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Rapid discrimination of several fungus species with FTIR spectroscopy and statistical analysis

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Abstract. The classification of fungal species that was based on statistical comparison of their infrared spectra is presented. The results obtained in the present study were compared with the classification hierarchy of Kingdom Fungi, according to contemporary phylogenetic studies. IR bands originating from the respective groups of chemicals provided a "spectroscopic fingerprint" of the fungal species analyzed. Statistical analysis of the spectra was made using a Hierarchical Cluster Analysis (HCA). The proper wavelength ranges in IR spectrum, crucial for species classification, were selected. The relations between species were evident in the dendrogram diagrams and they followed the systematic classification of the selected fungal species.
Keywords: fungi, infrared spectroscopy, HCA

1. Introduction

The infrared spectra of fungi show bands, which are specific to certain functional groups of biological origin. According to numerous reports [1-10], the three main regions can be distinguished:

— fatty acids (3050-2800 cm^{-1}),
— amide I and II (1700-1500 cm^{-1}),
— polysaccharides (1200-900 cm^{-1}).

All the regions listed above were observed in the spectra of the fungi examined in this study. The infrared spectra of fungi were shown in Figure 1.

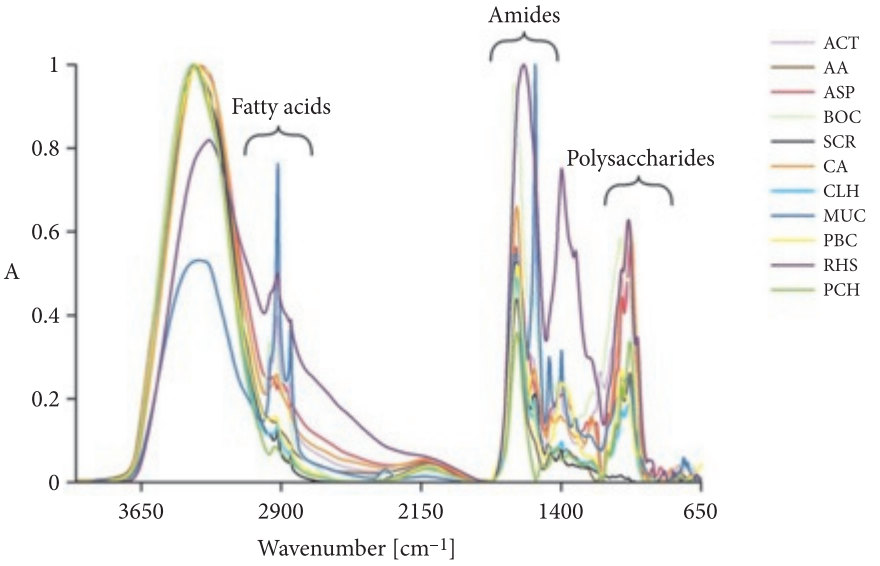

Fig. 1. IR spectra of different fungal strains

The spectral areas that corresponded to functional groups contributed important information about chemical composition of the fungal cells. However, information brought only by the analysis of these areas is being proved as insufficient for distinguishing individual species. Some parts of the IR spectrum, important for species identification, do not always belong to regions mentioned above. Presence of absorption bands in other regions is specific to the particular species of fungi. The analysis of the spectra in the ranges: 900-700 cm^{-1}, 1500-1200 cm^{-1}, 2800-1700 cm^{-1}, and 4000-3050 cm^{-1} gave the ability to determine similarities and differences between particular species of the fungi studied. As reported in the literature, some ranges have been recognized as a specific and justified for identification of microorganisms, e.g. a range of 700-900 cm^{-1} is referred to as a "fingerprint" region [1, 7, 11-13].

2. Material and Methodology

The fungal species analyzed in this study are presented in Table 1.

Table 1

Group	Species	Origin	Abbrev.
Fungi	*Penicillium chrysogeum*	ATCC 9179	PCH
	Alternaria alternata	ATCC 6663	AA
	Penicillium brevicompactum	ATCC 9056	PBC
	Mucor mucedo	ATTC 18356	MUC
	Acremonium strictum	ATCC 10141	ACT
	Botrytis cinerea	ATCC 26806	BOC
	Saccharomyces cerevisiae	ATCC 204983	SCR
	Rhizopus stolonifer	ATCC 14038	RHS
	Cladosporium herbarum	ATCC 28987	CLH
	Aspergillus flavus var. flavus	ATCC 16883	ASP
	Candida albicans	ATCC 18804	CA

The fungal species were grown as follows: *M. mucedo*, *A. strictum*, *B. cinera*, *R. stolonifer*, *C. herbarum* on a Potato Dextrose Agar (PDA) for: *P. chrysogeum*, *A. alternata*, *P. brevicompactum*, *A. flavus* var. *flavus* on a MEA solid medium, for *S. cerevisiae* — on a YEPD solid medium, *C. albicans* — a YPD solid medium according to ATCC recommendations [www.lgcstandards-atcc.org/TechnicalSupport]. The materials collected from the media were suspended in the sterile saline solution before measurement.

Spectroscopic studies were performed with a FTIR spectrometer GX Optica Perkin-Elmer. The samples were measured using the reflectance technique – HATR (Horizontal ATR) at IR range corresponding to 4000-650 cm^{-1}, with 4 cm^{-1} resolution. Samples were spread on the ZnSe crystal as a thin layer, and placed in the horizontal position in the spectrometer. The results of each sample were averaged from 150 scans in order to minimize noise and to improve separation of bands.

IR spectra of fungi were analyzed with Statistica software (StatSoft). Discrimination between species in a hierarchical tree form was made with multi-dimensional HCA using Ward's algorithm. HCA results were compared to PCA (Principal Component Analysis) results.

3. Results and discussion

In the presented study, 11 different species of fungi were examined, some of them belonged to closely related taxa. Therefore, we expected that these fungal species might show similarities in certain areas of their infrared spectra. The statistical analysis (HCA) was performed for four selected areas: i) the area I — three functional groups (fatty acids 3050-2880 cm^{-1}, amides 1700-1500 cm^{-1}, polysaccharides 1200-900 cm^{-1}); ii) the area II — non-specific ranges (900-700 cm^{-1}, 1500-1200 cm^{-1}, 2800-1700 cm^{-1});

iii) the area III — single continuous band (4000-3050 cm^{-1}); iv) the area IV — entire measurement range (4000-650 cm^{-1}).

The classification trees obtained for each area were compared and analyzed in order to find a region best suited for distinguishing between different species of fungi.

The specific dendrograms for areas I, III, and IV were shown in Figures 2 to 4.

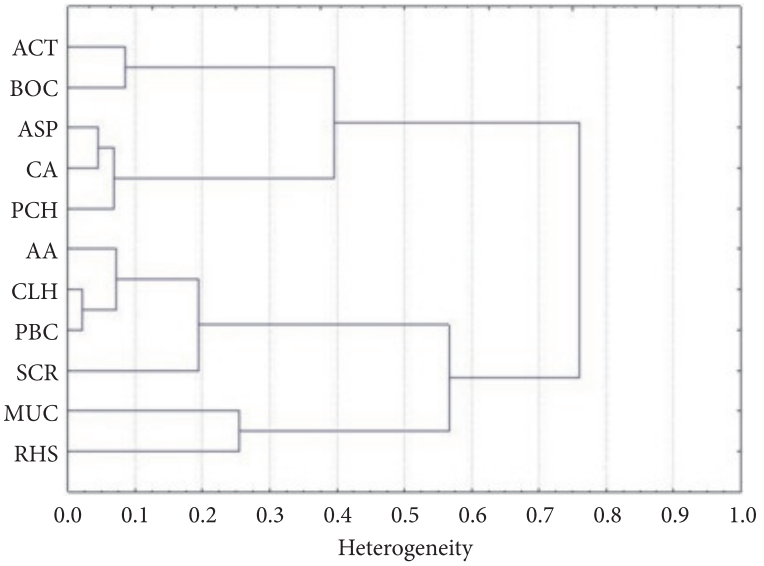

Fig. 2. The HCA tree based on the analysis of the area I

Absorption bands in this range (related to the area I) corresponded to chemical groups presented in living fungi. Each sample showed absorption in all three bands. No significant differences between species were found. The subtle differences between spectra turned out to be negligible in the statistical analysis. Thus, for better species discrimination the analysis of bands outside this area seemed necessary to be performed.

The IR spectra in the area II showed great diversity for each species, and it was observed not only in the "fingerprint" band (900-700 cm^{-1}), but also in the remaining two bands. However, it should be noted that absorption intensity in the "fingerprint" region was small for most of microorganisms. For the best classification results, all three bands should be included in analysis.

In the area III, an intense, wide band originating from an OH group was observed at a wavenumber of about 3300 cm^{-1}. This band was present in spectra of all the species examined. This band could originate from water molecules presented in the cytoplasm or from extracellular water, e.g. from the moisture in the air. Therefore, the area III

(4000-3050 cm^{-1}) is not suitable for identification of fungi since it may cause some confusion in interpretation of the results. The presence of the dominant water band made all fungi nearly identical in this spectral range. In addition, the statistical analysis revealed that this area added nothing substantial in the differentiation of the biological material (Fig. 3). As one can observe, the distance between species of fungi was close to zero.

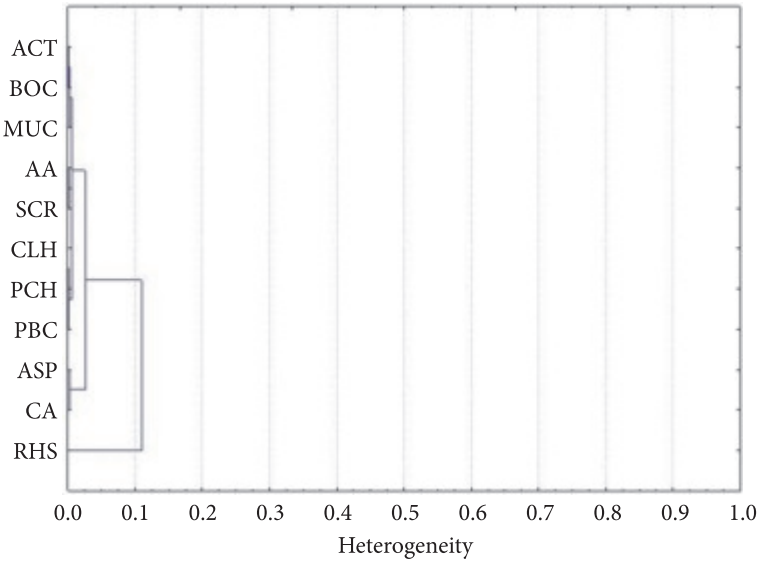

Fig. 3. The HCA tree based on the analysis of the area III (4000-3050 cm^{-1})

The results of analysis of the area IV were showed in Figure 4. There was no clear distinction between species probably as a result of the strong OH absorption band dominating in IR spectrum. Figure 5 showed the result of analysis after excluding the OH region (4000-3050 cm^{-1}). After this operation, the separate clusters of analyzed species of the fungi could be observed.

In our opinion, IR absorption spectrum in 3050-650 cm^{-1} range is best suited for the purposes of species classification, and includes: i) the area of functional groups, which is typical for biological materials and has similar characteristics for most species of fungi; ii) the area lying outside the scope of the functional groups showing high diversity, expanding the possibility of discrimination between the different species; iii) the exclusion of the 4000-3050 cm^{-1}range.

The grouping of the fungal species in the dendrogram (presented in Figure 5) was consistent with contemporary systematic classification included in the "Catalogue of Life: 2009 Annual Checklist" [14]. This classification is based on the information of phylogeny and consanguinity of fungi, as well as morphological, anatomical,

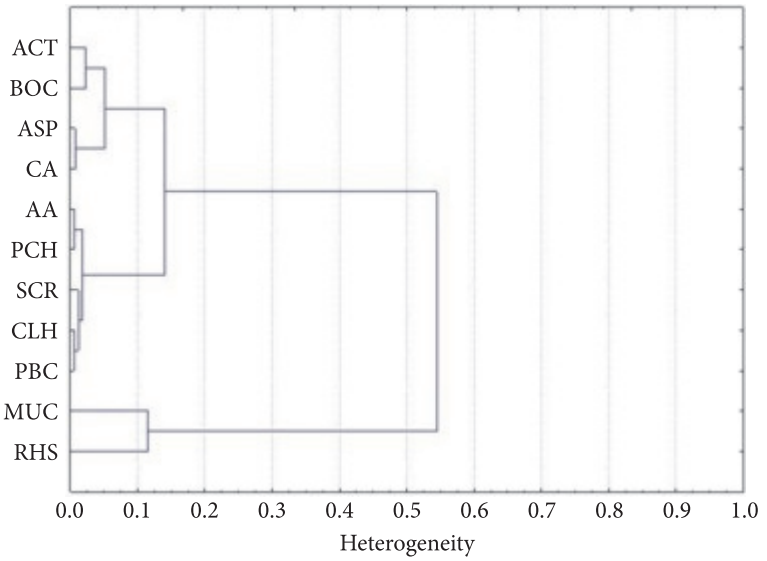

Fig. 4. The HCA tree based on the analysis of the area II (4000-650 cm^{-1})

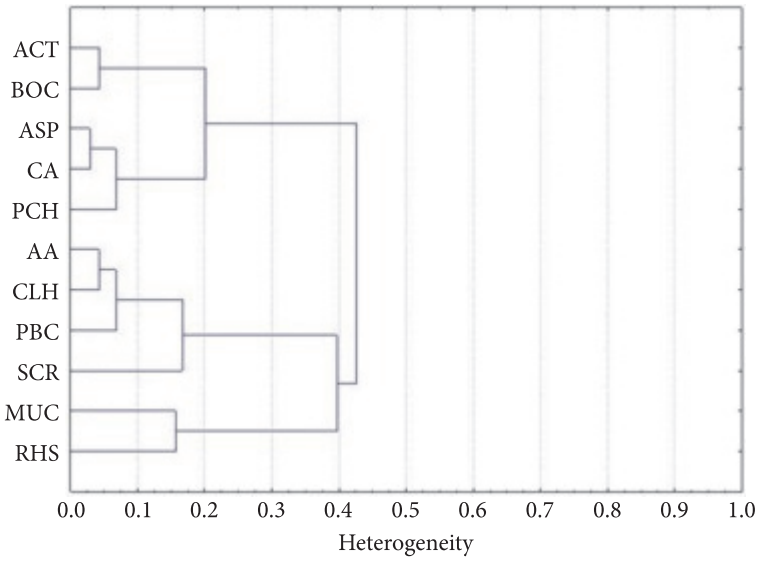

Fig. 5. HCA tree based on the 3050-650 cm^{-1} range

and biochemical data. In terms of systematic classification, fungal species are divided into eight groups and two kinds not belonging to any of these groups (*incertae sedis*). Within a framework of every group, other taxonomy units can be distinguished: class, order, family, kind, and species. Systematic classification dictates some sort

of hierarchical grouping of the objects showing similarities. IR spectra of the fungi submitted into HCA analysis in this study showed grouping of the matter with similar features, pictured on HCA tree as separate clusters [15].

The species of fungi in this study belonged systematically to two out of eight groups: *Ascomycota* and *Zygomycota* [14]. With regard to this level of classification, one would notice a separable cluster in the HCA dendrogram (in Figure 5), which grouped the species MUC and RHS. Both species belong to the group of *Zygomycota*, and in the systematic hierarchy were classified to the same order of *Mucorales* and the common family *Mucoraceae* [14]. Based on their similar characteristics in IR spectrum and statistical analysis, both species could be identified and distinguished form other groups of fungi.

Other species of fungi represent the group of *Ascomycota* but in further classification they belong to different classes and orders. In the dendrogram presented in Figure 5, two more clusters can be observed: (1) ACT and BOC, (2) ASP and CA.

The first cluster is represented by ACT belonging to *Sordariomycetes* class and BOC belonging to the *Leotiomycetes* class [14]. Those species, although belonging to different classes, showed biochemical similarities in the IR absorbance bands. According to the literature, *Sordariomycetes and Leotiomycetes* are the most morphologically similar species pairs within *Ascomycota* group [16]. This could explain the presence of these two fungal species in a single cluster on the dendogram.

The second cluster grouped species belong to *Saccharomycetes* (CA) and *Eurotiomycetes* (ASP) classes [14]. Both species were similar in anatomic and morphological constitution and they cause allergic reactions in humans. In the cells of both fungal species, similar antigenic components are observed, which react most frequently with immunoglobulin E (IgE) antibodies of allergic patients [17, 18].

SCR, belonging to the *Sacchcaromycetes* class, did not share the cluster with CA. SCRs biochemical characteristic of cells structure is well known in literature because of being one of the most thoroughly researched eukaryotic microorganisms. It is extremely important as a model organism in modern cell biology research. Many proteins building eukaryotic cells were first discovered by studying their homologues in SCR [19, 20, 21]. The differences between SCR and CA, that have been visible on HCA dendrogram, may be caused by dissimilar set of proteins in cells structure. The results obtained in the present study are suitable to phylogenetic tree representing the evolutionary relationships between sequenced Candida and Saccharomyces species adapted from Marcet-Houben & Gabaldón, 2009. The tree is based on the maximum likelihood analysis of concatenated alignments of 1137 protein families.

AA and CLH formed a single cluster and they both belong to the *Dothideomycetes* class. Additionally, both species possess antigens in cells structures which react with the patient serum causing the same type of allergic reactions. Similar antigenic components are observed in PBC from *Eurotiomycetes* class. As reported in

the literature, AA, CLH, and PBC species are also similar in their metabolites production [14]. They all lied close to each other in the HCA results of this study.

PBC, PCH, and ASP were classified to different clusters in the HCA dendogram, although they belong to the same class (*Eurotiomycetes*), and even the lower taxonomy units (order *Eurotiales*, family *Trichocomaceae*) [14]. Based on the literature data, *Eurotiomycetes* class was found to be most diverse out of any other classes of fungi. This class contains organisms considerably different morphologically and biochemically such as human pathogens and molds. The way they affect human organism depends on the kind of antigens present in fungal cells [22, 23].

In Figure 6, the results of Principal Components Analysis performed on 3050-650 cm^{-1} range in this study were presented.

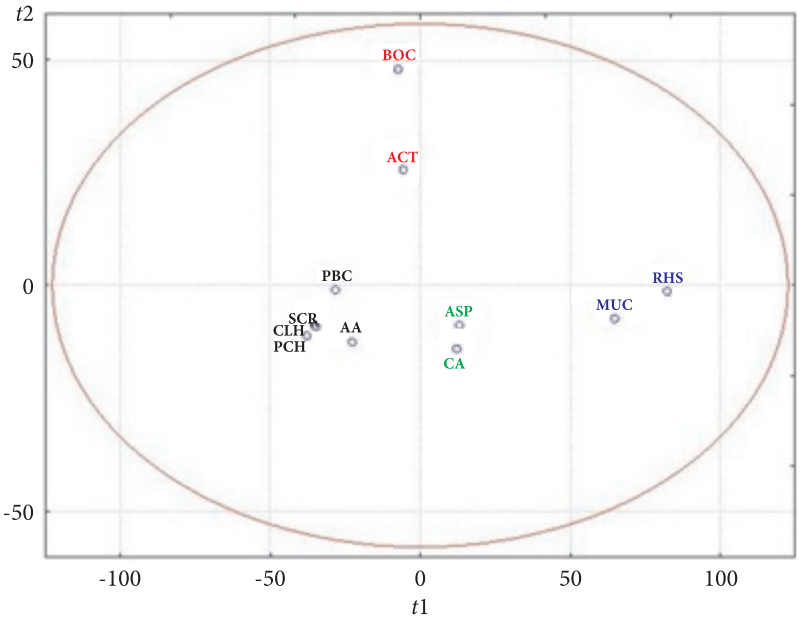

Fig. 6. The map of the PCA results

The PCA map confirmed our earlier observations made with the HCA analysis. Among the species examined, the four main clusters can be distinguished, allowing separation of the fungal species.

4. Summary

The statistical HCA classification, in the selected ranges of the IR spectrum was consistent with a systematic classification of several fungal species. The usefulness

of the discussed spectral range for differentiation of similar species of fungi was also confirmed by the results of PCA analysis. The results of both statistical analysis confirm the possibility of classification of fungi belonging to different species on the basis of spectral range of 3050-650 cm^{-1}.

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Szybkie rozróżnianie niektórych gatunków grzybów przy pomocy spektroskopii FTIR I analizy statystycznej

Streszczenie. W pracy zaprezentowano próbę klasyfikacji różnych gatunków grzybów w oparciu o statystyczną analizę widm podczerwieni badanych materiałów. Autorzy pracy dokonali porównania uzyskanych wyników z klasyfikacją systematyczną królestwa grzybów według współczesnego systemu klasyfikacji. Spodziewano się, że systematyczne podobieństwa w fizjologii i naturze biologicznej przedstawicieli królestwa grzybów, a także ich wspólne pochodzenie powinny przekładać się na pewne podobieństwa w widmie podczerwieni. Obecność odpowiednich pasm w widmie IR pochodzących od odpowiednich ugrupowań chemicznych jest cechą osobniczą i charakteryzuje biochemiczną strukturę poszczególnych gatunków grzybów. Analizy statystycznej widm grzybów pod kątem ich wzajemnych podobieństw i możliwości różnicowania dokonano przy użyciu metody HCA (*Hierarchical Component Analysis*). Dla potrzeb klasyfikacji za pomocą analizy HCA przeanalizowano i dokonano selekcji odpowiednich zakresów w widmie IR — region „odcisku palca", który wnosi ważne informacje dla celów identyfikacji. Uzyskany w toku analizy dendrogram HCA badanych gatunków grzybów w znacznym stopniu pokrywa się z ich klasyfikacją systematyczną.

Słowa kluczowe: grzyby, spektroskopia w podczerwieni, HCA