Antonie van Leeuwenhoek (2010) 98:93–101
DOI 10.1007/s10482-010-9433-5

ORIGINAL PAPER

Ogataea cecidiorum sp. nov., a methanol-assimilating yeast isolated from galls on willow leaves

Anna M. Glushakova · Irina A. Maximova · Aleksey V. Kachalkin · Andrey M. Yurkov

Received: 20 November 2009 / Accepted: 22 March 2010 / Published online: 7 April 2010

Abstract Ten strains of a new endophytic ascospore-forming, methanol-assimilating yeast were isolated from the galls induced by sawflies on the leaves of willows in the Losiny Ostrov National Park (Moscow region). Standard phenotypical tests and phylogenetic analyses of 18S rRNA gene, 5.8S-ITS gene region and 26S rRNA gene (D1/D2 domains) sequences showed that the species belongs to the genus *Ogataea*. We describe it as *Ogataea cecidiorum* and designate type culture KBP Y-3846 (= CBS 11522^T = VKM Y-2982^T = VKPM Y-3482^T = MUCL 52544^T = NCAIM Y.01965^T) as the type strain. The new species was registered in MycoBank under MB 515233.

Keywords New yeast species · Methylotrophic yeast · Molecular systematics · Endophytes · *Ogataea* · *Ogataea cecidiorum*

Electronic supplementary material The online version of this article (doi:10.1007/s10482-010-9433-5) contains supplementary material, which is available to authorized users.

A. M. Glushakova (✉) · I. A. Maximova · A. V. Kachalkin
Faculty of Soil Science, Lomonosov Moscow State University, Leninskie Gory 1, Moscow, Russia 119991
e-mail: soilyeast@mail.ru; andrey.yurkov@gmail.com

A. M. Yurkov
Geobotanik, Fakultät für Biologie und Biotechnologie, Ruhr-Universität Bochum, Universitätsstraße 150, 44801 Bochum, Germany

Introduction

Methanol-assimilating yeasts have been observed from the various plant-related substrates, like slime fluxes (Péter et al. 2006), tree bark, rotten wood material (Péter et al. 2003), and leaves (Péter et al. 2007). Recently they have been detected on about 45% of leaves collected in Hungary (Péter et al. 2007). Methanol produced inside the leaves and emitted primarily through stomata (Fall and Benson 1996) can obviously support development of these ascomycetous yeasts although the most common phylloplane colonisers are of basidiomycetous affinity (Péter et al. 2007). Consequently, inner plant tissues could be even more promising habitats for detection of the yeasts with rare nutritional abilities, including the assimilation of methanol. Indeed, endophytous yeast communities have been reported recently to be significantly distinct from those formed on plant surfaces (Isaeva et al. 2009). Abnormal growths formed from plant tissues and due to parasitic activity of another organism, known as galls (Redfern and Shirley 2002) are annually observed on leaves of deciduous plants. They can be induced by fungal or bacterial infections as well as the activity of different invertebrates. Sawflies are well known gall-inducing insects colonising willows in Eurasia with an extensive and opportunistic host shifts (Price 2005). The wide ecological distribution of willows and the proximity of plant species in the field enable frequent host shifts and radiation of sawflies, but also make galls, as a

substrate, more abundant in temperate zone. While plant surfaces have been recognized as an important habitat for yeasts and filamentous fungi for a long time, galls, as a substrate received very little attention so far.

During the survey aimed to analyse yeasts found as endophytes in insect galls of willow leaves in comparison with regular epiphytic yeast community, a novel methanol-assimilating yeast was isolated repeatedly. This species is described here as *Ogataea cecidiorum*.

Materials and methods

The survey was performed at Losiny Ostrov (Elk park) National Park, Moscow, Russia (approximate coordinates: 55.85N; 37.75E). Particular attention was given to the analysis of leaves that formed galls on their surface, which were induced by sawflies (*Pontania* spp. and *Euura* spp., Order Hymenoptera, Familia Tenthredinidae). Leaves of willows (*Salix myrsinifolia* Salisb.) were collected between May and September 2008, as long as galls were not destroyed due to elaboration of adult insects. Seventy intact leaves and 70 leaves containing a gall were collected and analysed.

Phylloplane yeast community was analysed by plating of leaf washings. Leaves were placed in 50 ml plastic tube, suspended 1:50 (w/v) in sterile demineralised water and vortexed. An aliquot of 0.1–0.2 ml was distributed on the surface of acidified with 4 ml/l lactate (final pH 4–4.5) glucose-yeast extract-peptone (GPY) agar, containing 20 g/l glucose, 5 g/l yeast extract, 10 g/l peptone, 20 g/l agar. Each sample was plated in triplicates. Pre-cultivation sample treatment was performed to isolate yeasts from the internal gall layers. For this, leaf surfaces were sterilised with ethanol and the epidermis was removed. The internal part of each gall was placed in a 50 ml plastic tube, diluted 1:50 (w/v) in sterile demineralised water, vortexed and plated in triplicates on acidified GPY agar as described above. Plates were incubated at room temperature and checked after one and two weeks. Grown yeast colonies were differentiated into macromorphological types using a dissection microscope, counted, and 1–2 representatives of every colony type per plate were purified. Phenotypic characterization of purified isolates was carried out according to Yarrow (1998).

DNA was extracted using the protocol of Sampaio et al. (2001) with the modification of cell lysate centrifugation at room temperature for 15 min, with 14,500 rpm. Mini- and microsatellite-specific PCR fingerprints and Restriction Fragments Length Polymorphism analysis were performed as described before (Yurkov and Chernov 2005; Kachalkin et al. 2008). For sequencing of the D1/D2 region of the 26S ribosomal gene and the internal transcribed spacers (ITS), DNA fragments were amplified with the ITS1f (5′-CTT GGT CAT TTA GAG GAA GTA) and NL4 (5′-GGT CCG TGT TTC AAG ACG G) primer pair using a program consisting of an initial denaturation step of 2 min at 96°C, followed by 35 cycles of 20 s at 96°C, 50 s 52°C and 1.5 min at 72°C and a final extension step of 7 min at 72°C. The 18S ribosomal RNA gene region was amplified according to White et al. (1990). PCR products were purified with myBudget Double Pure kit (Bio-Budget Tech., Germany) and sequenced using an ABI3130xl sequencer using the amplification primers. The assembly and editing of sequence data was performed using Sequencher 4.7 (Gene Codes Corp., USA). Alignments were made using the MAFFT algorithm (Katoh et al. 2002). The model of DNA substitution (GTR + G+I) and parameters for maximum likelihood analysis were derived by using Modeltest, version 3.7 (Posada and Crandall 1998). Maximum likelihood analysis was performed using RAxML, version 7.0.3 with 1,000 rounds of bootstrap replicates (Stamatakis et al. 2008; Felsenstein 1985).

Constrained trees based on several hypotheses for the phylogenetic relationships of the novel species and the other members of the *Ogataea* genus were constructed with PAUP 4.0b10 (Swofford 2002) and the Kishino–Hasegawa test (Kishino and Hasegawa 1989) was performed to evaluate the significance of those hypotheses. Therefore congruence between best maximum likelihood and constrained tree topologies also was examined by the Kishino–Hasegawa test.

Results and discussion

Ecology

The total abundance of yeasts inhabiting the leaf surface estimated by the plating technique was 2.5×10^5 CFU/g, in average and maximal 5.0×10^5 CFU/g

in June. Galls contained less yeasts, 5.0×10^4 CFU/g in average, but the total yeast quantity gradually increased through the sampling period reaching a maximum of 1.5×10^5 CFU/g in August.

The internal gall yeast community observed at the beginning of gall formation resembled very closely the epiphytic community with a dominance of filobasidious cryptococci. One month later, galls contained mainly ascomycetous yeasts, in contrast to the phylloplane, which was inhabited mainly by the basidiomycetous yeasts *Cryptococcus* spp. (Magnus clade, Filobasidiales), *Rhodotorula glutinis* sensu lato and *Cystofilobasidium capitatum*. Ascomycetes were very rare on the leaf surface. Besides *Candida oleophila*, which had been reported to be a regular phylloplane yeast (Glushakova et al. 2007), *Wickerhamomyces anomalus* (*Pichia anomala*) was observed on the leaf surface from June to September.

The internal gall yeast community was dominated by the novel methanol-assimilating yeast with an average relative abundance of 94–100% in June, July and August. However, the novel species was rarely observed at the initial stage of the gall formation in May (<1%) and after elaboration of adult insects, when galls dried out (9%). These observations imply that the environmental conditions formed in the galls during the development of sawfly's larvae are favourable for methanol-assimilating yeast. This could be, partly, explained by the fact that methanol produced in the plant tissues (Fall and Benson 1996) can support the development of the yeast population in a confined environment like a gall. Péter et al. (2007) recently reported the regular occurrence of methanol-assimilating yeasts on leave surfaces in Hungary, however, often in a low abundance. Regular isolation of *O. cecidiorum* and its high relative abundance in galls allow us to treat this habitat as a probable ecological niche for this species. A possible function of the gall-inducing insects as vectors for the yeast is considered and will lead to additional studies.

Identification, characterisation and distinction from related species

A total of about 100 cultures were isolated and studied in the survey. The physiological properties of the novel species resembled those of methanol-assimilating yeasts, including members of genera *Ogataea*, *Pichia* and related *Candida* species. The most similar species was *Pichia methanolica*, from which it differed in the assimilation of galactose, dulcitol and salicin (Kurtzman and Fell 1998).

We reduced the number of strains involved in the molecular studies by taking two to three isolates when they were obtained from the same sampling time, but also displayed identical growth responses. Thereby, 10 isolates obtained from different galls collected at different times were chosen to represent the novel species and were used in further analyses. They were grouped by PCR-fingerprinting with minisatellite (MS)-specific oligonucleotide derived from the core sequence of bacteriophage M13 with the sequence given by Sampaio et al. (2001) and the microsatellite (MS)-specific oligonucleotides $(GTG)_5$, $(ATG)_5$ and $(GAC)_5$ as single PCR primer. This technique has been repeatedly demonstrated to distinguish closely related species and populations of ascomycetous yeasts (Inácio et al. 2004; Naumova et al. 2004; Yurkov and Chernov 2005; Fidalgo-Jiménez et al. 2008). All of the studies strains showed high similarity of MS-fingerprints suggesting conspecificity (Fig. S1). Their conspecificity was additionally tested by Restriction Fragment Length Polymorphisms of the 5.8S-ITS rDNA region (Esteve-Zarzoso et al. 1999). All strains showed identical restriction profiles, namely, fragments of 540 + 250 (*Hae*III), 480 + 320 (*Hinf*I), and 400 + 330 (*Cfo*I) nucleotides. However, no match was found between observed and previously recorded restriction profiles (Esteve-Zarzoso et al. 1999; Villa-Carvajal et al. 2006).

The nearest sequence match among currently recognized species was obtained with *Pichia trehalophila* showing four nucleotide substitutions and one gap in the D1/D2 domains of LSU rRNA, one nucleotide substitution and one gap in SSU rRNA gene, and 16 nucleotide substitutions and three gaps in the 5.8S-ITS fragment. Phylogenetic analysis of pairwise sequence similarity for the small subunit rRNA gene, ITS region (ITS1-5.8S rRNA gene-ITS2) and the large subunit rRNA gene (D1/D2 domains) suggested relatedness of the newly described species with *Pichia methanolica*, *Pichia trehalophila*, and *Williopsis salicorniae* (Fig. 1, Fig. S2), also referred to the *Pichia methanolica* group (Nagatsuka et al. 2008). This group has repeatedly been mentioned to belong to the phylogenetic clade of methanol assimilating yeasts, comprising, amongst others, also *Pichia angusta* (*Ogataea polymorpha*) (Kurtzman and

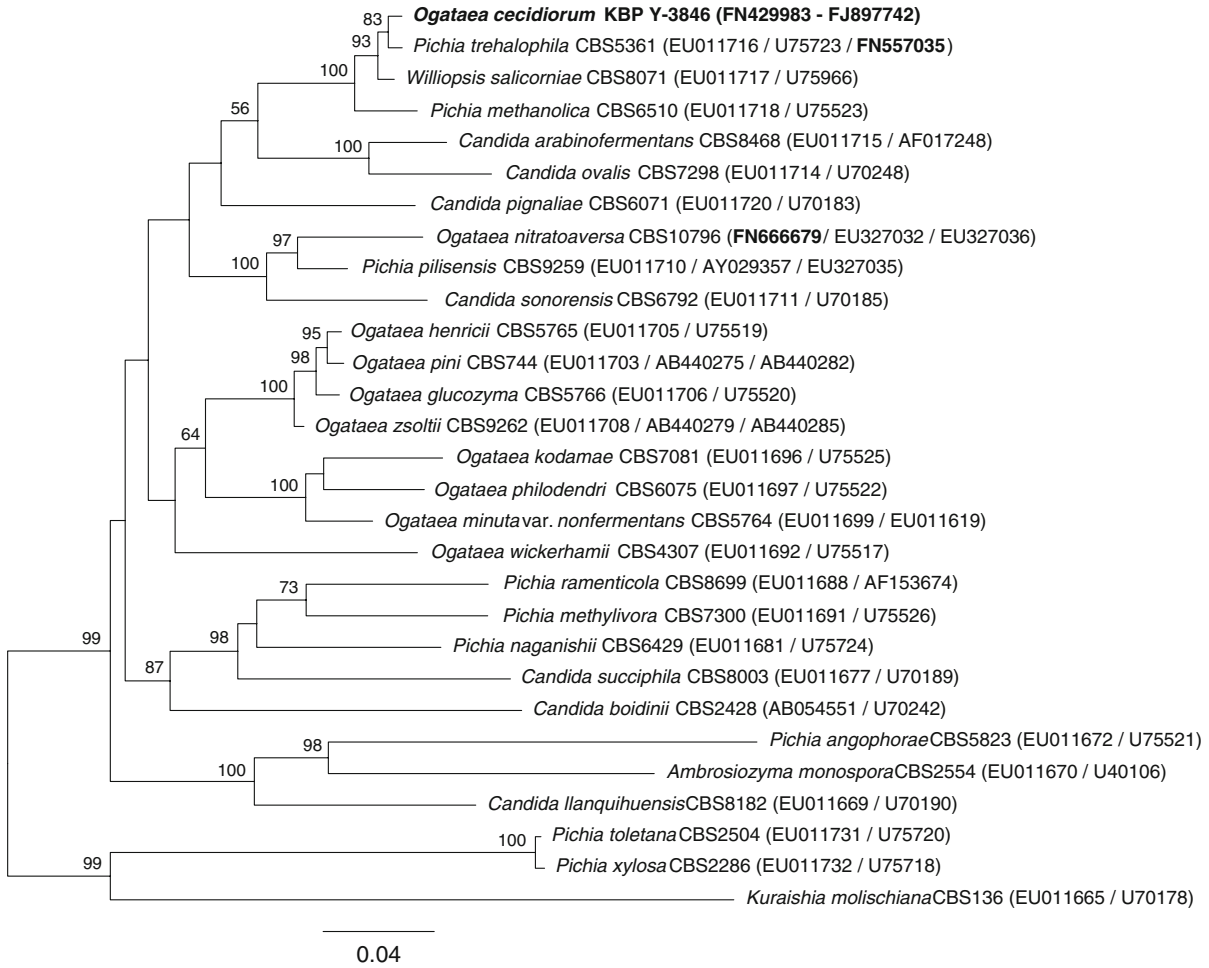

Fig. 1 Unrooted tree illustrating phylogenetic placement of *O. cecidiorum* obtained by maximum likelihood analysis of 18S rRNA, 26S rRNA (D1/D2 domains) genes and the ITS region. The *numbers* given on branches are frequencies (>50%) with which a given branch appeared in 1,000 bootstrap replications. The scale indicates the number of expected substitutions accumulated per site. Sequence accession numbers of type strains are listed; sequences determined in this study are given in bold

Robnett 1998; Morais et al. 2004). Recently, Kurtzman and Robnett (2010) proposed the transfer of the *Pichia methanolica* group and the sister species, *Pichia pilisensis*, *P. naganishii*, *P. methylivora* and *P. ramenticola* to the genus.

Analysis of the large subunit (D1/D2 domains) and the small subunit rRNA genes showed high similarities among the type strains of the "*Pichia methanolica*" group (Nagatsuka et al. 2008), including the proposed species *O. cecidiorum*, while ITS sequences clearly distinguished them (Table 1). High sequence similarities of ribosomal gene regions, not exceeding five nucleotide positions in the D1/D2 domains, were also reported for the species pair *Pichia pilisensis–Ogataea nitratoaversa* (Péter et al. 2008) and species, forming another subgroup in the *Ogataea* clade, related to *Ogataea glucozyma* (Nagatsuka et al. 2008).

The following physiological tests, namely positive assimilation of methyl-α-D-glucoside, inability to ferment trehalose and the maximal growth temperature, provide phenotypic differentiation of the novel species from *Pichia trehalophila*. All members of the *Pichia methanolica* group were considered as independent species on the basis of physiological tests. The first described species, *Pichia trehalophla* was isolated from tree fluxes (Phaff et al. 1964). Later, *Pichia methanolica* and *Pichia cellobiosa* were isolated from

Table 1 Pairwise comparison of 26S (D1/D2 domains, 570 bp), 18S (1670 bp) rRNA genes and the ITS1-5.8S-ITS2 region (730 bp) sequence data derived from alignment (MAFFT algorithm): substitutions and gaps, illustrating relationship within *P. methanolica* cluster

		P. methanolica	*W. salicorniae*	*P. trehalophila*
O. cecidiorum	26S, D1/D2	5 + 2	5 + 1	4 + 1
	18S	3 + 0	1 + 0	1 + 1
	5.8S-ITS	67 + 6	27 + 3	16 + 3
P. methanolica	26S, D1/D2		7 + 1	6 + 1
	18S		3 + 0	3 + 1
	5.8S-ITS		64 + 2	68 + 7
W. salicorniae	26S, D1/D2			5 + 0
	18S			0 + 1
	5.8S-ITS			23 + 4

the soil in Japan (Kato et al. 1974; Lee and Komagata 1980). Conspecificity of these species was demonstrated in DNA–DNA reassociation experiments by Kurtzman (1992) and *P. methanolica* has priority over the name *P. cellobiosa*. Interestingly, *P. methanolica* and *P. cellobiosa* differed in the original descriptions only in assimilation of L-sorbose and DL-lactate and fermentation of maltose. However, Lee and Komagata (1980) designated *Pichia castillae* as the closest species on the basis of physiological tests. In the original description *Pichia methanolica* significantly differs from *Pichia trehalophila* in assimilation of seven out of 30 carbon sources. The former species also grew above 37°C. Therefore, the recognition of *Pichia methanolica* was well-supported on the basis of a phenotypic approach. The last species, *Williopsis salicorniae*, was compared in physiological properties with the other members of *Williopsis* genus. This species did not assimilate methanol, and its relationship with methanol-assimilating yeasts was not recognised in the original description (Hinzelin et al. 1991). Close relationship of these taxa and other methanol-assimilating yeasts was not revealed before the analyses of partial 26S rRNA (Kurtzman and Robnett 1998) and 18S rRNA (James et al. 1998; Suzuki and Nakase 1999) genes sequence data.

Latin diagnosis of *O. cecidiorum* Glushakova, Maximova, Kachalkin et Yurkov sp. nov.

In medio liquido cum extracto malti (YM) post dies tres ad 25°C cellulae singulae, binae, in catenis brevis, aut congregationibus; cellulae ovoidae aut elongatae (1.8–4.8 × 2.5–5 μm). Post mensem unum sedimentum et pellicula (exigue) formantur. Cultura in striis in agaro cum dextroso et peptono et extracto levidinis (GPY) post unum mensem ad 25°C candida ad canita, obscura, laevis, margine integri. In agaro cum extracto malti post 3–5 dies ad 25°C cellulae sphaeroideae ad ovoideae, singulae, binae vel aggregatae, multilateraliter gemmantes. Post hebdomades tres in agaro farinae Zea mays vel agaro YM ad 20–25°C pseudohyphae vel hyphae verae desunt. Status teleomorphicus post cultivationem 3–7 dies ad 25°C in agaro cum extracto Solani tuberose et dextroso (PD) vel in agaro cum extractis levidinis et malti observatus, homothallicus. Asci conjugatione cellularum vel conjugatione cellularum gemmarumque oriuntur. Asci continentens 2–4 galeiformes aut pileiformes sporas. Glucosum fermentatur (procrastinater, post 3–4 dies) at non D-galactosum, sucrosum, D-maltosum, trehalosum, D-xylosum, nec raffinosum. L-sorbosum, trehalosum, D-xylosum (variabiliter), L-arabinosum (variabiliter), D-arabinosum (variabiliter), D-ribosum, L-rhamnosum (interdum exigue), methanolum, ethanolum, glycerolum, erythritolum, ribitolum, sorbitolum, D-mannitolum, methyl-α-D-glucosidum, arbutinum (variabiliter) et acidum succinicum (variabiliter) assimilantur at non D-galactosum, sucrosum, D-maltosum, cellobiosum, lactosum, melibiosum, raffinosum, melezitosum, inulinum, amylum solubile, D-glucosaminum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum, acidium citricum, acidium acidum D-glucuronicum, nec inositolum. Assimilatio nitro-compositorum: kalium nitricum (aliquando exigue) et lisinum. Materia amyloidea iodophila non formantur. Ureum non finditur. Diazonium caeruleum B est negativum.

Temperatura maxima crescentiae: 32°C. Cultura typica KBP Y-3846 isolata ex cecidium *S. myrsinifolia*, viva et exsiccata numero CBS 11522^T (= VKM Y-2982^T = VKPM Y-3482^T = MUCL 52544^T = NCAIM Y.01965^T) in collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, Hollandia, sustentat.

Description of *Ogataea secidiorum* Glushakova, Maximova, Kachalkin et Yurkov sp. nov.

In liquid malt extract (YM) medium after three days at 25°C cells are ovoid to elongated (1.8–4.8 × 2.5–5 μm), occurring singly, in pairs or in small clusters. After 1 month sediment and pellicle were formed. On Glucose Peptone Yeast extract Agar (GPYA), after 1 month at 25°C, the streak culture is whitish to pale-grey, dull and smooth. The margin is entire. After growth on YM agar for 3–5 days at 25°C, cells are spheroidal to short ovoidal (1.8–4.8 × 2.5–5.0 μm), occur singly, in pairs or in small clusters and proliferate by multilateral budding (Fig. 2). Pseudohyphae and true hyphae are not observed after three weeks in plate culture on YM agar or on Corn Meal (CM) agar at 20–25°C. On modified Gorodkowa agar cells are angular. The teleomorphic stage was obtained independently for all 10 strains on different media recommended for ascospore formation (van der Walt and Yarrow 1984; Yarrow 1998). Ascospores were observed on McClary's acetate, potato-dextrose (PD, Difco), Gorodkowa, and YM agars after 3–7 days at 25°C. Strain
KBP Y-3846 produced ascospores on GPY agar after replating from PD agar. Copulation tubes were frequently observed on McClary's acetate and potato-dextrose agars. Ascus formation may be preceded by either conjugation between a parent cell and a bud or by conjugation between independent cells. Asci contain two or four hat-shaped ascospores, and after maturation, ascospores are liberated from the ascus and tend to agglutinate (Fig. 2).

Glucose fermentation is delayed and starts after 3–4 days. No fermentation of galactose, sucrose, maltose, trehalose, xylose and raffinose was detected. Assimilation of carbon compounds: L-sorbose, trehalose, D-xylose (variable), L-arabinose (variable),

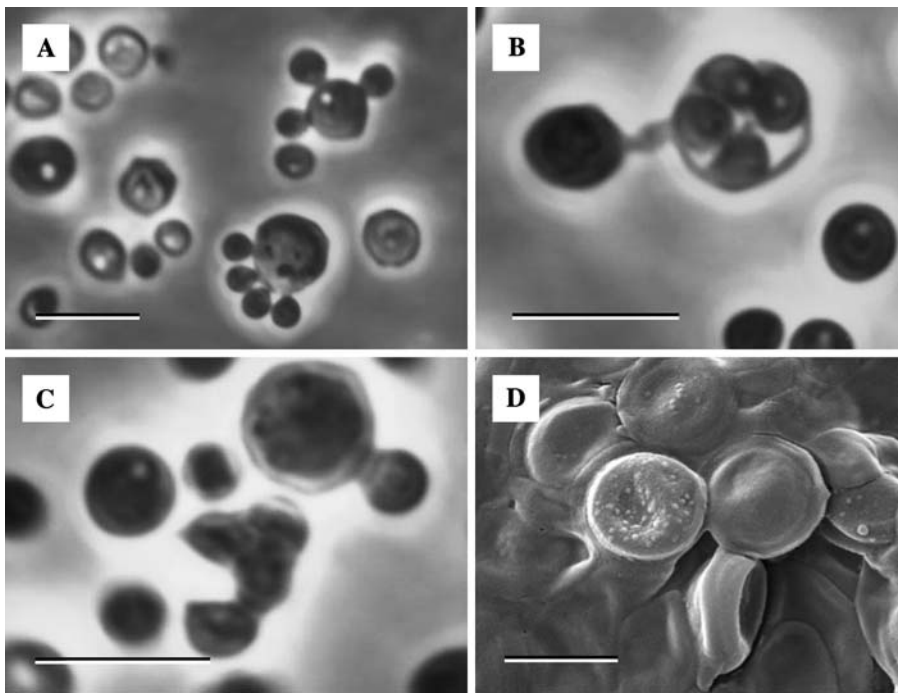

Fig. 2 Phase contrast micrographs (**a**–**c**) and scanning electron microscopic image (**d**) of *O. cecidiorum* KBP Y-3846. Vegetative cells reproducing by multilateral budding (**a**) after 7 days on YM agar at room temperature. Conjugated ascus with ascospores and copulation tube (**b**); hat-shaped ascospores are liberating from the ascus (**c**); McClary acetate agar, 14 days at room temperature, bar = 10 μm. Bar on scanning electron microscopic image (**d**) = 2 μm

D-arabinose (variable), D-ribose, L-rhamnose (occasionally weak), methanol, ethanol, glycerol, erythritol, ribitol, sorbitol, D-mannitol, methyl-α-D-glucoside, arbutin (variable) and succinic acid (variable) are assimilated. No growth occurs on galactose, sucrose, maltose, cellobiose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-glucosamine, 2-ketogluconic acid, 5-ketogluconic acid, DL-lactic acid, citric acid, D-glucuronic acid and inositol. Assimilation of nitrogen compounds: potassium nitrate (occasionally weak) and L-lysine. Starch-like compounds are not produced. Growth at 20, 25, 28, 30 and 32°C is positive. Growth at 34, 37 and 40°C is negative. Urease activity is negative. The Diazonium Blue B reaction is negative. Growth on vitamin-free medium is positive. Growth on YM agar with 10% sodium chloride is negative. Growth in 50% glucose/yeast extract (0.5%) is weak. Growth on 1% acid acetic medium is negative. Growth in the presence of 0.1% cycloheximide is positive.

The habitat is galls induced by sawflies on leaves of willows. Type strain KBP Y-3846 was isolated from leaf sample collected in the Losiny Ostrov National Park, Moscow, Russia. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 11522^T (= VKM $Y\text{-}2982^T$ = VKPM $Y\text{-}3482^T$ = MUCL 52544^T = NCAIM $Y.01965^T$). The specific epithet *cecidiorum* refers to the substrate, galls (cecidium) from which the species has been isolated.

The sequence of the ITS region (ITS1-5.8S-ITS2) and 26S rRNA (D1/D2 domain) is deposited in GenBank under the accession number FJ897742. The sequence of the 18S rRNA gene is deposited in GenBank under the accession number FN429983.

Phylogenetic placement

Despite the observation that most methanol-assimilating yeasts appear closely related (Kurtzman and Robnett 1998), the genus *Ogataea* has not been well recognised until recently (Suh et al. 2006). Kurtzman et al. (2008) demonstrated the polyphyletic nature of the genus *Pichia* from multigene sequence analysis and proposed to use the genus name *Pichia* only for species closely related to *Pichia membranifaciens*, the type species. Several studies have proposed the transfer of certain *Pichia* species to *Ogataea* (Limtong et al. 2008; Nagatsuka et al. 2008) and the genus diagnosis has been modified several times to accommodate phenotypic differences among phylogenetically related *Pichia* species (Péter et al. 2007, 2008; Nagatsuka et al. 2008).

Recently, a study of the *Ogataea–Ambrosiozyma–Kuraishia* clade resulted in the designation of three sub-clusters, even though the tree had weak branch support (Nagatsuka et al. 2008). From these results, *Ogataea nitratoaversa* was found to be related to a sub-cluster that included *Pichia methanolica*, *P. trehalophila*, *Williopsis salicorniae*, *P. pilisensis*, *Candida piceae* and *C. sonorensis*. The support for relatedness of that cluster to the other *Ogataea* species was weak. Nevertheless, due to the placement of *Ogataea nitratoaversa* apart from other recognised *Ogataea* spp., but in the "*P. methanolica*" clade, the authors suggested exclusion of the latter from the genus *Ogataea*.

Phylogenetic analysis of ribosomal small and large (D1/D2 domains) subunit genes and the ITS region performed in the current study using the maximum likelihood algorithm clearly suggests relatedness of the newly described species with a species group that includes *Pichia methanolica*, *P. trehalophila* and *Williopsis salicorniae*, and additionally reveals relationship between this group and *C. arabinofermentans* and *C. ovalis* (Fig. 1; Fig. S2). The clade comprised of *Pichia pilisensis*, *Candida piceae*, *C. sonorensis* and the recently described *Ogataea nitratoaversa* appeared to be the sister group to the "*P. methanolica*" clade.

The Kishino–Hasegawa test of constrained maximum likelihood trees was used to find out whether the novel species *O. cecidiorum* and the members of the "*P. methanolica*" clade form a monophyletic group with the other known *Ogataea* species (except for *O. nitratoaversa*) and thus could be classified in the latter genus. Therefore, the statistical estimation of phylogenetic tree topologies corresponding to the three different hypotheses of the "*P. methanolica*" clade placement were submitted to the Kishino–Hasegawa test: (i) when that clade was constrained with the *Candida cidri*, *C. hungarica*, *Kuraishia molischiana*; (ii) when it was constrained with the *Ambrosiozyma* clade (including *Pichia angophorae*); (iii) when it was constrained with the other described *Ogataea* species. After the statistical comparison of the tree topologies the first two constrained trees were rejected in favour

the tree when "*P. methanolica*" clade forms a monophyletic group with the other *Ogataea* species (diff −ln L: 124.69 and 216.41 respectively, $P < 0.001$). Our results suggest that members of "*P. methanolica*" clade should be rather classified in the genus *Ogataea*, than as in any other genera. Additionally, the properties of these species do not contradict the diagnosis of the genus *Ogataea* emended by Péter et al. (2008) and Nagatsuka et al. (2008).

Our observations are supported by the four-gene phylogenetic study of Kurtzman & Robnett, simultaneously performed with our investigation, which revealed strong support for the separate position of *Ambrosiozyma* clade and brought all *Ogataea* species and related *Pichia*, *Williopsis* and *Candida* species in into a single clade (Kurtzman and Robnett 2010). Based on these results, authors suggested the transfer of *Pichia methanolica*, *P. trehalophila*, *P. pilisensis* and *Williopsis salicorniae* to the genus *Ogataea* (Kurtzman and Robnett 2010). Phylogenetic analysis performed in our study supports relatedness of these species (Fig. 1). Therefore, we support the novel combinations *Ogataea trehalophila*, *O. methanolica*, *O. pilisensis* and *O. salicorniae* proposed by Kurtzman and Robnett (2010) and report a novel member of this clade for which the name *O. cecidiorum* is proposed.

Acknowledgements The authors are grateful to Chernov, I.Yu. (Moscow State University, Russia) and Daniel, H.-M. (BCCM/MUCL, Belgium) for their valuable suggestions on the manuscript. We also thank Prokhorov, V. P. (Moscow State University, Russia) for correcting the Latin diagnosis of the novel species. Schäfer, A. M. and Maier, W. (Ruhr-Universität Bochum, Germany) are acknowledged for various assistance. This work was supported by the Russian Foundation for Basic Research, project no. 07-04-00481, and the Russian Federation President's Program to support the Russian Ph.D. research scientists, project no. MK-5278.2008.4. Andrey Yurkov holds a Post-doc fellowship (A/07/94549) from German Academic Exchange Service (DAAD, Germany). We thank Kurtzman C.P. and Robnett C.J. for sharing unpublished data and suggestions to the manuscript. The type strains of *Ogataea nitratoaversa* and *Pichia trehalophila* were kindly provided by Péter G (NCAIM, Hungary) and Daniel H.-M. (BCCM/MUCL, Belgium).

References

Esteve-Zarzoso B, Belloch F, Uruburu A, Querol A (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int J Syst Evol Microbiol 49:329–337

Fall R, Benson A (1996) Leaf methanol—the simplest natural product from plants. Trends Plant Sci 1:296–301

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fidalgo-Jiménez A, Daniel H-M, Evrard P, Decock C, Marc-André Lachance M-A (2008) *Metschnikowia cubensis* sp. nov., a yeast species isolated from flowers in Cuba. Int J Syst Evol Microbiol 58:2955–2961

Glushakova AM, Yurkov AM, Chernov IYu (2007) Massive isolation of anamorphous ascomycete yeasts *Candida oleophila* from plant phyllosphere. Microbiology 76(6): 799–803

Hinzelin F, Kurtzman CP, Smith MTh (1991) *Williopsbs salicorniae* sp. nov. Antonie van Leeuwenhoek 59:125–127

Inácio J, Rodrigues MG, Sobral P, Fonseca Á (2004) Characterisation and classification of phylloplane yeasts from Portugal related to the genus *Taphrina* and description of five novel *Lalaria* species. FEMS Yeast Res 4:541–555

Isaeva OV, Glushakova AM, Yurkov AM, Chernov IYu (2009) The yeast *Candida railenensis* in the fruits of English oak (*Quercus robur* L.). Microbiology 78(3):355–359

James SA, Roberts IN, Collins MD (1998) Phylogenetic heterogeneity of the genus *Williopsis* as revealed by 18s rRNA gene sequences. Int J Syst Bacteriol 48:591–596

Kachalkin AV, Glushakova AM, Yurkov AM, Chernov IYu (2008) Characterization of yeast groupings in the phyllosphere of *Sphagnum* mosses. Microbiology 77(4): 474–481

Kato K, Kurimura Y, Makiguchi N, Asai Y (1974) Determination of methanol strongly assimilating yeasts. J Gen Appl Microbiol 20:123–127

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform (describes the FFT-NS-1, FFT-NS-2 and FFT-NS-i strategies). Nucleic Acids Res 30:3059–3066

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. J Mol Evol 29:170–179

Kurtzman C (1992) DNA relatedness among phenotypically similar species of *Pichia*. Mycologia 84:72–76

Kurtzman CP, Fell JW (1998) Yeasts, a taxonomic study, 4th edn. Elsevier, Amsterdam

Kurtzman C, Robnett C (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73:331–371

Kurtzman C, Robnett C (2010) Systematics of methanol assimilating yeasts and neighboring taxa from multigene sequence analysis and the proposal of *Peterozyma* gen. nov., a new member of the Saccharomycetales. FEMS Yeast Res. doi:10.1111/j.1567-1364.2010.00625.x

Kurtzman C, Robnett C, Basehoar-Powers E (2008) Phylogenetic relationships among species of *Pichia*, *Issatchenkia* and *Williopsis* determined from multigene sequence analysis, and the proposal of *Barnettozyma* gen. nov., *Lindnera* gen. nov. and *Wickerhamomyces* gen. nov. FEMS Yeast Res 8:939–954

Lee JD, Komagata K (1980) *Pichia cellobiosa*, *Candida cariosilignicola*, and *Candida succiphila*, new species of

methanol-assimilating yeasts. Int J Syst Bacteriol 30:514–519. doi:10.1099/00207713-30-2-514

Limtong S, Srisuk N, Yongmanitchai W, Yurimoto H, Nakase T (2008) *Ogataea chonburiensis* sp. nov. and *Ogataea nakhonphanomensis* sp. nov., thermotolerant, methylotrophic yeast species isolated in Thailand, and transfer of *Pichia siamensis* and *Pichia thermomethanolica* to the genus *Ogataea*. Int J Syst Evol Microbiol 58:302–307

Morais R, Teixeira L, Bowles J, Lachance M-A, Rosa C (2004) *Ogataea falcaomoraisii* sp. nov., a sporogenous methylotrophic yeast from tree exudates. FEMS Yeast Res 5:81–85

Nagatsuka Y, Saito S, Sugiyama J (2008) *Ogataea neopini* sp. nov. and *O. corticis* sp. nov., with the emendation of the ascomycete yeast genus *Ogataea*, and transfer of *Pichia zsoltii*, *P. dorogensis*, and *P. trehaloabstinens* to it. J Gen Appl Microbiol 54:353–365

Naumova ES, Gazdiev DO, Naumov GI (2004) Molecular divergence of the soil yeasts *Williopsis* sensu stricto. Microbiology 73(6):768–776

Péter G, Tornai-Lehoczki J, Fulop L, Dlauchy D (2003) Six new methanol assimilating yeast species from wood material. Antonie van Leeuwenhoek 84:147–159

Péter G, Dlauchy D, Tornai-Lehoczki J (2006) *Candida floccosa* sp. nov., a novel methanol-assimilating yeast species. Int J Syst Evol Microbiol 56:2015–2018

Péter G, Tornai-Lehoczki J, Dlauchy D (2007) *Ogataea allantospora* sp. nov., an ascomycetous yeast species from phylloplane. Antonie van Leeuwenhoek 92:443–448

Péter G, Tornai-Lehoczki J, Dlauchy D (2008) *Ogataea nitratoaversa* sp. nov., a methylotrophic yeast species from temperate forest habitats. Antonie van Leeuwenhoek 94:217–222

Phaff HJ, Miller MW, Spencer JFT (1964) Two new species of *Pichia* isolated from slime fluxes of deciduous trees. Antonie van Leeuwenhoek 30:132–140

Posada D, Crandall K (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14(9):817–818

Price PW (2005) Adaptive radiation of gall-inducing insects. Basic Appl Ecol 6:413–421

Redfern M, Shirley P (2002) British plant galls: identification of galls on plants and fungi. Field Stud 10:207–531

Sampaio J, Gadanho M, Santos S, Duarte F, Pais C, Fonseca A, Fell J (2001) Polyphasic taxonomy of the basidiomycetous yeast genus *Rhodosporidium*: *Rhodosporidium kratochvilovae* and related anamorphic species. Int J Syst Evol Microbiol 51:687–697

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web-servers. Syst Biol 75(5):758–771

Suh S, Blackwell M, Kurtzman C, Lachance M (2006) Phylogenetics of *Saccharomycetales*, the ascomycete yeasts. Mycologia 98:1006–1017

Suzuki M, Nakase T (1999) A phylogenetic study of ubiquinone Q-8 species of the genera *Candida*, *Pichia*, and *Citeromyces* based on 18S ribosomal DNA sequence divergence. J Gen Appl Microbiol 45:239–246

Swofford DL (2002) PAUP: phylogenetic analysis using parsimony, version 4.0b10. Illinois Natural History Survey, Champaign

Van der Walt JP, Yarrow D (1984) Methods for the isolation, maintenance, classification and identification of yeasts. In: Kreger-van Rij NJW (ed) The yeasts. A taxonomic study, 3rd edn. Elsevier, Amsterdam, pp 45–105

Villa-Carvajal M, Querol A, Belloch C (2006) Identification of species in the genus *Pichia* by restriction of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal DNA gene. Antonie van Leeuwenhoek 90:171–181

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M et al (eds) PCR protocols: a guide to methods and applications. Academic Press, Orlando, pp 315–322

Yarrow D (1998) Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman CP, Fell JW (eds) The yeasts. A taxonomic study, 4th edn. Elsevier, Amsterdam, pp 77–100

Yurkov AM, Chernov IYu (2005) Geographical races of certain species of ascomycetous yeasts in the Moscow and Novosibirsk regions. Microbiology 74(5):597–601