Mode of vegetative reproduction of the bipolar budding yeast species *Wickerhamomyces pijperi* and related strains

Yumi Imanishi,^{1†} Sasitorn Jindamorakot,² Savitree Limtong³ and Takashi Nakase^{1,2}

Correspondence

Yumi Imanishi

Imanishi-yumi@office.chiba-u.jp

¹NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2-5-8, Katusakamatari, Kisarazu-shi, Chiba 292-0818, Japan

²National Center for Genetic Engineering and Biotechnology (BIOTEC) NSTDA, Thailand Science Park, 113 Phaholyothin Rd, Klong 1, Klong Luang, Pathumthani 12120, Thailand

³Department of Microbiology, Faculty of Science, Kasetsart University, 50 Phaholyothin Rd, Bangkok 10900, Thailand

To clarify the budding pattern of *Wickerhamomyces pijperi*, the vegetative cells were observed by scanning electron microscopy. The cells grew by bipolar budding, but cells that budded from the shoulder of a mother cell were occasionally observed. We examined the cell morphology and phylogeny of five strains of *Wickerhamomyces* sp. isolated in Thailand as well as seven *W. pijperi* and three *Wickerhamomyces* sp. strains that were preserved in culture collections. Phylogenetic analysis based on three different nucleotide sequences (D1/D2 domain of 26S rDNA, the actin gene *ACT1* and the elongation factor 2 gene *EF2*) indicated that all the strains belonged to the genus *Wickerhamomyces* and were neighbours of the type strain *W. pijperi* NBRC 1290^T. The strains fell into two groups in this analysis. The budding patterns of the strains were carefully observed by staining the bud scars, and these patterns were categorized into three groups: types I–III. Type I included cells that grew by bipolar budding and formed multiple scars, type III included cells that grew by multilateral budding and formed a single scar, and type II included cells that exhibited a mixture of type I and type III patterns. Among the 15 strains, 12 strains, including *W. pijperi* NBRC 1290^T, mainly exhibited type I or type II budding patterns; these strains belonged to group 1 of the phylogenetic analysis. The remaining three strains, which belonged to group 2, exhibited either type II or type III patterns. Thus the phylogenetic relationship and budding patterns are related. Moreover, some cells also exhibited budding characteristics that were intermediate between bipolar and multilateral budding.

Received 16 March 2009

Revised 24 May 2009

Accepted 30 May 2009

INTRODUCTION

The taxonomy of *Wickerhamomyces pijperi* has long been a matter of debate because this species reproduces by bipolar budding (Ditlevsen & Hjort, 1964; Kreger-van Rij, 1984; van der Walt & Tscheuschner, 1957). *W. pijperi* was first described by van der Walt & Tscheuschner (1957), who isolated a yeast from buttermilk and proposed it to be *Pichia pijperi* on the basis of its biochemical and

physiological characteristics. Later, Ditlevsen & Hjort (1964) categorized it under the genus *Hanseniaspora* because they found that this isolate reproduced by bipolar budding, which is the major characteristic of this genus. Kreger-van Rij (1984) concluded that this species belonged to the genus *Pichia* because its budding pattern differed from that of *Hanseniaspora* when observed by transmission electron microscopy. Kurtzman *et al.* (2008) reorganized the genera *Pichia*, *Issatchenkia* and *Williopsis* into three different genera – *Barnettozyma*, *Lindnera* and *Wickerhamomyces* – based on the analysis of three genes coding for large- and small-subunit rRNAs and the translation elongation factor 1 α . Thus, *W. pijperi* was reclassified in the new genus *Wickerhamomyces*, which comprises 17 species, even though *W. pijperi* reproduces by bipolar budding and not by multilateral budding as observed in other *Wickerhamomyces* species.

[†]Present address: Medical Mycology Research Center (MMRC), Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8673, Japan.

Abbreviation: SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the sequence data for this study are: D1/D2, AB449691–AB449700; *EF2*, AB449701–AB449717; *ACT1*, AB449718–AB449733.

A supplementary table of strains is available with the online version of this paper.

Cell polarity has been the subject of many studies because it is an essential character not only for the development of an individual organism but also for the evolution of species in all organisms (Harkins *et al.*, 2001; Nanninga, 2001; Ni & Snyder, 2001; Powell *et al.*, 2003; Tsai *et al.*, 2008). In taxonomic studies of yeast, the budding pattern is one of the key characteristics considered in the classification of genera. For example, bipolar budding and the formation of multiple scars are characteristics of the genera *Hanseniaspora*, *Kloeckera*, *Nadsonia*, *Saccharomyces*, *Schizoblastosporion* and *Wickerhamia* (Kurtzman, 1998a, b).

In this study, we carefully examined the cell morphology of *W. pijperi* and related strains and found that some strains exhibited characteristics that were intermediate between bipolar and multilateral budding.

METHODS

Strains. The yeast strains used in this study are listed in Supplementary Table S1, available with the online version of this paper. *Candida solani* NBRC 0762^T was used as a reference strain; it is phylogenetically very closely related to *W. pijperi*. *Saccharomyces ludwigii* NBRC 0798^T and *Hanseniaspora valbyensis* NBRC 10834^T were used as model species showing bipolar budding.

Wickerhamomyces sp. strains BCC 7703, 7704, 7730, 11810 and 15138 were isolated using the methods described by Jindamorakot *et al.* (2004). *W. pijperi* strains NBRC 1290^T, 1791 and 1887, and *Wickerhamomyces* sp. strains NBRC 10040, 10041 and 10042 have been preserved in a culture collection at the National Institute of Technology and Evaluation (NITE), Biological Resource Center (NBRC), Japan. *W. pijperi* strains NBRC 102058, 102059, 102060 and 102061 were obtained from the Centraalbureau voor Schimmelcultures (CBS), The Netherlands.

Nucleotide sequence and phylogenetic analysis. A loopful of cells was inoculated into 5 ml yeast-peptone-dextrose (YPD) broth (10 g yeast extract l⁻¹, 20 g glucose l⁻¹, 20 g peptone l⁻¹) and grown at 25 °C overnight with shaking at 120 r.p.m. Cells were collected in a 2 ml test tube by centrifugation and used for genomic DNA preparation. Genomic DNA was extracted with Dr GenTLE for Yeast (Takara), according to the manufacturer's protocol. PCR was performed according to methods described for the amplification of the D1/D2 domain of 26S rDNA (D1/D2) (Kurtzman & Robnett, 1997). The actin gene *ACT1* and the elongation factor 2 gene *EF2* were amplified with KOD FX polymerase (Toyobo Co.) using the primers *ACT1* (5'-TACCCAATTGAACACGGTAT-3') and *ACT2* (5'-TCTGAATCTTTCGTTACCAAT-3') for *ACT1* and EF1F1 (5'-AAGTCTCCAAACAAAGCATAAC-3') and EF1R2 (5'-GGGAAA-GCTTGACCACCAGTAGC-3') for *EF2*. PCR was performed according to the method described by Diezmann *et al.* (2004). The PCR products were purified with AMPure (Agencourt Bioscience Co.), according to the manufacturer's protocol. Sequence analysis was performed as described previously (Imanishi *et al.*, 2007). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using the CLUSTAL W software (Thompson *et al.*, 1994). Bootstrap values (Felsenstein, 1985) were calculated from 1000 trials. The sequence data were deposited in the GenBank database under the following accession numbers: D1/D2, AB449691–AB449700; *EF2*, AB449701–AB449717; and *ACT1*, AB449718–AB449733.

Observation of the surface structures of vegetative cells. A loopful of cells was inoculated into 5 ml YPD broth and grown at

25 °C for 1 day. The cells were collected and washed twice with 1 ml phosphate buffer (0.1 mol l⁻¹; pH 7.2). The cells were then fixed and dehydrated using a method described previously (Mikata & Nakase, 1997). The surface structures were observed by scanning electron microscopy (SEM; model S-5200; Hitachi).

Bud scar staining and observations of budding sites. Cells were grown on a yeast extract-mannitol (YM) slant (10 g glucose l⁻¹, 5 g peptone l⁻¹, 3 g yeast extract l⁻¹, 3 g malt extract l⁻¹, 15 g agar l⁻¹) at 25 °C for 3 days. A loopful of cells was inoculated into 5 ml YPD broth and grown at 25 °C for 1 day with shaking at 120 r.p.m. Then, 0.5 ml of the culture was transferred into 5 ml YPD broth and grown at 25 °C for 5–6 h with shaking at 120 r.p.m. The cells were washed with 1 ml distilled water and fixed with 50% (w/v) ethanol. The chitin components of the cell wall were stained with Fungiflora Y (Biomate Inc.), according to the manufacturer's protocol. The morphology of the cells and the stained bud scars were observed using a microscope with an Axioplan 2 imaging system (Carl Zeiss). Only cells that had undergone at least four budding events were examined. For each sample, approximately 300 cells were counted during at least three independent experiments, and the mean and standard deviation of the cell number were calculated.

RESULTS AND DISCUSSION

To clarify the budding pattern of *W. pijperi*, the vegetative cells of the type strain NBRC 1290^T were observed by SEM and were compared with those of *C. solani*, *Saccharomyces ludwigii* and *H. valbyensis*. Fig. 1(a, b) shows the daughter cells of *W. pijperi* NBRC 1290^T just prior to budding at exactly the same site from where the elder daughter cells had budded and separated, leaving multiple scars (arrows); the daughter cells of this strain were sometimes found to bud from the shoulder of the mother cell (Fig. 1c). This shows that daughter cells are produced by a mixed mode of budding: bipolar and multilateral. On the other hand, *C. solani* NBRC 0762^T cells never bud at the same site as the older cells (Fig. 1d, e) and reproduce only by multilateral budding as observed in the case of *Saccharomyces cerevisiae* and other species of the *Wickerhamomyces* clade, except for *W. pijperi*. *Saccharomyces ludwigii* NBRC 0798^T and *H. valbyensis* NBRC 10834^T both exhibit bipolar budding characteristics and have multiple scars at the same site with the birth site and the old budding site (Fig. 1f, g). *H. valbyensis* occasionally exhibited multilateral budding, but this was not observed in *Saccharomyces ludwigii*.

Five strains of *Wickerhamomyces* sp. that were isolated in Thailand and 10 *W. pijperi*/*Wickerhamomyces* sp. strains that were deposited in culture collections were subjected to phylogenetic analysis. According to the nucleotide sequences of D1/D2, the strains were all found to be related to *W. pijperi* but were classified into two groups (Fig. 2): group 1 contained NBRC 1290^T, NBRC 1791, NBRC 1887, NBRC 102058, NBRC 102059, NBRC 102060, NBRC 102061, BCC 7703, BCC 7704, BCC 7730, BCC 11810 and BCC 15138; group 2 contained NBRC 10040, NBRC 10041 and NBRC 10042. When the sequences of *EF2* and *ACT1* were considered, these strains were found to be segregated into the same two groups as derived by the

Cell morphology of *W. pijperi* Cell morphology of *C. solani*

Typical genera having characteristics of bipolar budding

Fig. 1. Scanning electron micrographs of vegetative cells grown on YM agar for 1 week at 25 °C. (a, b, c) *W. pijperi* NBRC 1290^T, (d, e) *C. solani* NBRC 0762^T, (f) *Saccharomyces ludwigii* NBRC 0798^T, (g) *H. valbyensis* NBRC 10834^T. Scales are indicated in the micrographs.

D1/D2 sequences; however, there were minor differences in the branching pattern within each group (Fig. 3). The nucleotide differences of the three genes were compared against NBRC 1290^T and are summarized in Table 1. In group 2, more than 20 differences were observed but the number of differences was smaller than that for each of the genes in *C. solani*. This finding may suggest that *W. pijperi* is independent from group 2.

The budding patterns of the strains were examined by the Fungiflora Y-stained bud scars. The cells were classified into the three following types depending on the budding patterns: type I, bipolar budding, wherein the daughter cells bud at exactly the same site from where the older cells were produced and separated, leaving multiple scars; type II, the daughter cells bud not only at the same site from where the older daughter cells were produced and

Fig. 2. Phylogenetic relationships of *Wickerhamomyces* species inferred from the nucleotide sequences of the D1/D2 domain of 26S rDNA by the neighbour-joining method. Bootstrap values were calculated from 1000 replicates, and values below 50 % were omitted. All the sequences were edited to the longest common region (540 bp). The GenBank sequence accession numbers are shown in parentheses. The bar indicates a sequence dissimilarity value of 0.01 substitution per site. *Starmera caribaea* NRRL Y-17468^T was used as an outgroup.

separated but also from the shoulder of the mother cell, i.e. a mixture of bipolar budding and multilateral budding; type III, multilateral budding (Fig. 4). The results are summarized in Table 1. In group 1, more than 90 % of the observed cells were classified as either type I or type II, and a very small number of cells exhibited the type III budding

pattern. On the other hand, more than 90 % of the cells in group 2 exhibited type II or type III budding patterns, even though a certain proportion of the population reproduced by bipolar budding. We confirmed the differences in the two groups among the different species. *C. solani* NBRC 0762^T cells exhibited only type III budding, and no

Fig. 3. Phylogenetic relationships inferred from the nucleotide sequences of *EF2* (a) and *ACT1* (b) by the neighbour-joining method. Bootstrap values were calculated from 1000 replicates, and values below 50 % were omitted. The sequences of *EF2* were edited to a region of 501 bp, and sequences of *ACT1* to 490 bp, each of which was the longest common region. The bars indicate a sequence dissimilarity value of 0.002 substitution per site in *EF2* and 0.005 substitution per site in *ACT1*. *Candida solani* NBRC 0762^T was used as an outgroup.

Table 1. Nucleotide differences compared to *W. pijperi* NBRC 1290^T and budding pattern

Strain	Gene			Budding pattern (%) [*]		
	D1/D2	<i>ACT1</i>	<i>EFH</i>	Type I	Type II	Type III
Group 1						
NBRC 1290 ^T	0	0	0	44.9 ± 3.6	51.0 ± 5.5	4.1 ± 6.3
BCC 7703	0	4	4	71.2 ± 13.0	28.5 ± 13.1	0.3 ± 0.6
BCC 7704	0	4	4	52.0 ± 6.2	47.8 ± 6.2	0.3 ± 0.5
BCC 7730	2	4	4	40.3 ± 22.4	52.0 ± 17.5	7.7 ± 5.1
BCC 11810	3	3	5	56.4 ± 4.4	43.6 ± 4.4	0 ± 0
BCC 15138	2	3	0	68.6 ± 12.7	30.1 ± 12.0	1.3 ± 2.3
NBRC 1791	7	0	1	49.2 ± 6.2	47.5 ± 1.7	3.3 ± 5.7
NBRC 1887	8	0	1	48.2 ± 2.9	49.1 ± 1.5	2.7 ± 3.8
NBRC 102058	3	6	2	68.5 ± 22.0	29.8 ± 21.5	1.7 ± 2.9
NBRC 102059	6	5	4	42.2 ± 21.0	55.1 ± 16.6	2.6 ± 4.6
NBRC 102060	6	6	5	37.0 ± 22.0	59.7 ± 24.8	3.3 ± 3.5
NBRC 102061	6	0	4	44.4 ± 12.3	55.6 ± 12.3	0 ± 0
Group 2						
NBRC 10040	23	36	20	6.2 ± 3.4	29.0 ± 13.0	64.9 ± 15.6
NBRC 10041	23	37	20	2.9 ± 2.8	24.7 ± 4.7	72.4 ± 6.0
NBRC 10042	23	37	20	9.8 ± 4.8	56.9 ± 8.4	33.3 ± 12.0
<i>C. solani</i>						
NBRC 0762 ^T	24	38	22	0 ± 0	0 ± 0	100 ± 0

^{*}Mean ± SD of cell numbers counted from at least three independent experiments.

Fig. 4. Photographs of the budding patterns of *W. pijperi* NBRC 1290^T. Cells with more than four bud scars were classified as type I, exhibiting bipolar budding (a), type II, exhibiting a combination of type I and type II patterns (b), and type III, exhibiting multilateral budding (c).

multiple scars were observed either by bud scar staining or by SEM (Fig. 1d, e). Comparison of the budding mode and the phylogenetic relationship revealed that the findings of the phylogenetic analysis and the budding patterns were closely related (Fig. 1, Table 1). In the phylogenetic tree, other species of the *Wickerhamomyces* clade, including *C. solani* NBRC 0762^T, exhibited only multilateral budding. These results suggest that *W. pijperi* belongs to a genus other than *Wickerhamomyces*. Moreover, the budding pattern of group 2 appears to be intermediate between multilateral and bipolar budding.

Studies on the cellular biology of yeast have revealed that cell polarization involves the asymmetrical organization of the cytoskeleton, secretory system, etc. (Nanninga, 2001; Ni & Snyder, 2001; Tsai *et al.*, 2008). *Saccharomyces cerevisiae* cells reproduce by multilateral budding, and the cells have polarity. In the case of typical a/α haploid strains, budding is observed at axial sites, and mother cells form new buds near the bud scar, while daughter cells form new buds near the birth scar (Ni & Snyder, 2001). In the case of a/α diploid strains, daughter cells bud at distal poles (180° from the birth site), whereas mother cells bud either distal or proximal to the birth site. In *W. pijperi* and *C. solani*, budding occurred at the axial site, at the preceding division site, or at the opposite pole (Figs 1 and 4). Thus, the budding modes of *W. pijperi* or *C. solani* differ from that of *Saccharomyces cerevisiae*.

Many budding pattern mutants have been developed and investigated to clarify the mechanism underlying polarized cell division (Ni & Snyder, 2001). The phenotypes of the mutants were of three types – unipolar, axial-like and random – and several genes were involved in budding site selection. None of the mutants exhibited bipolar budding. Although the birth scars observed in aged *Saccharomyces cerevisiae* cells resembled a double ring of chitin (Powell *et al.*, 2003), the budding pattern seems to be different from the pattern of bipolar budding. Unknown genes may tightly regulate budding site selection in bipolar budding cells, and the budding pattern seems to be a major key character considered for yeast taxonomy.

ACKNOWLEDGEMENTS

We are very grateful to Dr K. Tanaka of NBRC for providing valuable suggestions for our manuscript. We also thank Mr Tanaka, Miss Toyama and Mr Ninomiya for providing technical support.

REFERENCES

- Diezmann, S., Cox, C. J., Schönián, G., Vilgalys, R. J. & Mitchell, T. G. (2004). Phylogeny and evolution of medical species of *Candida* and related taxa: a multigenic analysis. *J Clin Microbiol* **42**, 5624–5635.
- Ditlevsen, E. & Hjort, A. (1964). *Hanseniaspora pijperi* (van der Walt et Tschuschner) nov. comb. *C R Trav Lab Carlsberg* **34**, 347–357.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Harkins, H. A., Page, N., Schenkman, L. R., Virgilio, C. D., Shaw, S., Bussey, H. & Pringle, J. R. (2001). Bud8p and Bud9p, proteins that may mark the sites for bipolar budding in yeast. *Mol Biol Cell* **12**, 2497–2518.
- Imanishi, Y., Ueda-Nishimura, K. & Mikata, K. (2007). Two new species of *Kazachstania* that form ascospores connected by a belt-like intersporal body: *Kazachstania zonata* and *Kazachstania gamospora*. *FEMS Yeast Res* **7**, 330–338.
- Jindamorakot, S., Am-in, S., Thuy, T. T., Duy, N. D., Kawasaki, H., Potacharoen, W., Limtong, S., Tanticharoen, M. & Nakase, T. (2004). *Candida easanensis* sp. nov., *Candida pattaniensis* sp. nov. and *Candida nakhonratchasimensis* sp. nov., three new species of yeasts isolated from insect frass in Thailand. *J Gen Appl Microbiol* **50**, 261–269.
- Kreger-van Rij, N. J. W. (1984). *Pichia* Hansen. In *The Yeasts, a Taxonomic Study*, 2nd revised and enlarged edn, pp. 455–554. Edited by J. Lodder. Amsterdam: North-Holland Publishing.
- Kurtzman, C. P. (1998). Discussion of teleomorphic and anamorphic ascomycetous yeasts and a key to genera. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 111–121. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Kurtzman, C. P. (1998). *Pichia* E.C. Hansen emend. Kurtzman. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 273–352. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Kurtzman, C. P. & Robnett, C. J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* **35**, 1216–1223.
- Kurtzman, C. P., Robnett, C. J. & Basehoar-Powers, E. (2008). Phylogenetic relationships among species of *Pichia*, *Issatchenkia* and *Williopsis* determined from multigenic sequence analysis, and the proposal of *Barnettozyma* gen. nov., *Lindnera* gen. nov. and *Wickerhamomyces* gen. nov. *FEMS Yeast Res* **8**, 939–954.
- Mikata, K. & Nakase, T. (1997). Surface structure of ascospores of genus *Nadsonia* Sydow. *Microbiol Cult Collect* **13**, 97–102.
- Nanninga, N. (2001). Cytokinesis in prokaryotes and eukaryotes: common principles and different solutions. *Microbiol Mol Biol Rev* **65**, 319–333.

Ni, L. & Snyder, M. (2001). A genomic study of the bipolar bud site selection pattern in *Saccharomyces cerevisiae*. *Mol Biol Cell* **12**, 2147–2170.

Powell, C. D., Quain, D. E. & Smart, K. A. (2003). Chitin scar breaks in aged *Saccharomyces cerevisiae*. *Microbiology* **149**, 3129–3137.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W improving the sensitivity of progressive multiple sequence alignment

through sequence weighting position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tsai, P. C., Lee, S. W., Liu, Y. W., Chu, C. W., Chen, K. Y., Ho, J. C. & Lee, F. J. S. (2008). Afilp functions as an Arf3p polarization-specific docking factor for development of polarity. *J Biol Chem* **283**, 16915–16927.

van der Walt, J. P. & Tscheuschner, I. T. (1957). Three new yeasts. *Antonie Van Leeuwenhoek* **23**, 184–190.

Edited by: D. Burke