Phylogeny of Metschnikowia Species Estimated from Partial rRNA Sequences

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Phylogenetic relationships of species assigned to the genus Metschnikowia were estimated from the extents of divergence among partial sequences of rRNA. The data suggest that the aquatic species (Metschnikowia australis, Metschnikowia bicuspidata, Metschnikowia krissii, and Metschnikowia zobellii) and the terrestrial species (Metschnikowia hawaiiensis, Metschnikowia lunata, Metschnikowia pulcherrima, and Metschnikowia reukaufii) form two groups within the genus. M. lunata and M. hawaiiensis are well separated from other members of the genus, and M. hawaiiensis may be sufficiently divergent that it could be placed in a new genus. Species of the genus Metschnikowia are unique compared with other ascomycetous yeasts because they have a deletion in the large-subunit rRNA sequence that includes nucleotides 434 to 483.

The ascomycetous yeast genus Metschnikowia is defined by the presence of multilateral budding of vegetative cells and by the production of one or two needle-shaped ascospores in elongate asci. There are relatively few differences in physiological traits among the species of the genus (21). Species isolated from terrestrial habitats are typically associated with flowers or fruits and insect vectors, and species isolated from aquatic habitats are often parasitic in invertebrates but can also be isolated as free-living forms in water. Isolations of Metschnikowia species have been sporadic, but the unusual needlelike ascospore morphology of these organisms and their associations with other organisms have attracted the attention of taxonomists and ecologists. Metschnikoff (20) first described a species of this group as Monospora bicuspidata and also noted the function of its needle-shaped spores in the parasitic invasion of Daphnia magna. The name Metschnikowia was introduced by Kaminski in 1899 (9), and descriptions of anamorphs of two terrestrial Metschnikowia species, Candida pulcherrima (16) and Candida reukaufii (7), followed. Taxonomic work on this genus was stimulated by the isolation of the aquatic species Metschnikowia zobellii and Metschnikowia krissii reported in 1961 (30), of Metschnikowia bicuspidata (the type species) in 1964 (32), and of additional aquatic strains of Metschnikowia species by Spencer et al. (28) and Fell and Hunter (2). Needle-shaped ascospores were observed in strains of C. pulcherrima and C. reukaufii, showing that these organisms are members of the genus Metschnikowia (24). In vivo hybridization experiments defined an aquatic group of three species and a predominantly terrestrial chlamydospore-forming group containing two species (4, 24, 25). Metschnikowia lunata, another chlamydospore-forming species, was isolated from flowers and differed from other Metschnikowia species in having lunate vegetative cells (6). DNA reassociation studies clarified the delineation of species belonging to the aquatic group. M. bicuspidata, M. zobellii, and M. krissii were shown to be distinct from Metschnikowia australis, and M. australis was shown to be a sibling species of M. bicuspidata (19). M. zobellii exhibited slightly higher levels of DNA relatedness to the aquatic species than to terrestrial taxa, although the interspecific values were 15% or less.

A yeast that formed unusually large asc and needle-shaped ascospores was isolated from flowers and Drosophila sp. and was described as Metschnikowia hawaiiensis by Lachance et al. (14). Although this new species had a terrestrial origin, it did not form chlamydospores, occurred in nature in the haploid condition, and was shown to differ from Metschnikowia reukaufii and Metschnikowia pulcherrima in DNA base sequence complementarity. The lack of chlamydospores suggests that M. hawaiiensis could be more closely related to the aquatic group. DNA relatedness data and genetic hybridization data have clearly defined eight species in the genus Metschnikowia. These taxa can be separated into a group of four species that are predominantly associated with aquatic environments and a second group of four species that are associated predominantly with terrestrial environments.

Comparisons of levels of DNA relatedness allow good separation of strains at the species and sibling species level, but do not allow resolution of relationships above this level (10). RNA sequence comparisons have been used to determine phyllogenetic relationships above the species level (33). At this time complete sequencing of small- and large-subunit rRNAs is impractical for comparisons of large numbers of strains, but partial sequences of bacterial rRNAs were found by Lane et al. (15) to yield phylogenetic trees similar to those derived from complete sequences. For yeasts, partial sequencing of 18S and 25S rRNAs having different degrees of conservation has allowed resolution of species relationships in several genera (3, 8, 12, 13, 22, 23, 34, 35). In an effort to better understand species relationships in the genus Metschnikowia, we determined partial rRNA sequences for the type strains of all of the species that are assigned to this genus at this time.

MATERIALS AND METHODS

Yeast strains. The strains of Metschnikowia species and the comparative taxa used are listed in Table 1. All of these strains are maintained in the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Ill.

rRNA extraction and purification. Cells were grown at

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25°C in YM liquid medium (31) on a rotary shaker at 200 rpm for approximately 16 h and were harvested by centrifugation. The cells were suspended in 2x SSE buffer (29) at a concentration of 1 g (wet weight) per 10 ml of buffer; an equal volume of 80% phenol in water containing 0.1% 8-hydroxyquinoline, 2% (vol/vol) dimethyl sulfoxide, and 2% (vol/vol) xylene was added to this buffer as described by Kurtzman and Liu (12). The cells in the mixture were broken with a Braun cell homogenizer by using 0.5-mm-diameter glass beads. The suspension of broken cells was transferred to a flask and was made 1 M in sodium perchlorate and 1% N-lauroyl sarcosine. An equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) was added to the suspension, and the mixture was emulsified by swirling and then placed in an ice bath on a reciprocating shaker for 30 min. The emulsion was separated by centrifugation for 20 min at 20,000 × g, and the top aqueous layer containing rRNA was removed with a pipette for further purification by using the method of Chirgwin et al. (1). The physical integrity of purified rRNA was assessed by performing nondenaturing agarose gel electrophoresis.

**Sequencing reactions and sequence comparisons.** Sequencing of rRNA was accomplished by using specific oligonucleotide primers and the dideoxynucleotide chain termination method (15, 27). Two regions were sequenced from the large (25s) subunit. The large-subunit primers (and first bases of the rRNA sequences copied) were as follows: 5'-GGTC GTGTAC and base 1627, respectively. The nucleotide numbers for 18s rRNA segment 1627 and the 25s (25s) subunit, and one region was sequenced from the small (18s) subunit. The large-subunit primers (and first bases of the RNA sequences copied) were as follows: 5'-GGTC CTGCTTTCAAGACGG (base 635) and 5'-TTGGAGACCT GCTGCGG (base 1841). The small-subunit primer and first base of the RNA sequence copied were 5'-ACGGGCAGGT GTGTAC and base 1627, respectively. The nucleotide numbers given below are equivalent to the nucleotide numbers in the primary structure of *Saccharomyces cerevisiae* (5, 18, 26), but the numbers in the aligned sequences differ somewhat from the numbers in the *S. cerevisiae* sequence because of insertions and deletions in the other species. For easier reference below, the three primers described above are designated 25s-635, 25s-1841, and 18s-1627, respectively. The primers were made with an Applied Biosystems model 381A DNA synthesizer. The nucleotide fragments generated in the chain termination reactions were separated on 8% acrylamide–8 M urea gels and were visualized by autoradiography.

Sequences were manually aligned, and dendrograms of the sequence data were calculated by using each of the following three methods: DNAML, version 3.42, a phylogeny inference, maximum-likelihood program (J. Felsenstein, University of Washington, Seattle); PAUP, version 2.4.1, a parsimony analysis program (D. Swoford, Illinois Natural History Survey, Champaign); and the neighbor-joining method, in which the reproducibility of tree nodes was analyzed by using a bootstrapping program (PHYLIP, version 3.42; J. Felsenstein). For the initial data treatment we used the maximum-likelihood option. A total of 100 bootstrap trees were generated and examined.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for 18s rRNA segment 1627 and the 25s rRNA segments 635 and 1841 are, respectively, L10684, L10646, and L10743 for *M. australis*; L10685, L10644, and L10744 for *M. bicuspidata*; L10686, L10647, and L10745 for *M. hawaiiensis*; L10687, L10648, and L10746 for *M. krissii*; L10688, L10650, and L10747 for *M. lunata*; L10689, L10679, and L10748 for *M. pulcherrima*; L10690, L10680, and L10749 for *M. reukaufii*; L10691, L10681, and L10750 for *M. zobelli*; L10682, L10643, and L10741 for *S. cerevisiae*; and L10683, L10649, and L10742 for *D. hansenii*.

### RESULTS AND DISCUSSION

Aligned sequences for the three rRNA segments which were examined are shown in Fig. 1 through 3. The most notable feature of these sequences is a large deletion in the 25s-635-initiated region (nucleotides 434 to 483) that is characteristic of all *Metschnikowia* species but is not found in other genera of ascomycetous yeasts (11). In this region or differ by no more than 2 nucleotides among the approximately 300 nucleotides examined (11). In this study additional strains of *M. australis* and *M. pulcherrima* were compared, and for each species the sequences were identical to the sequence of the type strain. A matrix of the nucleotide differences among *Metschnikowia* spp. is shown in Table 2. The extent of nucleotide divergence within the three regions which were sequenced is
greater than the extent of divergence found in any other ascomycetous yeast genus examined so far (12, 13, 17, 22). When *M. hawaiensis* is removed from consideration, the remaining species still constitute a genus that is highly divergent, but one that is comparable in variety to the genus *Issatschenkia*, *Schizosaccharomyces*, and *Williopsis*. In contrast, the levels of species nucleotide divergence in the genera *Debaryomyces*, *Lipomyces*, *Saccharomyces*, and *Saturnospora* are about one-half the levels in the genus *Issatschenkia*, *Schizosaccharomyces*, and *Williopsis*. The variability observed in this study raises the question of whether the species that are currently assigned to the genus *Metschnikowia* are all members of the same genus. In the studies cited above, each genus was characterized by unique

FIG. 2. Alignment of the 25S rRNA partial sequences initiated at position 635 from *Metschnikowia* species and the reference taxa *S. cerevisiae* and *D. hansenii*. The sequence of *S. cerevisiae* is given in its entirety for this region and serves as a reference, but there is a departure from correct numbering in this and the other regions examined because of insertions and deletions that occur in the taxa compared. The dots indicate nucleotides identical to nucleotides in the reference species; the dashes indicate that no base is present at a position; and *N* indicates an unidentified base.

![Image of aligned sequences](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAA...)

FIG. 1. Alignment of the 25S rRNA partial sequences initiated at position 635 from *Metschnikowia* species and the reference species *S. cerevisiae* and *D. hansenii*. The sequence of *S. cerevisiae* is given in its entirety for this region and serves as a reference, but there is a departure from correct numbering in this and the other regions examined because of insertions and deletions that occur in the taxa compared. The dots indicate nucleotides identical to nucleotides in the reference species; the dashes indicate that no base is present at a position; and *N* indicates an unidentified base.
FIG. 3. Alignment of 18S rRNA partial sequences initiated at position 1627 from *Metschnikowia* and reference species. For further explanation see the legend to Fig. 1.

signature nucleotides that separated it from other genera (11-13, 17, 22). If genera are aggregates of closely related species which arose from a common ancestor, signatures presumably represent conserved nucleotides that reflect this shared phylogeny. Signatures are evident for the genus *Metschnikowia* if *M. hawaiiensis* is removed, and the genus is even more homogeneous if *M. lunata* is also removed.

Species relationships are shown in Fig. 4. This phylogenetic tree was calculated by the neighbor-joining method. The data set used included all three regions sequenced in order to introduce sufficient nucleotide differences to position closely related species more accurately. The large deletion in region 25S-635 in *M. hawaiiensis* had little impact on the branch lengths of trees generated after this species was removed (data not shown). Table 2 shows that *M. hawaiiensis* is also highly divergent in the other two regions examined. The relationships which were determined indicate that the aquatic *Metschnikowia* species are separate from the terrestrial species. The low level of detectable DNA-DNA complementarity among aquatic species (19) is consistent with the close relationships revealed by our rRNA comparison.

Bootstrapping of the data suggests that some branch nodes shown in Fig. 4 are weak; for example, a 56% confidence level was obtained for the *M. reukaufii* branch. Analyses of the data by DNAML and PAUP gave similar overall tree topologies. Both analyses showed that *M. lunata* and *M. hawaiiensis* are outliers and that the latter species is the species that is most distantly related to other members of the genus *Metschnikowia*. An analysis of individual rRNA regions by the three programs used in this study demonstrated that there is some shifting of species associations. In some treatments of the data, *M. reukaufii* fell in the aquatic species group and *M. pulcherrima* fell at the edge of the aquatic species group. Distances among *M. lunata*, *M. hawaiiensis*, and the reference taxa (*S. cerevisiae* and *D. hansenii*) may

### Table 2. Matrix of number of nucleotide differences in three rRNA regions for *Metschnikowia* species and the reference taxa *S. cerevisiae* and *D. hansenii*

<table>
<thead>
<tr>
<th>Species</th>
<th>S. cerevisiae</th>
<th>D. hansenii</th>
<th>M. australis</th>
<th>M. bicuspidata</th>
<th>M. hawaiiensis</th>
<th>M. krissi</th>
<th>M. lunata</th>
<th>M. pulcherrima</th>
<th>M. reukaufii</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. hansenii</em></td>
<td>57, 18, 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. australis</em></td>
<td>114, 41, 42</td>
<td>103, 35, 38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. bicuspidata</em></td>
<td>106, 42, 41</td>
<td>95, 35, 38</td>
<td>24, 4, 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. hawaiiensis</em></td>
<td>164, 76, 49</td>
<td>156, 72, 39</td>
<td>111, 70, 50</td>
<td>119, 71, 50</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><em>M. krissi</em></td>
<td>110, 37, 36</td>
<td>100, 30, 32</td>
<td>35, 7, 8</td>
<td>35, 7, 6</td>
<td>119, 70, 49</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. lunata</em></td>
<td>127, 71, 56</td>
<td>118, 66, 52</td>
<td>56, 53, 39</td>
<td>61, 54, 39</td>
<td>92, 87, 64</td>
<td>60, 55, 39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. pulcherrima</em></td>
<td>113, 39, 44</td>
<td>108, 35, 40</td>
<td>37, 19, 11</td>
<td>45, 20, 11</td>
<td>109, 68, 48</td>
<td>51, 18, 14</td>
<td>47, 43, 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. reukaufii</em></td>
<td>103, 38, 44</td>
<td>96, 35, 40</td>
<td>39, 9, 11</td>
<td>31, 9, 11</td>
<td>113, 68, 53</td>
<td>35, 8, 12</td>
<td>59, 53, 45</td>
<td>40, 14, 12</td>
<td></td>
</tr>
<tr>
<td><em>M. zobellii</em></td>
<td>104, 39, 36</td>
<td>95, 34, 34</td>
<td>29, 6, 2</td>
<td>27, 6, 2</td>
<td>113, 68, 47</td>
<td>32, 4, 7</td>
<td>64, 52, 37</td>
<td>46, 14, 11</td>
<td>35, 5, 9</td>
</tr>
</tbody>
</table>

* For each comparison the first number is the number of nucleotide differences in region 25S-635, the second number is the number of nucleotide differences in region 25S-1841, and the third number is the number of nucleotide differences in region 18S-1627. The following numbers of nucleotides were compared: region 25S-635, 300 nucleotides; region 25S-1841, 256 nucleotides; and region 18S-1627, 204 nucleotides. Deleted areas were included in these calculations.
FIG. 4. Phylogenetic tree calculated for species of the genus Metschnikowia and reference taxa by using the neighbor-joining method. The numbers at the branch nodes are frequencies in a set of 100 trees generated by bootstrapping. Branch lengths were derived from an analysis of the consensus tree data by using the program PAUP, version 2.4.1, and are proportional to the observed differences in nucleotides.

have been underestimated because the large number of nucleotide differences shown in Table 2 suggest that mutable sites were saturated and may have undergone multiple substitutions (23).

The extent of rRNA sequence diversity among Metschnikowia species is surprising considering the relative phenotypic homogeneity of these taxa. This suggests that the genus is very old or that it underwent rapid evolution. Since these characteristics from an analysis of the consensus tree data by using the program method. The numbers at the branch nodes are frequencies in a set of nucleotide differences shown in Table 2 suggest that mutable have been underestimated because the large number of

ACKNOWLEDGMENTS

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