Molecular-genetic diversity of the ascomycetous yeast genus *Arthroascus*: *Arthroascus babjevae* sp. nov., *Arthroascus fermentans* var. *arxii* var. nov. and geographical populations of *Arthroascus schoenii*

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Using molecular and genetic analyses, 28 *Arthroascus* strains were analysed, isolated from widely different geographical localities in Europe, North America, Far-East Asia and Hawaii. Most of the strains have been assigned to the species *Arthroascus schoenii*. PCR-RAPD revealed two Japanese *Arthroascus* strains (UCD 67-278 and IFO 10138) to have peculiar patterns. Comparative rDNA (D1/D2 26S, ITS1 and ITS2) sequence analysis showed that the two strains respectively represent a novel species and a novel variety. Based on the results of sequence analysis, genetic hybridization and DNA–DNA reassociation, two new members of the genus *Arthroascus* are formally described, *Arthroascus babjevae* sp. nov. (type strain UCD 67-278^T = CBS 9167^T) and *Arthroascus fermentans* var. *arxii* var. nov. (type strain IFO 10138^T = CBS 9168^T). These results show that *A. schoenii* has a worldwide distribution, while the species *Arthroascus javanensis* is represented only by the type culture CBS 2555^T, isolated in Indonesia. Cluster analysis revealed a correlation between PCR-RAPD fingerprints and geographical origin of the *A. schoenii* strains. Despite this molecular differentiation, *A. schoenii* strains collected in different regions of the world formed predominantly fertile hybrids, with normal recombination of control markers.

INTRODUCTION

The biological species concept, initially introduced for higher eukaryotes, has been successfully applied to some yeast genera, including *Metschnikowia* (Pitt & Miller, 1970; Lachance & Bowles, 2002, 2004), *Saccharomyces* (Naumov, 1996; Naumov *et al.*, 2000), *Kluyveromyces* (Naumov & Naumova, 2002; Naumova *et al.*, 2004), *Williopsis* and *Zygowilliopsis* (Naumov, 1987). Also, the genus *Arthroascus* is genetically well defined, as it includes closely related species that share a common mating type system (Naumov *et al.*, 1985). These predatory yeasts are able to penetrate cells

of ascomycetous and basidiomycetous yeasts and kill them by digesting the cell content (Kreger-van Rij & Veenhuis, 1973; Lachance, 1995; Lachance *et al.*, 2000). The biological aspects of this phenomenon are of great interest, in particular for biological control studies (Pimenta *et al.*, 2004).

The genus *Arthroascus* was proposed by von Arx (1972) for a single species, *Arthroascus javanensis*. Initially, this yeast was known as *Endomyces javanensis*, and it was later assigned to the genus *Endomycopsis* (Stelling-Dekker, 1931; Lodder & Kreger-van Rij, 1952; Kreger-van Rij, 1970). The second species, *Arthroascus schoenii*, was initially also introduced as a member of the genus *Endomyces*, by Nadson & Krassilnikov (1932). Genetic hybridization established the affiliation of *A. schoenii* to *Arthroascus* (Naumov *et al.*, 1985; Bab'eva *et al.*, 1986), which was later confirmed using DNA–DNA reassociation (Smith *et al.*, 1990) and rDNA sequencing (Naumov *et al.*, 2003). More recently, three strains isolated from soil in Taiwan were assigned to the genus *Arthroascus* on the basis of morphological and physiological properties (Lee *et al.*, 1994); based on DNA–DNA

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 and ITS sequences obtained in this study are detailed in Table 1.

Results of genetic analysis between three *A. fermentans* strains and ex-type cultures of *A. javanensis* and *A. schoenii*, results of genetic analysis of intrastain and intrapopulation hybrids of *A. schoenii* strains and DNA–DNA reassociation results between *Arthroascus* strains are available as supplementary material in IJSEM Online.

reassociation data, the species *Arthroascus fermentans* was described.

Kurtzman & Robnett (1995), using sequence data of the D1/D2 domain of the large subunit (26S) rDNA, considered *Arthroascus* to be congeneric with the genus *Saccharomycopsis*. The latter genus also contains predatory yeasts. Kurtzman & Robnett (1998) and Kurtzman & Smith (1998) followed this classification. Recently, the relatedness of 16 species assigned to the *Saccharomycopsis* clade has been reconsidered on the basis of molecular and genetic data, resulting in the reinstatement of the genus *Arthroascus* (Naumov *et al.*, 2003). Phylogenetic analysis of the D1/D2 rDNA region showed that the *Saccharomycopsis* clade is not monophyletic and apparently consists of a complex of separate genera, one of which is *Arthroascus*. The species *A. javanensis*, *A. schoenii* and *A. fermentans* have the same system of mating types responsible for their crossing and form a distinct lineage (bootstrap value, 100 %) with low phylogenetic affinity to other members of the *Saccharomycopsis* clade. The *Arthroascus* species can be well distinguished from the remaining taxa in the clade by the perforation of hyphal septa: cross walls with a closure line, i.e. the remnants of plasmalemma after centripetal closure during development of the wall (Kreger-van Rij, 1984a, b). The morphological peculiarity of forming ascospores with a ledge within swollen hyphal cells unites the genetically related species *A. javanensis*, *A. schoenii* and *A. fermentans*.

In the present paper, we examine *Arthroascus* strains of different origins. Comparative studies using PCR fingerprinting reveal four endemic populations within the species *A. schoenii*: European, North American, Far-East Asian and Hawaiian. On the basis of genetic hybridization, DNA–DNA reassociation studies and sequencing of the D1/D2 domain of 26S rDNA and the internal transcribed spacers ITS1 and ITS2, two novel taxa are reported: *Arthroascus babjevae* sp. nov. and *Arthroascus fermentans* var. *arxii* var. nov. In addition, *A. fermentans* is redefined on the basis of genetic criteria.

METHODS

Yeast strains, culture conditions and genetic hybridization.

The strains used and their origins are listed in Table 1. Yeast cells were grown at 28 °C on YPD complete medium containing (l⁻¹): glucose, 20 g; peptone, 10 g; yeast extract, 10 g; agar, 20 g. The minimal medium contained (l⁻¹): yeast nitrogen base without amino acids (Difco), 6.7 g; glucose, 20 g; agar, 20 g. Because all strains of *Arthroascus* studied in this work had natural methionine auxotrophy, methionine was added to the minimal nutritional medium. Spore formation was induced by cell incubation for 1–2 days on a culture medium containing (l⁻¹): maltose, 30 g; agar, 20 g. Auxotrophic mutations were induced in haploid cells by UV light on a complete agar medium. To obtain hybrids, mass crossing of haploid cells marked with complementary auxotrophic mutations on the sporulation medium was performed. After 1 day of incubation, the resulting mixture of two strains was lawn-plated on the minimal medium. After 3 days of incubation, prototrophic hybrid colonies were additionally cloned on the minimal medium and then transferred to YPD medium. After 1 day of incubation, the colonies

were transferred to the sporulation medium. Sporulation of hybrids was monitored at all stages of plating. Spores were isolated using a micromanipulator. Ascus walls were preliminarily destroyed using the stomach juice of the garden snail *Helix pomatia*. In addition to tetrad analysis, random spore analysis was done using diethyl ether treatment of sporulating cultures (Dawes & Hardie, 1974).

Phenotypic characterization. Fermentation ability and utilization of carbon and nitrogen compounds were tested according to the methods described by Yarrow (1998).

DNA extraction and PCR fingerprinting. DNA extraction was done as described previously (Naumov *et al.*, 1997a). The primers used included (GTG)₅ and M13 core sequence (5'-GAGGGTG-GCGTTCT-3'). PCR amplification was performed using an MJ Research PTC-100 Programmable Thermal Controller in a total volume of 25 µl containing 1 × PCR buffer, 2 mM MgCl₂, 2 U *Taq* polymerase, 0.2 mM each dNTP, 30 pmol primer and 25 ng genomic template DNA. The thermal cycle was programmed for 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C and a final extension of 10 min at 72 °C. PCR products were analysed by electrophoresis in 1.2 % agarose gels. Gels were stained with ethidium bromide and photographed under UV light.

For the analysis of fingerprints, the amplified bands for each strain and two primers (separately) were encoded as '0' (band absent) and '1' (band present). The binary matrices were combined to form a composite fingerprint for each strain and were then converted into simple matching coefficient data [$S_{SM} = (a + d)/n$; a = both characters are '1', d = both characters are '0', n = number of characters]. Corresponding distance values ($D = 1 - S_{SM}$) for each strain were finally used to construct a dendrogram by implementing the neighbour-joining (NJ) method from the TREECON package (Van de Peer & De Wachter, 1994).

Isolation of DNA, G+C content determinations and DNA–DNA complementarities. Isolation of nuclear DNA, G + C content calculations and determinations of DNA–DNA relatedness were performed as described previously (Smith *et al.*, 1995).

DNA sequencing analysis of the D1/D2 domain of 26S rDNA and the internal transcribed spacer (ITS) region. The divergent D1/D2 domain at the 5' end of the 26S rRNA gene was amplified with the primers NL-1 and NL-4 as described by Kurtzman & Robnett (1997).

The entire ITS region, including ITS1, ITS2 and the intervening 5.8S rRNA gene, was amplified using primers V9D and LS266 as described previously (Gerrits van den Ende & de Hoog, 1999). The amplified DNAs were purified using GFX columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Direct sequencing of both strands of the D1/D2 and ITS regions was performed using capillary electrophoresis on an ABI Prism 3700 automated DNA sequencer (PE Biosystems). The nucleotide sequences determined for *Arthroascus* strains have been deposited in GenBank (see Table 1 for details). Existing sequences for the type cultures of *Arthroascus* species and other members of the *Saccharomycopsis* clade (Kurtzman & Robnett, 1995; Kurtzman, 1999; Lachance *et al.*, 2000) were retrieved from GenBank.

Alignment and phylogenetic analysis. Sequences were edited and assembled by using the SeqMan package (DNASTar Inc.). A preliminary automatic alignment was generated using BioNumerics version 1.50 (Applied Maths) and adjusted manually. The TREECON package was used to generate an NJ tree using Kimura's two-parameter correction. A total of 100 bootstrap replicates were used for analysis. The topology was verified using several algorithms (parsimony, Ward's averaging, UPGMA).

RESULTS AND DISCUSSION

In this study, we compared 28 *Arthroascus* strains isolated from widely different geographical localities in Europe, Far-East Asia, Indonesia, North America and Hawaii (Table 1) in order to assess the genetic diversity of the yeasts. Most of the strains studied were originally designated as *Arthroascus* (*Saccharomycopsis*) *javanensis*. In our approach, we combined genetic hybridization analysis with various molecular methods: PCR fingerprinting, DNA–DNA reassociation and sequencing of variable rDNA regions.

The biological species *A. fermentans*

The results of the genetic circumscription of the biological species *A. fermentans* are presented in Supplementary Table S1 in IJSEM Online. Strains CBS 7830, CBS 7831 and CBS 7832 showed high ascospore viability of 89, 89 and 100 %, respectively. Their monosporic cultures carrying various auxotrophic mutations were used for intra- and interspecies crosses. High ascospore viability of intrastain and interstrain hybrids and normal digenic segregation of control auxotrophic markers confirmed the assignment of strains CBS 7830, CBS 7831 and CBS 7832 to a single biological species *A. fermentans*. Intraspecies hybrids of *A. schoenii* and *A. javanensis* were also fertile. In contrast, interspecies crosses between *A. fermentans*, *A. schoenii* and *A. javanensis* yielded sterile hybrids with ascospore viabilities of 0–3 %. Thus, *A. fermentans*, *A. schoenii* and *A. javanensis* represent reproductively isolated genetic populations. According to the biological species concept as applied to higher eukaryotes, these entities should be viewed as separate species.

Geographical populations of *A. schoenii*

PCR fingerprinting. Initially, all strains studied were characterized by PCR fingerprinting using two primers, (GTG)₅ and M13. Microsatellite primer (GTG)₅ generated complex banding patterns in the strains studied. The type cultures of *A. schoenii* (CBS 7223^T), *A. javanensis* (CBS 2555^T) and *A. fermentans* (CBS 7830^T) displayed clearly distinct profiles (Fig. 1, lanes 1, 23 and 24, respectively). Sixteen isolates originally designated as *A. javanensis* produced PCR fingerprints very similar to those of six *A. schoenii* strains, including the type culture CBS 7223^T (Fig. 1, lanes 1–22). Three *A. fermentans* strains showed mutually identical profiles (Fig. 1, lanes 24–26). Within the *A. schoenii* profile type, some polymorphisms were observed. For example, two major bands of about 400 bp were seen in all four European strains (Fig. 1, lanes 1–4). PCR profiles of Hawaiian isolates were also slightly different from the others (Fig. 1, lanes 19–22). Similar results were obtained with primer M13 (not shown).

Combined cluster analysis of the fingerprints generated by primers (GTG)₅ and M13 is presented in Fig. 2. The 22 *A. schoenii* strains formed a separate cluster, which can be further subdivided into four groups corresponding to four geographical populations: European, North American,

Hawaiian and Far-East Asian. The only exception was North American isolate UCD 52-317.54, which was found between two Far-East-Asian strains (Fig. 2). Thus, the cluster analysis revealed a correlation between the fingerprints and geographical origin of *A. schoenii* strains.

Genetic hybridization of *A. schoenii* isolates. Representative strains from four geographical populations were subjected to genetic hybridization analysis. First, fertile monosporic lines were obtained from all strains, except non-sporulating strain UCD 71-182, in which we isolated only a monocellular clone. The strains cloned were marked by various auxotrophic mutations. Hybrids were formed in all crossing combinations. Each hybrid was analysed for fertility and segregation of control markers. Intrastain and intrapopulation hybrids were highly fertile (73–96 % ascospore viability) with regular digenic segregation of control markers. Fertility of interpopulation hybrids was slightly lower: 51–89 %. Nevertheless, the latter hybrids showed normal meiotic segregation of control markers (for details see Supplementary Table S2 in IJSEM Online).

We performed a series of DNA–DNA reassociation experiments (see Supplementary Fig. S1 in IJSEM Online). High reassociation values of 89–100 % were observed between *A. schoenii* strains isolated in Europe, Far-East Asia and North America. These results agree well with the data obtained by genetic hybridization analysis (see Supplementary Table S2 in IJSEM Online). Both approaches confirmed the conclusion based on the PCR fingerprinting that the strains studied belonged to the same biological species, *A. schoenii*. Judging from the high percentages of fertility of the interpopulation hybrids and high reassociation values, there is no significant divergence between the *A. schoenii* populations inhabiting Europe, Far-East Asia, North America and the Hawaiian islands. Unlike *A. schoenii*, geographical populations of *Kluyveromyces lactis* and *Saccharomyces paradoxus* yield semi-fertile hybrids and, therefore, are partly reproductively isolated (Naumov & Naumova, 2002; Naumov *et al.*, 1993, 1997b, 1998). Our results show that the species *A. schoenii* has a worldwide distribution: strains collected in different regions of the world are homogeneous from a classical genetic point of view. In this respect, it is similar to another cosmopolitan, panmictic yeast, *Saccharomyces cerevisiae*. Populations of the latter species from different geographical and ecological origins produce highly fertile hybrids with regular segregation of control markers (Naumov *et al.*, 1993, 1998; Liti *et al.*, 2005). *A. javanensis* is a separate species and is represented only by the type culture, CBS 2555^T, isolated from Java in Indonesia.

Determination of taxonomic status of two *Arthroascus* strains having specific PCR fingerprints

Among the 28 *Arthroascus* strains studied, two Japanese isolates, IFO 10138 and UCD 67-278, displayed peculiar patterns with primers (GTG)₅ and M13 (Fig. 1, lanes 27 and 28, respectively). Based on the fingerprints, the two strains

Table 1. *Arthroascus* strains studied

Acronyms for culture collections are: CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; IFO, Institute for Fermentation, Osaka, Japan; KBP, Department of Soil Biology, Faculty of Soil Sciences, Moscow State University, Russia; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA; VKM, All-Russian Collection of Microorganisms, Moscow, Russia; UCD, Herman J. Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California, Davis, CA, USA; UWO, Department of Plant Sciences, University of Western Ontario, London, Ontario, Canada. All sequences shown were obtained in this study, except those deposited under accession numbers U40107, U40126 and U73600. T, Ex-type culture.

Strain	Other designation	Source of isolation	Reference or isolator	GenBank accession no.	
				D1/D2	ITS
<i>A. javanensis</i>					
CBS 2555 ^T	VKM Y-1069 ^T	Soil, Java, Indonesia	Klöcker (1909)	U40107	DQ361058
<i>A. fermentans</i>					
CBS 7830 ^T	NRRL Y-17710 ^T	Soil of orchards, Taiwan	Lee <i>et al.</i> (1994)	U73600	DQ361059
CBS 7831	NRRL Y-17711	Soil of orchards, Taiwan	Lee <i>et al.</i> (1994)		
CBS 7832	NRRL Y-17712	Soil of orchards, Taiwan	Lee <i>et al.</i> (1994)		
<i>A. schoenii</i>					
CBS 7223 ^T	VKM Y-1073 ^T	Exudate of <i>Quercus robur</i> , Kaluga, Russia	N. A. Krassilnikov	U40126	DQ361045
CBS 7048	KBP 2695	Exudate of <i>Q. robur</i> , Moscow district, Russia	Bab'eva <i>et al.</i> (1986)		DQ361046
CBS 10273	KBP 3099	<i>Pachyiulus flavipes</i> , Yalta, Crimea, Ukraine	V. N. Thanh		
CBS 7425		<i>Panagrellus zymosiphilus</i> , Italy	C. Schann	AY165962	DQ361047
CBS 6423*		Soybean-protein factory, Japan	Nakase (1971)	AY165964	DQ361051
CBS 6449		Rotten tree trunk, Japan	K. Kodama	AY165963	DQ361057
CBS 9154	UCD 52-317.54	Exudate of <i>Quercus kelloggii</i> , California, USA	Phaff & Knapp (1956)		DQ361056
CBS 9155	UCD 71-182	Flux of <i>Myoporum sandwicense</i> , Ahumoa, HI, USA	H. J. Phaff	AY165961	DQ361050
CBS 9156	UCD 72-139	Flux of <i>M. sandwicense</i> , Ahumoa, HI, USA	H. J. Phaff	AY165960	
CBS 9157	UCD 72-142	Flux of <i>M. sandwicense</i> , Ahumoa, HI, USA	H. J. Phaff		DQ361048
CBS 9158	UCD 72-156	Flux of <i>M. sandwicense</i> , Ahumoa, HI, USA	H. J. Phaff		DQ361049
CBS 9159	UWO (PS) 80-91	<i>Quercus rubra</i> , Ontario, Canada	Bowles & Lachance (1983)	AY165958	DQ361053
CBS 9160	UWO (PS) 80-92	<i>Q. rubra</i> , Ontario, Canada	Bowles & Lachance (1983)		
CBS 9161	UWO (PS) 80-93	<i>Q. rubra</i> , Ontario, Canada	Bowles & Lachance (1983)		
CBS 9162	UWO (PS) 91-123.2	<i>Quercus alba</i> , Ontario, Canada	M.-A. Lachance		
CBS 9163	UWO (PS) 91-125.1	<i>Q. rubra</i> , Ontario, Canada	M.-A. Lachance		DQ361055
CBS 9164	UWO (PS) 92-247.1	<i>Drosophila</i> sp., tequila factory, Mexico	Lachance (1995)	AY165959	DQ361052
CBS 9165	UWO (PS) 99-507.6	<i>Q. rubra</i> , Ontario, Canada	M.-A. Lachance		
CBS 9166	UWO (PS) 99-531.3	<i>Q. rubra</i> , Ontario, Canada	M.-A. Lachance		DQ361054
NRRL YB-1542		Soil in apple orchard, Peoria, IL, USA			
NRRL YB-2401		Rotten apple, Carbondale, IL, USA			
NRRL YB-2404		Rotten apple, Carbondale, IL, USA			
<i>A. babjevae</i>					
CBS 9167 ^T	UCD 67-278 ^T	<i>Quercus acutissima</i> , Kawaminami, Japan	Phaff <i>et al.</i> (1972)	DQ361062	DQ361061
<i>A. fermentans</i> var. <i>arxii</i>					
CBS 9168 ^T	IFO 10138 ^T	Flower of <i>Tricyrtis flava</i> , Miyazaki, Japan	Mikata (1985)	DQ361063	DQ361060

*Ex-type culture of *Pichia nonfermentans* Nakase.

Fig. 1. PCR fingerprints of *Arthroascus* strains generated with the microsatellite primer (GTG)₅. Lanes: 1–22, *A. schoenii* strains CBS 7223 (1), CBS 7048 (2), CBS 7425 (3), KBP 3099 (4), CBS 6423 (5), CBS 6449 (6), UWO 80-91 (7), UWO 80-92 (8), UWO 80-93 (9), UWO 91-123.2 (10), UWO 91-125.1 (11), UWO 92-247.1 (12), UWO 99-507.6 (13), UWO 99-531.3 (14), UCD 52-317.54 (15), NRRL YB-1542 (16), NRRL YB-2401 (17), NRRL YB-2404 (18), UCD 71-182 (19), UCD 72-139 (20), UCD 72-142 (21) and UCD 72-156 (22); 23, *A. javanensis* CBS 2555; 24–26, *A. fermentans* strains CBS 7830 (24), CBS 7831 (25) and CBS 7832 (26); 27, *Arthroascus* sp. IFO 10138; 28, *Arthroascus* sp. UCD 67-278. Lanes M, Molecular size marker (SmartLadder; Eurogentec); sizes are indicated in base pairs.

could not be assigned to any of the known *Arthroascus* species. To elucidate their taxonomic status, we used sequence analysis of the D1/D2 and ITS regions of rDNA, genetic hybridization analysis and DNA–DNA reassociation.

The D1/D2 nucleotide sequences obtained for strains IFO 10138 and UCD 67-278 were aligned with the corresponding sequences of *A. schoenii*, *A. fermentans* and *A. javanensis*, as well as 13 other species currently included in the *Saccharomycopsis* clade. Fig. 3 shows the phylogenetic relationship between these strains and other members of the genus *Arthroascus*. The *Arthroascus* strains showed close relatedness to one another and formed a well-separated cluster (bootstrap value, 100 %) within the *Saccharomycopsis* clade. The cluster could be subdivided into four groups. The first group included *A. schoenii* strains from different geographical populations. They had identical D1/D2 sequences, except

for the Hawaiian isolates, which exhibited two base differences. The second group consisted of the type culture of *A. javanensis*. Strain IFO 10138 and the type culture of *A. fermentans*, having identical D1/D2 sequences, formed the third group. The fourth group comprised strain UCD 67-278 only. Comparative sequence analysis revealed that the latter strain differed by 7, 11 and 22 bases, respectively, from the type cultures of *A. schoenii*, *A. javanensis* and *A. fermentans*. These results suggest that it belongs to an undescribed species of the genus *Arthroascus*. Close 18S rDNA relatedness of IFO 10138 and the type culture of *A. fermentans* can be seen from the published data (Yamada *et al.*, 1998). However, the latter authors incorrectly assigned the strain to *A. javanensis*.

Seventeen *Arthroascus* strains were further compared in the ITS region and found to be separated into five lineages:

Fig. 2. Neighbour-joining tree showing differentiation of *A. schoenii* strains based on combining the fingerprints obtained with primers (GTG)₅ and M13. Bar, dissimilarity of 0.02 (corresponding to the fraction of average fragment differences between PCR fingerprints).

Fig. 3. Neighbour-joining tree showing phylogenetic relationships between the 16 species within the *Saccharomycopsis* clade based on large subunit D1/D2 rDNA sequences. Bootstrap values from 100 replications are shown (only values >90 are indicated). Bar, 20 estimated base substitutions per 1000 nucleotide positions. T, Ex-type culture.

A. schoenii strains, UCD 67-278, the type culture of *A. fermentans*, IFO 10138 and the type culture of *A. javanensis* (data not shown). The differences between *A. schoenii* strains from four geographical locations did not exceed 4 base substitutions. The ITS sequence analysis showed that IFO 10138 can be differentiated from the type culture of *A. fermentans* on the basis of 11 base substitutions. The ITS region of UCD 67-278 differed by more than 30 base substitutions.

Strains IFO 10138 and UCD 67-278 were crossed with one another and with the type cultures of *A. schoenii*, *A. javanensis* and *A. fermentans*. Homothallic strain IFO 10138 sporulated well and showed 97 % ascospore viability. Strain UCD 67-278 had lower sporulation. The monosporic culture of IFO 10138 and the single-cell clone of UCD 67-278 were marked with auxotrophic mutations. Intrastrain crosses of both strains showed high ascospore viability (100 and 83 %, respectively) and regular digenic segregation of control markers (Tables 2 and 3, hybrids number 46, 58 and 59). IFO 10138 and UCD 67-278 yielded hybrids with each other and with all type cultures of described *Arthroascus* species, confirming their assignment to the same genus. The resulting hybrids were analysed for ascospore viability and recombination of control auxotrophic markers. Strain IFO 10138 formed almost sterile hybrids with the type cultures of *A. javanensis* and *A. schoenii* (Table 2, hybrids number 47–54). Most of the surviving ascospores of hybrids number 47–54 were prototrophic and did not carry parental auxotrophic markers. This suggests that they are not the products of meiotic division: such spores apparently represent hybrid heterozygotic diploids. Hybrids between IFO 10138 and *A. fermentans* showed low ascospore viability of 6–31 %. Although the majority of surviving segregants were prototrophic, there were also double auxotrophic recombinants (ab), indicating a possibility of genetic exchange

between strain IFO 10138 and *A. fermentans* (Table 2, hybrids number 55–57). On the other hand, strain UCD 67-278 formed sterile hybrids with IFO 10138 and with the type cultures of *A. schoenii*, *A. javanensis* and *A. fermentans* (Table 3, hybrids number 60–68). Random spore analysis of the hybrids revealed some surviving ascospores, which were characterized by irregular meiotic segregation of control auxotrophic markers with predominance of prototrophic segregants and auxotrophic markers of only one parental type. The absence of double auxotrophic recombinants (ab) in the progeny of the hybrids indicates that strain UCD 67-278 is genetically isolated from IFO 10138 and the type cultures of *A. schoenii*, *A. javanensis* and *A. fermentans* and, therefore, does not belong to any of these species.

IFO 10138 showed an intermediate reassociation value with the type culture of *A. fermentans* (65 %) and low rates of 19, 23 and 31 %, respectively, with the type cultures of *A. schoenii*, *A. javanensis* and strain UCD 67-278. DNA reassociation values between UCD 67-278 and the type cultures of *A. schoenii*, *A. javanensis* and *A. fermentans* did not exceed 30 % (Supplementary Fig. S1).

Taken together, the results of sequence analysis, genetic hybridization and DNA–DNA reassociation strongly suggest that strain UCD 67-278 represents a novel species of the genus *Arthroascus*, while IFO 10138 represents a novel variety of the species *A. fermentans*.

***I. Arthroascus babjevae* G. Naumov & M. Th. Smith sp. nov.**

Latin diagnosis of *Arthroascus babjevae* G. Naumov et M. Th. Smith sp. nov.

In medio liquido post dies 3 cellulae ellipsoidae vel cylindricae (2.0–4.0 × 6.0–35.0 µm), singulae vel binae vel structurae

Table 2. Genetic analysis of hybrids of *Arthroascus* sp. IFO 10138 with *A. fermentans* CBS 7830^T, *A. schoenii* CBS 7223^T and *A. javanensis* CBS 2555^T

For hybrid 48, data for three independent hybrids are summarized. (1), (2) and (3) represent independently obtained hybrids.

Hybrid	Markers	Spores isolated (<i>n</i>)	Proportion of spores of hybrid viable (%)	Ratio of tetrads*	Segregation of control markers†
				P:N:T:(+)	AB:aB:Ab:ab
<i>Arthroascus</i> sp. IFO 10138 × <i>Arthroascus</i> sp. IFO 10138					
46	<i>ade15</i> × <i>leu2</i>	60	100	1:1:11:0	13:13:12:13
<i>Arthroascus</i> sp. IFO 10138 × <i>A. schoenii</i> CBS 7223 ^T					
47	<i>ade15</i> × <i>ade12</i>	94	12	0:0:0:1	11:0:0:0
48	<i>leu2</i> × <i>ade12</i>	235	0	—	—
49	<i>leu2</i> × <i>lys4</i> (1)	100	6	—	3:0:3:0
	<i>leu2</i> × <i>lys4</i> (2)	15	0	—	(160:0:0:0)
<i>Arthroascus</i> sp. IFO 10138 × <i>A. javanensis</i> CBS 2555 ^T					
50	<i>leu2</i> × <i>his33</i>	200	2	—	2:0:0:0
51	<i>leu2</i> × <i>thr10</i> (1)	100	1	—	1:0:0:0
	<i>leu2</i> × <i>thr10</i> (2)	44	2	—	(64:0:0:0)
52	<i>ade15</i> × <i>his33</i>	112	0	—	—
53	<i>leu2</i> × <i>val11</i>	83	0	—	—
54	<i>ade15</i> × <i>val11</i>	100	0	—	—
<i>Arthroascus</i> sp. IFO 10138 × <i>A. fermentans</i> CBS 7830 ^T					
55	<i>leu2</i> × <i>ura15</i> (1)	52	8	—	2:0:0:0
	<i>leu2</i> × <i>ura15</i> (2)	144	18	0:0:0:2	21:0:0:0
	<i>leu2</i> × <i>ura15</i> (3)	112	14	2:0:0:2	8:4:4:0 (61:27:11:8)
56	<i>leu2</i> × <i>ade20</i> (1)	148	31	3:0:0:3	16:10:10:0
	<i>leu2</i> × <i>ade20</i> (2)	96	7	0:0:0:1	4:0:2:0
	<i>leu2</i> × <i>ade20</i> (3)	352	12	1:0:0:4	23:5:12:1
57	<i>ade15</i> × <i>ura15</i> (1)	52	6	—	2:0:0:0
	<i>ade15</i> × <i>ura15</i> (2)	244	24	0:1:0:5	44:3:1:6
	<i>ade15</i> × <i>ura15</i> (3)	176	10	2:0:0:1	8:6:4:0 (74:39:22:8)

*P, N, T and (+) represent respectively tetrads of parent ditype (2aB:2Ab), non-parent ditype (4AB:0ab), tetratype (1aB:1Ab:1AB:1ab) and prototrophic tetrads (4AB). a and b are the auxotrophies of the first (before the crossing mark) and second parent, respectively. A and B are the prototrophies.

†Random spore analysis data obtained by micromanipulator are presented. The results of random spore analysis using diethyl ether treatment are given in parentheses.

pseudomycelii. Post 7 dies sedimentum adest. Species homothallica. Asci conjugati vel inconjugati, habentes 2–4 sporae. Asci deliquescentes. Ascosporae pustulatae, globosae vel oblatae cum annulum in (sub)equatorio. Fermentatio nulla. Glucosum, sucrosum, trehalosum, glycerolum, acidum succinicum et ethanolum assimilantur (lente). Non assimilantur galactosum, L-sorbosum, D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, maltosum, methyl α -glucosidum, cellobiosum, salicinum, arbutinum, melibiosum, lactosum, raffinose, melizitosum, inulinum, amyllum solubile, erythritolum, ribitolum, xylitolum, L-arabinitolum, D-glucitolum, D-mannitolum, galactitolum, inositolum, glucono- δ -lactonum, acidum gluconicum, acidum glucuronicum, acidum DL-lacticum, acidum citricum, methanolum, lysinum, ethylaminum, cadaverinum, natrium nitricum et natrium nitrosum. Augmentum in 30 °C at non in 35 °C. Ad crescentiam vitaminarum externarum necessariae sunt.

Crescit (lente) in medio addito 0.01 % cycloheximido. G + C acidi deoxyribonucleati 30.4 mol%.

Typus vivus et exsiccatus CBS 9167^T (CBS H-18731) praeservatus in collectione Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum.

Description of *Arthroascus babjevae* G. Naumov & M. Th. Smith sp. nov.

Arthroascus babjevae [bab.je'vae. N.L. fem. gen. n. *babjevae* in honour of Dr I. P. Bab'eva (also transliterated as Babjeva), for her considerable contributions to yeast taxonomy and ecology].

In glucose/peptone/yeast extract (GPY) medium after 3 days, the cells are ellipsoidal to cylindrical (2.0–4.0 × 6.0–35.0 μ m), single, in pairs or in pseudomycelial structures.

Table 3. Genetic analysis of hybrids of *Arthroascus* sp. UCD 67-278 with *A. fermentans* CBS 7830^T, *A. schoenii* CBS 7223^T, *A. javanensis* CBS 2555^T and *Arthroascus* sp. IFO 10138

For hybrids 60–65, data for several independent hybrids are summarized. (1), (2), (3) etc. indicate independently obtained hybrids.

Hybrid	Markers	Spores isolated (<i>n</i>)	Proportion of spores of hybrid viable (%)	Segregation of control markers*
				AB : aB : Ab : ab
<i>Arthroascus</i> sp. UCD 67-278 × <i>Arthroascus</i> sp. UCD 67-278				
58	<i>lys1</i> × <i>arg5</i>	208	82	36:25:24:31†
59	<i>lys1</i> × <i>arg3</i>	104	84	14:14:14:14†
<i>Arthroascus</i> sp. UCD 67-278 × <i>A. fermentans</i> CBS 7830 ^T				
60	<i>lys1</i> × <i>ura15</i> (1)	244	5	7:3:2:0
	<i>lys1</i> × <i>ura15</i> (2)	—	—	(88:103:0:0)
61	<i>arg5</i> × <i>ade20</i> (1)	302	8	19:4:2:0
	<i>arg5</i> × <i>ade20</i> (2)	—	—	(78:94:0:0)
62	<i>arg3</i> × <i>ade20</i> (1–6)	269	1	3:1:0:0 (118:0:0:0)
<i>Arthroascus</i> sp. UCD 67-278 × <i>A. schoenii</i> CBS 7223 ^T				
63	<i>lys1</i> × <i>ade12</i> (1, 2)	116	0	(180:0:0:0)
64	<i>arg3</i> × <i>lys4</i> (1, 2)	112	2	5:0:0:0 (96:0:0:0)
<i>Arthroascus</i> sp. UCD 67-278 × <i>A. javanensis</i> CBS 2555 ^T				
65	<i>lys1</i> × <i>thr10</i> (1, 2)	112	0	(106:0:0:0)
66	<i>arg3</i> × <i>his33</i> (1, 2)	112	0	(167:0:11:0)
<i>Arthroascus</i> sp. UCD 67-278 × <i>Arthroascus</i> sp. IFO 10138				
67	<i>lys1</i> × <i>ade15</i> (1, 2)	116	2	0:0:2:0 (65:1:29:0)
	<i>lys1</i> × <i>ade15</i> (3)	40	0	(67:115:0:0)
68	<i>arg3</i> × <i>leu2</i> (1–3)	152	1	0:2:0:0 (23:60:0:0)

*a and b are the auxotrophies of the first (before the crossing mark) and second parent, respectively. A and B are the prototrophies. Random spore analysis data obtained by micromanipulator are presented. The results of random spore analysis using diethyl ether treatment are given in parentheses.

†Ratio of tetrads P:N:T in hybridizations 58 and 59 were 0:5:14 and 1:1:9, respectively, where P, N and T are the tetrads of parent ditype (2Ab:2Ab), non-parent ditype (4AB:0ab) and tetratype (1aB:1Ab:1AB:1ab), respectively.

After 1 week, sediment and an incomplete tiny ring are present. On Dalmau plate cultures on GPY agar, a well-developed pseudomycelium and septate mycelium is formed. Blastoconidia are not produced. Asci are formed directly from single cells or following conjugation between independent cells. Asci become deliquescent at maturity. Each ascus forms two to four warty ascospores that are globose to oblate with a narrow (sub)equatorial ledge (Fig. 4). Ascosporeulation after 3 days on 5 % Difco malt, V8 and GPY agars. Fermentation is absent. Glucose, sucrose, trehalose, glycerol, succinic acid and ethanol are assimilated (slowly). Growth does not occur on galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, maltose, methyl α -glucoside, cellobiose, salicin, arbutin, melibiose, lactose, raffinose, melezitose, inulin, starch, erythritol, ribitol, xylitol, L-arabinitol, D-glucitol, D-mannitol, galactitol, inositol, glucono- δ -lactone, D-glucuronate, D-glucuronate, DL-lactate, citrate, methanol, nitrite, nitrate, lysine, ethylamine or cadaverine. Growth occurs at

Fig. 4. Asci with ascospores of *Arthroascus babjevae* sp. nov. UCD 67-278^T. Ascosporeulation occurred after 7 days on Difco malt agar at 25 °C. Bar, 5 μ m.

30 °C and in 0.01 % cycloheximide (slowly); growth does not occur at 35 °C or in medium without vitamins. DNA G + C content is 30.4 mol%.

The type strain, UCD 67-278^T (= CBS 9167^T) (dried CBS H-18731, holotype), has been deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Ila. *Arthroascus fermentans* C.-F. Lee, F.-L. Lee, Hsu & Phaff var. *fermentans*

Int J Syst Bacteriol (1994) **44**, 305.

Synonym: *Saccharomycopsis fermentans* (C.-F. Lee, F.-L. Lee, Hsu & Phaff) Kurtzman & Robnett, *Can J Bot* (1995) **73**, S829.

Ex-type strain: CBS 7830 (ex-holotype), isolated from soil of a papaya orchard, Taiwan.

Ilb. *Arthroascus fermentans* var. *arxii* G. Naumov & M. Th. Smith var. nov.

Arthroascus fermentans var. *arxii* (ar'xi.i. N.L. masc. gen. n. *arxii* in honour of Dr J. A. von Arx, who introduced the genus *Arthroascus* in 1972).

Varietas Arthroascus fermentans var. *arxii* differt: *sucrosum assimilatur. Typus vivus et exsiccatus* CBS 9168^T (CBS H-18732) *praeservatus in collectione* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum*.

Arthroascus fermentans var. *arxii* differs from variety *fermentans* by the presence of growth on sucrose. The type strain, IFO 10138^T (= CBS 9168^T) (dried CBS H-18732, holotype), isolated from a flower of *Tricyrtis flava* in Japan, has been deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

III. *Arthroascus javanensis* (Klöcker) von Arx

Antonie van Leeuwenhoek (1972) **38**, 306.

Basionym: *Endomyces javanensis* Klöcker, *C R Trav Lab Carlsberg* (1909) **7**, 267.

Synonyms: *Schwanniomyces javanensis* (Klöcker) Zender, *Bull Soc Bot Geneve* (1926) **17**, 208; *Endomycopsis javanensis* (Klöcker) Dekker (in Stelling-Dekker, *Die Sporogenen Hefen, Verh K Ned Akad Wetensch Afd Natuurk Sect II*, 265, 1931); *Schizosaccharomyces javanensis* (Klöcker) Streiblová, *Folia Microbiol* (1963) **8**, 174; *Saccharomycopsis javanensis* (Klöcker) Kurtzman & Robnett, *Can J Bot* (1995) **73**, S829.

Type strain: CBS 2555 (ex-holotype), isolated from soil, Java, Indonesia, and described by Klöcker (1909), was sent to the CBS Yeast collection by Guilliermond.

IV. *Arthroascus schoenii* (Nadson & Krassilnikov) Babjeva, Vustin, Naumov & Vinovarova

Mikrobiologiya (1985) **54**, 727.

Synonyms: *Endomyces schoenii* Nadson & Krassilnikov, *Dokl Akad Nauk SSSR* (1932) **13**, 313; *Saccharomycopsis schoenii* (Nadson & Krassilnikov) Kurtzman & Robnett, *Can J Bot* (1995) **73**, S829; *Pichia nonfermentans* Nakase, *J Gen Appl Microbiol* (1971) **17**, 469.

Type strain: CBS 7223 (ex-holotype), isolated from exudates of oak (*Quercus* sp.).

Key to the species

1.	a. Glucose fermented	2
	b. Glucose not fermented	3
2.	a. Sucrose assimilated (slowly)	<i>Arthroascus fermentans</i> var. <i>arxii</i>
	b. Sucrose not assimilated	<i>Arthroascus fermentans</i> var. <i>fermentans</i>
3.	a. L-Sorbose assimilated	4
	b. L-Sorbose not assimilated	<i>Arthroascus babjevae</i>
4.	a. N-Acetyl-D-glucosamine assimilated	<i>Arthroascus javanensis</i>
	b. N-Acetyl-D-glucosamine not assimilated	<i>Arthroascus schoenii</i>

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