MATERIALS AND METHODS

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The ecological factors like rainfall, high humidity, low temperature and high altitude are confined at the south western part of Maharashta State which are favourable for growth of various plants and fungi.These environmental conditions are present at western ghats, and provides good amount of saprophytic and parasitic mycological collections. Some area have been studied by diferent mycologist but the Discomyceteous fungi are not studied seriously so far. There fore the systematic investigation has been undertaken. These fungi are not easily accessible due to their ephimeral nature, microscopic forms and inaccessible terrain during rainy season.

The methods have been adopted here in acordance with florestic and taxonomic study of discomyceteous fungi. The Discomycetous fungi which were collected in the form of ascocarps or fruiting bodies i.e. the apothecia, from different localities of Western ghats particularly in the monsoon season and occasionally through out the year (parasitic forms).

The Discomycetous fungi were collected from Koyananagar and Mahableshwar well known localities of the Western ghats of Satara District.

COLLECTION:

Systematic collection which provides base for classification. Variation in the habitat and the morphology of fungi is caused by the environmental factors. Their different habits, appearans i.e smaller or larger, predominance of particular group range of variations in the species in an area could be recognised by field observation. Workers in the field must give attention to variations in habitats, parasites and their hosts etc. This is possible by frequent visit to sites for many times.

Different methods are used for collecting the fungi. The fungi show different types of habitates as folicolous, lignicolous, and coprophilous etc. In the present study the apothecia or ascocarps were collected and brought in laboratory for their study.

The delicate ephimeral and variously coloured apothecia or ascocarps were collected frequently and seasonally throughout the year. A quite large number of Discomycetous forms were collected from humus rich soils of vallies, planes, open lands, dead plant parts, dead grass and dungs and dropings of various animal and bird.

Due to high rain fall locality like Radhanagari, Gaganbawda, Koyananagar provided rich collection of the order Helotiales and Pezizales. Mostly species of Genera Pulvinula, Scutllinia, Ascobolus, Trichoglossum and Geoglossum were

collected. Ecological features influence mycological flora from region to region and from locality to locality. During our frequent visits we have collcted ascocarps/apothecia of Peziza, Pulvinula and Lamprospora from the open and burned grounds in Mansoon. While some of the leathery and dry forms like Dasyscyphus, Mollisia and Lophodermium were collected on dead wood and culms of grasses throughout the year. The availability of fresh ascocarps of coprophilous forms depend on the ecological forms like rainfall, humidity and temperature etc. Dung samples (dry or fresh) were collected from different localities to study.

For collecting ephimeral forms of Perizales, regular and careful search was made from time to time and diffrent localities. Accurate deteminations of the material and their substrata were made during field collection. Each and every selected area for the collection of fresh apothecia was screened carefully.

The fresh delicate apothecia or ascocarps were collected from the field, the ~~same~~ locality, altitude, date of collection, colour of ascocarp their distribution and substrata etc. were recorded in the field note book. These collected material was ~~were~~ brought in bottles, polythene bags or specimen tubes etc. with great care, separately along with their substrata and in large quantity if available with various sizes and different stages of development. After

preliminary observations photographs were taken to reflect their natural colours and habits. Each specimen bottle was labelled in the field with required information. Tentative identification of the genera was made with the help of natural key characters. Fleshy ascocarps or fruits of Helotiales were easiest fungi to observe in the field. Spathulate large ascocarps like Trichoglossum, Geoglossum. etc. with black, brown flesh colour and their exudate were used for identification. Hymenoscyphus and Octospora were identifiedd by their leathery, brightly coloured, stalked or stipitate apothecia growning on dead parts of higher plants.

In addition to this, other forms like Ascobolus, Lasiobolus and Ascodesmis were also collected from various dungs and droppings of animal and bird.

LABORATORY OBSERVATIONS:-

After collection of various fungi from the various selected localities, tentatively labelled or unlabelled specimens were brought into the laboratory for further observations. Firstly all the spcimens were cleaned and washed, then all the specimens were sorted into different groups.

Cleaned and mature ascocarps were used to examine by usual laboratory methods . Different methods are used to identify the orders Helotiales, Phacidiales and Pezizales.Important characters are recorded such as texture, colour, secretion, size, substrate and dominance. Generally in coloured fungi, colour of ascocarp when it is fresh, is recorded, as fading of colour generally occurs(pezizales and Helotiales.)Special attention was also given to habit of ascocarp and their position in or on the subtrata, presence or absence of hairs, setaes etc. Detailed external morphology of specimens were kept uptodate for further use and identification.

LABORATORY TECHNIQUES :-

After preliminary laboratory observations, ascocarps were observed with eyelens, Microscopicaly, features were studied by mounting a piece of it either by sectioning or scrapping or by squashing and staining with cotton blue. Thin hand cut sections of apothecia were prepared and mounted them first in water to study colour , wings of the ascospore and other ornamentation of spores, presence or absence of gelatinous, unilateral or bilateral wings of ascopores which are useful characters for identification of genus Ascobolus.

SECTIONING OF ASCOCARPS :- To study detailed anatomical structure of ascocarps, transverse and vertical sections are necessary. All the Discomycetous fungi were studied by taking transverse or vertical sections of fertile and sterile parts of the matured ascocarp.

The sectioning was made by following methods :-

1) Simple handcut method 2) Freezing microtome or routine microtome (Rotary) (i) Hand cut sectioning :- Adequate T.S. or V.S. of the ascocarp were taken by hand using single or dcuble edged razor blade or cut through razor and holding the material between some tissue such as pith or under the dissecting microscops. Most of the delicate and soft forms of the Pezizales and the Helotiales were studied by this method.

(ii) Cryostat Sectioning **(Freezing microtome) method :-**

Freezing microtome form is an essential part of a mycological laboratory equipment which consist of a fixed stage cooled to below the freezing point of water (either by carbon dioxide or a 'pelcool' cooled water system) and a movable blade calibrated to cut material frozen on the stage with a drop of dilute gum arabic in water at a range of thickness viz. 5,10,15, or 20 µm (10µm thick sections are most commonly used). The sections were collected with fine point brush as they were cut and placed directly in a drop of mounting fluid on a slide or in water in a watch glass. Freezing technique of sectioning was used generally for leathery or for very soft ascocarps like Hymenoscyphus, Dascyphus, Octospora etc.

(iii) Squash Method :-

This is very simple method of sectioning to study the details of individual part of ascocarp squash. Preparation were made with the help of a single edged razor blade and mounted in different mounting media and stains. This method is used for observing asci, ascospores.

paraphyses, setae, hairs etc.

Staining Techniques :-

The different stains and the staining techniques are used to study ascus layers, ascus tips, ascospores and tissue of ascospores, ascocarp or apothecia. The colouring agents used for microtechniques are various kinds but majority of them are dyes so called stains. Stains are classified on their moleculer weight or on their chemical behaviour as acidic, basic and neutral. Different stains have different chemical action against the chemical compounds of a cell.

Mycological stains :-

The different fungal structures like mycelium, ascus, ascospore, conidium, conidiophore, hymenium, pseudoparaphyses, periphyses, cells of ascus wall sterigmata, basidium, basidiospore etc. can be studied with the help of different colouringdyes or stains. In the present study different stains are used such as Melzer's reagent, Cotton blue etc.

1. Melzers Reagent (Nylander, 1869)

This contains :-

Iodine	0.5 gm.
Potassium iodide	1.5 gm.
Chloral hydrate	20.0 gm.
Distilled water	20.0 ml.

This reagent was prepared by dissolving 0.5 gm of iodine and 1.5 gm of potassium iodide in 20 ml of distilled water separately in two diffeent containers and both the solutions were mixed in 20 ml of chloral hydrate this mixture was filtered before use.

The
Melzers reagent is used to check ~~to~~ iodine reaction of the ascus wall, while chloral hydrate is added to this as a clearing reagent. This reagent was used as a general differential stain and to test iodine reaction of the ascus apex or ascus pore. The apex of the ascus may be blue, or a ring of plug may blue. Rarely it stains whole length of ascus or even portion of hymenium may turn blue or violet.

2. Cotton blue : - It is an acidic stain used to study the fungal structures. It is a cytoplasmic stain. to Cytoplasm turns blue leaving the hyphae or spore and septa hyaline. It was prepared by dissolving 1 g. of cotton blue in 100 ml. of lactophenol. 1 % stain is generally used for fungal staining.

To study ornamentation in pezizales, a mixture of cotton blue or Aniline blue and lactic acid is used. This helps to study warts, spines and reticulated thickning on outer surface of ascospores which is deeply stained while walls of ascospores remain colourless (Le Gal, 1947). The bitunicate nature ofascus can be observed by this staining.

3. Phloxine & dye : This is an excellent cytoplasmic stain, usually used in a 1% aqueous form. This stain is useful to distinguish septa in ascospores and hyphae. A more critical examination of ascospores can be made if the phloxine is removed from the mount and replaced it with 5% aqueous glycerine, which removes the colour from the background.

4. Aqueous Solution of KOH :-

A 2% or 3% aqueous solution of KOH is useful for swelling dried material and may also be used in combination with phloxine, mixing a drop of each at the time of the mounting.Some Discomycetes liberate quantities of a purple dye like substance into dilute KOH.

5. Congo red :- A 1% of solution in 10% aqueous NH_4OH or in 2% KOH, is used for differential staining of ascus wall layers in Pezizales and also as a general wall staining.

6. Acid Fuchsine Stain :- This stain was prepared by dissolving 1 gm. of Acid fuchsin in 100 c.c. of distilled water. This stain is good when combined with methyl green. The combination may be used for wood sections.

It is an amylloid stain and when employed the inner layer of ascus wall coloured pink or red. This stain is used to study the bitunicate nature of ascus wall in Thecothens Boud. and Thelebolus.The outer layer was stained in congo red and inner in acid fuchsin (Kimbrough, 1966, Van Brummelen, 1967).

7. Lactophenol :- This is used as mounting medium. The semipermanent preperations were mounted in lactophenol and slides were slightly warmed so as to remove the air bubbles. This was prepared by adding 1 part of phenol, 39 parts of glycerine and 1 part of lactic acid in 9 parts of distilled water. the solution was filtered before use. Lactophenol is used because of its swelling and preservative properties of lactic acid and phenol, while glycerine keeps material free from rapid dehydration.

MICROPREPARATIONS :- During the study the slides were made semi-permanent by Sealing them with wax or nail polish paint. The permenant slides of micropreparation were mounted in canada balsum or DPX. The mounting medium for semipermanent microprepartion was Amann's lactophenol including a stain cotton blue. In some cases permanent preparation was found better than temperary mounts. The slides in lactophenol wanted to preserved were warmed for an hour or so to ensured that all the air bubbles were completely eliminated and any excess mounting fluid, throughly cleaned off. Semipermanent slides made with lactophenol were preserved by ringing with nail varnish or was and lebelled properly and placed in wooden cabinate. Each and every semipermanent slide was identified and lebelled properly.

MEASUREMENTS : Micrometry is an important and essential part in fungal taxonomy. Measurements of various structures are useful for identification and classification of the different taxa. The metric units are used for macro and micro measurements i.e. cm, mm, µm. In the present study the unit of measurement is µm. (Micrometer) instead of µ . micron).

Microscopic measurements were made with micrometer eye piece. The microscope was calibrated by placing a graduated slide (i.e. a slide with a scale accurately divided into division, each of which is 10 µm. . on the microscope stage and measuring the number of divisions on the eye piece scale which correspond to one or more divisions on the eye piece scale which correspond to one or more divisions on the graduated slide. From this information the distance, each division of piece scale represents can be easily calculated. With this knowledge, a seperate set of caliberation were prepared and used for further measurements of element with particular objectives. The largest and smallest mature structures were measured to ascertain the range of variations occured.

Measurements of asci ascospores, paraphyses, setaes, and sterile tissues from apothecia were made by Earnst Leitz Wetzlar occular with 10 x , 45 x and 100 x objectives Critical details of ascospore ornamentaion in species of Peziza, Scutellinia, Lamprospora, Ascobolus and Ascodesmis were appriciable with oil immersion lenses at magnification in the range of 1500X.

All the characters are useful in the classification and were distinctive such as colour, size, shape and cells of apothecia or fruting body, types of setaes, hairs , asci, ascospors, septation, and paraphyses have been dscribed as per the mycological terminology.

The genera species and varieties of the Discomycetes were indentified with the help of recent upto date literature published by many workers. World wide or regional keys and monographs of different mycologist were used to identify the genera and species of discomycetes fungi.

ILLUSTRATIONS : It constitutes, drawings, graphs, maps, plates and tables :

1. Camera lucida drawings : - The drawings of the micropreperations were made with pencil by drawing aid (camera lucida) attached to the microscope. Drawing were drawn with 5x, 10x, and 15x magnification eyepieces in combination with 10x, 45x, and 100x objectives. If necessary the asci, ascospores, setaes and paraphyses were drawn with variable magnification according to the size of

ii) Drawings and textplates : The essential requirement of any drawing is necessory, pencil drawings are not adequate for publication. It sould be in black water proof Indian Ink. It is done by transfering the pencil sketches on the standard sized white hard paper(22.5 x 29 cms or 9 x 11.5 inches). Traced out sketches as per sequence were again sketched by pencil and then were inked for getting uniform thickening of rotering pens of various thickness as 0.1 mm, 0.2 mm or 0.3 mm were used. The scales are directly drawn near each drawing or are incorporated along with the legend.

iii) Tables :- For summerising the statistical data of the taxa tables are valuable in the taxanomic studies. They have typed on separate pages whenever necessary and have legends which are explainatory without referring the text. In present investigation tables were made to show comparison between original species and the present collections, whenever the new variety has been proposed as well as to summerize the florestic pattern.

iv) Keys :- For indentification of various taxa of the different status keys plays very important role. It is very important part of the monograph and floras. In discomycetes the adequate keys do not exist and taxanomists have to construct their own keys to facilitate the identification of their material.

v) Maps :- The known distribution of species can be easily and accurately presented by maps. They form an important part of monographs an revision and are being increasingly employed in floras. In present study maps were produced accurately as much as possible show the distribution of locality from where the fungus species were collected.

vi) Photography :- Photographs of material show the natural habits. The closeup photographs of ascocarps have great value in reflecting natural habit, colour and substrate. The shape, colour and attachment of apothecia are clearly seen at a glance.

Photomicrography of the micropreparations were made with the help of automatic orthoplan microscope MPS 45. Coloured filters as orange or bluewere preferred to obtain the contrast. The photomicrography was carried out with 3.2x, 6.2x, 5x, 10x and 15x magnification of eyepieces in combination with 3.2x, 5x, 10x, 45x, and 100x with oil immersion objectives as the nature of th object being photomicrographed. Ascopores were photomicrographed under oil immersion to study detail structure. The selected snaps from the -ve were used for prints for prepavation of plates.

vii) Plates of the photographs and photomicrographs.

Drawings, photographs of material and photomicrographs are required to be set into plates. Selection of prints of photoraphs are very important. Before setting it, the size of the plate is to be considered. sufficient space is kept for any legend, numbering of plates, writing of scale etc. Before making a place the items are trimmed and dry mounted on the paper to locate them. Bykeeping sufficient space photographs finally mounted with the help of adhesive. They are properly numbered.

Scales were drawn directly as to line drawing, in case of photographsthey were mentioned on seperate page. Particular attention is given to scalesand spore shapes etc.

viii) Citation and References.

The accepted system of citation and reference are generally followed. Thereferences were cited by giving the list of the references or bibliography at the end in which the precise title of the article, volume, first and last pages are given. The list ofreferences or bibliography given at the end were arrangedalfabetically.

ix) Preservation and deposition of the material :-

Preservation of the material is an important part in the fungal taxonamy. The herbariums of the fungal specimen are prepared by variousmethod but that depends upon

the nature of material. The fresh, well cleaned material in the form of ascocarps or apothecia were well preserved by different methods for future chemotaxonomical, biochemical, physiological, serological, pathological or genetical studies. Fleshy forms of Discomycetes of the present investigation are well preseved in 2-4 % Formaldehyde in glasstube or container. Then they are prcperly labelled giving the information regarding the taxon such as class, order, family, genus, species, variety, locality and date of collection. They were arranged as genera, family, order and deposited in Mycological Herbarium, Botany Department, Yashwantrao Chavan College of Science, Karad under the codenumber WIF i.e.Fungiof western India.

x) Preservation & Deposition of Micropreparations :-

The semipermanant and permanant micropreparations were properly labelled withblack Indian Ink and arranged genus wise in wooden cabinates and were deposited in the Mycological Herbarium of Botany Department, Yashwantrao Chavan College of Science, Karad.

Orders	Families	Total genera studied	Total No.of Species	Varieties	New species (Proposed)	New varieties	Sp. New to India	Var. N. to India	Sp.N. State	Var N. State.	Remarks
I.Pezizales	Ascobolaceae.	-	-	-	-	-	-	-	-	-	-
	Pezizaceae.	2	2	-	1	1	-	1	-	1	-
	Pyrenomycetaceae	1	3	-	-	1	-	1	-	1	-
II.Phacidiales.	Rhytismataceae	1	2	-	-	-	-	-	-	-	-
	Cryptomycetaceae	-	-	-	-	-	-	-	-	-	-
III. Helotiales	Geoglossaceae.	-	-	-	-	-	-	-	-	-	List of recorded species is attached.
	Hyaloscyphaceae.	1	1	-	-	1	-	1	-	1	-
	Leotiaceae	-	-	-	-	-	-	-	-	-	List of recorded species is attached.
3	8	5	8	-	1	3	-	3	-	3	