associated insects examined in Hawai'i and Costa Rica would suggest that the flowers and their insects are not a primary habitat of the species, but demonstrate its broad geographical distribution in the neotropics.

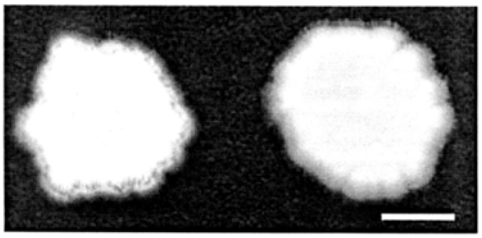

Fig. 1. Colonies of *C. leandrae* after 3 days on yeast extract/malt extract agar. (Left) Rugose colony, strain UNESP 00-64R^T, and (right) smooth colony, strain UNESP 00-64L. Bar, 0·5 mm.

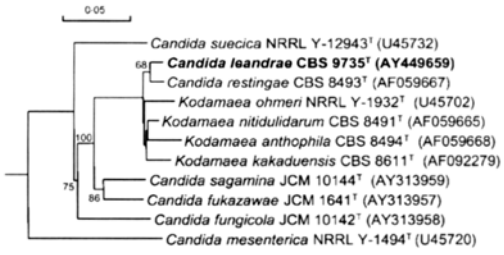

Fig. 2. Neighbour-joining phylogram based on the D1/D2 divergent domains of the large-subunit rRNA gene of *C. leandrae* and related species. Percentage bootstrap values were obtained from 1000 replicates. Only values above 50 % are shown. Bar, 5 % sequence divergence.

Identification

C. leandrae is easily distinguished from *C. restingae* based on the assimilation of inulin, growth on 50 % glucose, and the absence of growth on melezitose, at 37 °C, or in the presence of 10 % NaCl. These characters combined with the utilization of succinic acid and the lack of growth on melibiose allow separation from all other similar species. Confirmation of identity by D1/D2 sequencing is prudent.

Latin diagnosis of *Candida leandrae* Ruivo, Pagnocca, Lachance *et* Rosa sp. nov.

In medio liquido post dies tres ad 25 °C, *cellulae ellipsoideae aut elongatae, singulae aut in catenis brevis* (2–4 × 3–6 µm).

Cultura in agaro extracta malti et levidinis continente post 4 dies ad 25 °C, candida, aliquando cum lineis radiabus, glabra aut rugosa, butyrosa aut firma. In agaro farinae Zea mays post dies 14 pseudomycelium formatur. Asci non formantur. Glucosum fermentatur. Glucosum, inulinum (interdum exigue), sucrosum, raffinosum (interdum exigue), galactosum, trehalosum, maltosum, methyl α-D-glucosidium, L-sorbosum, D-xylosum (interdum lente), D-ribosum (variabile), ethanolum, glycerolum, ribitolum, xylitolum, mannitolum, glucitolum, acidum succinicum et acidum citricum (interdum exigue), acidum gluconicum (variabile), glucono-δ-lactonum, 2-ketogluconatum, N-acetylglucosaminum, et n-hexadecanum (exigue) assimilantur. Non assimilantur melibiosum, lactosum, melezitosum, amylum solubile, cellobiosum (interdum exigue), salicinum (variabile), L-rhamnosum, L-arabinosum, D-arabinosum, methanolum, erythritolum, galactitolum, L-arabinitolum, meso-inositolum, acidum glucuronicum, acidum lacticum, 5-keto gluconatum et glucosaminum (variabile). Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Ad crescentiam vitaminae externae necessariae sunt. Augmentum in 35 °C, at non 37 °C. Materia amyloidea non formantur. Crescit in agaro extrato fermenti confecto 50 partes glucosi per centum. Non crescit in medio 100 μg ml^{-1} cycloeximido addito. Ureum non finditur. Diazonium caeruleum B negativum.

Habitat fructus Leandra reversa Cogn. (Melastomataceae) *flores Convolvulacearum et insecta juncta. Typus stirps* UNESP 00-64R^{T}. *In collectione zymotica* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum, sub* no. CBS 9735^{T}, *typus stirps deposita est.*

Description of *Candida leandrae* Ruivo, Pagnocca, Lachance & Rosa sp. nov.

Candida leandrae (le.an.d'rae. N.L. gen. n. *leandrae* of *Leandra reversa*).

In yeast extract (0·5 %) glucose (2 %) broth after 3 days at 25 °C, the cells are ellipsoidal to elongate (2–4 × 3–6 μm), occur singly, in budding pairs or in short chains (Fig. 3). On YM agar after 4 days at 25 °C, the colonies are white, convex, sometimes fringed, glabrous or membranous, smooth or rugose, butyrous to tough due to filamentous growth. After 2 weeks in Dalmau plate culture on cornmeal agar, a rudimentary pseudomycelium is formed. Asci are not formed on common sporulation media. Glucose fermentation is complete after 2 days to 2 weeks. Assimilation of carbon compounds: glucose, inulin (sometimes weak), sucrose, raffinose (sometimes weak), galactose, trehalose, maltose, methyl α-D-glucoside, L-sorbose, D-xylose (sometimes slow), D-ribose (variable), ethanol, glycerol, ribitol, xylitol, D-mannitol, D-glucitol, succinic

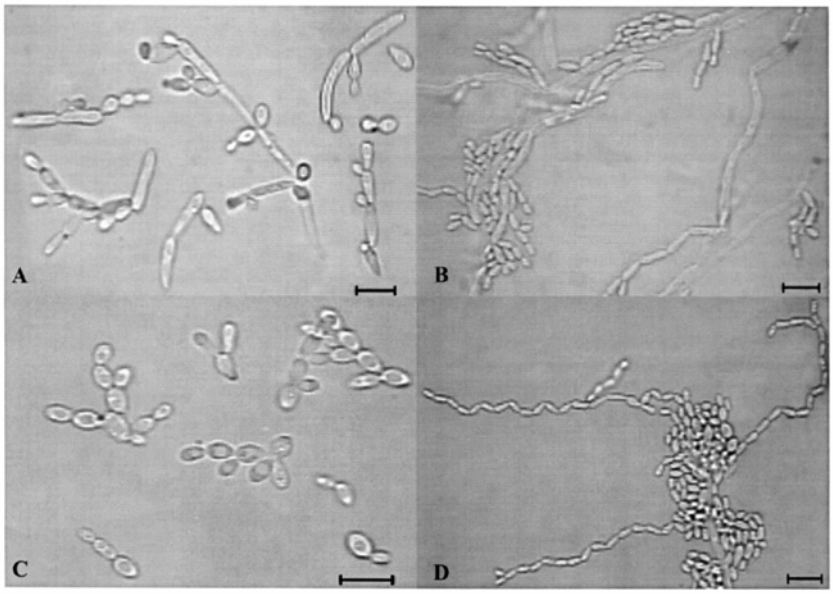

Fig. 3. Photomicrographs of cells of *C. leandrae* strain UNESP 00-64R^{T} (rugose colony) after (A) 3 days on 5 % malt extract at 25 °C and (B) 2 weeks on cornmeal agar at 25 °C. Cells of *C. leandrae* strain UNESP 00-64L (smooth colony) after (C) 3 days on 5 % malt extract at 25 °C and (D) 2 weeks on cornmeal agar at 25 °C. Bars, 10 μm.

acid, citric acid (sometimes weak), D-gluconic acid (variable), glucono-δ-lactone, 2-ketogluconic acid, *N*-acetyl-D-glucosamine and hexadecane (sometimes weak) are assimilated; no growth occurs on melibiose, lactose, melezitose, starch, cellobiose (occasionally weak), salicin (variable), L-rhamnose, L-arabinose, D-arabinose, methanol, erythritol, L-arabinitol, galactitol, *myo*-inositol, D-glucuronic acid, DL-lactic acid, 5-ketogluconic acid or D-glucosamine (variable). Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth in vitamin-free medium is negative. Growth in amino acid-free medium is positive. Growth at 35 °C is positive and at 37 °C negative. Acid formation on chalk agar is slow, weak or absent. Urease activity and diazonium blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50 % glucose/yeast extract agar is positive. Growth on YM agar with 5 % NaCl is positive; 10 % NaCl negative. Growth in the presence of 0·01 % cycloheximide is negative. Growth in the presence of 1 % of acetic acid is negative.

The type culture is strain UNESP 00-64R^T. It was isolated from a decaying fruit of *L. reversa* in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 9735^T (=NRRL Y-27757^T).

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3. CAPÍTULO 2:

***Candida bromeliacearum* sp. nov. and *Candida ubatubensis* sp. nov., two yeast species isolated from the water tanks of *Canistropsis seidelii* (Bromeliaceae).**

Texto editado nas normas da revista: **International Journal of Systematic and Evolutionary Microbiology** - enviado para publicação.

Abstract

Two new yeasts species, *Candida bromeliacearum* and *Candida ubatubensis*, were isolated from the bromeliad tank of *Canistropsis seidelii* (Bromeliaceae) in a sandy coastal plain (restinga) ecosystem site at an Atlantic rainforest of southeastern Brazil. These species were genetically isolated from all other currently accepted ascomycetous yeasts based on their sequence divergence in the D1/D2 domain of the large subunit rDNA and in the small subunit rDNA. The species occupy basal positions in the Metschnikowiaceae. The type strains are *Candida bromeliacearum* UNESP 00-103^T (=CBS 10002^T = NRRL Y-27811^T) and *Candida ubatubensis* UNESP 01-247R^T (= CBS 10003^T = NRRL Y-27812^T).

The GeneBank/EMBL/DDBJ accession numbers for the large-subunit rRNA sequences of strains UNESP 00-103^T - SSU: AY695396, D1D2: AY695394 and UNESP 01-247R^T - SSU: AY695397, D1D2: AY695395.

Key Words: bromeliad water tank, new yeast species, tropical plant, phytotelmata

Introduction

The Bromeliaceae constitutes a large plant family that is endemic to the neotropics and exhibits a high species richness in southeastern Brazil. The plants are mostly epiphytes that form phytotelms or water tanks. The water accumulated in the tanks of some species may function as a microhabitat for aquatic organisms including plants, animals, and microorganisms (Hagler et al. 1993; Araújo et al., 1998). These waters have a high nutrient concentration, creating an adequate environment for the development of a complex microbial community (Benzing, 1972). During a survey of yeasts associated with plants in tropical ecosystems of the southeastern Brazil, two new yeast species were isolated from the water tanks of the bromeliad *Canistropsis seidelii.* The sequences of the D1/D2 domains of the large subunit rDNA showed that these species are genetically distinct from all currently accepted ascomycetous yeasts. The novel yeast species *Candida bromeliacearum* and *Candida ubatubensis* are described herein.

Yeast isolation and characterization

Three strains each of *C. bromeliacearum* and *C. ubatubensis* were isolated from water tanks of the bromeliad *Canistropis seidelii* in the Picinguaba site in the "Serra do Mar" State Park, São Paulo State, Brazil (23º22' S and 44º48' W). This State Park contains one of the largest continuous areas of the remaining Brazilian Atlantic Forest in eastern São Paulo State and is located 230 kilometres from the city of São Paulo. For the isolation of *C. bromeliacearum* strains, the water tanks of six plants were collected during spring (September) 2000, whereas the strains of *C. ubatubensis* were collected

during summer (February) 2001, at which time water reservoirs of five plants were examined. The plants occurred along a large area in the forest.

Before collection, the water was stirred with a sterile loop and streaked in triplicate, onto YM agar (1% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2% agar) containing 100 mg 1^{-1} chloramphenicol (Trindade *et al*., 2002). The plates were incubated at 25ºC for 3-5 days, and one of each distinct morphotype were isolated and stored on YM slants at 6-8°C and in a -80ºC freezer. The yeasts were characterized by standard methods (Yarrow, 1998) and the identifications were carried out using the keys of Kurtzman & Fell (1998) and the computer program Yeasts of the World (Boekhout *et al*., 2002).

DNA sequence analysis

Yeast DNA was extracted and purified according to a protocol recommended for the Genomic Prep. Cells and Tissue DNA isolation Kit (Amersham Pharmacia Biotech Inc.). The divergent D1/D2 domain of the large subunit rDNA gene was amplified with NL1 and NL4 primers (O'Donnell, 1993). Each PCR reaction was performed with the Ready-To-Go™ Kit (Amersham Pharmacia Biotech Inc) using 1.5 μl solution containing approximately 100ng DNA; 1.6 μl NL1 primer; 1.1 μl NL4 primer (6 pmol each) and 17.8 μl MilliQ water. The sequence products were resolved in an ABI Prism® 377 DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) at the Centro de Estudos de Insetos Sociais – UNESP, Rio Claro, São Paulo. Alternatively, the DNA was amplified directly from whole cells and sequenced as described by Lachance *et al*. (1999). The small subunit ribosomal DNA was amplified by this method, using primers SSU1f (3'-CTG GTT GAT CCT GCC AGT AGT CAT A-3') and SSU2r (5'-

ATG ATC CTT CCG CAG GTT CAC-3'). The amplification products were purified on Qiagen columns and sequenced using the same primers as for amplification plus the primers SSU3f (5'-TGG AGG GCA AGT CTG GTG CCA-3') and SSU4r AAC TAA GAA CGG CCA TGC ACC A-3'). Sequencing was performed with an ABI automated sequencer at the Robarts Research Institute, London, Ontario. Sequence alignment and tree construction were done with the program DNAMAN 4.1 (Lynnon Biosoft, Vaudreuil, Québec).

Species delineation, classification and ecology

The physiological characteristics of the new species are typical of those of other species in the Metschnikowiaceae clade, although the absence of growth on galactose and the weak utilization of trehalose observed in *C. bromeliacearum* are not common. The isolates of *C. bromeliacearum* and *C. ubatubensis* were examined after growth on most common sporulation media (5% malt extract agar, corn meal agar, Fowell acetate agar and dilute V8 agar), but asci were not formed. Mixed pairs showed no signs of conjugation, suggesting that these species occur in nature in the asexual form.

Assignment of the isolates to new species was based principally on DNA sequence analyses. The D1/D2 LSU rDNA sequences (514 and 509 bases respectively for *C. ubatubensis* and *C. bromeliacearum*) of both species were so divergent that a credible placement near any particular species in the Metschnikowia clade was not possible. For this reason, the SSU rDNA sequences (1698 and 1662 bases respectively for *C. ubatubensis* and *C. bromeliacearum*) were determined and added to the analysis (Fig. 1). Trees obtained separately based on the two DNA regions were mostly but not entirely congruent. Removal of gapped regions did not alter the trees significantly.

Although *C. bromeliacearum* is shown as a sister species to *C. haemulonii*, the species are positioned at the ends of relatively long internodes and joined together by a much shorter internode. As the SSU sequence for "*C. haemulonii* type II", an undescribed variant of the species, was not available, the variant was not included in the tree. However, an analysis of the D1/D2 region showed that *C. bromeliacearum* is more or less equidistant to the two sisters species currently assigned to *C. haemulonii.*

One can therefore suspect that the addition of yet undiscovered taxa to the analysis may well cause a different branching order to appear in spite of the high bootstrap values. As for *C. ubatubensis,* a specific placement within the clade would be purely speculative.

Because all isolates of these species were obtained from the same site, it is not possible to assume that they are directly associated with the bromeliad water tank, which possesses a dynamic influx of a diversity of arthropod and amphibian visitors. As only three isolates of *C. bromeliacearum* and three of *C. ubatubensis* were recovered from a collection that comprises 29 isolates, the role of these species in the bromeliad yeast community remains obscure. In a similar approach Hagler et al. (1993) and Araujo et al. (1998) described the yeast communities of the water tanks of the bromeliad species *Quesnelia quesneliana, Q. arvensis, Neoregelia cruenta, Nidularium procedurum, Aechmea nudicaulis* and *Vriesia procera* occurring in mangrove and sand dune ecosystems. The yeast communities found in those works and in our study are quite different as they only share *C. famata* and *C. intermedia,* two widespread, generalistic species.

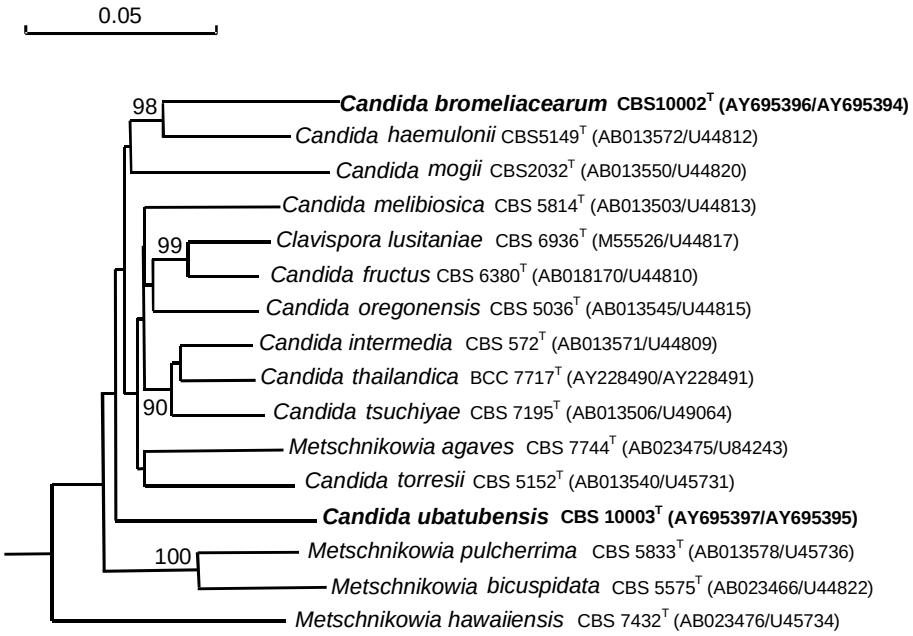

Fig. 1. Neighbour-joining phylogram showing the position of *Candida bromeliacearum* and C*andida ubatubensis* among related species. The tree was constructed from concatenated sequences of the small subunit rDNA and the D1/D2 region of the large subunit rDNA. The species included were chosen to represent the various subclades of the Metschnikowiaceae clade, subject to availability. Bootstrap values from 1000 pseudoreplicates are shown for values above 70% only. The tree was rooted by inclusion of the sequences for *Schizosaccharomyces pombe* as an outgroup (not shown).

Latin diagnosis of *Candida bromeliacearum* Ruivo, Pagnocca, Lachance et Rosa sp. nov.

In medio liquido post dies tres ad 25ºC, cellulae ellipsoideae aut elongatae, singulae aut in catenis brevis (3-5 x 4-6 μm). Cultura in agaro extracta malti et levidinis continente post dies 4 ad 25ºC, albida cremae et butyrosa. In agaro farinae *Zea mays* post dies 14 mycelium nec pseudomycelium non formantur. Asci non formantur. Glucosum fermentatur. Glucosum, sucrosum, trehalosum (exigue), maltosum, melezitosum, methyl α-D-glucosidium, cellobiosum, salicinum, L-sorbosum, D-

xylosum, ethanolum, glycerolum (variabile), erythritolum (variabile), ribitolum, xylitolum, mannitolum, glucitolum, acidum citricum (exigue et variabile), acidum gluconicum (variabile), glucono-δ-lactonum, 2-ketogluconatum, D-glucosaminum, N-acetylglucosaminum, et n-hexadecanum (lente) assimilantur. Non assimilantur inulinum, raffinosum, melibiosum, galactosum, lactosum, amylum solubile, L-rhamnosum, L-arabinosum, D-arabinosum, D-ribosum, methanolum, galactitolum, meso-inositolum, acidum lacticum, acidum succinicum, 5-keto gluconatum, L-arabinitolum nec acidum glucuronicum. Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35ºC, non crescit in 37°C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Crescit (lente) in agaro extrato fermenti confecto 50 partes glucosi per centum. Non crescit in medio 100 μm mL^{-1} cycloeximido addito. Habitat aquam in *Canistropsis seidelii* (L.B. SM.) Leme. (Bromeliaceae). Typus stirps UNESP 00-103^{T}. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 10002, typus stirps deposita est.

Description of *Candida bromeliacearum* Ruivo, Pagnocca, Lachance & Rosa sp. nov.

Candida bromeliacearum (bro.me.lia.ce.a'rum. N.L. gen. n. *bromeliacearum* of Bromeliaceae, referring to the plant from which the yeast was isolated).

In yeast extract (0.5%) glucose (2%) broth after 3 days at 25ºC, the cells are ellipsoidal to elongate (3-5 x 4-6 μm), occur singly, in budding pair, or in short chains. Buds are produced multilaterally (fig. 2A). On YM agar after 4 days at 25ºC, the colonies are white to cream, smooth and butyrous. After 2 weeks in Dalmau plate

culture on corn meal agar, pseudomycelium or true mycelium are not formed. Asci are not formed on common sporulation media. Glucose fermentation is complete after 2-5 days. Assimilation of carbon compounds: glucose, sucrose, trehalose (weak), maltose, melezitose, α-methyl D-glucoside, cellobiose, salicin, L-sorbose, D-xylose, ethanol, glycerol (variable), erythritol (variable), ribitol, xylitol, D-mannitol, D-glucitol, citric acid (weak and variable), D-gluconate (variable), glucono-δ-lactone, 2-ketogluconic acid, glucosamine, *N*-acetylglucosamine, and hexadecane (slow) are assimilated. No growth occurs on inulin, raffinose, melibiose, galactose, lactose, starch, L-rhamnose, L-arabinose, D-arabinose, D-ribose, methanol, galactitol, *meso*-inositol, lactic acid, succinic acid, 5-ketogluconic acid, L-arabinitol, saccharate, and D-glucuronate. The following nitrogen compounds are assimilated: Lysine, and ethylamine, cadaverine; nitrate and nitrite are negative. Growth at 35ºC is positive and negative at 37°C. Acid formation on chalk agar is positive. Urease activity and Diazonium Blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50% glucose-yeast extract agar is slow. Growth on YM agar with 10% NaCl is positive. Growth in the presence of 1% of acetic acid is negative. Growth in the presence of 0.1% and 0.01 % cycloheximide is negative. The type strain, UNESP 00-103^T, has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 10002^T (= NRRL Y-27811^T).

Latin diagnosis of *Candida ubatubensis* Ruivo, Pagnocca, Lachance et Rosa sp. nov.

In medio liquido post dies tres ad 25ºC, cellulae globosae aut ovoidae, singulae aut binae (3-4,5 x 4-6 μm). Cultura in agaro extracta malti et levidinis continente post 4

dies ad 25ºC, albida cremae et butyrosa. In agaro farinae *Zea mays* post dies 14 mycelium nec pseudomycelium non formantur. Asci non formantur. Glucosum fermentatur. Glucosum, galactosum, sucrosum, trehalosum, maltosum, melezitosum, methyl α-D-glucosidium, cellobiosum, salicinum, L-sorbosum, L-rhamnosum (exigue), D-xylosum, D-ribosum, ethanolum, glycerolum, erythritolum, ribitolum, xylitolum, L-arabinitolum, mannitolum, glucitolum, acidum gluconicum, glucono-δ-lactonum (variabile), N-acetylglucosaminum et n-hexadecanum assimilantur. Non assimilantur inulinum, raffinosum, melibiosum, lactosum, amylum solubile, L-arabinosum, D-arabinosum, methanolum, galactitolum, meso-inositolum, acidum lacticum, acidum succinicum, acidum citricum, 2-ketogluconatum, 5-keto gluconatum nec D-glucosaminum. Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35ºC, non crescit in 37°C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Non crescit in agaro extrato fermenti confecto 50 partes glucosi per centum. Non crescit in medio 100 μm mL^{-1} cycloeximido addito. Habitat aqua in *Canistropsis seidelii* (Bromeliaceae). Typus stirps UNESP 01-247R^T. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 10003, typus stirps deposita est.

Description of *Candida ubatubensis* Ruivo, Pagnocca, Lachance & Rosa sp. nov.

Candida ubatubensis (u.ba.tu.ben'sis N.L. gen. fem. adj. *ubatubensis* of Ubatuba, referring to a town near which the yeast was isolated.

In yeast extract (0.5%) glucose (2%) broth after 3 days at 25ºC, the cells are spheroidal to ovoid (3-4,5 x 4-6 μm), occur singly or in budding pair. Buds are produced multilaterally (fig. 2B). On YM agar after 4 days at 25ºC, the colonies are white to cream, smooth and butyrous. After 2 weeks in Dalmau plate culture on corn meal agar, pseudomycelium or true mycelium are not formed. Asci are not formed on common sporulation media. Glucose fermentation is complete after 2-5 days. Assimilation of carbon compounds: glucose, galactose, sucrose, trehalose, maltose, melezitose, α-methyl D-glucoside, cellobiose, salicin, L-sorbose, L-rhamnose (weak), D-xylose, D-ribose, ethanol, glycerol, erythritol, ribitol, xylitol, L-arabinitol, D-mannitol, D-glucitol, D-gluconate, glucono-δ-lactone (variable), N-acetylglucosamine, and hexadecane are assimilated. No growth occurs on inulin, raffinose, melibiose, lactose, starch, L-arabinose, D-arabinose, methanol, galactitol, *meso*-inositol, lactic acid, succinic acid, citric acid, 2-ketogluconic acid, 5-ketogluconic acid, and glucosamine. The following nitrogen compounds are assimilated: lysine, ethylamine, and cadaverine; nitrate and nitrite are negative. Growth at 35ºC is positive and negative at 37°C. Urease activity and Diazonium Blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50% glucose-yeast extract agar is negative. Growth on YM agar with 10% NaCl is positive. Growth in the presence of 1% of acetic acid is negative. Growth in the presence of 0.1% and 0.01 % cycloheximide is negative.

The type strain, UNESP 01-247R^{T}, has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 10003^{T} (= NRRL Y-27812^{T}).

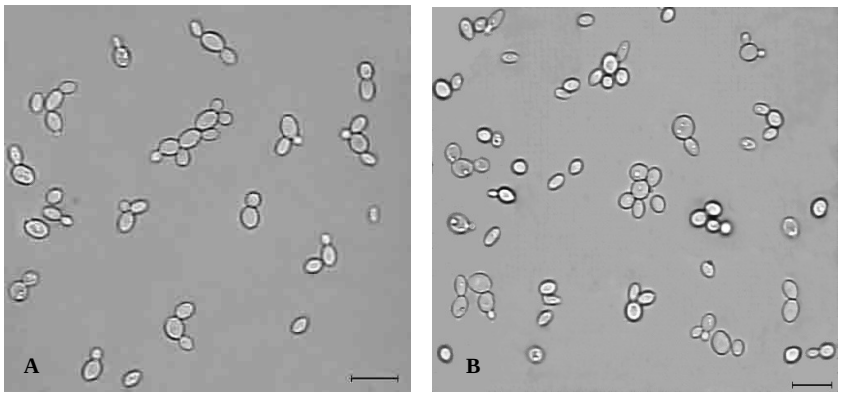

Fig. 2. Photomicrographs of cells of *Candida bromeliacearum* strain UNESP 00-103^T (**A**), and *Candida ubatubensis* strain UNESP 01-247R^T (**B**), In yeast extract, glucose broth after 3 days at 25ºC. Bars, 10 μm.

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4. CAPÍTULO 3:

Candida heliconiae, Candida picinguabensis and *Candida sanpauloensis*, three new ascomycetous yeasts isolated from *Heliconia velloziana* (Heliconiaceae).

Texto editado nas normas da revista **International Journal of Systematic and Evolutionary Microbiology** à qual será enviado para publicação.

Abstract

Four strains of *Candida heliconiae,* three of *Candida picinguabensis* and two of *Candida sanpauloensis* were isolated in 2000 from the flower bract water of *Heliconia velloziana* L. Emigd. (Heliconiaceae), found in a restinga ecosystem site at an Atlantic rainforest site of southeastern Brazil. Based in morphological and physiological tests, the strains *C. picinguabensis* and *C. sanpauloensis* are closely related each other but the sequencing of the D1/D2 domain of the large-subunit rDNA showed that they differ by 18 bases substitutions and 2 gaps. However *C. heliconiae* species are determined to be genetically isolated from all other currently accepted ascomycetous yeasts based on their sequence divergence in the species-variable D1/D2 domain of the 26S.

The GeneBank/EMBL/DDBJ accession numbers for the large-subunit rRNA sequences of strains: UNESP 00-91C1^{T} - AY566406, UNESP 00-89^{T} - AY566407 e UNESP 00-99 AY695398

Palavras chave: *Candida heliconiae, Candida picinguabae, Candida sanpauloensis, Heliconia velloziana*.

Introduction

The family Heliconiaceae has a single genus, *Heliconia* L. with 250 and so species dispersed in Neotropical areas since the North of Mexico through the South of Brazil (Santos, 1978; Dahlgren et al., 1985; Kress, 1990). A small Paleotropical group, with eight species occurs in islands of the South Pacific (Tomlinson, 1969; Kress, 1985). Approximately 40 Brazilian species are known occurring mostly at the Amazon basin and at the Atlantic coastal forest (Kress, 1990). *H. velloziana* is an endemic species from the Atlantic forest and occurs from the southeast through the South of Brazil (Mello-Filho, 1975; Santos, 1978; Citadini-Zanette & Baptista, 1989). A characteristic of the genus *Heliconia* is the rapidly decaying floral parts enclosed by the massive, still living bracts. The nectar in the bracts is throught to be responsible by the development of communities of yeasts and bacteria (Schnittler & Stephenson, 2002). In this paper, we describe the occurrence of the new yeasts species, *C. heliconiae, C. picinguabensis* and *C. sanpauloensis* in *H. velloziana*.

Yeast isolation and characterization

Nine yeast strains being four of *C. heliconiae,* three of *C. picinguabensis* and two of *C. sanpauloensis* were formerly isolated from flower bract water of *Heliconia velloziana,* collected in Picinguaba area, an Atlantic Rain Forest site at the "Serra do Mar" State Park in São Paulo state, Brazil. All the tree species were isolated during the spring (September) 2000, sampled from the flower bract water of 14 plants. The water accumulated in the bract was stirred with sterile loop before streaked in triplicate onto YM agar (1% glucose, 0.5% peptone, 0.3% malt extract, 2% agar) containing 100 mg l^{-1} chloramphenicol (Trindade *et al*., 2002). Plates were incubated at 25ºC for 3-5 days.

Selected representative colonies were pecked up and maintained in YM slants and at –80ºC. Yeasts were characterized by standard methods (Yarrow, 1998) and their identification was carried out using the keys of Kurtzman & Fell (1998) and the computer program Yeasts of the World (Boekhout *et al*., 2002).

DNA sequence analysis

Yeast DNA was extracted and purified according to a protocol recommended for the Genomic Prep. Cells and Tissue DNA isolation Kit (Amersham Pharmacia Biotech Inc.). The divergent D1/D2 domain (nucleotides 63-642 for *Saccharomyces cerevisiae*) at the 5´ end of the large subunit rDNA gene was symmetrically amplified with primers NL-1 and NL-4 (O'Donnell, 1993). Each PCR reaction was performed with the Ready-To-Go™ Kit (Amersham Pharmacia Biotech Inc) following the manufacturer's recommendations."

The sequence products were resolved in an ABI Prisma 377 DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) at the Centro de Estudos de Insetos Sociais – UNESP, Rio Claro, São Paulo. Alternatively, the DNA was amplified directly from whole cells and sequenced as described by Lachance et al. (1999). Sequence alignment and tree construction were done with the program DNAMAN 4.1 (Lynnon Biosoft, Vaudreuil, Québec).

Species delineation, classification and ecology

All strains were examined after growth on common sporulation media, alone or in pair-wise mixtures. Conjugation or ascus formation was not observed.

In the absence of a sexual cycle, species delineation relied on genetic divergence. Analysis of the large subunit rDNA D1/D2 domains, *Candida picinguabensis* and *Candida sanpauloensis* are sister species with an affinity with the *Metschnikowia* clade. Both strains differed each other by 18 substitutions and 3 gaps, which supports treating them as separate species (Kurtzman & Robnett 1998). The species included in Fig. 1 (A) are representatives of neighbouring clades, chosen to identify the approximate phylogenetic position of the new species. A reliable connection with any known species within the Metschnikowiaceae could not be identified. *Candida heliconiae* has no identifiable sister species and occupies a basal position in a clade that contains *Pichia nakazawae*, *Pichia mexicana*, and related *Candida* species. The species in Fig. 1 (B) were selected to represent neighbouring clades and assist in localizing *Candida heliconiae* phylogenetically. Despite this three new yeasts species have been isolated from water accumulated in bracts of *Heliconia velloziana* it is not possible to conclude that they are only associated with this plant, because the *Heliconia* species possesses a dynamic influx of hummingbirds, attracted by the flower's nectar (Stiles, 1975). Indeed, it has also been isolated from this species some strains of *Candida azyma*, *Candida restigae* and *Metschnikowia koreensis*, which are usually found in flowers (Lachance, 1989; Rosa, 1999; Hong, 2001). Since *C. picinguabensis* and *C. sanpauloensis* presents similar phisiology and morphology, is expected that they will just be found in similar microhabitats.

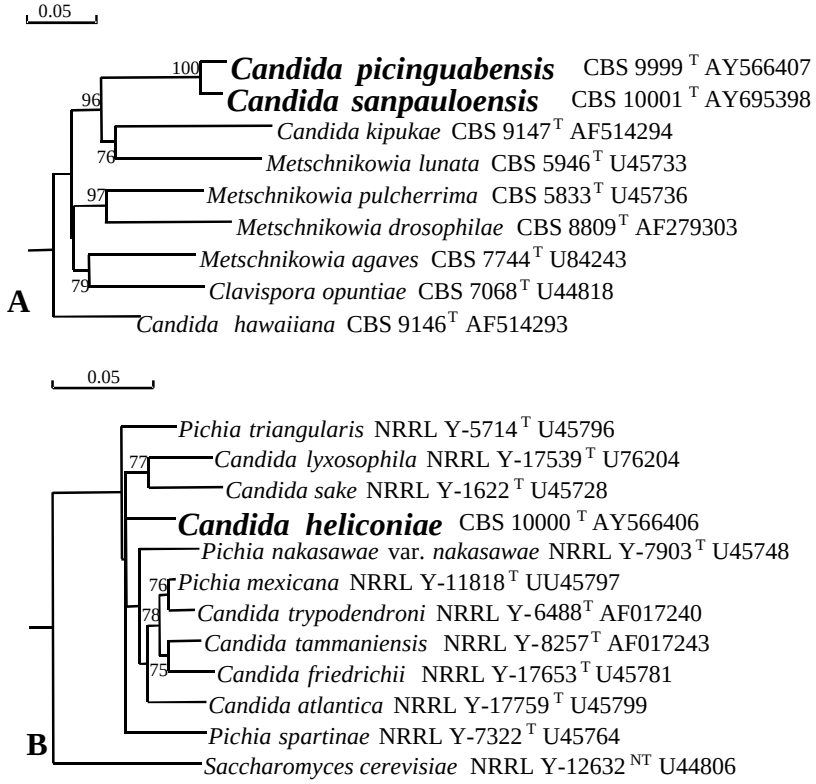

Fig 1. Neighbour-joining dendrograms depicting the approximate phylogenetic position of *Candida picinguabensis*, *Candida sanpauloensis* (A), and *Candida heliconiae* (B). Bootstrap values determined from 1000 iterations are shown. The scale bar represents 5% sequence divergence. Root placement is based on the inclusion of *Schizosaccharomyces pombe* (NRRL Y-12796 U40085, not shown) as outgroup.

Latin diagnosis of *Candida heliconiae* Ruivo, Pagnocca, Lachance et Rosa sp. nov.

In medio liquido post dies tres ad 25ºC, cellulae globosae aut ovoidae, singulae aut binae (3-5.5 x 3-5 μm). Cultura in agaro extracta malti et levidinis continente post 4 dies ad 25ºC, albida cremae et butyrosa. In agaro farinae *Zea mays* post dies 14 pseudomycelium non formatur. Asci non formantur. Glucosum fermentatur. Glucosum, galactosum, L-sorbosum, sucrosum, maltosum, cellobiosum, melezitosum, D-xylosum, L-arabinosum (variabile), D-arabinosum, D-glucosaminum (variabile), N-acetylglucosaminum, ethanolum, glycerolum, ribitolum, mannitolum, glucitolum,

methyl α-D-glucosidium, acidum gluconicum (lente), salicinum (exigue), et glucono-δ-lactonum assimilantur. Non assililantur trehalosum, lactosum, melibiosum, raffinosum, inulinum, amylum solubile, D-ribosum, L-rhamnosum, methanolum, erythritolum, galactitolum, acidum lacticum, acidum succinicum, acidum citricum, meso-inositolum, N-hexadecanum (lente), 2-ketogluconatum, 5-keto gluconatum et xylitolum. Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35ºC, at non 37°C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Non crescit agaro extrato fermenti confecto 50 partes glucosi per centum. Crescit in medio 100 μL^{-1} cycloeximido addito. Habitat aqua et *Heliconia velloziana* L. Emigd. (Heliconiaceae). Typus stirps UNESP 00-91C1^{T}. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 10000, typus stirps deposita est.

Description of *Candida heliconiae* Ruivo, Pagnocca, Lachance & Rosa sp. nov.

In yeast extract (0.5%) glucose (2%) broth after 3 days at 25°C, the cells occur singly or in budding pair. The cels are spheroidal to ovoid (3-5.5 x 3-5 μm). Buds are produced multilaterally (fig. 2A). On YW agar after 4 days at 25°C cream-coloured or white, low convex, smooth and butyrous. After 2 weeks in Dalmau plate culture on corn meal agar pseudomycelium or true mycelium are not formed. Glucose fermentation is complete after 2–5 days. Assimilation of carbon compounds: glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, melezitose, D-xylose, L-arabinose (variable), D-arabinose, D-glucosamine (variable), N-acetil-D-glucosamine, ethanol, glycerol, ribitol, mannitol, glucitol, methyl-α-D-glucoside, D-gluconic acid (slow), salicin (weak), and

glucono-δ-lactone are assimilated. No growth occurs on trehalose, lactose, melibiose, raffinose, inulin, starch, D- ribose, L-rhamnose, methanol, erythritol, galactitol, DL-lactic acid, succinic acid, citric acid, *myo*-inositol, hexadecane, 2-keto-D-gluconate, 5-keto-D-gluconate or xylitol. Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth at 35°C is positive and at 37°C is negative. Acid formation on chalk agar is weak or absent. Urease activity and diazonium blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50% glucose/yeast extract agar is negative. Growth on YM agar with 10% NaCl is negative. Growth in the presence of 100 µg cycloheximide mL^{-1} is positive. Growth in the presence of 1% of acetic acid is negative.

The type culture is strain UNESP 00-91C1^T. It was isolated from water accumulated on flower bract of *Heliconia velloziana* in Brazil. It has been deposited in the collection of the Yeast Division of Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 10000^T (= NRRL Y-27813^T).

Latin diagnosis of *Candida picinguabensis* Ruivo, Pagnocca, Lachance et Rosa sp. nov.

In medio liquido post dies tres ad 25ºC, cellulae globosae, singulae aut binae (3-7 x 4-8 µm). Cultura in agaro extracta malti et levidinis continente post 4 dies ad 25ºC, albida cremae et butyrosa. In agaro farinae *Zea mays* post dies 14 pseudomycelium non formatur. Asci non formantur. Glucosum fermentatur. Glucosum, L-sorbosum, sucrosum, maltosum, trehalosum, melezitosum, D-xylosum, ethanolum, ribitolum, mannitolum, glucitolum, methyl α-D-glucosidium, acidum gluconicum (variabile), acidum lacticum (lente), acidum succinicum (exigue), acidum citricum (exigue), N-

hexadecanum (lente), glucono-δ-lactonum, 2-ketogluconatum et xylitolum. assimilantur. Non assililantur galactosum, cellobiosum, lactosum, melibiosum, raffinosum, inulinum, amylum solubile, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum, N-acetylglucosaminum, methanolum, glycerolum, erythritolum, galactitolum, salicinum, meso-inositolum et 5-keto gluconatum. Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35ºC, at non 37°C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Crescit agaro extrato fermenti confecto 50 partes glucosi per centum et lente. Crescit in medio 10 μm mL^{-1} cycloeximido addito, at non 100 μm mL^{-1} cycloeximido addito. Habitat aqua et *Heliconia velloziana* L. Emigd. (Heliconiaceae). Typus stirps UNESP 00-89^{T}. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 9999, typus stirps deposita est.

Description of *Candida picinguabensis* Ruivo, Pagnocca, Lachance & Rosa sp. nov.

In yeast extract (0.5%) glucose (2%) broth after 3 days at 25°C, the cells occur singly or in budding pair. The cels are spheroidal (3-7 x 4-8 μm). Buds are produced multilaterally (fig. 2B). On YW agar after 4 days at 25°C cream-colored or white, low convex, smooth and butyrous. After 2 weeks in Dalmau plate culture on corn meal agar pseudomycelium or true mycelium are not formed. Glucose fermentation is complete after 2–5 days. Assimilation of carbon compounds: glucose, L-sorbose, sucrose, maltose, trehalose, melezitose, D-xylose, ethanol, ribitol, mannitol, glucitol, methyl-α-D-glucoside, D-gluconic acid (variable), DL-lactic acid (slow), succinic acid (weak),

citric acid (weak), hexadecane (slow), glucono-δ-lactone, 2-keto-D-gluconate and xylitol are assimilated. No growth occurs on galactose, cellobiose, lactose, melibiose, raffinose, inulin, starch, L-arabinose, D-arabinose, D- ribose, L-rhamnose, D-glucosamine, N-acetil-D-glucosamine, methanol, glycerol, erythritol, galactitol, salicin, *myo*-inositol or 5-keto-D-gluconate. Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth at 35°C is positive and at 37°C is negative. Acid formation on chalk agar is positive. Urease activity and diazonium blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50% glucose/yeast extract agar is slow. Growth on YM agar with 10% NaCl is negative. Growth in the presence of 10 µg cycloheximide ml^{-1} is positive and in 100 µg cycloheximide ml^{-1} is negative. Growth in the presence of 1% of acetic acid is negative.

The type culture is strain UNESP 00-89^T. It was isolated from water accumulated on flower bract of *Heliconia velloziana* in Brazil. It has been deposited in the collection of the Yeast Division of Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 9999^T (= NRRL Y-27814^T).

Latin diagnosis of *Candida sanpauloensis* Ruivo, Pagnocca, Lachance et Rosa sp. nov.

In medio liquido post dies tres ad 25ºC, cellulae globosae, singulae aut binae (3-6.5 x 4-7 µm). Buds are produced multilaterally. Cultura in agaro extracta malti et levidinis continente post 4 dies ad 25ºC, albida cremae et butyrosa. In agaro farinae *Zea mays* post dies 14 pseudomycelium non formatur. Asci non formantur. Glucosum fermentatur. Glucosum, galactosum, L-sorbosum, sucrosum, maltosum, trehalosum,

melezitosum, D-xylosum, ethanolum, ribitolum, mannitolum, glucitolum, methyl α-D-glucosidium, acidum gluconicum, acidum lacticum, acidum succinicum (exigue), acidum citricum (exigue), N-hexadecanum, glucono-δ-lactonum, 2-ketogluconatum et xylitolum. assimilantur. Non assililantur cellobiosum, lactosum, melibiosum, raffinosum, inulinum, amylum solubile, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum, N-acetylglucosaminum, methanolum, glycerolum, erythritolum, galactitolum, salicinum, meso-inositolum et 5-keto gluconatum. Lysinum, ethylaminum et cadaverinum assimilantur at non natrium nitrosum nec natrium nitricum. Augmentum in 35ºC, at non 37°C. Ureum non finditur. Diazonium caeruleum B negativum. Materia amyloidea non formantur. Crescit agaro extrato fermenti confecto 50 partes glucosi per centum raro exigue. Non crescit in medio 100 μm mL^{-1} cycloeximido addito. Habitat aqua et *Heliconia velloziana* L. Emigd. (Heliconiaceae). Typus stirps UNESP 00-99^{T}. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 10001, typus stirps deposita est.

Description of *Candida sanpauloensis* Ruivo, Pagnocca, Lachance & Rosa sp. nov.

In yeast extract (0.5%) glucose (2%) broth after 3 days at 25°C, the cells occur singly or in budding pair. The cels are spheroidal (3-7 x 4-8 μm). Buds are produced multilaterally (fig. 2C). On YW agar after 4 days at 25°C cream-colored or white, low convex, smooth and butyrous. After 2 weeks in Dalmau plate culture on corn meal agar pseudomycelium or true mycelium are not formed. Glucose fermentation is complete after 2–5 days. Assimilation of carbon compounds: glucose, galactose, L-sorbose, sucrose, maltose, trehalose, melezitose, D-xylose, ethanol, ribitol, mannitol, glucitol,

methyl-α-D-glucoside, D-gluconic acid, DL-lactic acid, succinic acid (weak), citric acid (weak), hexadecane, glucono-δ-lactone, 2-keto-D-gluconate and xylitol are assimilated. No growth occurs on cellobiose, lactose, melibiose, raffinose, inulin, starch, L-arabinose, D-arabinose, D- ribose, L-rhamnose, D-glucosamine, N-acetil-D-glucosamine, methanol, glycerol, erythritol, galactitol, salicin, *myo*-inositol or 5-keto-D-gluconate. Assimilation of nitrogen compounds: L-lysine, ethylamine and cadaverine are positive; nitrate and nitrite are negative. Growth at 35°C is positive and at 37°C is negative. Acid formation on chalk agar is positive. Urease activity and diazonium blue B reaction are negative. Production of amyloid compounds is negative. Growth on 50% glucose/yeast extract agar is weak or absent. Growth on YM agar with 10% NaCl is negative. Growth in the presence of 100 μg cycloheximide ml^{-1} is negative. Growth in the presence of 1% of acetic acid is negative.

The type culture is strain UNESP 00-99^T. It was isolated from water accumulated on flower bract of *Heliconia velloziana* in Brazil. It has been deposited in the collection of the Yeast Division of Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 10001^T (= NRRL Y-27815^T).

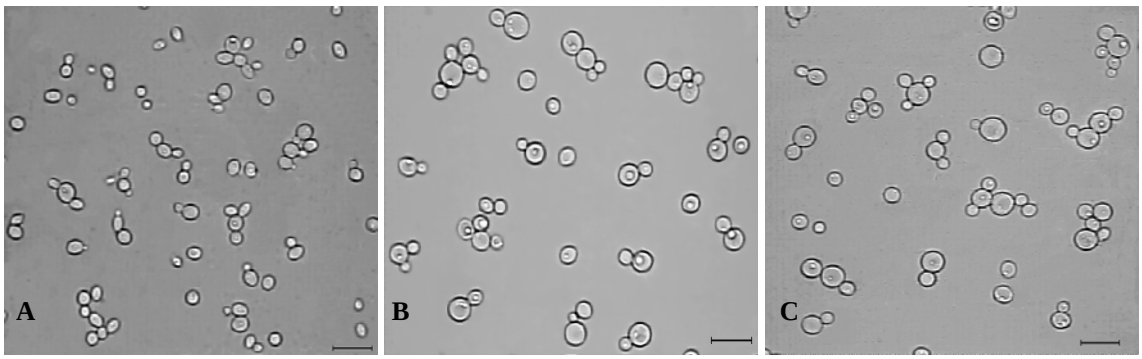

Fig. 2. Photomicrographs of cells of *Candida heliconiae* strain UNESP 00-$91C1^T$ (**A**), *Candida picinguabensis* strain UNESP 00-89^T (**B**) and *Candida sanpauloensis* (**C**) UNESP 00-99^T. In yeast extract-glucose broth after 3 days at 25ºC. Bars, 10 μm.

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5. CAPÍTULO 4:

Comunidades de Leveduras em Flores e Frutos de Espécies Vegetais Nativas de Mata Atlântica da Região Sudeste do Brasil.

Texto editado nas normas da revista **Microbial Ecology**, à qual será enviado para publicação.

Resumo

Trezentas e onze flores, setenta e dois frutos foram coletados de 19 espécies vegetais, ainda, amostras de água acumulada em outras duas espécies foram analisadas no período de março de 1999 a fevereiro de 2002, em áreas de restinga, planície, mata de encosta e beira mar.Trezentas e vinte e seis linhagens de leveduras foram isoladas, das quais, 75,8% apresentaram afinidade ascomicética e 24,2% basideomicéticas. O gênero *Candida* foi predominante, seguido por *Metschnikowia*, *Hanseniaspora*, *Bullera* e *Cryptococcus*. O gênero *Candida* apresentou o maior número de espécies identificadas nos diferentes substratos. Entre os ascomicetos e seus anamorfos, 37 espécies diferentes foram identificadas, sendo que *Hanseniaspora uvarum* foi predominante, com 22 isolados de flores e 20 de frutos. Alguns biotipos designados como *Candida* sp. A, B, C, D, E, F, G, H, I e *Debaryomyces* sp. A, B, C, entre outros, não corresponderam às características das espécies-padrão descritas na literatura. Dentre esse grupo, foram encontradas 6 novas espécies originárias de frutos de *Leandra reversa* (*Candida leandrae),* água de tanque de *Canistropsis seidelii* (*Candida ubatubensis e Candida bromeliacearum)* e água acumulada com néctar nas brácteas florais de *Heliconia velloziana* (*Candida heliconiae*, *Candida picinguabensis e Candida sanpauloensis).* Esses resultados confirmam a riqueza da biodiversidade microbiana em áreas tropicais, cujas matas nativas foram preservadas.

Introdução

Comunidades de leveduras têm sido descritas em vários tipos de substratos vegetais constituindo microhabitats como: tecidos em decomposição, flores, frutos, exudatos, néctares, frutos, túneis de insetos e água acumulada em brácteas e folhas (10, 17, 30, 31, 33-35). As flores e frutos são considerados excelentes habitats para leveduras devido à concentração elevada de açúcares, oferecendo uma sucessão de microhabitats durante o desenvolvimento e deterioração. Diversos trabalhos sobre comunidades de leveduras demonstraram que estes microrganismos fazem parte da microbiota natural de flores e frutos onde espécies pertencentes aos gêneros *Candida, Cryptococcus, Hanseniaspora, Aureobasidium, Rhodotorula, Pichia, Kloeckera* e *Metschnikowia* são comumente encontradas (28, 31, 35, 38). A colonização desses microhabitats ocorre devido à intensa visitação por insetos que agem como vetores de disseminação das espécies de leveduras (30, 40). A constituição química do hospedeiro, vetores de disseminação e interações microrganismo-microrganismo são fatores importantes para o estabelecimento e distribuição das espécies de leveduras no ambiente (16, 40).

O Parque Estadual da Serra do Mar – Núcleo Picinguaba (São Paulo, Brasil) é uma das poucas regiões remanescentes de Mata Atlântica do país quase intocada. Sendo considerada uma das formações florestais mais importantes do mundo, a Mata Atlântica contém uma grande variedade de organismos endêmicos, muitos ainda desconhecidos, o que torna importante os estudos sobre levantamento da diversidade tanto da macrobiota quanto da microbiota.

Este estudo teve como objetivo catalogar as linhagens de leveduras presentes junto à várias espécies vegetais que ocorrem neste local. As linhagens isoladas foram caracterizadas, identificadas e incorporadas à coleção de culturas do Centro de Estudos de Insetos Sociais (CEIS) da Universidade Estadual Paulista – UNESP, Campus de Rio Claro – SP.

Material e Métodos

Área estudada

No litoral norte do estado de São Paulo, o Parque Estadual da Serra do Mar - Núcleo Picinguaba (23° 31' a 23° 34' LAT S - 45° 02' a 45° 05' LONG W), abrange uma área de aproximadamente 47.000 ha, totalmente inserido no município de Ubatuba, fronteira com o Estado do Rio de Janeiro. Na região da Mata Atlântica, são encontrados diversos ecossistemas como as florestas tropicais pluviais de encosta e de planície, campo de altitude, costão rochoso, mata de encosta, restinga, manguezal e praia arenosa (11, 23, 24, 36).

As amostras foram coletadas no período de março de 1999 a fevereiro de 2002, ao longo das trilhas 'Estrada da Casa da Farinha', 'Trilha do Corisco' (regiões de planície e mata de encosta), 'Picadão da Barra' e 'Praia da Fazenda' (regiões de restinga e beira mar).

Processamento das amostras

Os frutos e flores foram colhidos e colocados em sacos plásticos estéreis, sendo processados imediatamente após a coleta. No caso das espécies que acumulam água nas

brácteas (*Heliconia velloziana*) e entre as folhas (*Canistropsis seidelii*), as amostras foram colhidas em triplicata e a semeadura em placa foi realizado imediatamente, mergulhando a alça diretamente na água. Para o isolamento a partir das flores, o nectário foi gentilmente raspado com uma alça de inoculação e esta estriada em meio YMA contendo 1% glicose, 0.5% peptona, 0.3% extrato de malte, 0.3% extrato de levedura, 2% ágar, acidificado para o pH 3.7 – 4.0 com HCl (12, 15) e 100 mg/L de cloranfenicol para inibir contaminantes bacterianos (1, 44).

Os frutos, normalmente pequenos, foram colocados inteiros em tubos de ensaio e triturados com um bastão de vidro em 2.0 mL de água destilada esterilizada e 0.1mL da suspensão resultante foi semeada em meio YMA.

As placas foram incubadas a 25ºC, por um período de 3 a 5 dias. As colônias morfologicamente distintas de cada placa, foram purificadas e estocadas a -80ºC e em meio "GYMP" (9) contendo 2% glicose; 0.5% extrato de levedura; 1% extrato de malte; 0.2% NaH_2PO_3; 2% ágar.

Identificação dos isolados

A identificação das linhagens foi realizada através de testes fisiológicos e morfológicos padrões conforme descritos por Yarrow (1998), Barnett et al. (2000) e Kurtzman & Fell (1998). Algumas linhagens não puderam ser identificadas pelos métodos tradicionais, sendo submetidas ao seqüenciamento do domínio D1/D2 do rRNA subunidade 26S, região esta que tem sido usada com sucesso para a identificação molecular de leveduras (6, 13, 18-20). O processo de extração e purificação do DNA foi realizado conforme o protocolo Genomic Prep. Cells and Tissue DNA isolation Kit (Amersham Pharmacia Biotech Inc.). Para a amplificação por PCR foi adotado o

protocolo descrito por Lachance et al., 2000, utilizando os seguintes primers: “forward” - NL1 (5’ GCATATCAATAAGCGGAGGAAAAG) e o “reverse” - NL4 (5’ GGTCCGTGTTTCAAGACGG) (25). A seqüência da região D1/D2 (400 a 600 pares de bases) foi obtida com o ABI Prism® 377 DNA sequencer (Applied Biosystems Inc., Foster City, Calif.). As seqüências obtidas foram comparadas com aquelas disponíveis no banco de dados do GenBank e submetidas a um alinhamento através do aplicativo ClustalW 1.4 (43). A análise filogenética foi realizada pelo método de máxima parcimônia, utilizando o programa PAUP* 4.0b4a (42) e os resultados comparados àqueles obtidos por “maximum likelihood”. O suporte dos ramos das árvores obtidas foi analisado por “Bootstrap” (7) e também pelo índice de Bremer (3), calculado com o programa TreeRoot 2.0 (39).

Resultados e Discussão

Foram analisados um total de 72 frutos, 311 flores e 75 amostras de água de tanque de 21 espécies vegetais, resultando em 326 linhagens de leveduras isoladas.

Os 311 exemplares de flores foram obtidos de 13 espécies vegetais diferentes, resultando em 193 linhagens isoladas, distribuidas em 25 espécies de ascomicetos e 14 de basídeomicetos (tabela 1).

Os setenta e dois exemplares de frutos foram obtidos de 6 espécies vegetais diferentes dos quais foram isoladas 77 linhagens de leveduras, havendo entre elas 9 espécies de ascomicetos e 10 de basídeomicetos.

Duas espécies vegetais que armazenam água, tanto entre as folhas (bromélia) quanto em brácteas florais (helicônia) também foram analisadas, resultando em 56

linhagens, distribuídas em 18 espécies, todas com afinidade ascomicética. Em estudo semelhante, Hagler et al., (1993) obtiveram vários isolados, além de ascomicetos, diversas espécies de basídeomicetos, especialmente de *Cryptococcus* e *Rhodotorula.*

Em relação as flores e frutos, os ascomicetos e seus anamorfos representaram 70,7% dos isolados contra apenas 29,3% de basideomicetos. Esta predominância de espécies ascomicéticas já foi determinada em flores e frutos por outros autores (17, 31, 35, 37). Em diversos estudos anteriores (5, 32, 35, 37) a espécie *Aureobasidium pullulans,* muitas vezes referida como "black-yeast" ou "yeast-like", foi freqüentemente encontrada, mas no presente estudo nenhuma linhagem semelhante foi isolada.

O gênero *Candida* apresentou o maior número de linhagens isoladas (34.6%), seguido por *Metschnikowia* (19.3%), *Hanseniaspora* (19.3%), *Bullera* (9.8%), *Cryptococcus* (7%), *Pseudozyma* e *Debaryomyces* (3,6%). Alguns biotipos, com apenas um isolado, designados como *Candida* sp. A, B, C, D, F, G, H, *Debaryomyces* sp. A, B, C e outros que representaram (6.7%), não corresponderam às descrições das espécies padrão encontradas na literatura. O seqüenciamento de DNA foi realizado em linhagens com mais de um represntante, por este motivo, os biotipos citados acima ainda não foram submetidos a tal análise.

O gênero *Candida* apresentou um grande número de espécies em flores (n = 12), frutos (n = 4) e água de tanque (n = 12), de maneira similar ao que foi encontrado em outros estudos semelhantes em outros ecosistemas (10, 31, 35, 37).

Dentre o total de espécies isoladas, as seguintes foram as 10 mais representativas, apresentadas em ordem decrescente de freqüência: *Hanseniaspora uvarum, Bullera unica, Metschnikowia koreensis, Metschnikowia bicuspidata, Metschnikowia continentalis, Candida intermedia, Candida silvae, Candida restingae,*

Candida azyma e *Candida leandrae*. Essas espécies corresponderam a aproximadamente 56% (n = 170) do total de linhagens identificadas ao nível de espécie (n = 303).

Várias espécies (aproximadamente 53 %), foram encontradas associadas a apenas uma espécie vegetal. Entretanto, como o número de amostras de cada planta foi variável e às vezes muito reduzido, não podemos concluir, que as leveduras delas isoladas apresentem uma relação específica com uma planta em particular.

A espécie *Hanseniaspora uvarum* foi a mais freqüente, tanto em flores quanto em frutos, confirmando se tratar de um microrganismo generalista, pois ela e seu anamorfo *Kloeckera apiculata* já foram encontrados em ambientes diversos como solo, água, salmoura de pepino, moluscos bivalvos, caranguejos, camarões, uvas, banana, cacau, processos fermentativos, moscas de frutas e flores (2, 4, 14, 21, 22, 26, 31, 35, 41). Embora seja raro, já houve registro desta espécie em fezes humanas (8).

Dentre os basideomicetos, o gênero *Bullera* foi mais freqüente em flores do que em frutos, tendo as espécies *B. unica* (n = 23) e *B. sinensis* (n = 6) predominado. Este fato reforça a tese de Phaff et al. (1978), os quais associaram este gênero à microbiota das flores.

Metschnikowia é um gênero comumente encontrado em flores e frutos (27), a qual foi muito freqüente neste estudo, sendo *M. koreensis* a espécie de maior ocorrência (n = 20). As espécies *M. bicuspidata* e *M. continentalis*, esta última já relacionada anteriormente com flores (15), foram significativamente isoladas tanto em flores quanto em frutos.

Tabela 1: Leveduras isoladas de flores, frutos e água armazenada em brácteas e entre folhas (água de tanque) de diferentes espécies vegetais de Mata Atlântica.

Espécies isoladas	Flores													Frutos						Água de tanque		Total
	Co n20	Hc n43	Hm n20	Hp n18	Ipc n28	Jac n49	Juc n14	Mf n10	Pn n20	Pp n28	St n28	Tc n30	Tp n12	Ca n6	Ee n16	Gs n12	Lr n10	P n22	S n6	Cs n11	Hv n14	
Bullera coprosmaensis *		1									1											2
Bullera oryzae																1						1
Bullera sinensis								2			1					2			1			6
Bullera unica						10					9	1		1		2						23
Candida apis												1										1
Candida azyma					3	2			1	1	1										2	10
Candida boidini																					1	1
Candida bromeliacearum **																				3		3
Candida drosophilae *						4			1			1										6
Candida ernobii										1												1
Candida etchellsii						1																1
Candida famata																		2		3		5
Candida fermentati						2																2
Candida heliconiae **																					4	4
Candida intermedia *		4	6				2													2		14
Candida leandrae **																	8					8
Candida magnífica *										3												3
Candida natalensis *															4							4
Candida parapsilosis		1																				1
Candida picinguabensis **																					3	3
Candida pseudointermedia *																					2	2
Candida restingae *								5		4											2	11
Candida sanpauloensis **																					2	2
Candida silvae *		2																		9	1	12
Candida ubatubensis **																				3		3
Candida vini															1							1
Candida ssp. A, B		2																				2
Candida sp. C						1																1
Candida sp. D							1															1
Candida sp. E													1									1
Candida sp. F																		1				1
Candida spp. G, H																				2		2
Cryptococcus aerius						1										4						5
Cryptococcus albidus																2						2
Cryptococcus arrabidensis						3								2		1						6
Cryptococcus flavus *						2																2
Cryptococcus hungaricus																2						2
Cryptococcus laurentii						3										1						4
Cryptococcus sp A																1						1
Cryptococcus sp B																		1				1
Debaryomyces etchellsii																					1	1
Debaryomyces hansenii	2	2							2													6
Debaryomyces vanrijiae					1						1											2
Debaryomyces sp. A																		1				1
Debaryomyces spp. B, C																					2	2
Hanseniaspora uvarum *		17				1				1	2	1					1	19			1	43
Kluyveromyces sp. A		1																				1
Kluyveromyces sp. B																					1	1
Kodamaea kakaduensis *				3					3												1	7
Kodamaea ohmeri													4									4
Metschnikowia bicuspidata					5							3	1		4				2			15
Metschnikowia colocasiae *						2						3										5
Metschnikowia continentalis		1	1		3										1	1		3		4		14
Metschnikowia koreensis *										4	11							3			2	20
Metschnikowia rekaufii *	6																				1	7
Metschnikowia sp. A																				1		1
Metschnikowia sp. B																					1	1

Continua

Continuação da tabela 1:

Espécies isoladas	Flores													Frutos						Água de tanque		Total
	Co n20	Hc n43	Hm n20	Hp n18	Ipc n28	Jac n49	Juc n14	Mf n10	Pn n20	Pp n28	St n28	Tc n30	Tp n12	Ca n6	Ee n16	Gs n12	Lr n10	P n22	S n6	Cs n11	Hv n14	
															1							*1*
Pichia sp. A									1													1
Pichia sp. B																				1		1
Pseudozyma antarctica					2																	2
Pseudozyma prolifica					4		1	1														6
Pseudozyma rugulosa						1					3											4
Pseudozyma sp											1											1
Rhodotorula fujisanensis																1						1
Rhodotorula glutinis													*1*									*1*
Rhodotorula mucilaginosa						2			1		1											4
Sporidiobolus ruineniae					1									1		1			1			1
Sporobolomyces rubescens												1										4
Wicherhamiella occidentalis											1											1
Wickerhamiella cacticola					2																	2
Wickerhamiella domercqiae			1																			1
Wickerhamiella sp																				1		1
Williopsis spp. A, B											2											2
Total de isolados	8	31	8	3	21	35	4	8	9	14	34	11	7	4	11	19	9	30	4	29	27	326

Flores: **Co** = *Couepia ovalifolia* (Schott) Benth. (Chrysobalanaceae), **Hc** = *Hedychium coronarium* Koen. (Zengiberaceae), **Hm** = *Herpetacanthus macahensis* Nees (Acanthaceae), **Hp** = *Hibiscus pernambucensis* Arruda (Malvaceae), **Ipc** = *Ipomoeae pes-caprae* (L.) Sweet. (Convolvulaceae), **Jac** = *Jacquemontia ciliata* Sandwith. (Convolvulaceae), **Juc** = *Justicia carnea* Lindl. (Acanthaceae). Mf = *Mandevilla funiformes* Vell. (Apocinaceae), Pn = *Psychotria nuda* (Cham. & Schltdl.) Wawra. (Rubiaceae), Pp = *Psychotria pubigera* Schlecht. (Rubiaceae), St = *Sophora tomentosa* L. (Fabaceae), Tc = *Tabebuia cassinoides* (Lam.) DC. (Bignoniaceae), Tp = *Tibouchina pulchra* Cogn. (Melastomataceae). **Frutos**: Ca = *Chiococca alba* (L.) Hitchc. (Rubiaceae), Ee = *Euterpe edulis* Mart. (Arecaceae), Gs = *Gomidesia schaueriana* O. Berg (Myrtaceae), Lr = *Leandra reversa* (DC.) Cogn. (Melastomataceae), P = *Paulinia* sp Gled. (Sapindaceae), S = *Smilax* sp L. (Smilacaceae). **Água de tanque:** Cs = *Canistropsis seidelii* (LB.Smith & Reitz) Leme. (Bromeliaceae), Hv = *Heliconia velloziana* L. Emygd. (Heliconiaceae). **n** = quantidade amostrada. **Quadros em cinza:** representam a ocorrência da mesma espécie de levedura em mais de um substrato (flor e fruto e água de tanque). *** =** espécies que tiveram a identidade confirmada por seqüenciamento de DNA. ** = espécies novas encontradas durante esta pesquisa.

Enquanto que as espécies com afinidade basideomicética *B. unica*, *B. sinensis*, *C. albidus*, *C. arrabidensis* e *C. laurentii* ocorreram em flores e frutos, as espécies ascomicéticas *C. azyma*, *C. intermedia*, *C. restingae* e *C. silvae*, além de estarem presentes nas flores, ainda foram encontradas na água de tanque. Contudo, *M. continentalis, M. koreensis* e um exemplar de *H. uvarum* foram as únicas espécies a ocorrer em todos os três substratos. De acordo com Skinner et al. (1980), os gêneros identificados neste estudo são comuns em flores e frutos.

As espécies marcadas com um asterisco (*) foram submetidas ao seqüenciamento de DNA para confirmar a identificação. Com isso algumas tiveram suas identidades confirmadas, como foram os casos de: *Candida drosophilae, Candida*

intermedia, *Kodamaea kakaduensis* e *Metschnikowia rekaufii*, enquanto outras tiveram suas identidades modificadas de *Hanseniaspora guilliermondii* e *Kloeckera lindneri* para *Hanseniaspora uvarum* e 6 novas espécies foram encontradas. Essas novas espécies são: *C. leandrae*, *C. bromeliacearum*, *C. picinguabensis*, *C. heliconiae*, *C. sanpauloensis* e *C. ubatubensis*, cujas descrições encontram-se nos capítulos ateriores.

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6. CONCLUSÕES

A partir das flores e frutos analisados, foram isoladas 270 linhagens de leveduras, sendo 70,7% de ascomicetos e 29,3% de basideomicetos. O estudo da água contida em tanques axilares de bromélias e em brácteas florais de helicônias, embora pouco explorada em pesquisas semelhantes, resultou no isolamento de 56 linhagens de leveduras ascomicéticas, além disso, 5 das 6 novas espécies descritas nos capítulos anteriores foram obtidas a partir desse material.

A maioria das linhagens isoladas foram dos gêneros: *Candida, Metschnikowia* e *Hanseniaspora*, seguidos por *Bullera*, *Cryptococcus*, *Pseudozyma* e *Debaryomyces*. O gênero *Candida*, destacou-se entre os ascomicetos por ter apresentado o maior número de espécies identificadas e pela grande variedade de substrados em que foram encontrados.

A descrição de 6 novas espécies de leveduras, apenas neste trabalho, bem como a identificação de outras 33 já conhecidas, confirmam o fato da Mata Atlântica ser

considerada um ecosistema com uma grande diversidade, não somente animal e vegetal, como também microbiana.

Os resultados evidenciaram a riqueza da microbiota existente junto às nossas matas, mostrando que o inventário das espécies microbianas brasileiras ainda é muito incipiente, carecendo de maiores investimentos não somente para o desenvolvimento de projetos temáticos e sistematizados de inventariamento, como também para a formação de recursos humanos especializados que possam melhor explorar o potencial que elas representam.

7. APÊNDICE

Apêndice 1: Outro trabalho publicado durante o período de realização do curso de Doutoramento em Microbiologia Aplicada no Instituto de Biociências da UNESP.

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Sympodiomyces attinorum sp. nov., a yeast species associated with nests of the leaf-cutting ant *Atta sexdens*

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Four strains of a novel yeast species were isolated from laboratory nests of the leaf-cutting ant *Atta sexdens* in Brazil. Three strains were found in older sponges and one was in a waste deposit in the ant nests. Sequencing of the D1/D2 region of the large-subunit rRNA gene showed that the novel species, named *Sympodiomyces attinorum* sp. nov., is phylogenetically related to *Sympodiomyces parvus*. Unlike *Sympodiomyces parvus*, *Sympodiomyces attinorum* can ferment glucose, assimilate methyl α-D-glucoside, salicin and citrate, and grow at 37 °C, thus enabling these two species to be distinguished. Differentiation from other related species is possible on the basis of other growth characteristics. The type strain of *Sympodiomyces attinorum* is UNESP-S156^T (=CBS 9734^T=NRRL Y-27639^T).

Yeasts are frequently isolated from nests of the leaf-cutting ant *Atta sexdens*. This ant uses plant materials to cultivate a symbiotic filamentous fungus, which is the main food for the broods. The yeasts grow in association with the fungus garden, which consists of mycelium growing on the plant material carried to the nests by the ants. Yeasts are also found in waste deposits, the material discharged by the ants (Carreiro *et al.*, 1997). The role of the yeasts in the symbiosis is not clear, but the ability of many yeast strains to degrade some plant polysaccharides may contribute to the availability of carbon sources for the symbiotic filamentous fungus (Carreiro, 2000).

During a survey of yeasts associated with laboratory nests of *A. sexdens*, Carreiro *et al.* (1997) found several yeasts, among which the dominant species were identified physiologically as *Candida homilentoma*, *Debaryomyces hansenii* and *Torulaspora delbrueckii*. However, sequence analysis of the D1/D2 domain of the large-subunit rRNA gene showed that most of the isolates identified as *Debaryomyces hansenii* and *Torulaspora delbrueckii* were, in fact, representatives of *Pichia guilliermondii*. Three strains previously identified as *Torulaspora delbrueckii*-like and one identified as *Pichia mexicana*-like (Carreiro *et al.*, 1997) were found to represent a novel species related to *Sympodiomyces parvus*. In this paper, the novel species *Sympodiomyces attinorum* sp. nov. is described.

Strains UNESP-S47, UNESP-S49 and UNESP-S156^T were isolated from the fungal garden (older sponge) and strain UNESP-S78 was recovered in a waste deposit; all four strains were isolated from laboratory nests of *A. sexdens* maintained at the Centro de Estudos de Insetos Sociais (CEIS), UNESP, Rio Claro, São Paulo, Brazil, as described by Carreiro *et al.* (1997). The yeasts were characterized by standard methods (Yarrow, 1998).

DNA templates were prepared as described by De Barros Lopes *et al.* (1996) and the PCR conditions followed were those given by Pataro *et al.* (2000). Primer EI1 (5′-CTGGC-TTGGTGTATGT-3′) is complementary to intron splicing sites found in mutable regions of the *Saccharomyces* genome (De Barros Lopes *et al.*, 1996). PCR products were analysed by 1% agarose gel electrophoresis. Authentic strains of *Debaryomyces hansenii*, *Pichia guilliermondii* and *Torulaspora delbrueckii* were used for comparison.

The D1 and D2 variable domains of the large-subunit rRNA

The GenBank/EMBL/DDBJ accession number for the large-subunit rRNA gene sequence of strain UNESP-S156^T is AY442294.

gene of a strain of *Sympodiomyces attinorum* were amplified by PCR from whole cells as described previously (Lachance *et al.*, 1999). Amplified DNA was concentrated and cleaned on QIAquick PCR columns (Qiagen) and sequenced in an ABI sequencer at the John P. Robarts Research Institute, London, Ontario, Canada. The D1/D2 regions of large-subunit rRNA genes of *Debaryomyces hansenii* UNESP-F46, *Pichia guilliermondii* UNESP-S170 and *Torulaspora delbrueckii* UFMG-B40.2 were sequenced in an ABI Prism 377 DNA sequencer at the Laboratory of Microbiology, CEIS. Sequences were edited with the program DNAMAN version 4.0 (Lynnon BioSoft). Existing sequences for other yeasts were retrieved from GenBank. The CLUSTAL_W (Thompson *et al.*, 1994) algorithm provided in the DNAMAN package was used to align sequences and construct a neighbour-joining tree with 1000 bootstrap iterations. The sequences were edited and aligned with CLUSTAL_W (Thompson *et al.*, 1994) and compared with existing sequences for other yeasts retrieved from GenBank.

Three yeast strains isolated from laboratory leaf-cutting ant nests were originally identified physiologically as *Torulaspora delbrueckii*-like (UNESP-S47, UNESP-S49, UNESP-S78) and one was identified as *Pichia mexicana*-like (UNESP-S156^T) by Carreiro *et al.* (1997). Characterization by PCR fingerprinting with primer EI1 showed that these strains were similar to one another, but different from *Debaryomyces hansenii*, *Pichia guilliermondii* and *Torulaspora delbrueckii* (Fig. 1). De Barros Lopes *et al.* (1998) showed that primer EI1 can be used to differentiate yeast species. The PCR method is simple and can be used for rapid species differentiation in large numbers of yeast strains before sequencing.

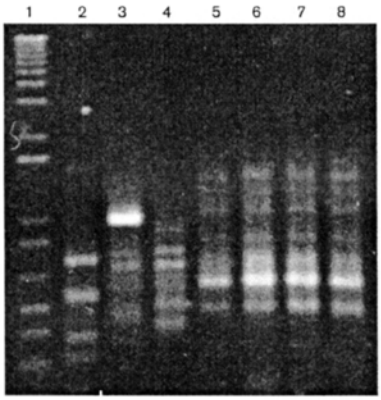

Fig. 1. PCR fingerprints of *Debaryomyces hansenii*, *Pichia guilliermondii*, *Torulaspora delbrueckii* and *Sympodiomyces attinorum* strains using primer EI1. Lanes: 1, 1 kbp ladder; 2, *Debaryomyces hansenii* UNESP-F46; 3, *Pichia guilliermondii* UNESP-S170; 4, *T. delbrueckii* UFMG-B40.2; 5, *S. attinorum* UNESP-S47; 6, *S. attinorum* UNESP-S49; 7, *S. attinorum* UNESP-S78; 8, *S. attinorum* UNESP-S156^T.

Sequencing of the D1/D2 region of the large-subunit rRNA gene of strain UNESP-S156^T showed that the strain differed by 29 base substitutions from *Sympodiomyces parvus*, indicating that the two species are phylogenetically distinct (Fig. 2). These species are also related to the recently described *Sympodiomyces indianaensis* and its ascogenous sister species, *Trichomonascus petasosporus* (Kurtzman, 2004). Kurtzman & Robnett (1995) had suggested that the genera *Arxula*, *Blastobotrys* and *Sympodiomyces* should be treated as a single genus, as they were thought to represent anamorphs of *Stephanoascus* species. However, they later showed that *Stephanoascus* species are highly divergent (Kurtzman & Robnett, 1998) and Kurtzman (2004) recently described the genus *Trichomonascus* as an ascogenous counterpart to *Sympodiomyces*. Accordingly, this novel species is being assigned to the genus *Sympodiomyces*. The current description of the genus (Fell & Statzell-Tallman, 1998) gives a very detailed description of sympodial conidiation in *Sympodiomyces parvus*, characterized by the accumulation of denticulate scars along a growing conidiophore. Like Kurtzman (2004), we do not construe the description to be so restrictive as to preclude the addition of species whose conidiation does not fit every detail. Formation of protuberances without conspicuous denticles (Fig. 3) is viewed as sufficiently compatible with the description not to require emendation of the genus.

The isolates of *Sympodiomyces attinorum* were examined after growth on common sporulation media (5% malt extract agar, corn meal agar, Fowell acetate agar and GY agar), but asci were not seen. The isolates were also mixed in pairs and no signs of conjugation were observed. It is concluded at this time that *Sympodiomyces attinorum* occurs in nature in the asexual form. The related species *Trichomonascus petasosporus* was isolated in the form of haploid mating types (Kurtzman, 2004), indicating that *Sympodiomyces attinorum* may be a heterothallic, ascogenous

Fig. 2. Neighbour-joining phylogram based on the D1/D2 divergent domains of the large-subunit rRNA gene of *Sympodiomyces attinorum* and related species. Percentage bootstrap values were obtained from 1000 iterations. Bar, 5% sequence divergence.

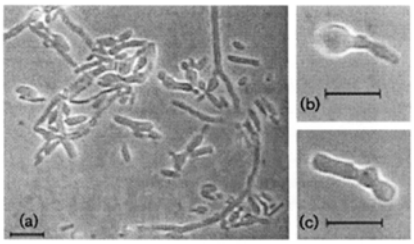

Fig. 3. Phase-contrast micrographs of *Sympodiomyces attinorum* UNESP-S156^T. Vegetative cells and true mycelium (a) and conidiophore formation (b, c) after 4 days on 5% malt extract at 25 °C are shown. Bars, 10 μm.

species. Unlike *Sympodiomyces parvus*, *Sympodiomyces attinorum* can ferment glucose, assimilate methyl α-D-glucoside, salicin and citrate, and grow at 37 °C, thus enabling these two species to be distinguished. The species differs from *Trichomonascus petasosporus* and *Sympodiomyces indianaensis* by its lack of growth on trehalose and the utilization of several carbon compounds, including L-rhamnose, D-ribose, galactitol and DL-lactic acid.

Sympodiomyces parvus is considered to be indigenous to marine waters, as all strains were isolated from open Antarctic waters in the southern Indian and Pacific Oceans (Fell & Statzell, 1971; Fell, 1976). Strains were isolated from depths of 16–748 m, at water temperatures of 3·1–10·6 °C and salinities of 33·9–34·9‰. *Sympodiomyces indianaensis* and *Trichomonascus petasosporus* were isolated in a white fungus growing on a fallen tree and in insect frass, respectively. The four strains of *Sympodiomyces attinorum* were isolated from laboratory nests of *A. sexdens*. The nests were maintained at about 25 °C and about pH 5·0 in the older sponges and pH 6·0 in the waste deposits (Carreiro, 1994; Carreiro *et al.*, 1997). Three strains were isolated from the fungal garden (older sponge) and one was from a waste deposit. Most yeast species in this community have the ability to degrade the plant polysaccharides pectin, starch and carboxymethylcellulose. All four strains of *Sympodiomyces attinorum* degraded soluble starch and strains UNESP-S47, UNESP-S78, UNESP-S156^T degraded pectin and polygalacturonic acid. Strain UNESP-S156^T, however, was able to degrade carboxymethylcellulose (Carreiro, 2000). These results show that *S. attinorum* can contribute to the degradation of plant polysaccharides present in the ant nest.

Latin diagnosis of *Sympodiomyces attinorum* Carreiro, Pagnocca, Rosa et Lachance sp. nov.

In medio liquido post dies 4 *cellulae singulae, binae, aut in catenis brevis; cellulae ovoidae aut elongatae* (5·5–7·0 × 2·0–3·5 μm). *Cultura in agaro malti post tres dies* (25 °C) *convexa, glabra, cremea et butyrosa. In agaro farinae Zea mays post dies* 14 *pseudomycelium et mycelium verum formantur. Glucosum et maltosum* (*variable*) *fermentatur. Glucosum, galactosum,* L-*sorbosum, glucosaminum,* D-*ribosum,* D-*xylosum,* L-*arabinosum,* D-*arabinosum,* L-*rhamnosum, maltosum, methyl* α-D-*glucosidum, cellobiosum, salicinum, amylum solubile, glycerolum, erythritolum, ribitolum, xylitolum,* L-*arabinitolum, glucitolum, mannitolum, galactitolum, N-acetylglucosaminum, meso-inositolum et acidum lacticum assimilantur, at non sucrosum, trehalosum, melibiosum, lactosum, raffinosum, melezitosum, inulinum,* D-*glucono-*1,5-*lactonum,* 2-*ketogluconatum,* 5-*ketogluconatum, acidum gluconicum, acidum glucuronicum, acidum succinicum, acidum citricum, methanolum, ethanolum nec hexadecanum. Lysinum, cadaverinum et ethylaminum assimilantur, at non natrium nitricum nec natrium nitrosum. Ureum non finditur. Diazonium caeruleum* B *negativum. Ad crescentiam vitaminae externae necessariae sunt. Augmentum in* 37 °C. *Materia amyloidea non formatur. Crescit in agaro extracto fermenti cum* 50% *glucoso et in agaro extracto fermenti cum* 10% NaCl. *Crescit in medio* 10 μm *cycloheximido* ml^{-1} *addito. Habitat nidum Attae sexdentis,* Sao Paolo, Brazil. *Typus* UNESP-S156^T. *In collectione zymotica* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum, sub* no. CBS 9734^T *typus stirps deposita est.*

Description of *Sympodiomyces attinorum* Carreiro, Pagnocca, Rosa et Lachance sp. nov.

Sympodiomyces attinorum (N.L. gen. masc. n. *attinorum* of Attini, referring to the tribe Attini, to which *Atta sexdens* belongs).

In yeast extract (0·5%)-glucose (2%) broth after 4 days at 25 °C, cells are elongate, ovoid and subglobose and occur singly, in pairs or in short branched chains (5·5–7·0 × 2·0–3·5 μm), with formation of true hyphae (Fig. 3). On yeast extract-malt extract agar after 2 days at 25 °C, colonies are dull cream, convex, rugose and butyrous. In Dalmau plate cultures on cornmeal agar after 2 weeks, true mycelium is formed with distinct septa and clusters of conidia. Glucose and maltose (variable) are fermented. Assimilates the following carbon compounds: glucose, galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, maltose, methyl α-D-glucoside, cellobiose, salicin, soluble starch (weak), glycerol, erythritol, ribitol, xylitol, L-arabinitol, glucitol, mannitol, galactitol, inositol, *N*-acetyl-D-glucosamine, D-gluconate (weak), DL-lactate. No growth occurs on sucrose, trehalose, melibiose, lactose, raffinose, melizitose, inulin, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-glucuronate, succinate, citrate, methanol, ethanol or hexadecane. Does not assimilate nitrate or nitrite, but does assimilate ethylamine, lysine and cadaverine. Urease activity and Diazonium blue B reaction are negative. Growth in amino-acid-free medium is positive, but negative in vitamin-free medium and in 1% acetic acid. Growth at 37 °C is positive. Acid formation on chalk agar and casein hydrolysis are positive. Starch formation is negative. Growth on 50% glucose-yeast extract agar and YM

agar with 10 % NaCl is positive. Growth in the presence 0·1 % cycloheximide is positive.

Isolated from nests of the leaf-cutting ant *Atta sexdens* in Brazil. The type strain of *Sympodiomyces attinorum* is strain UNESP-S156^T (=CBS 9734^T=NRRL Y-27639^T). This strain was isolated in 1996 from older sponge of a laboratory nest of *A. sexdens* maintained at the Centro de Estudos de Insetos Sociais (CEIS), UNESP, Rio Claro, São Paulo, Brazil.

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Apêndice 2: Relação de espécies vegetais e linhagens de leveduras identificadas, isoladas a partir de flores, frutos e água de tanque.

Flores		
Espécie vegetal / Família / Quantidade amostrada	**Códigos das linhagens**	**Espécies isoladas**
Couepia ovalifolia **Chrysobalanaceae (20 flores)**	UNESP – 428, 433	*Debaryomyces hansenii*
	UNESP – 429, 431, 432, 434, 435, 436	*Metschnikowia rekaufii*
Hedychium coronarium **Zengiberaceae (34 flores)**	UNESP – 329	*Bullera coprosmaensis*
	UNESP – 300, 316, 326, 440	*Candida intermedia*
	UNESP – 304	*Candida parapsilosis*
	UNESP – 323	*Candida prolifica*
	UNESP – 68, 322	*Candida silvae*
	UNESP – 321	*Candida sp*
	UNESP – 328, 301	*Debaryomyces hansenii*
	UNESP – 67, 69, 70, 302, 303, 305, 306, 307, 308, 309, 310, 311, 313, 318, 319, 320, 325	*Hanseniaspora uvarum*
	UNESP – 71	*Kluyveromyces sp*
	UNESP – 299	*Metschnikowia continentalis*
Herpetacanthus macahensis **Acanthaceae (20 flores)**	UNESP – 210, 211, 214, 215, 216, 218	*Candida intermedia*
	UNESP – 212	*Metschnikowia continentalis*
	UNESP – 213	*Wickerhamiella domercqiae*
Hibiscus pernambucensis **Malvaceae (18 flores)**	UNESP – 425, 426, 427	*Kodamaea kakaduensis*
Ipomoeae pes-capre **Convolvulaceae (28 flores)**	UNESP – 383, 393, 395	*Candida azyma*
	UNESP – 389	*Debaryomyces vanrijiae*
	UNESP – 372, 375, 376, 378, 385	*Metschnikowia bicuspidata*
	UNESP – 396-398-400-	*Metschnikowia continentalis*
	UNESP – 387, 401	*Pseudozyma antarctica*
	UNESP – 382, 386, 392, 394	*Pseudozyma prolifica*
	UNESP – 399	*Sporidiobolus ruineniae*
	UNESP – 390, 397	*Wickerhamiella cacticola*
Jacquemontia ciliata **Convolvulaceae (49 flores)**	UNESP – 118, 128, 136, 331, 332, 336, 342, 343, 346, 347	*Bullera unica*
	UNESP – 131, 139	*Candida azyma*
	UNESP – 120, 121, 122b, 123	*Candida drosophilae*
	UNESP – 138	*Candida etchellsii*
	UNESP – 341, 345	*Candida fermentati*
	UNESP – 134	*Candida sp*
	UNESP – 127	*Cryptococcus aerius*
	UNESP – 124, 140, 125	*Cryptococcus arrabidensis*
	UNESP – 135, 137	*Cryptococcus flavus*
	UNESP – 122c, 126, 130	*Cryptococcus laurentii*
	UNESP – 119	*Hanseniaspora uvarum*
	UNESP – 129, 133	*Metschnikowia colocasiae*
	UNESP – 132	*Pseudozyma rugulosa*
	UNESP – 142, 143	*Rhodotorula mucilaginosa*

Apêndice 2: Continuação

Flores		
Espécie vegetal / Família / Quantidade amostrada	**Códigos das linhagens**	**Espécies isoladas**
Justicia carnea **Acanthaceae (14 flores)**	UNESP – 296-297	*Candida intermedia*
	UNESP – 298	*Candida sp*
	UNESP – 295	*Pseudozyma prolifica*
Mandevila funiformes **Apocinaceae (10 flores)**	UNESP – 208, 209	*Bullera sinensis*
	UNESP – 200, 201, 202, 203, 204	*Candida restingae*
	UNESP – 205	*Pseudozyma prolifica*
Psychotria nuda **Rubiaceae (20 flores)**	UNESP – 34	*Candida azyma*
	UNESP – 36	*Candida drosophilae*
	UNESP – 266	*Debaryomyces hansenii*
	UNESP – 271	*Debaryomyces hansenii*
	UNESP – 272, 273, 265	*Kodamaea kakaduensis*
	UNESP – 270	*Pichia sp*
	UNESP – 268	*Rhodotorula mucilaginosa*
Psychotria pubigera **Rubiaceae (28 flores)**	UNESP – 81	*Candida azyma*
	UNESP – 73	*Candida ernobii*
	UNESP – 79, 74, 75	*Candida magnifica*
	UNESP – 72, 82, 76, 85	*Candida restingae*
	UNESP – 77	*Hanseniaspora uvarum*
	UNESP – 84, 86, 87, 78	*Metschnikowia koreensis*
Sophora tomentosa **Fabaceae (28 flores)**	UNESP – 185	*Bullera coprosmaensis*
	UNESP – 196	*Bullera sinensis*
	UNESP – 160, 162, 165, 166, 168, 174, 192, 193, 194	*Bullera unica*
	UNESP – 198	*Candida azyma*
	UNESP – 164	*Candida sp*
	UNESP – 184	*Debaryomyces vanrijiae*
	UNESP – 177, 190	*Hanseniaspora uvarum*
	UNESP – 172, 173, 176, 178, 181, 186, 187, 188, 191, 195	*Metschnikowia koreensis*
	UNESP –157, 158, 161	*Pseudozyma rugulosa*
	UNESP – 183	*Pseudozyma sp*
	UNESP – 163	*Rhodotorula mucilaginosa*
	UNESP – 182	*Wicherhamiella occidentalis*
	UNESP – 156, 159	*Williopsis sp*
Tabebuia cassinoides **Bignoniaceae (12 flores)**	UNESP – 152	*Bullera unica*
	UNESP – 145	*Candida apis*
	UNESP – 148	*Candida drosophilae*
	UNESP – 150	*Hanseniaspora uvarum*
	UNESP – 146, 147, 151	*Metschnikowia bicuspidata*
	UNESP – 144, 149, 154	*Metschnikowia colocasiae*
	UNESP – 153	*Sporobolomyces rubescens*
Tibouchina pulchra **Melastomataceae (30 flores)**	UNESP – 421	*Candida sp*
	UNESP – 417, 418, 420, 424	*Kodamaea ohmeri*
	UNESP – 422	*Metschnikowia bicuspidata*
	UNESP – 414	*Rhodotorula glutinis*

Apêndice 2: Continuação

Frutos		
Espécie vegetal / Família / Quantidade amostrada	**Códigos das linhagens**	**Espécies isoladas**
Chioccoca alba **Rubiaceae (6 frutos)**	UNESP – 32	*Bullera unica*
	UNESP – 30, 31	*Cryptococcus arrabidensis*
	UNESP – 29	*Sporidiobolus ruineniae*
Euterpe edulis **Arecaceae (16 frutos)**	UNESP – 287, 288, 289, 290	*Candida natalensis*
	UNESP – 291	*Candida vini*
	UNESP – 284, 285, 292, 293	*Metschnikowia bicuspidata*
	UNESP – 281	*Metschnikowia continentalis*
	UNESP – 286	*Pichia pijperi*
Gomidesia shaueriana **Myrtaceae (12 frutos)**	UNESP – 12	*Bullera oryzae*
	UNESP – 7, 28	*Bullera sinensis*
	UNESP – 21, 23	*Bullera unica*
	UNESP – 5, 10, 14, 20	*Cryptococcus aerius*
	UNESP – 13, 19	*Cryptococcus albidus*
	UNESP – 15	*Cryptococcus arrabidensis*
	UNESP – 2, 27	*Cryptococcus hungaricus*
	UNESP – 4	*Cryptococcus laurentii*
	UNESP – 17	*Cryptococcus sp*
	UNESP – 6	*Metschnikowia continentalis*
	UNESP – 1	*Rhodotorula fujisanensis*
	UNESP – 3	*Sporidiobolus ruineniae*
Leandra reversa **Melastomataceae (10 frutos)**	UNESP – 63L, 63R, 64L-64R-65R-65L-66L-66R-	*Candida leandrae*
	UNESP – 62	*Hanseniaspora uvarum*
Smilax sp Smilacaceae (6 frutos)	UNESP – 42	*Bullera sinensis*
	UNESP – 39, 40	*Metschnikowia bicuspidata*
	UNESP – 38	*Sporidiobolus runeniae*
Paulinia **sp Sapindaceae (22 frutos)**	UNESP – 232, 240	*Candida famata*
	UNESP – 244	*Candida sp*
	UNESP – 233	*Cryptococcus sp*
	UNESP – 219	*Debaryomyces sp*
	UNESP – 53, 54, 57, 220, 221, 222, 223, 224, 226, 227, 228, 229, 230, 231, 235, 237, 238, 242, 243	*Hanseniaspora uvarum*
	UNESP – 51, 55, 56	*Metschnikowia continentalis*
	UNESP – 234, 239, 241	*Metschnikowia koreensis*

Apêndice 2: Continuação.

Água de Tanque		
Espécie vegetal / Família / Quantidade amostrada	**Códigos das linhagens**	**Espécies isoladas**
Heliconia velloziana **Heliconiaceae (14 plantas)**	UNESP – 98, 102	*Candida azyma*
	UNESP – 258	*Candida boidini*
	UNESP – 91C1, 91C2, 91T1, 91T2	*Candida heliconiae*
	UNESP – 88.2, 89, 94	*Candida picinguabensis*
	UNESP – 259, 263	*Candida pseudointermedia*
	UNESP – 255, 262	*Candida restigae*
	UNESP – 98.2, 99	*Candida sanpauloensis*
	UNESP – 96	*Candida silvae*
	UNESP – 90	*Debaryomyces etschellsii*
	UNESP – 257, 264	*Debaryomyces sp*
	UNESP – 100	*Hanseniaspora uvarum*
	UNESP – 97	*Kluyveromyces sp*
	UNESP – 95	*Kodamaea kakaduensis*
	UNESP – 101	*Metschnikowia sp*
	UNESP – 92, 93	*Metschnikowia koreensis*
	UNESP – 88	*Metschnikowia rekaufii*
Canistropsis seidelii **Bromeliaceae (11 plantas)**	UNESP – 103, 104.2, 110.2	*Candida bromeliacearum*
	UNESP – 249, 250, 251	*Candida famata*
	UNESP – 438, 439	*Candida intermedia*
	UNESP – 115, 248	*Candida sp*
	UNESP – 245, 247R, 248R	*Candida ubatubensis*
	UNESP – 111	*Metschnikowia*
	UNESP – 106, 108, 109, 113	*Metschnikowia continentalis*
	UNESP – 114	*Pichia*
	UNESP – 246	*Wickerhamiella sp*

Apêndice 3: Listagem dos principais bancos de culturas de fungos filamentosos e leveduras.

Coleções de cultura	Localização	Número de linhagens
American Type Culture Collection (ATCC)	Manassas, Virgina USA	Fungos filamentosos e Leveduras - 27,000
Agricultural Research Culture Collection (NRRL)	Peoria, Illinois, USA	Fungos filamentosos - 55,000 Leveduras - 10,000
Centraalbureau voor Schimmelcultures (CBS)	Utrecht, The Netherlands	Fungos filamentosos - 28,000 Leveduras - 4,500
Industrial Yeasts Collection (DBVPG)	Dipartimento di Biologia Vegetale e Biotecnologia Agroambientale - Perugia, Italy	Leveduras - 4,500
Culture Collection of Yeasts (CCY)	Slovak Academy of Sciences	Leveduras - 3,800
NITE Biological Resource Center (NBRC)	National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan	Fungos filamentosos - 8,000 Leveduras - 3,150 (incluindo linhagens IFO)
National Collection of Yeast Cultures	Institute of Food Research Norwich, UK	Leveduras - 3,000
The Spanish Type Culture Collection (CECT)	University of Valencia Valencia, Spain	Fungos filamentosos - 1,500 Leveduras - 2,500
All-Russian Collection of Microorganisms (VKM)	Moscow, Russia	Fungos filamentosos - 3,300 Leveduras - 2,300
Japan Collection of Microorganisms (JCM)	RIKEN (The Institute of Physical and Chemical Research), Saitama, Japan	Fungos filamentosos - 1,200 Leveduras - 2,100
Portuguese Yeast Culture Collection (PYCC)	Faculty of Sciences and Technology, New University of Lisbon - Lisbon, Portugal	Leveduras - 2,000
Labatt Culture Collection - Labatt Brewing Company	London, Ontario, Canada	Leveduras - 2,000
Yeast Collection of the Department of Soil Sciences, (YBP)	Moscow State University Moscow, Russia	Leveduras - 2,000
ZIM Culture Collection of Industrial Microorganisms	University of Ljubljana Ljubljana, Slovenia	Leveduras - 1,700
Bioresource Collection and Research Center (BCRC, formerly CCRC)	Hsinchu, Taiwan	Fungos filamentosos - 3,000 Leveduras - 1,500
National Collection of Agricultural and Industrial Microorganisms	Szent István University Budapest, Hungary	Fungos filamentosos - 300 Leveduras - 1,100
VTT Biotechnology, Culture Collection	VTT Biotechnology and Food Research Finland	Fungos filamentosos - 800 Leveduras - 800
Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)	Braunschweig, Germany	Fungos filamentosos - 2,400 leveduras - 500

Fonte: Modificado de [1]KURTZMAN e FELL (1998), [2]McCLUSKEY (2003).

[1]KURTZMAN, C. P., FELL, J. W. **The Yeasts – A Taxonomy Study**. 4 ed. Amsterdam: Elsevier Science B. V., 1998. 1055 p.

Apêndice 4: Principais bases de dados on-line para acesso aos bancos de culturas.

Banco de Dados	Acesso	Enfoque
World Data Centre for Microorganisms (WDCM)	www.wdcm.nig.ac.jp/	Compreende as bases de dados e coleções de culturas mundiais
WDCM Agent to Help Microbial Information Integration (AHMII)	www.wdcm.nig.ac.jp/AHMII/ahmii.html	Base de dados da WDCM
Microbial Germplasm Database (MGD)	www.nacse.org/ocid/prospct3.html	Pequenas coleções
The United Kingdom National Culture Collection (UKNCC)	www.ukncc.co.uk/	Coleções do reino unido incluindo o banco de linhagens
Common Access to Biological Resources and Information (CABRI)	www.cabri.org	Acesso a 26 coleções na união européia
USDA GRIN	www.ars-grin.gov/	Links dos sites da USDA
Canadian Collection of Fungal Cultures	http://sis.agr.gc.ca/brd/ccc/	Listagem das fontes Canadenses
Collnet	www.collnet.cnrb.it/	Centros de recursos biológicos Italianos
All-russian Collection of Microorganisms - VKM	www.vkm.ru/	Catalogo de culturas microbianas presentes na coleção Russa (não médica)

Fonte: Modificado de: McCluskey, 2003

[2]McCLUSKEY, K. Fungal germplasm and databases. In: KHACHATOURIANS, G. G.; ARORA, D. K. (Ed.). **Applied Mycology & Biotechnology**. An International Series. Volume 3: Fungal Genomics. San Diego: San Diego Technical Books, Inc., 2003. Pages 295-310

Apêndice 5: Foto das espécies vegetais das quais foram isoladas as novas espécies.

Figura 1: *Heliconia velloziana* (Heliconiaceae)

Figura 2: *Canistropsis seidelii* (Bromeliaceae)

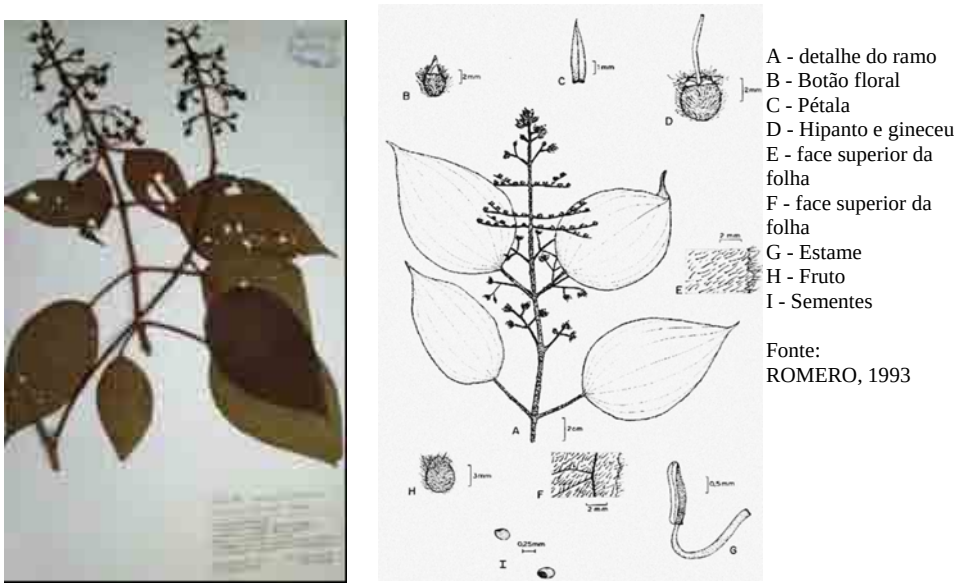

Figura 3: *Leandra reversa* (Melastomataceae)

ROMERO, R. **Florística da família Melastomataceae na planície litorânea de Picinguaba, município de Ubatuba, Parque Estadual da Serra do Mar, SP**. 1993. Dissertação (Mestrado em Botânica) – Instituto de Biociências, Universidade Estadual Paulista - UNESP, Rio Claro, SP. 1993.

Apêndice 6: Fotos das principais áreas de coleta.

Figura 3: Estrada da casa da farinha

Figura 5: Praia da Fazenda – região de duna (beira mar)

Figura 6: Trilha do Corisco

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