RESOLUTION OF VARIETAL RELATIONSHIPS WITHIN THE SPECIES HANSENULA ANOMALA, HANSENULA BIMUNDALIS, AND PICHIA NAKAZAWAE THROUGH COMPARISONS OF DNA RELATEDNESS

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SUMMARY

The varietal relationships within the species Hansenula anomala, H. bimundalis, and Pichia nakazawae were determined through comparisons of DNA relatedness. H. anomala var. anomala and H. anomala var. schneggii at 94% DNA relatedness were considered to be the same taxon, whereas the 19% relatedness between H. bimundalis var. bimundalis and H. bimundalis var. americana indicates the latter variety to be a separate species. The varieties of P. nakazawae exhibited 41% DNA relatedness, and it is proposed that their varietal designations be retained.

INTRODUCTION

Variatel designations within fungus taxa often are established on the basis of unique morphological features (Bisby, 1953). Among the yeasts, varieties are formed on physiological characteristics as well as on novel morphology. However, genetic and molecular studies have shown many physiological and morphological features

1 The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.
to be determined by either one or only a few genes and therefore are not representative of the whole genome (Lindegren and Lindegren, 1949; Winge and Roberts, 1949; Wickerham and Burton, 1954; Kurtzman and Smiley, 1976; Meyer et al., 1975; Starmer et al., 1978). For example, in comparisons of DNA relatedness between species of Saccharomyces, Price et al. (1978) showed only 11% base sequence complementarity between Saccharomyces microellipsodes Osterwalder var. microellipsodes and Saccharomyces microellipsodes Osterwalder var. osmophilus van der Walt, thereby demonstrating the two taxa to be distinct species. Conversely, the two physiological varieties of the invalidly described Pichia vini shared 93% DNA relatedness and were regarded as members of the same taxon (Kurtzman and Smiley, 1979). Price et al. (1978) suggested that strains showing 80% or greater DNA relatedness be considered conspecific. However, Kurtzman et al. (1980a, b) provided evidence that this figure probably can be much lower.

Within the genera Hansenula and Pichia, varieties have been described for H. anomala (Hansen) H. et P. Sydow, H. bimundalis Wickerham et Santa Maria and P. nakazawae Kodama. These varieties were designated primarily on physiological characteristics. The relationship of these species with their varieties has been compared through estimates of DNA base sequence complementarity, and the taxonomic implications of these results are reported here.

MATERIALS AND METHODS

Yeast strains. Cultures of the strains studied are maintained in the Agricultural Research Service Culture Collection (NRRL), Northern Regional Research Center, and their designations and nuclear DNA base composition are given in Table 1.

DNA purification, base composition determination, and conditions for reassociation. Nuclear DNA was extracted and purified by a combination of the procedures of Marmur (1961) and Bernardi et al. (1970) as described by Price and coworkers (1978). The guanine + cytosine (G+C) content of the DNA was calculated from buoyant density in cesium chloride by using the equation of Schildkraut et al. (1962). Determinations were made
<table>
<thead>
<tr>
<th>Culture Collection No.</th>
<th>NRRL</th>
<th>CBS</th>
<th>Taxonomic Designation</th>
<th>Mol% G+C&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-366&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5759</td>
<td></td>
<td><em>Hansenula anomala</em> (Hansen) H. et P. Sydow var. <em>anomala</em></td>
<td>36.3 ± 0.23</td>
</tr>
<tr>
<td>Y-1773</td>
<td></td>
<td></td>
<td><em>H. anomala var. anomala</em> (<em>Candida pelliculosa</em> Redaelli)</td>
<td>36.9 ± 0.10</td>
</tr>
<tr>
<td>Y-1783</td>
<td>605</td>
<td></td>
<td><em>H. anomala var. anomala</em> (<em>C. pelliculosa</em>)</td>
<td>35.8 ± 0.17</td>
</tr>
<tr>
<td>Y-102</td>
<td></td>
<td></td>
<td><em>Hansenula anomala</em> (Hansen) H. et P. Sydow var. <em>schneggi</em> (Weber) Wickerham</td>
<td>36.9 ± 0.09</td>
</tr>
<tr>
<td>Y-945</td>
<td></td>
<td></td>
<td><em>H. anomala var. schneggi</em></td>
<td>37.4 ± 0.27</td>
</tr>
<tr>
<td>Y-993&lt;sup&gt;b&lt;/sup&gt;</td>
<td>113</td>
<td></td>
<td><em>H. anomala var. schneggi</em></td>
<td>36.7 ± 0.08</td>
</tr>
<tr>
<td>Y-5343&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5642</td>
<td></td>
<td><em>Hansenula bimundalis</em> Wickerham et Santa Maria var. <em>bimundalis</em></td>
<td>42.1 ± 0.10</td>
</tr>
<tr>
<td>YB-2805</td>
<td></td>
<td></td>
<td><em>H. bimundalis var. bimundalis</em></td>
<td>41.5 ± 0.09</td>
</tr>
<tr>
<td>Y-2156&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5644</td>
<td></td>
<td><em>Hansenula bimundalis</em> Wickerham et Santa Maria var. <em>americana</em> Wickerham</td>
<td>44.0 ± 0.24</td>
</tr>
<tr>
<td>YB-2444</td>
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<td></td>
<td><em>H. bimundalis var. americana</em></td>
<td>43.2 ± 0.12</td>
</tr>
<tr>
<td>Y-7903&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6700</td>
<td></td>
<td><em>Pichia nakazawae</em> Kodama var. <em>nakazawae</em></td>
<td>39.4 ± 0.08</td>
</tr>
<tr>
<td>Y-7904&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6701</td>
<td></td>
<td><em>Pichia nakazawae</em> Kodama var. <em>akitaensis</em> Kodama</td>
<td>39.9 ± 0.17</td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard deviation calculated from 3-5 determinations.

<sup>b</sup>Type strain.
with a Spinco Model E analytical ultracentrifuge equipped with an electronic scanner. The extent of DNA renaturation was determined spectrophotometrically by using essentially the method of Seidler and Mandel (1971) as described by Kurtzman et al. (1980a).

Single spore isolation. Pichia nakazawae and its variety akitaensis were determined to be homothallic through examination of single ascospore isolates obtained from four-spored asci. Ascospores were isolated by micromanipulation.

RESULTS AND DISCUSSION

Hansenula anomala and its variety schneggii were separated by Wickerham (1970) because the former is less capable of fermenting sucrose than the latter, and it does not grow in osmotic medium (10% sodium chloride plus 5% glucose in Difco yeast nitrogen base). In addition, the variety schneggii does not assimilate raffinose. Certain morphological differences between the varieties are evident. The variety schneggii produces cylindrical and often markedly elongated thread-like cells while the variety anomala does not. However, sexual reactions between these heterothallic taxa tend to obscure the other differences. Mating reactions between strains of the variety schneggii are weak, but mixtures that contain one strain of each variety show stronger reactions.

Type strains of the varieties anomala and schneggii show 94% DNA relatedness which is comparable to the complementarity shown between strains within each variety (Table 2). DNA comparisons also clearly demonstrate Candida pelliculosa to represent the imperfect form of H. anomala. In view of the high DNA relatedness between the varieties, as well as the considerable intervarietal fertility, it is proposed that the variety schneggii be considered a synonym of the variety anomala.

Hansenula bimundalis var. bimundalis is associated with coniferous trees in Europe and Asia, whereas strains of H. bimundalis var. americana are found with coniferous trees of the southwestern United States (Wickerham, 1965). The variety bimundalis assimilates D-arabinose and grows at 37°C, but the variety americana
Table 2. Extent of DNA relatedness between varieties of Hugomala anomala, H. bimundalis and Pichia nakazawae.

<table>
<thead>
<tr>
<th>Species/NHRL No.</th>
<th>Y-366&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Y-993&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Y-5343&lt;sup&gt;b&lt;/sup&gt;</th>
<th>YB-2805</th>
<th>Y-2156&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Y-7903&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. anomala var. anomal a Y-1773</td>
<td>97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. anomala var. anomal a Y-1783</td>
<td>93</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. anomala var. schneggi Y-102</td>
<td>97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. anomala var. schneggi Y-945</td>
<td>100</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>H. anomala var. schneggi Y-993&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. bimundalis var. bimundalis Y-5343&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. bimundalis var. bimundalis YB-2805</td>
<td></td>
<td>94</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. bimundalis var. americana Y-2156&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td></td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. bimundalis var. americana YB-2444</td>
<td></td>
<td></td>
<td>18</td>
<td>89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. nakazawae var. akitaensis Y-7904&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard deviation ≤ ±5% calculated from 2-6 determinations.

<sup>b</sup>Type strain.
does not. Wickerham (1965) also noted that hyphae of the variety *bimundalis* are about twice as long as hyphae of the variety *americana*. Both varieties are heterothallic. Intervarietal crosses form zygotes but ascospores are not produced. Fuson et al. (1979) noted that the G+C content of the variety *americana* was 2.5% higher than that of the variety *bimundalis*; on the basis of previous studies (Price et al., 1978), they suggested that the varieties might represent distinct species.

As seen in Table 2, the extent of DNA relatedness between the two varieties of *H. bimundalis* is only 19%, consistent with the suggestion that they are separate species. Unrelated species may be expected to show less than 10% DNA complementarity (Price et al., 1978; Kurtzman et al., 1980a, b), and the slightly greater percentage shown here is in keeping with the limited mating response. These data also indicate that this pair represents only recently evolved sibling species whose divergence may be attributed to allopatry. Earlier we had shown about 25% DNA relatedness between *Pichia amylophila* Kurtzman et al. and *P. mississippiensis* Kurtzman et al., heterothallic species that exhibited good interspecific mating, but which produced only poorly formed and infertile ascospores (Kurtzman et al., 1980a). A somewhat different relationship was reported for *Issatchenkia scutulata* (Phaff et al.) Kurtzman et al. var. *scutulata* and *I. scutulata* (Phaff et al.) Kurtzman et al. var. *exigua* (Phaff et al.) Kurtzman et al. where DNA relatedness between the varieties was about 25%, but a few fertile progeny resulted from the intervarietal crosses and varietal designations were maintained (Kurtzman et al., 1980b). Consequently, *H. bimundalis* provides one more example among the yeasts that the extent of DNA relatedness parallels mating competence and fertility, and that the resolution afforded by whole genome DNA comparisons goes no further than to sibling species.

In keeping with the data presented here, it is proposed that the variety *americana* be elevated to species level.

Hansenula *americana* (Wickerham) Kurtzman comb. nov. Basionym: *Hansenula bimundalis* Wickerham et Santa

The remaining taxa under study, Pichia nakazawae and its variety akitaensis, were described from Japan (Kodama, 1975). The variety nakazawae was isolated from exudate of Quercus myrsinaefolia Blume, whereas the variety akitaensis came from exudate of Salix sp. In standard physiological tests, the variety akitaensis differs from the variety nakazawae by its failure to ferment galactose, by the presence of a weak and slow sucrose fermentation, and by its inability to assimilate L-rhamnose and lactic acid. Another difference is the formation of moderately well-developed pseudohyphae by the variety akitaensis. In the present study, single-ascospore isolates from four-spored asci showed both varieties to be homothallic, thus complicating verification of relatedness through mating studies.

The extent of DNA relatedness between the varieties of P. nakazawae was 41% (Table 2). As discussed earlier, limited intervarietal fertility might be found even at this low level of DNA relatedness; for this reason, it is suggested that the varietal designations be maintained rather than elevating the variety akitaensis to specific rank.

DNA base sequence complementarity provides a means for assessing relatedness between taxa not amenable to resolution by other methods. Correlation of DNA relatedness with mating reactions is essential if results from this technique are to be equated with actual biological phenomena. To date, there have been few studies investigating this aspect of molecular evolution (Fuson et al., 1979; Kurtzman et al., 1980a, b), and the present work with H. bimundalis provides additional guidance. Work with other taxa will be required before firm guidelines can be established. However, it appears that the degree of mating response parallels the extent of base sequence divergence. Exceptions to this trend, not yet detected, would include amphidiploidy and such chromosomal changes that would genetically isolate populations despite relatively high DNA relatedness.
ACKNOWLEDGMENTS

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LITERATURE CITED


