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Identification of phylloplane yeasts from paper mulberry (*Broussonetia papyrifera* (L.) L'Hér. ex Vent.) in Java, Indonesia

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ABSTRACT

Aims: *Broussonetia papyrifera* (Saeh plant) has many qualities, the inner bark was the material for 'dluwang' papers on which Indonesian historical manuscripts were written, and the leaves have bioactive constituents of medicinal value, and antifungal activities. We investigated the diversity of yeast species associated with leaves from 6 months and 1.5 year-old plants, which is prerequisite to understand the phylloplane yeasts and plant interaction.
Methodology and results: The yeasts were isolated from fresh leaves by washing and membrane filtration methods. A total of 16 leaf samples of 6 months and 1.5 year-old plants were collected from four locations in Java, Indonesia, and 2,543 yeast isolates were obtained. Based on similarity of colony morphology, 82 representative yeast isolates were selected and identified based on the sequence analyses of internal transcribed spacer (ITS) regions of rDNA. The identification result showed that they consisted of 17 genera and 32 species. Thirty six of representative yeast isolates belong to 11 genera (18 species) of the phylum *Ascomycota* and forty six isolates belong to 6 genera (14 species) of the phylum *Basidiomycota*. Phylogenetic trees showed that the yeast isolates are phylogenetically diverse and distributed in the phyla of *Ascomycota* (classes *Saccharomycetales* and *Dothideomycetes*) and *Basidiomycota* (classes *Microbotryomycetes*, *Tremellomycetes*, and *Ustilaginomycetes*).
Conclusions, significance and impact of study: The phylloplane yeasts of *B. papyrifera* (Saeh plant) were taxonomically heterogeneous. This is the first report of the isolation and identification of phylloplane yeasts from *B. papyrifera*. Phylloplane yeasts may possess antagonistic activity to fungal plant pathogens in their natural habitats.

Keywords: Broussonetia papyrifera, ITS regions of rDNA, phylloplane yeast

INTRODUCTION

Broussonetia papyrifera (L.) Vent. (*Moraceae*) (paper mulberry) is a plant which grows naturally in Asian and Pacific countries (Thailand, China, Myanmar, Laos, Japan, Korea, and Indonesia) (Whistler and Elevitch, 2006). In Indonesia this plant was commonly found in Java, Sulawesi, Kalimantan, Bali, New Guinea, and Maluku islands (Permadi, 2010). This plant is known by several vernacular names, e.g. 'saeh' (Sunda), 'galugu' or 'glugu' (Java), 'dhalubang or dhulubang' (Madura), 'sepukau' (Basemah, Sumatera), 'kembala, 'rowa' (Sumba), and 'linggowas' (Banggai) (Permadi, 1998).

Broussonetia papyrifera has been widely exploited by humans (Whistler and Elevitch, 2006). The most significant part of this plantis its strong fibrous bark which was used as a raw material for paper-making and textiles for centuries throughout East and Southeast Asia, and Polynesia (Teijgeler, 1995; Permadi, 2005). According to Teijgeler(1995), in the past the inner bark of *B. papyrifera* was used by the Indonesians for making high-quality paper known as 'daluang' ('dluwang'). Islamic manuscripts are written especially on 'daluang'. At the turn of last century the only production centres left were Tunggilis Village, Garut (West Java) and Tegalsari, Ponorogo (East Java). After World War II only one family in Tunggilis, near Garut, was still engaged in making 'daluang'. Teijgeler (2000) reported that 'daluang' was used for clothing in Java. Old Javanese literature mentioned that in the pre-Islamic era, roughly before A.D. 1550, clothes made from 'daluang' were worn by clergymen, especially ascetics. Oetari *et al*. (2010) stated that nowadays, the plant is in scarcity and the traditional knowledge for 'daluang' paper making is not known anymore.

All parts of this plant (root, leaves, bark, and fruit) are used in traditional Chinese medicines (Sun *et al*., 2012).

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At present there is an increasing interest in findings the bioactive constituents of medicinal value from all parts of *B. papyrifera*. Lee *et al*. (2001) found aromatase inhibitors from the whole parts of *B. papyrifera*. Various types of flavonoids (flavans, flavanones, chalcones, and flavones) were found in the roots of *B. papyrifera* (Ko *et al.*, 1998) and antimicrobial activity were identified (Ho-Yong *et al*., 2010). Xu *et al*. (2010) reported antioxidant activity in the stem bark. Antifungal activity (Zafar *et al*., 2002) and antioxidant activity (Xu *et al*., 2010) were found from the leaves. The leaves of *B. papyrifera* can be used as food for both human and animal consumption (Whistler and Elevitch, 2006). Sun *et al*. (2012) reported chemical composition and antioxidant activity from the fruit of *B. papyrifera.* However, there was only one report on isolation of microorganisms from *B. papyrifera* and there is no information on the bioactive compounds isolated from its microorganisms. De Errasti *et al*. (2010) isolated fungal endophytes from *B. papyrifera* and they found *Acremonium* Link., *Cladosporium* Link., *Penicillium* Link. ex. Fr., and *Trichoderma hamatum* (Bonord.) Bain. At present, there is no report on yeasts isolated from this plant.

Plant surfaces have been recognized as important habitats for yeasts (Hong *et al*., 2002; Fonseca and Inácio, 2006; Crestani *et al*., 2009). There were many reports on the occurence of yeasts on plants. Ahansal *et al*. (2008) reported that *Bullera variabilis* Nakase Her. & M. Suzuki, *Rhodotorula glutinis* (Fresenius) F.C. Harrison, and *Pichia angusta* (Teunisson, Hall, & Wickerman) Kurtzman, were found on the leaf surface of *Argania spinosa* Skeels. Nix-Stohr *et al.* (2008) reported that plant leaves of *Festuca sasanqua* Schreb were dominated by yeasts of the genera of the phylum *Basidiomycota*, especially *Cryptococcus* Vuillemin and *Sporobolomyces* Kluyver & van Niel. Carvajal *et al*. (2006) reported that yeasts are common on the surfaces of leaves with the most common genera being *Candida* Berkhout, *Cryptococcus*, *Pichia*, *Rhodotorula*, and *Trichosporon* Behrend. Sjamsuridzal *et al*. (2010) reported the phylloplane yeasts in Indonesia,and they found the following genera from various plant leaves e.g. *Aureobasidium*, *Cryptococcus*, *Pseudozyma* Bandoni emend. Boekhout, *Rhodotorula*, *Sporidiobolus* Nyland, and *Ustilago* (Pers.) Roussel. So far, there is no report on the phylloplane yeasts from *B. papyrifera.*

In this study, we isolated yeasts from fresh leaves of *B. papyrifera*, collected from plants of different ages (6-month and 1.5-year old) and various locations in Java island, Indonesia. Identification of the isolated yeasts was carried out based on the sequence data of ITS regions of rDNA.

MATERIALS AND METHODS

Samples and sampling location

Samples of fresh leaves of *B. papyrifera* were obtained from 6-months and 1.5 year-old plants, the age of plant that was used by 'daluang' artists for making high quality paper. Each leaf sample was the fifth from the top of the main branch, and was selected for yeast isolation because it represented mature or nearly mature leaf which may provide more exudates and higher nutrient source than younger or senescent leaves. A total of sixteen leaf samples were collected for yeast isolation. Leaf samples were collected at four locations (Figure 1), e.g. Dago Pojok Village, Bandung (West Java) (April, 2009); Sukadanu Village, Garut (West Java) (April, 2009 and March, 2011); Tunggilis Village, Garut (West Java) (April, 2009); and Bejijong Village, Trowulan (East Java) (July, 2009).

Isolation of yeasts

Yeasts were isolated from fresh leaves by washing and membrane filtration methods based on Sjamsuridzal *et al*. (2009). The use of two isolation methods was intended to obtain as much as possible of phylloplane yeast species. All parts of leaves were weighed-in (1 g) and cut into pieces. The leaf pieces were washed with 30 mL sterile distilled water (in 50 mL conical tube) and vortexed at 2000 rpm for 10 min. Washed pieces of leaves were placed directly onto isolation medium containing 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.5% agar (YM agar), and added with 0.05% tetracycline after sterilization. After that, 0.1 mL of leaf suspension was inoculated directly onto YM agar medium in three replicates. The leaf suspension was filtered using 0.45 µm membrane filter (Millipore), and the membrane filter was placed onto the YM agar medium. Plates were incubated at room temperature (27-28 °C), and after three days all single colonies were picked up using sterile toothpicks and placed into new plates to create colony libraries. The representative colonies of each morphological type were purified at least two times on yeast malt extract agar (YMA), maintained on potato dextrose agar (PDA) slants, and stored at –80 °C. The cultures from this study were deposited in the Universitas Indonesia Culture Collection (UICC), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Indonesia.

Identification of yeasts

Yeast isolates

Eighty two representative yeast isolates were selected for molecular identification. The selection of isolates was based on the colors and textures of colonies from four different locations e.g. Dago Pojok Village, Bandung (West Java) (20 isolates); Sukadanu Village, Garut (West Java) (22 isolates), Tunggilis Village, Garut (West Java) (20 isolates), and Bejijong Village, Trowulan (East Java) (20 isolates).

DNA isolation

The yeast cultures were cultivated in YM agar for DNA isolation. Cells were collected from the logarithmic phase

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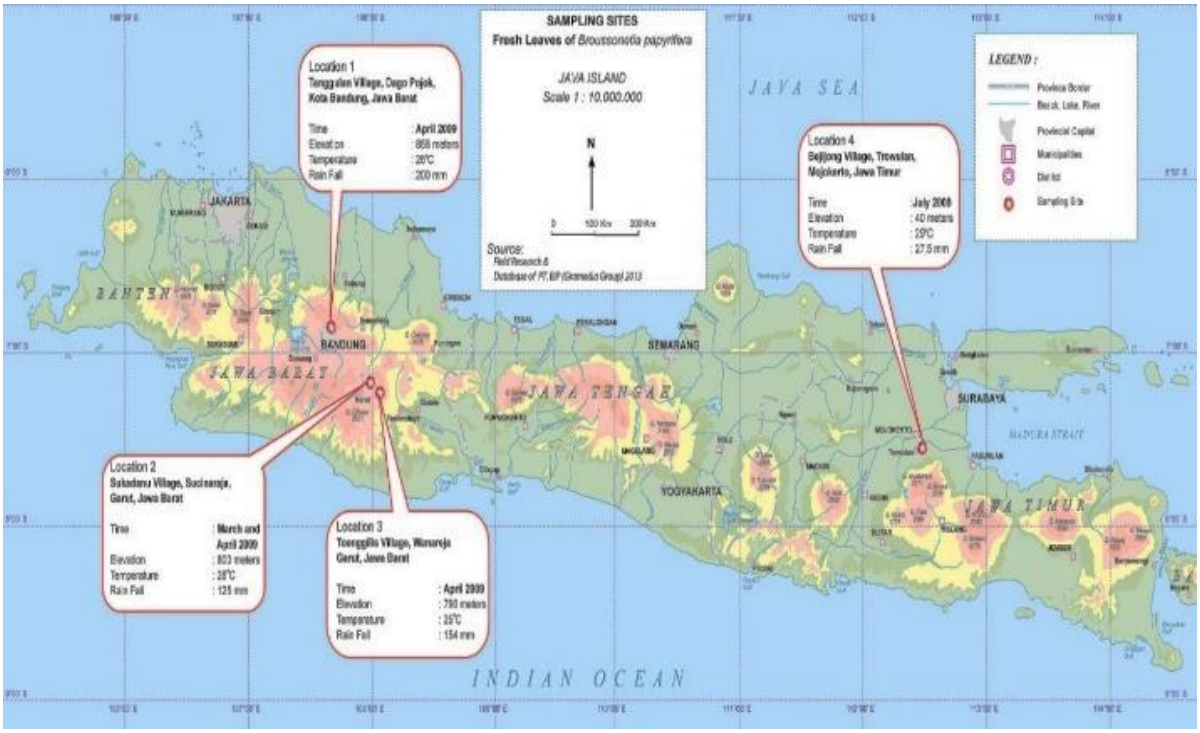

Figure 1: Location of four sampling sites, e.g. Dago Pojok Village, Bandung (West Java); Sukadanu Village, Garut (West Java); Tunggilis Village, Garut (West Java); Bejijong Village, Trowulan (East Java), Indonesia.

of growth. For extraction of DNA, one loopful of cells was suspended in 300 µL MilliQ water in microtubes and were homogenized using vortex (Bio Rad: BR 2000, California, USA). The DNA was extracted by using boiling method of Sjamsuridzal and Oetari (2003)

PCR reaction

The ITS regions of rDNA of yeasts were amplified using primer pairs ITS4 (5'-TCCTCCGCTTATTGATATGC-3'),ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al*., 1990). The PCR reaction was performed using PuReTaq™ Ready-To-Go™ PCR Beads (GE Healthcare) in a total of 25 µL reaction volume which contained 15 µL nuclease free water (NFW) diluted in PuReTaq™ Ready-To-Go (RTG) PCR beads (GE Healthcare), 10 pmol for each primer ITS4 and ITS5, and ca. 100 ng DNA template. PCR condition was as follows: 95 °C for 2 min (1 cycle); 94 °C for 15 sec, 56 °C for 30 sec, 68 °C for 1 min (40 cycles); 68 °C for 10 min for final extension (1 cycle).

Visualization of PCR products

The gel electrophoresis of PCR products was performed using Tris Acetate EDTA (TAE) buffer solution at 100 Volt for 25 min. The 100 bp DNA MW marker was used as a molecular size marker. The PCR products were analyzed on 2% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light using the Gel doc (Sambrook and Russell, 2001). When amplified bands were confirmed, PCR products were purified using ethanol precipitation method. Purified PCR products were measured for their quality and quantity using Nanodrop Spectrophotometer.

Sequencing of ITS regions of rDNA

The nucleotide sequences of ITS regions of rDNA were determined with Big Dye Terminator v3.1 cycle sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions. The PCR cycle sequencing reaction was performed in a total reaction volume of 10 µLwhich contained 1 µL product of PCR (100 ng/µL), 1 µL Big Dye, 0.5 µL primer (10 µM/µL), 7 µLsequencing buffer, and 0.5 µLnuclease free water. The PCR cycle sequencing condition was as follows: 96 °C for 2 min (1 cycle); 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min (30 cycles). The products of PCR cycle sequencing were purified by using Pellet Paint® NF Co-Precipitant (Novagen). The sequence analysis was performed using the ABI 310 Prism Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis

The sequence data were sent to online international DNA database for homology search by Basic Local Algorithm Search Tools (BLAST) program (Altschul *et al*., 1997). The sequences of ITS regions of rDNA of the yeast isolates

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were aligned with other ITS regions of rDNA sequences available on the online database on the basis of similarity of the sequences. Sequences of the ITS regions of rDNA gene were manually edited and assembled using MEGA version 4 software (Tamura *et al*., 2007). The gaps were excluded in our phylogenetic analyses. The distance matrix for the aligned sequences was calculated using two-parameter method Kimura. The neighbor-joining (NJ) method was used to construct all phylogenetic trees (Saitou and Nei, 1987). The robustness for individual branches was estimated by bootstrapping with 1000 resamplings (Felsenstein, 1985).

Description of morphological characteristics of the identified strains

The morphological characteristics of the yeast strains were examined using the methods described by Yarrow (1998).

RESULTS AND DISCUSSION

Isolation of yeasts from fresh leaves of *B. papyrifera*

A total of 2,543 isolates were obtained from four sampling locations (Table 1), i.e. from Dago Pojok Village, Bandung (West Java) (756 isolates); Sukadanu Village, Garut (West Java) (182 isolates); Tunggilis Village, Garut (West Java) (578 isolates); Bejijong Village, Trowulan (East Java) (1,027 isolates). The colonies of the representative yeast isolates (82) from *B. papyrifera* showed a variety of colors and textures. The color and textures of yeast colonies, and their percentages are as follows: white butyrous (27%), white mucoid (16%), cream butyrous (7%), cream mucoid (29%), red butyrous (5%), red mucoid (10%), and light pink butyrous (6%) on YMA after 3 days incubation at room temperature (27-28 °C) (Table 2). In this study, pigmented yeast colonies were dominant from *B. papyrifera.* The leaf surface is exposed to fluctuating temperature and sun exposure, besides providing limited nutrient resources, and therefore pigmented microorganisms dominate the leaf surfaces (Lindow and Brandl, 2003).

The phylloplane yeasts are dominated by yeasts with colored colonies e.g. cream and light pink (Fonseca and Inácio, 2006). Production of pigments e.g. pink-red (astaxanthin), black (melanin), orange red (torularhodin), and red (β-carotene) have been reported from the yeast isolates (Joshi *et al*., 2003; Dufossé, 2006). Yeast pigments prevent the yeasts from light intensity, and UV radiation (Azeredo *et al*., 1998). Many yeast isolates (*Crytococcus* sp., *Phaffia rhodozyma* and *Rhodotorula*) are good source of microbial pigment (Joshi *et al*., 2003).

Table 1: Yeast isolates obtained from leaves of *B. papyrifera.*

Sampling location, and time of sampling	Condition of sampling location	Temperature of the location (°C)	Humidity (%)	Total number of collected isolates	Number of selected isolates for identification
Dago Pojok Village, Bandung, West Java 11.00 a.m., April 2009	The lowland and crowded with human population	26	54	756	20
Sukadanu Village, Garut, West Java 12.10 a.m., April 2009	The plateau and sparse with human population	28	53	182	22
Tunggilis Village, Garut, West Java 11.38 a.m., April 2009	The lowland and crowded with human population	25	56	578	20
Bejijong Village, Trowulan, East Java 6.28 p.m., July 2009	The lowland and crowded with human population	29	53	1.027	20
				2.543	82

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Table 2: Morphology of yeast isolates on Yeast Malt Agar (YMA) after 3 days incubation at 27-28 °C.

Location	Total number of isolates	White butyrous	White mucoid	Cream butyrous	Cream mucoid	Red butyrous	Red mucoid	Light pink butyrous
		Number of isolates	Number of isolates	Number of isolates	Number of isolates	Number of isolates	Number of isolates	Number of isolates
Dago Pojok Village, Bandung (West Java)	20	5	6	2	4	2	1	-
Sukadanu Village, Garut (West Java)	22	7	4	1	9	1	-	-
Tunggilis Village, Garut (West Java)	20	7	1	3	6	1	2	-
Bejijong Village, Trowulan (East Java)	20	3	2	0	5	-	5	5
Total	82	22 (27%)	13 (16%)	6 (7%)	24 (29%)	4 (5%)	8 (10%)	5 (6%)

Identification and phylogenetic placements of yeast species from different sampling location

A total of 82 yeast isolates were identified based on ITS regions of rDNA. The isolates were identified as *Cryptococcus* (22), *Rhodotorula* (12), *Candida* (9), *Debaryomyces* (4), *Trichosporon* (4), *Hanseniaspora* (4), *Wickerhamomyces* (4), *Pseudozyma* (4), *Aureobasidium* (4), *Meyerozyma* (3), *Hyphopichia*(3), *Hannaella* (3), *Kodamaea* (1), *Geotrichum* (1), *Pichia* (1), *Bullera* (1), and *Saccharomycetales* (1).

Based on sequence data of ITS regions of rDNA, all of the yeast isolates from fresh leaves of *B. papyrifera* showed a high degree of similarity (98-100%) to their closest species (Tables 3-6). Twelve yeast isolates from four different locations (UICC Y-462, UICC Y-454, UICC Y-491, UICC Y-502, UICC Y-506, UICC Y-516, UICC Y-524, UICC Y-525, UICC Y-526, UICC Y-515, UICC Y-522, and UICC Y-530) have 98% similarity of their sequences to *Saccharomycetales* sp. LM434, *R. glutinis* IWBT-Y824, *Cryptococcus rajasthanensis* 15L, *Tr. asahii* KDLYL4-1, *Aureobasidium* sp. Cs/3/6, *C. flavescens* IMUFRJ 51986, *C. flavescens* IMUFRJ 52043, *C. flavescens* IMUFRJ 51986, *R. mucilaginosa* L3, and *Pseudozyma thailandica* M9933, respectively. Seven yeast isolates (UICC Y-471, UICC Y-487, UICC Y-484, UICC Y-485, UICC Y-479, UICCY-483, and UICC Y-519) have 100% similarity of their sequences to *Kodamaea ohmeri* WM10.220, *Candida saopaulonensis* SJ18, *Hanseniaspora uvarum* NT0103, *Cryptococcus* sp. LCF-06 strain GG2M05, *Trichosporon asahii* clone (15) 10, and *Aureobasidium pullulans* UTHSC 09-1190, respectively. Sugita *et al*. (1999) who worked on *Trichosporon* mentioned that the overall ITS similarity of identical species is more than 99%.

The relationship between yeast isolates from *B. papyrifera* and their closely related species are shown in the phylogenetic tree using NJ method (Figures 2-5). Our NJ tree showed that the yeast isolates are phylogenetically diverse and distributed in the phylaof *Ascomycota* and *Basidiomycota.* Fonseca and Inácio (2006) reported that phylloplane yeasts from various regions of the world are members of the *Basidiomycetous* and *Ascomycetous* yeasts.

We found that the phylloplane yeast isolates of *B. papyrifera* were taxonomically heterogeneous and represent several classes from the phyla of *Basidiomycota* and *Ascomycota*. Yeast isolates from *B. papyrifera* include classes of *Dothideomycetes*, *Saccharomycetes*, *Microbotryomycetes*, *Tremellomycetes* and *Ustilaginomycetes*. The yeasts that belong to the phylum *Ascomycota* are within the classes *Saccharomycetes* (32 isolates) and *Dothideomycetes* (4 isolates). Meanwhile, the yeasts that belong to the phylum *Basidiomycota* are within the classes *Microbotryomycetes* (12 isolates), *Tremellomycetes* (30 isolates), and *Ustilaginomycetes* (4 isolates). Inácio *et al*. (2005) reported that the yeast isolates from the leaves collected at the Arrabida natural Park (Portugal), were mostly of the phylum *Basidiomycota* in the class *Tremellomycetes.*

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Table 3: Identification of yeast isolates from Dago Pojok Village (West Java) based on ITS region of rDNA.

Isolate	UICC number	Closely related species	Accesion number	Similarity (%)	Identities	Gaps
BD2S1.MF1.3	Y-469	*Candida quercitrusa*	DQ665264	99	541/548 (99 %)	2/548 (0 %)
BD2S1.D.11	Y-470	*Candida quercitrusa*	DQ665264	99	534/538 (99 %)	1/538 (0 %)
BD1S1.WM1.5	Y-456	*Debaryomyces nepalensis*	JQ665426	99	309/314 (98 %)	1/314 (0 %)
BD2S2.D1.15	Y-462	*Saccharomycetales* sp.	EF060740	98	162/165 (98 %)	2/165 (1 %)
BD2S2.WM1.20	Y-465	*Hyphopichia burtonii*	KC621079	99	317/319 (99 %)	1/319 (0 %)
BD2S2.WM1.32	Y-468	*Hyphopichia burtonii*	AM420292	99	322/324 (99 %)	1/324 (0 %)
BD2S2.D.12	Y-471	*Kodamaea ohmeri*	JN183449	100	326/326 (100 %)	0/326 (0 %)
BD2S1.WM.34	Y-472	*Kodamaea ohmeri*	JN183448	99	377/381 (99 %)	1/381 (0 %)
BD1S1.DM 5	Y-455	*Wickerhamomyces anomalus*	HF952837	99	254/255 (99 %)	0/255 (0 %)
BD1S1.WM1.7	Y-453	*Wickerhamomyces anomalus*	JQ241272	99	254/255 (99 %)	0/256 (0 %)
BD2S2.WM1.3	Y-460	*Wickerhamomyces anomalus*	JN839959	97	376/381 (99 %)	1/380 (0 %)
BD1S1.DM1	Y-454	*Rhodotorula glutinis*	JQ993380	98	191/194 (98 %)	3/194 (1 %)
BD1S2.WM 1.2	Y-458	*Cryptococcus rajasthanensis*	AM262325	99	305/309 (99 %)	2/309 (0 %)
BD2D2.W.25	Y-467	*Cryptococcus rajasthanensis*	AM262325	99	459/461 (99 %)	0/461 (0 %)
BD2S2.WM1.6	Y-461	*Cryptococcus luteolus*	AM176652	99	530/531 (99 %)	1/531 (0 %)
BD2D1.1.W1.20	Y-463	*Hannaella zeae*	JQ754020	99	396/398 (99 %)	0/398 (0 %)
BD2D1.1.W2.15	Y-464	*Hannaella zeae*	JQ754020	99	426/428 (99 %)	0/428 (0 %)
BD1S1.WM1.8	Y-457	*Rhodotorula dairenensis*	NR_073270	99	214/215 (99 %)	0/215 (0 %)
BD2S2.WM2.4	Y-466	*Rhodotorula mucilaginosa*	AF321544	99	472/477 (99 %)	1/477 (0 %)
BD1S2.WM2.1	Y-459	*Pseudozyma aphidis*	JQ743064	99	406/409 (99 %)	3/409 (0 %)

Table 4: Identification of yeast isolates from Sukadanu Village (West Java) based on ITS region of rDNA.

Isolate	UICC number	Closely related species	Accesion number	Similarity (%)	Identities	Gaps
SUK2S2.WM1.1	Y-473	*Candida metapsilosis*	JQ585714	99	329/330 (99%)	1/330 (0%)
SUK2S1.WM1.1	Y-474	*Candida metapsilosis*	JQ585714	99	448/449 (99%)	1/449 (0%)
SUK2S1.WM1	Y-475	*Candida pseudojiufengensis*	GU291263	99	430/434 (99%)	1/434 (0%)
SUK1D1.2.W1.8	Y-487	*Candida saopaulonensis*	FJ515172	100	298/298 (100%)	0/298 (0%)
SUK1D1.WM.8	Y-492	*Candida saopaulonensis*	FJ515172	99	297/298 (99%)	0/298 (0%)
SUK2D1.2.W1.81	Y-494	*Candida saopaulonensis*	FJ515172	99	289/293 (99%)	0/293 (0%)
SUK1D1.2.W1.16	Y-488	*Debaryomyces hansenii*	AB305097	99	343/348 (99%)	3/348 (0%)
SUK2D2.2.W1.14	Y-480	*Hanseniaspora thailandica*	AB501150	99	186/188 (99 %)	0/188 (0 %)
SUK1D2.2.W1.2	Y-481	*Hanseniaspora thailandica*	AB501150	99	293/295 (99 %)	0/295 (0 %)
SUK1D2.2.W1.8	Y-484	*Hanseniaspora uvarum*	KC349934	100	294/297 (99 %)	3/297 (1 %)

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SUK1D1.2.W1.4	Y-485	*Hanseniaspora uvarum*	AB469379	100	271/271 (100%)	0/271 (0%)
SUK2S2.DM2	Y-490	*Pichia veronae*	DQ414543	99	436/441 (99%)	2/441 (0%)
SUK2D2.2.W1.13	Y-477	*Cryptococcus luteolus*	FJ591129	99	389/391 (99%)	0/391 (0%)
SUK1D1.2.W1.20	Y-493	*Cryptococcus luteolus*	AM176654	99	401/404 (99%)	0/404 (0%)
SUK1D2.1.W1.5	Y-486	*Cryptococcus luteolus*	AM176656	99	400/402 (99%)	0/402 (0%)
SUK2D2.1.W1.1	Y-479	*Cryptococcus* sp.	HQ623595	100	392/392 (100%)	0/392 (0%)
SUK1D1.2.W1.12	Y-482	*Cryptococcus rajasthanensis*	HQ623596	99	421/427 (99%)	3/427 (0%)
SUK1S2.DM1	Y-489	*Cryptococcus rajasthanensis*	HQ832836	99	434/440 (99%)	1/440 (0%)
SUK1S2.DM.5	Y-491	*Cryptococcus rajasthanensis*	AM262325	98	419/424 (99%)	3/424 (0%)
SUK2S2.WM 2.4	Y-476	*Rhodotorula mucilaginosa*	AF321544	99	472/477 (99%)	1/477 (0%)
SUK2D2.2.W1.15	Y-478	*Hannaella zeae*	JQ754020	99	393/396 (99%)	1/396 (0%)
SUK1D2.2.W1.7	Y-483	*Trichosporon asahii*	KC127676	100	464/464 (100%)	0/464 (0%)

Table 5: Identification of yeast isolates from Tunggilis Village (West Java) based on ITS region of rDNA.

Isolate	UICC number	Closely related species	Accesion number	Similarity (%)	Identities	Gaps
T2S2.MF3	Y-495	*Geotrichum candidum*	FN376417	99	302/306 (99%)	0/306 (0%)
T.D1.2.W1.16	Y-514	*Debaryomyces hansenii*	KC111444	99	500/505 (99%)	0/505 (0%)
T1S2.MF4	Y-503	*Debaryomyces nepalensis*	JQ665426	99	326/327 (99%)	0/327 (0%)
T2S2.WM1.4	Y-496	*Hypopichia burtonii*	AM420292	99	296/299 (99%)	0/299 (0%)
T2S1.MF8	Y-497	*Wickerhamomyces anomalus*	KC568565	99	508/514 (99%)	1/514 (0%)
T1.D1.2.W1	Y-509	*Bullera sinensis*	AF314989	99	404/406 (99%)	0/406 (0%)
T2S2.WM2.9	Y-498	*Cryptococcus rajasthanensis*	AM262325	99	475/481 (99%)	2/481 (0%)
T2.D2.1.W1	Y-502	*Cryptococcus rajasthanensis*	AM262325	98	488/496 (98%)	4/496 (0%)
T1S1.WM1.3	Y-504	*Cryptococcus rajasthanensis*	AM262325	99	309/313 (99%)	1/313 (0%)
T1.D2.1.W1.1	Y-508	*Hannaella kunmingensis*	JN181160	99	398/405 (98%)	3/405 (0%)
T1.D1.2.W1.2	Y-510	*Hannaella zeae*	JQ754020	99	428/430 (99%)	0/430 (0%)
T1.D.1W1.4	Y-511	*Hannaella zeae*	JQ754020	99	406/407 (99%)	0/407 (0%)
T2S1.MF6	Y-499	*Rhodotorula mucilaginosa*	KC544481	99	320/323 (99%)	1/323 (0%)
T1.D1.2.W1.11	Y-513	*Rhodotorula mucilaginosa*	AF321544	99	534/536 (99%)	0/536 (0%)
T1S2.MF2.3	Y-500	*Rhodotorula dairenensis*	NR_073270	99	338/343 (99%)	3/343 (0%)
T1S1.MF2	Y-505	*Trichosporon asahii*	JX174411	99	300/304 (99%)	4/304 (1%)
T1S2.MF	Y-506	*Trichosporon asahii*	JX174411	98	303/306 (99%)	3/306 (0%)
T1S2.MF2	Y-507	*Trichosporon asahii*	JX174411	99	300/304 (99%)	4/304 (1%)
T1.D1.2.W1.12	Y-512	*Pseudozyma aphidis*	HQ832804	99	363/366 (99%)	3/366 (0%)
T2S2.WM2.3	Y-501	*Pseudozyma aphidis*	JQ743064	99	423/424 (99%)	1/424 (0%)

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Table 6: Identification of yeast isolates from Bejijong Village,Trowulan (East Java) based on ITS region of rDNA.

UICC number	Closely related species	Accesion number	Similarity (%)	Identities	Gaps
Y-516	*Aureobasidium* sp.	JN585936	98	460/469 (98%)	3/469 (0%)
Y-519	*Aureobasidium pullulans*	GU475133	100	511/511 (100%)	0/511 (0%)
Y-527	*Aureobasidium pullulans*	HM855960	99	480/485 (99%)	3/485 (0%)
Y-528	*Aureobasidium pullulans*	JQ235065	99	499/505 (99%)	2/505 (0%)
Y-533	*Candida orthopsilosis*	KC544482	99	412/413 (99%)	0/413 (0%)
Y-517	*Meyerozyma caribbica*	JN183445	99	523/528 (99%)	3/528 (0%)
Y-518	*Meyerozyma caribbica*	JX910353	99	523/525 (99%)	1/525 (0%)
Y-529	*Meyerozyma caribbica*	JX910353	99	524/525 (99%)	0/525 (0%)
Y-523	*Cryptococcus flavescens*	FN428902	99	449/451 (99%)	1/451 (0%)
Y-524	*Cryptococcus flavescens*	FN428902	98	433/443 (98%)	5/443 (1%)
Y-525	*Cryptococcus flavescens*	FN428920	98	334/335 (99%)	1/335 (0%)
Y-526	*Cryptococcus flavescens*	KC294323	98	186/190 (98%)	0/190 (0%)
Y-515	*Cryptococcus flavescens*	FN428902	98	438/441 (99%)	1/441 (0%)
Y-534	*Cryptococcus flavus*	EU177576	99	450/452 (99%)	0/452 (0%)
Y-531	*Rhodotorula mucilaginosa*	FN428899	99	472/476 (99 %)	0/476 (0%)
Y-532	*Rhodotorula mucilaginosa*	FN428885	99	469/473 (99 %)	1/473 (0%)
Y-521	*Rhodotorula mucilaginosa*	FN428885	99	469/473 (99 %)	1/473 (0%)
Y-522	*Rhodotorula mucilaginosa*	FN428886	98	469/473 (99 %)	1/473 (0%)
Y-520	*Rhodotorula glutinis*	JQ425397	99	436/440 (99 %)	1/440 (0%)
Y-530	*Pseudozyma thailandica*	AB089354	98	430/438 (98 %)	3/438 (0%)

In our study, the phylogenetic tree constructed from rDNA-ITS sequencing showed that the yeasts from classes *Saccharomycetes* and *Tremellomycetes* were found from all sampling locations (Figures 2-5). Yeast isolates from classes *Microbotryomycetes* and *Ustilaginomycetes* were found in Pojok Dago Village, Bandung (West Java), Tunggilis Village, Garut (West Java), and Bejijong Village, Trowulan (East Java) (Figures 2, 4, 5). Yeast isolates from the class *Dothideomycetes* was only found in Bejijong Village, Trowulan (Figure 5).

Class *Saccharomycetes* was represented by species *Hyphopichia burtonii* (Figures 2, 4, 5), *Debaryomyces nepalensis* (Figures 2, 4), *D. hansenii* (Figures 3-4). Class *Dothideomycetes* was represented by species *A. pullulans* (Figure 5). Class *Tremellomycetes* was represented by species *C. rajasthanensis* and *Hannaella zeae* (Figures 2-4), *T. asahii* (Figures 3-4), *Bullera sinensis* (Figure 4), *C. flavescens* and *C. flavus* (Figure 5). Class *Microbotryomycetes* was represented by species *Rhodotorula mucilaginosa* (Figures 2, 4, 5) and *R. dairenensis* (Figure 4). Class *Ustilaginomycetes* was represented by species *Pseudozyma aphidis* (Figures 2, 4) and *P. thailandica* (Figure 5). We found *R. mucilaginosa* in West Java and East Java, while *C. rajasthanensis, R. mucilaginosa,* and *H. zeae* were found in three locations in West Java (Pojok Dago Village, Sukadanu Village, and Tunggilis Village).

Yeasts from fresh leaves of *B. papyrifera* plants of different ages

A total of 32 yeast species from leaves of plants of different ages (6 month-old and 1.5 year-old) of *B. papyrifera* belong in the phyla *Ascomycota* and *Basidiomycota*. As shown in Table 7, the number of yeast species from 1.5 year-old *B. papyrifera* plants is higher than from 6 month-old plants.

We obtained 20 yeast species from 6 month-old plants. Ten yeast species belong to the phylum *Ascomycota*, e.g. *Aureobasidium* sp., *A. pullulans*, *C. saopaulonensis*, *C. orthopsilosis*, *D. nepalensis*, *D. hansenii*, *Hanseniaspora thailandica*, *H. uvarum*, *Meyerozyma caribbica*, and *Wickerhamomyces anomalus.* Another ten yeast species belong to the phylum *Basidiomycota*, e.g. *B. sinensis*, *C. rajasthanensis*, *C. luteolus*, *C. flavescens*, *H. zeae*, *P. aphidis*, *Rhodotorula dairenensis*, *Rhodotorula mucilaginosa*, *R. glutinis*, and *T. asahii.*

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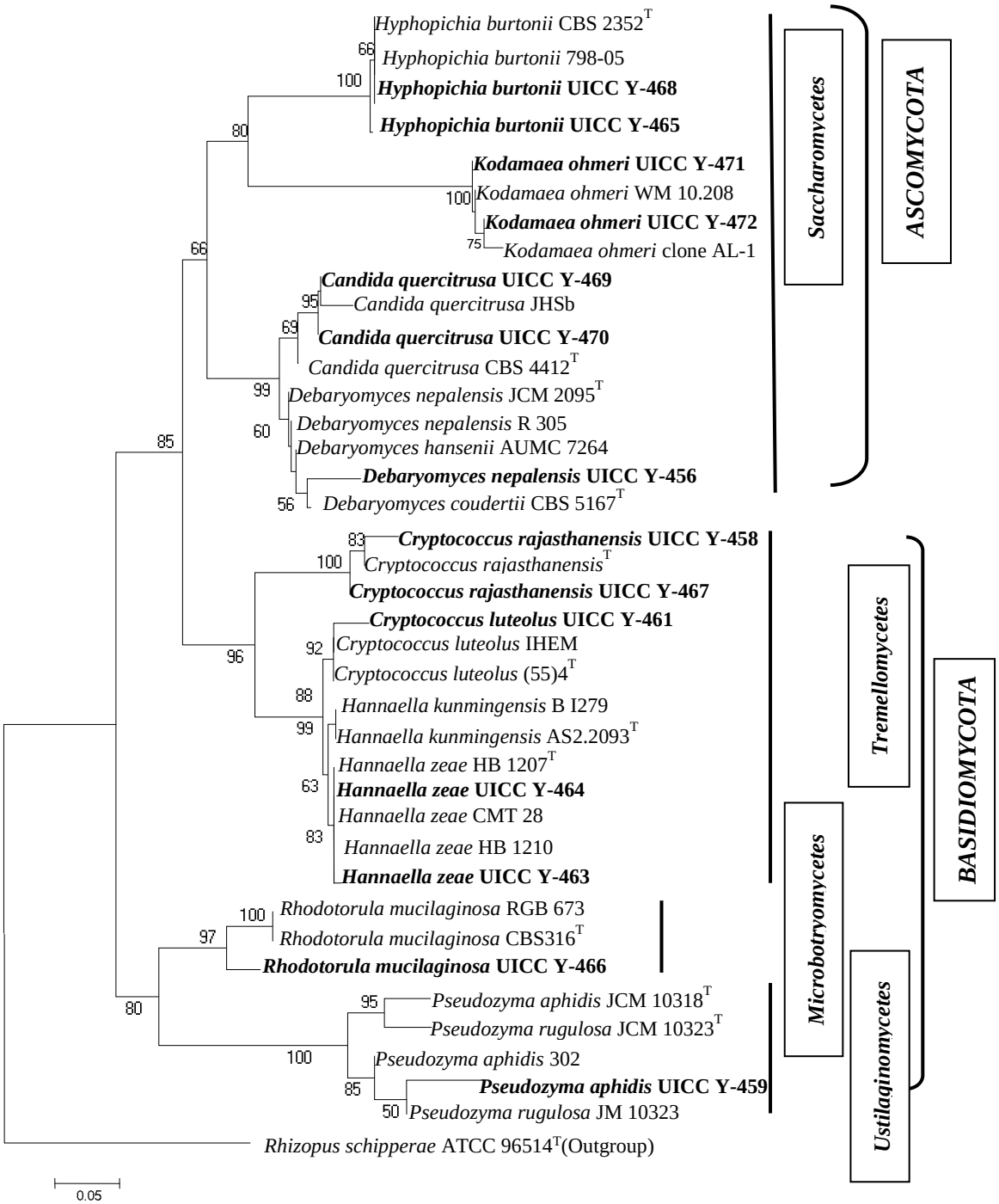

Figure 2: Phylogenetic tree of yeast isolates from Dago Pojok Village, Bandung was reconstructed by neighbor-joining algorithm based on the distance calculated by Kimura's two-parameter model from sequences of ITS regions of rDNA. Bootstrap values greater than 50% from 1,000 replicate bootstrap resamplings. *Rhizopus schipperae* ATCC 96514^T was used as an outgroup.

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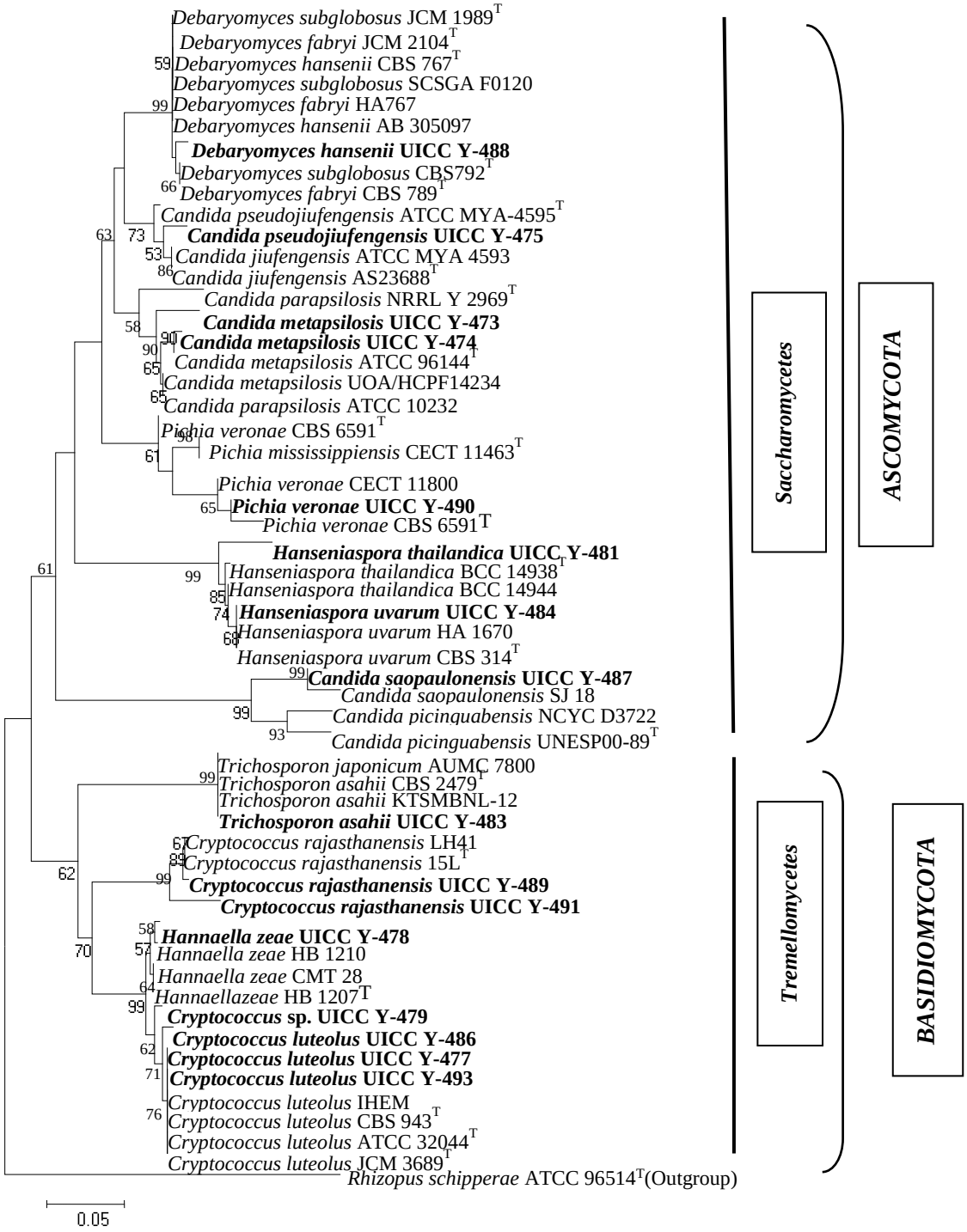

Figure 3: Phylogenetic tree of yeast isolates from Sukadanu Village, Garut was reconstructed by neighbor-joining algorithm based on the distance calculated by Kimura's two-parameter model from sequences of ITS regions of rDNA. Bootstrap values greater than 50% from 1,000 replicate bootstrap resamplings. *Rhizopus schipperae* ATCC 96514^T was used as an outgroup.

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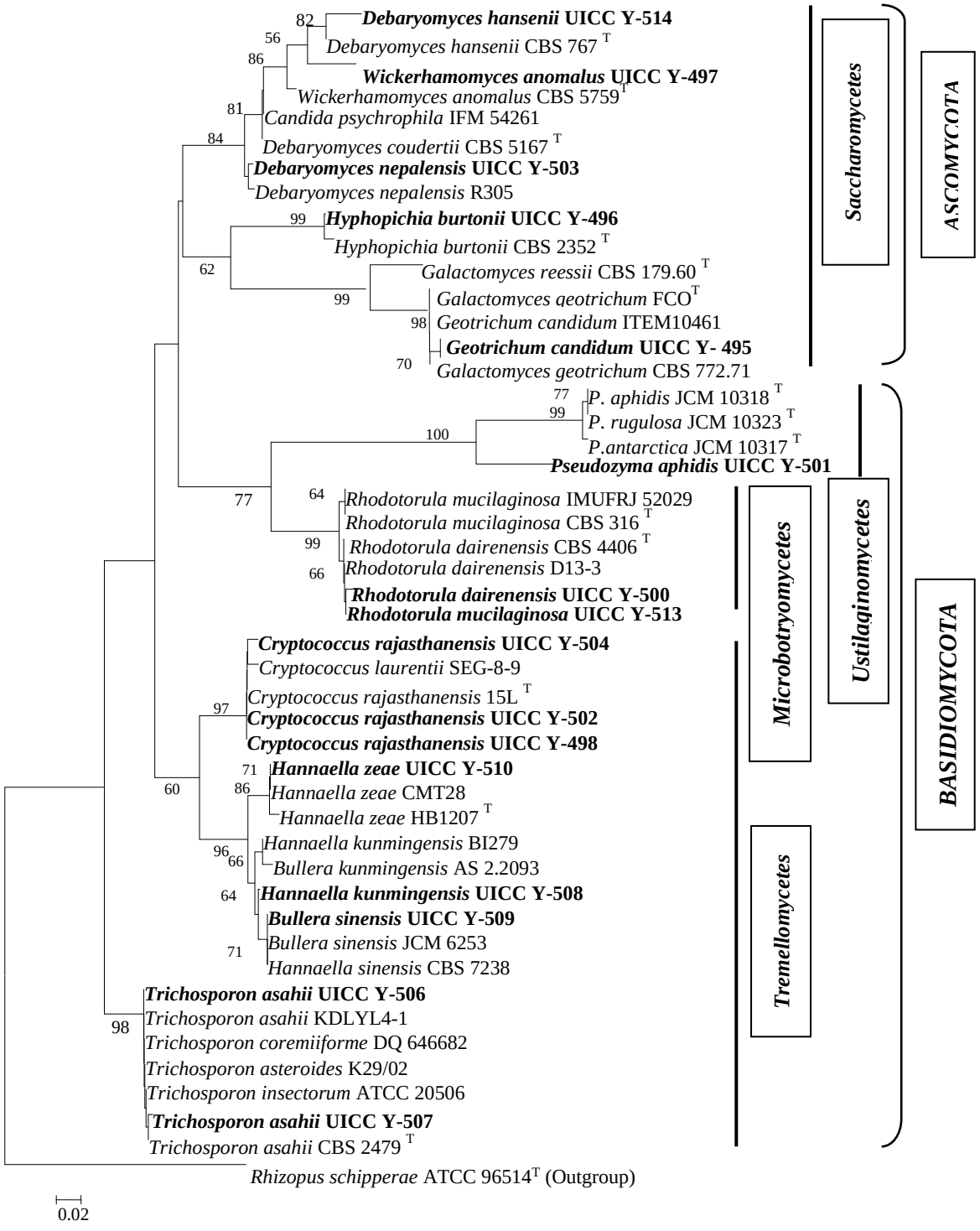

Figure 4: Phylogenetic tree of yeast isolates from Tunggilis Village, Garut was reconstructed by neighbor-joining algorithm based on the distance calculated by Kimura's two-parameter model from sequences of ITS regions of rDNA. Bootstrap values greater than 50% from 1,000 replicate bootstrap resamplings. *Rhizopus schipperae* ATCC 96514^T was used as an outgroup.

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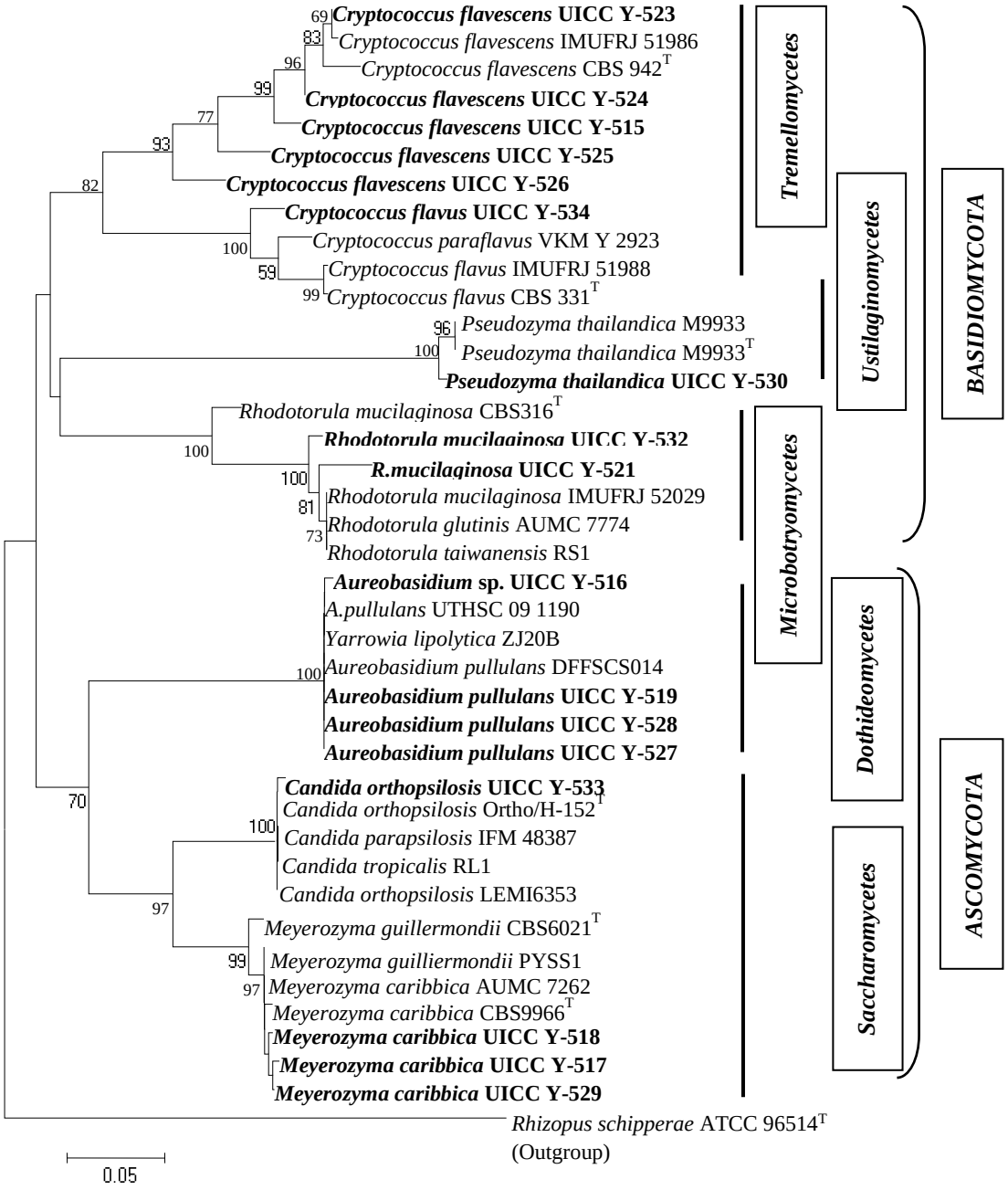

Figure 5: Phylogenetic tree of yeast isolates from Bejijong Village, Trowulan was reconstructed by neighbor-joining algorithm based on the distance calculated by Kimura's two-parameter model from sequences of ITS regions of rDNA. Bootstrap values greater than 50% from 1,000 replicate bootstrap resamplings. *Rhizopus schipperae* ATCC 96514^T was used as an outgroup.

A total of 23 yeast species was obtained from 1.5 year-old plants. Thirteen yeast species belong to the phylum *Ascomycota*, e.g. *A. pullulans*, *C. saopaulonensis*, *C. quercitrusa*, *C. metapsilosis*, *C. pseudojiufengensis*, *Geotrichum candidum*, *H. thailandica*, *Hyphopichia burtonii*, *Kodamaea ohmeri*, *M. caribbica*, *Pichia veronae*, *Saccharomycetales* sp. and *W. anomalus.* Ten yeast species belong to the phylum *Basidiomycota*, e.g. *C. flavus*, *C. rajasthanensis*, *C. luteolus*, *C. flavescens*, *Cryptococcus* sp., *H. zeae*, *P. aphidis*, *P. thailandica*, *R. dairenensis*, and *R. mucilaginosa* (Table 7).

Various studies on phylloplane yeasts have been reported. Alamri (2008) reported that the number of yeasts from old leaves was higher than the young leaves of

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Juniperusprocera. Kumaresan and Suryanarayanan (2002) reported that leaf age influences the phylloplane yeast colonies. According to their findings, the number of species and colonies of endophytic fungi isolated from leaves will increase with the increase of leaf age. Glushakova and Chernov (2007), Pennycook and Newhook (1981) reported that younger leaves show a lower density of yeasts than in mature leaves. Glushakova and Chernov (2004) found that younger leaves provide less exudates causing a lower nutrient source that is available for the growth of a large population of microorganisms. Redford *et al*. (2010) stated that the variables influencing the phylloplane community are leaf age, structure, and composition of smooth cuticles, the chemical composition, and volatile organic molecules released by leaf. Nevertheless, outside factors may also influence the community structure of the phyllosphere.

We noted that 12 yeast species were commonly found from 6 months and 1.5 year-old of *B. papyrifera*. Five yeast species belong to the phylum *Ascomycota*, e.g. *A. pullulans*, *C. saopaulonensis*, *H. thailandica, M. caribbica*, and *W. anomalus*. Seven yeast species belong to the phylum *Basidiomycota*, e.g. *C. rajasthanensis*, *C. luteolus*, *C. flavescens*, *R. dairenensis*, *R. mucilaginosa*, *P. aphidis*, and *H. zeae.* The common yeasts genera from the leaves of *B. papyrifera* were *Candida*, *Cryptococcus*, *Rhodotorula*, *Hannaell*a, and *Trichosporon.*

Our result showed that the phylloplane yeasts were dominated by the genera *Cryptococcus*, *Rhodotorula*, *Candida*, and *Trichosporon*, which is consistent with previous studies, i.e. Azeredo *et al.* (1998) and Slavikova *et al*. (2009) on their reports that the genera *Cryptococcus*, *Rhodotorula*, *Trichosporon*, *Aureobasidium*, *Debaryomyces*, *Hanseniaspora*, *Hyphopichia*, and *Saccharomyces* are often found from phylloplane. Wang *et al*. (2006) reported that genera *Pseudozyma*, *Rhodotorula*, and *Cryptococcus* were found from the leaves of *Rhododendron oroedoxa* in China*.* Yurkov (2005) reported that the most common genera of yeasts isolated from birch leaves in Rusia and Western Siberia were *Cryptococcus* and *Rhodotorula.* Chanchaichaovivat *et al.* (2007) reported that the genus *Rhodotorula* can be isolated frequently from water, soil, plants, and animals. Jager *et al.* (2001) reported that saprophytic yeasts are common on the surfaces of leaves with the most common genera being *Candida*, *Cryptococcus*, *Rhodotorula*, and *Trichosporon.*

Table 7: Yeasts from differentage of fresh leaves of *B. papyrifera* collected from four locations in Java, Indonesia.

6 month-old host plant	1.5 year-old host plant	Common species
Aureobasidium sp. (UICC Y-516)	-	-
Aureobasidium pullulans (UICC Y-519)	*Aureobasidium pullulans*(UICC Y-527, UICC Y-528)	*Aureobasidium pullulans*
Candida saopaulonensis (UICC Y-487, UICC Y-492)	*Candida saopaulonensis* (UICC Y-494)	*Candida saopaulonensis*
-	*Candida metapsilosis* (UICC Y-473, UICC Y-474)	-
-	*Candida pseudojiufengensis* (UICC Y-475)	-
-	*Candida quercitrusa* (UICC Y-469, UICC Y-470)	-
Candida orthopsilosis (UICC Y-533)	-	-
Debaryomyces nepalensis (UICC Y-503, UICC Y-456)	-	-
Debaryomyces hansenii (UICC Y-514, UICC Y-488)	-	-
-	*Geotrichum candidum* (UICC Y-495)	-
Hanseniaspora thailandica (UICC Y-481)	*Hanseniaspora thailandica* (UICC Y-480)	*Hanseniaspora thailandica*
Hanseniaspora uvarum (UICC Y-484, UICC Y-485)	-	-
-	*Hyphopichia burtonii* (UICC Y-465, UICC Y-468, UICC Y-496)	-
-	*Kodamaea ohmeri* (UICC Y-471, UICC Y-472)	-
Meyerozyma caribbica (UICC Y-517, UICC Y-518)	*Meyerozyma caribbica* (UICC Y-529)	*Meyerozyma caribbica*
-	*Pichia veronae* (UICC Y-490)	-
-	*Saccharomycetales* sp. (UICC Y-462)	-
Wickerhamomyces anomalus (UICC Y-453, UICC Y-455)	*Wickerhamomyces anomalus* (UICC Y-497, UICC Y-460)	*Wickerhamomyces anomalus*
Bullera sinensis (UICC Y-509)	-	-

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-	*Cryptococcus flavus* (UICC Y-534)	-
Cryptococcus rajasthanensis (UICC Y-504, UICC Y-482, UICC Y-489, UICC Y-491, UICC Y-458)	*Cryptococcus rajasthanensis* (UICC Y-498, UICC Y-502, UICC Y-467)	*Cryptococcus rajasthanensis*
Cryptococcus luteolus (UICC Y-486, UICC Y-493)	*Cryptococcus luteolus* (UICC Y-461, UICC Y-477)	*Cryptococcus luteolus*
Cryptococcus flavescens (UICC Y-515)	*Cryptococcus flavescens* (UICC Y-523, UICC Y-524, UICC Y-525, UICC Y-526)	*Cryptococcus flavescens*
-	*Cryptococcus* sp. (UICC Y-479)	-
Hannaella zeae (UICC Y-510, UICC Y-511)	*Hannaella zeae* (UICC Y-478, UICC Y-463, UICC Y-464)	*Hannaella zeae*
Hannaella kunmingensis (UICC Y-508)	-	-
Trichosporon asahii (UICC Y-505, UICC Y-506, UICC Y-507, UICC Y-483)	-	-
Pseudozyma aphidis (UICC Y-512, UICC Y-459)	*Pseudozyma aphidis* (UICC Y-501)	*Pseudozyma aphidis*
-	*Pseudozyma thailandica* (UICC Y-530)	-
Rhodotorula dairenensis (UICC Y-457)	*Rhodotorula dairenensis* (UICC Y-500)	*Rhodotorula dairenensis*
Rhodotorula mucilaginosa (UICC Y-521, UICC Y-522, UICC Y-513)	*Rhodotorula mucilaginosa* (UICC Y-531, UICC Y-499, UICC Y-476, UICC Y-466, UICC Y-532)	*Rhodotorula mucilaginosa*
Rhodotorula glutinis (UICC Y- 520, UICC Y-454)	-	-

CONCLUSION

The identification results based on ITS rDNA sequence data reveal that 82 representative yeast isolates from fresh leaves of *B. papyrifera* are taxonomically heterogeneous; they consist of 17 genera and 32 species. Phylogenetic analysis showed that phylloplane yeasts from *B. papyrifera* are phylogenetically diverse and distributed into two classes (*Dothideomycetes* and *Saccharomycetes*) from the phylum *Ascomycota* and three classes (*Microbotryomycetes*, *Tremellomycetes*, and *Ustilaginomycetes*) from the phylum *Basidiomycota*. We found the yeasts species in 1.5 year-old plants was more diverse (23 species) compared to 6 month-old plants (20 species). Our results showed that *B. papyrifera* harbors a variety of yeast species which indicate that this plant serves as important fungal habitat.

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