

**APHYLLOPHORALES AND THEIR
MORPHOTYPES IN PAKISTAN**

**A THESIS SUBMITTED TO THE UNIVERSITY OF THE
PUNJAB IN FULFILMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**



IN

BOTANY

BY

MUHAMMAD HANIF

**DEPARTMENT OF BOTANY
UNIVERSITY OF THE PUNJAB, LAHORE**

JULY 2011



DEDICATED

To

My great & caring
FATHER 'MY IDEAL'

&

My sweet and endeared
LATE MOTHER

They are always the source of inspiration and
motivation for me and

Whose love is

Precious boon

That God has conferred me with

And for which

I am destitute to defray.

CONTENTS

TITLE	Page No.
ACKNOWLEDGEMENTS	i
SUMMARY	ii
Chapter 01	
INTRODUCTION	01
AIMS AND OBJECTIVES	09
Chapter 02	
MATERIALS AND METHODS	11
2.1 Description of the sampling site	12
2.2 Sampling of the Basidiomata	13
2.3 Collection of Morphotypes	13
2.4 Morphological Characterization	13
2.5 Molecular Characterization	14
2.5.1 DNA Extraction and Amplification Protocols	14
2.5.2 Polymerase Chain Reaction and Sequence analysis	15
2.5.3 Analysis of PCR Products	16
2.5.4 Cycle Sequencing Reaction	16
2.5.5 Purification of PCR Products	17
2.5.6 Editing of Sequences & BLAST Analysis of ITS sequences	17
2.5.7 Alignments of ITS Sequences	17
2.6 Phylogenetic analysis	18
2.7 Biodiversity Calculation	18
2.8 Analysis of above and below ground community structure	19
2.9 Chemicals and Reagents used	19
Chapter 03	
RESULTS	21
3.1.1 Enumeration of taxa based on morpho-anatomic and molecular Methods	22
3.1.2 <i>Cantharellus</i> spp.	23
3.1.3 <i>Clavariadelphus subfastigiatus</i>	35

3.1.4	<i>Clavulina</i> spp.	39
3.1.5	<i>Gomphus</i> spp.	45
3.1.6	<i>Hydnum</i> spp.	51
3.1.7	<i>Ramaria</i> spp.	58
3.1.8	<i>Sparassis crispa</i>	68
3.1.9	<i>Thelephora</i> spp.	72
3.2.1	Phylogenetic Analysis of Aphylophorales	78
3.2.2	Phylogenetic analysis of <i>Cantharellus</i> spp.	79
3.2.3	Phylogenetic analysis of <i>Clavariadelphus</i> spp.	82
3.2.4	Phylogenetic analysis of <i>Clavulina</i> spp.	86
3.2.5	Phylogenetic analysis of <i>Gomphus</i> spp.	89
3.2.6	Phylogenetic analysis of <i>Hydnum</i> spp.	92
3.2.7	Phylogenetic analysis of <i>Ramaria</i> spp.	95
3.2.8	Phylogenetic analysis of <i>Sparassis</i> spp.	99
3.2.9	Phylogenetic analysis of <i>Thelephora</i> spp.	102
3.3.1	Community structure and community analysis	105
3.4.1	Host Range and diversity of MOTUs/species	136
Chapter 04		
	DISCUSSION	142
	Conclusion and recommendations	167
	REFERENCES	169
	Annexure (1-7)	185
	Datasheets generated by Estimate-S indicating different Species richness and Biodiversity Indices	

LIST OF PLATES

Sr. No.	TITLE	Page No.
1	Plate 01. <i>Cantharellus albiceratus</i> nom. prov.	25
2	Plate 02. <i>Cantharellus cf. cibarius</i>	28
3	Plate 03. <i>Cantharellus lutescenioides</i> nom. prov.	31
4	Plate 04. <i>Cantharellus pakistanicus</i> nom. prov.	34
5	Plate 05. <i>Clavariadelphus subfastigiatus</i>	38
6	Plate 06. <i>Clavulina cf. cinerea</i>	42
7	Plate 07. <i>Clavulina minor</i> nom. prov.	44
8	Plate 08. <i>Gomphus pukhtunkhawensis</i> nom. prov.	47
9	Plate 09. <i>Gomphus macrocarpus</i> nom. prov.	50
10	Plate 10. <i>Hydnum repandum</i>	54
11	Plate 11. <i>Hydnum pileoelevari</i> nom. prov.	57
12	Plate 12. <i>Ramaria flavescenioides</i> nom. prov.	61
13	Plate 13. <i>Ramaria cymeoides</i> nom. prov.	64
14	Plate 14. <i>Ramaria alba</i> nom. prov.	67
15	Plate 15. <i>Sparassis crispa</i>	71
16	Plate 16. <i>Thelephoara horrida</i> nom. prov.	75
17	Plate 17. <i>Thelephora palmata</i>	77

LIST OF TABLES

Sr. No.	TITLE	Page No.
01	Table. 01 Observed and % Abundance for ectomycorrhizal MOTUs/species of Stand 01	110
02	Table. 02 Observed and % Abundance for ectomycorrhizal MOTUs/species of Stand 02	114
03	Table. 03 Observed and % Abundance for ectomycorrhizal MOTUs/species of Stand 03	118
04	Table. 04 Observed and % Abundance for ectomycorrhizal MOTUs/species of Stand 04	122
05	Table. 05 Observed and % Abundance for ectomycorrhizal MOTUs/species of Stand 05	126
06	Table. 06 Observed and % Abundance for ectomycorrhizal MOTUs/species of Stand 06	130
07	Table. 07 Observed and % Abundance for ectomycorrhizal MOTUs/species of Stand 07	134
08	Table. 8 Species Diversity and Species Richness Indices	140
09	Table. 09 Comparative analysis of MOTUs/Species found with ectomycorrhizal Host trees in Forests of Pakistan	157
10	Table. 10 Stand Wise Communities formed by MOTUs/species	160

LIST OF FIGURES

Sr. No.	TITLE	Page No.
1	Fig. 01. Phylogenetic tree of <i>Cantharellus</i> ssp.	81
2	Fig. 02. Phylogenetic tree of <i>Clavariadelphus</i> ssp.	85
3	Fig. 03. Phylogenetic tree of <i>Clavulina</i> ssp.	88
4	Fig. 04. Phylogenetic tree of <i>Gomphus</i> ssp.	91
5	Fig. 05. Phylogenetic tree of <i>Hydnum</i> ssp.	94
6	Fig. 06. Phylogenetic tree of <i>Ramaria</i> ssp.	98
7	Fig. 07. Phylogenetic tree of <i>Sparassis</i> ssp.	101
8	Fig. 08. Phylogenetic tree of <i>Thelephora</i> ssp.	104
9	Fig.09. Abundance and frequency (%) of ECM Morphotypes for Stand 01	108
10	Fig.10. Community Composition of ECM Morphotypes for Stand 01.	108
11	Fig.11. Diversity Indices for Stand 01	109
12	Fig.12. Rarefied Species Accumulation Curve based for Stand 01	109
13	Fig.13. Abundance and frequency (%) of ECM Morphotypes for Stand 02	112
14	Fig.14. Community Composition of ECM Morphotypes for Stand 02.	112
15	Fig.15. Diversity Indices for Stand 02	113
16	Fig.16. Rarefied Species Accumulation Curve based for Stand 02	113
17	Fig.17. Abundance and frequency (%) of ECM Morphotypes for Stand 03	116
18	Fig.18. Community Composition of ECM Morphotypes for Stand 03.	116
19	Fig.19. Diversity Indices for Stand 03	117
20	Fig.20. Rarefied Species Accumulation Curve based for Stand 03	117
21	Fig.21. Abundance and frequency (%) of ECM Morphotypes for Stand 04	120

22	Fig.22. Community Composition of ECM Morphotypes for Stand 04.	120
23	Fig.23. Diversity Indices for Stand 04	121
24	Fig.24. Rarefied Species Accumulation Curve based for Stand 04	121
25	Fig.25. Abundance and frequency (%) of ECM Morphotypes for Stand 05	124
26	Fig.26. Community Composition of ECM Morphotypes for Stand 05.	124
27	Fig.27. Diversity Indices for Stand 05	125
28	Fig.28. Rarefied Species Accumulation Curve based for Stand 05	125
29	Fig.29. Abundance and frequency (%) of ECM Morphotypes for Stand 06	128
30	Fig.30. Community Composition of ECM Morphotypes for Stand 06.	128
31	Fig.31. Diversity Indices for Stand 06	129
32	Fig.32. Rarefied Species Accumulation Curve based for Stand 06	129
33	Fig.33. Abundance and frequency (%) of ECM Morphotypes for Stand 07	132
34	Fig.34. Community Composition of ECM Morphotypes for Stand 07.	132
35	Fig.35. Diversity Indices for Stand 07	133
36	Fig.36. Rarefied Species Accumulation Curve based for Stand 07	133
37	Fig.37. Diversity Comparison of ECM Morphotypes for Pakistan	141
38	Fig.38. % Abundance of Aphylophorales MOTUs in all stands	151
39	Fig. 39. Species richness of Ectomycorrhizal MOTUs/species belonging to Aphylophorales with different host trees	154

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I sincerely thank my supervisor, Dr. A. N. Khalid, Associate professor, Department of Botany, University of the Punjab, Lahore, for his unparalleled guidance throughout this project. He not only fulfilled the role of scientific advisor, but also acted as my spiritual and moral mentor, helping build my trust and self confidence in my own work. I am also thankful to Dr. Martin I. Bidartondo, Imperial College, London, UK for bringing me into his laboratory at Imperial College, London and at Royal Botanical Gardens, KEW, UK. I remain thankful to him for his co-operation, for teaching me the intricacies of Molecular Mycology and Bioinformatics. I am grateful to Prof. Dr. Khan Rass Masood, Chairman, Department of Botany, University of the Punjab, Lahore for providing conducive environment in the Department for research. I express my gratitude to chairperson DPCC for financial assistance throughout the programme and Higher Education Commission of Pakistan for financial support for a period of six months to get training about molecular fungal ecology and Bioinformatics from Imperial College, London and Jodrell Laboratory, Royal Botanical Gardens, KEW, UK

I express my thanks to my senior lab fellows (Dr. Abdul Rehman and Dr. N. S. Afshan) and junior lab fellows (Abdul Razaq, Binte Zahra, Nousheen Yousaf , Sobia Illyas, Tayyaba Ashraf, and many significant others*) for their unconditional support, assistance and prayers. Their love and unconditional support makes me enable to complete this thesis on time.

I remain thankful to Ms Samina Bilal and Ms Malka Saba for helping me finalizing the thesis. I also express my thanks to International collaborators/ Scientists at Imperial College, London and Jodrell Laboratory, Kew, UK (Dr. Brian Spooner, Dr Paul Canon, Dr. Richard Waterman, Dr. Flipa Cox and Dr. Bigonia Augoir for their memorable company at Jodrell Laboratory, UK. Dr. Ron H. Petersen (USA), Dr. Methven (USA), Dr. Admir Giachini (USA), Dr. Terry Henkle (USA) for their valuable suggestions regarding identification and phylogeny. I would like to pay my special gratitude to my friends Mr Muhammad Irfan, Rana Sohail Shareef and Mr. Kashif Fida to whom I shared my problems for solution. Last but not the least, I address my warm thanks to all my family members who greatly encouraged me always.

MUHAMMAD HANIF

SUMMARY

SUMMARY

The main objective of this study is characterization and identification of Aphyllophorales and their morphotypes based on rDNA-ITS usage and morpho-anatomical approaches. The attempts have also been made to assess the diversity of Aphyllophorales associated with different host trees and below ground ectomycorrhizal structures, in Himalayan Moist Temperate forests (HMTF) of Pakistan distributed in seven stands. Other objectives were to find out the connections between above and below ground mycoflora, community structure of Aphyllophorales and to find out the phylogenetic relationship among the members of these fungi.

In the present investigation, sixty one (61) basidiomata of Aphyllophorales were analyzed. These were grouped into seventeen (17) species belonging to eight (08) genera viz; four of *Cantharellus* (*C. albiceratus* nom. prov., *C. cf. cibarius*, *C. lutescenioides* nom. prov., *C. pakistanicus* nom. prov.), one of *Clavariadelphus* (*C. subfastigiatus*), two of *Clavulina* (*C. cf. cinerea*, *C. minor* nom. prov.), two of *Gomphus* (*G. pukhtunkhawensis* nom. prov., *G. macrocarpus* nom. prov.), two of *Hydnum* (*H. repandum*, *H. pileoelevari* nom. prov.), three of *Ramaria* (*R. flavescenioides* nom. prov., *R. cymeoides* nom. prov., *R. alba* nom. prov.), one of *Sparassis* (*S. crispa*), two of *Thelephora* (*T. horrida* nom. prov. and *T. palmata*). All species except five (*C. cf. cibarius*, *C. cf. cinerea*, *Hydnum repandum*, *Sparassis crispa* and *Thelephora palmata*) are new to science while *Clavariadelphus subfastigiatus* is first time reported from Pakistan. These Aphyllophorales were identified using morpho-anatomic features and partial Internal Transcribed Spacer regions (ITS1 and ITS2), complete 5.8s gene and sometimes with partial sequences of 18s and 28s genes. For this purpose, 97% cut-off similarity has been used as a proxy for a species level identification.

A total of 97 molecular operational taxonomic units (MOTUs)/species belonging to 35 genera from roots of different host trees were identified. Most of them were found associated with *conifers* while few were associated with deciduous trees.

The treated MOTUs/species fall in following 35 genera; *Amphinema* (01 species), *Bisporella* (01 species), *Cenococcum* (01 species), *Chalara* (01 species),

Clavulina (02 species), *Cortinarius* (01 species), *Davidiella* (01 species), *Entoloma* (01 species), *Helvella* (03 species), *Humaria* (01 species), *Hyaloscypha* (01 species), *Hydnobolites* (02 species), *Hymenoscyphus* (02 species), *Inocybe* (17 species), *Marasmius* (01 species), *Pachyphloeus* (01 species), *Peziza* (03 species), *Phialocephala* (01 species), *Phoma* (01 species), *Piloderma* (02 species), *Pochonia* (01 species), *Pseudotomentella* (01 species), *Rhizoscyphus* (02 species), *Russula* (09 species), *Sebacina* (05 species), *Sistotrema* (01 species), *Suillus* (01 species), *Tarzetta* (01 species), *Thelephora* (03 species), *Tomentella* (16 species), *Trichophaea* (03 species), *Tuber* (05 species), *Tylospora* (01 species) and *Wilcoxina* (01 species).

All these ninety-seven (97) MOTUs/species are first time reported as ectomycorrhizal from Himalayan Moist Temperate forests of Pakistan. On the whole, communities of all the stands are heterogeneous and composed of ectomycorrhizal species. Stand 01 was colonized by five (05) species which were not represented in rest of the sampling sites. These species include; *Helvella* sp. MHLAC-01, *Hymenoscyphus* sp. MHHYM-01, *Inocybe* spp., MHMUR-01, MHOB-01, MHTAR-01 and MHUMB-01, *Lactarius* sp. MHSEM-01, *Peziza michelii*, *Peziza* sp. MHSUC-01, *Piloderma* sp. MHFAL-02, *Pseudotomentella* sp. MHTRI-01, *Rhizoscyphus* sp. MHERI-01 and *Russula livescens*.

Twenty six (26) species were found to be limited in occurrence in stand 02 viz; *Cenococcum* sp. MHGEO-01, *Clavulina* sp. MHCIN 01, *Cortinarius leucopus*, *Entoloma* sp. MHAME-01, *Helvella* sp. MHELA-02, *Inocybe* spp., MHGLA-01, MHNIT-01, MHSQU-01 and MHUMB-01, *Lactarius* sp. MHSEM-01, *Pachyphloeus* sp. MHMEL-01, *Peziza* sp. MHSUCS-01, *Piloderma* sp. MHFAL-02, *Russula cessans*, *Russula* spp., MHDIS-01, MHFOE-01, MHHET-01 and MHNAU-01, *Suillus sibiricus*, *Tarzetta* sp. MHCAT-01, *Thelephoraceae* sp. MHTBR-01, *Tomentella* sp. MHFER-01, *Tomentella* sp. MHVIR-01, *Trichophaea* sp. MHCFH-02, *Tuber aestivum* and *Wilcoxina rehmi*.

From Stand 03, *Hyaloscypha* sp. MHAUR-01, *Inocybe* sp. MHCRY-01, *Inocybe* sp. MHHIR-01 and *Tomentella* sp. MHSUB-01 were found to be unique as these did not occur in any other stand.

Ten (10) exclusive MOTUs/species were found in stand 04. These included; *Chalara* sp. MHSP-01, *Clavulina* sp. MHCRI 02, a member of *Corticaceae* sp.

MHCOR-01, *Davidiella tassiana*, *Humaria* sp. MHHEM-01, *Hydnobolites* sp. MHCER-01, *Inocybe* sp. MHPRA-01, *Phialocephala* sp. MHFOR-01, *Piloderma* sp. MHOLI-01 and *Pochonia* sp. MHSUC-01.

From Stand 05, nine (09) ITS unique sequences were identified. These included; *Bisporella* sp. MHCIT-01, *Inocybe* sp. MHMIC-01, *Sebacina aff. epigaea*, *Sebacina* spp., MHAFE-01 and MHSEB-02, *Sistotrema* sp. MHALB-01, *Tomentella* sp. MHSTU-02, *Tuber whetstonense* and *Tylospora* sp. MHTYL-01.

From Stand 06, six (06), unique ITS sequences were identified and these included; *Inocybe* spp., MHPSE-01 and MHRUF-01, *Sebacina epigaea*, a member of *Sebacinaceae* sp. MHSEB-01, *Thelephora* sp. MHGAN-01 and *Tomentella* sp. MHFUS-01.

From Stand 07, seven (07) unique ITS sequences were identified and these included; *Hydnobolites* sp. MHCER-02, *Lactarius sanguifluus*, *Marasmius* sp. MHHAE-01, *Russula lutea*, *Tomentella galzinii*, *Tomentella* spp., MHFUS-02 and MHGAL-01.

Most of the communities studied were found to be dominated by species belonging to Aphyllphorales (17-40.22%). Among them, members of Thelephoraceae were found to be more prominent and formed ectomycorrhizal association. The species richness of Stand 06 was observed maximum (3.53) and minimum for PU Suite and Stand 02, while the maximum diversity was observed for Stand 01 (0.037).

A total of twenty three (23) MOTUs/species are being reported in the present work belonging to Aphyllphorales. Three (03) species of Aphyllphorales were isolated and identified from stand 01, five (05) from stand 02, four (04) from stand 03, three (03) from stand 04, nine (09) from stand 05, eight (08) from stand 06 and five (05) of Aphyllphorales from stand 07, with different host trees. Stand 05 represented the maximum number of MOTUs/species of Aphyllphorales.

Among the hosts plants, *Abies pindrow* showed the greater affinity than other hosts towards ectomycorrhizal fungi (45 MOTUs/species) including 15 Aphyllphorales and this number reduced with other host trees.

Pakistan's part of Himalayan Moist Temperate Forests (HMTF) is rich in Aphyllophorales and many more new taxa are expected to be there if sampling effort are extended to less frequently surveyed areas.

For species richness and underground community of ectomycorrhizal MOTUS/species, there is much more need to obtain sample to reach maximum richness and complete community structure. This is the first serious attempt to assess the diversity and community structure of ectomycorrhizal fungi and Aphyllophorales in HMTF of Pakistan using above ground fruiting surveys as well ectomycorrhizal morphotypes. We recovered 17 species from fruiting bodies and 97 MOTUs/species from soil with no overlap between above and below ground communities. It is very essential to sample more from these sites to find the belowground Aphyllophorales matching the above ground community and vice versa. Documentation of Aphyllophorales is yet to complete which requires to explore less frequently surveyed areas.

CHAPTER 01
INTRODUCTION

INTRODUCTION

Pakistan is an oblong stretch of land between the Arabian Sea and Karakoram mountains lying between 24° N and 37°N latitude and 61°E and 75°E longitude, covering an area of 87.98 m hac (Pakistan Online Encyclopaedia, 2011).

The forests of Pakistan reflect great physiographic, climatic and edaphic contrasts in the country. There are twelve types of forests based on the climate, altitude and composition of forest species. These range from Tropical wet evergreen to sub-alpine and alpine forests (Champion *et al.*, 1968).

The Moist temperate forests are found along the moist Himalayan Mountains between the dry temperate and sub alpine zone (1,200-3,000 m). These are found in Kashmir, Murree-Hazara hills tracts, Swat, Dir, upper reaches of Kurram Agency, Gilgit and Baltistan districts. In moist Himalayan mountains, precipitation is derived from the South-Western monsoon, which falls during the months of July to September and due to westerly disturbances it also takes place during the winter and spring seasons. Rainfall ranges between 650-750mm or some times to about 1500mm. In these areas, large amount of precipitation takes place in the form of snow and is a factor of considerable importance in determining the types of forests, particularly where summer rainfall is low. Gradual melting of snow in early summer prolongs the season during which adequate moisture is available. The mean maximum temperature in the months of December-February is 9°C, -4°C and -1°C, respectively while mean minimum temperature in these months is -2°C, -4°C and -1°C. In the summer months of June, July and August mean maximum temperature is 30°C, 31°C and 30°C and 17°C as mean minimum temperature. Average humidity is 57% (Pakistan Meteorological Department, 2011).