Project report to DEFRA 4: April 2012

Systematics, barcoding and ecology of fungi from waxcap grasslands in Britain

1. Milestones

This report describes activities since October 2011, and covers Milestones 6 (Second phase of sample acquisition complete, probe testing on fruiting populations complete; end Nov 2011 – **see sections 2-6**) and 7 (Final phylogenetic tree available, description of cryptic species complete; end March 2012 – **see sections 7-8**).

2. Sample acquisition

The 2011 field season ended with a grand total of 656 fresh samples collected under the auspices of the project (i.e. not including the dried collections accessed at Kew and Edinburgh). These were contributed by 79 separate individuals and/or recording groups from all regions of Great Britain – further evidence of the wide scale of support from the volunteer community for our research. In addition to the fresh collections, we have around 170 ITS sequences derived from GB dried reference collections, and acquired nearly 200 further reference sequences via our collaborations with the USDA Forest Service. With the publically accessible sequences submitted to GENBANK (including around 50 from Hungarian collections which provide a useful comparison between British and Central European populations), we now have a very substantial body of phylogenetic data covering our species of interest.

The samples we have worked on directly still represent a fairly small proportion of those accessible in the major collections; Kew and Edinburgh together possess around 1000 waxcap collections made since 1990 (and probably therefore with easily extractable DNA) and the collection at the University of Aberystwyth numbers around 3800. All of the Kew and Edinburgh specimen data are already incorporated into the NBN, and the Aberystwyth specimens have now been databased as an accessory output of the project, with the intention that these also will be included in the NBN datasets.

Unsurprisingly, species known to be rare at commencement of the project have continued to be so, meaning that phylogenetic analysis of certain species is still inadequate. This position can only be resolved through further sampling and monitoring programmes. We are confident that our volunteer collaborators will continue with this task after the formal conclusion of the project, and we shall do our best to support them within the restrictive confines of our core funding.

3. Molecular detection of *Hygrocybe* populations

Solid progress has been made towards this task, and the research has been extended beyond the original objectives of the project. As agreed at the outset of the project, this work focused on Welsh sand dune communities harbouring populations of *Hygrocybe conica* s.l.

Given the suspicion that *Hygrocybe* spp. are potentially mycorrhizal and the occurrence of *H. conica* in these unfixed dunes often amongst rather sparse vegetation with a low diversity of plant hosts present, there is the potential to investigate plant-fungus interactions in a rather simpler context that those found in nutrient-poor (waxcap) grassland which often contain 20+ potential angiosperm hosts.

Additionally *H. conica* is a 'pioneer' waxcap species, often being the first to fruit in formerly disturbed sites. Therefore four additional sites in gardens were identified where *H. conica* is found. As with sand dunes, the plant diversity is also low relative to more established semi-natural grasslands. They have the benefit relative to dunes of being more easily accessible, allowing pegs marking basidiocarp positions to be easily relocated.

Fig. 1. *H. conica* and golf tee marker at the Aelybryn garden site (Caernarfon). (3rd Oct 2010).


Fig. 2. *H. conica* (23rd Sept 2011) at Aelybryn within 30cm of position of fruit-bodies in 2010


Fig. 3. *H. conica* at the Dolcoed garden site (Aberystwyth) (28th July 2010).


Fig. 4. *H. conica* at the Quebec Terrace garden site (Aberystwyth) (4th Nov 2011).


Fig. 5. *H. conica* in fixed dunes at Dinas Dinlle.


Fig. 6. *H. conica* in less well-established dunes at Dinas Dinlle.

The target amplicon was within the ITS1 spacer region of the rDNA gene. Primers specific to *H. conica* were designed based on 17 ITS sequences obtained from *H. conica* samples in the Aberystwyth fungarium as well as 10 sequences from Genbank. The aim was to design primers generating an amplicon of 100-300 bp but specific to the *H. conica* species complex. The ideal

approach might have been to design primers specific to cryptic species within the *H. conica* aggregate, especially as we have found that some *Hygrocybe* species segregates are sympatric with others in the aggregate, but (a) definition of the cryptic species was not well established when the work had to be carried out, and (b) differences between cryptic species in their ITS sequence may not be sufficiently great to allow reliable molecular detection.

The primers designed are listed below and their position on an alignment of 27 *H. conica* sequences (including 10 from Genbank) is shown in Fig. 7. These seven primers designed to anneal within the ITS1 region were tested in combination with each other or with the more standard fungal–specific ITS1F primer.

CTTGGTCATTTAGAGGAAGTAA	ITS1F	(in 18S; 68-88bp from ITS1 start)
CAATTCACAATTTCCATCACACC	HconF136	(ITS1fwd 136-150)
GGTGTGATGGAAATTGTGAATTG	HconR136	(ITS1rev 136-150)
AATGGTGCTCTTGAACGCATCT	HconF250	(ITS1fwd 250-271)
AGATGCGTTCAAGAGCACCATT	HconR250	(ITS1rev 250-271)
GAAAGTTGTTTCTGTGATTACT	HconR296	(296-318 rev ITS1/5.8S boundary)
CTTGTGCACATCTTGTAGGTGC	HconF160	(ITS1rev 160-181)
CACCTACAAGATGTGCACAAG	HconR160	(ITS1fwd 160-181)

Predicted amplicon sizes ranging from 69 bp to 309 bp are possible with various combinations of these primers. Following initial tests of the nine possible combinations of these primers, the strongest bands were obtained with ITS1F/HconR250 (261 bp) and HconF136/HconR250 (125 bp) combinations. These tests were conducted with DNA samples from six *H. conica* basidiocarps and also an unrelated basidiomycte (*Bjerkandera adusta*), plus water (no DNA) control (Fig. 8), initially using a low 54ºC annealing temperature.

The HconF136/HconR250 primer pair was found to be the most suitable; it amplified all *H. conica* samples and did not amplify *B. adusta*. However, even when the annealing temperature was raised to 59ºC, there was some weak amplification from DNA of other *Hygrocybe* spp. (Fig. 9) It is likely that higher annealing temperatures would reduce/eliminate such non-specific amplification.

Small 15 mm soil cores were obtained from the Dolcoed garden site (a fairly new lawn <20 yrs since reseeding, with only *H. conica* present; mostly *Lolium*/*Agrostis* and very few herbs). Coring was conducted within 3 cm of the position of basidiocarps recorded in July 2011. Soil was removed and roots were extensively washed. 200 mg samples of roots were used for DNA extraction. A set of plant-specific primers (NS1P/NLBP) was initially used to check that the DNA did not contain compounds that would inhibit PCR. The presence of strong bands for all samples indicated that this was not the case.

Amplification with the HconF136/HconR250 primer pair and an annealing temperature of 59ºC or 60ºC was conducted. Also included was DNA from basidiocarps of *H. conica* and *H. miniata*. Amplification products were obtained from all root samples (Fig. 10) suggesting the presence of *H. conica* DNA within the root tissues. Increasing the annealing temperature to 60ºC did not diminish the intensity of these bands but did reduce amplification of the *H. miniata* DNA. This suggests that further increase in the annealing temperature would give rise to an assay procedure, specific to *H. conica*.

However, without actually visualising the hyphae of *H. conica* inside the roots, it is difficult to provide strong evidence that the DNA giving rise to the bands observed in Fig. 10 is from endophytic hyphae, rather than hyphae adhering to the root surface. Furthermore, DNA extraction is destructive so it is not possible to examine root tissues having established a positive result by PCR. The risk of false positive results is also potentially misleading with this approach. In these circumstances, visualisation of the hyphae is considered to be important, so a further detection method based on fluorescent in-situ hybridisation (FISH) was designed, as described below.

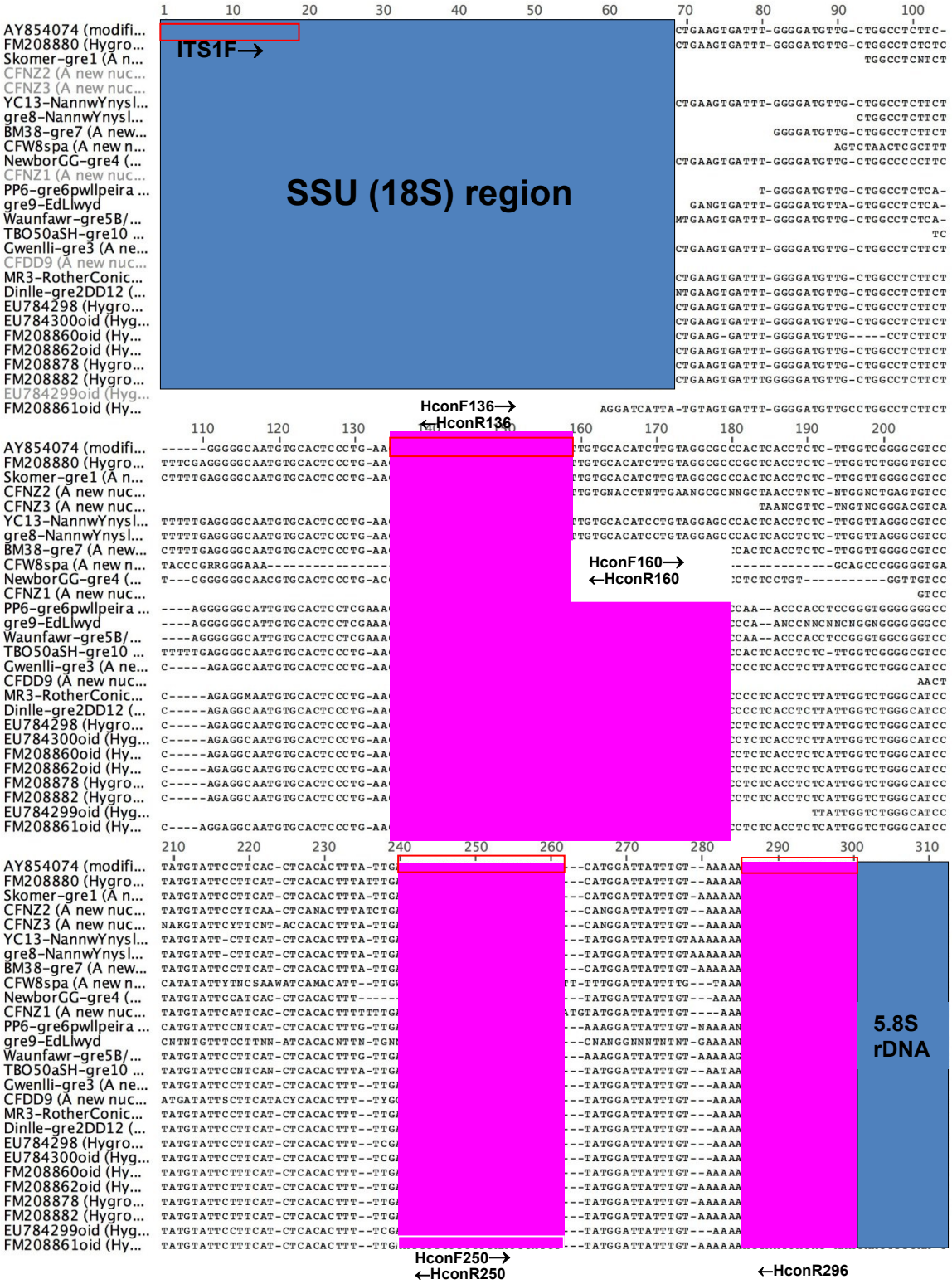
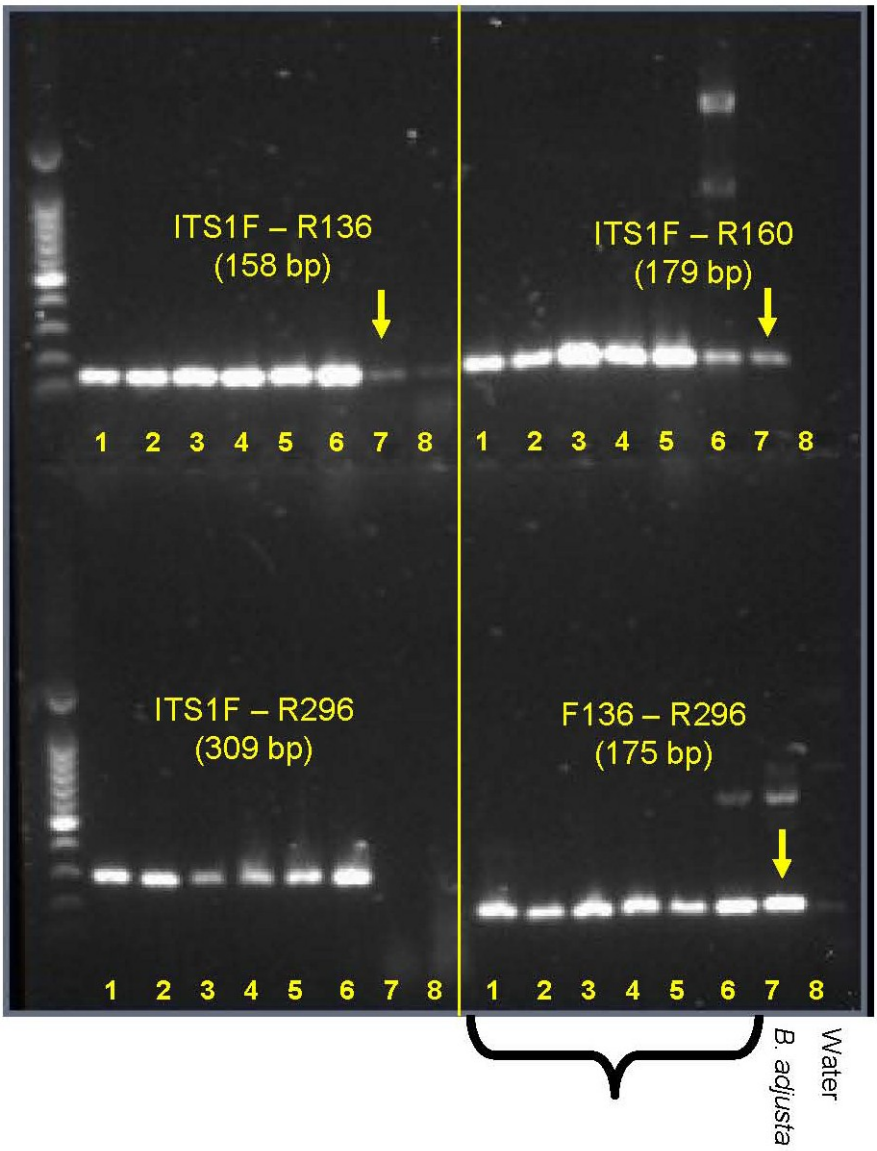


Fig. 7. Design of *H. conica* specific primers based on the ITS region

Fig. 8. Testing of Hygrocybe conica-specific primers

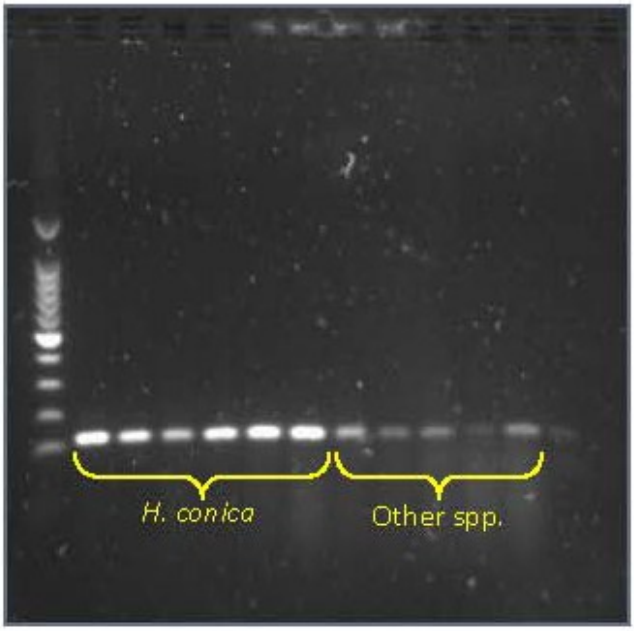


LANE ORDER:-

1. *H. conica* Ynyslas (YC13)
2. *H. conica* Botany Gardens (AD10-05)
3. *H. conica* Pwllpeiran (PP6-gre
4. *H. conica* Dolcoed (DC10-1)
5. *H. conica* Edward Llwyd (gre9)
6. *H. conica* Rhosgadfan (RG10-1)
7. *Bjerkandera adusta*
8. No DNA (Water)

Six *H. conica* samples (plus two negative controls - no DNA and an unrelated basdiomycete) were tested against a range of primer combinations, initially with 54˚C annealing. Other primer permutations showing specificity and amplification of all *H. conica* samples were ITS1F/HconR250 (261 bp amplicon) and HconF136/HconR250 (125 bp amplicon) (not shown). Non-specific amplification of *B. adusta* indicated by arrows.

Fig. 9. Test of the specificity of the HconF136/HconR250 primer pair. At an annealing temperature of 59˚C, there was good amplification of *H. conica* samples but still some amplification from other *Hygrocybe* species.



LANE ORDER:-
1. *H. conica* Ynyslas (YC13)
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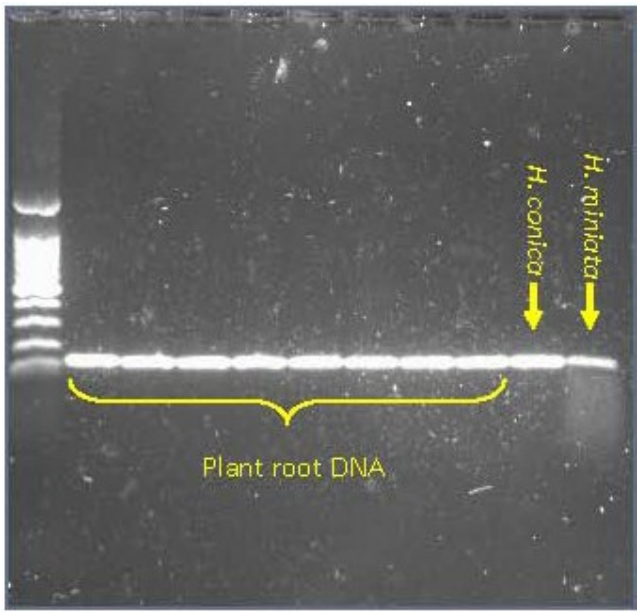
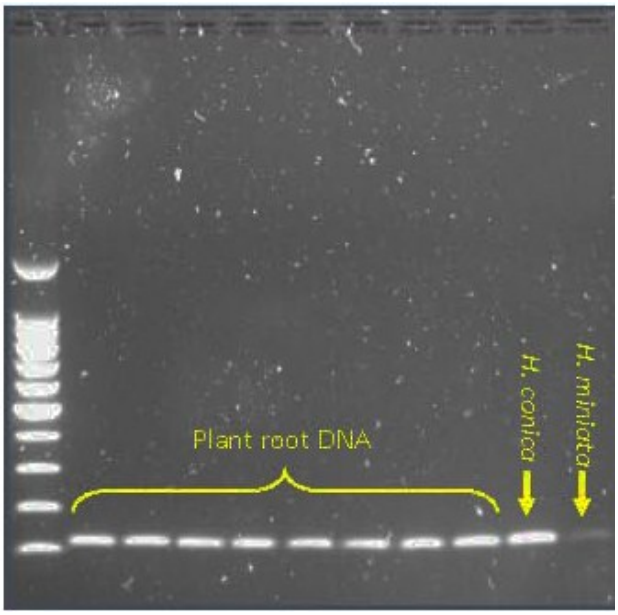
4. *H. conica* Dolcoed (DC10-1)
5. *H. conica* Edward Llwyd (gre9)
6. *H. conica* Rhosgadfan (RG10-1)
7. *H. pratensis*
8. *H. chlorophana*
9. *H. coccinea*
10. *H. irrigata*
11. *H. miniata*

Fig. 10. Amplification from basidiocarp DNA and root samples using *H. conica* specific PCR primers. DNA was extracted from root samples were taken from beneath the basidiocarps from the Dolcoed garden site.Specificity of the PCR was improved at 60˚C relative to 59˚C



Annealing at 59°C



Annealing at 60°C

4. Other studies using environmental sequencing techniques

There have been several studies published in the last few years using environmental sequencing methods that have recovered sequences attributable to *Hygrocybe* species, and some at least of these are valuable in understanding species limits and distributions. Notable here is a study of the impact of grazing on mown and unmown grasslands in the French Alps (Mouhamadou et al., *Fungal Diversity* **47**: 55-63, 2011). Some of their results appear surprising (especially the apparent lack of overlap between the fungal communities associated with grass roots in mown and unmown ecosystems), but we have no reason to doubt their detection of sequences from the *Hygrocybe spadicea* aggregate. These were obtained from roots that did not show evidence of typical ectomycorrhizal colonization, leading the authors to suppose that the species was a saprotroph.

Other studies detecting environmental *Hygrocybe* sequences include:

- basidiomycete diversity in Michigan agricultural soils (Lynch & Thorn, *Applied and Environmental Microbiology* **72**(11): 7050-7056, 2006).
- above- and below-ground diversity of forest agarics in Ontario, Canada (Porter et al., *Molecular Ecology* **17**(13): 3037-3050, 2008)
- fungal diversity in the rhizosphere of endemic Canarian plants (Zachow et al., *ISME Journal* **3**: 79-92, 2009).
- ectomycorrhizal fungus diversity relative to a nitrogen gradient in forests of eastern USA (Lilleskov et al., *Fungal Ecology* **4**: 174-183, 2011; see also Vineis et al., abstracted in *Bulletin of the Ecological Society of America* **68**, 2010).
- research into fungal communities in beech forests in Thuringia, Germany (Krüger et al., *PLoS ONE* **7**(2): e32139, 2012 – primarily focused on methodology)

Taken together with the work described in section 3 above, we believe that proof of concept has been well-established for direct detection of *Hygrocybe* species in soil and root samples. While further research is needed (especially into the patchiness of distribution within individual localities, and thus the sampling regime needed), it would be entirely feasible to assess waxcap diversity using molecular methods (454/Illumina or Sanger sequencing) rather than traditional sampling. Potentially, this could provide a powerful new and more objective tool for assessing the conservation quality of grassland sites in the UK.

5. Fluorescent in-situ hybridisation (FISH)

Whilst a PCR-based approach as described above was successful in demonstrating the presence of *H. conica* in root tissues, it would be difficult to prove unequivocally that the fungus was not simply adherent to the root surface. Ideally it would be good to be able to see the structure of any mycorrhizal interface but our previous attempts to detect *Hygrocybe* spp. in stained roots have been unsuccessful due to the abundance of other fungal structures. The hyphae and sclerotia of ascomycetous dark septate endophytes and the hyphae, arbuscules and vesicles of arbuscular mycorrhizal fungi (Glomeromycota) are far more easily visible than the hyaline and narrow hyphae of most agaric fungi.

FISH (fluorescent in situ hybridisation) is a method widely used in bacterial ecology, wherein a fluorescent dye is conjugated to an oligonucleotide, allowing sequence specific staining of bacterial cells. The usual targets are the large or small rRNA components of the ribosome with the probe being designed to bind (as the complementary strands) to particular regions of the SSU/LSU. In contrast to its widespread application in bacterial ecology, the method has received little attention from mycologists. One exception is Baschien *et al*. (*Appl. Env. Microbiol*. **74**(20): 6427-6436, 2008) who designed a FISH probe complementary to the transcript of the 18S rRNA gene to detect aero-aquatic fungi. This 15 bp probe called MY1574 (TCCTCGTTGAAGAGC; position 1474-1489 on *Saccharomyces cerevisiae* sequence) is specific to fungi so should bind to the SSU of a range of fungal taxa.

Bioinformatic analysis of the SSU regions of *Hygrocybe* spp. from Genbank confirmed that this probe would bind to the SSU of all the species for which SSU data are present (and probably many other fungi). A key requirement for any probe to be used to stain hyphae *in planta* is that it

does not bind to plant ribosomes. Therefore alignment of SSU genes from several grasses (*Poaceae*; the likely host group in this case) was also conducted.

Within the region spanned by Baschien's MY1574 probe, there was only one mismatch to the grass SSU sequences. Whilst hybridisation conditions can be altered to make FISH probes highly specific (ie higher hybridisation temperature and more stringent washing conditions), it was decided to design a slightly longer probe, in order to incorporate a second mismatch in the primer. This primer (EumycGG2; TCCTCGTTGAAGAGCAAT; 18 bp; position 1474-1493 on *Saccharomyces cerevisiae* sequence; Fig 11) has been synthesised (conjugate to FAM fluorophore) but has not yet been tested. It is planned for this work to be carried out within the next three months.

The target material to be used will be the mesocosm cores collected in autumn 2010 and 2011 from Dinas Dinlle and Ynyslas dunes and kept outdoors at the AU Botany Gardens, and soil cores to be taken fresh beneath where *H. conica* basidiocarps were observed in summer/autumn 2011, as shown in Fig. 4. Golf tee markers have been placed to mark the exact location of the *H. conica* basidiocarps at these sites.

6. Whole genome analysis of *Hygrocybe conica*

The relationship between *Hygrocybe* species and plant roots has been considered as enigmatic for years, and recent research combining studies using stable isotopes and phylogenetics (Seitzman et al., *Mycologia* **103**: 280-290, 2011) has confirmed this enigmatic status without being able to provide clear evidence that waxcaps are either mycorrhizal fungi or saprotrophs.

Matched funding from a private source has enabled us to initiate a complementary project to carry out an analysis of the entire genome of two *Hygrocybe* species, including a British representative of the *H. conica* aggregate. The objective is to search for genes that are associated with mycorrhizal symbiosis. This in itself will not demonstrate that such genes are functional, but will at least indicate the potential for symbiotic associations, and will provide the impetus for further studies on gene expression. Combined with the FISH approach described above, we will be several steps closer to understanding the linkages between waxcaps and the grassland plants with which they are associated.

Time constraints mean that this research must be delayed until autumn 2012, so the results will not directly feed into the DEFRA programme.

7. Phylogenetic analysis and detection of cryptic species

Since submission of the project report in October 2011, we have substantially increased the number of samples sequenced, primarily from collections made in the 2011 field season, focusing on some of the major species aggregates and resolving anomalies in taxon placement within the global phylogenetic tree. The latest version is presented as a separate pdf file, and contains 739 sequences (of which 293 were derived directly via Project Waxtongue).

The tree demonstrates the existence of a series of major clades and, as genera such as *Hygrophorus* (which has historically been treated as a separate genus) cluster within *Hygrocybe sensu lato.*This means that it is logical to separate *Hygrocybe* into a series of about five segregate genera. This will lead to some name changes for well-known taxa, but the familiar red/yellow species will be retained in *Hygrocybe* and continued use of the traditional names by field mycologists will not create insuperable problems of communication. More serious will be the fragmentation caused by recognition of cryptic species within the taxa we now know to be species aggregates. Explanation of the newly recognized segregates and their significance to the field mycology community will be an important task. For practical reasons (the extent of diversity detected, and a natural gravitation by many field mycologists to the autumn as the major period of activity) this will not be complete until after the formal conclusion of the project as currently planned. We shall nevertheless take this task very seriously, as it will affect the overall impact of the research.

Fortunately, perhaps, not all the *Hygrocybe* species traditionally recognized in Britain have proved to be species aggregates; some may be demonstrated to be homogenous monophyletic

taxa, and there are also cases where perceived morphological variation within these taxa can be shown not to have independent evolutionary origins according to our methods. We still have experimental material oustanding for some of these, but examples are *H. chlorophana* (including an orange morph sometimes recognized as a separate variety), *H. aurantiosplendens*, *H. calyptriformis* (excluding the poorly known var. *domingensis*), *H. citrinovirens*, *H. coccinea*, *H. intermedia*, *H. phaeococcinea*, *H. salicis-herbaceae* and *H. vitellina*.

In some cases, previously detected apparent genetic heterogeneity has been due to mixed or misidentified samples, and considerable efforts have been made since submission of the last project report to weed out these inconsistencies. 63 specimens have been re-examined in 2012, selected on the basis of mismatches between the name on the packet and the sequence-based placement. These included 31 from those sent to us as fresh collections, and 32 dried specimens at Kew. 15 of the fresh samples and 12 of the Kew specimens have been re-determined as a result of this work, and studies will continue over the next three months to resolve the remaining anomalies.

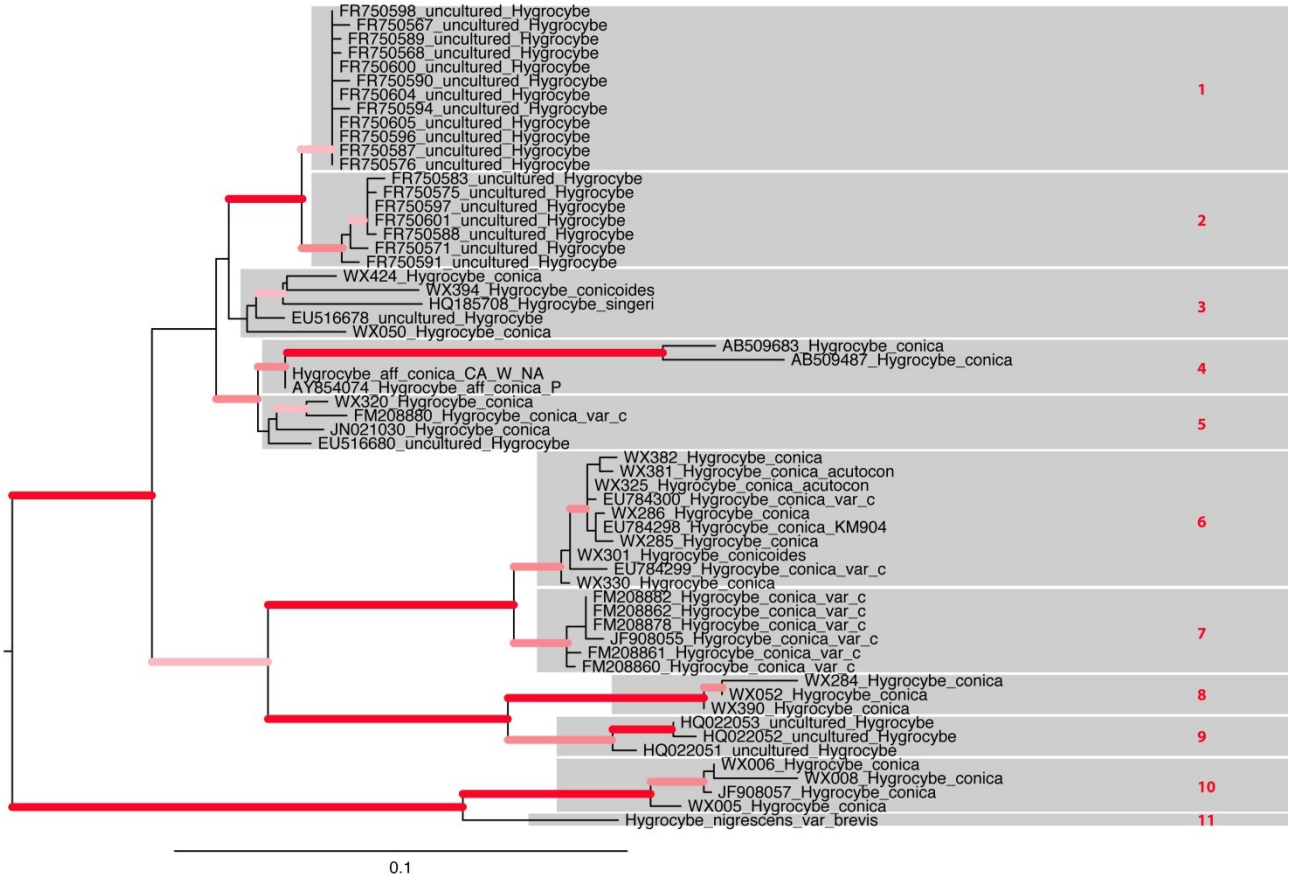
Fig. 11. *Hygrocybe* aff. *psittacina*, an undescribed species from Derbyshire

Molecular studies have informed the recognition of new taxa with distinctive morphological features, and the distribution of taxa newly recorded from Britain. An example of the former is an apparently undescribed species from Derbyshire with some morphological similarities to *Hygrocybe psittacina* but with a purplish cap (see fig. 11) and a distinct ITS sequence. Its status as a novel taxon still needs to be confirmed, with further comparisons with European specimens. *H. sciophanoides* could possibly provide a name for this species, but no authentic material remains and the name is of rather uncertain application. Taxa new to the UK include *H. subpapillata*, a species first described from France in 1979 with “very few collections since” (Boertmann, *The Genus Hygrocybe* edn 2, 2010) was recorded and confirmed by Boertmann as new to Britain with a sequenced voucher K(M)54960 collected from lawn of Dunwood Hall in 1997. This was not assessed for inclusion on the 2006 unofficial Red Data List (RDL authors stated that 10y had to elapse between 1st record and 1st assessment). No material clustering with this seq was collected or received during the current project. Since 1997 there have been 5 recorded sites but none are verified or backed by vouchers in RGB, Kew. A similar number of unverified GB site records exist pre-1997. It should certainly be included in the next RDL assessment, although it would currently be considered as Data Deficient due to lack of verified records. There is one other unverified K collection (K(M)63393) from W. Glos. 1986, which we shall attempt to sequence later this year.

Most of the work on phylogenetics since October 2011 has been focused on the major species aggregates, defining cryptic species and attempting to correlate the taxa as defined by sequence with morphological, ecological or distributional traits. In many cases we cannot be confident that our sampling programme has captured the entire UK diversity at cryptic species level, and we expect that further sampling will yield even more unique taxa. As explained previously, this work is taking substantially longer than expected due to the diverse nature of the species complexes, but we have made good progress.

In the images of phylogenetic trees that follow, thick red branches indicate very strong support (95-100% bootstrap value), light red branches are strongly supported (90-95%) and pink branches are moderately supported (85-90%).

A. *Hygrocybe conica* clade



According to the data we have available, *Hygrocybe conica* is a species aggregate containing at least 11 well-supported cryptic species. This diversity is not altogether surprising as the taxon has long been considered to be polymorphic, and its members occupy highly diverse ecological niches in waxcap terms. There is potentially a good case to be made for resurrecting the taxa *H. conicoides* (treated as a variety of *H. conica* in Boertmann, 2010) and perhaps also *H. nigrescens*, but the former name appears to be applied to at least two and possibly three clades within the *H. conica* aggregate, and further samples of the *H. nigrescens* clade are needed. All of the names need to be lectotypified/epitypified to fix their application in sequence-based systems, and we also need to establish which clade represents *H. conica sensu stricto*. The species was originally described from southern Germany, and it may well be that none of the British collections belong to the species in its narrowly defined sense.

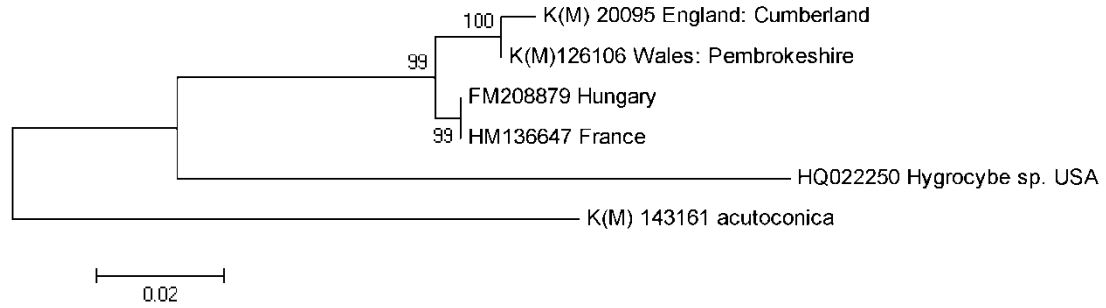
The clades are described as follows:

1. Only known from environmental samples, from central Germany.
2. A distinct clade, again containing only environmental samples from the same German study.
3. A rather poorly defined clade, containing a sequence of an American species *H. singeri*, with two British collections, one from dune slacks in Anglesey labelled as *H. conicoides* (possibly because of the habitat) and the other from a cemetery (unimproved grassland) in Surrey. Two further samples may belong here, one from unimproved grassland in Pembrokeshire and the other an unpublished environmental sequence from Austria. The *H.* "*conicoides*" collection has been further studied, and its morphology does not conform to that of *H. singeri*. Bootstrap values are not strong, and it may be that the clade represents several species.
4. This is a North American clade with collections from the USA and Canada, again possibly containing multiple taxa.
5. Clade 5 is not well supported internally. Sequences from Austria and Hungary cluster weakly with a collection from sand dunes in Anglesey, and with one from Ontario (Canada).

It is unlikely that this represents a monophyletic unit. The Anglesey specimen needs re-sequencing to confirm its identity; only a forward sequence was obtained.

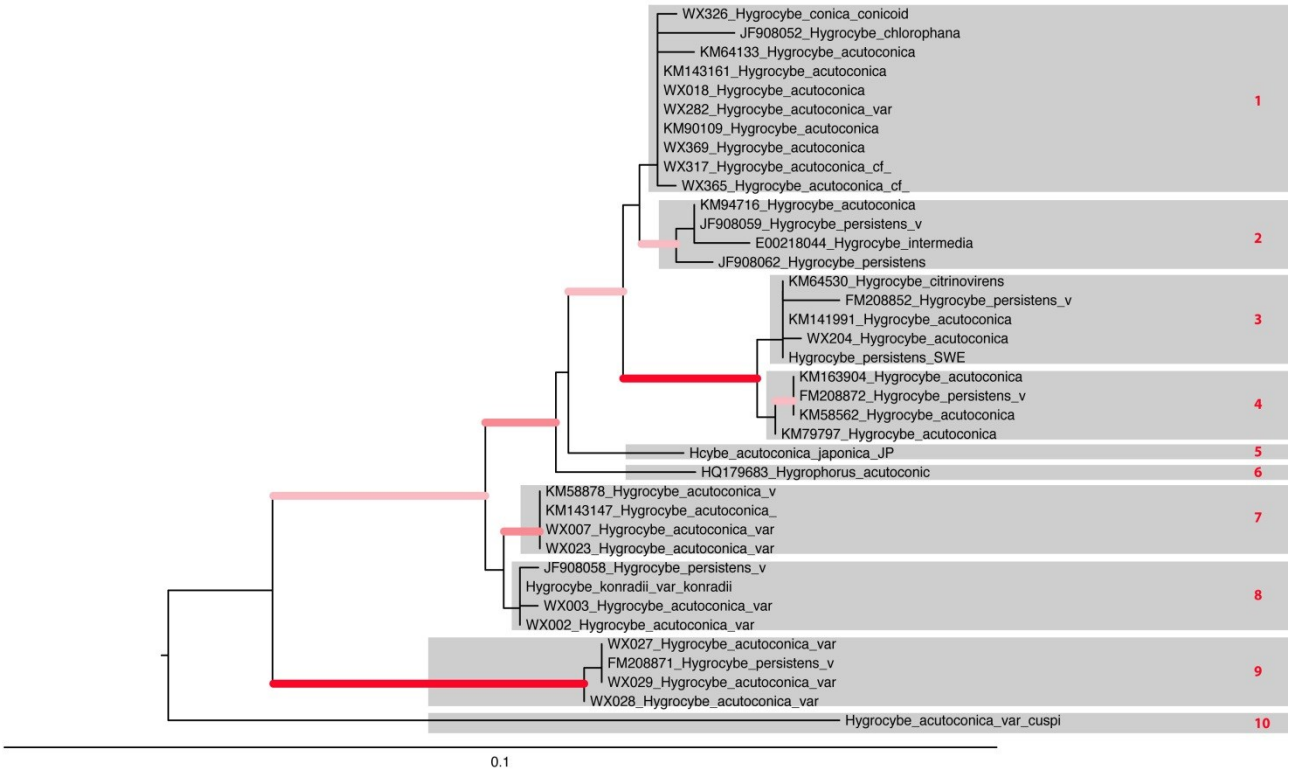
6. This is one of two clades containing sequences from specimens referable to *H. conicoides*. Both are well-supported but are collectively monophyletic. Clade 6 contains exclusively British fungi, and as the type of *H. conicioides* originates from Somerset, it would be logical to epitypify the species with one of the Waxtongue project samples assuming that the morphology matches adequately well. The samples sequenced come from dune grassland in East Sussex and Westmorland (England) and Anglesey, Cardiganshire and Merioneth (Wales), providing good indications that the species is habitat-specific. However, it does appear that other clades of the *H. conica* aggregate may also occupy dune environments.
7. Clade 7 contains sequences of specimens from Hungary and Italy identified as *H. conicioides*. Their ecological niche has not been recorded in the published literature. It seems likely that this clade represents a cryptic, possibly recently evolved taxon with a Central European rather than oceanic distribution.
8. Clade 8 is well-supported, but may not be homogenous in genetic terms. The three samples from which sequences were obtained come from dune grassland in Merioneth, coastal limestone grassland in Caernarvonshire, and inland unimproved grassland in E Sussex. All three have bright yellow-orange (rather than red) fruit-bodies that blacken strongly following maturity.
9. This clade contains three sequences known only from environmental samples, all from New Hampshire (USA).
10. Here we have three British sequences, two originating from specimens collected in Surrey (actually Kew) and one from Northamptonshire, along with a single sequence from an Italian collection. It may be that this represents a Continental species with its westernmost outpost in SE England. The British collections have large, bright red and strongly blackening fruit-bodies, and may represent the currently abandoned species *H. nigrescens*. That species was originally described from the Alpes Maritimes (SE France).
11. Clade 11 contains a single sequence, from a specimen identified as *H. nigrescens* var. *brevispora*. Its provenance is unknown but is presumably distinguishable morphologically from the type variety.

B. *Hygrocybe spadicea*



We have rather few sequences of this BAP-listed species. We did receive collections from Derbyshire and a new Scottish site adjacent to a known locality, but they arrived too late to be sequenced and included in this report. We shall be working on this as a matter of priority, and on another collection that is congruent with morphological definitions of the species but appears to have a contaminated sequence. On current evidence, we suspect that the species is an aggregate of two taxa, one from montane central Europe and the other represented in the UK. The species was originally described from Austria, so on the balance of probabilities the UK species will require a new name. Bearing in mind the consequences for BAP and Sections 41 & 42 listing of this action, we will need to sequence further material from the UK and Europe before describing the new taxon.

C. *Hygrocybe acutoconica* clade



The *acutoconica* clade has broad morphological similarities to the *conica* aggregate, but typically has yellow/orange fruit-bodies that do not blacken or merely become somewhat greyed with age/bruising. Misidentifications based on field observations do occur occasionally, especially when young samples of the *conica* clade are concerned. Like that group, the *acutoconica* clade is a complex containing multiple segregate taxa, though not all are sufficiently well-defined using ITS sequences to be sure of their status. We have found some morphological data that correlate with the ITS clusters.

H. acutoconica was originally described from North American material. It is unlikely to be conspecific with any of the British collections, and we have not been able to obtain sequences from specimens likely to belong to this taxon in its strict sense. The name was published in the same year as (but earlier than) *H. persistens*, a species described from southern Germany, and the aggregate was referred to under this name until the second edition of Boertmann's monograph in 2010. We need to find authentic collections of *H. persistens* (or at least material from the same region that corresponds with its description) before we can apply that name to any of the clades listed below.

Seven of the ten clades provisionally recognized here contain British representatives. They are characterized as follows:

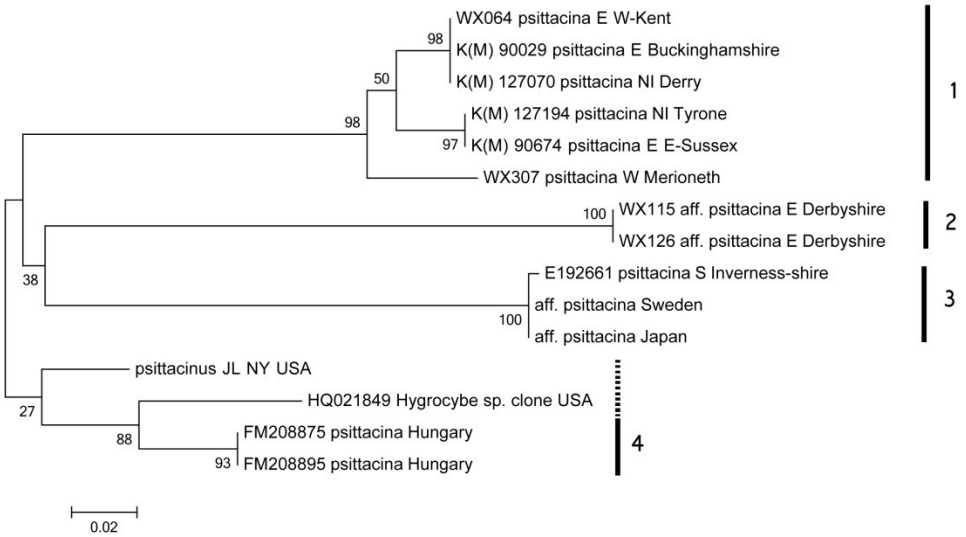
1. A species of sand dunes with clamped 4-spored basidia and relatively elongate spores (Q[1] = 1.8-2.6). It may well be referable to *H. aurantiolutescens*, which was described from Cornish dunes (Orton, *Notes R. Bot. Gdn Edinburgh* **29**: 75-128, 1969), and possibly also to a misapplication of the American taxon *Hygrophorus cuspidatus* by Arnolds (*Persoonia* **13**: 137-160, 1986; see Boertmann, 2010). Our samples originated from Northumberland and Westmorland (England), and Anglesey, Cardiganshire, Glamorgan, Merioneth and Pembrokeshire (Wales). An Italian specimen identified as *H. chlorophana* also clusters here but with a number of bp differences, and we are not convinced that it truly belongs to this clade.
2. Clade 2 is fairly well-supported, but seems to contain a somewhat disparate set of fungi. Included are two Scottish specimens, a collection from Perthshire (Kindrogan) in the Kew fungarium and one from dunes in Wester Ross in Edinburgh. The Kew collection has

[1] Q is the acronym commonly used for length/breadth ratio of spores in *Hygrocybe* systematics

unclamped 2-spored basidia and spores with a Q value of 1.6-2.3, and corresponds to the Danish species *H. langei* (see Orton, *Trans. Br. mycol. Soc.* **43**: 254, 1960). The Edinburgh specimen was identified as the somewhat similar species *H. intermedia,* and needs re-examination. Also included in clade 2 are sequences from two Italian collections; further details of their provenance have not been obtained.

3. Clades 3 and 4 are strongly supported collectively. Clade 3 contains fungi that are morphologically similar to Clade 2 (unclamped 2-spored basidia with spores with a Q value of 1.6-2.0(-2.5)) and may also be good candidates for *H. langei*. Collections come from inland grassland sites in Easterness (Scotland) and Carmarthenshire (Wales), along with sequences from Hungary and Sweden. A further sequence from a specimen identified as *H. citrinovirens* needs confirmation.
4. Again, this group fits *H. langei* from morphological evidence, with 2/3-spored unclamped basidia and spores with a Q value of 1.6-2.5. Collections from inland or coastal turf, from Buckinghamshire and Surrey (England) and the Clyde Is (Scotland), along with a sequence from Hungary.
5. Clade 5 comprises a single sequence from Japan, from a specimen identified as *H. acutoconica* forma *japonica*.
6. This contains a single sequence, from a specimen identified as *H. acutoconica* var. *microspora* originating from Tennessee (USA). There would be a strong argument for separating this (and also the Japanese taxon) from *H. acutoconica* at species level.
7. Clades 7-9 all have broadly ellipsoidal to subglobose spores, and could be identified using morphological methods as *H. acutoconica* var. *konradii*. This has been accepted quite widely in the past at species rather than varietal level, but the challenge remains to decide which of the three clades the name should properly refer to. Clade 7 is well-supported, and contains sequences from four British collections, from E Kent, Northamptonshire and Oxfordshire (England) and Carmarthenshire (Wales). An appropriate name for this clade might be *H. subglobispora*; that species was described by Orton (1960) from Surrey, and one of the collections was originally identified as this taxon. That has clamped 4-spored basidia and rather small spores, in contrast to the other collections from clade 7 that have unclamped, 2-spored basidia.
8. Clade 8 contains sequences from two English collections (from E Kent and Northamptonshire), along with samples from Denmark and Italy. The clade is not well supported, and could possibly end up being combined with clade 7.
9. This clade is very well supported, and includes three GB-derived sequences (all from the same locality in N Somerset, along with one from Hungary. The British material has unclamped 2-spored basidia and spores with a Q value of 1.2-1.4(-1.6).
10. Clade 10 is occupied by a single sequence from a specimen from North Carolina (USA) identified as *H. acutoconica* var. *cuspidata* - this should be the taxon in its original sense rather than Arnold's misinterpretation (see clade 1 above).

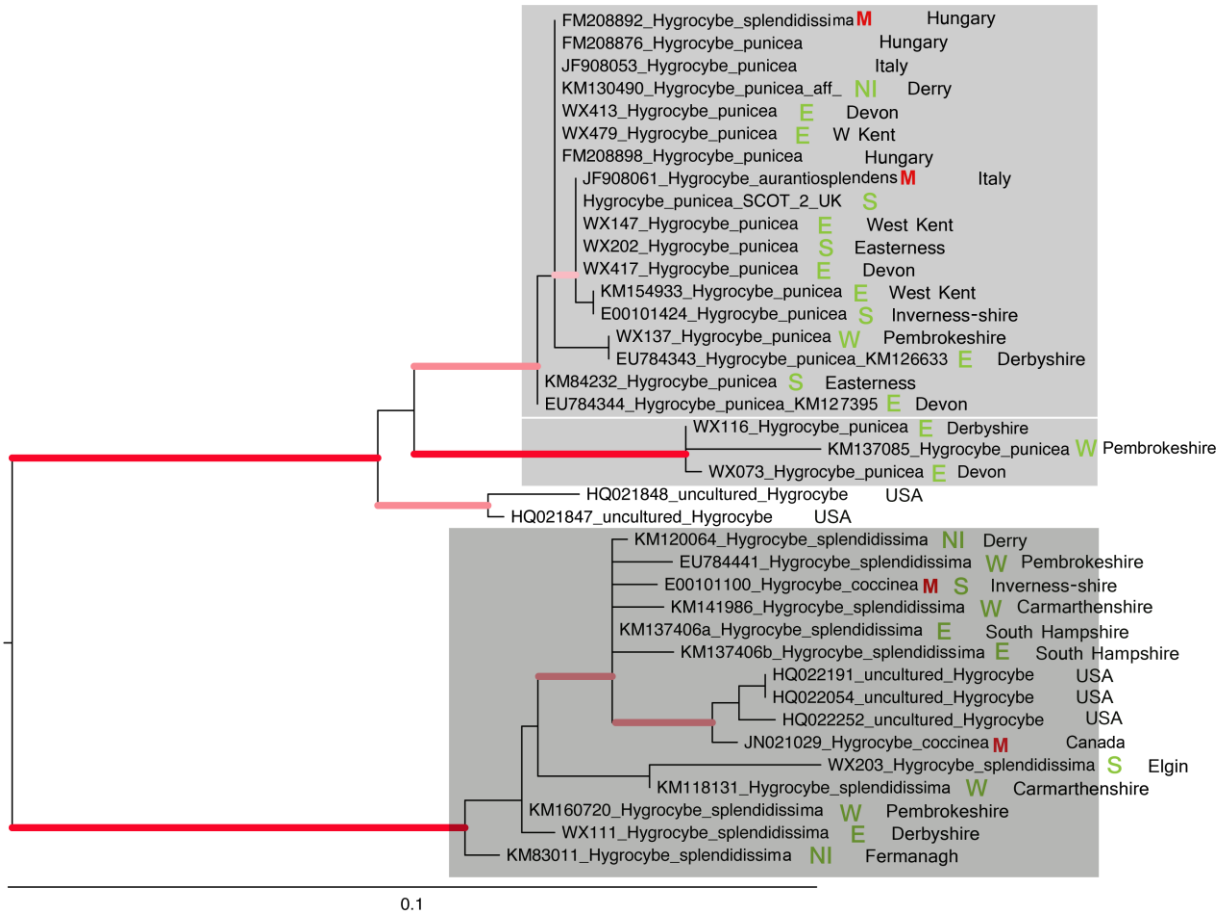
D. *Hygrocybe psittacina* clade



Hygrocybe psittacina as currently accepted is a variable but highly distinctive species with highly glutinous, bright green and yellow fruit-bodies. Several other fungi assumed to be colour morphs of *H. psittacina* have been placed within the aggregate, and these may well deserve status as distinct species. Four clades have been recognized, all of which are highly distinct in molecular terms.

1. Clade 1 appears to comprise the "standard" British species, with samples from Buckinghamshire, W Kent and E Sussex (England), Merioneth (Wales) and Derry and Tyrone (N Ireland).
2. Clade 2 contains the purple-capped species referred to earlier in this report as a probable undescribed species (see Fig. 11). Two collections have been found, both from Derbyshire.
3. Clade 3 could have a circumboreal distribution, but we need more information about the collection localites. One Scottish sample, from an arctic-alpine environment on Creag Megaidh, clusters with sequences from Swedish and Japanese collections. Images of the fresh collection (in the Edinburgh fungarium) do not appear to be noticeably distinct from the species as normally circumscribed in the UK.
4. Clade 4 contains sequences from two collections from Hungary, with two outliers from North America that may well not belong. As *H. psittacina* was originally described from Bavaria, it is possible that the species in its narrow sense occupies this clade, and new names will be required for all of the British taxa within this aggregate.

E. *Hygrocybe punicea* and *splendidissima clades*



M indicates a misidentification; the green letters denote the UK region.

Hygrocybe punicea and *H. splendidissima* are two large and spectacular species with bright red caps, that to date have not been suspected to be polyphyletic. *H. punicea* contains two very well-supported clades, which should probably be recognized as distinct species. However, we have not been able to find well-correlated morphological differences between the two taxa, and in Devon at least, they are sympatric with specimens from the segregates found in different fields on the same farm.

Hygrocybe splendidissima forms a well-supported clade with several daughter clades; most of the GB material examined fits in one well-defined group, which is sister to a clade containing sequences of specimens from North America. The species was originally described from Devon. In addition, there are five separate sequences that do not fit into either of these clades, but their inter-relationships are uncertain. Further research is needed to elucidate their evolutionary affinities, which we shall carry out as time permits.

8. Further areas for research

There is a number of small species clusters for which we have some information on their constituents and phylogenetic structure, but where our research is currently incomplete. These include:

- *H. inspida* – with two clades, one of which seems to lack the intensification of pigment at the stipe apex that is characteristic of the species (and might be referable to *H. subminutula*), along with two further clusters that may be nearer to *H. constrictospora*.
- *H. constrictospora* – the application of this name is in doubt, with possible confusions with *H. inspida*, *H. ceracea* and *H. calciphila*.
- *H. coccineocrenata* – further sequences are needed for this species and *H. turunda*, which appears to be closely related.
- *H. coccinea* – a clade close to this species seems to be confused with *H. marchii*; the application of the latter name needs clarification.
- *H. pratensis* var. *pallida* – a clade containing sequences from collections identified as this taxon may be better treated as *H. berkeleyi* but further work is needed to eliminate pale specimens of other species (e.g. from the *H. fornicata* and *H. virginea* complexes)
- *H. lacmus*, *H. flavipes* and *H. radiata* - these species are easily confused in morphological terms, but we have provisionally identified clades occupied by each of the three taxa.

The work on the *Geoglossaceae* has had to be put on the back burner in favour of the studies on *Hygrocybe* and we still have a substantial number of sequences to generate. We will work on these as soon as possible, focusing on the "*Thuemenidium*" and *Microglossum* complex.

9. Plans for dissemination

The major academic output currently planned is a paper to be published in a high-impact journal detailing the extreme diversity of species in the *Hygrocybe* complex. This could not be completed earlier (i.e. without the final ITS tree) but should be submitted well before the formal end of the project. A further publication will be aimed at the field collector (probably submitted to *Field Mycology*) which will address the conflicts between species concepts as defined by field characters and DNA, and explain which taxa can continue to be recognized using morphological methods and which need laboratory analysis. Again, this should be submitted before the formal end of the project. Depending on results from the FISH analysis, a third paper may well be submitted to an academic journal detailing methods for visualization of *Hygrocybe* mycelium in or on plant roots. We would also expect to publish a paper on the species complexes within the *Thuemenidium* and *Microglossum* clades, and this is also likely to be accompanied by a field-oriented publication. As detailed previously, we have also made contributions to papers with colleagues from North America on the generic limits of both the *Hygrocybe* and *Geoglossaceae* complexes.

The project will be featured in a special symposium on the science that underpins fungal conservation at the Mycological Society of America meeting at Yale University in July 2012. There are no formal UK-based scientific meetings being held before the formal end of the project, but funds permitting we hope to provide oral presentations to field-focused groups in the autumn of this year. Kew will make a commitment to support this work further if appropriate financial support is available; these fungi can be regarded as a flagship group for fungal conservation and it is a high priority that we can provide robust assessments of the species and their need for protection.

Report submitted by:

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