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Bioinformatic design and pilot validation of a PCR assay for species-level identification of *Candida* spp. in biological samples

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Abstract. Species-level identification of representatives of the genus *Candida* in complex biological samples of natural, agrobiological and technological origin is necessary for the accurate analysis of mixed microbial communities, the control of microbial contamination and the assessment of potential sources of fungal pathogen dissemination. This study aimed to provide bioinformatic justification, design and preliminary laboratory verification of a polymerase chain reaction system for the specieslevel differentiation of *Candida glabrata*, *Candida parapsilosis* and *Candida auris*. The study included analysis of the ribosomal cluster, selection of reference sequences, in silico design of primers and hydrolysis probes, assessment of their specificity, optimisation of the reaction mixture and testing on control samples of DNA and archived isolates of biological origin. The internal transcribed spacer region of the ribosomal cluster was selected as the main target, as it combines conserved flanking fragments with variable internal sequences. Primer-probe sets were developed for *Candida glabrata*, *Candida parapsilosis* and *Candida auris*, with amplicon sizes of 82, 91 and 118 base pairs, respectively. The amplification reaction was performed in a final volume of 25 µl. The final concentration of each primer was 0.28 µM; the concentration of the hydrolysis probe was 0.2 µM, MgC_2 3 mM, dNTPs 0.3 mM and betaine 1.5 M. Positive amplification was obtained for 40 positive control samples, including 29 *Candida* spp. samples, 7 *Candida glabrata* samples and 4 *Candida parapsilosis* samples. No cross-reactivity was detected in the negative control panel, which included 43 samples of non-target microorganisms. The proposed system confirmed its suitability for primary qualitative analytical species-level identification of selected representatives of the genus *Candida* under controlled laboratory conditions. The developed approach may serve as a basis for further sanitary and biological screening of complex matrices of soil, aquatic and plant microbiota, for biosafety control in the agro-industrial sector, and for subsequent adaptation to the early molecular detection of fungal pathogens relevant to invasive infections

Keywords: ribosomal cluster; yeast-like fungi; probes; screening; microbial contamination; microorganisms

INTRODUCTION

Species-level identification of yeast-like fungi of the genus *Candida* in biological samples of different origin remains a methodologically complex task, since these microorganisms may form part of the accompanying microbiota, act as markers of contamination and microbial transfer between different ecological niches, or represent potential infectious agents in people at increased risk. In such

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matrices, genus-level detection of *Candida* does not always provide sufficient interpretative accuracy, as individual species differ in their capacity for survival, surface colonisation, biofilm formation and interaction with other components of microbial communities. Therefore, ecological and agrobiological analysis requires not only confirmation of the presence of yeast deoxyribonucleic acid, but also clarification of the species affiliation of the detected taxon. Environmental matrices, including soil, water, plant and technologically modified samples, are examples of objects in which the presence of *Candida* may have different origins and therefore requires cautious interpretation. According to M. Sautour *et al*. (2021), target *Candida albicans* deoxyribonucleic acid was detected in only 7 of the 460 soil samples examined, that is, in 1.5% of cases, which indicates a low but confirmed possibility of detecting this species in soil. At the same time, *Candida albicans* was found to persist for up to 30 days in 80% of the soils studied. Its short-term survival was associated with specific physicochemical soil properties, particularly cation-exchange capacity and clay content. This points not to soil as the principal matrix, but rather to the possibility of temporary preservation of cells or their DNA under certain physicochemical conditions. Accordingly, soil should not be regarded merely as an incidental contamination matrix: under certain conditions, it may support the temporary persistence of yeast cells or their deoxyribonucleic acid, which increases the importance of species-level identification. The issue of environmental reservoirs of *Candida* is not limited to soil. A.B. Akinbobola *et al*. (2023) showed that representatives of this genus may be isolated from freshwater and seawater, plant substrates, soil environments and technologically modified sources. On the basis of these data, the authors concluded that non-anthropogenic and anthropogenically transformed environments may support yeast persistence and facilitate their transfer between ecological niches. For *Candida auris*, this issue has an additional dimension: according to I. Silva *et al*. (2024), the potential reservoirs of this species include seawater, sand, salt marshes, estuaries, plant materials and other environments. However, the available reports remain heterogeneous in terms of geography, sample type and confirmation methods. Accordingly, such objects require standardised molecular tools capable of providing species-level, rather than merely genus-level, detection. D. Corrêa-Moreira *et al*. (2024) confirmed the possibility of detecting *Candida* in wastewater, which accumulates microbial material from various sources and may reflect changes in the biological status of a particular territory or facility. In addition, M.M. Oliveira *et al*. (2022) established that marine isolates of *Candida parapsilosis* are capable of early adhesion to high-density polyethylene and may contribute to the formation of microbial associations on polymer substrates. These findings are important not as evidence that every case of human infection originates directly from the environment, but as a justification for the need for rapid screening of potential transmission links for fungal pathogens. Soils, water, plant materials, wastewater and technological surfaces may accumulate or transfer yeast cells and their DNA. When barrier mechanisms are disrupted, or in the context of invasive procedures, catheterisation or immunosuppression, individual *Candida* species acquire clinical significance as potential agents of invasive infections, candidaemia and septic conditions. For this reason, molecular detection in complex matrices logically connects agro-environmental monitoring with the tasks of biosafety and clinically oriented diagnostics. It is also relevant to aquatic and technological systems, where polymer surfaces may serve as stable zones of colonisation and where the presence of *Candida parapsilosis* requires species-level interpretation.

The agrobiological context in this study is considered as one possible area of application, rather than as a self-contained concept of soil-based identification. In plant systems, yeasts do not always have an unequivocally negative significance, since they may participate in interactions with plants, other microorganisms and substrates. J. Kowalska *et al*. (2022) concluded that yeasts can colonise plant surfaces, compete for space and nutrients, produce biologically active compounds and contribute to the suppression of certain phytopathogens. This indicates that the detection of yeasts in plant matrices cannot automatically be interpreted as contamination, because their role depends on the species, abundance, localisation and environmental conditions. At the same time, S. Zhu *et al*. (2021) showed that rhizosphere soils of fruit agrocenoses contain diverse yeast

communities, the structure of which varies depending on the region, climatic factors and soil parameters. A relationship was established between the composition of yeast communities and precipitation, humidity, solar radiation, electrical conductivity, total phosphorus and potassium; these findings emphasise the dynamic nature of such systems. Plant microbiota are generally regarded as a multi-level system of interactions between the plant, soil, microorganisms and environmental factors. G. Berg & T. Cernava (2022) emphasised that the plant microbiome is involved in nutrition, stress resistance and the maintenance of the functional stability of the plant organism; therefore, its composition is important for sustainable agriculture. At the same time, M. Chialva *et al*. (2022) showed that microbiota is shaped by plant species, organ, soil type, environmental conditions and interactions among the microorganisms themselves. This means that the presence of *Candida* in plant or rhizosphere samples requires analysis not merely at the level of general yeast detection, but at the level of species identification within a specific biological matrix. Under such conditions, culture-based methods do not always provide rapid or sufficiently accurate differentiation of species in mixed microbial communities. Molecular approaches based on analysis of the ribosomal cluster make it possible to improve both the speed and specificity of detection. A.A. Rivera-Zizumbo *et al*. (2024) confirmed the suitability of internal transcribed spacer 1 metabarcoding for analysing soil microeukaryotic diversity, while also emphasising that the results depend on marker selection, the reference database and interpretation criteria. For *Candida*, this supports the use of multicopy regions of the ribosomal cluster, namely the internal transcribed spacers, which combine conserved flanking regions for primers with variable internal fragments for species-level discrimination.

Thus, the available data indicate that *Candida* may be present in various biological systems. However, its ecological, sanitary-biological or clinical significance depends on species affiliation, matrix type and the context of detection. This study aimed to justify the selection of molecular targets and to perform a primary analytical assessment of a primer-probe approach for differentiating selected representatives of the genus *Candida* in biological material, using control deoxyribonucleic acid matrices and archived isolates of biological origin. In a broader perspective, this approach may be useful both for monitoring potential environmental sources of contamination and for subsequent adaptation to the early molecular detection of fungal pathogens relevant to invasive infections and sepsis diagnostics.

MATERIALS AND METHODS

The research was conducted as a methodological study involving the analysis of scientific publications and bioinformatic databases, together with pilot laboratory verification of a PCR assay designed for species-level identification of selected representatives of the genus *Candida* in biological samples. *C. glabrata*, *C. parapsilosis* and *C. auris* were selected as the target taxa because these species require accurate differentiation from related yeast taxa and can be used as model objects for testing the discriminatory capacity of a multiplex PCR assay. The theoretical and analytical part of the study was based on scientific publications used to justify the choice of molecular targets and to interpret fungal diversity in biological matrices. Environmental and technological materials may serve as sources of contamination or transfer of fungal DNA, whereas, in the clinical context, specieslevel differentiation of *Candida* is important for the further interpretation of invasive infections. In particular, the study took into account the findings of C. Djemiel *et al*. (2024) concerning the spatial variability of the soil mycobiome and the influence of ecological factors on the structure of fungal communities. It also considered the conclusions of C.L. Schoch *et al*. (2012) regarding the suitability of the internal transcribed spacer region of the ribosomal cluster as a universal marker for fungal identification. The ITS1/ITS2 regions of the ribosomal cluster and the 18S rRNA and 28S rDNA sequences were considered as the main molecular targets. The latter were treated as auxiliary regions for genus-specific screening, confirmation that the amplified material belonged to fungal DNA, and potential further expansion of the test system. Reference sequence selection, *in silico* primer and probe design, specificity assessment and compatibility testing in a multiplex format were performed using bioinformatic analysis, with consideration of melting temperature, GC content, amplicon length, spatial positioning of the

hydrolysis probe and the risk of undesirable secondary structure formation.

Bioinformatic analysis was performed using open resources of the National Center for Biotechnology Information (NCBI), including nucleotide sequence databases and search tools for comparing reference fragments, which provide access to standardised genomic and taxonomic data described by E.W. Sayers *et al*. (2024a; 2024b). Nucleotide sequences of the target loci and reference genomic sequences of the relevant microorganisms were selected from the GenBank database, which operates as an open international database of nucleotide sequences and is regularly updated within the NCBI resources described by E.W. Sayers *et al*. (2024a; 2024b). For *C. glabrata*, *C. parapsilosis* and *C. auris*, the available ribosomal cluster sequences were analysed to identify regions suitable for the design of primers and TaqMan probes. The first stage involved comparing homologous regions of the ribosomal cluster and identifying fragments in which conserved regions could be used for primer binding, while internal variable regions could be used for probe-based species discrimination. The ITS1-5.8S-ITS2 fragments were prioritised because of their suitability for amplification in a real-time PCR format and the presence of sequences useful for differentiating individual taxa of the genus *Candida*. The 18S rRNA and 28S rDNA sequences were considered auxiliary loci for assessing the stability of the selected regions and for providing methodological justification for genus-level detection. Oligonucleotide design and melting temperature calculations were performed using the OligoAnalyzer tool (Integrated DNA Technologies, USA). The probability of secondary structure formation was assessed using the standard programme parameters for the specified reaction conditions. For each candidate primer pair, the following parameters were evaluated: oligonucleotide length, calculated melting temperature, GC content, expected amplicon size, primer orientation, probe position within the amplicon and the presence of potential sites of self-dimerisation, heterodimerisation or hairpin formation.

To assess the specificity of the selected primers and probes, they were checked *in silico* using the NCBI BLAST tool (USA). The assessment was based on the identification of complete and partial matches with target and non-target sequences, including closely related yeast taxa and model nontarget microorganisms. Selection was carried out with consideration of several parameters: oligonucleotide length, expected amplicon size, melting temperature, GC-pair content, spatial positioning of the probe and compatibility of all components within a single reaction mixture. This approach made it possible to compare the requirements for an individual primer pair with those for the multiplex system as a whole. Particular attention was paid to the potential complementarity of the 3′ ends of primers to non-target sequences, as such interactions may create conditions for non-specific amplification. Primer-probe combinations that met the established physicochemical criteria and showed no predicted non-specific binding to non-target sequences during the *in silico* assessment were selected for subsequent laboratory testing. The genetic targets were selected with regard to the need to combine genus-level detection of yeast-like fungi with the possibility of species-level differentiation of selected representatives of the genus *Candida*. For this purpose, the ITS1, 5.8S rRNA, ITS2, 18S rRNA and 28S rDNA regions were analysed. The ITS1-5.8S-ITS2 fragment became the main working target for constructing the speciesspecific panel, because its flanking regions were suitable for primer binding, while its internal variable sequences allowed the placement of hydrolysis probes. For each target species, the presence of regions suitable for generating short amplicons appropriate for real-time PCR was assessed. The absence of large insertions or deletions within the target sequences, which could affect amplification stability, was checked separately. The compatibility of candidate primers and probes with the other components of the future multiplex reaction was also considered during selection, since all targets had to be amplified under a single temperature profile. The study used control DNA samples of commercial or synthetic origin together with archived isolates of biological origin from the internal collection of Laboratory X, which had been deposited as reference strains. The material was considered solely as an analytical matrix for pilot verification of the PCR assay. No clinical biomaterials were used in the study, no blood samples were analysed, and no personal data were accessed.

The species affiliation of the archived isolates was established before the start of the study using culture-based and molecular methods in accordance with internal laboratory procedures. At the beginning of the experiments, the cells were washed twice with phosphate-buffered saline, standardised by concentration and used to obtain the DNA template. Positive control samples and archived isolates of biological origin were used for *C. glabrata* and *C. parapsilosis*, whereas synthetic control material was used to assess the species specificity of *C. auris*. To assess specificity and cross-reactivity, a control panel was formed, which included both positive samples of *Candida* spp., *C. glabrata* and *C. parapsilosis*, and negative control samples containing DNA from non-target microorganisms. The positive panel consisted of 29 *Candida* spp. samples, 7 *C. glabrata* samples and 4 *C. parapsilosis* samples. The negative control panel included *Staphylococcus aureus* (n = 3), *Streptococcus pneumoniae* (n = 2), *Streptococcus pyogenes* (n = 2), *Escherichia coli* (n = 5), *Klebsiella pneumoniae* (n = 2), *Pseudomonas aeruginosa* (n = 2), *Acinetobacter baumannii* (n = 2), *Mycoplasma hominis* (n = 5), *Mycoplasma genitalium* (n = 5), *Ureaplasma parvum* (n = 5), *Ureaplasma urealyticum* (n = 5) and *Gardnerella vaginalis* (n = 5). The negative samples were used as a model non-target panel to determine whether the selected primers and probes generated a non-specific fluorescent signal in the presence of accompanying microbial DNA. Real samples of soil, aquatic and plant microbiota were not examined at this stage. These matrix types were considered a potential area for further application of the developed system after completion of the primary laboratory testing on control DNA samples and archived isolates of biological origin. PCR was performed in a final volume of 25 µl in a buffer solution containing 50 mM Tris, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% non-ionic detergent and 4% glycerol. This buffer composition was selected to maintain a stable ionic environment, provide suitable conditions for Taq DNA polymerase activity and reduce the effect of potential amplification inhibitors. The concentrations of $MgCl_2$, dNTPs, primers, probes and enzyme were optimised separately, taking into account the requirements of the multiplex reaction format. The final concentration of each primer was 0.28 µM, while the concentration of the TaqMan probes was 0.2 µM. The concentrations of $MgCl_2$ and dNTPs were determined empirically and used at 3 mM and 0.3 mM, respectively. The optimal composition of the master mix was determined on the basis of the shape of the amplification curves, Ct values, fluorescent signal amplitude, stability of the internal control and absence of signal in the negative control samples. Betaine was added to the reaction at a concentration of 1.5 M. The appropriateness of its use was assessed by changes in Ct values, the shape of the amplification curve and the preservation of reaction specificity.

To monitor amplification and detect possible PCR inhibitors in samples with a complex matrix, the pUC19 plasmid vector (GenScript Biotech, China), containing a cloned heterologous control sequence, was added to the reaction. This construct was used as an internal control to monitor the course of the reaction and reduce the risk of false-negative interpretation of the results. The control target was detected using the following primers and probe: FWD: GAGAAGTCTGCCGTTACTGC; REV: GATAGCAACCTGCCCAGGG; PRB: HEX-TGGGGCAAGGTGAACGTGGA-BHQ1. The internal control served as a technical indicator of whether the reaction was suitable for result interpretation. If the control signal was present, this indicated that amplification had proceeded normally and had not been affected by inhibitors. In such cases, the absence of a signal from the target sequence could therefore be regarded as a true negative result. Nucleic acid amplification was performed using the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA) in a two-phase thermal cycling mode. The amplification protocol included initial denaturation at 94°C for 3 minutes, followed by 9 cycles consisting of denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 30 seconds. A further 38 cycles were then performed with shorter step durations: 94°C for 15 seconds, 58°C for 15 seconds and 72°C for 15 seconds. Fluorescence was measured during the elongation step. Amplification results were assessed according to the presence or absence of a specific amplification curve in the relevant detection channel, the Ct value, the amplitude of the fluorescent signal, the stability of the internal control and the absence of signal in the negative control samples. For positive control samples, the

expected result was the presence of a specific signal in the corresponding detection channel. For the negative control panel, the expected result was the absence of amplification in the channels specific to *Candida* spp. The assessment of the results was qualitative and analytical in nature and was intended to confirm the functionality of the primer–probe combinations, analytical specificity and the absence of detectable cross-reactivity within the constructed control panel. Quantitative determination of the limit of detection, construction of calibration curves, calculation of amplification efficiency as a percentage and comparison with sequencing data were not performed at this stage and are regarded as subsequent stages in the validation of the proposed PCR assay.

RESULTS

Justification of the selection of molecular targets for species-level identification of representatives of the genus *Candida*

Following the systematisation of molecular targets suitable for the identification of yeast-like fungi, the ITS1-5.8S-ITS2 fragment of the ribosomal cluster was found to be the most informative region for differentiating the selected representatives of the genus *Candida*. Its advantage lies in the combination of three features important for the construction of a primer-probe system: multicopy occurrence, the presence of relatively conserved flanking regions and sufficient interspecies variability of the internal sequences. This was also established in the study by C. Djemiel *et al*. (2024), in which the authors demonstrated the high informativeness of fungal molecular markers for analysing the spatial variability of the soil mycobiome. It is also supported by the conclusions of C.L. Schoch *et al*. (2012) regarding the suitability of the internal transcribed spacer region as a universal marker for fungal identification. Assessment of the ribosomal cluster showed that 18S and 28S ribosomal deoxyribonucleic acid are more conserved and may be useful for confirming the fungal origin of amplified material or for genus-level detection. At the same time, their variability was insufficient for reliable differentiation of closely related *Candida* species within the selected panel. These loci should therefore be regarded as auxiliary, whereas the ITS1-5.8S-ITS2 region performs the main species-specific function. For *Candida glabrata*, *Candida parapsilosis* and *Candida auris*, fragments suitable for the placement of hydrolysis probes in the internal part of the amplicon were identified within ITS1-5.8S-ITS2. This target structure provided a dual level of specificity: the flanking regions allowed primer binding, while the internal variable sequences enabled species-level discrimination by means of the probe. As a result, primer-probe combinations were generated with expected amplicons of 82 base pairs for *Candida glabrata*, 91 base pairs for *Candida parapsilosis* and 118 base pairs for *Candida auris*.

For *Candida glabrata*, the selected target proved effective in distinguishing this species within the genus *Candida* without relying solely on genus-specific conserved regions. This was important for testing the discriminatory capacity of the system, since a positive signal had to be generated only in the presence of the target species sequence. For *Candida parapsilosis*, the selected target also enabled species-level identification, which is important given the potential presence of this species in aquatic, surface-associated or technological biological matrices. For *Candida auris*, the suitability of the selected sequence for inclusion of this species in the multiplex panel was confirmed using a synthetic control, without the need for additional biological isolates. The results obtained indicate that ITS1-5.8S-ITS2 is the most suitable target for constructing a test system aimed at species-level identification of the selected representatives of the genus *Candida*. Unlike the 18S and 28S ribosomal regions, which are mainly auxiliary, the ITS region provides the necessary balance between the stability of primer binding and species-level variability in the probe-binding regions. This combination of properties provided the basis for the subsequent construction of a multiplex primer-probe system and its pilot laboratory testing. The initial *in silico* assessment using BLAST showed that correct primer selection should be based not only on complete matching with target sequences, but also on the analysis of partial complementarity to non-target organisms. After comparison of reference sequences in databases and assessment of their specificity using BLAST, regions were selected that proved to be the most suitable for designing primers and hydrolysis probes. On the basis of the *in silico* analysis, a set of primers and

probes was generated for the detection and differentiation of selected representatives of the genus *Candida*. The characteristics of the selected oligonucleotides are presented in Table 1.

Table 1. Characteristics of the selected primers and TaqMan probes for detecting selected representatives of the genus *Candida*

Species/component	Sequence	Length, nt	Target region	Amplicon size, bp	Modification
Candida parapsilosis, Fprimer	TTG GTT CTC GCA TCG ATG	18	ITS1-5.8S-ITS2	91	–
Candida parapsilosis, Rprimer	ATG TGC GTT CAA AGA TTC G	19	ITS1-5.8S-ITS2	91	–
Candida parapsilosis, TaqMan probes	CGA AAT GCG ATA CGT AAT ATG AAT TGC AGA	30	Internal region of the amplicon	91	5′-Cy-5; 3′-BHQ3
Candida glabrata, Fprimer	CCA TTT CTC CTG CCT GC	17	ITS1-5.8S-ITS2	82	–
Candida glabrata, Rprimer	CAC TCC CAG GTC TTT GTC	18	ITS1-5.8S-ITS2	82	–
Candida glabrata, TaqMan probes	TGC GCG GTT GGT GGG TGT T	19	Internal region of the amplicon	82	5′-FAM; 3′-BHQ1
Candida auris, Fprimer	GAT GAA GAA CGC AGC GAA	18	ITS15.8SITS2	118	–
Candida auris, R-primer	ATC ACG CTC AAA CAG GCA	18	ITS1-5.8S-ITS2	118	–
Candida auris, TaqMan probes	GCA CAT TGC GCC TTG GGG T	20	Internal region of the amplicon	118	5′-ROX; 3′-BHQ2

Source: compiled by the author

Analysis of the data in Table 1 shows that comparatively short amplicons were selected for the three target taxa: 82 bp for *C. glabrata*, 91 bp for *C. parapsilosis* and 118 bp for *C. auris*. This size range corresponds to the aim of developing a real-time PCR system, in which shorter amplicons are generally better suited to rapid amplification and to work with samples in which DNA may be partially fragmented. Primer length was maintained within the range of 17-19 nt, which allowed annealing conditions to be compared within a single temperature programme. The length of the TaqMan probes was set at 19-30 nt, with each probe located in the internal region of the corresponding amplicon. Signal differentiation in the multiplex format was made possible by the use of different fluorophores and quenchers for the three targets. The design criterion for the primers and probes was the ability of all targets to function within a single unified temperature profile. To achieve this, the calculated melting temperature of the primers was optimised within a narrow range of 60-62°C, ensuring similar annealing conditions for all target sequences. In a multiplex format, this parameter is methodologically important, since substantial differences in Tm between primer pairs may lead to uneven amplification efficiency, competition between reactions and preferential accumulation of individual amplicons. The melting temperature of the hydrolysis probes was designed to be higher than the Tm of the corresponding primers, mainly within the range of 68-70°C. The probes were positioned within the amplicons in regions containing discriminatory nucleotide differences, while local regions with excessive GC content or a potential tendency to form secondary structures were avoided. Within the developed system, this arrangement provided a combination of two levels of specificity: primer binding to the target region and probe-based detection of the internal fragment of the amplicon.

A two-phase thermal cycling mode was selected to allow the sequential formation of a specific product followed by signal accumulation. The extended annealing and elongation steps in the first cycles promoted stable formation of the primary amplicon, which is important for matrices with complex secondary structures or a low amount of target DNA. The shortened steps in the second cycling block ensured the accumulation of the already formed specific product without excessively increasing the duration of the reaction. Thus, the amplification mode was aligned with the logic of the primer-probe design and the multiplex format.

Experimental optimisation of the reaction mixture was aimed at aligning amplification sensitivity, specificity and reproducibility. The most significant parameters were the concentrations of $MgCl_2$, dNTPs, primers, probes and betaine. During optimisation, the shape of the amplification curves, Ct values, fluorescent signal amplitude, the presence or absence of signal in the negative control, and the stability of the internal control were assessed. This set of criteria made it possible to evaluate not only whether amplification occurred, but also the quality of the reaction under conditions of potential competition between several primer–probe systems. The multiplex PCR format imposed stricter requirements on component compatibility than a monoplex system. Therefore, during panel development, consideration was given not only to the specificity of each individual primer pair but also to the overall thermodynamic compatibility of all reaction components. The risks of self-dimer, heterodimer and hairpin formation were assessed, since such interactions could reduce primer availability for the target template and affect the intensity of the fluorescent signal. Betaine at a concentration of 1.5 M was added to improve amplification of GC-rich fragments.

Assessment of specificity and cross-reactivity

A panel of positive and negative control samples was formed for experimental assessment of specificity. The negative control samples included model non-target bacterial microorganisms and representatives of the accompanying microbiota, which were used to assess the cross-reactivity of the test system. This structure of the control panel made it possible to determine whether a fluorescent signal was generated only in the presence of target fungal DNA and to assess the behaviour of the system in the presence of DNA from organisms that do not belong to the genus *Candida*. Positive samples were used to confirm the capacity of the system to detect *Candida glabrata* and *Candida parapsilosis*. Within the methodological study, this made it possible to maintain control over the experimental component without expanding the object of investigation beyond control DNA samples and archived isolates of biological origin. The negative panel was used to exclude cross-reactivity. Testing of the negative control panel showed no amplification in the relevant detection channels. All samples containing DNA from non-target microorganisms, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma parvum*, *Ureaplasma urealyticum* and *Gardnerella vaginalis*, produced negative results. This indicates the absence of detectable cross-reactivity with the non-target microorganisms examined within the constructed test panel. The results are consistent with the preliminary *in silico* analysis, according to which the primer–probe systems were selected for variable regions of the target ITS1-5.8S-ITS2 region. The absence of amplification in the negative panel confirms that the selected oligonucleotides do not generate a significant non-specific signal in the presence of DNA from non-target microorganisms. At the same time, positive amplification in *C. glabrata* and *C. parapsilosis* samples indicates that the system retained analytical sensitivity to the target sequences (Table 2).

Table 2. Results of the assessment of the specificity and cross-reactivity of the PCR assay

Sample category	Microorganism/control	n	Amplification result
Negative control	*Staphylococcus aureus*	3	Negative
	Streptococcus pneumoniae	2	Negative
	Streptococcus pyogenes	2	Negative
	Escherichia coli	5	Negative
	Klebsiella pneumoniae	2	Negative
	Pseudomonas aeruginosa	2	Negative
	Acinetobacter baumannii	2	Negative
	Mycoplasma hominis	5	Negative
	Mycoplasma genitalium	5	Negative
	Ureaplasma parvum	5	Negative
	Ureaplasma urealyticum	5	Negative
	Gardnerella vaginalis	5	Negative

Table 2. Continued

Sample category	Microorganism/control	n	Amplification result
Positive samples	*Candida* spp.	29	Positive
	Candida glabrata	7	Positive
	Candida parapsilosis	4	Positive
Synthetic positive control	*Candida auris*	1	Positive

Source: compiled by the author

Analysis of the data in Table 2 shows that all samples in the negative control panel produced negative amplification results. This applied both to Gram-positive and Gram-negative bacteria and to microorganisms that may form part of the accompanying microbiota in complex biological matrices. Thus, within the tested panel, no signals were recorded that could indicate cross-binding of primers or probes to non-target DNA. The 29 positive *Candida* spp. samples, as well as the separate positive samples of *C. glabrata* (n = 7) and *C. parapsilosis* (n = 4), produced positive amplification results. This distribution confirms the correspondence between the presence of target fungal DNA and the generation of a fluorescent signal in the system. Taken together, the results allow the developed PCR assay to be considered a methodological basis for species-level identification of selected representatives of the genus *Candida* in biological samples. The bioinformatic stage enabled the selection of ITS1-5.8S-ITS2 regions suitable for primer-probe design, while the experimental assessment confirmed the absence of detectable crossreactivity within the control panel used. The persistence of a positive signal for target samples and a negative result for non-target microorganisms indicates consistency between the *in silico* prediction and the laboratory testing. From a methodological standpoint, the data obtained demonstrate that the proposed approach may be used for further screening of complex matrices in which *Candida* may be a component of the microbiota, a marker of contamination or a potentially clinically significant taxon. What matters is not only the detection of *Candida* spp. DNA, but also the ability to distinguish the target signal from the background DNA of other microorganisms. The results of control panel testing show that the selected combination of primers, probes, temperature profile and reaction mixture composition provide a functional basis for such differentiation under the tested conditions. Thus, the study results confirm the consistency of the methodological approach: from the selection of the ribosomal ITS region as the main target to laboratory verification of specificity using control samples. The developed system does not go beyond the tasks of primary analytical testing, but it provides a basis for further application in the monitoring of biological systems, the control of potential sources of fungal contamination, biosafety, and subsequent testing in matrices relevant to the diagnosis of invasive infections.

DISCUSSION

The results should be considered within the methodological logic of the staged development of a PCR assay for species-level identification of yeast-like fungi. At this stage, primary analytical testing was performed, including bioinformatic selection of molecular targets, design of primers and TaqMan probes, assessment of their compatibility in a multiplex format, and testing using a control panel. This approach is consistent with the principles of molecular test validation for environmental monitoring, according to which transition to real multi-component matrices should take place only after preliminary confirmation of specificity, inhibition control and reaction reproducibility. In the readiness and environmental DNA analysis scale proposed by B. Thalinger *et al*. (2021), the initial stages include laboratory testing of specificity and sensitivity, whereas application in routine monitoring requires further levels of validation. In this context, the results of the present study should not be interpreted as complete ecological validation of the PCR assay. Their methodological significance lies elsewhere: the selected oligonucleotides were shown to provide specific detection of target DNA templates and to produce no detectable cross-signal within the negative panel used. This level of evidence corresponds to primary laboratory testing and is necessary before moving to environmental, technological or clinical matrices,

where sample composition, PCR inhibitors and accompanying DNA may substantially affect the result. The selection of the ITS1-5.8S-ITS2 region as the main molecular target is justified by the balance between conservation and variability within the ribosomal cluster. In environmental studies of fungal diversity, R. Winand *et al*. (2025) showed that ITS1 and ITS2 may produce different results depending on the reference database, classification algorithm and composition of the mock community. For the interpretation of the present study, this means that the selection of the ITS region cannot be automatic; it requires prior sequence comparison and assessment of discriminatory regions. In addition, H. Orsud *et al*. (2025) compared 18S, ITS1 and ITS2 as markers for fungal profiling and demonstrated that different ribosomal regions have unequal taxonomic resolution. Therefore, inclusion of ITS1/ITS2, 18S rRNA and 28S rDNA in the bioinformatic stage made it possible to assess not only the main species-specific target, but also auxiliary regions for potential genus-level screening.

It should be emphasised that, in the present study ITS1-5.8S-ITS2 was used not as a universal marker for describing the entire mycobiota, but as a basis for targeted species-level identification of predefined *Candida* taxa. This fundamentally distinguishes the proposed approach from metabarcoding, where the outcome is a broad taxonomic profile of the fungal community. For targeted PCR, the key requirement is not maximum coverage of all fungi, but the generation of a specific signal only in the presence of target DNA. In plant samples, this requirement is particularly important, as C. Viotti *et al*. (2024) showed that primer pairs, PCR conditions and additional approaches to blocking non-target sequences can alter the profile of detected fungal diversity. Thus, the results of the present study provide a basis for further testing of the system on plant matrices, but do not replace that stage. The methodological appropriateness of using TaqMan probes is supported by current practice in the development of species-specific PCR assays for *Candida*. A. Lima *et al*. (2019) described a TaqMan-based real-time PCR approach for identifying *Candida auris* using ribosomal DNA as the target region. For the present study, the principle of dual specificity is particularly relevant: amplification is determined by the primer pair, while detection is determined by hybridisation of the probe with the internal region of the amplicon. This is consistent with the selected design, in which the probes were positioned in discriminatory fragments of ITS1-5.8S-ITS2. At the same time, even for a single target species, the characteristics of different PCR assays may differ substantially. J.B. Buil *et al*. (2025) compared several real-time PCR assays and considered analytical sensitivity, specificity and the limit of detection as separate assessment parameters. Therefore, the interpretation of the results in the present study is appropriately limited to the level of primary testing: functionality and the absence of detectable cross-reactivity within the control panel were confirmed, but full analytical validation was not claimed. Compared with approaches based on melting-curve analysis, the TaqMan format has an additional advantage in multiplex detection. E.R. Tavares *et al*. (2025) showed that melting-curve-based real-time PCR may be suitable for differentiating selected *Candida* species; however, such identification depends on the thermal characteristics of the amplicons. In the system presented here, differentiation of the target taxa is ensured not only by amplification parameters, but also by separate fluorescent channels for the corresponding probes. Interpretation of a positive signal in DNA-based PCR should be limited to the detection of the target nucleic acid. B.L. Freitas *et al*. (2022) justified the use of RT-qPCR for rapid detection of viable *C. auris* cells in environmental samples, demonstrating the difference between DNA- and RNA-based approaches. In the present study, a DNA template was used; therefore, the results confirm the presence of target DNA and the functionality of the primer–probe system, but do not allow conclusions about cell viability or the metabolic activity of *Candida* spp. in a biological environment.

The potential application of the developed system to aquatic microbiota is methodologically justified, but requires further experimental confirmation. P. Pham *et al*. (2024) considered fungal communities in agricultural aquatic systems as a dynamic component shaped by ecological and anthropogenic factors. This highlights the value of targeted methods for rapid screening of selected fungal taxa in aquatic environments. At the same time, experience with molecular monitoring of

Candida in wastewater demonstrates the connection between environmental screening and epidemiological and clinical contexts. C. Barber *et al*. (2023) described community-level wastewater surveillance for *C. auris*, in which control of sample preparation, test specificity and result reproducibility were key conditions for interpretation. K.M. Babler *et al*. (2023) also showed the possibility of detecting *C. auris* in hospital and municipal wastewater, confirming the suitability of aquatic environments for molecular detection of yeast DNA. For the present study, these data are not direct evidence that the proposed system is ready for aquatic monitoring, but rather provide a rationale for the next stage of matrix-specific testing. Further scaling of such approaches requires standardisation. A. Rossi *et al*. (2023) linked the detection of *C. auris* in wastewater with an epidemiological context, providing an example of the transition from laboratory detection to applied monitoring. A. Zulli *et al*. (2024) investigated *C. auris* nucleic acids in wastewater solids across a broad set of treatment facilities, which highlights the importance of standardised protocols, replicates and spatial coverage. For the PCR assay presented here, this means that, after primary testing, it will be necessary to determine the limit of detection, assess inter-run reproducibility, test different DNA extraction methods and examine samples with varying levels of inhibition. For plant microbiota and agrobiological systems, the key issue is not only the presence of target DNA, but also the accurate differentiation of *Candida* spp. from the broad background of endophytic, epiphytic, soil-associated and technologically associated microorganisms. A.E. Fadiji *et al*. (2025) emphasised that plant microbiome studies require standardised sampling protocols, metadata control, reproducible extraction methods and verification of results under different soil and climatic conditions. This is fully consistent with the limitations of the present study: real plant, soil and water matrices were not examined, and should therefore be regarded as the next stage rather than as an already confirmed field of application. The ecological presence of representatives of the genus *Candida* requires cautious interpretation. A.D. Valério *et al*. (2025) summarised data on the detection of *Candida albicans* outside its usual biotopes, including in water, soil and plant material, but also emphasised that the ecology of this species in natural environments remains insufficiently understood. In the broader agro-industrial context, species-level identification of yeast-like fungi may form part of a biosafety system.

M.C. Fisher *et al*. (2024) considered the problem of fungal antimicrobial resistance within a One Health framework, in which natural, agricultural, hospital, technological and anthropogenically altered environments are interconnected. J.C. Allison & E.M. Hyland (2025) additionally drew attention to the role of agricultural use of azole compounds in creating ecological conditions for the selection of resistant fungal variants. Resistance was not assessed in the present study; however, the proposed species-specific PCR assay may be used as a first-level screening tool in broader monitoring programmes, to which resistance testing, sequencing or population analysis may subsequently be added. The bioinformatic component of this study also has independent methodological value. F.J. Ambrosio III *et al*. (2023) emphasised the role of standardised workflows for fungal genomic characterisation, since the reproducibility and transparency of computational procedures determine the quality of subsequent laboratory or genomic interpretation. In the present study, the bioinformatic stage performed a similar function at the level of primer-probe design: it enabled preliminary assessment of specificity, compatibility and the risk of non-specific oligonucleotide binding. Thus, the laboratory testing was not an isolated procedure, but was based on a previously established computational framework. Overall, the results confirm that the developed PCR assay has the status of a primarily tested methodological tool for species-level identification of selected representatives of the genus *Candida*. Its strengths include the combination of *in silico* target selection, the use of TaqMan probes, a multiplex format, an internal amplification control, and testing on positive and negative control panels. At the same time, the limits of the study remain clearly defined: the limit of detection was not determined, calibration curves were not constructed, amplification efficiency was not calculated quantitatively, comparison with sequencing was not performed, and real soil, water or plant samples were not tested.

CONCLUSIONS

The study confirmed the appropriateness of developing a PCR assay for species-level identification of *Candida* spp. as a methodological tool for analysing complex biological samples, controlling potential sources of fungal contamination and further adapting the system to biosafety tasks. The transition from general genus-level detection to species-level differentiation is important for interpreting results in complex microbial consortia, where yeast-like fungi may be components of the microbiota, symbionts, accompanying colonisers, contaminants or potentially clinically significant pathogens. Under such conditions, genus-level detection does not always provide sufficient information about the structure of the fungal component, whereas a species-specific approach allows the biological significance of the detected signal to be assessed more accurately. Based on the *in silico* analysis, primers and TaqMan probes were selected for the detection of *Candida glabrata*, *Candida parapsilosis* and *Candida auris*. The selected oligonucleotides had compatible physicochemical parameters, similar melting temperatures and suitability for use within a unified temperature profile for multiplex real-time PCR. The choice of the ITS1-5.8S-ITS2 region of the ribosomal operon as the main molecular target was justified by the combination of conserved flanking regions and variable internal fragments suitable for species-level discrimination. The developed and optimised multiplex PCR approach demonstrated analytical specificity, satisfactory amplification performance and suitability for detecting fungal DNA in control samples and archived isolates of biological origin. The absence of amplification in the negative control panel indicated no detectable cross-reactivity with the non-target microorganisms examined. The use of TaqMan probes, an internal amplification control and optimised reaction conditions improves the reproducibility of result interpretation in complex biological matrices. Thus, the proposed approach is suitable for monitoring biological systems and may be integrated into sanitary and biological screening of complex matrices, biosafety control in the agroindustrial sector, assessment of microbial contamination in technological environments, and further adaptation to the early molecular detection of fungal pathogens in clinically significant materials. At the same time, a limitation of the study is the use of control DNA samples, synthetic controls and archived isolates of biological origin without extensive testing on real multi-component environmental matrices. Future research should focus on increasing the number of species-specific targets, testing the system on samples of soil, aquatic and plant microbiota, assessing its reproducibility under different laboratory conditions, and comparing PCR identification results with sequencing data and, where appropriate, with clinical diagnostic algorithms for invasive mycoses.

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Біоінформатичний дизайн та пробна апробація ПЛР-системи для видової ідентифікації *Candida* spp. у біологічних зразках

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Аспірант

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Анотація. Видова ідентифікація представників роду *Candida* у складних біологічних зразках природного, агробіологічного та технологічного походження є необхідною для коректного аналізу змішаних мікробних угруповань і контролю мікробної контамінації та оцінювання потенційних джерел поширення грибкових патогенів. Метою дослідження було біоінформатичне обґрунтування, конструювання та пробна лабораторна верифікація системи полімеразної ланцюгової реакції для видової диференціації *Candida glabrata*, *Candida parapsilosis* і *Candida auris*. Дослідження передбачало аналіз рибосомного кластера, добір референтних послідовностей, комп'ютерне конструювання праймерів і гідролізних зондів, перевірку їх специфічності, оптимізацію реакційної суміші та тестування на контрольних зразках ДНК й архівних ізолятах біологічного походження. Як основну мішень було визначено ділянку внутрішніх транскрибованих спейсерів рибосомного кластера, що поєднувала консервативні фланкувальні фрагменти та варіабельні внутрішні послідовності. Для *Candida glabrata*, *Candida parapsilosis* і *Candida auris* було сформовано праймерно-зондові набори з розмірами ампліконів 82, 91 і 118 пар основ відповідно. Ампліфікаційну реакцію проводили в кінцевому об'ємі 25 мкл. Кінцева концентрація кожного праймера становила 0,28 мкМ, гідролізного зонда – 0,2 мкМ, $MgCl_2$ – 3 мМ, дНТФ – 0,3 мМ, бетаїну – 1,5 М. Позитивна ампліфікація була отримана для 40 позитивних контрольних зразків, серед яких було 29 зразків *Candida* spp., 7 зразків *Candida glabrata* і 4 зразки *Candida parapsilosis*. У негативній контрольній панелі, що включала 43 зразки нецільових мікроорганізмів, перехресної реактивності не виявлено. Запропонована система підтвердила придатність для первинної якісно-аналітичної видової ідентифікації окремих представників роду *Candida* у контрольованих лабораторних умовах. Розроблений підхід може бути використаний як основа для подальшого санітарно-біологічного скринінгу складних матриць ґрунтової, водної та рослинної мікробіоти, контролю біологічної безпеки в агропромисловості та подальшої адаптації до задач ранньої молекулярної детекції грибкових патогенів, важливих для інвазивних інфекцій

Ключові слова: рибосомний кластер; дріжджоподібні гриби; зонди; скринінг; мікробна контамінація; мікроорганізми