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Research Article

RESEARCH ARTICLE

Diversity of yeast species from Dutch garden soil and the description of six novel Ascomycetes

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One sentence summary: Dutch children collected soil that was then used to study the yeast biodiversity and led to the description of novel species, named after the children who collected the particular samples.

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ABSTRACT

A Citizen Science initiative by the Westerdijk Fungal Biodiversity Institute and the Utrecht University Museum gave rise to a project where fungal and yeast isolates were obtained and identified from Dutch soil samples. During the current study, 386 yeast strains were isolated from 157 different locations in the Netherlands. These strains were identified using sequence data of the large-subunit rRNA gene (D1/D2 region) and the internal transcribed spacer 1 and 2 regions. A total of 53 different yeast species were found as well as 15 potentially novel species. Six novel ascomycetous species are described during this study that include *Hanseniaspora mollemarum* sp. nov., *Ogataea degrootiae* sp. nov., *Pichia gijzeniarum* sp. nov., *Saccharomycopsis oosterbeekiorum* sp. nov., *Trichomonascus vanleenenius* sp. nov. and *Zygoascus flipseniorum* sp. nov. This study made it possible to incorporate numerous yeast isolates into the CBS collection without any restrictions, which make these isolates readily available for use by others. Many of the isolates represented species of which only a few isolates or even only a single ex-type strain were available. Therefore, it is a clear indication that such biodiversity-orientated Citizen Science projects can enrich the pool of available yeasts for future research projects.

Keywords: Ascomycetes; Basidiomycetes; Citizen Science Project; culture collections; soil ecosystem; yeasts

INTRODUCTION

A number of studies and reviews investigated and discussed the frequency of the occurrence, diversity and importance of yeasts associated with soil worldwide (De Ruiter, Neutela and Mooreb 1998; Sálviková and Vadkertiová 2000, 2003a,b; Ekelund, Rønn and Christensen 2001; Botha 2006, 2011; Fierer *et al.* 2007; Yurkov, Kemler and Begerow 2011, 2012a; Yurkov *et al.* 2012b, 2016; Vadkertiová *et al.* 2017; Yurkov 2018). It has been reported that Basidiomycetous yeasts such as *Apiotrichum* Berkhout, *Saitozyma* Xin Zhan Liu, F.Y. Bai, M. Groenew. and Boekhout and *Solicoccozyma* Xin Zhan Liu, F.Y. Bai, M. Groenew. and Boekhout are in general more abundantly found in soil samples than Ascomycetous yeasts (Vishniac 2006; Connell *et al.* 2008; Vadkertiová, Dudášová and Balaščáková 2017; Yurkov 2018). However, Ascomycetous yeasts were often found in

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managed soils exposed to human intervention, mostly vegetation and land use (Abdel-Sater, Moubasher and Soliman 2016; Vadkertiová, Dudášová and Balaščáková 2017), such as *Barnettozyma* Kurtzman, Robnett and Bas.-Powers, *Cyberlindnera* Minter, *Hanseniaspora* Zikes, *Lipomyces* Lodder and Kreger-van Rij, *Metschnikowia* T. Kamieński, *Schwanniomyces* Klöcker and *Torulaspora* Lindner. Analyses done by Vishniac (2006), Yurkov, Kemler and Begerow (2011, 2012a) and Yurkov *et al.* (2012b, 2016) on the diversity of yeasts collected from different types of soil and a variety of sampling sites, including woodlands and grasslands, showed that the majority of yeasts characterised were from the class Basidiomycetes. However, during several studies a diversity of ascomycetous yeasts was found to be present in agricultural and managed soils (reviewed in Vadkertiová, Dudášová and Balaščáková 2017). Abdel-Sater, Moubasher and Soliman (2016) found during a survey of soil mycobiotia from agricultural soil surrounding citrus and grapevine plantations in Egypt the majority of the yeast species isolated belonged to Ascomycetes. Numerous novel yeast species were isolated and identified during the above-mentioned studies, and several of these studies suggested that the soil environment represents an important niche for the discovery of novel yeast species.

The majority of the known fungal species thus far described are likely to occur in soil, as many of the known inhabitants of soil can facilitate the transfer of yeasts to the soil ecosystem at some stage (Botha 2011). Therefore, it has been suggested that some of the yeast diversity found in soil originate from other habitats. The occurrence of yeasts in a variety of soil inhabitants such as beetles and other insects has also been noted (Suh *et al.* 2003; Suh, McHugh and Blackwell 2004; Suh, Nguyen and Blackwell 2006; Nguyen, Suh and Blackwell 2007; Stefani *et al.* 2016). However, many yeasts are perceived to be autochthonous soil inhabitants that include ascomycetous and basidiomycetous species (Kurtzman and Fell 1998; Lachance and Starmer 1998). Yeast species occurring in soil are seen as widely dispersed, with a large number of species known to be present in soil, but only a few species are shared between sampling sites, even from the same region (reviewed in Yurkov 2018). From what is presently known, more than 20% of the yeast strains, preserved in internationally renowned public culture collections world-wide, were isolated specifically from soils obtained from different environments including aquatic, extreme cold and warm habitats, forests and mountains (Groenewald *et al.* 2017a). The known biodiversity preserved in these culture collections is limited comparing to what can be found in natural environments (Lachance 2006; Kurtzman *et al.* 2015).

It has been reported that filamentous fungi and bacteria outcompete yeasts in most soil environments. However, it has been known for quite some time that yeast abundances are higher in fertilised agricultural and managed soils since plants and spoiled fruit deposited in the soil may act as nutrient-rich yeast inoculum (Phaff, Miller and Mrak 1966; Botha 2011; Yurkov 2017). Moawad *et al.* (1986) found a positive correlation between soil yeast population size and both the organic carbon and organic nitrogen content of the soil. Research also showed that soil yeasts have a positive effect on the soil structure, nutrient recycling and even plant growth (reviewed in Botha 2011; Yurkov 2017). The simple organic carbon compounds associated with root exudates are readily assimilated by yeasts (Badr El-Din *et al.* 1986; Cloete *et al.* 2009). Characteristics that commonly occur among yeast species frequently encountered in soil are the ability to utilise L-arabinose, D-xylose and cellobiose aerobically. These are products of hydrolytic enzymes from bacteria or moulds, which act on lignocellulosic plant material (Bisaria and Ghose 1981; Tomme *et al.* 1995; Botha 2006). In the early 2000's, agriculturalist have tried to improve soil conditions and crop performance with some success by including viable yeasts to soil in order to investigated their potential as biofertilisers (Mohamed and Gomaa 2005; Gomaa and Mohamed 2007; Botha 2011).

A Citizen Science initiative by the Westerdijk Fungal Biodiversity Institute and the Utrecht University Museum gave rise to a project where fungi and yeasts were isolated from Dutch garden soil samples. These samples were collected by children in their home gardens and sent them back to the Westerdijk Institute for analysis. During this study, hundreds of different isolates obtained from 292 soil samples in the Netherlands were sequenced and many known as well as yet to be identified fungi were recovered. During the current study, 386 yeast strains were isolated from 157 different samples in the Netherlands. Different yeast morphotypes from each soil sample were selected, and 335 strains were identified using the sequence data of the large-subunit rRNA gene (D1/D2 region) and the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions. During this study, 67 different species were identified including 15 potential novel species. Six of these represented ascomycetous species that belong to *Hanseniaspora*, *Ogataea* Y. Yamada, K. Maeda and Mikata, *Pichia* E.C. Hansen, *Saccharomycopsis* Schiønning, *Trichomonascus* H.S. Jacks and *Zygoascus* M.T. Sm., respectively, which were described during this study.

MATERIALS AND METHODS

Sampling and isolation

Samples were collected from garden soil of different localities in the Netherlands (Fig. 1) during March to May 2017. The soil was taken from the top 15 cm, avoiding stones and organic materials as much as possible, and then collected into two 20 ml plastic tubes with screw-lids. These were sent by postal mail the same day that the soil was collected and received by the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands within one or two working days. The soil samples were kept at 4°C until further analyses were done. From one of these tubes, 1 g of soil was subjected to a serial dilution in sterile distilled water as described by Domsch, Gams and Anderson (2007) and Crous *et al.* (2009). Dilutions ranging from 10^{-2}–10^{-4} were plated in duplicate onto solid 2% (w/v) malt extract agar (MEA, Oxoid, Landsmeer, The Netherlands), supplemented with 50 ppm penicillin-G (Sigma-Aldrich, Zwijndrecht, The Netherlands) and streptomycin (Sigma-Aldrich, Zwijndrecht, The Netherlands) and incubated at room temperature (17°C–20°C) in the dark for 3–7 days. The soil in the second tube was mixed with 10% (v/v) of glycerol (Sigma-Aldrich, Zwijndrecht, The Netherlands) and stored at –80°C for future use.

The colonies with yeast-like appearance were transferred to fresh glucose-peptone-yeast extract agar (GPYA, Oxoid, Landsmeer, The Netherlands) plates and incubated at room temperature for 3–7 days to obtain single colonies. Single yeast colonies were transferred to fresh GPYA plates. From each soil sample, a maximum of three colonies with the same macromorphological features were selected and used in further analyses. One isolate of each of the species found in a specific soil sample as well as the new yeast species were deposited in the yeast collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. Taxonomic information and nomenclature for new species were deposited in MycoBank (www.MycoBank.org; Crous *et al.* 2004).

Figure 1. The areas in the Netherlands where the garden soil was collected from.

Molecular analyses for species identification

Yeast isolates were grown on GPYA at 24°C for 3 days, and the DNA were extracted using the internal prepman protocol (internal protocol available on request). The ITS region and D1D2 domains were amplified and sequenced with the primer pairs ITS5/ITS4 (White *et al.* 1990) and LR0R/LR5 (Vilgalys and Hester 1990; Vilgalys and Sun 1994), respectively. The translation elongation factor 1-alpha (TEF1) regions were amplified and sequenced with the primer pair EF1-983f: GCY CCY GGH CAY CGT GAY TTY AT and EF1-1567R: ACH GTR CCR ATA CCA CCR ATC TT.

ITS and D1/D2 PCR reactions and program conditions were performed as described by Stielow *et al.* (2015). For the TEF1 amplifications, the same cycle conditions were applied as for the D1/D2 region but with an annealing temperature of 54°C. PCR products were directly purified using FastAP thermosensitive alkaline phosphatase and shrimp alkaline phosphatase (Thermo Fisher Scientific, Waltham, MA, USA). Cycle-sequencing reactions were performed with ABI BigDye Terminator (BDT) v. 3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol but using a fifth of the recommended volumes of BDT, followed by bidirectional sequencing with a 3730xl DNA Analyser (Thermo Fisher Scientific, Waltham, MA, USA). Sequences were archived, bidirectional reads assembled and manually corrected for sequencing artefacts using BioloMICS software v. 8.0 (www.bio-aware.com; Vu *et al.* 2012). All sequences of the studied strains were blasted against sequences in GenBank at NCBI, DataBank of Japan (DDBJ) and the European Nucleotide Archive (ENA) (http://blast.ncbi.nlm.nih.gov/), publically available sequences of strains present in the CBS sequence database (http://www.westerdijkinstitute.nl/Collections/; Robert *et al.* 2008) as well as those in MycoBank (www.MycoBank.org) to identify the species the strains belong to.

The ITS, D1/D2 and for some genera the TEF1 sequences obtained from strains representing novel species as well as those sequences from their closest relatives obtained from GenBank were used in phylogenetic analyses. Subsequent alignments for each locus were generated separately in MAFFT v. 7.110 (Katoh *et al.* 2017), and the ambiguously aligned regions of both ends were manually truncated. Each partition was assessed for incongruence before being concatenated by checking their individual phylogenies for conflicts between clades with significant maximum likelihood (ML) and Bayesian inference (BI) support (Mason-Gamer and Kellogg 1996; Wiens 1998). Before being concatenated the congruency of the loci was tested using the 70% reciprocal bootstrap criterion (Mason-Gamer and Kellogg 1996; Wiens 1998).

Phylogenetic analyses of the individual gene regions and the combined dataset were based on BI, ML and maximum parsimony (MP). For BI and ML, the best evolutionary models for each locus were determined using MrModeltest (Nylander 2004) and incorporated into the analyses. MrBayes v. 3.2.1 (Ronquist and Huelsenbeck 2003) was used for BI to generate

phylogenetic trees under optimal criteria for each locus. A Markov Chain Monte Carlo (MCMC) algorithm of four chains was initiated in parallel from a random tree topology with the heating parameter set at 0.3. The MCMC analysis lasted until the average standard deviation of split frequencies was below 0.01 with trees saved every 1000 generations. The first 25% of saved trees were discarded as the 'burn-in' phase and posterior probabilities (PP) were determined from the remaining trees.

The ML analyses were performed using RAxML v. 8.0.9 (randomised accelerated (sic) ML for high performance computing; Stamatakis 2014) through the CIPRES website (http://www.phylo.org) to obtain another measure of branch support. The robustness of the analysis was evaluated by bootstrap support (BS) with the number of bootstrap replicates automatically determined by the software. For MP, analyses were done using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford 2003) with phylogenetic relationships estimated by heuristic searches with 1000 random addition sequences. Tree-bisection-reconnection was used, with branch swapping option set on 'best trees' only. All characters were weighted equally and alignment gaps treated as fifth state. Measures calculated for parsimony included tree length, consistency index, retention index and rescaled consistence index. Bootstrap analyses (Hillis and Bull 1993) were based on 1000 replications. All new sequences generated in this study were deposited in GenBank (Figs 3–6).

Morphological and physiological characterisation of novel species

A total of six isolates, representing the new ascomycetous yeast species, were submitted for morphological and physiological characterisation by using methods and protocols previously described by Kurtzman, Fell and Boekhout (2011). Cell morphology was determined after 3–7 days of growth on GPYA at 25°C. Using the ID 32C microtiter system (bioMérieux, Marcy-l'Etoile, France), the physiological characteristics were recorded after 1, 2 and 3 weeks of incubation at 25°C. Fermentation of glucose and assimilation of nitrogen compounds were tested using the methods of Kurtzman, Fell and Boekhout (2011). Results were recorded after 7–10 days. Growth at different temperatures ranging from 25°C–42°C was determined by incubation on GPYA for 7 days. All tests were done in duplicate.

Production of ascospores by strains of each of the novel species was examined either on one or on a selection of the following media (Kurtzman, Fell and Boekhout 2011): V8 juice agar (V8, Oxoid, Landsmeer, The Netherlands), yeast extract-malt extract-peptone-glucose agar (Becton Dickinson, Vianen, The Netherlands), Difco malt extract agar (DMA, Becton Dickinson, Vianen, The Netherlands), Potato-dextrose agar (PDA, Oxoid, Landsmeer, The Netherlands), MacClary acetate agar (Oxoid, Landsmeer, The Netherlands), 2% Malt extract agar (MA2, Becton Dickinson, Vianen, The Netherlands) and Corn meal agar (CMA, Becton Dickinson, Vianen, The Netherlands). Cultures were incubated at 25°C and examined at regular intervals during 2 months.

RESULTS

Sampling and isolation of yeasts

During the Citizen Science initiative by the Westerdijk Institute, nearly 2000 fungal isolates (including filamentous fungi and yeast) were obtained and identified at genus or species level (data not shown). The isolates were recovered from 292 soil samples taken in different places of the Netherlands, mainly from the South Holland and Utrecht provinces (Fig. 1). A total of 386 yeast isolates were obtained, representing 67 yeast species isolated from 157 of the samples. In the remainder of the samples no yeasts were isolated as in some samples no yeast morphotypes were observed but mostly due to the overgrowth from filamentous fungi.

Species delineation and classification

For 11 of the recovered species, less than three strains were previously deposited in the CBS yeast collection (Table 1) with six species having only the ex-type strains available. Different yeast morphotypes from each soil sample were selected for sequence analyses and it was found that 231 of the 335 sequenced isolates were unique and do not represent possible clones found in one specific sample. Of these, 195 isolates were included in the CBS yeast collection and most are now publically available for future use, except for the isolates that represent species that still have to be described. Due to the isolation conditions, only yeasts that were able to grow on MYA at 17°C–20°C were isolated. The yeast colonies were isolated from the same culture plates on which the filamentous fungi were cultivated, and therefore in some soil samples yeasts were not observed and/or no pure colonies could be obtained due to faster filamentous fungal growth on many of the plates after 2–7 days, depending on the fungal species present.

During our study, basidiomycetous species were obtained from more than 87% of the sites where yeasts were isolated (Table 1) with *Saitozyma podzolica* (Babeva and Reshetova) Xin Zhan Liu, F.Y. Bai, M. Groenew. and Boekhout (35% of soil samples) the most common species found during this study in Dutch garden soil, followed by *Apiotrichum dulcitum* A.M. Yurkov and Boekhout (16% of soil samples) and *Tausonia pullulans* (Lindner) Xin Zhan Liu, F.Y. Bai, M. Groenew. and Boekhout (10% of soil samples). Among the ascomycetous yeast, strains from the *Debaryomyces hansenii/fabryi* complex were the most abundantly found (11% of soil samples). However, since these two species cannot be distinguished through D1/D2 and ITS regions, additional loci are necessary to separate them (Groenewald *et al.* 2008; Quintilla *et al.* 2018)

The ability of the species isolated during this study to utilise L-arabinose, D-xylose and cellobiose aerobically, three carbon compounds, which are abundantly found in soil ecosystems utilised by many soil associated yeasts (Botha 2006), are listed in Table 1.

Characterisation and description of novel species

In addition to the known species found in the soil samples (Table 1), several potential new yeast species were also found during this survey. Six of these are new ascomycetous species belonging to *Hanseniaspora*, *Ogataea*, *Pichia*, *Saccharomycopsis*, *Trichomonascus* and *Zygoascus*. The grouping of sexual and asexual forms within a clade using DNA characters led to a change in the nomenclature rules. The International Code of Nomenclature for algae, fungi and plants (Turland *et al.* 2018) ended 'dual' nomenclature for fungi and requires inclusion of sexual and asexual morphs within monophyletic groups, which then have a common generic name. In the current study, these rules were retained and forma asexualis (f.a.) is indicted below to the species name where no sexual structures were observed as a reminder that a sexual state is not known yet (Lachance 2012). As *Trichomonascus* (Jackson 1947), the sexual morph genus, has priority over the asexual morph genus *Blastobotrys* Klopotek

Table 1. Isolated yeast species and their ability to assimilate L-arabinose, D-xylose and cellibiose.

				Ability of species to assimilate (+,w,s,v, –)		
Order	Genus (40 in total)	Epithet (67 in total)	Present in number of samples	L-arabinose	D-xylose	Cellibiose
Basidiomycetes	*Apiotrichum*	*dulcitum*	25	Yes	Yes	Yes
		laibachii	2	Yes	Yes	Yes
		porosum	6	Yes	Yes	Yes
		sporotrichoides	1	Yes	Yes	Yes
		[a]*xylopini*	1	Yes	Yes	Yes
		[b]Species 1	1	ND	ND	ND
	Cryptococcus	[a]*cerealis*	1	Yes	Yes	Yes
		terreus	1	Yes	Yes	Yes
	Colacogloea	[b]Species 1	1	ND	ND	ND
	Cutaneotrichosporon	*moniliiforme*	2	Yes	Yes	Yes
		terricola	1	Yes	Yes	Yes
		[b]Species 1	1	ND	ND	ND
		[b]Species 2	1	ND	ND	ND
	Cystobasidium	*slooffiae*	5	Yes	Yes	Yes
	Cystofilobasidium	*capitatum*	1	Yes	Yes	Yes
	Holtermanniella	*wattica*	9	No	Yes	No
	Leucosporidiella	*fragaria*	1	Yes	Yes	Yes
		[a]*muscorum*	2	Yes	Yes	Yes
		yakutica	1	No	Yes	Yes
	Leucosporidium	*scottii*	2	Yes	Yes	Yes
		[b]Species 1	1	ND	ND	ND
	Myxozyma	[b]Species 1	1	ND	ND	ND
	Piskurozyma	[a]*filicatus*	3	Yes	Yes	Yes
		[a]*taiwanensis*	3	Yes	Yes	Yes
	Pseudohyphozyma	[a]*pustula*	1	Yes	Yes	Yes
	Rhodosporidiobolus	*colostri*	1	No	No	Yes
	Rhodosporidium	*babjevae*	1	No	Yes	Yes
	Rhodotorula	*toruloides*	1	Yes	Yes	Yes
		dairenensis	1	Yes	Yes	Yes
	Saitozyma	*podzolica*	55	Yes	Yes	Yes
	Sakaguchia	[b]Species 1	1	ND	ND	ND
	Slooffia	[b]Species 1	2	ND	ND	ND
		[b]Species 2	2	ND	ND	ND
	Solicoccozyma	*terricola*	4	Yes	Yes	Yes
	Sporobolomyces	*lactosus*	1	No	Yes	Yes
	Tausonia	*pullulans*	15	Yes	Yes	Yes
	Vishniacozyma	*foliicola*	1	Yes	Yes	Yes
Ascomycetes	*Barnettozyma*	*californica*	4	No	Yes	Yes
	Blastobotrys	[a]*malaysiensis*	2	Yes	Yes	Yes
	[d]*Candida*	*glaebosa*	1	No	Yes	Yes
		railenensis	2	No	Yes	Yes
		sake	5	No	Yes	Yes
		[a]*subhashii*	6	Yes	Yes	Yes
		vartiovaarae	2	No	Yes	Yes
	Cyberlindnera	*misumaiensis*	3	No	Yes	Yes
		saturnus	1	No	Yes	Yes
	Debaryomyces	*fabryi\hansenii*	17	Yes	Yes	Yes
		marama	2	Yes	Yes	Yes
		vindobonensis	1	Yes	Yes	Yes
	Hanseniaspora	[c]*mollemarum*	1	No	No	Yes
		uvarum	1	No	No	Yes
	Kregervanrija	*fluxuum*	1	No	No	No
	Nadsonia	[a]*commutata*	3	No	No	No
	Ogataea	[c]*degrootia*	1	Yes	Yes	Yes
		methanolica	1	Yes	Yes	Yes
	Pichia	[c]*gijzeniarum*	1	No	Yes	No
		membranifaciens	1	No	Yes	No
	Priceomyces	*carsonii*	1	Yes	Yes	Yes
	Saccharomycopsis	[c]*oosterbeekiorum*	1	No	No	No

Table 1. continued.

Order	Genus (40 in total)	Epithet (67 in total)	Present in number of samples	Ability of species to assimilate (+,w,s,v, –) L-arabinose	D-xylose	Cellibiose
		schoenii	1	No	Yes	No
	Saturnispora	[a]*zaruensis*	1	No	No	No
	Schizoblastosporion	*starkeyi-henricii*	1	No	No	No
	Schwanniomyces	[a]*capriottii*	1	Yes	Yes	Yes
		polymorphus	1	Yes	Yes	Yes
	Torulaspora	*delbrueckii*	1	No	Yes	No
	Trichomonascus	[c]*vanleenenius s*	7	Yes	Yes	Yes
	Zygoascus	[c]*flipseniorum*	1	Yes	Yes	Yes

Yes: + = positive, w = positive weak, s = positive slow, v = positive variable (highlighted in grey).
No: – = negative.
ND: not determined.
[a]Species of which there were less than three strains in the CBS yeast collection.
[b]Potential novel Basidiomycetous species isolated in this study.
[c]Novel Ascomycetous species isolated and described in this study.
[d]Clade affiliation of *Candida* species: *C. glaebosa* = *C. glaebose clade*; *C. railenensis* = *Kurtzmaniella* clade; *C. sake* = unaffiliated; *C. subhashii* = unaffiliated; *C. vartiovaarae* = *Wickerhamomyces* clade.

(von Klopotek 1967), we have described the new species in *Trichomonascus* and not *Blastobotrys*, even though the sexual morph was not found. The new species are described below as *Hanseniaspora mollemarum* f.a. sp. nov., *Ogataea degrootiae* sp. nov., *Pichia gijzeniarum* f.a. sp. nov., *Saccharomycopsis oosterbeekiorum* sp. nov., *Trichomonascus vanleenenius* f.a. sp. nov. and *Zygoascus flipseniorum* f.a. sp. nov.

Phylogenetic inference of the six sequence datasets was each rooted to *Candida vanderwaltii* (Vidal-Leir.) S.A. Mey. and Yarrow (CBS 5524). Based on the results of MrModelTest, dirichlet base frequencies and the GTR+I+G model was used for the BI analyses of each sequence dataset. The best ML tree for each sequence dataset confirmed the consensus tree topologies obtained from the BI and MP analyses, and therefore only the best ML tree is presented for each sequence dataset (Figs 3–8). Statistical information on each sequence dataset is presented in Table S1, Supporting Information, including number of polymorphic and parsimony informative sites.

All new species could be distinguished from their closest relatives on their D1/D2 and/or ITS and/or TEF1sequence identities (Figs 3–8). The novel species *H. mollemarum*, *P. gijzeniarum* and *Z. flipseniorum* showed more than 25 nucleotide (nt) differences (5%) in the ITS regions with respect to their closest relatives. As the ITS region of the novel species *O. degrootiae*, *S. oosterbeekiorum* and *T. vanleenenius* showed only 17–18 nt differences with their closest relatives, sequences of the TEF1 region were added to the analyses. The total nucleotide differences between the novel species and their closest relatives in ITS and TEF1 were 34, 32 and 36 nucleotides for *O. degrootiae*, *S. oosterbeekiorum* and *T. vanleenenius*, respectively. These values are the same or even more than those found among other closely related species belonging to these genera such as between *O. cecidiorum* I.A. Maximova, Kachalkin and A.M. Yurkov and that of *O. salicorniae* (Hinzelin, Kurtzman and M.T. Sm.) Kurtzman and Robnett (34 nt) and *O. trehalophila* (Phaff, M.W. Mill. and J.F.T. Spencer) Kurtzman and Robnett (20 nt) and those found between *Blastobotrys mokoenaii* (Mokwena, E. Jansen and Myburgh) Kurtzman and Robnett and that of *Blastobotrys illinoisensis* Kurtzman (29 nt) and *Blastobotrys malaysiensis* Kurtzman (21 nt). In addition, several physiological characters were found distinguishing each new species and their closest phylogenetic relatives (Table 2).

Description of *H. mollemarum* M. Groenew. G. Péter, S.A. James, Dlauchy and M.T. Sm., sp. nov.

Cell morphology

After 7 days at 25°C on GPYA, cells proliferating by bipolar budding are apiculate, ovoid to elongate, 2–5.2 × 3–10.5 μm, occurring singly, in pairs or short chains (Fig. 2A). Ascospores were not formed at 25°C on DMA, V8, YM and PDA neither by the ex-type strain nor by the con-specific strains NCAIM Y.02204 or NCYC 3553.

Fermentation and growth reactions

Glucose is fermented. Positive growth was observed on D-glucose, α,α-trehalose (delayed), cellobiose D-gluconate (delayed) ethylamine (variable), L-lysine (variable) and cadaverine (variable). Growth is positive in the presence of 0.01% cycloheximide. Negative growth is observed on D-galactose, L-sorbose, D-glucoseamine, D-ribose, D-xylose, L-arabinose, L-rhamnose, maltose, sucrose, methyl-α-D-glucopyranoside, melibiose, lactose, raffinose, melezitose, glycerol, erythritol, D-glucitol, D-mannitol, 2-keto-D-gluconate, myo-inositol, D-glucuronate, N-acetyl glucosamine, DL-lactate and on nitrate, nitrite and D-glucoseamine HCl as sole sources of nitrogen. No growth obtained at 30°C.

Type

Holotype: The Netherlands, Meteren, from garden soil, March 2017, collected by Marit and Lizanne Mollema, CBS 15034, preserved in metabolically inactive condition. Culture ex-type CBS 15034. MycoBank number: MB824953.

Con-specific strains, which were also used for the species description, were NCAIM Y.02204 and NCYC 3553.

Etymology

The species epithet *mollemarum* pertains to Marit and Lizanne Mollema who collected the soil sample from where the ex-type strain was isolated from.

Notes

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequences are *Hanseniaspora valbyensis* Klöcker (GenBank KY103576; identities = 616/648

Table 2. Growth tests that can be used to distinguish the novel species from their close relatives.

	D-galactose	α,α-trehalose	Methyl-α-D-glucopyranoside	Cellobiose
Ogataea degrootiae[a]	–	+	–	+
Ogataea methanolica[b]	+	+	–	+
Ogataea salicorniae[b]	–	–	–	–
Ogataea cecidiorum[b]	–	+	+	–
Ogataea trehalophila[b]	–	+	–	–
	Glucose ferm.	L-sorbose	N-acetyl glucosamine	30°C
Saccharomycopsis oosterbeekiorum	–	+	–	–
Saccharomycopsis babjevae[d]	–	–	nd	+
Saccharomycopsis fermentans[b]	+	v	–	+
Saccharomycopsis javanensis[b]	–	+	+	+[a]
Saccharomycopsis schoenii[a]	–	V	–	+
	α,α-trehalose	0.01% cycloheximide	30°C	37°C
Hanseniaspora mollemarum	+/L	+	–	–
Kloeckera lindneri[a]	–	+	+	–
Hanseniaspora valbyensis[b]	–	+	v	–
Hanseniaspora singularis[e]	–	–	+	+
	Sucrose	Melezitose	L-rhamnose	Lactic acid
Zygoascus flipseniorum	–	–	–	–
Zygoascus biomembranicola[f]	–	L	+	s
Zygoascus polysorbophila[b]	+	+	–	+
Zygoascus bituminiphila[b]	+	–	–	+
	Lactose	α,α-trehalose	Melezitose	Lactic acid
Trichomonascus vanleenenius	–	+	–	+
Blastobotrys mokoenaii[b]	–	+	–	–
Blastobotrys malaysiensis[b]	+	+	+	+
Blastobotrys illinoisensis[b]	–	–	–	–

Data taken from:
[a]The current study.
[b]Kurtzman, Fell and Boekhout (2011).
[c]Glushakova *et al.* (2010).
[d]Naumov *et al.* (2006).
[e]Jindamorakot *et al.* (2009).
[f]Nagatsuka *et al.* (2016).
L = delayed positive, V = variable growth

(95%), 22 substitutions, 10 gaps) and *Hanseniaspora lindneri* Zikes (GenBank NR138190; identities = 616/640 (96%), 19 substitutions, 5 gaps). The hits found for the same species using the D1/D2 sequences are *H. valbyensis* (GenBank U73596; identities = 538/549 (98%), 10 substitutions, 1 gap) and *H. lindneri* (GenBank U84226; identities = 537/548 (98%), 11 substitutions). Phylogenetic inference placed *H. mollemarum* in a well-supported clade (Fig. 3; ML BS = 96; MP BS = 100; PP = 0,99), distinct from *Hanseniaspora jakobsenii* Ouoba, Diawara and J.P. Sutherl. (YAV17), *Hanseniaspora singularis* Jindam., Ninomiya, Limtong, H. Kawas. and Nakase (ST 244), *H. valbyensis* (CBS 479) and *Kloeckera lindneri* (Klöcker) Janke (CBS 285).

Description of *O. degrootiae* M. Groenew. and M.T. Sm., sp. nov.

Cell morphology

After 7 days at 25°C on GPYA, cells proliferating by multilateral budding are round to oval, 1.5–6 × 2–8 μm, occur singly, in pairs or in small clusters (Fig. 2B). Asci with ascospores were observed on GPYA and YM after 7 days at 25°C. Asci are formed between cells or by mother-bud conjugation, producing 2–4 hat- to saturnoid-shaped ascospores (Fig. 2B). Glucose is fermented.

Fermentation and growth reactions

Positive growth was observed on D-glucose, D-ribose, D-xylose, L-arabinose, α,α-trehalose, cellobiose, glycerol, erythritol, D-sorbitol, D-mannitol and on ethylamine, L-lysine and cadaverine as sole sources of nitrogen. Positive growth occurred in the presence of 0.01% cycloheximide and at 30°C. Negative growth was observed on D-galactose, L-sorbose, D-glucosamine, L-rhamnose, sucrose, maltose, methyl-α-D-glucopyranoside, melibiose, lactose, raffinose, melezitose, inositol, potassium-2-keto-D-gluconate, D-gluconate, D-glucuronate, lactic acid, N-acetyl glucosamine, and on nitrate, nitrite and D-glucosamine HCl as sole sources of nitrogen. No growth obtained at 36°C.

Type

Holotype: The Netherlands, Houten, from garden soil, March 2017, collected by Sonja de Groot, CBS 15033, preserved in metabolically inactive condition. Culture ex-type CBS 15033. MycoBank number: MB824955.

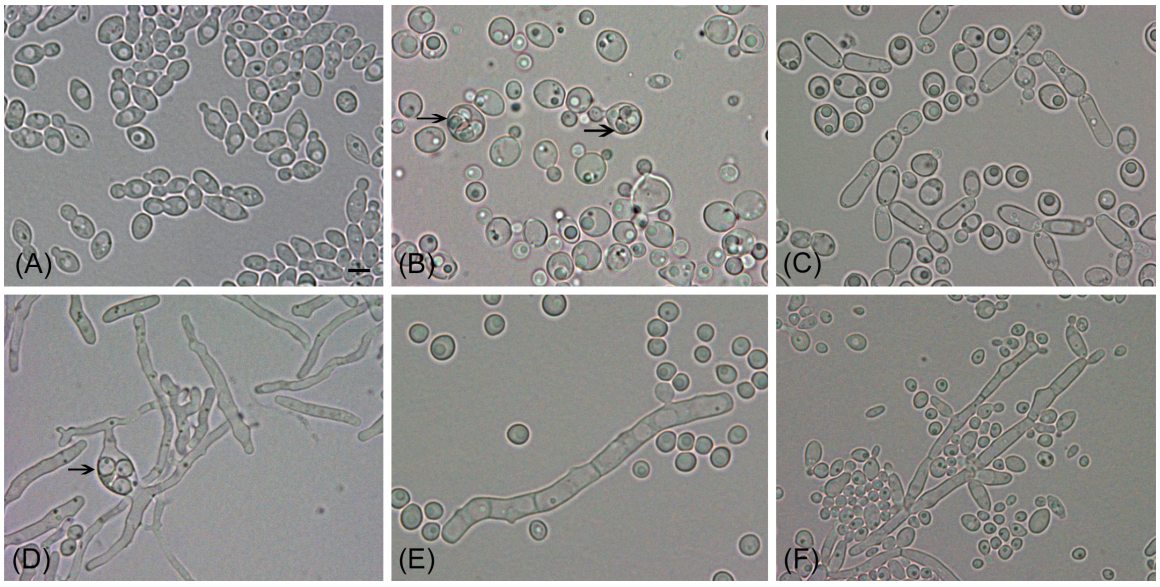

Figure 2. Growth at 25°C after 7 days on GPYA. (**A**) Vegetative cells of *H. mollemarum*, CBS 15034^T, (**B**) vegetative cells and ascospores in mother-bud-conjugated ascus (indicated with arrows) of *O. degrootiae*, CBS 15033^T, (**C**) vegetative cells and small clusters of pseudomycelium of *P. gijzeniarum*, CBS 15024^T, (**D**) vegetative cells and ascospores in ascus (indicated with arrow) of *S. oosterbeekiorum*, CBS 14943^T, (**E**) vegetative cells and pseudomycelium with blastoconidia on denticles of *T. vanleenenius*, CBS 14902^T and (**F**) vegetative cells and pseudomycelium of *Z. flipseniorum*, CBS 14876^T. Scale bar 5 μm.

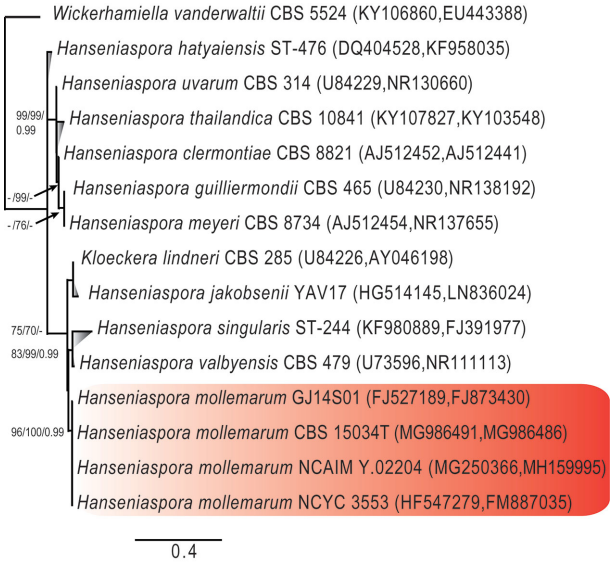

Figure 3. ML consensus tree representing the phylogenetic placement of *H. mollemarum* sp nov. Statistical information is presented in Table S1, Supporting Information. Type strains of all species were used in the analyses, and the type strains of *M. mollemarum* is indicated in bold and GenBank numbers of the D1/D2 and ITS sequences are indicated next to each taxon, respectively. *Wickerhamiella vanderwaltii* (CBS 5524) was used as outgroup.

Etymology

The species epithet *degrootiae* pertains to Sonja de Groot who collected the soil sample from where the ex-type strain was isolated from.

Notes

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the ITS, D1/D2 and TEF1 sequences is *O. methanolica* (Makig.) Kurtzman and Robnett with the ITS (GenBank KY104403) having 668/685 (97%) identities, 10 substitutions, 7 gaps, the D1/D2 (GenBank KY108680) having 829/835 (99%) identities, 6 substitutions and the TEF1 (GenBank EU014748) having 406/423 (96%) identities, 17 substitutions. *Ogataea degrootiae* (CBS 15033) clustered with *O. methanolica* in a highly supported clade (ML BS = 100; MP BS = 100; PP = 0.99; Fig. 4).

Description of *P. gijzeniarum* M. Groenew. and M.T. Sm., sp. nov.

Cell morphology

After 7 days at 25°C on GPYA, cells proliferating by multilateral budding are round to oval, occurring singly, in pairs or forming (small) clusters of pseudomycelium (Fig. 2C). Ascospores were not obtained on V8, YM, DMA, MA2 and MacClary acetate after 2 months of incubation.

Fermentation and growth reactions

Glucose is not fermented. Positive growth was observed on D-glucose, D-glucosamine, and on ethylamine, L-lysine, cadaverine and D-glucosamine HCl as sole sources of nitrogen. Variable growth was observed on L-sorbose and D-xylose. Negative growth was observed on D-galactose, D-ribose, L-arabinose, L-rhamnose, sucrose, maltose, α,α-trehalose, methyl-α-D-glucopyranoside, cellobiose, melibiose, lactose, raffinose, melezitose, glycerol, erythritol, D-sorbitol, D-mannitol, inositol, potassium-2-keto-D-gluconate, D-gluconate, D-glucuronate, lactic acid, N-acetyl glucosamine, and on nitrate and nitrite as sole sources of nitrogen. No growth occurred in the presence of 0.01% cycloheximide and at 30°C.

Type

Holotype: The Netherlands, Liempde, from garden soil, March 2017, collected by Lena and Vera Gijzen, CBS 15024, preserved in metabolically inactive condition. Culture ex-type CBS 15024. MycoBank number: MB824960.

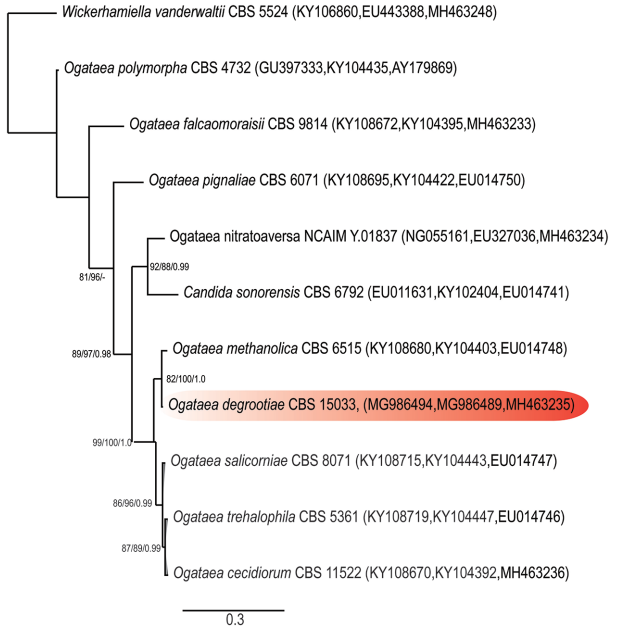

Figure 4. ML consensus tree representing the phylogenetic placement of *O. degrootiae* sp nov. Statistical information is presented in Table S1, Supporting Information. Type strains of all species were used in the analyses. GenBank numbers of the D1/D2, ITS and TEF1 sequences are indicated next to each taxon, respectively. *Wickerhamiella vanderwaltii* (CBS 5524) was used as outgroup.

Etymology
The species epithet *gijzeniarum* pertains to Lena and Vera Gijzen who collected the soil sample from where the ex-type strain was isolated from.

Notes
Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the D1/D2 sequence have less than 94% similarity. All megablast hits using the ITS sequence with sequences that derived from living cultures were lower than 90% with regions in the range of only 250 nt in length. However, 100% sequence identity was found with an environmental sequence from an unidentified, uncultured fungus (GenBank KT965037). This sequence was obtained during a study where the fungal community composition in the gut of rove beetles was studied (Stefani *et al.* 2016). *Pichia gijzeniarum* (CBS 15024) formed a single lineage, distinct from any other known *Pichia* species (Fig. 5).

Description of *S. oosterbeekiorum* M. Groenew. and M.T. Sm., sp. nov.

Cell morphology
After 7 days at 25°C on GPYA, cells are mostly elongated, 2.5–5 × 6–24.7 μm, and pseudomycelium is formed (Fig. 2D). Asci with ascospores were observed on GPYA after 7 days. The lytic asci, formed after conjugation of independent cells or between adjacent cells, were spindle shaped and contained four spherical ascospores with an equatorial or subequatorial ledge (Fig. 2D).

Fermentation and growth reactions
Glucose is not fermented. Positive growth was observed on D-glucose, L-sorbose, sucrose, α,α-trehalose and glycerol as well as in the presence of 0.01% cycloheximide. Negative growth was observed on D-galactose, D-glucosamine, D-ribose, D-xylose, L-arabinose, L-rhamnose, maltose, methyl-α-D-glucopyranoside, cellobiose, melibiose, lactose, raffinose, melezitose, erythritol, D-sorbitol, D-mannitol, inositol, potassium-2-keto-D-gluconate,D-gluconate, D-glucuronate, lactic acid, N-acetyl glucosamine, and on nitrate, nitrite, ethylamine, L-lysine, cadaverine and D-glucosamine HCl as sole sources of nitrogen. No growth obtained at 30°C.

Type
Holotype: The Netherlands, Houten, March 2017, collected by Tim and Mijke Oosterbeek, CBS 14943, preserved in metabolically inactive condition. Culture ex-type CBS 14943. MycoBank number: MB824962.

Etymology
The species epithet *oosterbeekiorum* pertains to Tim and Mijke Oosterbeek who collected the soil sample from where the ex-type strain was isolated from.

Notes
Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the ITS,D1/D2 and TEF1 sequences is *S. schoenii* (Nadson and Krassiln.) Kurtzman and Robnett with the ITS (GenBank KY105272) having 601/618 (97%) identities, 9 substitutions, 8 gaps, the D1/D2 (GenBank KY109506) having 820/826 (99%) identities, 4 substitutions, 2 gaps, and the TEF (GenBank EU057532) having 415/430 (96.5%) identities, 15 substitutions. *Saccharomycopsis oosterbeekiorum* (CBS 14943) clustered with *S. schoenii* (CBS 7223) in a well-supported clade (ML BS = 91; MP BS = 52; PP = 0.99) based on phylogenetic inference (Fig. 6).

Description of *T. vanleenenius* M. Groenew. and M.T. Sm., sp. nov.

Cell morphology
After 7 days at 25°C on GPYA, cells proliferating by multilateral budding are spherical, 2–4 μm diam., to elongated 2–3 × 2.5–8 μm. Pseudomycelium is formed, may disarticulate into arthroconidia and produces blastoconidia on denticles (Fig. 2E). Ascospores were not obtained on V8, YM, DMA, PDA and CMA after 2 months of incubation neither by the ex-type strain alone nor in pairwise mixtures with CBS 14819 and CBS 14867 of the same identity.

Fermentation and growth reactions
Glucose is fermented. Positive growth was observed on D-glucose, D-galactose, L-sorbose, D-glucosamine, D-xylose, L-arabinose, L-rhamnose, sucrose, maltose, α,α-trehalose, methyl-α-D-glucopyranoside, cellobiose, erythritol, D-sorbitol, D-mannitol, inositol, potassium-2-keto-D-gluconate, D-gluconate, D-glucuronate, lactic acid and N-acetyl glucosamine, and on ethylamine, L-lysine, cadaverine and D-glucosamine HCl as sole sources of nitrogen. Weak growth was observed on D-ribose and at 40°C. Positive growth in the presence of 0.01% cycloheximide. No growth was seen on melibiose, lactose, raffinose, melezitose, glycerol, and on nitrate and nitrite as sole sources of nitrogen. No growth observed at 42°C.

Type
Holotype: The Netherlands, Spijkenisse, March 2017, collected by Gina van Leenen, CBS 14902, preserved in metabolically inactive condition. Culture ex-type CBS 14902. MycoBank number: MB824963.

Con-specific strains, which were also used for the species description, were CBS 14819, CBS 14867 and CBS 15249.

Etymology

The species epithet *vanleenenius* pertains to Gina van Leenen who collected the soil sample from where the ex-type strain was isolated from.

Notes

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequences are *B. illinoisensis* Kurtzman (GenBank KY101751; identities = 540/560 (96%), 14 substitutions, 6 gaps), *B. mokoenaii* (Mokwena, E. Jansen and Myburgh) Kurtzman and Robnett (GenBank KY101754; identities = 511/535 (96%), 12 substitutions, 12 gaps) and *B. malaysiensis* Kurtzman (GenBank KY101753; identities = 543/561 (97%), 12 substitutions, 6 gaps). The hits using the D1/D2 sequences for the above mentioned species are *B. illinoisensis* (GenBank NG042438; identities = 855/859 (99%), 3 substitutions, 1 gap), *B. mokoenaii* (GenBank NG055335; identities = 854/859 (99%), 4 substitutions, 1 gap) and *B. malaysiensis* (GenBank NG042437; identities = 853/859 (99%), 5 substitutions, 1 gap). The hits using the TEF1 sequences for the above mentioned species are *B. illinoisensis* (GenBank MH463241; identities = 441/459 (96%), 18 substitutions), *B. mokoenaii* (GenBank MH463242; identities = 425/437 (97%), 12 substitutions) and *B. malaysiensis* (GenBank MH463243; identities = 438/460 (95%), 22 substitutions *T. vanleenenius* formed a distinct, well-supported clade (ML BS = 99; MP BS = 96; PP < 0.95) with *B. illinoisensis* (CBS 10339), *B. malaysiensis* (CBS 10336) and *B. mokoenaii* (CBS 8435) as sister clade (Fig. 7).

Description of *Z. flipseniorum* M. Groenew. and M.T. Sm., sp. nov.

Cell morphology

After 7 days at 25°C on GPYA, cells proliferating by multilateral budding are round to oval, 2.5–5 × 4–8.5 μm, occurring singly, in pairs or small clusters. Pseudomycelium is formed (Fig. 2F). Ascospores were not formed by the single strain of this species on V8, YM, DMA, PDA and CMA after 2 months of incubation.

Fermentation and growth reactions

Glucose is fermented. Positive growth was observed on D-glucose, D-galactose, L-sorbose, D-glucosamine, D-xylose, L-arabinose, maltose, α,α-trehalose, cellobiose, D-sorbitol, D-mannitol, inositol, D-gluconate(variable), D-glucuronate (variable) and N-acetyl glucosamine, and on cadaverine as sole source of nitrogen. Growth is positive in the presence of 0.01% cycloheximide. No growth was observed on D-ribose, L-rhamnose, sucrose, methyl-α-D-glucopyranoside, melibiose, lactose, raffinose, melezitose, glycerol, erythritol, potassium-2-keto-D-gluconate, lactic acid, and on nitrate, nitrite, ethylamine, L-lysine and D-glucosamine HCl as sole sources of nitrogen as well as at 30°C.

Type

Holotype: The Netherlands, Zoelen, March 2017, collected by Matthijs Flipsen, CBS 14876, preserved in metabolically inactive condition. Culture ex-type CBS 14876. MycoBank number: MB824965.

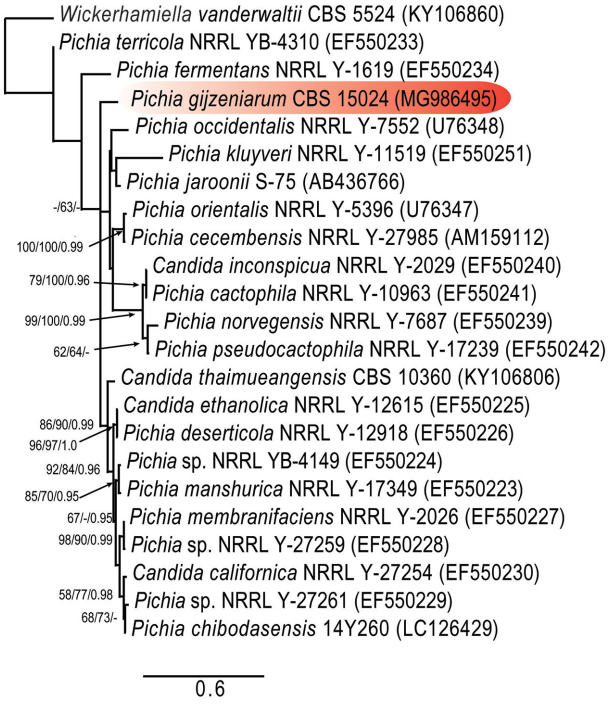

Figure 5. ML consensus tree representing the phylogenetic placement of *P. gijzeniarum* sp nov. Statistical information is presented in Table S1, Supporting Information. Type strains of all species were used in the analyses. GenBank numbers of the D1/D2 sequences are indicated next to each taxon. *Wickerhamiella vanderwaltii* (CBS 5524) was used as outgroup.

Etymology

The species epithet *flipseniorum* pertains to Sarah and Matthijs Flipsen who collected the soil sample from where the ex-type strain was isolated from.

Notes

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hit using the ITS and D1/D2 sequences are *Z. biomembranicola* Nagatsuka, Ninomiya, Kiyuna, Kigawa and Sugiyama and *Z. polysorbophila* (Kurtzman) with the *Z. biomembranicola* ITS (GenBank LC060997) having 515/540 (95%) identities, 9 substitutions, 16 gaps and the D1/D2 (GenBank LC060997) having 556/560 (99%) identities, 3 substitutions, 1 gap and with the *Z. polysorbophila* ITS (GenBank LC060996) having 509/549 (93%) identities, 19 substitutions, 21 gaps and the D1/D2 (GenBank LC060996) having 553/560 (99%) identities, 5 substitutions, 2 gaps. Based on phylogenetic inference, *Z. flipseniorum* clustered in a supported clade (ML BS = 72; MP BS = 96; PP = 0.96) with *Z. polysorbophila* (Kurtzman) Nagatsuka, Kiyuna and Sugiyama (T6517), sister to *Z. biomembranicola* (K61208; Fig. 8).

DISCUSSION

Watanabe (1994) suggested that the number of fungal species isolated from soil to be around 1200 taxa. With the non-culturing pyrosequencing technique, 249–408 fungal (including yeasts) taxonomic groups were found in a single soil sample (Buée *et al.* 2009). However, a large part of those sequences belonged to unclassified fungi from incompletely annotated environmental samples. It is clear from the Dutch Citizen Science project, where almost 2000 different culturable fungal species were found, that the number presented by Watanabe (1994) is far from accurate,

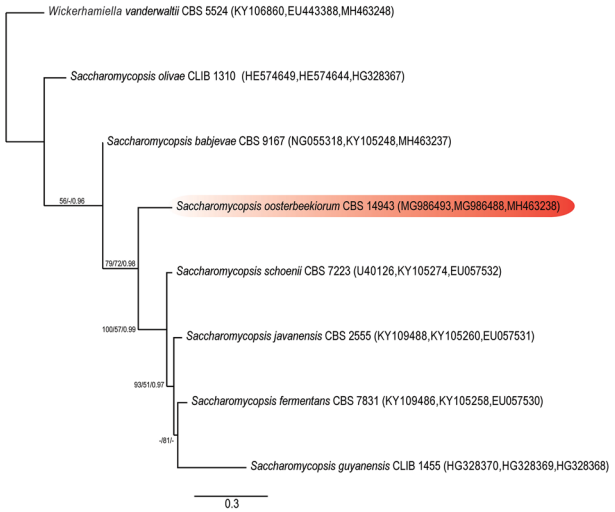

Figure 6. ML consensus tree representing the phylogenetic placement of *S. oosterbeekiorum* sp nov. Statistical information is presented in Table S1, Supporting Information. Type strains of all species were used in the analyses. GenBank numbers of the D1/D2, ITS and TEF1 sequences are indicated next to each taxon, respectively. *Wickerhamiella vanderwaltii* (CBS 5524) was used as outgroup.

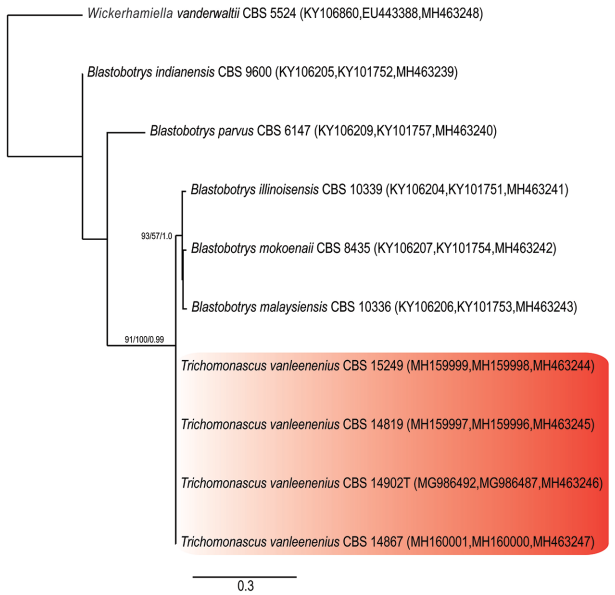

Figure 7. ML consensus tree representing the phylogenetic placement of *T. vanleenenius* sp nov. Statistical information is presented in Table S1, Supporting Information. Type strains of all species were used in the analyses. GenBank numbers of the D1/D2, ITS and TEF1 sequences are indicated next to each taxon, respectively. *Wickerhamiella vanderwaltii* (CBS 5524) was used as outgroup.

and that the numbers presented by Buée *et al.* (2009) remain debatable. As soil is a dynamic and multifunctional system, functioning as habitat for various living organisms such as animals, plants, insects and microorganisms, it is difficult to establish whether the yeasts present are true soil inhabitants or transferred to the soil by an external vector only to be eliminated from the soil environment in due course (Yurkov 2017, 2018).

As the project was initiated to obtain mainly filamentous fungi from the soil with yeasts not set as a priority, the first selection step using MEA was to ensure filamentous fungal growth. Although a large number of yeasts and a wide variety of species were isolated during this project, it is certainly not a

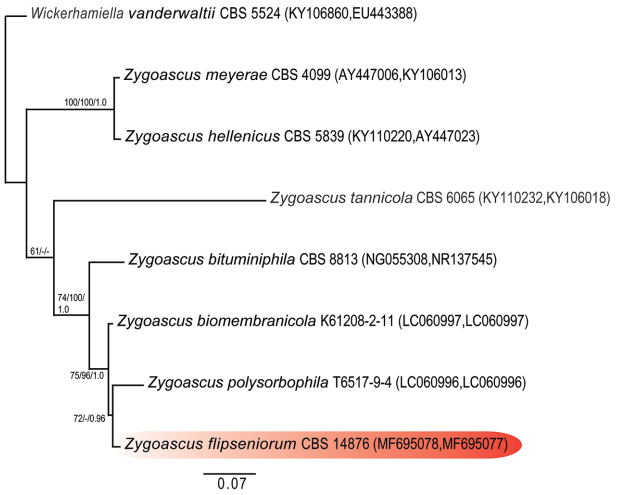

Figure 8. ML consensus tree representing the phylogenetic placement of *Z. flipseniorum* sp nov. Statistical information is presented in Table S1, Supporting Information. Type strains of all species were used in the analyses, and the type strain of *Z. flipseniorum* is indicated in bold. GenBank numbers of the D1/D2 and ITS sequences are indicated next to each taxon, respectively. *Wickerhamiella vanderwaltii* (CBS 5524) was used as outgroup.

realistic representation of yeast diversity that exist in the Dutch garden soil ecosystem, partly due to the isolation conditions that were used. Only culturable yeasts that were able to grow on MEA at 17°C–20°C were isolated. As the yeasts were cultivated from the soil samples together with the filamentous fungi on the same culture plates, some of the yeasts could not be isolated and/or no pure colonies could be obtained due to the fast growth of a number of filamentous fungi. Although the time between collecting the soil samples and the cultivation of the yeasts were kept to a minimum, it is possible that some of the yeasts present in the soil did not survive. Although the Dutch soil samples do not represent a wide variety in altitudes, temperature ranges and rainfall, the results of this pilot study in the Netherlands give valuable information on the diversity of yeast taxa associated with Dutch garden soil. Many of the yeast species found in the Dutch samples were also reported to be present in soil, particularly managed soils, collected from several sites in Europe, Brazil, Dagestan, Korea, Russia, New Zealand, South Africa, United Arab Emirates and the USA (Vadkertiová, Dudášová and Balaščáková 2017). Some of the genera found during this study and not by Yurkov, Kemler and Begerow (2011, 2012a), Yurkov *et al.* (2012b, 2016) or listed by Vadkertiová, Dudášová and Balaščáková (2017) included *Blastobotrys*, *Ogataea*, *Priceomyces* M. Suzuki and Kurtzman, *Pseudohyphozyma* Q.M. Wang, F.Y. Bai, M. Groenew. and Boekhout and *Saturnispora* Z.W. Liu and Kurtzman. However, it is quite difficult to compare genera found in the current study with that found in previous studies, as recently, numerous name changes have been introduced, especially in the Basidiomycetous yeasts (Liu *et al.* 2015; Wang *et al.* 2015a,b). Two of the most abundant species in the current study were *Saitozyma podzolica* and *Apiotrichum dulcitum*, two species that were also abundantly found in the studies of Yurkov, Kemler and Begerow (2011, 2012a) and Yurkov *et al.* (2012b, 2016), at that time known as *Cryptococcus podzolicus* and *Trichosporon dulcitum*, respectively. Also, in the 454-pyrosequencing study by Buee *et al.* (2009), a large number of sequence reads of the yeast *Saitozyma* (*Cryptococcus*) *podzolica* were obtained. These studies clearly show that this species is a dominant inhabitant of soil and may play an important role in soil ecosystems. It has been

reported that yeasts in soil overwhelmingly belong to the order Basidiomycetes (Vashniac 2006; Connell *et al.* 2008; Yurkov, Kemler and Begerow 2011, 2012a; Yurkov *et al.* 2012b, 2016). In our study, Basidiomycetes were found in more than 87% of the soil samples and more than 50% of the species identified belonged to genera from this class (Table 1). The success of many Basidiomycetes species, especially the formerly known *Cryptococcus* species, to occupy harsh diverse ecological niches such as soil, was linked to the presence of a capsule around the cells for many of these species (Vashniac 2006). However, *S. podzolica* and *A. dulcitum,* the two most abundantly found species during our study, do not produce extracellular polysaccharides and must have found other means to survive in the soil environment, which are not yet well understood.

In many of the studies mentioned above, only a low number, if any, Ascomycetous yeasts were isolated. Although Basidiomycetes are often found to be the most abundant in soil samples, Vadkertiová, Dudášová and Balaščáková (2017) listed several Ascomycetous yeast species that were often found in managed soils. The diversity of Ascomycetous yeasts found in the Dutch soil samples correlates with data obtained from yeast diversity studies on soils that were exposed to human intervention (Vadkertiová, Dudášová and Balaščáková 2017; Yurkov 2018), such as *Barnettozyma californica* (Lodder) Kurtzman, Robnett and Bas.-Powers (Yurkov, Kemler and Begerow 2012a; Yurkov *et al.* 2016), *Candida railenensis* C. Ramírez and A.E. González 1984 (Yurkov *et al.* 2016), *Debaryomyces hansenii* (Zopf) Lodder and Kreger-van Rij (Connell *et al.* 2008; Yurkov, Kemler and Begerow 2011, 2012a; Yurkov *et al.* 2016) and *Schwanniomyces polymorphus* (Klöcker) M. Suzuki and Kurtzman (Yurkov *et al.* 2016). Strains from the *D. hansenii/fabryi* complex were isolated from 17 locations, making it the most abundantly found ascomycetous species from our study. Connell *et al.* (2008), Yurkov, Kemler and Begerow (2012a) and Yurkov *et al.* (2016) used the rDNA regions for identification of their strains and it is therefore not clear if they also had a mixture of *D. hansenii and D. fabryi* isolates, as it is not possible to distinguish *D. hansenii and D. fabryi* from one another using the ITS and D1/D2 gene regions (Groenewald *et al.* 2008).

Several surveys to identify soil-associated yeasts in the past resulted in the identification of numerous known but also novel yeast species (Vishniac 2006; Connell *et al.* 2008; Buée *et al.* 2009; Yurkov, Kemler and Begerow 2012a; Yurkov *et al.* 2012b, 2016), indicating that soil is a rich source of known as well as yet undescribed yeast taxa. This was also substantiated by the current study where isolates of 52 currently known as well as 15 potential novel yeast species were obtained from soil samples in the Netherlands, of which six were described in this study. From the culture-based studies, it is clear that the soil ecosystem is very complex and that each type of soil and location may hold their own unique yeast diversity. However, the true diversity of yeasts in the many types of soil environments that can be found is yet unknown, as the current isolation techniques used in this study only result in the detection of those propagules that can grow on the specific isolation medium and conditions used, also substantiated by the study done by Buée *et al.* (2009). Sequences obtained and released in publically available sequence repositories from data obtained from environmental samples from uncultured fungi can be very useful as was also seen in our study. Although no culture is available for the GenBank sequence KT965037, linked to an unidentified, uncultured fungus, the availability of this sequence that was obtained from the gut of a rove beetle confirmed the presence of additional isolates of *Pichia gijzeniarum* in the soil ecosystem (Stefani *et al.* 2016).

Spencer and Spencer (1997) showed that nutrient-rich soils are able to support a wider diversity of yeast species than nutrient-poor soils, and that 25%–40% of yeasts in nutrient-rich moist soils are able to ferment carbohydrates. Although the soil properties have not been measured during this study, degraded plant material is in general always present in garden (managed) soil (Vadkertiová, Dudášová and Balaščáková 2017). Characteristics that commonly occur among yeast species frequently encountered in soil are the ability to utilise L-arabinose, D-xylose and cellobiose aerobically; three carbon compounds that are abundantly found in soil ecosystems that originate from degraded lignocellulosic plant material (Botha 2006). This was also observed during this study, where the majority of the investigated isolates belonged to species that are known to be able to utilise at least two of these compounds, with only a small number of strains that belonged to species that are not known to have this ability (Table 1). However, most soil yeasts are not the primary degraders of complex recalcitrant polymers, but they are able to assimilate the microbial degradation products of lignocellulose plant materials (Botha 2011).

In total, 178 isolates obtained during this study are now publically available in the CBS yeast culture collection and future studies to understand the specific role they play in soil ecosystems, e.g. their physiological properties and genomic profile are possible for future generations as well. A large number of the known fungal species so far described are likely to occur in the soil environment at some stage of their life cycle, as soil is part of an ecosystem containing plants, animals, insects and human activities that all can lead to the transfer of yeast strains to the soil environment at some stage. However, it is therefore difficult to predict the true identity of the autochthonous soil yeasts as it is mostly determined by plant, animal, insect and fungal hosts and vectors (Yurkov 2018). Many of the species obtained during the project were only isolated from one sample site that fits with the idea that yeast species distribution in soil is fragmented (reviewed in Yurkov 2018).The numerous potential novel species obtained during this study also confirms that the soil environment is an important niche for the discovery of novel yeast species. Only a small portion of the strains isolated from soil in the above mentioned yeast diversity studies has been deposited in public culture collections and are therefore not publicly available or preserved for future use. This is also true for many additional strains used in many other publications. The publicly availability of strains is declining even more due to recent international and national regulations that now apply to biological resources obtained from different countries. To protect local biodiversity many countries have national regulations or have signed the Nagoya Protocol (NP) on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilisation to the Convention on Biological Diversity (Herkenrath and Harrison 2011) that entered into force in October 2014. At the present time, access to isolates, even ex-types, collected in many countries after ratification of the NP or due to additional national laws, has become problematic (Overman and Scholz 2017). These aspects have negative effects on international collaboration and also complicate the research of local researchers. Many of the countries do not have public culture collections to preserve the strains for future use and deposition in culture collections outside of the country of origin is prohibited. Therefore, many of the strains are only available in personal research collections that may lead to the inaccessibility and even loss of many valuable isolates. As the Netherlands does not formally restrict access to their genetic resources, this study made it possible to incorporate numerous yeast isolates into the CBS

collection without any restrictions, which make these isolates readily available for use by the international community. Many of the isolates represented species of which only a few isolates or even only a single ex-type strain was available, stressing the importance of this niche for future research.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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Conflicts of interest. None declare.

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