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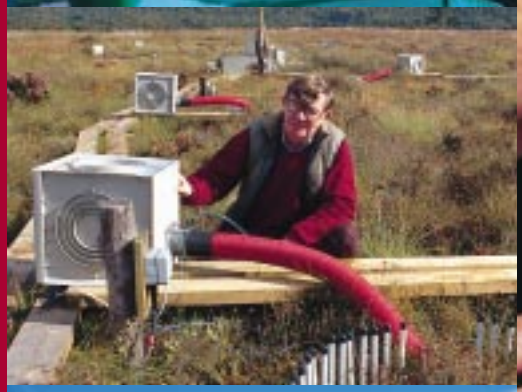

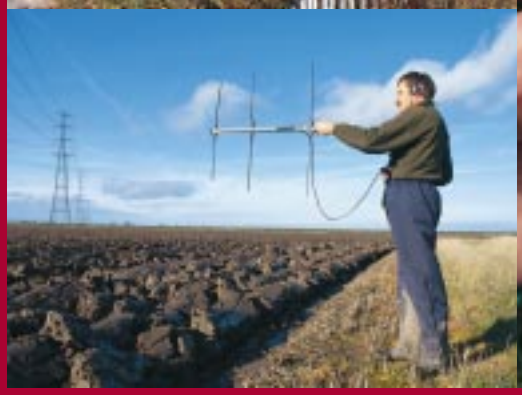

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Population diversity and speciation in *Hydnellum* and *Phellodon* species

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Executive summary

The stipitate hydnoid fungi are a group of ectomycorrhizal fungi associated with conifers and deciduous trees. Fourteen of the British species are listed in the Provisional British Red Data list as either endangered or vulnerable and 12 as extinct or endangered in the Provisional IUCN red data list of European Fungi. In the Biodiversity Action Plan for stipitate hydnoid fungi, the species *Hydnellum aurantiacum* is classified as critically endangered and *Bankera fuligineoalba, H. caeruleum, H. ferrugineum, Sarcodon glaucopus* and *S. scabrosus* are classified as endangered. *Hydnellum concrescens, H. spongiosipes, H. peckii, H. scrobiculatum, Phellodon confluens, P. tomentosus, P. melaleucus* and *S. imbricatus* are considered vulnerable within the BAP context. As a result of these categorisations a Priority Species Action Plan has been developed for the species of hydnoid fungi considered to be threatened.

The project was undertaken to provide baseline information as to the identity of the species of stipitate hydnoid fungi occurring in the UK, information on their genetic diversity, and possible mechanisms for the maintenance and spread of populations. The genera *Hydnellum* and *Phellodon* were selected for this study, and particular consideration was given to potential species pairs in *Hydnellum*. One hundred and two collections of stipitate hydnoid fungi were provided by collectors in the UK from the autumn 2001 and autumn 2002 collecting seasons. Of these, 57 were of species of *Hydnellum,* 36 were of species of *Phellodon,* and nine further collections represented species of *Sarcodon, Bankera* and *Hydnum* (see Table 1).

Genomic DNA was successfully extracted from 29 collections of *Hydnellum* and 6 collections of *Phellodon*. Sequencing of the internally transcribed spacer regions and the 5.8s gene of the ribosomal RNA gene cluster, from 12 collections, showed five distinct lines in the genus *Hydnellum* corresponding to the species *H. concrescens/H. scrobiculatum*, *H. aurantiacum, H. caeruleum*, *H. ferrugineum/H. spongiosipes* and *H. peckii*. Within these lines each individual species could be clearly separated. Comparison of these to database sequences of north American collections of *H. geogenium* and *H. diabolus* suggested that both species were most closely related to *H. peckii*, but the suggested synonymy of *H. diabolus* with *H. peckii* could not be confirmed.

Sequencing of the same regions, from six collections, did not allow detailed conclusions to be made for *Phellodon* species. Specimens of *P. confluens* and *P. melaleucus* were found to be closely related, but distinct, and *P. tomentosus* and *P. niger* also appeared as separate distinct lines. Specimens received as *Phellodon* sp. had sequences that were different from the named species, and different from each other. There are no publicly available reference sequences for any *Phellodon* species for comparison. The limited results obtained here suggest that either the sequences of the rRNA region are very variable in *Phellodon* species, or that a number of undescribed taxa may be present in the genus.

A simplified DNA fingerprinting method was used to investigate heterogeneity in populations of *H. concrescens, H. ferrugineum* and *H. aurantiacum* within and between sites. The results showed that at restricted sites the population of *H. concrescens* can be homogenous, indicating that spread is likely to have been by vegetative mycelial growth below ground. Larger sites supported heterogenous or distinct populations of *H. ferrugineum*

and *H. aurantiacum*, suggesting that mycelial spread may be limited to short distances, that spore spread occurs within sites, or that sites had only recently been colonised.

The results suggest that sites where hydnoid fungi occur will need to be maintained in entirety in order to maintain the genetic diversity of the population. Current classical species concepts in *Hydnellum* are supported by the molecular data, but further work is required to clearly identify the taxa occurring in *Phellodon*. Further sequencing work is also required using English material morphologically identified as *Hydnellum scrobiculatum* to assess similarities with the Scottish specimen sequenced and used in this study.

Contents

1. Project specification

Title – Population diversity and speciation in *Hydnellum* and *Phellodon* species

1.1 Scope of work

To investigate the genetic diversity of *Hydnellum scrobiculatum* and *Phellodon confluens* at and between distinct sites in SE England and Scotland, and to use the molecular diversity measures to suggest the relative roles of mycelial growth and fruiting body production in population maintenance and spread. Speciation within a part of the group will be investigated by comparison of specific DNA sequences.

1.2 Project Objectives

The work programme will focus on the use of molecular markers to determine the variability in local populations of *Hydnellum scrobiculatum* and *Phellodon confluens* at and between distinct sites in SE England and Scotland. DNA extractions will be made from fresh cortex material and where possible from mycelium grown *in vitro* from cortex. Variability will be assessed by obtaining microsatellite DNA fingerprints from multiple specimens collected from each site. Variation in the species concept will be estimated by obtaining DNA sequence data from the internally transcribed spacer (ITS) regions of the ribosomal RNA (rRNA) gene cluster, and comparing these to sequences obtained from a broader range of collections available as existing herbarium specimens. Further analysis of additional gene regions including the rRNA intergeneric spacer and total mitochondrial DNA will be dependent on the initial findings. Sequence information obtained for the ITS regions will also be considered for the potential future development of a species specific PCR amplification method for detection of the fungus in the absence of fruiting body production. Provided suitable fresh material becomes available this will be augmented by comparable sequences from the following ill defined species pairs and their closest relatives: *H. concrescens*/*H. scrobiculatum*, *H. ferrugineum/H. spongiosipes* and *H. auratile*/*H. aurantiacum*.

1.3 Products

The final report should include:

- A review of data from published sources.
- An assessment of the genetic variability of the fungus at the specified sites.
- An assessment of the genetic diversity between populations from different sites in the UK.
- An indication of the potential species of Hydnoid fungi present in the UK.
- Practical guidelines on the likely population and spread mechanisms prevailing at each site, for the sustainable maintenance of diversity.
- An estimation of the potential diversity present in the species, based on comparisons between the project collections and earlier herbarium material.
- The identification of ITS DNA sequences with the potential for further development as a specific molecular detection method.

- Interim and final reports should be produced in line with the attached *English Nature Research Reports - Guidance for authors.*
- Reports should be provided to English Nature on paper and electronically on disk in formats compatible with standard English Nature software (see point 6 ownership and storage of data).

2. Review of available information

The stipitate hydnoid fungi of conservation concern are a small group of stalked mushroom-like fleshy fungi of the order Thelephorales. They are ectomycorrhizal and occur with both conifers and deciduous trees. Fruitbodies usually form on the soil amongst leaf litter during autumn. They have a simple structure especially in hyphal construction and pileal surface, with no protective veil in the developmental stages. The hydnoid hymenophore comprises primarily of geotropic "teeth" that hang vertically below the sporophore (see Pegler *et al* 1997).

Most are not considered edible as they are too tough or bitter to be eaten raw but *Sarcodon imbricatus* has been described as of “esculent quality”(Cooke 1862). Species of the genera *Hydnellum* and *Phellodon* are restricted to the host range of their associated trees, and some species are considered rare. The exact conservation status of stipitate hydnoid fungi is uncertain. They are regarded as widely distributed in temperate regions and many species are found both in North America and Europe (Pegler *et al* 1997). In England they are reported as widely distributed, but rare outside their core areas in southern England (Marren 2000). However, stipitate fungi have been reported to be in decline in Europe during the last 20 years (e.g. Vesterholt & Knudsen 1990) and this has been documented among hydnaceous species in the Netherlands (Arnolds 1989), where five species of *Hydnellum* and *Phellodon tomentosus* are now considered to be extinct, and the Czech Republic (Hrouda 1999). Other studies have found that the decline in hydnaceous fungi may be limited to certain species. Gulden & Hanssen (1992) identified 3 species of *Hydnellum* as in decline in Norway, and Newton *et al* (2002) suggested that only 4 of the 17 species of stipitate hydnoids found in Scotland could be shown to be in decline.

Fourteen of the British species of stipitate hydnoids are listed in the Provisional British Red Data list in the endangered or vulnerable categories (Ing 1992) and 12 as extinct or endangered in the Provisional IUCN red data list of European Fungi (Lizon 1995). In the Biodiversity Action Plan for stipitate hydnoid fungi, *Hydnellum aurantiacum* is classified as critically endangered and *Bankera fuligineoalba, Hydnellum caeruleum, Hydnellum ferrugineum, Sarcodon glaucopus* and *Sarcodon scabrosus* are classified as endangered. *Hydnellum concrescens, Hydnellum spongiosipes, Hydnellum peckii, Hydnellum scrobiculatum, Phellodon confluens, Phellodon tomentosus, Phellodon melaleucus* and *Sarcodon inbricatus* are considered vulnerable within the BAP context. As a result of these categorisations a Priority Species Action Plan has been developed for the species of hydnoid fungi considered to be threatened (Anon 1999).

The distribution of stipitate hydnoid fungi has been studied in some detail in four recent studies. Surveys by the Hampshire Wildlife Trust in 1999 and 2000 found six species at 37 sites, but failed to find four species for which there were historical records from the New Forest area. (Ewald 2000, 2001). A survey of stipitate hydnoid sites in Berkshire detailed 8 species from 31 sites, although in this survey *Phellodon melaleucus* and *P. confluens* were

grouped as a single taxon (Green 2001). In all three studies the most commonly identified species were *H. concrescens*, *H. spongiosipes* and *P. melaleucus*. A fourth study of coniferous forests in Scotland identified 11 species occurring at 30 native forest sites and 11 plantation sites, largely in the east of Scotland. The most common species encountered in these surveys were *Bankera fuligineoalba, Hydnellum peckii, Phellodon tomentosus,* and *Sarcodon imbricatus*, species that are generally associated with conifers and that are rarely found with deciduous trees (Newton *et al* 2002).

Hydnellum

There are a total of 829 records of *Hydnellum* species in the British Mycological Society Foray Record Database (BMSFRD), assigned to 14 different species names. The identity of some of these species and collections is uncertain, and currently 7 species are recognised as occurring in the UK. The accepted species and synonyms as listed in Pegler *et al* (1997) are *H. caeruleum, H. peckii* (syn *H. diabolus*), *H aurantiacum, H. concrescens* (*H. zonatum, H. velutinum* var. *zonatum*), *H. scrobiculatum* (*H. velutinum* var. *scrobiculatum*), *H. ferrugineum* and *H. spongiosipes* (*H. velutinum* var. *spongiosipes*). The most commonly recorded species in the UK are *H. concrescens*, followed by *H. spongiosipes* and *H. scrobiculatum* (Ewald 2000, 2001; Green 2001), although all species with the exception of *H. concrescens* are regarded as rare or uncommon. *H. peckii* is considered to be confined to Scotland. Most species are associated with conifers, particularly pine and spruce, although *H. caeruleum* and *H. scrobiculatum* have been occasionally reported with beech, *H. concrescens* has been associated with both conifers and deciduous trees and *H. spongiosipes* has only been found associated with deciduous trees, particularly oak and beech (Pegler *et al* 1997).

Phellodon

The genus *Phellodon* is described as terricolous and mycorrhizal (Pegler *et al* 1997). There is a total of 601 records of *Phellodon* species in BMSFRD identified to the four species *P. confluens, P. melaleucus, P. tomentosus* and *P. niger*. Species are commonly found in old woodlands, generally associated with deciduous trees including oak, sweet chestnut, birch and beech, although some species occur occasionally with conifers and *P. tomentosus* has almost exclusively been found with conifers. The most commonly recorded species is *P. melaleucus* (Ewald 2000, 2001; Green 2001), which is considered rather common in England, Scotland and Wales (Pegler *et al* 1997) whereas *P. niger* is considered to be rare, and *P. tomentosus* and *P. confluens* are listed as endangered (Ing 1992). There has been some controversy regarding the taxonomy of *P. melaleucus* and *P. confluens* (Green 2001) but Pegler *et al* (1997) regard these as two distinct species.

3. Activity

3.1 Collection of material

The project commenced in late 2001. An initial announcement and request for materials was made informally, largely through British Mycological Society contacts. Collections of *Hydnellum* and *Phellodon* species, generally in fresh condition, were received at RBG, Kew. Collection information as given by original collector was recorded. All fresh material was sampled within 24h of receipt, and original fresh collections were maintained at –40 C.

3.2 Material received

The term of the project from autumn 2001 to spring 2003 included 2 autumn collecting seasons. A total of 102 specimens were received and these are listed in full in Annexe 1, and summarised in Table 1.

3.3 Sample preparation and handling

Clean internal material (approx. 25mm^3) was dissected from stipes or caps of all collections. The dissected material was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. DNA was extracted from ground material using a cetyl-trimethyl ammonium bromide (CTAB) buffer method (Cubero *et al* 1999; Annexe 2). All DNA samples were screened in agarose gels and stored in Tris-EDTA buffer at –20C.

3.4 Molecular characterisation

Two separate DNA based approaches were adopted to meet the project objectives. The relationship between putative species pairs, and species concepts was investigated by comparison of sequences from the internal transcribed spacer (ITS) regions and 5.8s gene in the ribosomal RNA gene cluster (see Figure 1). A direct polymerase chain reaction fingerprinting method was used to investigate genetic variability and population spread at individual sites.

Table 1 Summary of specimens examined

	No of collections	
Species	**2001-02**	**2002**
Hydnellum aurantiacum	1	9
Hydnellum caeruleum	3	
Hydnellum concrescens	13	
Hydnellum ferrugineum	2	8
Hydnellum peckii	6	3
Hydnellum scrobiculatum	5	1
Hydnellum spongiosipes	3	3
Phellodon confluens	9	
Phellodon melaleucus	5	
Phellodon niger	5	1
Phellodon tomentosus	2	4
Others		
Phellodon sp.	10	
Sarcodon scabrosus	1	1
Sarcodon imbricatus		3
Sarcodon glaucopus		2
Bankera fuligineoalba		1
Hydnum repandum	1	

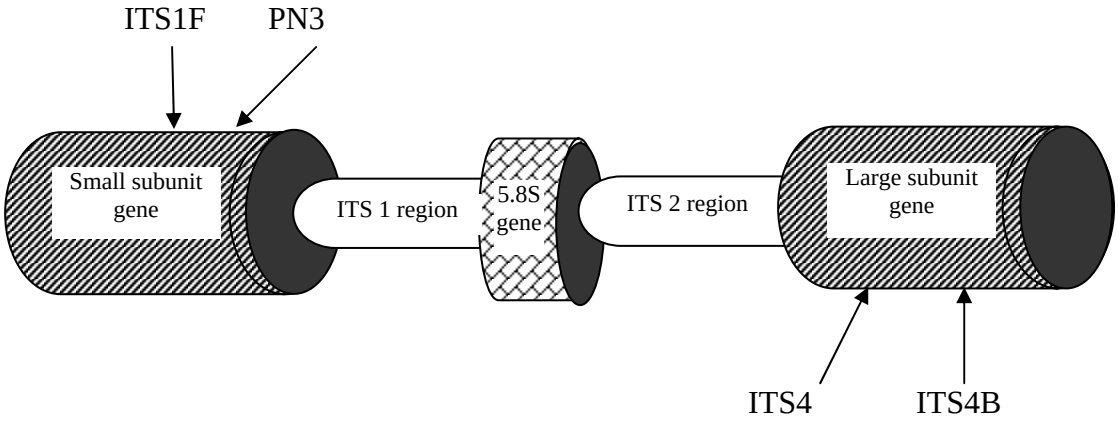

Figure 1 Schematic diagram of ribosomal RNA gene cluster showing location of primer sites

3.5 Ribosomal RNA gene cluster analysis

DNA solutions were used in polymerase chain reactions to obtain complete ITS1-5.8s-ITS2 regions of the ribosomal RNA gene cluster. Pairs of primers were used consisting of one 5' primer (PN3 or ITS1F) and one 3' primer (ITS4 or ITS4B). No single pair of primers was successful in amplifying DNA from all samples and so all 5'/3' combinations were used for each sample (Annexe 2). PCR products were screened in 1% agarose gels in TAE buffer and visualised under UV light after staining with ethidium bromide (0.5µg/ml). Small sections of agarose containing PCR products of the expected sizes (600-900bp) were cut from the gel and re-cast in 1% low melting point agarose and subjected to a further electrophoresis step. The final pure products were cut from the gels and purified with Wizard PCR purification kits (Promega). The final pure products were sequenced through a commercial sequencing service provided by Qiagen (Germany).

3.6 Sequence analysis

PCR products were sequenced in both directions and the two sequences were paired and compared for ambiguity. Final consensus sequences were constructed in 5' to 3' orientation. Where products were obtained from more than one primer pair this process was repeated. All sequences were initially screened through FASTA (Pearson & Lipman 1988) searches of the EMBL DNA database to ensure that the new sequences matched closely to other thelephoroid fungi. DNA sequences were initially aligned with the program CLUSTAL-W (Thompson *et a*l 1994), and then manually edited in JAL-VIEW to give the best alignments. Average distance trees were constructed in JAL-VIEW. Reference sequences for *Hydnellum diabolus* (AF351863), *H. aurantiacum* (AF351866) and *H. geogenium* (AF351868) were downloaded from EMBL to provide further comparisons. All sequence manipulations were carried out interactively through the facilities of the European Bioinformatics Institute (www.ebi.ac.uk).

3.7 Fingerprinting

Simple genetic fingerprints were obtained by direct PCR amplification with single primers corresponding to commonly occurring repetitive DNA sequences according to Bridge *et al* (1997); Annexe 2). PCR products were separated by electrophoresis in 3% NuSieve (2:1). Primers MR, RY and GACA4 were found to give reliable patterns consisting of between 5

and 10 bands per isolate. Final gels were photographed and comparisons were made by direct observation and measurement of band migration distances.

4. Results

4.1 Hydnellum species pairs

DNA sequences from the ribosomal RNA gene cluster were obtained from at least one specimen of all suggested species pairs in *Hydnellum*, with the exception of *H. auratile* for which no material was available.

Specimens sequenced are listed in Table 2. The length of unambiguous sequence obtained varied between 400 and 700bp among specimens, with most ambiguity occurring in the ITS2 regions. After sequences were trimmed to equal lengths for alignment, comparisons between all specimens were made on the basis of the ITS1 and 5.8s gene regions.

The *Hydnellum* sequences were aligned together with the reference sequences from *H. aurantiacum* and *H. geogenium*. The resulting average distance tree showed five main lines, with *H. diabolus* loosely linked at the base of the tree. The placement of *H. diabolus* is incorrect in this tree, as the EMBL database sequence did not contain a full ITS1 sequence and so the full sequence could not be adequately aligned with the others. This sequence is considered separately in the species variation section.

Table 2 Specimens of *Hydnellum* species sequenced

H6	*H. concrescens*
H6 (duplicate)	*H. concrescens*
H25	*H. concrescens*
H25 (duplicate)	*H. concrescens*
H26	*H. concrescens*
H29	*H. spongiosipes*
H47	*H. caeruleum*
H48	*H. caeruleum*
H50	*H. aurantiacum*
H51	*H. ferrugineum*
H52	*H. ferrugineum*
H55	*H. peckii*
H67	*H. scrobiculatum*
H98	*H. spongiosipes*

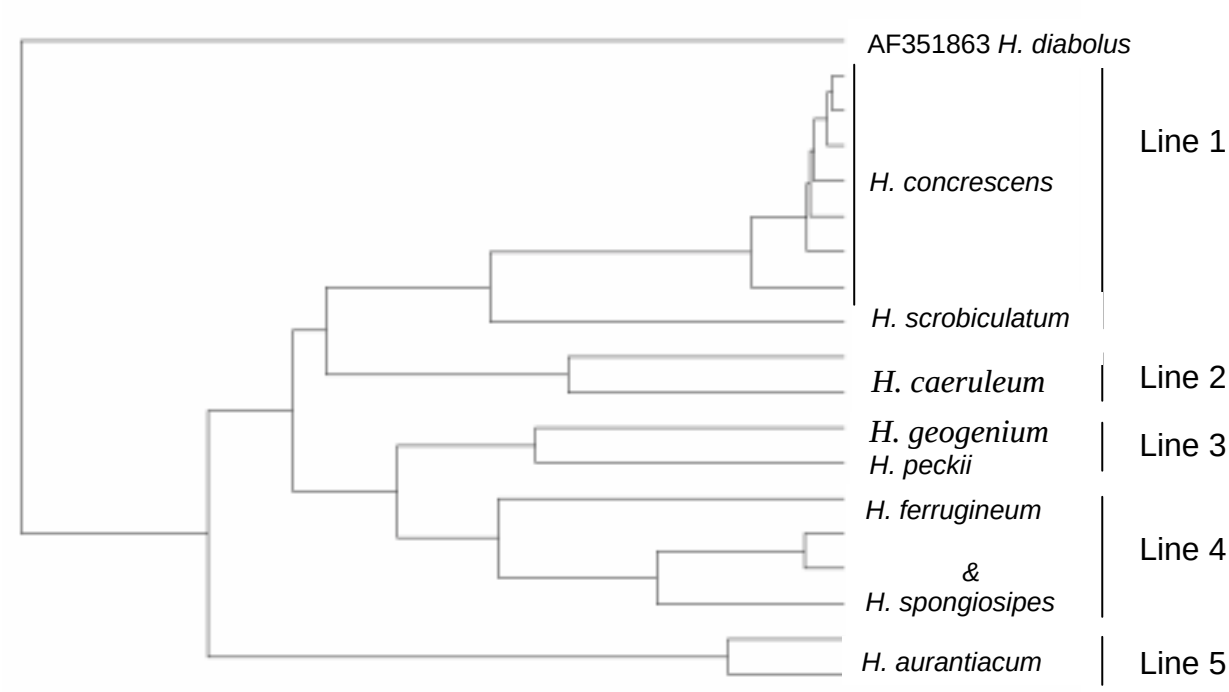

Figure 2 Alignment of *Hydnellum* specimens based on ITS1 and 5.8s sequences

Line 1 contained all sequences derived from *H. concrescens* specimens, together with the sequence from the specimen of *H. scrobiculatum* that was loosely linked to these. Line 2 contained the two sequences obtained from *H. caeruleum*. Line 3 comprised two relatively deep branches representing the sequence for *H. peckii* and the database sequence for *H. geogenium*. Line 4 contained two sequences from *H. ferrugineum* and the two sequences from *H. spongiosipes*. Line 5 was distinct from the other 4 lines and consisted of the sequence from *H. aurantiacum* and the database sequence of *H. aurantiacum*. As there was considerable variation between sequences in different lines that could affect close alignments, the relationships within each species line were considered by making further alignments restricted to only those specimens.

4.2 Hydnellum species concepts

Line 1 consisted of eight sequences derived from four specimens of *H. concrescens* and one from *H. scrobiculatum* that aligned near to them (see Figure 3). The sequences from different specimens of *H. concrescens*, and sequences derived from duplicated DNA extractions showed a small amount of variation, but overall the *H. concrescens* sequences were between 93 and 99% similar (see Table 3). Specimens 25 and 26 were collected in the New Forest, and specimen 6 was collected from Windsor Forest, and it would therefore seem likely that the 1-7% variation seen between these sequences is representative of both sequencing error, and within and between site variation. The sequence from *H. scrobiculatum* however differed quite markedly from these and was between 78 and 81% similar to the individual *H. concrescens* sequences. This result would suggest that *H. scrobiculatum* and *H. concrescens* are two closely related but distinct species.

Table 3 Similarity values between sequences in *H. concrescens*/*H. scrobiculatum* line.

H6							
H25H	97.5						
H25PN	97.9	98.2					
H26H	97.5	98.2	97.5				
H25HE	98.2	98.9	98.9	98.6			
H26PN	98.2	98.2	99.3	97.5	98.9		
H6PN	95.7	93.3	94.3	93.3	93.9	93.9	
H67	81.1	79.2	79.2	79.2	79.2	79.6	78.1
	H6	H25	H25PN	H26H	H25HE	H26PN	H6PN

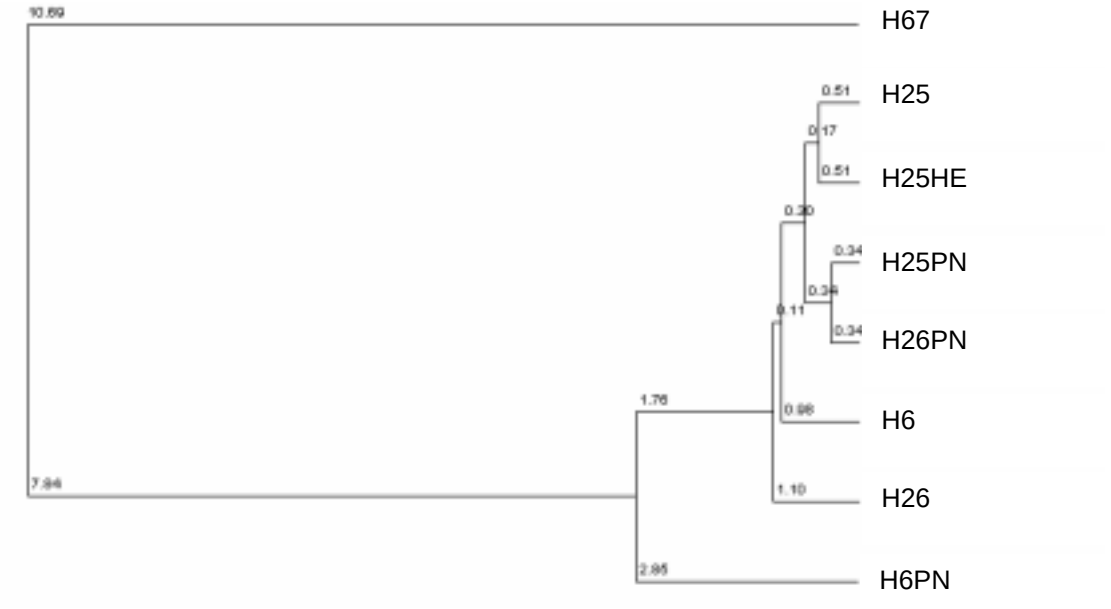

Figure 3 Average distance tree of sequences in *H. concrescens*/*H. scrobiculatum* line.

Line 2 consisted of two sequences derived from two specimens of *H. caeruleum* collected in Scotland. This species was most closely related to the *H. concrescens*/*H. scrobiculatum* line, but was clearly distinct from it. The two sequences were 97.2% similar, a figure that falls well inside the expected intra-specific variation seen in *H. concrescens*.

Line 3 consisted of two sequences, one derived from a specimen of *H. peckii*, and one obtained from the EMBL database from *H. geogenium*. The sequence from *H. diabolus* had originally grouped some distance from these sequences in the full alignment. This placement may however be due to the later primer used by the original depositors, that failed to amplify the beginning of the ITS1 region. *H. diabolus* has however been suggested as a synonym of *H. peckii* (Maas Geesteranus 1969) and this sequence was included in line 3 in the detailed alignment (see Figure 4). In this alignment the sequences of the three species were between 85 and 92% similar (see Table 4). These figures indicate that these three species are probably closely related. The higher figure seen for the comparison of sequences from *H. diabolus* and *H. peckii* gives some support to the synonymy of the two species, although the 92% similarity is a little low when compared to other species. However, in this case the sequence of *H. diabolus* was obtained from EMBL and had originally been derived from a collection in a US laboratory. It would seem reasonable to assume that there would be greater genetic variation between collections from different continents than collections from the same country, and

this may account for the reduced similarity. Conversely, the *H. diabolus* sequence did not include all of the variable ITS1 sequence, and as a result a greater proportion of the sequence compared was made up of the more conserved 5.8s gene sequence. This in turn could result in an artificially high similarity value, and would also account for the relatively high values seen for the comparisons with *H. geogenium*. It is therefore not possible to determine if *H. diabolus* and *H. peckii* are synonymous until further sequence data is available from the more variable regions of the gene cluster.

Table 4 Similarity values between sequences in *H. diabolus*/*H.peckii*/*H. geogenium* line.

H55 *H. peckii*			
H. diabolus	91.7		
H geogenium	84.9	87.1	
	H55 *H. peckii*	*H. diabolus*	*H. geogenium*

Figure 4 Average distance tree of sequences in *H. diabolus/H. peckii/H. geogenium* line.

Line 4 consisted of two sequences obtained from specimens of *H. spongiosipes* and two sequences obtained from specimens of *H. ferrugineum*. The *H. ferrugineum* sequences were obtained from two collections from the same location in Scotland, and were 99.2% similar to each other. The two *H. spongiosipes* sequences were obtained from collections from the New Forest and Kent, and these were 89.5% similar to each other. The similarities between the sequences of the two different species ranged from 81.7 to 86.8% (see Table 5).

Table 5 Similarity values between sequences in *H. ferrugineum*/*H. spongiosipes* line.

H51 *H. ferrugineum*				
H52 *H. ferrugineum*	99.2			
H29 *H. spongiosipes*	86.8	86.4		
H98 *H. spongiosipes*	81.7	82.1	89.5	
	H51	H52	H29	H98

Although the sequences were recovered as a mixed group in the overall alignment (Figure 2), they formed two distinct lines when aligned separately (see Figure 5). It would seem likely from this that as with the *H. concrescens*/*H. scrobiculatum* line, these two species are closely related but distinct taxa.

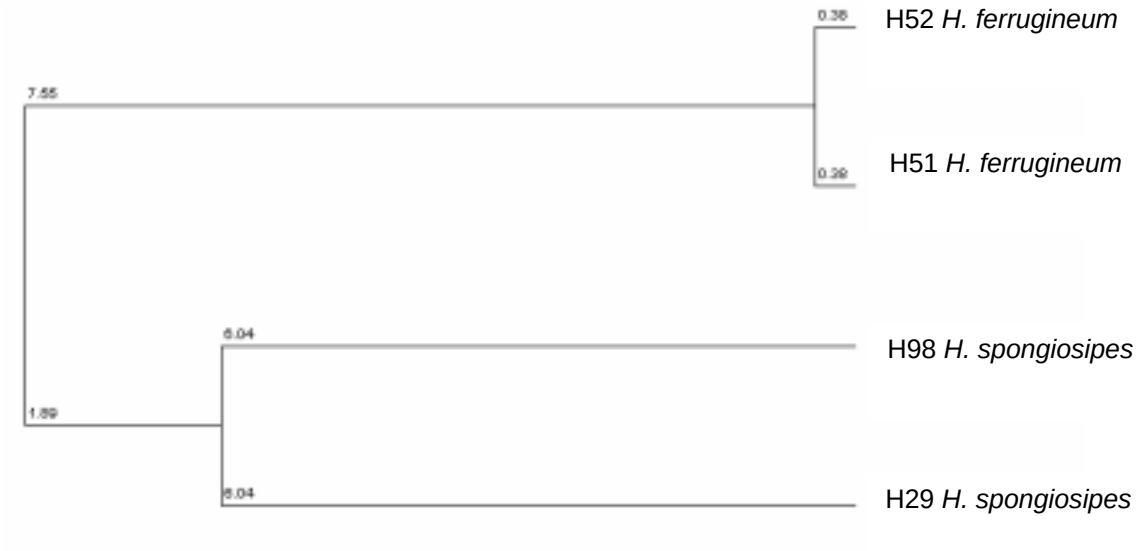

Figure 5 Average distance tree of sequences in *H. ferrugineum/H. spongiosipes* line.

Line 5 consisted of two sequences from *H. aurantiacum*, one derived from a collection from Scotland, and one database sequence derived from a collection in a US laboratory. The sequences were 90.3% similar when aligned.

Overall the results of the DNA sequencing can be used to provide some preliminary delineation of individual species and species pairs. It would appear that there may be some variation in the ITS1/5.8s regions of the ribosomal RNA gene cluster between isolates of the same species, and that this can be as high as 10% when specimens from different countries or continents are compared. Closely related species vary by approximately 15 to 20% in the same gene region, and overall the different species within the genus show around 30% variation. On this basis the species pairs of *H. concrescens*/*H. scrobiculatum* and *H. ferrugineum*/*H. spongiosipes* are two groups that each consists of two closely related, but distinct species. This finding may suggest that each species pair had a relatively recent common ancestor. These four species, together with *H. peckii, H. caeruleum* and *H. aurantiacum* comprise the seven species of *Hydnellum* that were collected in the UK during this study. The situation regarding *H. peckii* and *H. diabolus* is however less clear. Although collections of these two species from different continents had less than 10% sequence variation, this figure may be artificially low due to the restricted sequence data for the ITS1 region of the *H. diabolus* sequence available. As a result of this a definite conclusion cannot be made, but the results obtained here would suggest that these are closely related species, but more information is required before their possible synonymy can be determined.

4.3 Phellodon sequence analysis

In general the extraction and amplification of DNA from collections of *Phellodon* species was less successful than for *Hydnellum*. DNA extractions from *Phellodon* specimens were always strongly pigmented, and this pigmentation was not removed during DNA purification. This may indicate the presence of a co-extracted contaminating substance that may have prevented efficient DNA amplification. It was however possible to obtain usable PCR products from six specimens and two duplicate collections (see Table 6).

Table 6 Specimens of *Phellodon* species sequenced

H2	*P. melaleucus*
H2 (duplicate)	*P. melaleucus*
H32	*Phellodon* sp.
H32 (duplicate)	*Phellodon* sp.
H34	*Phellodon* sp.
H41	*P. confluens*
H85	*P. tomentosus*
H102	*P. niger*

The sequences from the *Phellodon* specimens were aligned together. Unlike the *Hydnellum* species there were no reference sequences available in EMBL databases for any *Phellodon* species. The initial alignment of sequences was used to construct an average distance tree, and the eight sequences were recovered in this as four distinct lines (see Figure 6).

Line 1 consisted of the sequence from the single specimen of *P. tomentosus*. This was the most divergent of the sequences obtained for this genus, and the sequence also showed little homology with others in the EMBL database. There was however sufficient homology in the 5.8s region to confirm its relation to the other sequences from *Phellodon* species obtained in this study.

Line 2 consisted of the three sequences obtained from unidentified specimens received as *Phellodon* sp. The two sequences from duplicate collections were 99.6% similar, but the similarity between the two different specimens was considerably lower at 89.4% (see Table 7). On the basis of the variability seen in the *Hydnellum* sequences this figure would be border-line for considering the two specimens as a single species.

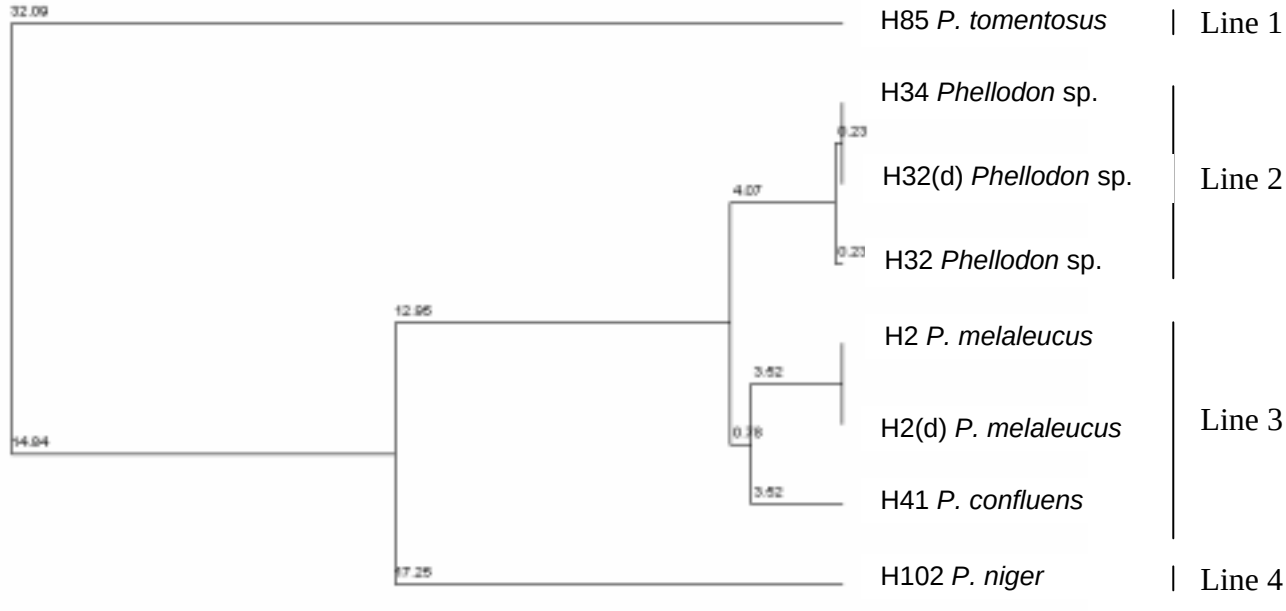

Figure 6 Average distance tree of sequences from *Phellodon* specimens.

Line 3 consisted of 2 sequences from duplicate collections of *P. melaleucus*, and the sequence obtained from the collection of *P. confluens*. The sequences from the duplicate collections of *P. melaleucus* were 99.2% similar and the *P. confluens* sequence was 85.7 and 86.1% similar to these (Table 7). These levels of similarity suggest that *P. confluens* is closely related to *P. melaleucus*, but that the two taxa are distinct. There was however some overlap in similarity and in some insertion sequences in these and the sequences from line 2, and an alternative explanation may be that the sequences in lines 2 and 3 represent the extremes of a single, highly variable taxon. Neither possibility can be unequivocally accepted until further sequences are available from a wider range of collections.

Table 7 Similarity values between sequences in *P. melaleucus*/*P. confluens* and *Phellodon* sp. lines.

H2	*P. melaleucus*						
H2E	*P. melaleucus*	99.2					
H32	*Phellodon* sp.	83.6	84.1				
H32E	*Phellodon* sp.	83.6	84.1	99.6			
H34	*Phellodon* sp	75.6	75.8	89.4	89.4		
H41	*P. confluens*	85.7	86.1	78.7	78.7	78.7	71.3
		H2	H2E	H32	H32E	H34	H41

Line 4 consisted of the single sequence from a collection of *P. niger*. This was distinct from the other species lines, and more similar to *P. melaleucus* than to *P. tomentosus*.

Overall the results from the analysis of the *Phellodon* species are less conclusive than those obtained with *Hydnellum*. This is due in part to the difficulties experienced in obtaining good DNA preparations from these species, and also the lack of any reliable reference sequences. It would appear that there is greater sequence variation between species in *Phellodon* than there is in *Hydnellum*, but this will need confirmation by sequencing from further named specimens. The distinct nature of the sequences from the specimens labelled as *Phellodon* sp. would suggest that some further basic taxonomic study is required in this genus.

When the sequences obtained here were compared against those in the EMBL database, the best matches were obtained against *Sarcodon* species. However the sequences from the *Sarcodon* species were distinct and when aligned with the *Phellodon* species (Figure 7), the two genera were recovered as two separate lines, with around 70-75% similarity between them.

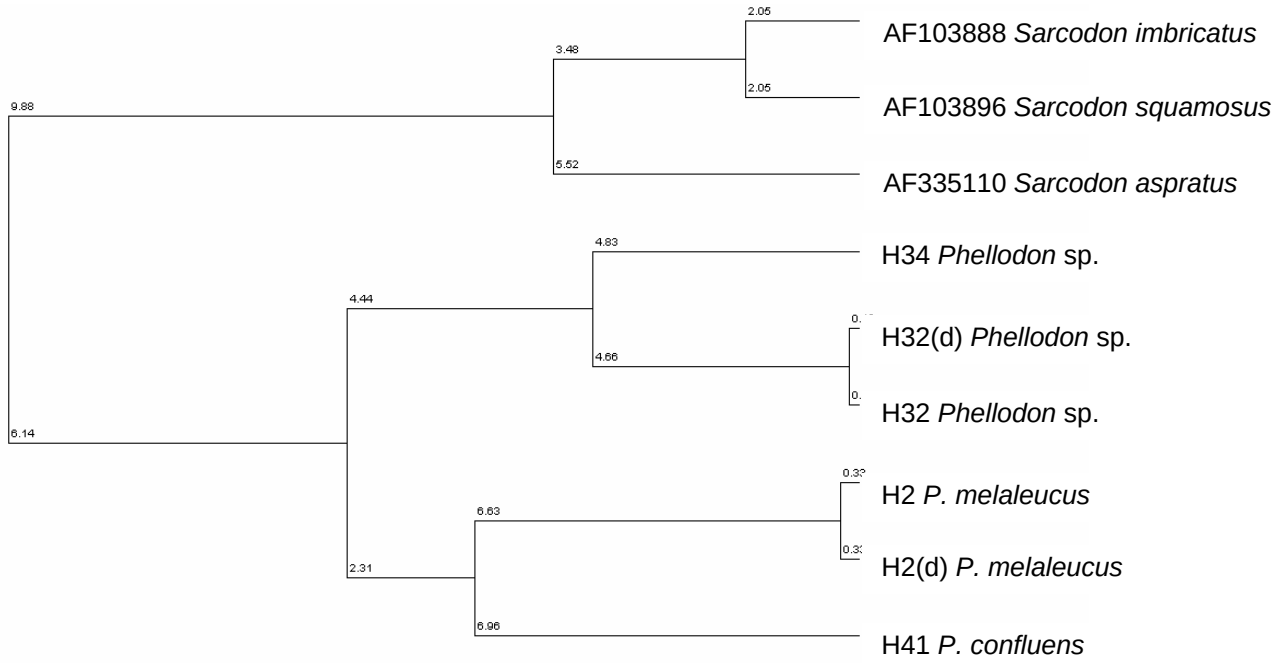

Figure 7. Average distance tree of sequences from *Phellodon* species aligned with reference sequences from *Sarcodon* species.

4.4 Population analysis

Sites were selected where several collections of the same species had been made. Simple DNA based fingerprints were derived for each of these collections in order to compare the diversity of the species at each site. The species analysed and the sites selected were *H. concrescens* from nearby sites in the New Forest (SU269081 & SU248067), and *H. aurantiacum* and *H. ferrugineum* from sites located around Loch an Eilein in Scotland.

Hydnellum concrescens

DNA was extracted from the four specimens (H23-H26) of *H. concrescens* obtained from site SU269081 (see Figure 8), and a further specimen of *H. concrescens* (H28) obtained from a separate site nearby (SU248067). Electrophoresis of the PCR amplification products for each of the primers RY, MR and GACA4 (see Annexe 2) gave between 7 and 12 brightly stained bands. The PCR products obtained for specimens H23-26 gave a single unique pattern with each primer, and these were different from the patterns obtained from H28 with each primer.

H. aurantiacum

H. aurantiacum specimens from 3 sites on the north shore of Loch an Eilein were analysed (sites 1, 2 & 4, Figure 9). The band patterns obtained with primer RY were the same for all isolates. Electrophoresis of the PCR products from amplification with primer GACA4 gave 4 different, but similar patterns. Specimens H82 & 83 from site 4 showed one pattern, that was similar to but different from that shown by specimens H71, 72 & 73 from the same site. Specimens H74 & 75 from site 1 showed a similar pattern to H71-73, but both differed from

this due to the absence of a single band. Specimen 76 showed a unique band pattern. These results were supported by the patterns obtained with primer MR. These results suggest that *H. aurantiacum* specimens at different sites are different from each other, and that a single site may contain a number of genetically distinct individuals, as with site 4.

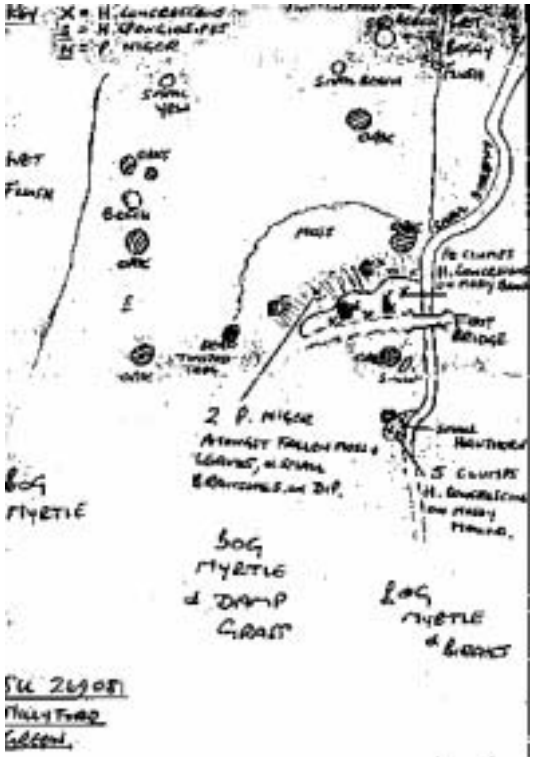

Figure 8 Sketch plan of site at SU269081 (supplied by N. Ewald).

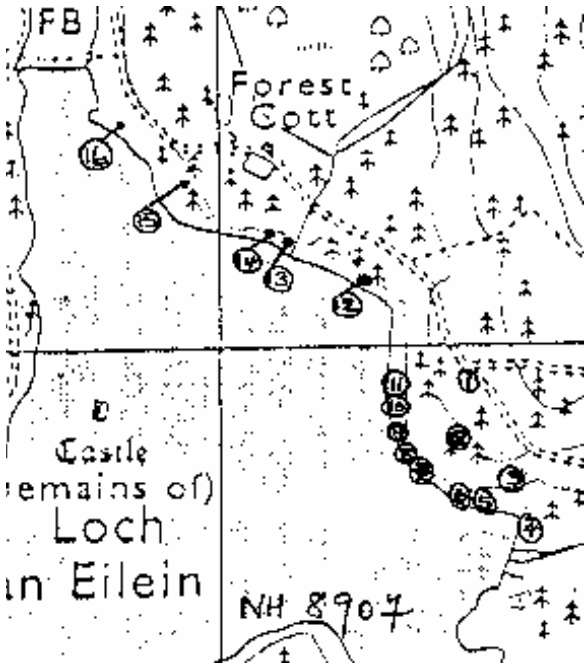

Figure 9 Map of sites for *Hydnellum* species at Loch an Eilein (supplied by E & V Emmett)

H. ferrugineum

Eight specimens of *H. ferrugineum* were analysed from seven sites on the north shore of Loch an Eilein (sites 5, 6, 8, 9, 10, 11 & 14, see Figure 9). Band patterns obtained with the three primers were very heterogeneous for the specimens. There were five patterns with primer MR, five patterns with GACA4 and seven patterns with primer RY. Results are shown in Figure 10, and are summarised in Table 8, and suggest that all of the specimens of *H. ferrugineum* collected, both at and between sites, are from genetically different origins.

Table 8 DNA fingerprinting results for *H. ferrugineum*.

Specimen	Site	Pattern with primer MR	Pattern with primer GACA4	Pattern with primer RY
H81	6	Unique	Unique	Unique
H88	14	Unique	Unique	Unique
H89	8	Unique	Unique	Unique
H90	8	Unique	Unique	Unique
H91	9	Pattern A	Pattern B	Unique
H92	10	Pattern A	Pattern B	Unique
H93	11	Pattern A	Pattern B	Unique
H94	5	Pattern A	Pattern B	Unique

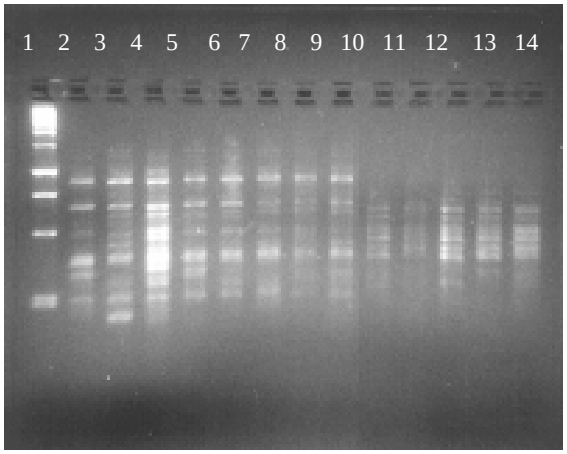

Figure 10a PCR products from DNA fingerprinting of *H. ferrugineum*. Lane 1, molecular size markers; lanes 2-9 specimens 81 & 88-94 with primer MR; lanes 11-16 specimens 81 & 88-91 with primer GACA4

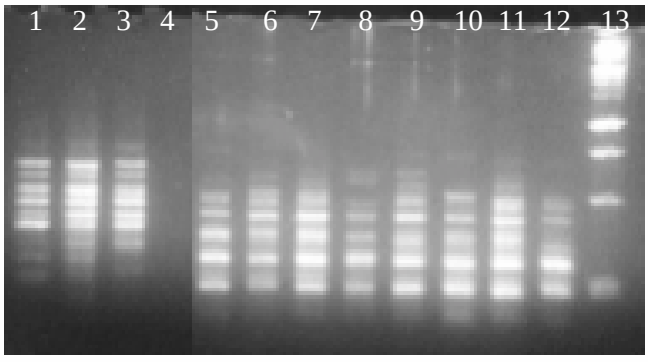

Figure 10b PCR products from DNA fingerprinting of *H. ferrugineum*. Lanes 1-3 specimens 92-94 with primer GACA4; lanes 5-12 specimens 81 & 88-94 with primer RY; lane 13 molecular size markers.

5. Conclusions

5.1 Species concepts

The different *Hydnellum* species names used for the specimens analysed here appear to represent distinct taxa. The suggested species pairs of *H. concrescens*/*H. scrobiculatum* and *H. ferrugineum*/*H. spongiosipes* are two pairs of closely related but distinct taxa. A fifth distinct species *H. peckii* was also collected during the study, and the preliminary analysis based on the single reference sequence available suggests that this species may be closely related to the North American species *H. diabolus*.

The taxonomic situation within the genus *Phellodon* is, however, less clear. Specimens received as *P. melaleucus* and *P. confluens* had similar but distinct rRNA sequences, a finding that supports their separation as two taxa. However further specimens identified to only genus level had sequences that were different from each other and also different from those of named collections of the four known British species. This suggests that either there is considerable sequence variability within *Phellodon* species, or that there are two or more taxa occurring in the UK that have yet to be described. Problems in obtaining good DNA preparations from the *Phellodon* specimens, and the lack of any published reference sequences for the genus prevent definitive conclusions being made.

5.2 Population analysis

The DNA fingerprinting method used is one that has previously been used to show clonal lines of fungal populations (see Bridge *et al* 1997), and would be expected to be affected by crossover, thus allowing interpretations to be made as to the roles of vegetative growth and recombination (Bridge *et al* 2003). The results obtained showed a number of different situations for each of the species studied. At a New Forest site all collections of *H. concrescens* were genetically identical, and different from a single collection made at a nearby site. Assuming spore production always produces genetically variable progeny in this species, the identical fruitbodies would suggest that the fungus was maintained by vegetative spread, probably as mycelium in the soil. This would seem the most likely explanation as all of the collections were made from a single mossy bank between two oak trees. The fingerprints from *H. aurantiacum* at Loch an Eilein also showed that fruitbody collections from different sites were from genetically different origins. However in this case more than one individual was present at a single site (site 4). This would suggest that the population at that site had either resulted from two or more initial colonisation events or that recombination was taking place, and that spore dispersal was a factor within the site. This finding was repeated with the *H. ferrugineum* collections where again different individuals were identified both at and between different sites.

There are some differences between the environments of the New Forest and Scottish sites that may provide some explanation for these results. The New Forest site was apparently isolated, and collections were made from a relatively small area on one moss bank. The Scottish sites were spread over a 800-900m length of shoreline, and individual sites were between 4 and 10m^2 in area (see Figures 9 & 11), all distances considerably greater than those assumed for the New Forest site. A possible explanation is that individual fungi spread at a single location by vegetative growth, and that this spread is over only a relatively limited

distance. Therefore a small site could represent a single colonisation event, and a larger site would be due to two or more colonisations that may have occurred at different times. The effect of time on sites may also be significant. It has been found for some basidiomycete fungi, such as *Heterobasidion annosum*, that the initial colonisation of an area is due to a spore inoculum that gives rise to considerable genetic heterogeneity. In time the faster growing and/or better adapted individuals from this population become dominant, and the subsequent maintenance and spread of these individuals leads to a reduction in the heterogeneity (see Korhonen & Stenlid 1998). In this study no information was requested on the length of time that *Hydnellum* species had been recorded at individual sites, and it may be that the New Forest site had been established for longer than the Scottish ones.

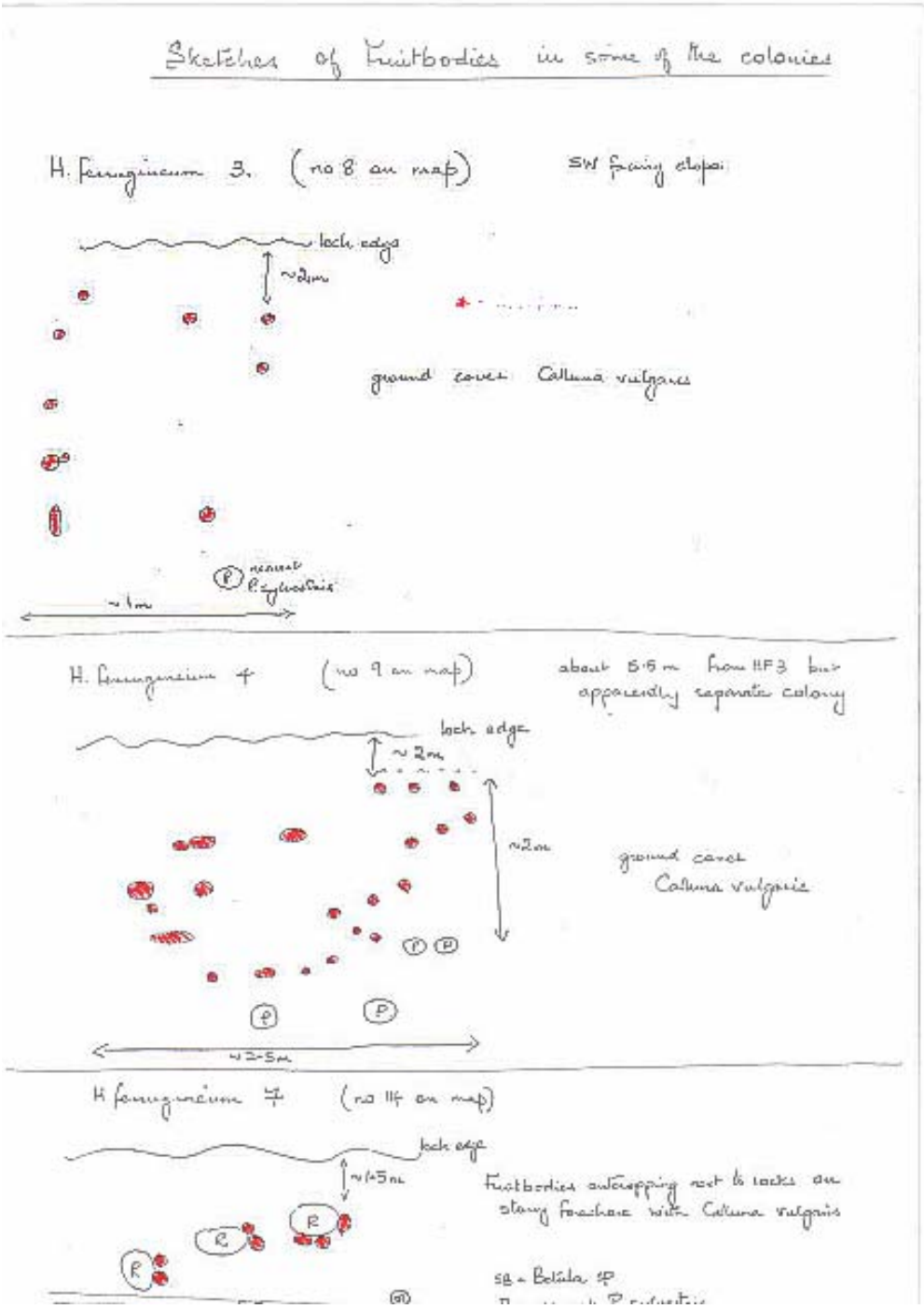

Figure 11 Details of selected Scottish sampling sites

5.3 Implications for conservation

The good correlation between the classical species names in *Hydnellum* and the molecular data indicates the accuracy of the current species concepts and identification methods. The clear differences in sequence similarity at the inter- and intra-specific level may suggest that species specific sequence may occur in the rRNA region of *Hydnellum* species, and that there may be potential to develop a relatively simple PCR based diagnostic for detecting *Hydnellum* species in soil. A limitation to this at present is the relatively small number of reference sequences available, and this is further complicated by the publication of several sequences as unidentified thelephoroid mycorrhizae.

In *Phellodon* the poor alignment and grouping of the available sequences and the lack of suitable reference sequences seriously limits the implications that can be made. The recovery of the *Phellodon* sequences as a single distinct group, separate from *Sarcodon*, shows the integrity of the genus. However the variability of the sequences obtained also suggests that species concepts are not clear, and this is supported by the inclusion of specimens received as *Phellodon* sp. The implication of these results is that further work is required to determine the taxa within the genus before conservation issues can be fully considered.

Previous studies with basidiomycete fungi have shown both heterogeneity and homogeneity at single sites (e.g. Korhonen & Stenlid 1998, Xu *et al* 2002). These results may be due to the initial colonisation of a site and the subsequent development of the population, and may also be expected to be dynamic in respect to time. The fungi considered here are largely ectomycorrhizal and so could be expected to spread by mycelial growth along roots. This mode of growth does not always lead to homogeneity among neighbouring fruitbodies and Perotto *et al* (1996), studying the ericoid mycorrhizal system, have shown that different populations of a single fungal species can be maintained on the roots of a single plant. The results obtained here have shown both homogeneity and heterogeneity, for different species. One explanation for this may be that not all of the species are entirely mycorrhizal, and that different mechanisms (spore dispersal vs vegetative spread) may be employed by different species. Notes included with some of the Scottish collections refer to different clumps of fruitbodies at some sites (see Figure 11), and another possible explanation is that mycelial spread is limited to the short distances required to form small groups of fruitbodies, and that spore dispersal is necessary for the fungus to spread from these. This explanation is also partly supported by the relative differences in sizes of the site where the homogenous populations were obtained, and the larger sites where heterogenous populations were obtained. Limited mycelial spread and spore dispersal over larger distances may have implications for collecting at sites, as removal of fruitbodies prior to sporulation would restrict the subsequent spread of the fungus in that area. The finding of populations of genetically different individuals at some sites may also have implications for site management, as the loss of part of a site may result in a loss of some of the genetic variation present in the population established there.

One question that could not be answered in a short project is the influence of time on the establishment and diversity of populations. An alternative explanation to the above may be the relative age of the colonisation, but this could only be investigated by long term monitoring and sampling of selected sites.

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Annexe 1 Fungal specimens received

Project number	Received as	Collector	Location	Notes
H1	*P. confluens*	M. Ainsworth	Windsor Forest	
H2	*P. melaleucus*	M. Ainsworth	Windsor Forest	
H3	*Phellodon sp.*	M. Ainsworth	Windsor Forest	
H4	*H. concrescens*	M. Ainsworth	Windsor Forest	
H5	*H. concrescens*	M. Ainsworth	Windsor Forest	
H6	*H. concrescens*	M. Ainsworth	Windsor Forest	
H7	*P. confluens*	M. Ainsworth	Windsor Forest	
H8	*Sarcodon scabrosus*	M. Ainsworth	Windsor Forest	
H9	*P. confluens*	M. Ainsworth	Windsor Forest	
H10	*H. concrescens*	M. Ainsworth	Windsor Forest	
H11	*H. concrescens*	M. Ainsworth	Windsor Forest	
H12	*P. confluens*	M. Ainsworth	Windsor Forest	
H13	*P. melaleucus*	M. Ainsworth	Windsor Forest	
H14	*P. niger*	M. Ainsworth	Windsor Forest	
H15	*P. confluens*	M. Ainsworth	Windsor Forest	
H16	*P. confluens*	M. Ainsworth	Windsor Forest	
H17	*P. confluens*			K(M) 90698
H18	*H. concrescens*			K(M) 90731
H19	*Hydnum repandum*			K(M) 90186
H20	*P. melaleucus*	N. Ewald	New Forest	Sketch map supplied A
H21	*P. melaleucus*	N. Ewald	New Forest	B
H22	*P. melaleucus*	N. Ewald	New Forest	C
H23	*H. concrescens*	N. Ewald	New Forest	SU269081 Site A
H24	*H. concrescens*	N. Ewald	New Forest	SU269081 Site B
H25	*H. concrescens*	N. Ewald	New Forest	SU269081 Site C
H26	*H. concrescens*	N. Ewald	New Forest	SU269081 Site D
H27	*H. spongiosipes*	N. Ewald	New Forest	SU269081 Site E
H28	*H. concrescens*	N. Ewald	New Forest	SU248067 Site A
H29	*H. spongiosipes*	N. Ewald	New Forest	SU248067 Site B
H30	*Phellodon sp.*	N. Ewald	New Forest	SU248067
H31	*Phellodon sp.*	N. Ewald	New Forest	SU248067
H32	*Phellodon sp.*	N. Ewald	New Forest	SU248067
H33	*Phellodon sp.*	N. Ewald	New Forest	SU248067
H34	*Phellodon sp.*	N. Ewald	New Forest	SU248067
H35	*Phellodon sp.*	N. Ewald	New Forest	SU248067
H36	*H. spongiosipes*	N. Ewald	New Forest	SU248067
H37	*P. niger*	S. Evans	New Forest	Rufus Stone
H38	*P. niger*	S. Evans	New Forest	Rufus Stone
H39	*H. concrescens*	N. Ewald	New Forest	3579398466
H40	*H. concrescens*	N. Ewald	New Forest	3572698466
H41	*P. confluens*	N. Ewald	New Forest	3572698429
H42	*P. confluens*	N. Ewald	New Forest	3572698429
H43	*Phellodon sp.*	P. Eade	Kent	Hosey Common
H44	*Phellodon sp.*	P. Eade	Kent	Hosey Common
H45	*Phellodon sp.*	P. Eade	Kent	Hosey Common
H46	*H. scrobiculatum*	E. Holden	Scotland	NH994199 Colln. A
H47	*H. caeruleum*	E. Holden	Scotland	Mar Lodge Colln. B
H48	*H. caeruleum*	E. Holden	Scotland	Mar Lodge Colln. C
H49	*H. caeruleum*	E. Holden	Scotland	Mar Lodge Colln. D
H50	*H. aurantiacum*	E. Holden	Scotland	Mar Lodge Colln. E
H51	*H. ferrugineum*	E. Holden	Scotland	Mar Lodge Colln. F

Project number	Received as	Collector	Location	Notes
H52	*H. ferrugineum*	E. Holden	Scotland	Mar Lodge Colln. G
H53	*H. peckii*	E. Holden	Scotland	Loch Fleet Colln. H
H54	*H. peckii*	E. Holden	Scotland	Nethy Bridge Colln. I
H55	*H. peckii*	E. Holden	Scotland	Mar Lodge Colln. J
H56	*H. peckii*	E. Holden	Scotland	Mar Lodge Colln. K
H57	*H. peckii*	E. Holden	Scotland	Dinnet Colln. L
H58	*H. peckii*	E. Holden	Scotland	Mosach Colln. M
H59	*H. scrobiculatum*	E. Holden	Scotland	Dinnet NJ454011 Colln. N
H60	*P. tomentosus*			K(M) 40055
H61	*P. tomentosus*			K(M) 40042
H62	*P. niger*			K(M) 40025
H63	*P. niger*			K(M) 40047
H64	*H. scrobiculatum*			K(M) 20362
H65	*H. scrobiculatum*			K(M) 38649
H66	*H. scrobiculatum*	E. Holden	Scotland	Inveray Youth Hostel NJ077897
H67	*H. scrobiculatum*	E. Holden	Scotland	Abernethy NJ024160
H68	*H. peckii*	E. Holden	Scotland	Abernethy NJ024160
H69	*H. peckii*	E. Holden	Scotland	Lairg NH923080
H70	*H. aurantiacum*	E. Holden	Scotland	Lairg NH921092
H71	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 4, spec 1
H72	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 4, spec 2
H73	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 4, spec 3
H74	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 1, spec 1
H75	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 1, spec 2
H76	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 2
H77	*Sarcodon imbricatus*	V&E Emmett	Scotland	Loch an Eilein site 12, spec 1
H78	*Sarcodon imbricatus*	V&E Emmett	Scotland	Loch an Eilein site 12, spec 2
H79	*P. tomentosus*	V&E Emmett	Scotland	Loch an Eilein site 13, spec 1
H80	*P. tomentosus*	V&E Emmett	Scotland	Loch an Eilein site 13, spec 2
H81	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 6
H82	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 4, spec 3
H83	*H. aurantiacum*	V&E Emmett	Scotland	Loch an Eilein site 4, spec 4
H84	*Sarcodon imbricatus*	V&E Emmett	Scotland	Loch an Eilein site 12, spec 2
H85	*P. tomentosus*	V&E Emmett	Scotland	Loch an Eilein site 7, spec 1
H86	*P. tomentosus*	V&E Emmett	Scotland	Loch an Eilein site 7, spec 2
H87	*H. peckii*	V&E Emmett	Scotland	Loch an Eilein site 16
H88	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 14
H89	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 8, spec 1
H90	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 8, spec 2
H91	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 9
H92	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 10
H93	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 11
H94	*H. ferrugineum*	V&E Emmett	Scotland	Loch an Eilein site 5
H95	*Sarcodon glaucopus*	V&E Emmett	Scotland	Loch an Eilein site 3, spec 1
H96	*Sarcodon glaucopus*	V&E Emmett	Scotland	Loch an Eilein site 3, spec 2
H97	*Bankera fuligineoalba*	V&E Emmett	Scotland	Loch an Eilein site 15
H98	*H. spongiosipes*	J. Pitt	Kent	Pembury walks
H99	*Sarcodon scabrosus*	J. Pitt	Kent	Pembury walks
H100	*H. spongiosipes*	J. Pitt	Kent	Hosey Common
H101	*H. spongiosipes*	J. Pitt	Kent	Hosey Common
H102	*P. niger*	J. Pitt	Kent	Hosey Common

Annexe 2 Molecular characterisation methods

DNA extraction protocol

DNA was extracted from fungal specimens following the method of Cubero *et al* (1999). Samples of dissected fungal material were frozen in liquid nitrogen and ground to a fine powder in an alcohol sterilised pre-cooled mortar and pestle. Routine extractions gave approximately 50-250 µg powder. The ground sample was suspended in 0.5ml CTAB buffer I, prewarmed to 60C. Samples were incubated at 65-70C for 30 minutes, after which an equal volume chloroform:isoamyl alcohol (24:1) was added and the components were mixed by inverting the tube. The aqueous and solvent phases were separated by centrifugation at 10000xG for 5 mins, and the upper aqueous layer was removed to a clean tube. The CTAB/DNA complex was precipitated by addition of 2 volumes CTAB buffer II and mixed by inversion for 2 minutes and the pellet was collected by centrifugation at 13000xG. The liquid was poured off the pellet and the pellet was resuspended in 350 µl of 1.2M NaCl. One volume chloroform:iso amyl alcohol was added and this was mixed by inversion. The aqueous and solvent phases were separated by centrifugation as before and the upper aqueous layer was transferred to a clean tube. DNA was precipitated from this by the addition of 0.6 volume cold iso propyl alcohol to form 2 layers, that were then mixed by inversion and incubated at –20C for 15 min. The DNA was collected as a pellet by centrifugation at 13000xG for 20 min, washed briefly in cold 70% ethanol, and dried under vacuum. The final dried DNA pellet was redissolved in 10-100µl TE buffer depending on volume.

TE buffer

10 mM Tris-HCl (pH 8)
1 mM EDTA

CTAB buffer I

1% Cetyl trimethyl ammonium bromide
1M NaCl
100mM Tris
20 mM EDTA
Sterilise buffer and add 1% poly vinylpolypyrollidone immediately prior to use

CTAB buffer II

1% Cetyl trimethyl ammonium bromide
50mM Tris HCl
10mM EDTA
40 mM NaCl

Polymerase chain reaction conditions

Methodology for rRNA gene amplification

Template DNA was diluted in ultra-pure water to give a working concentration of 50pg-50ng μl^{-1}. Typically original DNA extraction were diluted 1 in 10.

1-2µl of template DNA was mixed with 3µl 25mM $MgCl_2$, 31.5 µl water, 0.5U enzyme, 5µl 10X PCR buffer 4µl dNTPs (each at 5mM) and 2.5µl of each primer (25pmol) in a 50µl reaction mixture. All components were mixed and consolidated by centrifugation in micro-centrifuge tubes.

The polymerase chain reaction was carried out in an MJ Research PTC100 thermal cycler and conditions were an initial heating to 94 for 1.25 s, followed by 35s @ 95°C, 55s @ 55°C, 45s @ 72°C for 14 cycles, 35s @ 95°C, 55s @ 55°C, 2mins @ 72°C for 14 cycles, 35s @ 95°C, 55s @ 55°C, 3mins @ 72°C for 10 cycles, followed by 10mins @ 72°C.

Amplified DNA products are analysed in agarose gels (typically 1.2-1.5% agarose) with ethidium bromide.

TAE buffer

Tris base	4.84g
Na acetate	2.72g
EDTA	0.38g
H_20	1l

pH to 7.2 with acetic acid

Primer sequences

ITS1F	CTTGGTCATTTAGAGGAAGTAA
ITS4B	CAGGAGACTTGTACACGGTCCAG
PN3	CCGTTGGTGAACCAGCGGAGGGATC
ITS4	TCCTCCGCTTATTGATATGC

Primer sequence ITS4 from White *et al* (1990), ITS1F and 4B from Gardes & Bruns (1993) and PN3 from Mugnier (1994).

Methodology for direct PCR fingerprinting

Template DNA was used un-diluted and 1-2µl of template DNA was mixed with 3µl 25mM $MgCl_2$, 31.5 µl water, 0.5U enzyme, 5µl 10X PCR buffer 4µl dNTPs (each at 5mM) and 5µl of a single primer (25pmol) in a 50µl reaction mixture. All components were mixed and consolidated by centrifugation in micro-centrifuge tubes.

The polymerase chain reaction was carried out in an MJ Research PTC100 thermal cycler and conditions were 1min @ 95°C, 1min @50°C, 1min @ 72°C for 40 cycles, followed by 10min @72°C

Amplified DNA products were analysed in 3% NuSeive 2:1 in TAE and stained with ethidium bromide.

Primer sequences

RY	CAGCAGCAGCAGCAG
MR	GAGGGTGGCGGTTCT
GACA4	GACAGACAGACAGACA

Primer sequences RY and MR from Bridge *et al* (1997) and GACA4 from Weising *et al* (1989).

Annexe 3 Sequences used

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Front cover photographs:
Top left: Using a home-made moth trap.
Peter Wakely/English Nature 17,396
Middle left: Co_2 experiment at Roudsea Wood and Mosses NNR, Lancashire.
Peter Wakely/English Nature 21,792
Bottom left: Radio tracking a hare on Pawlett Hams, Somerset.
Paul Glendell/English Nature 23,020
Main: Identifying moths caught in a moth trap at Ham Wall NNR, Somerset.
Paul Glendell/English Nature 24,888