Temperature Adaptation Markedly Determines Evolution within the Genus Saccharomyces

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The present study uses a mathematical-empirical approach to estimate the cardinal growth temperature parameters (Tmax, the temperature below which growth is no longer observed; Topt, the temperature at which the μmax equals its optimal value; μopt, the optimal value of μmax; and Tmax, the temperature above which no growth occurs) of 27 yeast strains belonging to different Saccharomyces and non-Saccharomyces species. S. cerevisiae was the yeast best adapted to grow at high temperatures within the Saccharomyces genus, with the highest optimum (32.3°C) and maximum (45.4°C) growth temperatures. On the other hand, S. kudriavzevii and S. bayanus var. uvarum showed the lowest optimum (23.6 and 26.2°C) and maximum (36.8 and 38.4°C) growth temperatures, respectively, confirming that both species are more psychrophilic than S. cerevisiae. The remaining Saccharomyces species (S. paradoxus, S. mikatae, S. arboricolaus, and S. cariocanus) showed intermediate responses. With respect to the minimum temperature which supported growth, this parameter ranged from 1.3°C for S. cariocanus) to 4.3°C (S. kudriavzevii). We also tested whether these physiological traits were correlated with the phylogeny, which was accomplished by means of a statistical orthogram method. The analysis suggested that the most important shift in the adaptation to grow at higher temperatures occurred in the Saccharomyces genus after the divergence of the S. arboricolaus, S. mikatae, S. cariocanus, S. paradoxus, and S. cerevisiae lineages from the S. kudriavzevii and S. bayanus var. uvarum lineages. Finally, our mathematical models suggest that temperature may also play an important role in the imposition of S. cerevisiae versus non-Saccharomyces species during wine fermentation.

The estimation of the temperature range in which microorganisms are able to grow is very important for the food industry, to guarantee food safety or optimize fermentative conditions, for example, but also in ecological and taxonomic studies to classify and identify the different species of microorganisms. In this way, several works have shown the marked importance of temperature for the growth of industrial yeasts (1, 4, 22, 24), as well as the influence of this environmental factor in determining the natural distribution of wild species (12, 21, 23, 25). Specifically, there is an increasing interest in determining the influence of temperature in the adaptation of Saccharomyces species to both wild and fermentative environments (8, 21, 25).

The Saccharomyces genus includes several species associated only with natural habitats (S. cariocanus, S. kudriavzevii, S. mikatae, S. paradoxus, and S. arboricolaus) and others that are present in both fermentative and wild habitats (S. cerevisiae and S. bayanus). The ability of the latter to ferment a broad range of beverages (cider, beer, and wines) and foods (bread, vegetables, etc.) (19) has unconsciously favored their selection by humans for thousands of years. It is thought that temperature could play an important role in the imposition and presence of S. cerevisiae in human activities (8). Several studies have shown that S. cerevisiae is well adapted to grow at higher temperatures, while other species, such as S. bayanus and S. kudriavzevii, are better adapted to grow at lower temperatures (2, 3, 11, 15, 22). However, there is a lack of quantitative information on this respect, and many of these studies do not include the whole biological temperature range in which Saccharomyces yeasts are able to grow. Thus, a more detailed study of the influence of temperature on Saccharomyces growth is necessary, including a larger number of strains isolated from different origins.

In this endeavor, predictive microbiology could be a very useful tool. This discipline uses mathematical models to quantitatively describe the behavior of microorganisms as a function of environmental variables (14). In the specific case of temperature, a primary model (usually a sigmoidal function) is first required to estimate the yeast growth parameters under diverse isothermal conditions. Then, a secondary model is necessary to appropriately describe the effects in the whole biotemperature range assayed (dynamic conditions). Fortunately, temperature has been a factor widely studied, and diverse secondary mathematical models are available in the references (14, 18, 29). Specifically, we have used in the present study the cardinal temperature model with inflection (CTMI) developed by Rosso et al. (20), because of the simplicity of its use and the easy and direct biological interpretation of results.

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The study of the evolution of a determined phenotype (in our case, yeast response versus temperature changes) within a taxon (Saccharomyces genus) is essential to understand the evolutionary history of the group. A meaningful approach for a comprehensive understanding is to first generate a well supported molecular phylogenetic tree of the group and then interpret the evolution of morphological traits in the light of this phylogeny. The evolution of the trait may be plastic and stochastic or, on the contrary, may evolve according to a trend tightly linked to the phylogeny. Methods have been developed recently to detect phylogenetic dependence in comparative data (6, 17). These tests have the advantage that they only use the topological structure of the tree. Specifically, the orthogram method developed by Ollier et al. (17) represents a relevant approach because it detects and characterizes phylogenetic dependence of quantitative data and, at the same time, provides a comprehensive understanding of the evolutionary history of the group. A meaningful approach for understanding the evolutionary history of a genus is to first generate a well supported molecular phylogenetic tree and then interpret the evolution of morphological traits in the light of this phylogeny. The evolution of the trait may be plastic and stochastic or, on the contrary, may evolve according to a trend tightly linked to the phylogeny. Methods have been developed recently to detect phylogenetic dependence in comparative data (6, 17). These tests have the advantage that they only use the topological structure of the tree. Specifically, the orthogram method developed by Ollier et al. (17) represents a relevant approach because it detects and characterizes phylogenetic dependence of quantitative data and, at the same time, highlights different patterns of evolution along a phylogenetic tree.

The main goal of the present study was to determine the whole biological temperature range in which the different Saccharomyces species are able to grow. For this purpose, primary (reparameterized Gompertz equation) and secondary (CTMI) models are used to fit experimental data collected at different isothermal conditions. We also evaluate the phylogenetic dependence of the CTMI parameters by means of a canonical procedure which allows variance decomposition along the phylogenetic tree (orthogram method). Finally, CTMI models are also used to describe the effect of temperature on a hypothet-

### MATERIALS AND METHODS

**Yeast strains and inoculum preparation.** A total of 27 yeast strains belonging to different Saccharomyces and non-Saccharomyces species were used in the present study. Yeasts were selected to obtain representative isolates from natural (10 strains) and fermentative (17 strains) habitats where possible. Their origins and designations are listed in Table 1.

Inocula were prepared by introducing one single colony from a pure culture of each strain into 5 ml of YM broth medium (Difco; Becton Dickinson Company, Sparks, MD). After 48 h of incubation at room temperature (25 ± 2°C [mean ± standard deviation]), 1 ml from each tube was centrifuged at 9,000 rpm for 10 min and the pellet washed with sterile saline solution

### TABLE 1. Origin and designation of the 27 yeast strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Straina</th>
<th>Origin (country)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>CECT 10131</td>
<td>Centaurea alba flower (Spain)</td>
</tr>
<tr>
<td></td>
<td>T73</td>
<td>Wine fermentation (Spain)</td>
</tr>
<tr>
<td></td>
<td>PE35 M</td>
<td>Masato fermentation (Peru)</td>
</tr>
<tr>
<td></td>
<td>CPE7</td>
<td>Sugarcane fermentation (Brazil)</td>
</tr>
<tr>
<td></td>
<td>KYOKAI/CBS 6412</td>
<td>Sake fermentation (Japan)</td>
</tr>
<tr>
<td></td>
<td>TEMOHAYA-MI26</td>
<td>Agave fermentation (Mexico)</td>
</tr>
<tr>
<td></td>
<td>Oq231</td>
<td>Wine fermentation (Portugal)</td>
</tr>
<tr>
<td></td>
<td>TTAaM</td>
<td>Wine fermentation (France)</td>
</tr>
<tr>
<td></td>
<td>PDMaM</td>
<td>Wine fermentation (Spain)</td>
</tr>
<tr>
<td></td>
<td>RVAaM</td>
<td></td>
</tr>
<tr>
<td><em>S. paradoxus</em></td>
<td>CECT 1939NT/CBS 432NT</td>
<td>Tree exudate (Russia)</td>
</tr>
<tr>
<td></td>
<td>120 M</td>
<td>Pulque fermentation (Mexico)</td>
</tr>
<tr>
<td></td>
<td>K54</td>
<td>Wine fermentation (Croatia)</td>
</tr>
<tr>
<td><em>S. bayanus var. uvarum</em></td>
<td>CA111</td>
<td>Carpinus betulus exudate (Hungary)</td>
</tr>
<tr>
<td></td>
<td>NCAIM 789</td>
<td>Wine fermentation (Spain)</td>
</tr>
<tr>
<td><em>S. kudriavzevii</em></td>
<td>CA111</td>
<td>Quercus ilex bark (Spain)</td>
</tr>
<tr>
<td></td>
<td>CR85</td>
<td>Quercus ilex bark (Spain)</td>
</tr>
<tr>
<td></td>
<td>CR89</td>
<td>Quercus faginea bark (Spain)</td>
</tr>
<tr>
<td><em>S. mikatae</em></td>
<td>NBRC 1815T/CBS 8839T</td>
<td>Soil (Japan)</td>
</tr>
<tr>
<td><em>S. arboricus</em></td>
<td>CBS 10644T</td>
<td>Quercus fabri bark (China)</td>
</tr>
<tr>
<td><em>S. caroticus</em></td>
<td>CBS 8841T</td>
<td>Fruit fly (Drosophila sp) (Brazil)</td>
</tr>
<tr>
<td>Non-Saccharomyces</td>
<td>Hanseniaspora uvarum CECT 10389</td>
<td>Wine fermentation (Spain)</td>
</tr>
<tr>
<td></td>
<td>Candida stellata CECT 11108</td>
<td>Wine fermentation (Spain)</td>
</tr>
<tr>
<td></td>
<td>Tordaspora delbueckii CECT 11199</td>
<td>Wine fermentation (Spain)</td>
</tr>
<tr>
<td></td>
<td>Kluyveromyces marxianus CECT 10585</td>
<td>Wine fermentation (Spain)</td>
</tr>
<tr>
<td></td>
<td>Pichia fermentans CECT 10064</td>
<td>Wine fermentation (Spain)</td>
</tr>
</tbody>
</table>

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* Superscript annotations: T, type strain; NT, neotype strain; L, commercial strain from Lallemand, Inc.; M, commercial strain from Mauri Yeast Australia.
triplicate. Therefore, a total of 891 growth curves (11 levels of temperature times 27 strains times 3 replicates) were obtained and analyzed.

**Primary modeling.** Growth parameters were obtained from each treatment by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwieten et al. (28), which was originally introduced in predictive microbiology by Gibson et al. (7). It has the following expression:

\[ y = D \times \exp\left(-\exp\left(\frac{\mu_{\text{max}} \times e}{D} \times (\lambda - t) + 1\right)\right) \]  

where \( y = \ln(O\bar{D}/O\bar{D}_0) \), with \( O\bar{D}_0 \) being the initial OD and \( O\bar{D} \) the OD at time \( t; \ D = \ln(O\bar{D}_\infty/O\bar{D}_0) \), with \( O\bar{D}_\infty \) being the maximum optical density reached; \( \mu_{\text{max}} \) is the maximum specific growth rate (h^{-1}); and \( \lambda \) is the lag phase period (h). Growth data from each experiment and strain were fitted by a nonlinear regression procedure, minimizing the sum of squares of the differences between experimental data and the fitted model, i.e., loss function (observed – predicted^2). This task was accomplished using the nonlinear module of the Statistica 7.0 software package (StatSoft, Inc., Tulsa, OK) and its Quasi-Newton option.

**Secondary modeling.** The cardinal temperature model with infection (CTMI) (18, 20) was used to describe the \( \mu_{\text{max}} \) changes of yeasts as a function of temperature \( T \) (°C). CTMI is a descriptive model purely based on empirical observations and includes the three cardinal temperature values often used in microbiology. It has the following expression:

\[
\begin{align*}
\mu &= 0 & \text{if } T < T_{\text{opt}} \quad \text{or } T > T_{\text{min}} \\
\mu &= \mu_{\text{max}}(D/E) & \text{if } T_{\text{opt}} < T < T_{\text{max}} \\
D &= (T - T_{\text{max}})(T - T_{\text{min}})^2 \\
E &= (T_{\text{opt}} - T_{\text{min}})(T_{\text{opt}} - T_{\text{max}})(T - T_{\text{opt}}) \\
&\quad - (T_{\text{opt}} - T_{\text{max}})(T_{\text{opt}} - T_{\text{min}} - 2T)
\end{align*}
\]

where \( T_{\text{max}} \) is the temperature above which no growth occurs, \( T_{\text{min}} \) is the temperature below which growth is no longer observed, and \( T_{\text{opt}} \) is the temperature at which the \( \mu_{\text{max}} \) equals its optimal value (\( \mu_{\text{max}}^\text{opt} \)). As in the case of the primary modeling, the CTMI parameters were estimated by a nonlinear regression procedure using the Statistica 7.0 software package. The adequacy of the fit was checked by the proportion of variance explained by the model (R^2) with respect to the experimental data.

**ANOVA.** First, strains were checked for significant differences among them by means of analysis of variance (ANOVA) using the one-way ANOVA module of Statistica 7.0 software. The dependent variables introduced for the analysis were the CTMI parameters obtained from secondary modeling. The post hoc comparison was carried out using the Scheffé test, which is considered to be one of the most conservative post hoc tests (27). An alternative advantage of the Scheffé test is that it can also be used with unequal sample sizes.

A second ANOVA was then performed by grouping the different Saccharomyces strains as a function of their respective species. In this way, the CTMI average parameters for the species \( S. \text{ceresii} \), \( S. \text{paradoxus} \), \( S. \text{bayanus} \), \( S. \text{kudriavzevii} \), and \( S. \text{uvarum} \) and \( S. \text{kudriavzevii} \) were estimated from data for 10, 3, 2, and 4 strains, respectively, isolated from diverse origins. In this way, the biological temperature range obtained for each species represents a general behavior rather than a single-strain trait.

**Identification of phylogenetic dependence.** We use the orthogram method developed by Ollier et al. (17) to determine the existence of dependence among the CTMI model parameters (\( T_{\text{opt}}, T_{\text{max}}, \mu_{\text{max}}^\text{opt} \), and \( \mu_{\text{max}}^\text{opt} \)) and Saccharomyces phylogeny. This method, based on the variance decomposition along the phylogenetic tree, considers the null hypothesis to be the complete absence of phylogenetic dependence and uses the following statistics to corroborate this hypothesis (17): R2Max, which tests whether a significant change in the quantitative trait appeared at one node of the phylogenetic tree while being conserved in the deriving branches; SKR3k, which assesses to what extent the variance distribution across the abiotic environmental tree is skewed to the root or to the tips; Dmax, which corresponds to the Kolmogorov-Smirnov statistic; and SCE, which measures the average local variation of the orthogam values. The method consists essentially of three steps. First, phylogenetic relations between the units of the tree are described by means of dummy variables taking values equal to 1 (for descendant tips within a node) or 0 (for the remaining tips). In a second step, these dummy variables are ordered in decreasing phylogenetic dissimilarity. Finally, since these dummy variables are not linearly independent, an orthornormal decomposition is necessary to obtain a matrix of linearly independent vectors (orthonormal vectors) which are used as regressors against CTMI model parameters. The method provides two graphical tools, called orthogram and cumulative orthogram, obtained by plotting the squared coefficients and the cumulative squared coefficients against the orthonormal vectors, respectively, that are very useful to interpret and identify the possible phylogenetic dependence. A complete description of the procedure and interpretation of statistics can be found in Ollier et al. (17) and Covain et al. (6).

Orthograms and associated tests were conducted using the ade4 package (5) in R 2.4.0 software (10).

The topology of the phylogenetic tree of the genus Saccharomyces (Fig. 1) was obtained from Wang and Bai (26), who used sequences of the ITS-5.8S ribosomal DNA (rDNA) region and 26S rDNA D1/D2 domains to determine the phylogenetic relationships among Saccharomyces species. The phylogenetic positions of \( S. \text{paradoxus} \) populations from America (in our case, strain \( S. \text{paradoxus} \) 120 M) and Europe (strains \( S. \text{paradoxus} \) 1939 and \( S. \text{paradoxus} \) K54) were obtained from Liti et al. (13). Torulaspora delbrueckii was included as the outgroup.

**RESULTS**

**Estimation of the temperature range where yeasts are able to grow.** In this work, we have studied and compared the response of 27 yeast strains in a wide range of temperature values (from 4 to 46°C). For this purpose, yeasts were monitored by means of OD measurements, and their respective biological growth parameters (\( \mu_{\text{max}} \) and \( \lambda \)) estimated at each value. A total of 891 growth curves were fitted using a nonlinear regression procedure with the reparameterized Gompertz equation proposed by Zwieten et al. (28), which represents an empirical approach for the estimation of the growth parameters. In all cases the fit was good, with an \( R^2 \) ranging from 0.95 to 0.99. Although the lag phase duration was also calculated for all experiments, it was not possible to appropriately model this parameter as a function of this environmental variable. Even at low temperatures, the lag phase of the different yeast species was very short, always below 15 h (data not shown).

However, changes in \( \mu_{\text{max}} \) as a function of temperature could be fitted well by means of the CTMI secondary model.

As an example, Fig. 2 shows the fit of the CTMI model to \( \mu_{\text{max}} \) experimental data obtained for the wild strains \( S. \text{ceresii} \) 10131 and \( S. \text{kudriavzevii} \) CR85. Clearly, a considerable difference was noticed between the two microorganisms, which was especially evident with respect to the optimum and maximum growth temperatures. In this way, \( S. \text{ceresii} \) 10131 was able to grow at up to 45°C and had its optimum growth around 33°C, with an estimated \( \mu_{\text{max}} \) at this temperature of 0.45 h^{-1}. Conversely, \( S. \text{kudriavzevii} \) CR85 was not able to grow at 37°C and showed an optimum growth around 24°C, with a \( \mu_{\text{max}} \) of 0.29 h^{-1}. Below 20°C, we can see that both fits are practically overlapped. The minimum temperature to support growth was around 4°C in both cases (Fig. 2).
An advantage of the use of the CTMI model is that all its parameters have biological meanings, so the interpretation of this type of model is very easy and direct. Table 2 shows the temperature-dependent parameters obtained with the CTMI model for the 27 yeast strains assayed in this work. For each strain, average values were obtained from 3 independent experiments. The Scheffe post hoc comparison test was used to distinguish significant differences among strains for each parameter. Totals of 13, 11, and 5 different groups were obtained for parameters $\mu_{opt}$, $T_{opt}$, and $T_{max}$, respectively. Conversely,

![FIG. 2. Fit of the cardinal temperature model to experimental data obtained for strains S. cerevisiae CECT10131 (squares) and S. kudriavzevii CR85 (circles). The quantitative parameters of both fits are shown in Table 2.](image)

<table>
<thead>
<tr>
<th>TABLE 2. Estimated parameters ($\mu_{opt}$, $T_{max}$, $T_{min}$, and $T_{opt}$) of the cardinal temperature model for the 27 yeast strains assayed in this work</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
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<tr>
<td>------------------</td>
</tr>
<tr>
<td>S. cerevisiae</td>
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<tr>
<td>S. paradoxus</td>
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<td></td>
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<tr>
<td>S. bayanus var. uvarum</td>
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<td></td>
</tr>
<tr>
<td>S. mikatae</td>
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<td></td>
</tr>
<tr>
<td>S. cariocanus</td>
</tr>
<tr>
<td>Non-Saccharomyces</td>
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</tbody>
</table>

a Standard deviations for each parameter (in parentheses) were obtained from 3 independent nonlinear fits. Values within the same column followed by different letters are significantly different according to a Scheffe post hoc comparison test.
no significant differences were found among strains in the case of \( T_{\text{min}} \). These parameters ranged from 0.096 (\( \text{Candida stellata} \)) to 0.449 h\(^{-1}\) (\( \text{S. cerevisiae} \)) for \( \mu_{\text{opt}} \), from 23.1 (\( \text{S. kudriavzevii CA111} \)) to 38.7°C (\( \text{Kluyveromyces marxianus} \)) for \( T_{\text{opt}} \), from 36.7 (\( \text{S. kudriavzevii CR89} \)) to 46.3°C (\( \text{K. marxianus} \)) for \( T_{\text{max}} \), and finally, from 0.4 (\( \text{S. cerevisiae TTA} \)) to 5.0°C (\( \text{S. cerevisiae PE35M} \)) for \( T_{\text{min}} \). Figure 3 provides a graphical representation of the biological temperature range in which yeast strains were able to grow. In general, the \( \text{S. cerevisiae} \) strains exhibited the highest maximum and optimum growth temperatures within the \( \text{Saccharomyces} \) genus, with the exception of strain \( \text{S. cerevisiae PE35M} \), which had a lower \( T_{\text{opt}} \) (~30°C). However, \( \text{S. kudriavzevii} \) strains clearly showed the lowest maximum and optimum growth temperatures, although these were very similar to the values obtained for the \( \text{S. bayanus var. uvarum} \) strains. With the exception of the species \( \text{K. marxianus} \), whose results showed it to be the most thermotolerant microorganism assayed in this work (see Table 2), the rest of the non-\( \text{Saccharomyces} \) yeasts exhibited optimum growth temperatures ranging from 24 to 27°C and maximum growth temperatures from 36 to 40°C.

The second ANOVA, grouping strains according to their respective \( \text{Saccharomyces} \) species, showed that \( \text{S. cerevisiae} \) was the species with the statistically highest \( T_{\text{opt}} \) and \( T_{\text{max}} \) values (average of 32.3 and 45.4°C, respectively) (Table 3). Conversely, \( \text{S. kudriavzevii} \) exhibited the lowest \( T_{\text{opt}} \) and \( T_{\text{max}} \) values (significantly different with respect to the rest of the \( \text{Saccharomyces} \) species in the case of \( T_{\text{opt}} \)), with 23.6 and 36.8°C, respectively. Again, no significant differences were found among yeasts for their \( T_{\text{min}} \) values. A clear and more intuitive interpretation of the overall response of the \( \text{Saccharomyces} \) species as a function of temperature can be obtained by the

**Table 3. Results of ANOVA for the parameters of the cardinal temperature model (\( \mu_{\text{opt}}, T_{\text{max}}, T_{\text{min}}, \) and \( T_{\text{opt}} \), grouping strains according to their respective \( \text{Saccharomyces} \) species)**

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains/no. of cases included in ANOVA</th>
<th>( \mu_{\text{opt}} ) (h(^{-1}))</th>
<th>( T_{\text{opt}} ) (°C)</th>
<th>( T_{\text{max}} ) (°C)</th>
<th>( T_{\text{min}} ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{S. cerevisiae} )</td>
<td>10/30</td>
<td>0.368 (0.051) B</td>
<td>32.27 (1.45) D</td>
<td>45.39 (1.17) D</td>
<td>2.84 (1.91) A</td>
</tr>
<tr>
<td>( \text{S. bayanus var. uvarum} )</td>
<td>2/6</td>
<td>0.295 (0.016) A</td>
<td>26.24 (0.69) B</td>
<td>38.36 (1.47) BC</td>
<td>2.37 (1.61) A</td>
</tr>
<tr>
<td>( \text{S. kudriavzevii} )</td>
<td>4/12</td>
<td>0.258 (0.039) A</td>
<td>23.61 (0.35) C</td>
<td>36.80 (0.12) C</td>
<td>4.29 (0.74) A</td>
</tr>
<tr>
<td>( \text{S. paradoxus} )</td>
<td>3/9</td>
<td>0.319 (0.031) AB</td>
<td>29.92 (0.73) A</td>
<td>41.55 (0.93) A</td>
<td>1.69 (1.25) A</td>
</tr>
<tr>
<td>( \text{S. mikatae} )</td>
<td>1/3</td>
<td>0.318 (0.002) AB</td>
<td>29.20 (1.46) A</td>
<td>40.20 (0.10) AB</td>
<td>1.79 (0.95) A</td>
</tr>
<tr>
<td>( \text{S. arboricolus} )</td>
<td>1/3</td>
<td>0.328 (0.009) AB</td>
<td>28.14 (0.61) AB</td>
<td>39.80 (0.04) AB</td>
<td>2.26 (0.99) A</td>
</tr>
<tr>
<td>( \text{S. cariocanus} )</td>
<td>1/3</td>
<td>0.329 (0.006) AB</td>
<td>28.81 (0.73) AB</td>
<td>41.79 (0.06) A</td>
<td>1.31 (1.10) A</td>
</tr>
</tbody>
</table>

* Values within the same column followed by different superscript letters are significantly different according to a Scheffé post hoc comparison test.
Saccharomyces species, exhibiting the highest parameter was obtained by dividing the parameter of interest by the maximum growth rate at that temperature. The minimum temperatures to support growth were very similar among all Saccharomyces species, although S. kudriavzevii showed a slightly but not significantly higher T_{min} value. We also studied a possible relationship among the CTMI model parameters obtained for the diverse Saccharomyces species. In this way, we found a linear correlation (R^2 always above 0.90) between T_{opt} and T_{max}, and T_{max} and μ_{opt} and T_{opt}. However, there was no correlation between T_{opt} and T_{min} or μ_{opt} and T_{min}. This means that the species which supported the highest growth temperatures also had the highest T_{opt} and μ_{opt} values, but species with the lowest T_{opt} and μ_{opt} values did not exhibit the minimum growth temperatures. This fact can also be graphically observed in Fig. 4.

Figure 5 shows the theoretical evolution of the μ_{max} ratios of the Saccharomyces species as a function of temperature. This parameter was obtained by dividing the μ_{max} of S. cerevisiae by the μ_{max} of each of the rest of the Saccharomyces species and vice versa. A ratio of 1 is indicative of both yeasts growing with similar μ_{max} at a specific temperature value. On the other hand, a ratio of 2 means that one yeast grows 2-fold faster than the other. Thus, this parameter provides valuable information on the effects of temperature on a hypothetical sympatric association between S. cerevisiae and the rest of the Saccharomyces species. In all cases, the results showed S. cerevisiae to be the most competitive yeast at high temperatures. The models show that S. cerevisiae grows faster than S. bayanus var. uvarum above 24°C, and at 35°C, its μ_{max} almost doubles compared to that of S. bayanus var. uvarum (Fig. 5a). In the rest of the comparisons, S. cerevisiae grows faster than S. kudriavzevii, S. paradoxus, S. mikatae, S. arboricolas, and S. cariocanus above 22, 19, 20, and 25°C, respectively (Fig. 5b, c, d, e, and f). Below these values, S. cerevisiae progressively grows more slowly than the other species and is less competitive. In fact, at 5°C, the μ_{max} of S. bayanus var. uvarum is double the μ_{max} of S. cerevisiae (Fig. 5a).

We also determined the influence of temperature on the growth of S. cerevisiae and non-Saccharomyces strains isolated from wine fermentations. Figure 6 shows the μ_{max} evolution of 5 commercial S. cerevisiae strains and 4 non-Saccharomyces wine yeasts as a function of temperature. The S. cerevisiae strains were able to grow at up to 45°C, and their T_{opt} was around 32°C, with a μ_{opt} ranging from 0.35 to 0.42 h^{-1} (Table 2). However, the strains of T. delbrueckii and Pichia fermentans were unable to grow above 40°C, while this value was even lower (around 36°C) for the species Hanseniaspora uvarum and Candida stellata. The optimum growth temperatures of these 4 strains were included in the interval from 24 to 27°C, with a μ_{opt} that was always lower than for S. cerevisiae strains (be-
between 0.096 and 0.235 h⁻¹). As can be deduced from the results in Fig. 6, at temperatures above 20°C, the difference between the μ_max of S. cerevisiae and the μ_max of non-Saccharomyces strains increased progressively.

Analysis of phylogenetic dependence. The CTMI model parameters μ_opt, T_opt, and T_max, which are quantitative variables, were tested using the orthogram approach (17) to determine a possible relationship with Saccharomyces phylogeny. We did not analyze T_min because no significant differences were observed among strains. The topology of the phylogenetic tree, with node identifications (left side), and the corresponding values for each tested variable and strain (right side) are shown in Fig. 7A. The orthogram analysis showed that the orthonormal vector representing node 3 (which differentiates S. arboricolus, S. mikatae, S. cariocanus, S. paradoxus, and S. cerevisiae from S. kudriavzevii and S. bayanus var. uvarum) explained the greatest part of the variance for the three parameters analyzed (Fig. 7B, C, and D). This vector peaked in all cases outside the confidence limits (represented by dashed lines in Fig. 7B, C, and D, upper panels) and showed a strong departure from the value expected under the hypothesis of absence of phylogenetic dependence (represented by a solid straight line). Cumulative variance representation confirmed the preponderance of the orthonormal vector 3 in the variance distribution, and again, a significant departure from the null hypothesis was registered for parameters μ_opt, T_max, and T_opt (solid line in Fig. 7C and D, lower panels). The maximum deviations from the expected values were given for the sum of the first three and six orthonormal vectors for the T_opt and T_max parameters, respectively (vertical arrows in Fig. 7C and D, lower panels). Phylogenetic dependence was also confirmed by the results of the statistical tests R2Max, SkR2k, D_max, and SCE. For the T_opt and T_max parameters, all test results were significant, showing P values lower than 0.0001. In the case of μ_opt, 3 of the 4 tests displayed significant values (0.042, 0.030, and 0.017 for R2Max, SkR2k, and D_max respectively). Only the SCE test was not able to indicate a significant departure from the null hypothesis, with a P value of 0.061. For the three CTMI parameters, the results of the R2Max test indicated that a significant part of the variance was explained by a single vector’s contribution, indicative of a single punctual modification event having occurred in the evolutionary history of the genus. Relating this result to the orthogram plots, we determined that the significant change in the variables occurred after the divergence of the S. arboricolus, S. cariocanus, S. mikatae, S. paradoxus, and S. cerevisiae lineages from the S. kudriavzevii and S. bayanus var. uvarum lineages (Fig. 7A, node 3). Moreover, the decomposed variance plot and the SkR2k statistics also indicated that a significant variance explanation occurred in nodes toward the root. However, according to the orthogram plot and the cumulative orthogram plot of parameters T_max and T_opt (Fig. 7C and D), a secondary modification of parameters could also have occurred at node 6, after the divergence of the S. paradoxus and S. cerevisiae lineages, although the statistics tests did not support the presence of this second event. In any case, these results suggest that these three parameters have been modeled during the evolution of the Saccharomyces genus.

DISCUSSION

The influence of temperature on microorganism growth has been widely studied by microbiologists, and different mathematical models have been developed to quantify and predict its effects. Rosso et al. (20) and, more recently, Oscar (18) compared several temperature secondary models on the basis of different criteria (simplicity and biological significance of pa-
parameters, applicability, quality of fit, and ease of determination of parameters), concluding that the CTMI model was better than its competitors to fit a total of 48 data sets belonging to different species of microorganisms. In the present study, the CTMI model was also very useful to fit the experimental growth data of 27 yeast strains in the whole biological temperature range.

*S. bayanus* var. *uvarum* and *S. kudriavzevii* are considered the most psychrotrophic species of the *Saccharomyces* genus (3, 11, 22). Our results corroborate these observations, and *S. bayanus* var. *uvarum* and *S. kudriavzevii* were the yeasts with the lowest *T*\text{opt} and *T*\text{max} values. Unfortunately, little information is available in the literature to carry out a quantitative comparison with data obtained in this study. Serra et al. (22) mentioned that the optimum growth temperature for the wine strain *S. bayanus* var. *uvarum* P3 was attained around 28°C, close to the values obtained from this work for the average of the two *S. bayanus* var. *uvarum* strains assayed (26.3°C). In the case of *S. kudriavzevii*, Arroyo-López et al. (2) estimated, using response surface methodology, that the optimum growth temperature of the type strain IFO 1802\textsuperscript{T} was attained at 24°C, while in this study, the average obtained for four *S. kudriavzevii* strains was 23.6°C. Belloch et al. (3) mentioned that both species were able to grow at 30°C but not at 37°C. Sampaio and Gonçalves (21) reported that the maximum growth temperatures for wild *S. kudriavzevii* and *S. bayanus* var. *uvarum* strains were 35°C and 36°C, respectively. In this work, the *T*\text{max} values estimated were slightly higher, 36.8°C for *S. kudriavzevii* and 38.3°C for *S. bayanus* var. *uvarum*.

Conversely, *S. cerevisiae* was the most thermotolerant species within the genus *Saccharomyces*, with the highest optimum (32.3°C) and maximum (45.4°C) growth temperatures. Several authors (2, 22) have reported that the optimum growth temperature of *S. cerevisiae* wine strains was around 34°C. The association of *S. cerevisiae* with fermentations established by humans is well known, and preliminary data suggest that temperature could play an important role in the predominance of this species. Heard and Fleet (9) observed that *S. cerevisiae* dominated traditional grape juice fermentations when they were carried out at higher temperatures. Recently, Goddard (8) also showed that temperature was an important factor in the imposition of *S. cerevisiae* versus non-*Saccharomyces* yeasts during wine fermentations. An increase of temperature from 16 to 23°C as a consequence of the highly vigorous respirofermentative consumption of sugars (Crabtree effect) favored the rapid growth of *S. cerevisiae* cells and final imposition of the species, although the initial frequency of this species was very low (<1%) (8). Such data clearly correlated with our results. We found that at temperatures above 20°C, the \( \mu \text{max} \) of the *S. cerevisiae* wine strains increased faster than the \( \mu \text{max} \) of the non-*Saccharomyces* wine species. These differences were especially evident at 32°C. Even under the optimum growth temperature conditions of the non-*Saccharomyces* species.
(~25°C), the \( T_{\text{max}} \) values of the \( S.\ cervisiae \) wine strains were always significantly higher.

Unfortunately, our knowledge of the ecology and distribution of the \( Saccharomyces \) species in wild environments is still very limited, but the present study could shed some light on the influence of temperature on the ecological interactions among \( Saccharomyces \) species. Diverse studies have shown that \( S.\ cervisiae \) and its sibling species \( S.\ paradoxus \) occupy the same ecological niches (oak exudates, oak bark, and oak-associated soils) in widely separated woodland sites (16, 23). Sweeney et al. (25) reported that the growth temperature profiles of diverse \( S.\ paradoxus \) and \( S.\ cervisiae \) wild strains, isolated from a single natural site, were different. \( S.\ paradoxus \) wild isolates exhibited \( T_{\text{opt}} \) values of around 30°C (similar to those presented in this work), while for \( S.\ cervisiae \), \( T_{\text{opt}} \) was above 37°C. Sampaio and Gonçalves (21) also carried out an interesting study on the influence of temperature on the sympatric association of four \( Saccharomyces \) species (\( S.\ cervisiae, S.\ paradoxus, S.\ bayanus\ var.\ uvarum, \) and \( S.\ kudriavzevii \)) isolated from oak bark samples. Their study showed that temperature played a fundamental role in the interactions among the \( Saccharomyces \) species. They suggested that circadian temperature changes could provide a range of temperatures, allowing the

![FIG. 7. Phylogenetic dependence analysis for the cardinal temperature model parameters (\( \mu_{\text{opt}}, T_{\text{max}}, \) and \( T_{\text{opt}} \)) of the 22 \( Saccharomyces \) strains. Torulaspora delbrueckii was used as an outgroup. (A) The topology of the phylogenetic relationships of the different strains is depicted on the left, and the dot plot of temperature model parameters for each strain on the right. Sc, \( S.\ cervisiae \); Sp, \( S.\ paradoxus \); Scar, \( S.\ cariocanus \); Smik, \( S.\ mikatae \); Sarb, \( S.\ arboricolus \); Sk, \( S.\ kudriavzevii \); Su, \( S.\ bayanus\ var.\ uvarum \); Td, Torulaspora delbrueckii. (B, C, and D) Variance decomposition using orthograms (top) and cumulative orthograms (bottom) for \( \mu_{\text{opt}}, T_{\text{max}}, \) and \( T_{\text{opt}}, \) respectively. In the orthogram plots, abscissas correspond to the vector numbers associated with the nodes indicated in the phylogenetic topology of panel A, while ordinates show the contribution of each vector to the variance of the parameter by the squared regression coefficients (positive in white and negative in gray). Dashed lines correspond to upper confidence limits at 95%, deduced from Monte Carlo permutations, and solid lines represent the mean value. In the cumulative orthogram plots, ordinates show the cumulated contribution of successive vectors to the variance. Diagonal solid lines represent the expected values in the absence of phylogenetic dependence. Dashed lines correspond to the bilateral 95% confidence intervals. Circles show the observed values of cumulated squared regression coefficients. Vertical arrows mark the maximum deviations from the expected values (diagonal lines).]
sympatric association involving a species more adapted to grow at high temperatures (S. cerevisiae or S. paradoxus) and another species more adapted to grow at low temperatures (S. bayanus var. uvarum or S. kudriavzevii). Our results also support the hypothesis that adaptation to grow at different temperatures could be a very important factor in the ecology of Saccharomyces species. Mathematical models developed in the present study reveal that S. cerevisiae grows faster than the rest of the Saccharomyces species at high temperatures but exhibits a loss of competitiveness at low temperatures. Our models estimate the limits of the temperatures at which S. cerevisiae grows faster than the other species. These temperature values are 24°C for S. cerevisiae with respect to S. bayanus var. uvarum, 22°C with respect to S. kudriavzevii, 19°C with respect to S. paradoxus, 20°C with respect to S. mikatae, 26°C with respect to S. arboricola, and 26°C with respect to S. cariocanus. In this way, in a hypothetical sympatric association between the species S. cerevisiae and S. kudriavzevii, if temperature were the only limiting factor, the growth of S. cerevisiae would be selectively favored over that of S. kudriavzevii at temperatures above 22°C. However, circadian temperature changes around this value would favor the growth of both species even in the same ecological niche. It is worth noting that this is a hypothetical case based on theoretical models. However, these predictions confirm previous studies of relative fitness tests carried out by Sampaio and Gonçalves (21) with the species S. cerevisiae and S. kudriavzevii; the second event could now be occurring in the S. cerevisiae lineage after its divergence from S. paradoxus and S. cariocanus, which would explain the higher thermostolerance exhibited by this species. Another interesting point, although it was not revealed by the phylogenetic dependence analysis, is the progressive adaptation of S. kudriavzevii to grow at lower temperatures. This was evidenced because S. bayanus var. uvarum, the first species to diverge within the Saccharomyces genus, exhibited higher $T_{\text{opt}}$ and $T_{\text{max}}$ values than S. kudriavzevii. In light of these results, temperature has influenced the evolution of the Saccharomyces genus, favoring the adaptation of some species to grow at lower (S. kudriavzevii) and higher (especially S. cerevisiae) temperatures.

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