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THE NATURAL HISTORY OF SLAPTON LEY NATIONAL NATURE RESERVE

XXII : ISOLATION OF MICROFUNGI FROM SOIL AND WATER SAMPLES

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ABSTRACT

A first survey of microfungi isolated into culture from 11 samples of soil, water and other materials at Slapton Ley National Nature Reserve yielded 58 species, of which 42 were new records for Slapton, and one was a new record for the UK. Most species were commonly isolated saprobes, but others were important plant pathogens and some were rare species seldom isolated into culture. Shingle beach and stream margin had the greatest diversity and the most new records of the sites sampled. A total of 2,500 species of fungi predicted for this site is likely to be a conservative estimate. The importance of microfungi in biodiversity assessments and implications for calculation of species abundance are emphasised.

INTRODUCTION

In the British Isles, about 12,000 species of fungi have been recorded and about 50 additional species are added to this list each year. Many fungi remain to be discovered, but are often overlooked because they occur in specialised habitats, or have specific nutritional/environmental requirements and fail to grow under standard conditions. Collection and isolation of these organisms demands knowledge of the organisms themselves, of specialised habitats, and of a wide variety of techniques. The Slapton Ley National Nature Reserve has one of the best documented fungal biota in the world, with 2,185 recorded species at September, 1994 (Hawksworth, 1986; 1994). However, the majority of these species are either collectable by hand (mushrooms, toadstools and lichens), or visible with the naked eye, hand lens or dissecting microscope. To make a full inventory of the fungi at a site, it is also necessary to detect those which are either not visible to the naked eye, or are not visible in an identifiable state. This requires the isolation and culture of species present, but invisible, in water, soil, plants, insects etc. Isolation work has not previously been carried out at Slapton, and this paper reports a preliminary study to determine the microfungi in samples of soil, water and decaying plant material obtained from the Reserve, using a variety of standard and specialised techniques. The aims of this first study were to determine how many previously undetected species could be found, and whether there were any marked differences in the species found in water and soil from different sites in the Reserve.

MATERIALS AND METHODS

Sample Sites

Ten sites were examined from a total of 38 now used for biological recording in the reserve (Fig. 1 : Bates, Perry & Proctor, 1993): A shingle (bore); B shingle (ridge); C

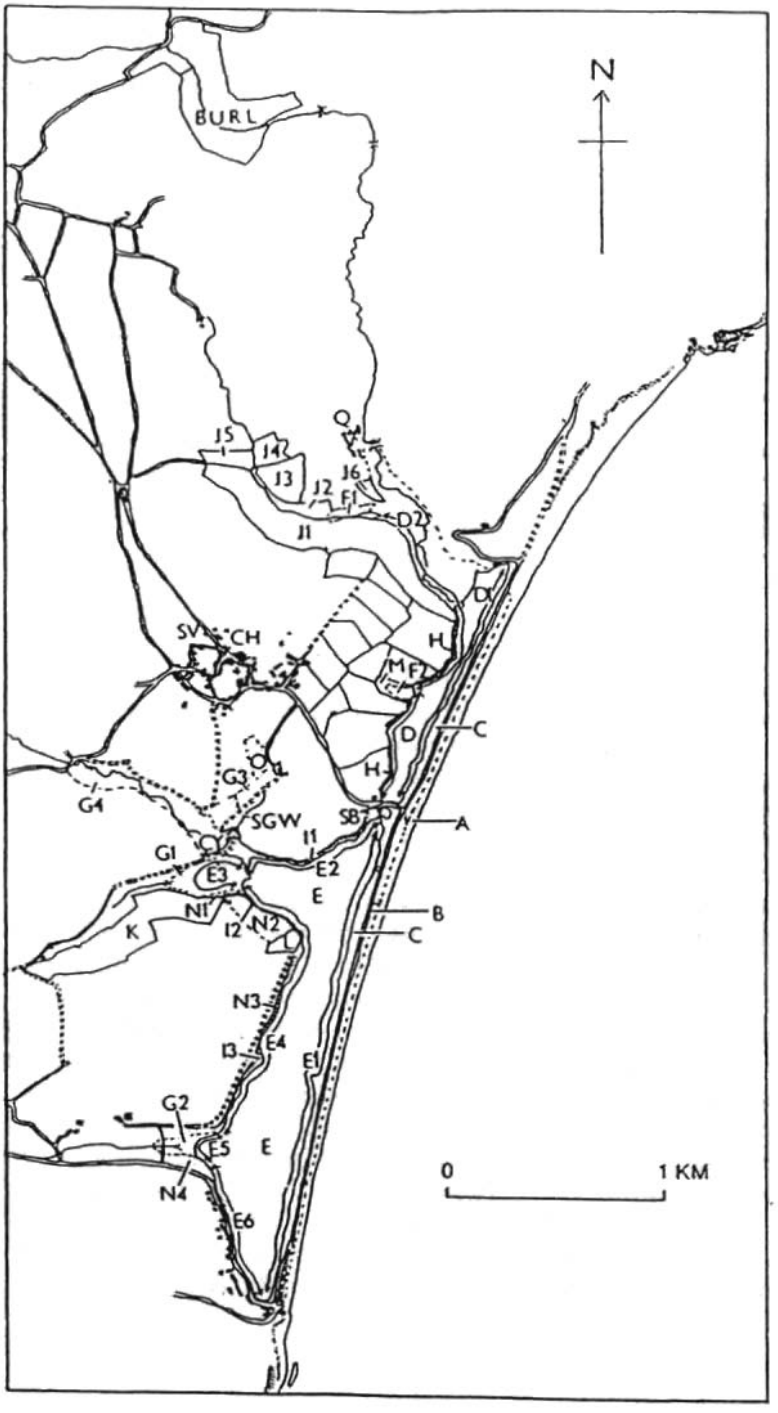

FIG. 1
Map of Slapton Ley National Nature Reserve, showing the position of the habitat units used for sampling (from Bates, *et. al.*, (1993).

backslope margin of the Higher and Lower Leys ; DM bank by marsh; E1 amongst the *Phragmites* (reed) stand; G1 marsh edge; I1 under *Ruscus* (butcher's broom) stand (A) and grazed cattle pasture (B); J1 under *Castanea* (sweet chestnut) trees; J2 stream margin; J3 under *Larix* (larch). Soil samples were obtained from sites A, B, C, DM, I1(A & B), J1, J2, and J3 between 13-14 September 1993, using a sterilised steel trowel and placed directly into sterilised containers at the sampling point. Samples of other substrates were obtained from sites A, DM, E1, G1, J1 and J2 between 2-3 October 1993, and were placed directly into sterilised sampling 3 l bottles or aluminium cannisters, as appropriate. Samples were transported from their collection sites within 24 h to the isolation laboratory at the International Mycological Institute (IMI) for processing. Methods for the preparation of agar media used are found in Smith & Onions (1994), Eggins & Pugh (1962) and Lange (1952), unless stated.

Soil Samples

A sub-sample of 1 g. soil from each sample taken was plated directly onto malt extract agar supplemented with 0.6 g/l each of penicillin G and streptomycin sulphate (MAB), malt extract agar plus 20% (w/v) sucrose (M20), cellulose agar (CELL), wheat straw-tap water agar (WS), horse dung agar (HD) and potato dextrose agar (PD). These plates were incubated at 25° C and 37° C.

Serial dilutions to 1 x 10^{-4} were made from another sub-sample of 1 g fresh soil from each sample collected using sterile 0.05% (v/v) 'Tween' (polyoxyethylene (20) sorbitan mono-oleate) diluent. For sites A-C, dilutions were spread onto corn meal agar plus 1.5% (w/v) sodium chloride (CMS) and incubated at 25°C. For the remaining sites, dilutions were all spread onto MAB and incubated at 25° C and 37° C. Serial dilutions of 1 g air dried soil from each site were made to 1 x 10^{-2} in sterile 0.05% (v/v) 'Tween', spread onto MAB and incubated at 25°C.

Plates from all methods were examined after 3 and 7 days and fungi isolated onto appropriate fresh agars.

Other Substrates

Seaweeds (Site A).

Material was rehydrated with 3.6% (w/v) sodium chloride for 2 h. Pieces of red, green and brown seaweed and kelp holdfast were placed on Petri plates of CMS, either fresh, or after surface-sterilisation with 1% (v/v) sodium chlorate for 1 min, folowed by three washes in sterile distilled water.

Decaying leaves of *Salix* in water (Site DM).

Surface water (*ca* 10 ml) was decanted into 2 Petri plates and baited with snakeskin, "Cellophane" (washed to remove plasticisers), split hemp seeds and whole clover seeds. Mud residue (2 x 1 g) was mixed with 10 ml sterile distilled water and baited with snakeskin, "Cellophane", split hemp and whole clover seeds. Washed decayed leaves were placed in 4 Petri plates in a humid chamber, and also on 4 Petri plates of Corn Meal Agar supplemented with 0.01% (w/v) yeast extract, 0.002% (w/v) ferric (= iron III) sulphate and 0.6 g/l each of penicillin G and streptomycin sulphate (CMAB+).

Water amongst *Phragmites* (Site E1),

from marsh edge (Site G1) and from stream margin (Site J2). Five aliquots of 200 ml water in conical flasks were baited with apple cubes, snakeskin, 'Cellophane' and split hemp seeds.

Mud by stream (Site J2).
Surface liquid (5 ml) was baited with split hemp seeds. Four mud samples, each of 1 g, were mixed with 10 ml sterile distilled water in Petri plates and baited with 'Cellophane', snakeskin, split hemp and whole clover seeds,. Washed leaf pieces and grass blades were incubated in four Petri plates in a humid chamber. Ten spread plates of two soil samples on glucose yeast phosphate sucrose agar (GYPS) were prepared using 1 ml of a 1:100 dilution in peptone water.

Roots of *Chrysosplenium* (Site J2).
Following washing under the tap in the laboratory, brown and white root segments were selected. Pieces were surface sterilised with 0.05% (v/v) sodium chlorate, washed three times with sterile distilled water and plated onto two Petri plates of CMAB+. Root pieces were soaked in 10% *(w/v) potassium hydroxide for 7 days, neutralized with 1M hydrochloric acid washed three times in sterile distilled water, and stained in lactic acid/trypan blue.

Moss frond (Site J2).
One frond of the moss, *Thuidium tamariscinum*, was cut up and plated onto two plates of CMAB+.

All material was incubated at 15° C and examined daily until the emergence of mycelium, which was then transferred to fresh plates of CMS or CM, as appropriate. Identification of isolates was checked by specialists at the IMI, following transfer to specialised media, if necessary.

RESULTS

Species isolated from each site are tabulated below. The number following each name refers to the isolation method used: 1, direct plating of fresh soil; 2, dilution plating of soil; 3, dilution plating of air-dried soil; 4, surface sterilisation and plating onto agar; 5, direct plating of sample onto agar; 6, baiting, followed by plating of the bait onto agar; 7, clearing of roots; 8, incubation in a humid chamber. Fungi which proved to be new records for Slapton are indicated by an asterisk, records new to Britain by a double asterisk.

Site A: shingle (bore)

Alternaria alternata	1,5
Alternaria sp.	5
**Ascochyta* sp.	5
Aspergillus fumigatus	1,2
Cladosporium cladosporioides	1,5
Cladosporium herbarum	5
Epicoccum nigrum	5
Fusarium graminearum	5
**Leptosphaerulina trifolii*	5
**Mortierella humilis*	5
Penicillium brevicompactum	3,5
**Penicillium chrysogenum*	5
**Penicllium herquei*	5
**Penicillium simplicissimum*	1
**Phoma complanata*	5
**Phoma exigua*	5
Phomopsis sp.	5
Pleospora herbarum	5
Rhizopus stolonifer	2
**Scolecobasidium arenarium*	5
**Scolecobasidium salinum*	4
**Trichoderma koningii*	1

Site B: shingle (ridge)

**Absidia corymbifera*	3
Aspergillus fumigatus	1
Cladosporium cladosporioides	1
**Penicillium janczewskii*	3

**Penicillium simplicissimum*	2
**Penicillium spinulosum*	2
Rhizopus stolonifer	2

Site C: leas margin

**Absidia corymbifera*	3
Aspergillus fumigatus	1
Penicilllium brevicompactum	3
Rhizopus stolonifer	3

Site DM: bank by marsh

**Absidia corymbifera*	3
Aspergillus fumigatus	1
**Byssochlamys nivea*	1
**Cylindrocarpon destructans*	6
Epicoccum nigrum	3
Mortierella sp.	6
Mucor circinelloides	3
Mucor hiemalis	1,3,5
**Neosartorya fischeri*	2
**Neosartorya spinosa*	1
**Penicillium janczewskii*	2
**Saprolognia parasitica*	6
**Trichoderma harzianum*	3
**Trichoderma koningii*	5
Trichoderma viride	5

Site E1: water amongst *Phragmites*

Fusarium avenaceum	5
Fusarium graminearum	5
**Saprolegnia parasitica*	6

Site G1: water from stream edge

Fusarium avenaceum	5
Pythium sp. Group HS	5
***Pythium salinum*	6

Site I1 (A): under *Ruscus* stand

Aspergillus fumigatus	1
**Cyphellophora* sp.	3
Mucor hiemalis	1,2,3
**Mortierellla isabellina*	3
**Neosartorya fischeri*	1,2
**Penicillium citrinum*	2
**Penicillium spinulosum*	3
**Trichoderma harzianum*	3,5
**Trichoderma koningii*	3
**Trichosporiella sporotrichoides*	2

Site I1 (B): cattle pasture (grazed)

**Absidia cylindrospora*	1
**Absidia spinosa*	2
Aspergillus fumigatus	2
**Chaetomium convolutum*	2
**Gelasinospora cerealis*	3
**Neosartorya fischeri*	1,2
**Neosartorya spinosa*	1
**Paecilomyces lilacinus*	3
**Penicillium janczewskii*	3
**Penicillium simplicissimum*	2
**Talaromyces flavus*	3

Site J1: under *Castanea*

Absidia cylindrospora	1
**Acrophialophora levis*	2
Aspergillus fumigatus	1
**Colletotrichum acutatum*	3
**Fusarium sporotrichoides*	1
Mortierella ramanniana	2,3
Mucor hiemalis	1,3
**Neosartorya spinosa*	1
Phomopsis sp.	2
Rhizopus stolonifer	3
**Trichoderma koningii*	1,2
**Trichoderma longibrachiatum*	2

Site J2: stream margin

**Absidia cylindrospora*	1
Aspergillus fumigatus	2
Cladosporium cladosporioides	3
**Diplodinia acerina*	2
Fusarium avenaceum	5
**Fusarium oxysporum*	5
Fusidium griseum	8
Gliocladium roseum	5
**Mortierella elongata*	2,5
Mucor hiemalis	1
**Neosartorya fischeri*	2
**Olpidium* sp.	7
Penicillium brevicompactum	6
**Penicillium simplicissimum*	2
**Pythium intermedium*	5,6
**Pythium irregulare*	5
**Pythium middletonii*	6
Pythium sp. Group HS	1
**Saprolegnia diclina*	6
**Saprlegnia ferax*	6

**Saprolegnia parasitica*	6	*Mucor hiemalis*	1
**Zygorhynchus moelleri*	6	**Neosartorya fischeri*	1
		**Penicillium simplicissimum*	2
Site J3: under *Larix*		**Penicillium spinulosum*	3
Gliocladium roseum	2	**Phialophora malorum*	2
**Mortierella ramanniana*	3	**Trichoderma koningii*	1
Mortierella sp.	3	*Trichoderma viride*	1

DISCUSSION

A total of 58 species of fungi were found, of which 42 (75%) were new records for Slapton, one being a new record for the UK; only 15 species had been previously recorded. While many of these 42 species are widespread fungi, commonly occurring as saprobes on decaying vegetation and in soil, several were important plant pathogens (*Colletotrichum acutatum, Fusarium avenaceum, Fusarium graminearum, Leptosphaerulina trifolii*) and some were unusual species which are rarely isolated into culture (*Acrophialophora levis, Chaetomium convolutum, Mortierella humilis, Scolecobasidium arenarium, Trichosporiella sporotrichoides*). Thirty four species were isolated from one site only, even when identical methods were used. These included some very common saprobes such as *Alternaria alternata, Penicillium citrinum, Cladosporium herbarum, Mucor circinelloides* and *Talaromyces flavus*. Remarkably, only eight species were isolated from two or more sites. Two genera, *Cyphellophora* and *Olpidium,* were recorded for the first time.

While no attempt was made to enumerate or identify all species present in a given sample, rather only those identificable in the 1g sub-sample, critical comparisons among sites are inappropriate. The differences discovered here between samples make it clear that major differences between samples can be expected, and that in order to approach a reasonably comprehensive list of culturable species, an intensive programme of sampling would be required. The shingle beach (and its component seaweeds) and stream margins had the most different species of fungi with many previously unrecorded species, and these sites in particular, would benefit from more detailed study.

The occurrence of the cereal pathogens *Fusarium avenaceum* and *F. graminearum* from these sites, especially from the water samples, indicates that these species may survive away from host crops. Jenkinson & Parry (1994) showed that five species of *Fusarium*, including *F. avenaceum* and *F. graminearum* which were isolated in this study, could be isolated from the roots of perennial plants occurring as weeds in crops in Shropshire. However, they did not find *F. sporotrichoides* (found at Slapton), even though they obtained 226 isolations from 1,346 plants.

Oomycete fungi are often overlooked when environments are sampled to estimate fungal biodiversity, but baiting water samples yielded four species of *Pythium* and three of *Saprolegnia*. The record of *Pythium salinum* is new for the UK and the only record of its occurrence since its description by Höhnk in 1953 at Kiel, Germany. The isolates of *Pythium* and *Saprolegnia* were most probably derived from zoospores, indicating that they were active in the water samples.

Interestingly, most of the *Penicillium* species previously found fruiting on substrata in the reserve (*P. cyclopium, P. digitatum, P. frequentans, P. funiculosum, P. patulum* and *P. variabile*) were not isolated here. This implies that the species isolated may have been present as dormant spores in the soil.

Using a variety of isolation and culture methods, many previously undetected taxa were found in the Reserve, increasing further still the species known at this site. As a consequence, the projected estimate of 2,500 species for this site (Hawksworth, 1994) must be regarded as conservative, and could merit a considerable upwards revision. The contribution of microfungi to estimates of species diversity in this area will be substantial, especially when other rarely examined groups such as entomogenous fungi, endomycorrhizas, endophytes, marine fungi, fungi from birds' nests, chytrids and yeasts have been examined.

If such a large number of previously unrecorded fungal species can be found at Slapton, one of the best-documented and intensively studied sites in the world, the number of species remaining to be found at those sites which have not been studied as intensively must be enormous. Indeed, the estimate of the number of species of fungi in the world (Hawksworth & Colwell, 1992) as 1.5 million is unlikely to be an overestimate.

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