Project report to DEFRA 2: 15 April 2011

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

1. Collaborations

The work since submission of the previous report has focused on DNA extraction and analysis from the samples provided by our volunteer cadre, along with samples from the Kew collection. The field collection season had finished by December 2010, but we received a number of further samples (some collected in 2010, others from personal collections), and are promised further material. Once our existing data are fully analysed (see Section 3 below) we will follow up collections of particular interest with their contributors.

Collaboration activities in 2011 will focus primarily on the autumn field season, with more strongyl focused requests for further samples, and support activities such as the workshops that have been planned for September (in Perthshire) and October 2011 (in North Wales). Preparations for the workshops have been initiated, with the Scottish work funded by the supplementary SNH grant (see section 6 below), and we have also received a small amount of matching funding from the British Mycological Society that will allow us to have a greater presence at the Welsh workshop. The Danish waxcap expert Dr David Boertmann has confirmed attendance at the Welsh workshop (subject to unavoidable work commitments), along with Dr Eef Arnolds from the Netherlands who is the primary Dutch expert on these species.

2. Collections

As noted above, we have not acquired substantial numbers of new specimens since the last report, but we have spent some time documenting existing collections. About 300 specimens of waxcaps and earthtongues (*Geoglossaceae*) at the Royal Botanic Garden, Edinburgh have been databased, mostly English material that was not included in a previous SNH-funded project to database their Scottish holdings.

Over 600 further collections of grassland fungi from the University of Aberystwyth have also been databased, and the data set will be made available via the NBN once editing is complete, along with that from Edinburgh. Nearly all of the Aberystwyth collection originates from Wales, between 2006-2010 (including 120 from the 2010 season) – these include 303 *Hygrocybe* spp. and 15 *Geoglossum* samples. The Welsh collections are mainly from the Bronydd Mawr and Pwllpeiran grassland fieldsites, Snowdon ECN grassland and Trawsgoed, Bala. Collections from elsewhere include those from Rothamsted (105 samples), Palaceleas, and Morpeth (England) and Co. Donegal (Northern Ireland). For the period between 2001-2005, the collection includes *ca* 1400 samples, including *ca* 700 from the Sourhope NERC Soil Biodiversity site in southern Scotland. In addition to these there are 679 samples from Aelybryn, Caernarfon (a 13 x 7m area in Dr Griffith's front garden) collected between 2002-2010. The samples themselves are stored desiccated at 4C in sealed plastic tubs with silica, having previously been frozen to kill insects/mites. The herbarium is now being reorganised in order to facilitate rapid sample retrieval and the various excel sheets containing sample information are being integrated.

These databasing activities constitute an additional output that was not formally offered in the project proposal, but we considered this a good opportunity to make the data available.

3. DNA sequence analysis

The molecular sequencing programme was delayed by several months due to difficulties in acquisition of key reagents; we are using a high throughput protocol that has not previously been used at Kew. Further delays were caused by foreign fieldwork that was not anticipated when the proposal was put together. Neverthess, we have been working very actively on the sequencing for about the last month.

The new protocol uses DNA extracted from samples in a 96-well plate system, and has proved to work effectively for our purposes. We have obtained good-quality ITS sequences from both the first pair of plates of *Hygrocybe* samples, one with an over 90% success rate and the other with only around 50% successful. The problem with the second plate is probably trivial in experimental terms, and we will be repeating the work shortly to retrieve the missing sequences. We also have a further four plates in the pipeline, two containing further *Hygrocybe* samples and the others containing *Geoglossaceae*. To date, we have selected material to sequence of 59 taxa (species, varieties etc.) of *Hygrocybe* and eighteen species of *Geoglossaceae*.

In early May, we intend to fill a further plate with samples from The Royal Botanic Garden, Edinburgh, to boost the number of Scottish collections in response to the extra cash from SNH. This should mean that we will have between 600 and 700 ITS sequences generated by the project by some time in June.

It must be emphasized that our results to date are preliminary, and that further analysis and "cleaning" of our sequences are necessary. We have found in a number of instances that some parts of the ITS domain in *Hygrocybe* appears to show allele-like polymorphism on an individual basis; this is not unusual amongst the *Basidiomycota* but it means that more extensive manual analysis of the trace files is necessary in order to achieve a reliable sequence.

To date, we have completed the analysis for about 130 sequences, belonging to around 40 species of *Hygrocybe*. Almost all of these originate from the samples submitted by our volunteer collectors in 2010, but the list also includes samples from the Kew collection of *H. salicis-herbaceae*, *H. splendidissima* and *H. spadicea*. The first of these is a recently recognized montane heathland species that in the UK is confined to the Scottish Highlands, and the third is BAP-listed.

Of the samples analyzed, fewer than 20 show a conflict between the field identification and the sequence-based diagnosis. This is a most heartening result, confirming independently that the field identification skills of our volunteer workforce are good. We will be analyzing these results in more detail over the next couple of months, to establish whether the original identification was mistaken or the reference sequence is incorrect, or that contamination in the molecular preparation work was responsible. In a number of cases we suspect that the conflicts are caused by the presence in our sample set of unrecognized taxa. Apparent complexities, conflicts or inconsistencies that we intend to explore include:

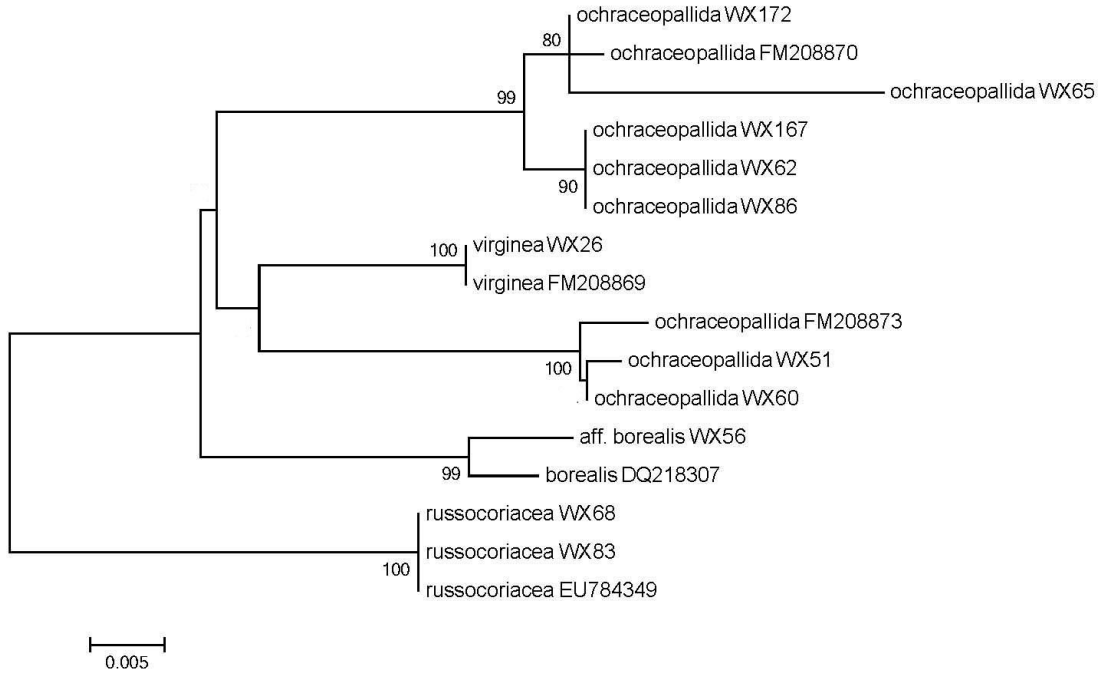
Hygrocybe virginea group

Most specimens identified as *H. virginea* var. *virginea* appear to have sequences previously identified as var. *ochraceopallida*, which appears to include two or three distinct cryptic species. This is in contrast to Boertmann's experience[1], who states that in overall terms var. *virginea* is commoner that var. *ochraceopallida*. In addition, a collection from Sussex identified as var. *virginea* showed a BLAST result most similar to the North American species *H. borealis*. Boertmann refers to divergent collections from Central Europe identified as this species; the Sussex collection may represent a north-western outlier of what could be an undescribed species.

In the tree below and in others in this report, sequences labelled with "WX" originate from this study, those with "EU" are Kew samples sequenced during previous projects, and those labelled "FM" were submitted to GENBANK by a Hungarian research group.

[1] Boertmann, D. (2010). *The genus Hygrocybe* edn 2. Danish Mycological Society, 200 pp.

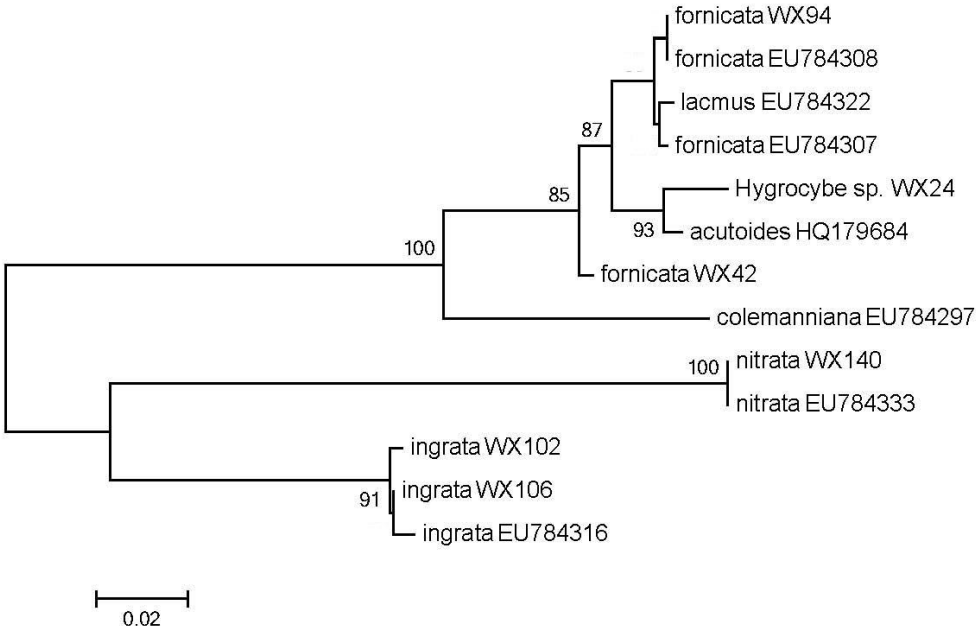
Fig. 1. ML Tree with 1000 bootstrap replicates of the *H. virginea* complex



***H. fornicata* group**

An unidentified *Hygrocybe* species collected from Perthshire (WX24; see image below) may be an undescribed/unsequenced species in the *H. fornicata* clade; from the very limited molecular data available it appears most similar to the poorly known North American species *Hygrophorus acutoides*. We also seem to have two further distinct species that are identifiable as *H. fornicata*. One of these may be specimens of *H. lacmus* misidentified as *H. fornicata*.

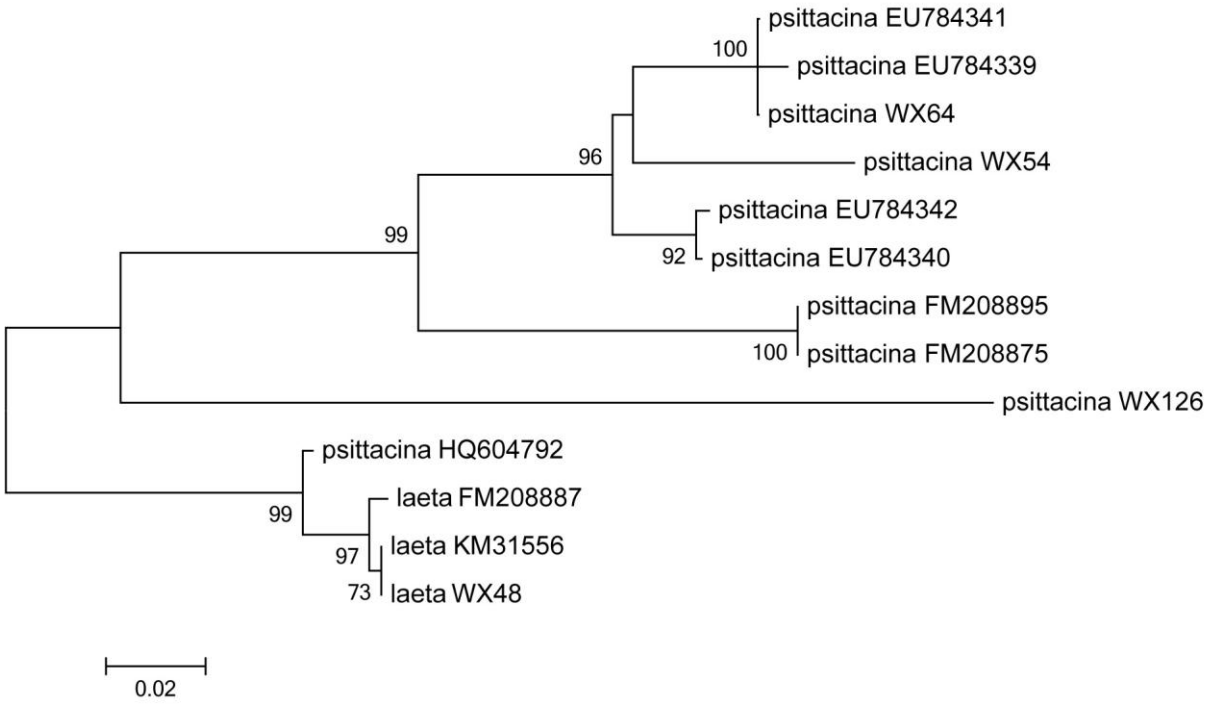
Fig. 2. ML Tree with 1000 bootstrap replicates of the *H. fornicata* clade



***H. psittacina* group**

Hygrocybe psittacina is known to be a highly variable species. Judging from preliminary molecular analysis, the species as defined in Hungary seems quite distinct from those in the UK. Several of the sequences from specimens identified as this species by our volunteer collectors were of poor quality and will be repeated in the next couple of months. Of those with sequences of acceptable quality, one corresponded well to one of the two previously sequenced UK clades, a second appears to occupy a distinct clade within the *H. psittacina* cluster, and a third is completely different and may not be a *Hygrocybe* at all. This last sample was described as distinct from *H. psittacina* in a number of characteristics including purplish pigmentation by the collector.

Fig. 3. ML Tree with 1000 bootstrap replicates of the *Hygrocybe psittacina* clade



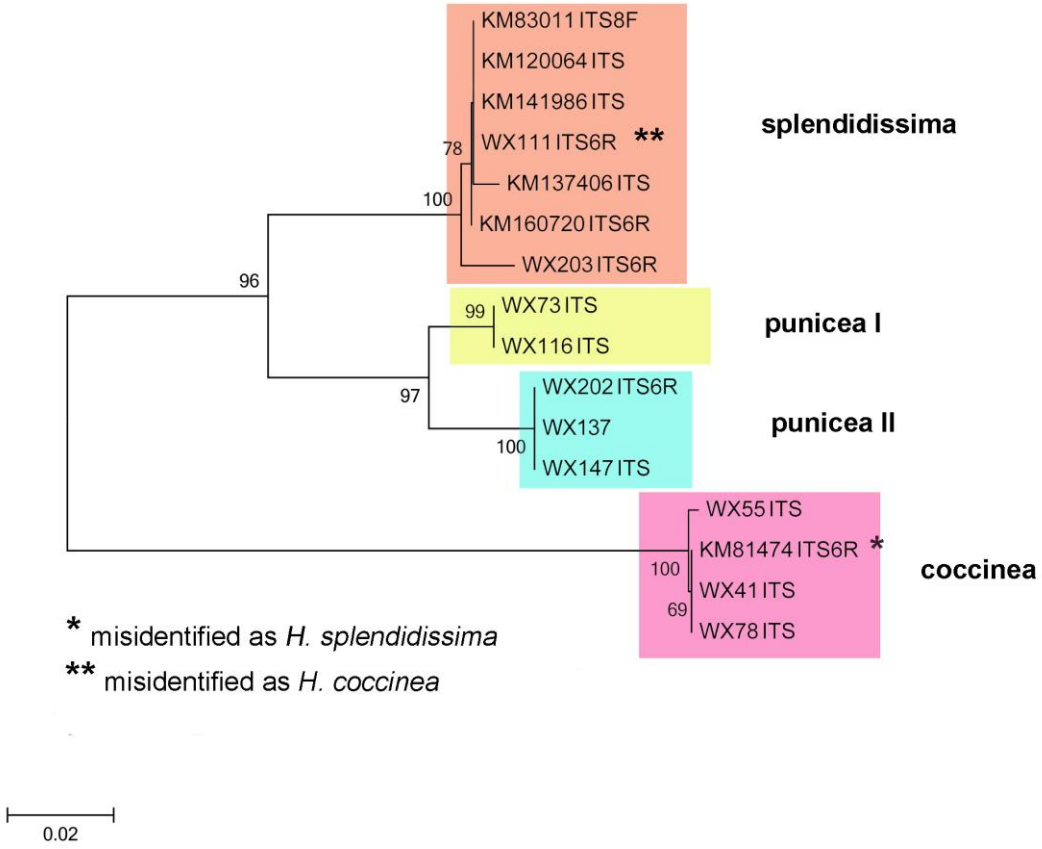
***H. spadicea* group**

We did not receive any specimens of the BAP-listed *Hygrocybe spadicea* from our volunteer collectors; this is not particularly surprising as the species is certainly rare and does not fruit reliably to our knowledge in any of its stations. Analysis of five dried specimens suggests that only three are correctly identified, though the identification of one from the sequence is so surprising that we suspect that the DNA had become contaminated. We will focus on the material we do have of this species in 2011, and are planning to carry out targetted sampling for *H. spadicea* in Scotland.

***H. punicea* group**

There are three quite common large bright red species of *Hygrocybe*, *H. coccinea*, *H. punicea* and *H. splendidissima*. Our studies of these species have shown that misidentifications can occur, both by our field collectors and in the dried collections. There have been suggestions by some authors that *H. punicea* and *H. splendidissima* are conspecific, but the molecular data indicate strongly that not only are the two species distinct, but that *H. punicea* itself is a complex of two cryptic species.

Fig. 4. ML Tree with 1000 bootstrap replicates of the *Hygrocybe punicea* clade



As indicated above, we do consider these results preliminary, but we have potentially identified around 10 novel taxa within the four groups studied so far, indicating that waxcap species richness in the UK is substantially greater than had previously been realized. There is a substantial amount of further work to be done before the new species can be accepted, especially in the identification of correlated morphological characters that could allow identification by traditional means, and assessment of ecology, distribution patterns and potential conservation issues. We do now have some lines of research to pursue, and will ask our volunteer collectors to assist in this process during the 2011 field season.

4. Ecology

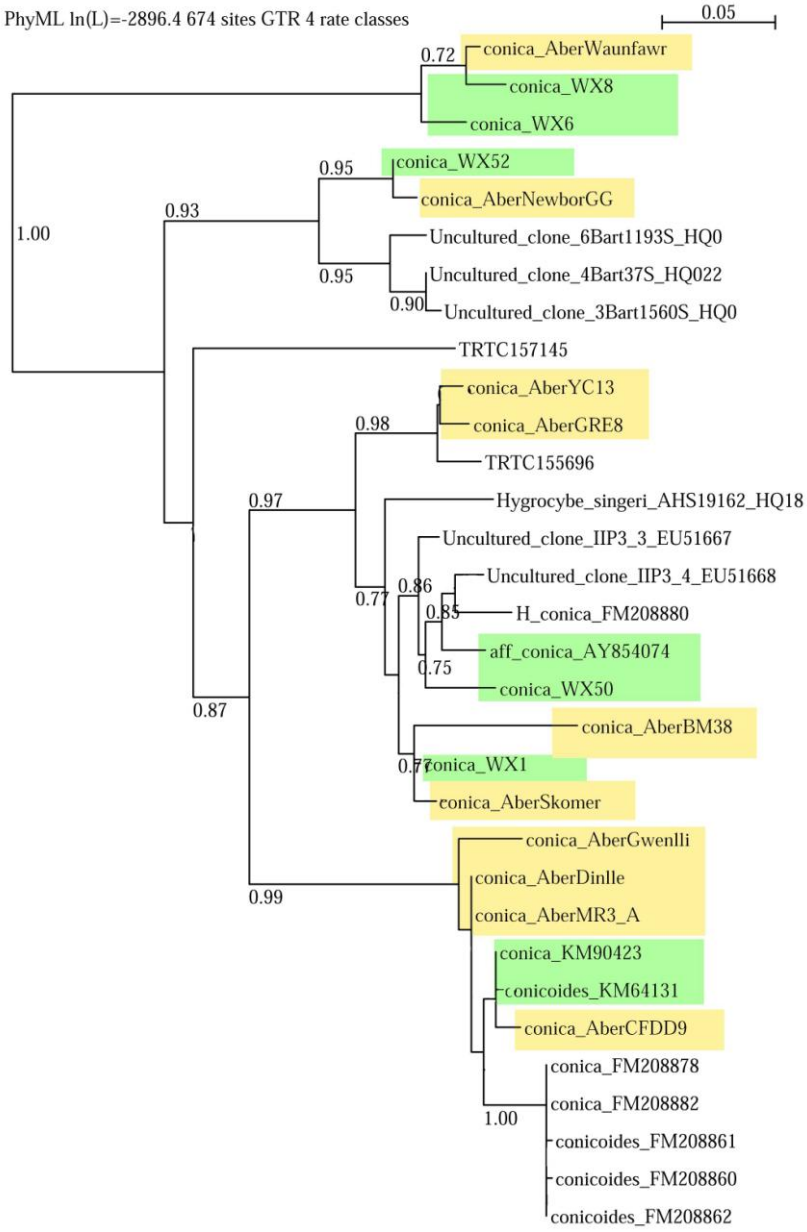
During autumn 2010, *Hygrocybe conica* and *Geoglossum cookeianum* samples were collected from Ynyslas (nr. Aberystwyth) and Dinas Dinlle (nr. Caernarfon) sand dune habitats. Intact cores (15cm^3; 8 of each species) with associated vegetation were collected and taken to the Botany Gardens Abersytwyth, where the exact position of fruitbody was marked, prior to drying and archiving of the herbarium sample. These cores (in pots) are currently stored outdoors with the vegetation intact, in readiness for destructive sampling and extraction of root samples. These roots will be used for trialling of the *H. conica* and *G. cookeianum*-specific PCR probes.

Based on ITS sequences obtained from 12 *H. conica* samples from Wales, including several from Ynyslas and Dinas Dinlle dune, a series of PCR primers have been designed and are currently being tested. Primer have been designed based on regions of the intronic ITS1 and ITS2 regions which are conserved amongst the *H. conica* samples. Several primer pairs have been designed and these are being validated against a range of *H. conica* samples. We will then move take the same approach in designing primers for specific detection of *G. cookeianum*. The process may be more complex than anticipated as preliminary analysis of *H. conica* sequences from the project have identified substantial diversity, suggesting that this too is a complex of cryptic species

(see below). Neverthless, it should be possible to develop primers that work at least on a local level and can potentially track associations with the surrounding vegetation. Research just published from the USA, admittedly inconclusive, suggests that *Hygrocybe* species most probably have biotrophic (presumably parasitic) rather than saprobic nutrition and points towards bryophytes as the most likely food source.

We have carried out a preliminary phylogenetic analysis of the samples of *H. conica* that we have so far analyzed, along with other reference sequences from Kew and GENBANK. The sequences proved difficult to align and may show unusually high levels of variation in the ITS region, but indications so far suggest that there are seven or eight species in the clade, almost all of which have been detected in Welsh sand dunes.

Fig. 5. ML Tree (not bootstrapped) of the *Hygrocybe conica* clade. Welsh sand dune collections are shaded yellow, other UK collections are shaded green.



5. Outreach

Further outreach activities have included a short article currently being prepared for the DEFRA Elements magazine, the project website has been updated and it now features on the Kew website. We plan to send an e-newsletter to the project partners/stakeholders shortly, to include much of the information in this report. A non-technical description of the project has been sent for appearance on the Scottish Fungi website (http://sites.google.com/site/scottishfungi/).

Further progress has been made on preparing for two workshops in autumn 2011, which will be the main outreach activity for volunteer records.

6. Scotland

In response to the work plan set by SNH for the Scottish component of the project, the following activities have taken place:

1. Preparation of a non-technical article on the project, which has been submitted to Dave Genney (SNH) for inclusion on the Scottish Fungi website (see above), and can be made more widely available on request.
2. Scottish collections of waxcaps and earthtongues to be included in the sequencing programme. There is currently DNA from 87 Scottish specimens in the seqencing pipeline, with 15 good-quality sequences obtained. The number will be augmented substantially in early May, when material from the collections at RBG Edinburgh will be accessed – probably at least 100 further samples. Focus will be on Scottish BAP species as specified, and on other rare and poorly known taxa that may be of conservation concern. We have obtained an ITS sequence of *Hygrocybe salicis-herbaceae* for the first time; this is an arctic-alpine species with its only UK stations in the Scottish Highlands, and should be the subject of further analysis for its conservation status.

   We have also been sent a sample from Perthshire of what appears to be an undescribed species in the *H. fornicata* aggregate, as detailed in Section 3 above and illustrated here. Further assessment will be made of its relationships, and we shall be asking that the site be monitored in the future.

3. Databasing has taken place of collections as described in Section 2 above. Waxcap and earthtongue records from Kew and Edinburgh are now databased in their entirety, and further Scottish samples currently at the University of Aberystwyth will be databased shortly. These constitute by far the largest and most significant collections of these fungi in the UK.
4. Preparations for the Scottish workshop have been advanced, in participation with Dave Genney (SNH); this will be held on 24/25 September at Mar Lodge near Braemar, with consultant mycologist Liz Holden as local organizer. The workshop will provide a mixture of basic support for those new to waxcap recording and matters of interest for the more specialist mycologists, in addition to reports back on progress to date and requests for targetted collection in the 2011 season. It has been advertised on the Scottish mycology

website with individual invitations going to those who we would particularly like to see present.

5. Survey of historical sites for Scottish BAP species has been planned; details remain to be clarified but the intention is to focus on *Hygrocybe spadicea* sites in Southern Scotland in the early part of the season, and localities for *Geoglossum atropurpureum* in NW Scotland later on (probably Skye). The latter species has turned out to incorporate two quite unrelated taxa (work published since the project began) and we will do our best to separate the historical records into the constituent species.

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