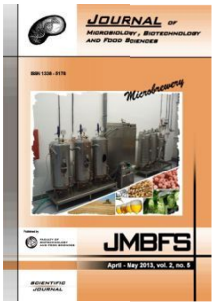

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SPECTROSCOPIC ANALYSIS OF FIVE PHYLOGENETICALLY DISTANT FUNGI (DIVISION: ASCOMYCETE) FROM VELLAR ESTUARY, SOUTHEAST COAST OF INDIA – A PILOT STUDY

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ABSTRACT

Fungal taxonomy is dynamically driven towards controversial discipline that consequently requires changes in nomenclature. Scarcity of microbiological expertise particularly for marine fungi is another major setback for these taxonomical differences. Here, five different species pharmacologically important marine fungi under Division Ascomycete were studied for their spectral variation. This work verified the practical applicability of FT-IR microspectroscopy technique for early and rapid identification of these species based on the spectral data showed striking difference with their major biomolecules such as lipids, proteins and nucleic acids produced by them. Spectra of all the species showed striking differences while individual peaks of each spectrum are parallel to each other in their respective spectral regions. Aspergillus oryzae have intense peaks in the lipid and nucleic acid spectral region and moderate bands in the amide spectrum. Phoma herbarum and Trichoderma piluliferum showed intense peaks in the protein spectral region but moderate peaks in the lipid and nucleic acid regions. Hypocrea lixii and Meyerozyma guilliermandii have less intense peaks in all the five spectral regions. This unique spectral representation is concordant with the cluster analysis dendrogram by minimum variance statistical method where low spectroscopic distance was found between H. lixii and M. guilliermondii whereas a higher spectroscopic distance was found between P. herbarum and T. piluliferum. FTIR spectroscopy delivers a combined advantage for efficient fungal classification as well as simultaneous visualization of chemical composition of samples as evident from this study.

Keywords: FTIR spectroscopy, fungal taxonomy, cluster analysis, Ascomycetes, marine fungi

INTRODUCTION

Fungal identification is achieved mainly based on their morphology, biochemical and nutritional criterion. This classification system consequently requires changes in nomenclature because of novel and controversial dynamic evidences. This dynamic controversy is evident for the large ecological group such as marine fungi, which has never been in this odds but their evolutionary existence in marine environment by origin or secondary adaptation is always under heated discussion. The conventional identification methods are laborious, time-consuming, and somewhat variable and provide insufficient taxonomic resolution. In contrast, molecular methods are universally applicable and the data are adding a new dimension to the understanding of the relationships among the different groups of microbes. One of the best phylogenetic trees to depict the evolutionary history of ascomycetes was published by **Berbee and Taylor (1994),** based on the morphological convergence. There has been no recent standardized conclusion **(Kohlmeyer and Volkmann- Kohlmeyer, 1991; Hyde *et al.*, 2000)** for the identification of marine fungi except the book of **Kohlmeyer and Kohlmeyer (1979).**

The internal transcribed spacer (ITS) region of nuclear DNA is the preferred DNA barcoding marker for the identification of fungi (**Vilgalys and Gonzalez, 1990**). Ribosomal genes (16S, 18S rRNA) also have been used to study the phylogenetic relationships of fungi **(Gardes and Bruns, 1993;Vineusa *et al.*, 2001)**. More than 100 000 fungal ITS sequences are generated and deposited in the International Nucleotide Sequence Databases and/or other databases **(Nilsson *et al.*, 2009)** which provides a large reference material for the identification of fungal taxa. However, these data are to some extent hampered by misidentifications or technical errors such as mixing of DNA templates or sequencing errors **(Nilsson *et al.*, 2006)**.

Secondary metabolites are mixture of closely related molecules which is neither essential for growth nor key intermediates of the organism's basic metabolism. They are considered as steroids, terpenes, alkaloids, cyclopeptides, and coumarins and some of these are mycotoxins with a peculiar and rare chemical structure (**Frisvad and Filtenborg, 1983**). The pattern of secondary-metabolite production has been used as identification key in ascomycete systematics because these organisms produce a vast array of such compounds **(Carlile and Watkinson, 1994)** which are used in chemotaxonomic studies **(Frisvad and Filtenborg, 1990; Frisvad, 1994; Whalley and Edwards, 1995)**. But it is least used identification key in fungal kingdom when compared to lichens (**Whalley and Edwards, 1995**). However, production of these compounds is controlled by the environmental conditions and hence the detection procedure consequently have some difficulties.

The identification method based on optical spectroscopic techniques would be the far-reaching, effective and confirmative approach in case of fungal taxonomy. Fourier transform infrared (FT-IR) and Raman spectra constitute a rapid, inexpensive and highly specific spectroscopic fingerprint of microorganisms by which they can be identified **(Dukor, 2001)**. Fourier transform infrared spectroscopy works based on the vibrational excitation of molecular bonds by absorption of infrared light energy. The sum vibrational spectra for a cell´s macromolecule content (nucleic acids, proteins, lipids, polysaccharides, etc.) can be thought of as a spectral "fingerprint" for that organism. These spectra can be used diagnostically in typing or identification of various microorganisms. The possible value of FT-IR spectroscope have successfully been carriedout to identify and characterize a number of bacteria and yeast at strain level **(Naumann *et al.*, 1991; Holt *et al.*, 1995; Timmins *et al.*, 1998; Lefier *et al.*, 2000; Schmalreck and Hotzel, 2000; Gomez and Montero, 2001; Irudayaraj *et al.*, 2002; Guibet *et al.*, 2003; Al-Qadiri., 2010; Lamprell *et al.*, 2006).** However, studies on the discrimination of marine fungi by FT-IR spectrum are very limited **(Adilson *et al.*, 1998; Erukhimovitch *et al.*, 2005; Fischer *et al.*, 2006).**

In the present study, pharmacologically potent five marine fungal species such as *Aspergillus oryzae, Phoma herbarum, Trichoderma piluliferum, Hypocrea lixii* and *Meyerozyma guilliermondii* were used for their FT-IR spectral variation. The secondary metabolites and their pharmacological activities of these species were well studied and reported (**Joel and Bhimba, 2010; 2012; Bhimba *et al.*, 2011a, b; 2012a, b**).

MATERIAL AND METHODS

Collection of isolates

The mangrove associated and pharmacologically potent marine fungi, A. oryzae, P. herbarum , T. piluliferum, H. lixii and M. guilliermondii were got from Department of Biotechnology, Sathyabama University, Chennai by request. All the five species were previously identified by ITS gene sequence and deposited in the genbank (HQ823764, JQ754707, GU815342, GU815341, and JF730118).

Sample preparation

Fungal samples for FT-IR had been taken from individual pure culture plates and subcultured into fresh mycological broth (Hi-Media, India) medium, incubated at 25 ±1 °C for 72 h. Mycelia were then separated and washed with saline for three times. About 2.5 mg of each sample was grounded into fine particles and mixed with 100 mg of potassium bromide (KBr) in a ball blender and dried for 2 hrs in microfuge tubes. KBr pellets were prepared by establishing pressure of 10kg/cm^2 for about 30sec and a pure KBr was used as blank.

FT-IR spectroscopy analysis

Infrared spectra of the samples were registered with a Perkin–Elmer Spectrum BX (Waltham, MA, USA) spectrometer with a resolution of 4 cm^{-1}, in the classic MIR (middle infrared) range of 4000 to 400 cm^{-1} (50 scans) which was expected to contain unique molecular fingerprint vibrational bands occurring at the wavelengths of bio-molecular functional groups. Freeze-dried biopolymers with no microbial contamination were used as reference material.

Statistical analysis

The spectra of the five fungal species were analyzed by minimum variance method **(Ward, 1963)** for the cluster analysis on different regions of the spectra. A total of 5 spectra in all the five species were used for cluster analysis.

RESULTS AND DISCUSSION

Each fungal sample was repeated for 5 times at different culture days and obtained the consistent spectra in all the species. Results of the molecular vibration spectral quantification difference of the five fungal species are given in Table 1. The spectra showed striking difference with their major biomolecules such as lipids, proteins and nucleic acids as shown in figures 1 and 2. The clear spectral differences were observed in *M. guilliermondii* (purple lines) when compared with other species. *Aspergillus oryzae* has intense bands in the lipids region (2800–3020 cm^{-1}) and also a large band with centroid at 2900 cm^{-1} which also arises from lipids absorption**.** There was a considerable spectral difference in the region 3200-3600 cm^{-1} (amide A) among the species. Particularly, *H. lixii* and *M. guilliermondii* showed high spectral difference. All the remaining species showed slight difference in that region.

Several studies have used this FTIR technique for phenotyping of microbial cultures based on their metabolic fingerprints **(Winder *et al.*, 2004; 2006).** FTIR spectrum has been analysed by multivariate statistical methods for the identification of microbes at sub-species level **(Naumann *et al*., 1991; Timmins *et al*., 1998; Maquelin *et al*., 2003)**. Each species showed specific spectroscopic fingerprint pattern and it simply reflects the phenotypic difference among the species.

In order to differentiate the five species, cluster analysis with Ward's algorithm was used. Figure 3 shows the clustering pattern of five species made by spectral observations with 4000 - 400 cm^{-1} wavenumbers region. To make an accurate analysis in this wave numbers region, all spectra should be baseline corrected and normalized, and then they were bisected in the desired region, background subtracted and the spectra were offset corrected. Thus it was insure that all the changes were contributed due to the inherent samples differences. Based on the dendrogram, low spectroscopic distance was found between *H. lixii* and *M. guilliermondii*. This might be the reason of long term phenotypic conservation has maintained by these organisms. Where as a higher spectroscopic distance was found between *P. herbarum* and *T. piluliferum.*

All the species showed striking differences in the major spectral regions like nucleic acids, lipids and proteins as depicted in Table 1. The quantitative difference in the peak area of the five species were simply reflects in the cluster analysis. *Phoma herbarum* and *T. piluliferum* were showed considerable variation in amide I and amide II regions. Whereas the *H. lixii* and *M. guilliermondii* showed high variation in the N-H stretching of proteins. The similar spectral differences were found in the previous spectroscopy studies on fungal phytopathogens (**Salman *et al*., 2010).** In that study, they found a clear spectral difference at specific regions such as lipids which was used to differentiate *Rhizoctonia* from *Colletotrichum* and *Verticillium* and carbohydrates region was used for differentiate the *Colletotrichum* and *Verticillium*. By the FTIR spectral analysis, the *P. herbarum* and *T. piluliferum* were clearly differentiated from the other species.

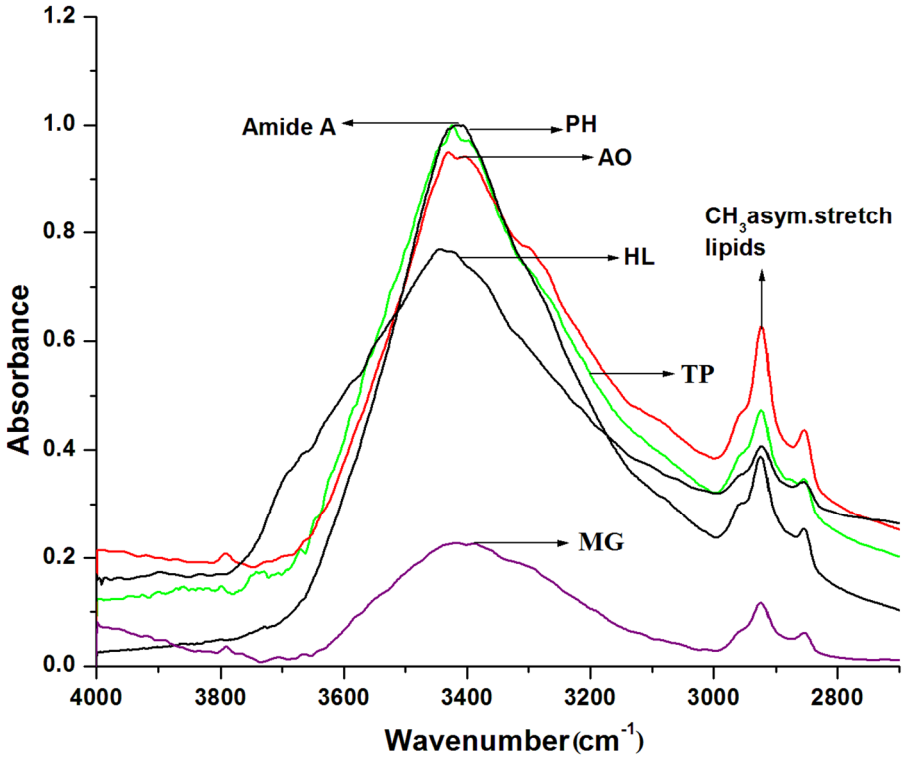

Figure 1 Mid infrared spectra in the region 4000 – 2800 cm− 1 of five fungal species: AO: Aspergillus oryzae; HL: Hypocrea lixii ; MG: Meyerozyma guilliermondii; PH: Phoma herbarum ; TP: Trichoderma piluliferum

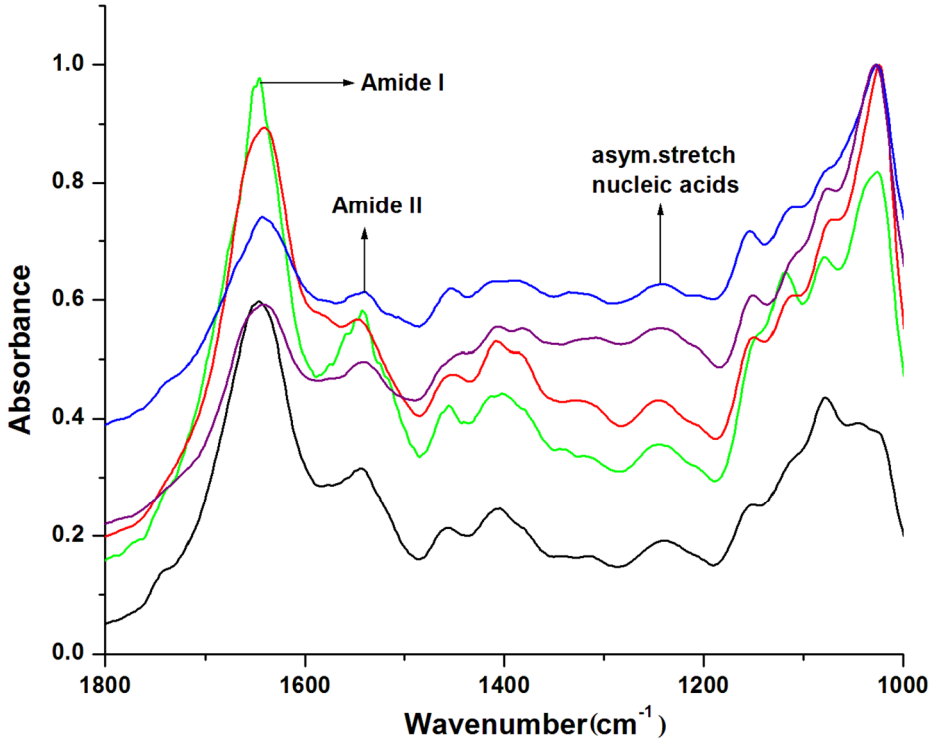

Figure 2 Mid infrared spectra in the region 1800 - 1000 cm^{-1} of five fungal species: (*Aspergillus oryzae*; *Hypocrea lixii* ; *Meyerozyma guilliermondii;Phoma herbarum* ; *Trichoderma piluliferum*)

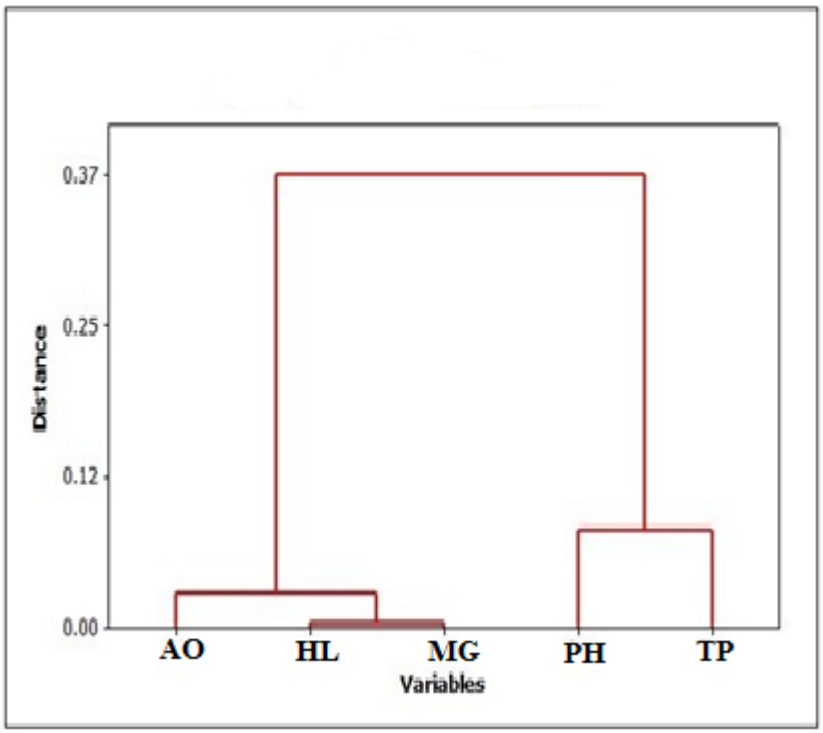

Figure 3 Dendogram of the five species based on Ward linkage,correlation co-efficient distance (AO: *Aspergillus oryzae*; HL: *Hypocrea lixii* ; MG: *Meyerozyma guilliermondii;* PH: *Phoma herbarum* ; TP: *Trichoderma piluliferum*).

Clustering pattern in the region of 4000 - 400cm^{-1} on the spectra, enables to differentiate *P. herbarum* and *T. piluliferum* in to a separate clade and the rest of them in separate clade.

Table 1 Spectral observations implying quantification difference in molecular vibrations (Peak area) in five fungal species

Molecular vibration	AO	HL	MG	TP	PH
CH_2 asymmetric stretch:lipids	4.318	1.679	1.289	2.775	2.881
Nucleic Acids	2.602	1.043	1.875	2.290	2.041
N-H stretching (proteins)	176.958	200.416	156.660	188.274	257.588
Amide I (protein)	28.093	13.382	12.516	33.995	24.543
Amide II (protein)	1.412	0.819	1.359	6.456	3.044

AO- *Aspergillus oryzae*, HL- *Hypocrea lixii*, M- *Meyerozyma guilliermondii,* TP- *Trichoderma piluliferum* and PH: *Phoma herbarum.*

CONCLUSION

There is a great potential of FTIR microscopy in tandem with appropriate mathematical tools for an easy and rapid discrimination and identification of various agriculturally and pharmocologically important fungal species. The simplicity of sample preparation, avoidance of chemical (i.e. costs and environmental impact), reliability and short measurement times (<1 min) compared to other available methods makes FTIR technique suitable for a large scale screening of fungal samples. These facts also encourage the possibility of developing FTIR spectroscopy as a reliable method for rapid identification of fungal species.

Combining the advantages of FTIR spectroscopy with other conventional techniques, offers the chance to improve the efficiency of fungal classification and identification **(Naumann *et al*., 2005; Naumann *et al*., 2007)**. Moreover, the chemical composition of the fungal species also can be simultaneously visualized. Hence, FTIR spectroscopy may help in understanding the complex chemical processes during their growth and substrate degradation.

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