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WHO LIVES IN OUR DISHWASHER? PRELIMINAR RESULTS OF FUNGAL METAGENOMIC ANALYSIS OF HOUSEHOLD DISHWASHERS

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Who lives in our dishwasher? Preliminar results of fungal metagenomic analysis of household dishwashers

In the last few years the advances in molecular biological methods, especially the development of next generation sequencing, have drastically changed and improved our view of microbial world. Progress in new molecular techniques enables us to overcome potential disadvantages of traditional microbiological techniques in fungal community identifications. It also enables us to evaluate the richness of fungal populations more efficiently and reliably. In the present study, we used the Ion Torrent PGM next generation sequencing platform to analyse fungi present in ordinary household dishwashers. The identification was based on massive parallel sequencing of the D2 LSU *rRNA* amplicon. The analysis revealed rich and diverse fungal communities present in our dishwashers. Interpretation of the results was based on previously published research by Zalar *et al.* (2011). The results of our study confirmed that the new technology in many ways surpasses classical methods used in fungal analysis by offering quicker, reliable, more sensitive and inexpensive high-throughput identification of microorganisms in entire communities.

Key words: molecular biology / molecular techniques / fungi / metagenomics / next generation sequencing / Ion Torrent PGM / household dishwashers

Kdo živi v našem pomivalnem stroju? Preliminarni rezultati metagenomske analize gliv v gospodinjskih pomivalnih strojih

Na področju metagenomike je napredek molekularno bioloških metod, predvsem razvoj naslednje generacije sekvenciranja, dramatično spremenil in razširil pogled na mikrobni svet. Napredek novih molekularnih tehnik nam omogoča premagovanje pomanjkljivosti tradicionalnih mikrobioloških tehnik, predvsem pri identifikacij populacij gliv. Z novim pristopom dobimo učinkovitejšo in zanesljivejšo ocenitev števila vrst gliv v določenih populacijah. V naši raziskavi smo uporabili tehnologijo naslednje generacije sekveniranja Ion Torrent za analizo prisotnosti gliv v gospodinjskih pomivalnih strojih. Identifikacija je temeljila na masivni paralelni določitvi nukleotidnega zaporedja podenote D2 LSU glivnega gena *rRNA*. S končno analizo smo potrdili bogate in raznolike skupnosti gliv v naših pomivalnih strojih, interpretacija rezultatov pa je temeljila na že objavljenih predhodnih raziskavah Zalarjeve in sod. (2011). Rezultati naše raziskave so potrdili, da nova tehnologija na mnogih področjih presega klasične mikrobiološke metode, ki se uporabljajo pri analizi skupnosti gliv in ponuja hitrejšo, zanesljivejšo, občutljivejšo ter cenejšo identifikacijo mikroorganizmov v skupnosti.

Ključne besede: molekularna biologija / molekularne tehnike / glive / metagenomika / naslednja generacija sekveniranja / Ion Torrent PGM / gospodinjski pomivalni stroji

1 INTRODUCTION

Fungi have a billion years of evolutionary history, number perhaps 1.5 million species, and are hence extremely diverse both phylogenetically and functionally, with a complex taxonomy.

In the last few years, several approaches have been proposed to study fungi in various environments. Each of the approaches (traditional or molecular) has their advantages and limitations. Classification of fungi that cannot be isolated in pure culture can be especially problematic. In recent years, development of the next generation sequencing (NGS) techniques has enabled sequenc-

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ing of whole genomes or only parts of genomes, which can be used for taxonomical studies. These techniques can also be used to investigate complex microbial communities with the metagenomics approach. Metagenomics analyses present different challenges as microbiome samples can contain thousands of species, often novel and closely related (Tringe *et al.*, 2005) and accessing the genetic information from an entire community of organisms represents a bioinformatical challenge. In the last years, many articles were published in the area of eukaryotic metagenomics (Venter *et al.*, 2004; Turnbaugh *et al.*, 2009; Qin *et al.*, 2010). Articles published since 2009, which describe fungal metagenomics studies performed using different NGS platforms, are listed in Supplement 1. These studies are based on the analysis of nucleotide sequences of whole genomes or use different genetic (ITS1, ITS2 or LSU). One of the possible approaches to fungal metagenomics analysis is to use the D2 expansion segment region; part of the gene which encodes the large subunit ribosomal 28S rRNA (LSU rDNA) (Amend *et al.*, 2010; Gottel *et al.*, 2011; Lekberg *et al.*, 2012; Tonge *et al.*, 2014). Especially the variations in the 5' end of LSU region are widely used for fungal phylogenetic analyses at or above the genus level. Recently, (Thomas *et al.*, 2012) published some guidelines about the entire workflow for microbial metagenomics studies, ranging from sampling to data analysis.

In our study, we were interested in fungal communities, which are in daily contact with humans at home. As it is well known, fungi are very diverse organisms living almost anywhere, including very extreme conditions. One of the possible extreme habitats in human homes is the dishwasher. It represents an interesting habitat, because it is rich in nutrients and water, but on the other hand, it is regularly exposed to extreme conditions, which include high temperatures, very fluctuating humidity levels and high detergent concentrations. (Zalar *et al.*, 2011) presented the study of fungal community in the dishwashers across the world. The focus of their study were polyextermotolerant fungi, which can be potentially pathogenic for humans. Certain generalist species are adjusted at adapting to different stressful environments and Zalar et. al (2011) have shown that this includes the dishwasher. Actually, most fungi are not dangerous, but some types can be harmful, like oligotrophic black fungi, which have been potential to cause human infection (Lian & de Hoog, 2010), or for example, *Exophiala dermatitidis,* which can cause potentially fatal systemic and brain infections (Zeng *et al.*, 2007). Furthermore, fungi have also been implicated in the sick building syndrome (Straus, 2009; Thrasher & Crawley, 2009). In the review of Gostinčar *et al.* (2011) some genera (*Exophiala*, *Aspergillus*, *Candida*, *Dipodascus*, *Fusarium*, *Penicillium*, *Pichia* and *Rhodotorula*) were found to form stable communities in dishwashers.

In the present study, we used the Ion Torrent PGM, NGS platform to analyse fungi present in ordinary household dishwashers. In a few previous studies, Ion Torrent technology was already used to identify fungal communities from different sources (Kemler *et al.*, 2013; Brown *et al.*, 2013; Tonge *et al.*, 2014; Geml *et al.*, 2014), but here we present the first report of the NGS metagenomic approach for analysis of fungal populations in the samples from four different dishwashers. Identification was based on NGS of the D2 LSU rRNA amplicon and it revealed rich, but also diverse fungal communities present in our dishwashers.

2 MATERIAL AND METHODS

2.1 MATERIAL

In our preliminary study, we collected samples from 4 different household dishwashers. The samples were collected with buccal swabs (Prionics, Switzerland) around the door-sealing O-ring.

2.2 METHODS

2.2.1 DNA EXTRACTION AND QUANTIFICATION

Before the isolation, the buccal swabs were vortexed in 1 ml of 1x PBS. The samples were divided into two tubes and centrifuged for 1 min at 100 x g. The DNA from half of the sample was isolated with the PrepMan® Ultra Sample Preparation Reagent (Life Technologies, USA) according to the manufacturer's instructions. The DNA from the other half of the samples was isolated using the MagMAX™ Total Nucleic Acid Isolation Kit with the MagMAX Express Magnetic Particle Processor (both Life Technologies) following the manufacturer's instructions. The concentrations and purities of the extracted DNA were determined using the LAMBDA Bio+ spectrophotometer (Perkin-Elmer, USA), and the DNA was diluted 100x with sterile deionized water.

2.2.2 PCR AMPLIFICATION, SIZE SELECTION AND QUANTIFICATION OF PCR PRODUCT

PCR amplification of the D2 LSU rDNA region of fungal DNA was done with the PCR MicroSeq module,

which is a part of the MicroSeq® D2 LSU rDNA Fungal Identification Kit (Life Technologies). The size selection of the specific approximately 350 bp long PCR products was done with the E-Gel® SizeSelect™ 2 % kit (Life Technologies). The concentrations of PCR products were determined using the dsDNA HS Assay Kit and the Qubit® 2.0 Fluorimeter (Life Technologies).

2.2.3 ION TORRENT LIBRARY PREPARATION AND SEQUENCING

The DNA library was prepared using the Ion Xpress™ Plus gDNA Fragment Library Preparation kit (Life Technologies) according to the manufacturer's protocol. Some modifications were made as the concentration of starting material was in the range between 10 to 20 ng and the step of fragmentation of gDNA with Ion Shear ™ Plus Reagent was skipped. The samples were barcoded according to the manual with Ion Xpress™ Barcode Adapters 1-16 Kit (Life Technologies). The amount and size distribution of library DNA fragments was determined with the Labchip GX instrument (Perkin-Elmer). Emulsion PCR and enrichment steps were carried out using the Ion PGM™ Template OT2 200 Kit and the Ion OneTouch™ 2 System as described in the manufacture's protocol. Assessment of the Ion Sphere particle quality was undertaken between the emulsion PCR and enrichment steps with the Ion Sphere quality control kit (Life Technologies) using a Qubit 2.0 fluorimeter. Libraries were sequenced on the Ion 316™ Chip v2 (Life Technologies) with the Ion PGM™ Sequencing 200 Kit v2 follow-

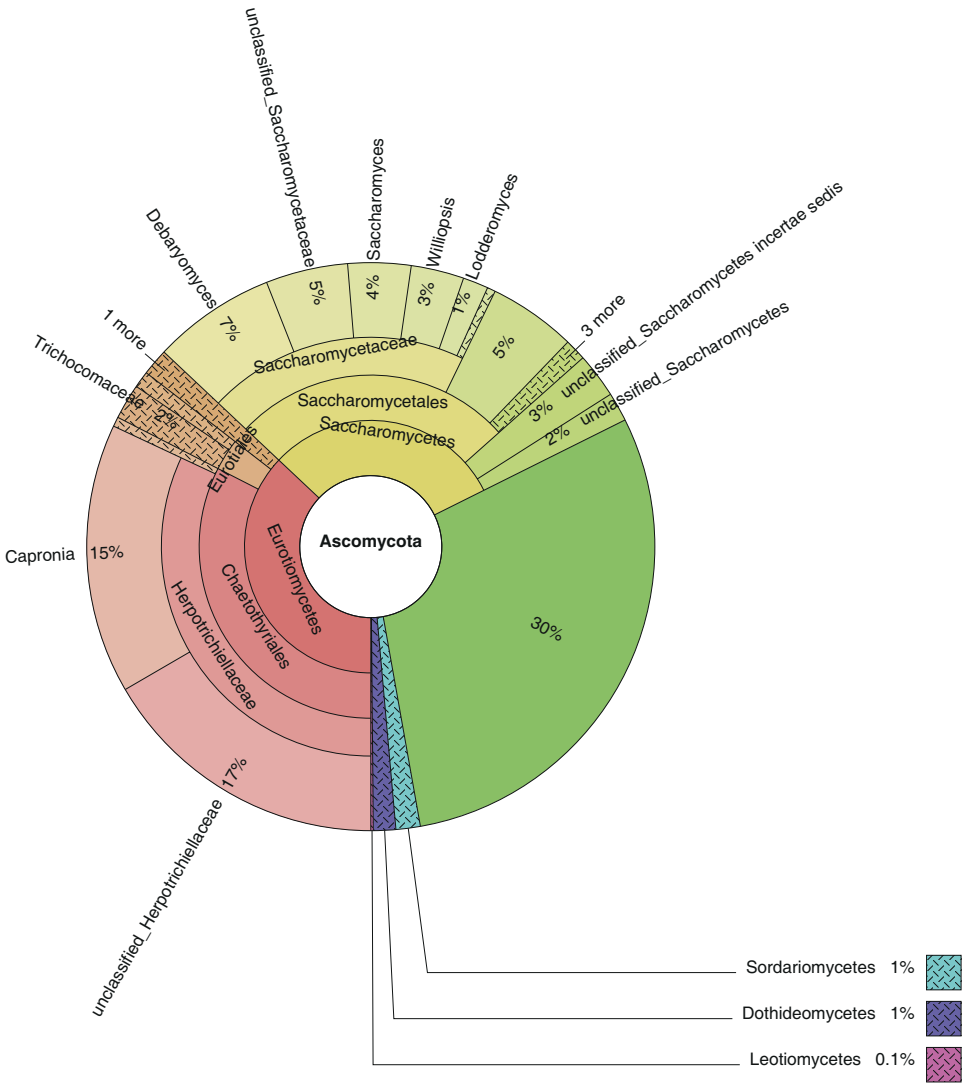

Figure 1a: *Distribution of the taxa for the most prominent phylum Ascomycota identified in the first dishwasher*
Slika 1a: *Porazdelitev taksonov v najbolj zastopanem deblu Ascomycota v prvem pomivalnem stroju*

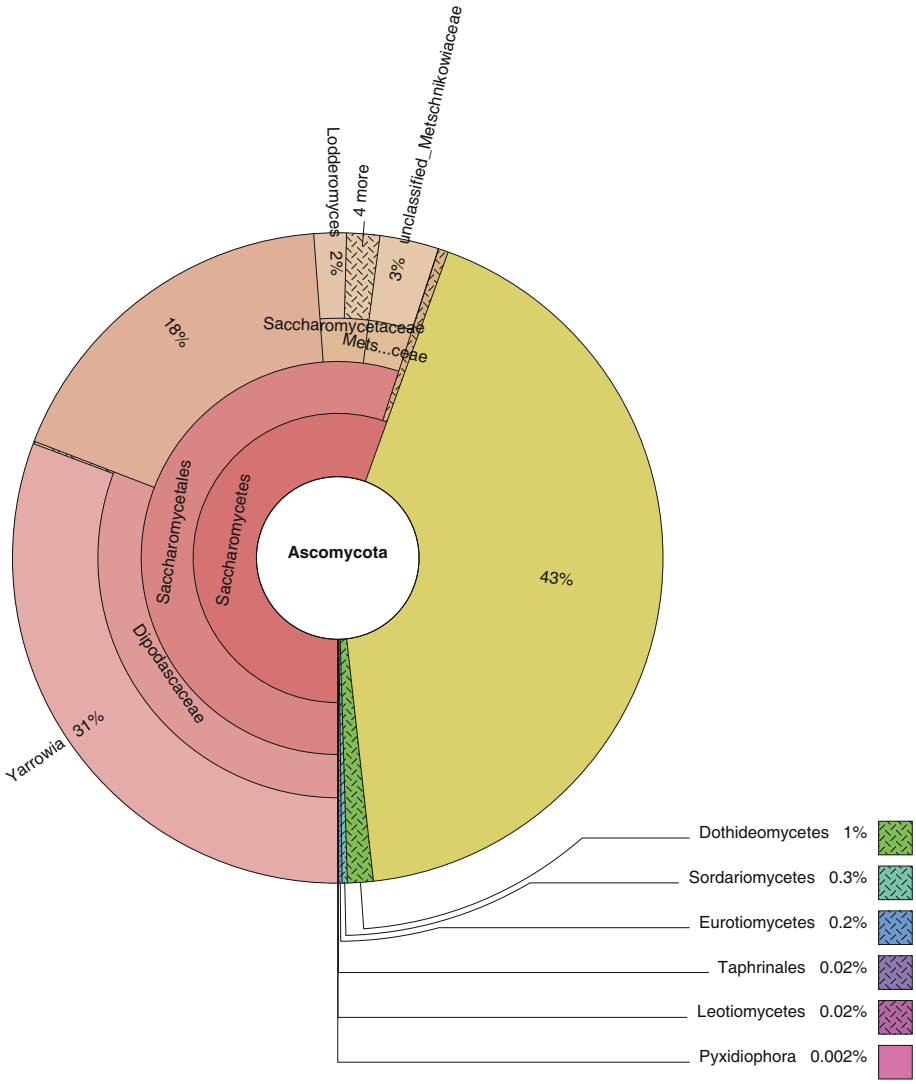

Figure 1b: *Distribution of the taxa for the most prominent phylum Ascomycota identified in the second dishwasher*
Slika 1b: *Porazdelitev taksonov v najbolj zastopanem deblu Ascomycota v drugem pomivalnem stroju*

ing the manufacturer's manual. Signal processing and base calling was performed with the Torrent Suite software version 4.0 (Life Technologies).

2.2.4 BIOINFORMATICS ANALYSIS

For the metagenomics analysis, all the reads that passed the default Torrent Suite quality thresholds were exported into FASTA format and classified using the fungal naïve Bayesian classifier available through the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Since the classifier requires sequences with at least 50 bp for good classification results, shorter sequences were not submitted for the analysis. The bootstrap confidence threshold of 80 % was used for classification, and the sequences not reaching this threshold were grouped into "unclassified" taxons. Interactive hierarchical data browser Krona (Ondov *et al.*, 2011) was used to display the resulting taxonomical data.

3 RESULTS

In total, 678,354 reads for four samples were sequenced with a mean length of approximately 119 bp and the longest reads over 300 bp long, resulting in 80.85 Mbp of sequencing data, from which 54.73 Mbp met the Q20 quality criteria (67.7 %).

Each sequence of fungal D2 LSU *rRNA* gene was classified from the phylum down to the order and in

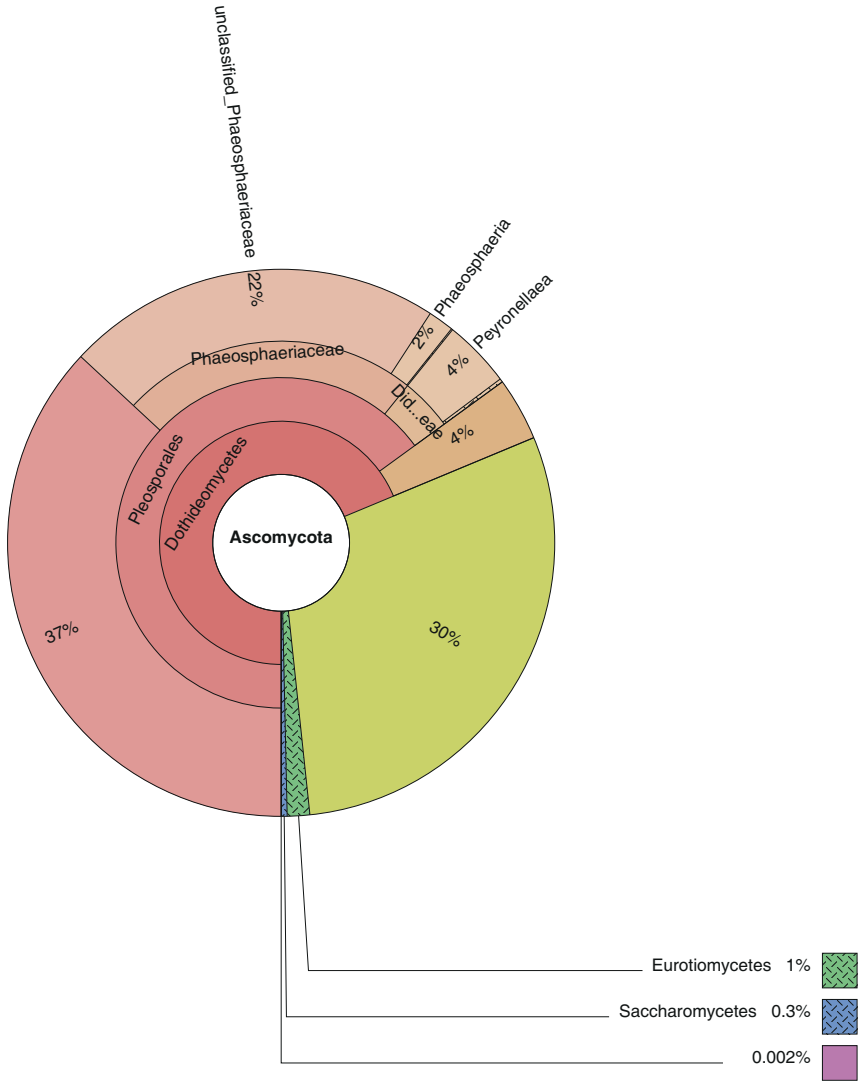

Figure 1c: *Distribution of the taxa for the most prominent phylum Ascomycota identified in the third dishwasher*
Slika 1c: *Porazdelitev taksonov v najbolj zastopanem deblu Ascomycota v tretjem pomivalnem stroju*

some cases also genus level using the Ribosomal Database Project.

The richness of fungal biomes present in the four sampled dishwashers differed.

The proportion of sequences assigned to unclassified fungi was between 11.9 and 36.0 %. In all four samples, only two phyla (*Ascomycota* and *Basidomycota*) were present. Taxonomic composition analysis for all samples is presented in Figure 1a–d. The *Ascomycota* were the dominant phylum in all samples tested. Of the total six classes of *Ascomycota* and four classes of *Basidiomycota* were observed. The three classes of the phylum *Ascomycota* commonly shared between the samples were the *Saccharomycetes*, *Eurotiomycetes* and *Dothideomycetes*. In contrast, *Sordariomycetes* and *Leotiomycetes* were found only in two samples and *Ascomycota incertae sedis* only in one sample. In addition, members of *Basidiomycota* were observed only in one sample (sample 4) at higher proportions – for this sample, 32 % of the sequences were assigned to this phylum, whereas in the other three samples, the percentages of *Basidiomycota* were much lower – 0.01, 0.4 and 4 %, respectively. Using the cut off of at least 10 reads, at the class level *Exobasidiomycetes* and *Tremellomycetes* were present only in one sample (sample 1 and sample 4, respectively); *Microbotryomycetes* and *Agaricomycetes* were present in two samples (sample 2/ sample 4 and sample 1/sample 4, respectively). The detailed further classification for all four samples can be interactively visualized in the hierarchical data browser Krona (supplemental information S2). In summary, a total of 46 different genera could be identified in the samples at the selected confidence threshold.

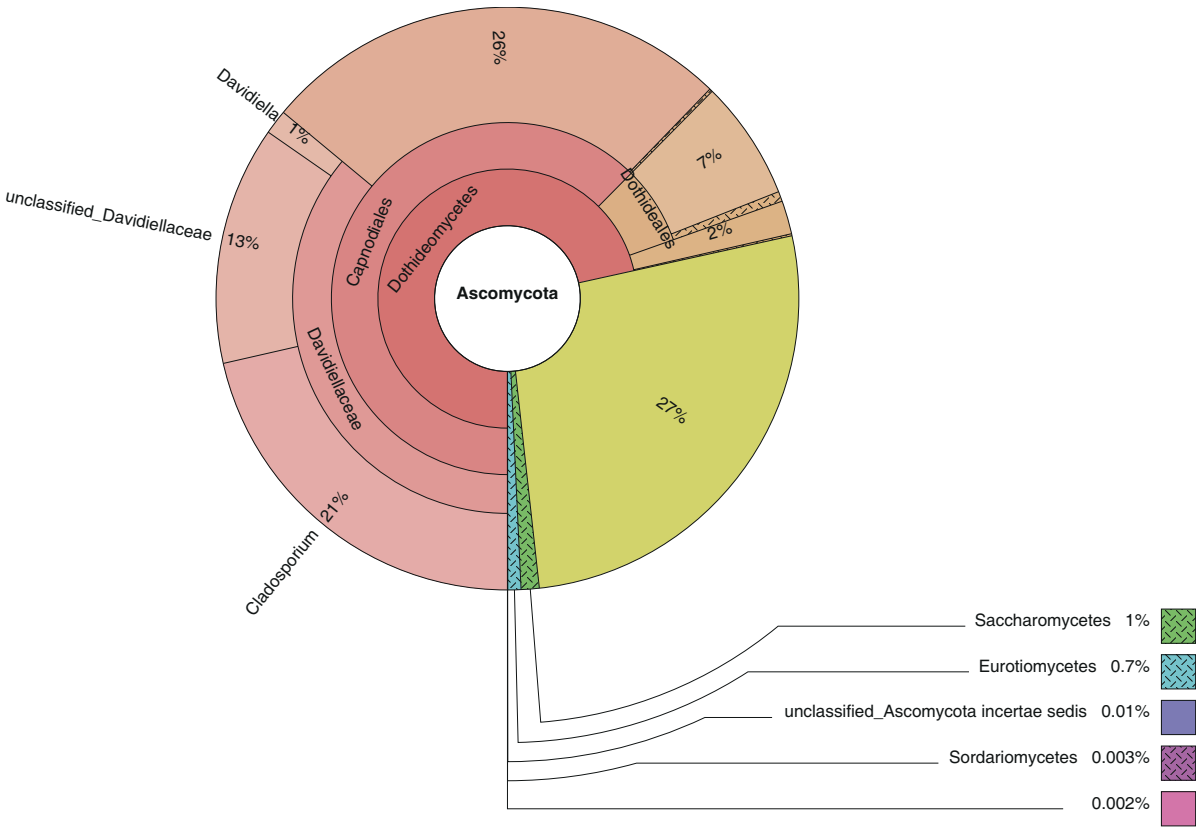

Figure 1d: *Distribution of the taxa for the most prominent phylum Ascomycota identified in the fourth dishwasher*
Slika 1d: *Porazdelitev taksonov v najbolj zastopanem deblu Ascomycota v četrtem pomivalnem stroju*

4 DISCUSSION

Indoor environments offer numerous different habitats for microorganisms. The most contaminated places are kitchens and bathrooms (Ojima *et al.*, 2002; Beumer & Kusumaningrum, 2003; Nishiuchi *et al.*, 2009; Feazel *et al.*, 2009). Zalar *et al.* (2011) observed the fungal flora inside the dishwasher by classic microbiology or the classical sequencing molecular biology approach, whereas in our study we aimed to capture a broader view of fungal communities using the massive parallel sequencing metagenomics approach.

Amend *et al.* (2010) showed that fungi are ubiquitous and diverse components of human indoor environments. The most cosmopolitan taxa reported on their studies were also present in our dishwasher samples: *Alternaria* (3 of 4 samples), *Cladosporium* (1 of 4 samples), *Penicillium* (2 of 4 samples), *Aspergillus* (2 of 4 samples) and *Sordariomycetes* (2 of 4 samples).

In all sample's class, *Saccharomycetes* was present, especially families *Metschnikowiaceae* (*Clavispora*), *Saccharomycetaceae* (*Lodderomyces, Saccharomyces, Debaryomyces*) and *Saccharomycodaceae* (*Dipodascaceae*, *Yarrowia*). *Saccharomycetes* are economically and environmentally important fungi and generally occupy damp or wet habitats that are high in organic material so the presence of a high number of different *Saccharomycetes* in all samples was not surprising. Some of the species of *Saccharomycetes* (*S. cerevisiae*) are used in food processing, production of macromolecular cellular components such as lipids, proteins, including enzymes, and vitamins. However, some evidence indicates also the involvement of *S. cerevisiae* in a range of superficial and systemic diseases (Murphy & Kavanagh, 1999). Interestingly, genus *Yarrowia*, which contains a single-species *Yarrowia lipolytica* was present in high numbers in one of the samples. The species has attracted a lot of interest because of its very high biotechnological potential, especially due to its lipid metabolism abilities (Gonçalves *et al.*, 2014), which are likely also useful for survival in the ecosystem of the dishwasher.

We also investigated the presence of potentially pathogenic fungi described by Zalar et. al (2011) in the dishwashers from our study. Members of the order *Chaetothyriales* were identified in 3 out of 4 samples, in one of them in very high numbers. On the other hand, genus *Exophiala*, which contains numerous potential opportunistic pathogens causing disease, mainly in immuno-

compromised humans and in cold-blooded animals (de Hoog *et al.*, 2011), was not confirmed. However, in the Ribosomal Database Project, most species of *Exophiala* are classified under the genus *Capronia,* and we did identify this genus in all tested dishwashers. Therefore, it is likely that the pathogenic species of *Exophiala*, found to be widespread in dishwashers in the study by Zalar *et al.*, were also present in dishwashers from our study. Genus Capronia also includes a group of fungi known as black yeast with some species responsible for important opportunistic infections in the vertebrata (de Hoog *et al.*, 2000).

We also identified some other potentially pathogenic fungi. In one sample, the genus *Alternaria* was present. It can also be found within the nose, mouth, and upper respiratory tract; they are common allergens in humans (O'Hollaren *et al.*, 1991). The same sample also contained fungi from the genus *Cladosporium*. Some *Cladosporium* species are pathogenic and toxigenic to humans, it has been reported to cause infections of the skin, as well as sinusitis and pulmonary infections (Tasić & Miladinović-Tasić, 2007).

In another sample genus, *Penicillium* was present. It is a very common species known for causing allergies and asthma; some species produce mycotoxins, one being the common antibiotic penicillin (Frisvad *et al.*, 2004; Watanabe, 2008; Bundy *et al.*, 2009).

One sample was also rich in the order *Pleosporales*. Species of this order occur in various habitats, and can be epiphytes, ednophytes or parasites of living leaves or stems, hyperparasits on fungi or insects or saprobes of dead plant material. Some species of this order contain both plant pathogens and food spoilage agents; some of them also contain enzymes that are biological control agents (Kruys *et al.*, 2006). Furthermore, it is known that some species invade homes, and they can cause plant diseases or hay fever and more serious infections in humans (Khan *et al.*, 2000).

NGS has revolutionised the field of metagenomic microbiology by providing a culture independent technique through which to identify and assess microbiological diversity. Furthermore, the availability of affordable "bench-top" sequencers has placed the ability to perform such studies in the hands of most laboratories.

We have tested our approach using four separate fungal communities. According to our data, the fungal biomes present in the dishwashers differed considerably in composition and richness, probably due to differences in models and programming of dishwashers, user's habits and sampling skills. Zalar *et al.* (2011) already suggested that extreme conditions like high temperature, detergent and pH fluctuations can provide an alternative habitat for species also known to be pathogenic to humans, and this was also confirmed in our study. Different stressful conditions can serve as preadaptations for fungal communities and drive their evolution towards pathogenicity. Gostinčar *et al.* (2011) investigated possible scenarios and mechanisms by which the extreme conditions play a role in this process.

The results from our study confirmed that the new technology in many ways surpasses classical methods used in the fungal analysis by offering quicker, reliable, more sensitive and inexpensive high-throughput identification for entire communities. In further studies, it would be interesting to extend the scope of this preliminary study by analysing more samples, increasing the number of reads and by sequencing additional targets, which would enable even deeper classification of fungi down to the level of species. Furthermore, comparison of the temperature protocols in the dishwasher instruments would also contribute valuable data on the role of extreme conditions for the composition of the biome.

5 ACKNOWLEDGEMENTS

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