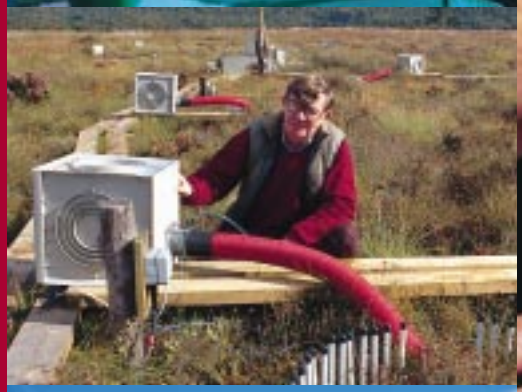
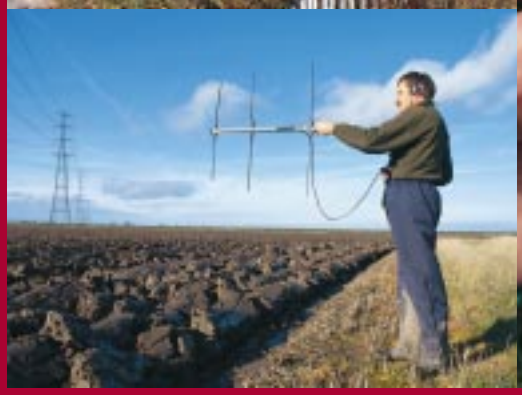
ENGLISH NATURE

Report Number
625

Synonymy between *Battarrea phalloides* and *B. stevenii*

English Nature Research Reports





working today
for nature tomorrow

English Nature Research Reports

Number 625

Synonymy between *Battarrea phalloides* and *B. stevenii*

Peter Jeffries and Lynton McLain
Department of Biosciences, University of Kent, Canterbury, Kent, CT2 6NJ

August 2004

ISSN 0967-876X

Summary

Battarrea phalloides, the sandy stiltball, is one of four non-lichenised fungi afforded legal protection by being included in Schedule 8 of the Wildlife and Countryside Act 1981. *Battarrea phalloides* is classified as endangered in the GB provisional Red Data List and is subject to a Priority Species Action Plan, under United Kingdom Biodiversity Action Plan. *Battarrea phalloides* and *B. stevenii* are morphologically similar, and limited molecular data has recently suggested they could be considered as synonyms. This study used traditional and molecular methods to compare field-collected English specimens and an extensive range of herbarium material in order to resolve the question of conspecificity.

Tissue from 78 specimens of *B. phalloides* and *B. stevenii* were obtained from the Herbarium of the Royal Botanic Gardens, Kew. These comprised the entire Kew collection of these species, one of the largest in the world. *Battarrea phalloides* specimens came largely from the UK, in similar habitats, whereas *B. stevenii* specimens came from a variety of world sites, with widely differing habitats. Other fresh samples were collected in Suffolk, from the area where the first specimen of *B. phalloides* was found in 1782.

Variability between specimens was assessed by comparing basidiocarp morphology and spore sizes. Molecular studies were based on SSCP (single-strand conformation polymorphism) analysis and sequence comparison of the internal transcribed spacer (ITS) regions of the rRNA gene cluster of the fungal genome. Morphological comparisons showed that the specimens examined formed a continuum, but that specimens would be assigned species identities on the basis on size or other variable characters. For example, there was a wide range of stipe and cap sizes between specimens of *B. stevenii* from a range of world habitats, while specimens labelled *B. phalloides* were generally more consistent in size and smaller than those of *B. stevenii*. Spore sizes also showed a continuum across the two species rather than a bi-modal distribution.

SSCP analysis showed that there was variability in sequence within the ITS region, but this did not correlate with the species designation. Phylogenetic analysis of ITS sequence data did not separate *B. phalloides* and *B. stevenii* specimens, thus suggesting they are conspecific.

In conclusion, we found little evidence to separate the two species. Specimens labelled *B. stevenii* came from a diversity of habitats, some of which might have influenced their morphology. Thus taller, scalier specimens would be assigned to *B. stevenii*, on the basis of environmentally-influenced morphology, even though the molecular evidence suggests they are conspecific with *B. phalloides*.

Contents

Acknowledgements
Summary

1. Background 9

2. History and classification 9

2.1 *Battarrea phalloides* 9
2.2 *Battarrea stevenii* 11
2.3 Comparison of *B. phalloides* and *B. stevenii* 12

3. Aim and objective 13

4. Materials and methods 13

4.1 Specimens examined 13
4.2 Morphological observations 14
4.2.1 Macromorphology 14
4.2.2 Micromorphology 14
4.3 Molecular analysis 15

5. Results 16

5.1 Macromorphology 16
5.2 Micromorphology 17
5.2.1 Molecular analysis 18

6. Discussion 22

7. References 23

Appendix 1. List of material examined 25

Appendix 2. Statistical analyses 31

1. Background

Battarrea phalloides, the sandy stiltball (Figure1), is one of four non-lichenised fungi afforded legal protection by being included in Schedule 8 of the Wildlife and Countryside Act 1981. The other endangered non-lichenised fungi in the British Isles also protected under the Act are *Boletus regius, Buglossoporus pulvinus* and *Hericium erinaceum*. *Battarrea phalloides* is classified as endangered on the provisional GB Red Data List and subject to a Priority Species Action Plan under United Kingdom Biodiversity Action Plan. This research project relates to *Battarrea phalloides* and the other main species in the genus*, B. stevenii*. Recent molecular evidence has suggested these two species might be synonyms (Martin & Johannesson 2000).

Information about the genetic diversity, speciation and the mode of spread of an organism are fundamental to the formation of Biodiversity Action Plans (English Nature 2002; Anon. HMSO 1995). These action plans develop policy initiatives for the long-term protection of endangered species and their habitats. These plans are developed by coordinating action by local authorities, government departments, local wildlife trusts, and agencies such as English Nature, responsible for managing the protection of endangered species and monitoring Sites of Special Scientific Interest, under Acts of Parliament, and by encouraging research into endangered species. Thus it is important to know whether fungi described under different names ought be synonymised in the Red Data List.

2. History and classification

2.1 *Battarrea phalloides*

Eumycota, Basidiomycotina, Gasteromycetes, Tulostomatales, Tulostomataceae, *Battarrea.* Type species: B. phalloides

Battarrea phalloides was discovered by a Mr Humphrey in Norfolk in 1782 (Woodward 1784) and described by Dickson (Dickson 1785) as *Lycoperdon phalloides*. The formal Linnean classification, the first description for the genus *Battarrea* and the type description of the species *B. phalloides*, was published by Persoon (Persoon 1801). A painting, entitled *Lycoperdon phalloides* is possibly the earliest representation of *B. phalloides*, and is in a book of coloured figures of fungi in the Mycology Library at the Royal Botanic Gardens, Kew (Sowerby 1803).

The first recorded reference to what was subsequently known as *Battarrea phalloides* appeared in the Philosophical Transactions of the Royal Society (Woodward 1784). The 'plant', was described under the name *Lycoperdon phalloides*: 'Its specific character is thus given, *Lycoperdon* deflexum campanulatum, supra pulverulentum, calyptratum, subtus glabrum, stipite volvato' ie the cap droops downwards and has a powdery spore mass on the outside of the cap. To this genus it must at present probably be referred, though the total want of an exterior coat prevents its agreeing with it so perfectly as it ought.' Woodward goes on to write that 'this extraordinary vegetable production arises from a volva, which is buried 6 or 8 inches deep in dry sandy banks; and consequently it is extremely difficult to detect in its earliest state'. The specific epithet refers to the similarity of the volva to the genus *Phallus*. 'At its first appearance above ground, the powdery head is covered with a loose campanulated cap, which does not adhere by any of the smallest filaments'. Woodward

supposed this to be the upper part of the volva, as both always appear ragged when taken up. 'When fully grown, in a few days after its first appearance above ground, the plant attains the summit of its growth, which is from 9 to 15 inches, more than half being generally buried in the ground. The stem become woody, though hollow, the bark still more ragged, and the whole plant much lighter, both volva and stem being now quite dry, and free from mucilage. At length the stalk appears with a naked, coriaceous, campanulated pileus, and considerably bleached, in colour and appearance not unlike a dry stalk of hemp.'

Figure 1. *Battarrea phalloides* at the protected hedgerow site at Blyford, Suffolk, November 2002

Woodward gave the date when he 'first met with it' as February or March 1783 'in its dry and withered state' (Woodward 1784). He suspected it to be a decayed *Agaricus procerus*, and wished to examine the root to see if it was bulbous. 'The bulbs of *Agaricus* are scarcely hidden under the surface', but he had to 'dig deep for the root of this plant, 7 or 8 inches, before discovering the volva, so unlike the fugitive one of the agaric,' that he was 'immediately convinced it must be something new'. Woodward's report gives no date for the discovery of this first specimen but Ramsbottom (Ramsbottom 1953) and Pegler (Pegler, Laessoe & Spooner 1995) wrote that it hadbeen gathered by W. Humphrey at Bungay, Suffolk in 1782. Suffolk, Norfolk and Kent remain the main sites in England for this rare fungus. Watling (Watling, Gucin & Isiloglu 1995) agreed that the fungus was collected by Humphrey in 1782 and shown to Woodward in spring 1783; Woodward then sent it to J. Dickson, who formally described the fungus (Dickson 1785). This description forms the basis for the first use of the name *Battarrea phalloides* in Persoon (Persoon 1801) as *B. phalloides* (Dicks.) Pers. The genus was named after A. C. J. Battarra, an Italian fungal biologist

> 'BATARREA. Voluatal stipitata. Pileus deflexus, campanulatus, villosus, pulueris strato obsitus, a volua calyptratus.
> Batar. phalloides: fuscescens.
> Lycoperdon phalloides, voluatum stipitatum, pileo deflexo campanulato suprapulueplunerulento calyptrato, infra glabro libero.
> Dicks. Pl. cryp. Brit. Fasc. 1. p.24. Woodward in Act. Angl. V.74. p423. t.26
>
> In aggeribus aronosis in Anglia prope Norwich inuenit G. Humphrey et circa Bungay Suffolciae T. Woodward.

Battarrea has a pronounced, wood-like stem which distinguished it from the other puffball-like fungi, although 'some species have a sterile base which resembles a stem, with the same origin as the fertile gleba, but is a portion of the gleba which does not carry on with its original purpose' (Ramsbottom 1953). Ramsbottom also identified three genera in the UK which have a true stem, distinct from the glebal tissue from its first appearance, and which he suggested were 'nearly related' to puffballs, *Tulostoma, Battarrea* and *Queletia*, each represented by one species. He described *B. phalloides* as a 'strange-looking fungus, usually about a foot (30 cm) high, rusty brown, with the spores enclosed in a concave-convex receptacle, which is borne on a hard tapering stem covered with long twisted fibres, seated in a loose white parchment-like volva. The receptacle splits around the rim to release the spores. The young fungus is ovate, whitish or slightly brownish, with a wall composed of an outer fleshy and almost membranous inner layer, the space between them filled with mucilage. As development proceeds, the young stem elongates rapidly and with some force, bursts through the soil carrying the whole of the inner and part of the outer layers of the volva on the top of the cap where it gradually dries.' This description has many features in common with the original account given by Woodward (Woodward 1784).

Contemporary descriptions of *Battarrea phalloides* emphasise many of the same features. For example, Mycoweb (Mycoweb 2002) reports 'The fungus has a fruiting body 4 – 7 cm broad, 2 – 3 cm thick; it is compressed-globose, white to cream and partially buried in the substrate. It has an exoperidium, ruptured by a spore sac and elongating stalk. The spore sac is 2.5 – 4.5 cm broad, 2 – 3 cm thick, convex and covered with a white membranous endoperidium, the latter splitting horizontally along the margin, exposing a sticky, brown spore mass. The stalk is 15 – 35 cm tall, 0.5 – 1.5 cm thick, equal to tapered at the base, with fibrous, rusty-brown scales. A membranous volva at the base shrivels with age. The spores are 5.5 – 6.5 μm, nearly round, warted. The spore print is rusty-brown'.

The most recent formal description is from Pegler (Pegler, Laessoe & Spooner 1995): '*Battarrea phalloides* has 'volva present, gelatinous, stem tall, hard, scaly, receptacle pendant, spirally thickened elators. It has a fruitbody egg 3 – 4 cm diameter, ovoid, whitish at first, becoming brown, buried in soil; peridium 2-layered. The exoperidium is gelatinous; the endoperidium has circumcissile dehiscence. The stipe at first has a gelatinous coat, which soon dries, becoming hollow and 9 – 30 cm high, 6 – 20 mm diameter, pale brown to brown or greyish brown, surface fibrous-scaly, often shaggy. The receptacle is pendant, convex to hemispherical, bearing the gleba, which is exposed by the shedding of the peridial cap.'

2.2 *Battarrea stevenii*

Battarrea stevenii was first described as *Dendromyces stevenii* by Liboschitz (Liboschitz 1814), but Fries attributed the species to *Battarrea* (Fries 1832). *Dendromyces* was originally an ascomycete genus, but has been synonymised with *Battarrea* (Eriksson & Hawksworth 1998). The two type descriptions vary in a number of morphological characters (Table 1), such as the degree of scaliness of the stipe, the size of the cap and whether the volva is gelatinous, as well as the geographical distribution. Fries (Fries 1832) concluded that 'plants of slender habit, with narrow scales on the stipe, represented *B. phalloides*, and plants usually more robust, with broad, ribbon-like scales, represented *B. stevenii*. (Libos.) Fr.'

2.3 Comparison of *B. phalloides* and *B. stevenii*

Based on a comprehensive iconography of specimens from around the world, Hollos (Hollos 1904) first suggested that *B. phalloides* and *B. stevenii* formed a single polymorphic species. However, Maublanc and Malencon (Maublanc and Malencon 1930) maintained that the important distinction between the species was the presence of abundant gelatinous material within the universal veil in the egg stage of *B. phalloides* and its absence in *B. stevenii*. They quoted observations of the dry volva (at the egg stage) by previous authors in fungi referred to as *B. stevenii,* which they considered to be identical with *B. guicciardiniana* (a species which they differentiated from *B. stevenii* by the lack of a volva) on which their work was done. These authors also suggested a link between habitat and morphological development, such that *B. phalloides,* the form with a gelatinous volva was confined to northern, cooler, more humid regions. In contrast, the *B. stevenii-guicciardiniana-gaudichaudii* group, with a dry volva was confined to southern, subtropical, hot, dry regions. Rea (Rea 1942) studied 25 specimens from southern California and also concluded that *B. stevenii* 'may be considered a synonym of *B. phalloides*' but admitted that his work 'contributes to, but does not solve, the problem of the limits of variation in *B. phalloides*'. Most of the specimens studied came from desert habitats yet 'some of the desert plants agree perfectly with the typical English *B. phalloides* in size, slender habit, fine scales and other characters. At the other end, two plants from the coast (with a very mild and equable climate) agree equally well with *B. stevenii*.' It was noted, however, that some of the desert specimens had coarser scales. Rea (Rea 1942) pointed out a fundamental problem with using the gelatinous, or dry volva as a distinguishing feature between the two species, since it requires the observation of the egg stage which Rea did not encounter. Watling (Watling, Gucin & Isiloglu 1995) considered this a significant character in separating the two species, and it was one of the important field characteristics observed by the early British collectors. These authors also considered that *B. phalloides* was distinguishable on the basis of the presence of sterile 'elater' cells, and in the more orange, tawny, slightly larger (5-6.5 x 5.75-7 μm), less ornamented basidiospores compared with *B. phalloides* (4.5-5.25 to 6 x 4.5-5.75 μm); much greater basidiome size; more scaly stipe and lack of mucilage in the volva and stipe medulla than *B. phalloides*. Reid (1985) confirmed the presence of the gelatinised tissue in material from the Channel Islands, as Watling (Watling, Gucin & Isiloglu 1995) also did with material collected in Turkey. Moreno and others (1995) identified collections from Mexico and Spain as *B. stevenii*, while expressing the view that they might be conspecific with *B. phalloides*. More recently, Martin and Johannesson (Martin and Johannesson 2000) did not support a separation of the two taxa on the basis of morphological and molecular data. Specimens of both species came from Austria, Burundi, France, Hungary, Italy, Mexico and Spain. They repeated the dictum that while *B. phalloides* had a gelatinous volva (but not in their specimens) and smaller basidiomata than *B. stevenii*, they noted that these characters were not enough to separate the taxa at the species level. The authors used scanning electron microscopy for spore examination, but they observed no differences in spore size between the two *Battarrea* species. Also, they found 'no other morphological characters to support the separation of the two taxa.' The work was also complemented by sequence analysis of the ITS region of the ribosomal gene cluster using 5 specimens of *B. phalloides* and 12 of *B. stevenii*, mainly collected from Spain. The sequence variability within *Battarrea phalloides*, was greater than the variability between the two species. Phylogenetic analysis showed that *B. phalloides* specimens did not cluster together; instead they appeared in different lineages with the *B. stevenii* specimens, alternating unevenly through different branches. The authors concluded that both taxa belong to the same species.

Table 1. Comparison of the type descriptions of *Battarrea phalloides* and *Battarrea stevenii*

Type specimen description. Earliest description (pre-*Battarrea*)	*Battarrea phalloides* (Dicks.) Pers. *Syn. Meth. Fungi*, p. 129, t. III, F. 1 (1801). *Lycoperdon phalloides* Dicks. Crypt. Brit. 1, p. 24 (1785).	*Battarrea stevenii* (Libosch.) Fries. *Syst. Myc.* 3: 7 (1832). *Dendromyces stevenii* Lib. Monogr. t. 1, 2. (1814)
Volva	The volva has two layers, is white, mucilagenous and is buried between 7 and 8 inches underground.	The dry volva is found in the ground.
Stipe	The stipe is cylindrical, bare, hollow and woody, with bark that is striped and lacerated; it stands about 1 foot in height.	More scaly
Cap	The cap is smooth, with a serated edge and is turned abruptly downwards.	Cap is generally larger
Veil	Above the cap is a veil, free and loose.	Similar to *B. phalloides*
Spores	The powdery spore mass is dark.	More orange, tawny, slightly larger
Habitat	The habitat of *Lycoperdon phalloides* is fields with sand (A*ggeribus arenosis)*, specifically Norwich, Norfolk and Bungay, Suffolk. *B. phalloides*, with a gelatinous volva, is confined to northern, cooler, more humid regions.	*B. stevenii*, with a dry volva is confined to southern, subtropical , hot, dry regions.

3. Aim and objective

The aim of the project was to clarify uncertainties about the taxonomic relationships between the two species. Are *Battarrea phalloides* and *Battarrea stevenii* conspecific?

The objective was to use a much more comprehensive collection of material than any previous study, and to compare both morphological and molecular data.

4. Materials and methods

4.1 Specimens examined

A total of 78 specimens were examined from sources around the world, of widely differing habitats, from the rift valley in Kenya, to Baghdad, and from Jersey to Suffolk in the UK, close to the site of the original type collection. The complete collection of herbarium samples at the Royal Botanic Gardens, Kew, was examined. This comprised 40 samples labelled *B. phalloides*, 24 samples labelled *B. stevenii*, and 4 samples labelled as other species of *Battarrea*. One additional specimen of *B. phalloides* was found in the collection and was returned to its original repository at Wisley. In addition, three fresh specimens were collected

from Blyford, Suffolk, and an additional 7 dried specimens donated by the Suffolk Wildlife Trust. See Appendix 1, tables 3-6.

4.2 Morphological observations

4.2.1 Macromorphology

Measurements were taken of complete specimens of both species at the RBG Kew Herbarium and from specimens from Suffolk. Measurements were taken of stipe length and diameter; cap diameter and height and also of the length and diameter of the volva, where this was present.

4.2.2 Micromorphology

Not all the specimens from the RBG Kew, Herbarium were preserved with spores and so could not be included in this part of the survey. For *B. phalloides*, spores from 19 specimens were measured, and for *B. stevenii* spores from 17 specimens were measured. An ethanol-sterilised scalpel blade was used to deposit spores on standard microscope slides. One drop of sterile Milli-Q water was added to the spores and a 13 mm diameter glass cover slip was placed on the liquid/spore suspension. The slip was pressed to expel excess water. One drop of immersion oil was added to the centre of the slip. Observations were carried out under a Zeiss Axioscop microscope, with an Achrostigmat 100/1.25 objective lens and 10X eyepiece lens, by moving the slide carriage along the 50 graticule mark from right to left. Spores that crossed the 50 graticule mark were measured for length and then the graticule was rotated through 90 degrees and spore breadths were measured. The mean length and breadth for 100 spores per specimen were calculated and the spore quotient (length/breadth) was calculated.

Figure 2. *Battarrea* species from RBG herbarium collected in Cape Province, South Africa UKC58 - *B. phalloides* or *B. stevenii?*

Graphs were drawn on MS Excel, including a graph of spore quotient *vs*. age of spores to assess whether or not there was any correlation between the age of spores (some more than 100 years old) and the spore quotient, to take account of the possible effect of shrinkage of spores with time, through dehydration. One-way ANOVA was used to compare differences between means.

4.3 Molecular analysis

Cap tissue, including spores, previously kept in cell lysis buffer (Puregene), was placed in a cryolite microcentrifuge tube with 400 µm Puregene cell lysis buffer, 4 µm RNaseA and 300 mg 0.5 mm diameter glass beads. After incubation at 65°C for 60 minutes, the material was disrupted with bead beating for 4 x 30 second cycles at maximum speed, 5,000 rpm.

DNA was then extracted with phenol/chloroform. Phenol (600 µl from the fridge, 4°C) was added to the incubated lysed cells in the eppendorf and mixed by hand for 5 minutes and centrifuged at 13,000 rpm for 10 minutes. The top layer was transferred to a new 2 ml eppendorf and 600 µl phenol added and mixed by hand for 5 minutes, before centrifuging at 13,000 rpm for 10 minutes. The top layer was transferred to a new 2 ml eppendorf tube and 600 µl cold chloroform added. The contents were mixed by hand for 5 minutes and then centrifuged at 13,000 rpm for 10 minutes. The top layer was transferred to a new 2 ml eppendorf tube and 600 µl chloroform added and mixed with a pipette tip for 5 minutes and then centrifuged at 13,000 rpm for 10 minutes. The top layer was again transferred to a new 2 ml eppendorf tube. The supernatant volume was estimated and 0.8 x this volume of isopropanol (from the -20°C freezer) was added to precipitate the DNA. The DNA/isopropanol mixture was then kept overnight in the -20°C freezer to precipitate the DNA and centrifuged at 13,000 rpm for 30 minutes. The supernatant was poured off and 100 µl of 70% sterile ethanol was added to the pellet of DNA and the tube centrifuged at 13,000 rpm for 10 minutes before being dried at room temperature and re-dissolved in 30 µl 1x TE buffer pH 8.0 for storage at 4°C overnight.

For amplification, basidiomycete fungal-specific primers, ITS1F and ITS4B, were used (Gardes & Bruns, 1993) with *Super Tth* from HT Biotechnology Ltd, Cambridge. PCR amplification used a standard master mix (with 3.0 µl of magnesium chloride per 1.0 µl sample of template DNA) and the Kew Herbarium PCR cycle for basidiomycete tissue PCR cycle in an MJ Research PTC-200 Peltier Thermal Cycle DNA Engine. This involved the following: denature 95°C (35 seconds.), anneal 48°C (55 seconds.), extend 72°C (45 seconds.) for 12 cycles, followed by a further 12 cycles as above with an extension time of 120 seconds, followed by a further 8 cycles as above with an extension time of 180 seconds. The mixture was then left at 72°C and then stored at 4 °C before use.

Amplified DNA from PCR was analysed for single strand conformation polymorphisms using the methods of Clapp and others (2001) and the band patterns analysed for homology between individual isolates of *B. phalloides* and *B. stevenii.* This procedure enables the multiple comparison of DNA fragments for sequence differences. If SSCP profiles are identical, then it can be assumed that the sequences are also identical. If SSCP profiles are different, then sequence differences are expected. Thus the protocol allows large numbers of samples to be associated into groups with identical sequences, and then one individual per group can be sequenced (Clapp and others 2001).

Once the representative samples had been identified, the amplified DNA was purified with a Wizard PCR Preps DNA Purification System (Promega) using a vacuum manifold. The purified DNA from 20 successful extractions and amplifications of Herbarium and field specimens of *B. phalloides* and *B. stevenii* was submitted to ABC (Advanced Biotechnology Centre) at Imperial College London for sequencing on an ABI Prism 310 system. Once sequences were returned, several were entered into BLAST to find near matches and a selection of matches and outlying sequences were selected for phylogenetic analysis. Sequences were aligned using CLUSTALW and trimmed in JALVIEW before neighbour-joining phylogenetic trees were constructed using PAUP 4.0b10 (Swofford 1999). Maximum Parsimony was used to produce a more conservative tree where only phylogenetically informative sites were analysed. Maximum parsimony analysis was performed using heuristic search options with 100 bootstrap replications (Felsenstein 1985) branch swapping by tree-bisection reconnection (TBR), branches able to collapse yielding polytomies with parsimony uninformative characters excluded.

5. Results

5.1 Macromorphology

Stipe dimensions: A comparison of 24 specimens identified as *B. phalloides* and 26 of *B. stevenii* by stipe size (Figure 3) showed that *B. phalloides* specimens all had long, narrow stipes, all less than 14 mm in diameter, but some approaching 350 mm long. In contrast, *B. stevenii* specimens became broader as they became taller. However, it should be noted that almost half the *B. stevenii* specimens examined overlapped *B. phalloides* in stipe length vs. stipe diameter. Statistical analysis (see appendix 2) indicated that there is no significant difference in stipe length between the two groups identified as different species, but those identified as *B. stevenii* had thicker stipes.

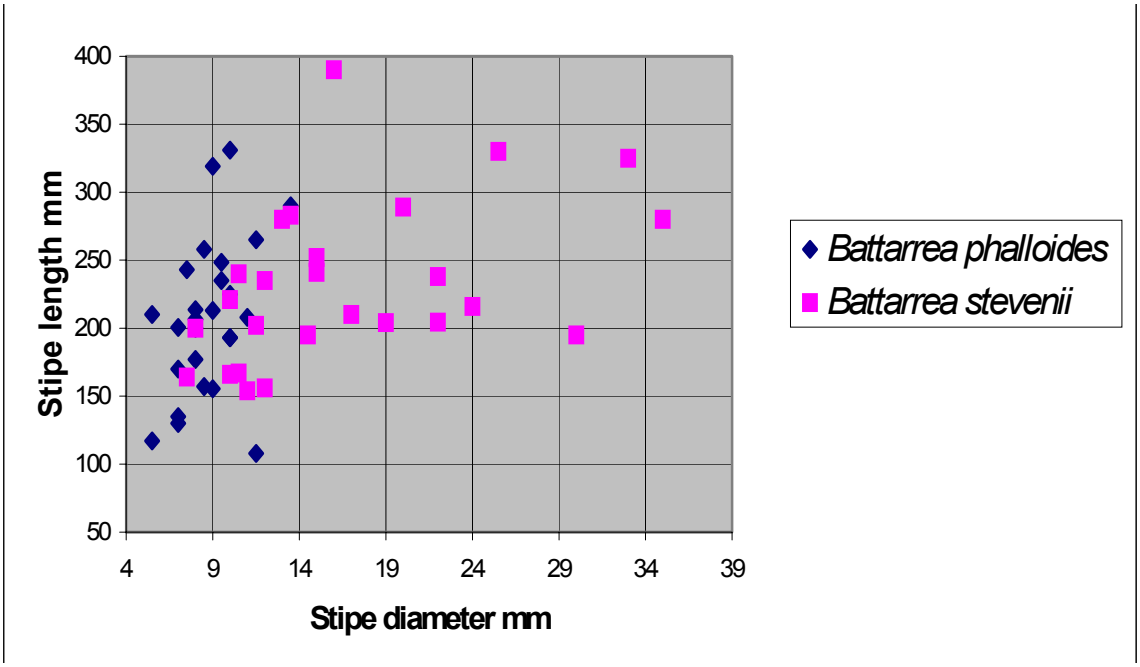


Figure 3. Species comparison by stipe size

Cap dimensions: Most specimens of both species had cap sizes from 20-40 mm diameter and 15-25 mm height (Figure 4). However, those with cap sizes above 40 cm tended to be labelled *B. stevenii*, while the smallest caps were usually on specimens identified as *B. phalloides*. Statistical analysis of cap dimensions indicates that cap size is significantly correlated with the separation into two species (see appendix 2).

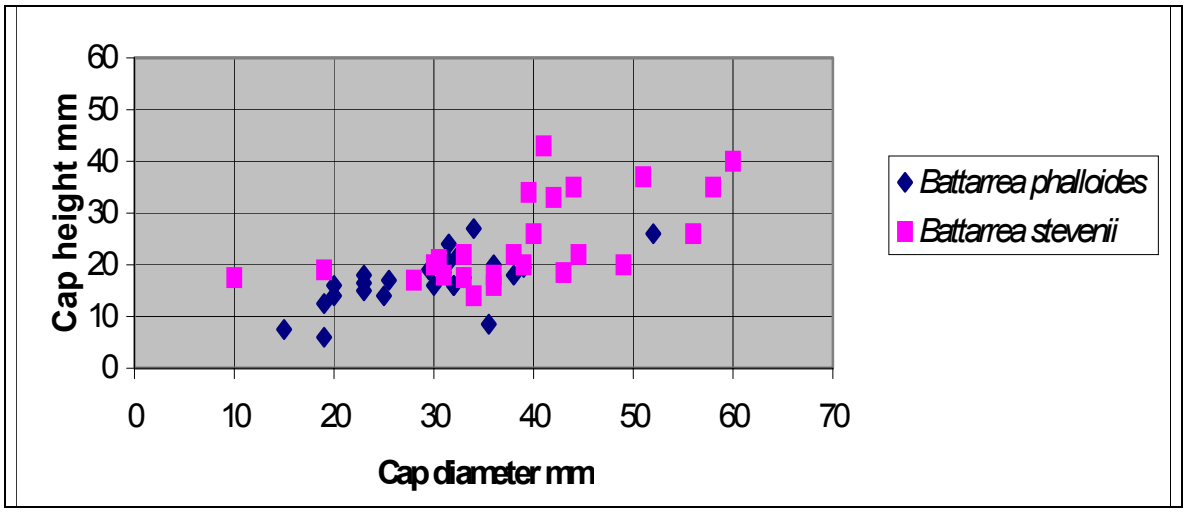


Figure 4 Species comparison by cap size.

5.2 Micromorphology

The dimensions of basidiospores from the 35 herbarium specimens measured are summarised in Figure 5. The spore sizes form a continuum, and most of the specimens have spores sizes in the overlap region. However, spores from specimens labelled *B. stevenii* tend to have larger spores than those labelled *B. phalloides* and the difference between the two groups was statistically significant ($p=0.002$ for length; $p = 0.004$ for breadth) ie there was a significant difference in mean spore size between species. Eighteen of the *B. phalloides* specimens from the RBG Kew Herbarium had no spores, including the oldest specimen, dated 1803. The age of specimens with spores ranged from 136 years old to one year old and to eliminate the possibility that spores got smaller with age, an analysis of age versus size was carried out. There was no correlation, thus spores did not shrink with age.

The colour, ornamentation and shape of the spores was also noted, but no clear association of any of these characters emerged. Most spores were round or oval, but others were pear-shaped, but this parameter was difficult to quantify objectively. Half the spores of *B. phalloides* and *B. stevenii* were round, but both species also had oval, pear-shaped and uneven-shaped spores. Ornamentation appeared pitted in all cases; no other detail was evident.

In summary, despite the overall means of spore size being different, this character is not useful to distinguish species as there is considerable overlap at the level of individuals.

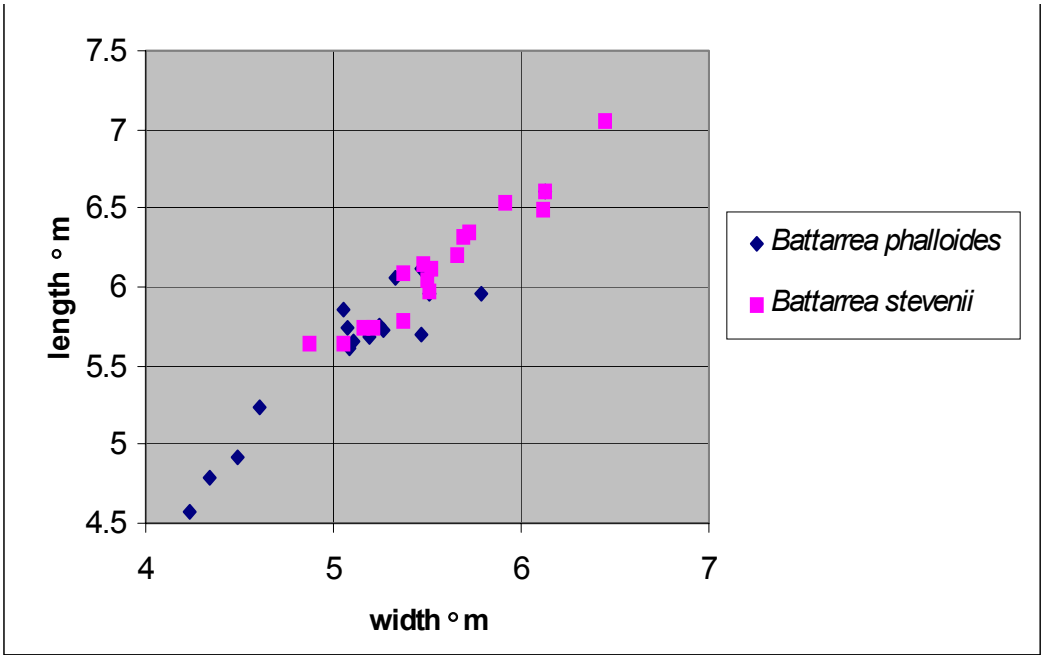


Figure 5 Species comparison by spore size

During the examination of spores, elaters were also found routinely in microscope examination of both *B. phalloides* and *B. stevenii*. The elaters found were semi-transparent, or lightly coloured brown, conical tubular structures, often twisted, and wider at an opening, which had a diameter slightly greater than the diameter of a spore, than at the other end, which appeared to be closed; each elater had circumferential, near-circular ribs with lateral structural elements, thicker towards the open end.

5.2.1 Molecular analysis

SSCP analysis (Figure 6) showed that there was considerable sequence divergence within the ITS region across the range of specimens examined. Some isolates could be grouped together with apparently similar sequences despite the fact that they were labelled as different species (for example *B. phalloides* UKC39 and *B. stevenii* UKC55 and 67), whereas others had different sequences yet belonged to the same species designation (for example *B.stevenii* UKC56, UKC57, UKC67 and UKC69).

Amplified products from the ITS region of the following individuals were sequenced, representing the range of SSCP profiles, and including representatives from both species:

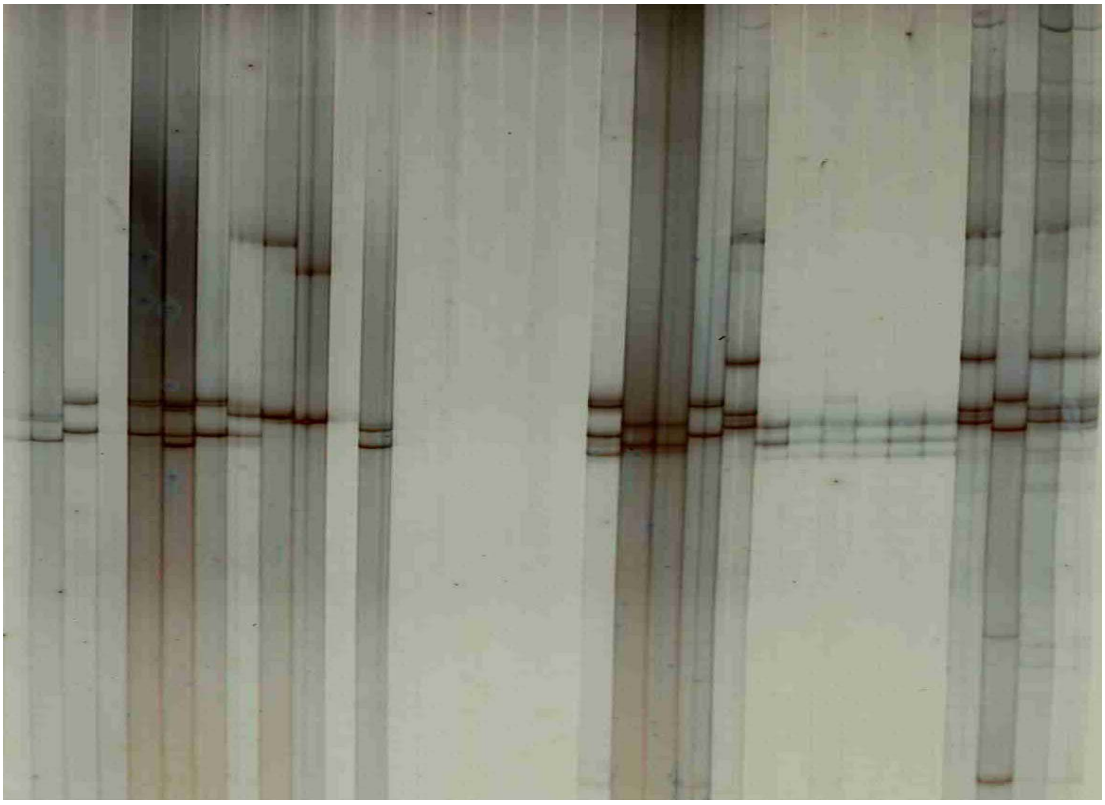
UKC11	*B. phalloides*	UK
UKC36	*B. phalloides*	Hungary
UKC39	*B. phalloides*	Cyprus
UKC46	*B. stevenii*	Kenya
UKC52	*B. stevenii*	Kenya
UKC55	*B. stevenii*	Israel
UKC56	*B. stevenii*	Israel
UKC57	*B. stevenii*	Israel

Products ranged from 590bp (*B. phalloides* UKC37) to 746bp (*B. stevenii* UKC52) and the sequences were aligned and trimmed to around 540bp for phylogenetic analysis. In total 29

sequences (including representatives from other genera and species obtained from GenBank) were assessed over 642 characters. 481 base positions were variable and of these 129 were phylogenetically uninformative. A neighbourhood-joining (NJ) tree (Fig. 7) shows that all the *Battarrea* sequences clustered together, but that the sequences from specimens identified as *B. phalloides* or *B. stevenii* were intermingled and did not form separate groups. There is no evidence on this basis for the separation into two species.

The *Battarrea* sequences clustered more closely to members of the Agaricales than they did to members of the Tulostomatales. This suggests that further molecular phylogenetic study is needed to ascertain its true taxonomic affinities.

During alignment, a visual scan was conducted for sequences shared by all the 18 *Battarrea* sequences used, but which had differences from all the other sequences entered. The following two regions would be suitable to design *Battarrea*-specific primers on this basis: 3'ATCACAGGC5' and 3'CAGCTTCTAA5'.

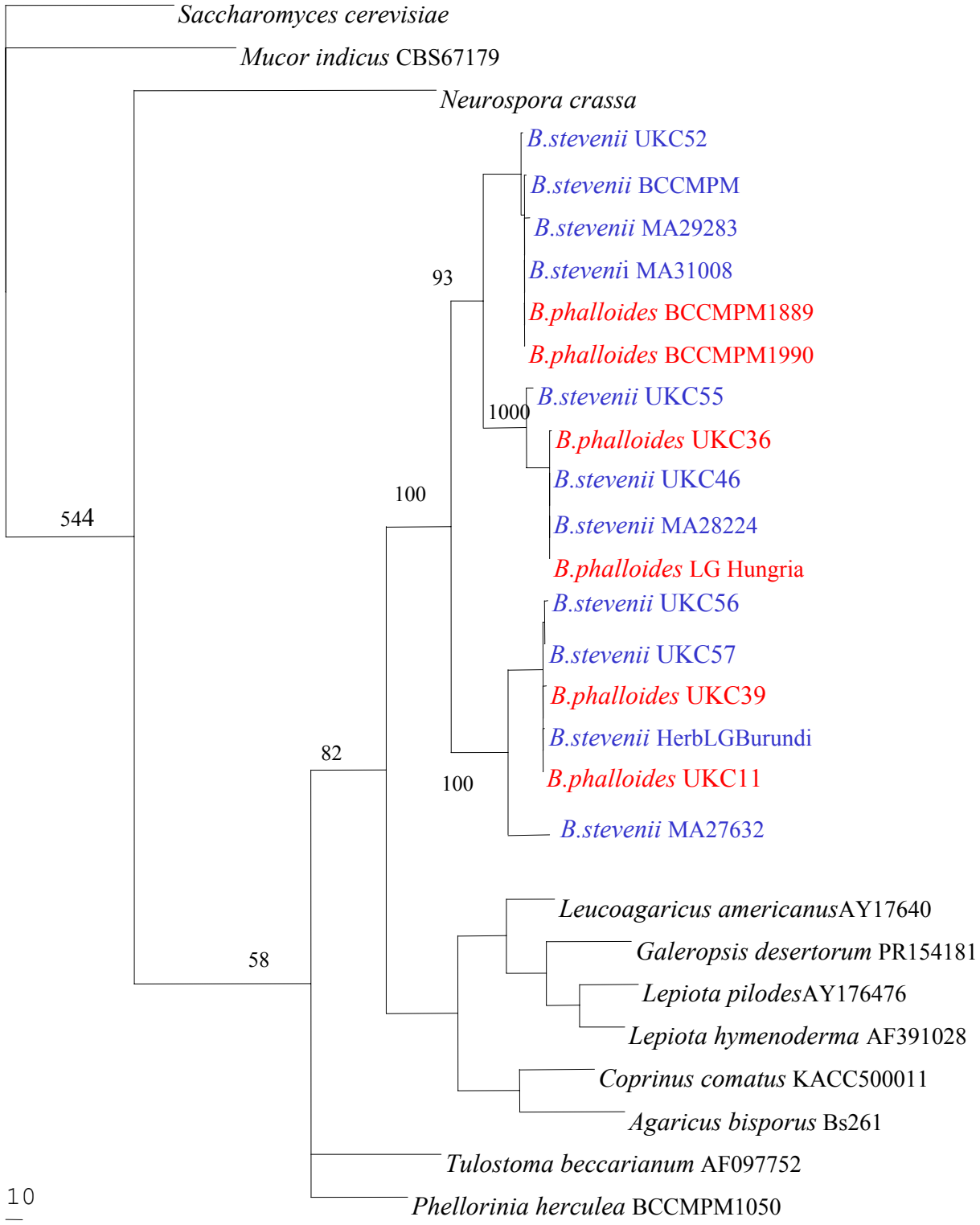


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

Figure 6. SSCP of PCR-amplified ITS region from 33 specimens of *Battarrea* from the RBG herbarium (UKC numbers refer to material listed in tables 3 and 5, Appendix 1 and list identity, country of origin and year of collection). Where the same specimen number applies to several lanes, different basidiomes were used for DNA extraction.

Key to lanes:

1.	UKC8	*B. phalloides*	UK	1969
2.	UKC37	*B. phalloides*	Hungary	1987
3.	UKC39	*B. phalloides*	Cyprus	1932
4.	UKC40	*B. phalloides*	UK	1969
5.	UKC67	*B. stevenii*	Cyprus	1998
6.	UKC69	*B. stevenii*	Cyprus	1997
7.	UKC55	*B. stevenii*	Israel	1950
8.	UKC56	*B. stevenii*	Israel	1959
9.	UKC57	*B. stevenii*	Israel	1951
10.	UKC58	*B. phall./stevenii*	South Africa	?
11.	UKC59	*B. stevenii*	South Africa	1939
12.	UKC61	*B. stevenii*	Kenya	1977
13.	UKC41	*B. gaudichandi*	Egypt	
14.	UKC43	*B. guicciardiniana*	Cyprus	1934
15.	UKC44	*B. guicciardiniana*	Cyprus	?
16.	UKC45	*B. phalloides*	Wisley ?	1993
17, 24.	UKC46	*B. stevenii*	Kenya	1973
18, 25	UKC47	*B. stevenii*	Iraq	1961
19, 26.	UKC48	*B. stevenii*	Kenya	1970
20, 21.	UKC3	*B. phalloides*	Channel Isles	1980
22, 29, 31, 32.	UKC52	*B. stevenii*	Kenya	1970
23, 30.	UKC53	*B. stevenii*	Greece	1973
27.	UKC49	*B. stevenii*	West Pakistan	1962
28.	UKC50	*B. stevenii*	Kenya	1972


Saccharomyces cerevisiae
Mucor indicus CBS67179
Neurospora crassa
B.stevenii UKC52
B.stevenii BCCMPM
B.stevenii MA29283
B.stevenii MA31008
B.phalloides BCCMPM1889
B.phalloides BCCMPM1990
B.stevenii UKC55
B.phalloides UKC36
B.stevenii UKC46
B.stevenii MA28224
B.phalloides LG Hungria
B.stevenii UKC56
B.stevenii UKC57
B.phalloides UKC39
B.stevenii HerbLGBurundi
B.phalloides UKC11
B.stevenii MA27632
Leucoagaricus americanusAY17640
Galeropsis desertorum PR154181
Lepiota pilodesAY176476
Lepiota hymenoderma AF391028
Coprinus comatus KACC500011
Agaricus bisporus Bs261
Tulostoma beccarianum AF097752
Phellorinia herculea BCCMPM1050
93
1000
100
544
82
100
58
10

6. Discussion

The aim of the project was to clarify uncertainties about the taxonomic relationships between *Battarrea phalloides* and *Battarrea stevenii* and to consider whether they should be considered conspecific. The results are typical of a modern taxonomic dilemma in which individuals may exhibit a wide range of morphological characters, yet their genomic similarity is very close. In this case, the *Battarrea* specimens which are taller, with scalier stipes and dry volva are identified as *B. stevenii*, whilst the shorter, smoother examples with or without a gelatinous volva are identified as *B. phalloides.*

This is in keeping with traditional taxonomy and relates to the original descriptions of these two species. However, in this study the examination of a large number of specimens, of widespread origin, revealed that all the morphological characters used to distinguish the two species form a continuum and thus are unreliable as absolute delimiters at a species level. Hence our statistical analysis of correlating morphology with species identity is flawed as the two factors are automatically linked and will almost inevitably result in an implied difference between two populations initially separated on the antithesis of the null hypothesis being tested (although even on this basis, stipe height did not differentiate the two groups!). There will thus always be a problem in assigning a specimen with basidiome and spores mid-range size, and in which a gelatinous volva was not found. The question of phenotypic characters, becomes more troublesome when considered in the context of habitat. **This suggests there is an inconsistency in the current concept of two species rather than one, when comparing morphology.**

Maublanc and Malencon (Maublanc and Malencon 1930) maintained that the important distinction between the species is the presence of abundant gelatinous material within the universal veil in the egg stage of *B. phalloides* and its absence in *B. stevenii*. However, since the present project was working almost exclusively with dry herbarium samples, no gelatinous volvas were evident, so this potentially differentiating parameter was unavailable. The authors suggested a link between habitat and species (or variety) development, with *B. phalloides* with a gelatinous volva confined to northern, cooler, more humid regions. In contrast, *B. stevenii*, with a dry volva is confined to southern, subtropical , hot, dry regions and it may be that under dry conditions the volva is not gelatinous. Dry conditions are also associated with warmer climates, and warmer climates may also favour the development of larger basidiomes than the cooler equivalents. Thus the difference in morphologies in the two 'species' may be directly related to climate, with '*B. stevenii'* taking over from '*B. phalloides'* as the site of collection moves nearer the equator! *Battarrea phalloides* is found most often in moist, mild or cooler habitats, such as on the coast of California or close to the coast in East Anglia, or on the Channel Island of Jersey, whereas *B. stevenii* comes from dry Mediterranean or sub-tropical climates.

Tl
st
B.
fo
de
br
ur
re

Fig.7. NJ tree was constructed using PAUP 4.0b10. Maximum parsimony was used to produce a more conservative tree where only phylogenetically informative sites were analysed. Maximum Parsimony analysis was performed using heuristic search options with 100 bootstrap replications branch swapping by tree-bisection reconnection (TBR), branches able to collapse yielding polytomies with parsimony uninformative characters excluded.
The 17 sequences for *Battarrea* are colour-coded for putative species identity as B.phalloides and B.stevenii. Sequences with UKC numbers were obtained form this study and refer to specimens described in Appendix 1.

The mean spore measurements were compared (Table 2) with those from recent research. Watling (Watling, Gucin & Isiloglu 1995) recorded spore dimensions close to those observed, for both species, in the current work, whereas Martin & Johannesson (Martin & Johannesson 2000) observed only round spores, compared with the range of shapes evident in the current project; the *B. phalloides* spores and those from *B. stevenii* were smaller than the spores observed in this project. Watling (Watling, Gucin & Isiloglu 1995) claimed that *B. phalloides* is a 'unique fungus' on the basis of the presence of 'elater' cells but these were found here in both species.

Table 2. Comparison of spore dimensions (figures represent range of spore length X width) in this study compared the figures quoted by Watling (Watling, Gucin & Isiloglu 1995) and Martin & Johannesson (Martin & Johannesson 2000).

Species	**Current project**	**Watling**	**Martin & Johan.**
B. phalloides	4.6-6.1 x 4.2-5.8	4.5-5.75 x 4.5-5.25	4.2-5.3 diam
B. stevenii	5.6-7.1 x 4.9-6.4	5.75-7.0 x 5.0-6.5	5.2-5.6 diam

When modern molecular methods are used to compare genomic differences between populations, separation of groups on the basis of ITS sequence differences has been used to justify the separation of species. However, the molecular evidence gathered here suggests that ***B. phalloides* and *B. stevenii* are not significantly different and that separation into two species in not justified.**

Sequence information has also suggested that *Battarrea* may be incorrectly placed in the Tulostomatales but further investigation is needed. The sequences analysed are closer to more typical 'agarics'. It was also evident that there are a number of short regions of ITS sequence which are conserved in the genus *Battarrea* but differ from other fungi. These could be used for the development of a species-specific PCR amplification method to detect the fungus in soil in the absence of the fruiting body.

7. References

ANON (HMSO). 1995. *Biodiversity: The UK Steering Group Report - Volume II: Action Plans*. HMSO, December 1995.

CLAPP, J.P., and others. 2001. Ribotyping of rhizobia nodulating *Acacia mangium* and *Paraserianthes falcataria* from different geographical areas in Indonesia using PCR-RFLP-SSCR (PRS) and sequencing. *Environmental Microbiol*, 3, 273-280.

DICKSON, J. 1785. *Lycoperdon phalloides* in *Fasc. Crypt. Plant. Brit.* (*Fasciculus plantarcum cryptogarricanum Britanniae*), 1, 24.

ENGLISH NATURE. 2002. Project synopsis, Biodiversity Programme Research Project - Sandy stilt puffball *Battarrea phalloides*. Colchester: English Nature.

ERIKSSON, O.E., HAWKSWORTH, D.L. 1998. Outline of the ascomycetes. *Systema Ascomycetum,* 16**,** 83-296.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution***, 39,** 783-793.

FRIES, N. 1832. *Battarrea stevenii* (Lib.) Fries, *Syst. Mycol*. 3, 7.

GARDES, M., BRUNS, T. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113-118.

HOLLOS, L. 1904. *Die Gasteromyceten Ungarns*. 278. XXIV. Tab. Leipzig (part of series: Gasteromycetes Hungariae, cum tabulus 31)

LIBOSCHITZ, J. 1814. *Dendromyces stevenii*, Lib. Monog. t. 1, 2. Beschreib. neu entdeckt. Pilzes. Wien

MAUBLANC, A., MALENCON, G. 1930. Rechercher sur le *Battarraea* Guicciardiana Ces. *Bull. Mycol. Soc. Fr*., 46, 43-73.

MORENO, G. and others. 1995. Contribution to the study of the Tulastomataceae in Baja California, Mexico I. *Mycologia,* 34, 96-120.

MARTIN, M.P. & JOHANNESSON, H. 2000. *Battarrea phalloides* and *B. stevenii*, insight into a long-standing taxonomic puzzle. *Mycotaxon,* 74, 67-75.

MYCOWEB. 2002. Available from: http://www.mykoweb.com/CAF/species/Battarrea_phalloides.html

PERSOON, D.C.H. 1801. *Battarrea phalloides. Syn. Meth. fung*. 129.

PEGLER, D.N., LAESSOE, T., SPOONER, B.M., 1995. British puffballs, Earthstars and stinkhorns, an account of the British gasteroid fungi. Kew: Royal Botanic Gardens.

RAMSBOTTOM, J. 1953. Mushrooms and Toadstools, a study of the activities of fungi. *The New Naturalist*, *a survey of British Natural History*. London: Collins.

REA, P.M. 1942. Fungi of Southern California. I. *Mycologia,* 34, 563 – 574.

REID, D.A. 1985. Further notes on the fungi of Jersey. *Trans. Brit. Mycol. Soc.*, 84. 715-722.

SOWERBY, J. 1803. Coloured figures of English fungi, or mushrooms. Plate 370, Vol. III, (RBG Kew).

SWOFFORD, D.L. 1999. *PAUP*. Phylogenetic analysis using parsimony (*and other methods).* Sunderland, MA, USA, Sinauer.

WATLING, R., GUCIN, F., ISILOGLU, M. 1995. *Battarrea phalloides* – its history, biology and extension to its distribution. *Nova Hedwigia*, 60, 13-18.

WOODWARD. T. 1784. An account of a new plant of the order Fungi. *Phil. Trans. Roy. Soc. London*, 74, 423 – 427.

Appendix 1. List of material examined

Table 3. *Battarrea phalloides* specimens from the Mycology Collection, the Herbarium, Royal Botanic Gardens, Kew.

RBG Herbarium referenceTulostomataceae 2.1.18.1.4 *Battarrea phalloides* (Pers. Dicks.)

UKC Project Number	K(M) Kew	Species	Packet or carp.	Collector number	Date collected	Site Country
1	58703 Box 2/2	*phalloides*	C	J. Revett	15 Sept. 1998	Grid ref. 221 055 Ipswich Rd A140 Norwich UK
2	54980a Box1/2	*phalloides*	C	N. La ffoley	16 Sept. 1980	under *Cupresses macro-carpa* hedge. St. Peter. Augerez, Channel Islands
3	54980b Box 1/2	*phalloides*	C	N. La ffoley	16 Sept. 1980	under *Cupresses macro-carpa* hedge. St. Peter. Augerez, Channel Islands
4	55001	*phalloides*	P	W. Spread-bury	21 Oct. 1954	nr. *Taxus*, Druid's Grove nr. Leather-head, Surrey
5	43180a Box 1/2	*phalloides*	C	B. Spooner	April 1996	Jersey, CI
6	43180b Box 1/2	*phalloides*	C	B. Spooner	April 1996	Jersey, CI
7	91300	*phalloides*	P	T.W. Dove	Nov. 2001	Lay-by with *Quercus*sp, Narborough, Norfolk
8	54989a Box 1/2	*phalloides*	C	Weller	18 Feb. 1969	Gravesend-Strood Rd. nr. Flagstaff Inn, Gads Hill, Kent
9	54989b Box 1/2	*phalloides*	C	Weller	18 Feb. 1969	Gravesend-Strood Rd. nr. Flagstaff Inn, Gads Hill, Kent
10	54989c Box 1/2	*phalloides*	C	Weller	18 Feb. 1969	Gravesend-Strood Rd. nr. Flagstaff Inn, Gads Hill, Kent
11	54990	*phalloides*	P	G. Sowerby	1803	Suffolk
12	54991 Box 1/2	*phalloides*	C	E.J. Noel	4 Oct. 1915	In hollow tree, Temple Guiting, Gloucs.
13	54983	*phalloides*	P	Miss Notley	1 Nov. 1944	Concealed by brickwork at base of dead *Ulnus* trunk in hedgerow, Callow Hill, Virginia Water, Surrey
14	54995	*phalloides*	P	H.G. Tunstall	31 Oct. 1962	Under yew trees, Druid's Grove, nr. Leather-head, Surrey
15	54993	*phalloides*	P	Miss Martin	7 Oct. 1955	Under thick yew hedge on Thanet Sand, grounds of Wickham College, West Wickham, Kent
16	54987	*phalloides*	C	C.H.S. Perceval	1872	Inside hollow ash tree, Nork Park, Surrey
17	54985	*phalloides*	P	-	Autumn 1953	Under yew, Box Hill, Surrey

UKC Project Number	K(M) Kew	Species	Packet or carp.	Collector number	Date collected	Site Country
18	54986	*phalloides*	P	W.J. Finnigan	24 Oct. 1956	At foot of large *Taxus* Druid's Grove, Boxhill, Surrey
19	54999a	*phalloides*	P	M.C. Cooke	1885	Dropmore, Bucks.
20	54999b	*phalloides*	P	M.C. Cooke	1885	Dropmore, Bucks.
21	55000a	*phalloides*	P	Herb. Hooker	1867	Dropmore, Bucks.
22	55000b	*phalloides*	P	Herb. Hooker	1867	Dropmore, Bucks.
23	54998	*phalloides*	P	Spencer Perceval	1873	Nork, nr. Epsom, Surrey
24	54997a	*phalloides*	P	Dawson Turner (Herb. Hooker)	1867	Norfolk
25	54997b	*phalloides*	P	Dawson Turner (Herb. Hooker)	1867	Norfolk
26	54997c	*phalloides*	P	Dawson Turner (Herb. Hooker)	1867	Norfolk
27	55003	*phalloides*	P	C. Perceval	12 Dec. 1872	Growing on the outside of a dead hollow ash pollard - three other specimens were growing on the light powdery soil in the middle of the tree, the largest of which was 12 inches high and 2.5 inches across the top. Nork, nr. Epsom, Surrey.
28	55002	*phalloides*	P	C. Perceval	12 Dec. 1872	On outside of dead hollow ash pollard, Nork, nr. Epsom, Surrey
29	40051 Box 1/2	*phalloides*	C	T.W. Doore	19 Aug. 1996	In hedgerow pine needle litter, A140 road to Norwich nr. Hall Road Marston Lane crossings, Norfolk
30	54996	*phalloides*	C	R.W.G.Dennis D.M. Dring	26 July 1968	By roadside nr. Strood, Kent
31	54838 Box 1/2	*phalloides*	C	R.A. Fortey	10 Oct. 1997	On dry bank nr. *Quecus*. Su743824 Pack & Prince Lane, Henley
32	54982	*phalloides*	C	J. Webb	2 Jan. 1981	Roadside verge, rural area nr. Cobham Kent.
33	81494 Box 2/2	*phalloides*	C	H.R. Arnold	12 Jan. 1999	Under *Fraxinus* covered in ivy, Bury Cambs.
34	54992 Box 1/2	*phalloides*	C	J. Ramsbottom	Nov. 1956	Anne's Walk, Coloma Training College, West Wickham, Kent
35	54988 Box 1/2	*phalloides*	C	D.M. Dring	Spring 1973	Nr Sir John Falstaff PH; wall just past Telegraph Hill. Strood-Gravesend rd., Rochester Kent.
36 (UKC HUN-1)		*phalloides*	C	Dr. Janos Zsolt	Sept. 1987	Hungary

UKC Project Number	K(M) Kew	Species	Packet or carp.	Collector number	Date collected	Site Country
37 (UKC HUN-2)		*phalloides*	C	-	Sept. 1987	Hungary
38 (UKC EQ-1)		*phalloides*	C	V. Fleming	14Sept. 1987	In puma cave, Mazan, Equador
39 (UKC CYP-1)		*phalloides*	C	B.TerPalma	Sept. 1932	Nursery gardens, Nicosia, Cyprus
40 (UKC Kent-1)		*phalloides*	C	White, Dennis, Dring	26 July 1969	By roadside, Strood, Kent

Table 4. *Battarrea* specimens from the Mycology Collection, the Herbarium, the Royal Botanic Gardens, Kew, excluding *B. phalloides* and *B. stevenii*. The table includes a single specimen of *B. phalloides* from the Royal Horticultural Society, Wisley, which has been returned to Wisley.

UKC Project Number	K(M) Kew	Species	Packet or carp.	Collector number	Date collected	Site Country
41 UKC Gaudi-1		*gaudichaudii* (Mont.)	C	Dr. Bromfield		Egypt
42 UKC Griff-1	Box collection 237 type specimen	*griffithsii*	C	David Griffiths 379	Autumn 1900	Tucson, Arizona, USA
43 UKC Guicci-1		*guicciardiniana*	C	R.M. Northass	Jan. 1934	Cyprus
44 UKC Guicci-2		*guicciardiniana*	C	-	-	Dept. Agriculture Nicosia, Cyprus
45	RHS Wisley (private specimen)	*phalloides*	-	-	1993	Nr. Coggeshall Essex

Table 5. *Battarrea stevenii* specimens from the Mycology Collection, the Herbarium, Royal Botanic Gardens, Kew.

RBG Herbarium reference Tulostomataceae 2.1.18.1.4 *Battarrea stevenii* (Liboschitz) Fries

UKC number	K(M) number Kew	Species	Packet or carp.	Collector number	Date collected	Country
46	B.stevenii Herb. Box Part 1/2 411	stevenii	C	-	13 Nov. 1973	West Rift, Lake Naivaska, Kenya alt. 6200ft
47	IMI 92311	stevenii	C	L.A. Debbagh	Dec. 1961	Gaseeba, 15km south of Baghdad, Iraq
48	6647(a)	stevenii	C	B.Mathew	6 June 1970	Open sandy area nr. River Kiero, Lokori 7ml south of Kangetet, alt. 1950ft, South Turkana, Kenya
49	----------	stevenii	C	S.Ahmad	23 Feb. 1962	Changa Manga, Lahore, West Pakistan

UKC number	K(M) number Kew	Species	Packet or carp.	Collector number	Date collected	Country
50	411c	stevenii	C	E.Polhill	10 Dec. 1972	Sparse understorybush in woodland of Acacia Xanth, grazed by stock. West Rift, Lake Naivaska, Kenya
51	----------	stevenii	C	E.Polhill	29 Oct. 1968	West Rift, Lake Naivaska, Kenya
52	6370	stevenii	C	----------	25 May 1970	Forest floor beneath Ficus and Acacia. Katilia Forest, alt.1900ft, 12ml NNE Kangetat, Kenya
53	----------	stevenii	C	A. J. Huxley	25 June 1973	Sandy hummock, west coast 20-25 km S of Nea Mamaras, Sithonia, N.Greece
54 UKC IS-1	B.stevenii Herb. Box Part2/2	stevenii	C	Prof.T Rayss	1950	Mishmar Ha-Emek, Israel
55 UKC IS-2	----------	stevenii	C	Prof.T Rayss	1950	Mishmar, Ha-Emek, Israel
56 UKC IS-3	----------	stevenii	C	Prof.T Rayss	Dec. 1959	Kinneseth, Israel
57 UKC IS-4	----------	stevenii	C	U.Aran	March 1951	Kibbutz, Merhavia, Israel
58 UKC SA-1	PHOTO 1 PHOTO 2	stevenii or phalloides	C	----------	----------	Cape Province, South Africa
59 UKC SA-	----------	stevenii	C	N.J.G. Smith 147	July 1939	Bottelgaat River, Tarkastad, East Cape, South Africa
60 UKC IS-5	----------	stevenii	C	Prof.T Rayss	----------	Negev, Israel
61 UKC-K-1	----------	stevenii	C	E.Polhill 498	27 July 1977	Open situation in cultivated soil, alt.6200 ft, West Rift, Lake Naivaska, Kenya
62 UKC-K-2		stevenii	C	E.Polhill 498	27 July 1977	Open situation in cultivated soil, alt.6200ft, West Rift, Lake Naivaska, Kenya
63	M5347	stevenii	C	Pill & Weightman	10 April 1989	On bank beside dirt road under Pistachia lentiscus, nr. Paleochova, Crete
64 UKC-K-3	----------	stevenii	C	R.W. Rayner	May 1948	On sandy ground with herbs and grass in wood-land of Acacia xantho-phloeia, nr..Lake Naivaska, Kenya
65 UKC PAK-1	----------	stevenii	C	S.Ahmad	23 Feb. 1962	Changa Manga, Lahore, West Pakistan

UKC number	K(M) number Kew	Species	Packet or carp.	Collector number	Date collected	Country
66 UKC-K-4	----------	stevenii	C	E.Polhill	5 Nov. 1968	14 in tall. volva remained in ground when stipe pulled., alt.6200ftWest Rift, Lake Naivaska, Kenya
67	B.stevenii Herb. box Part3/2 63045	stevenii	C	J.Christou	Dec. 1998	Nr. Cypressus (previous reports of growth in very dry conditions, often in olive groves). Pathos District, Polis, Cyprus
68	Cabinet collection 149	stevenii	C	J.W.Ash 359	3 May 1970	16 specimens in group, peppery smell, under shrubbery by lake margin, amongst large volcanioc rocks from pumice cliff, below Acacias in dense shade. Alt. 395 ft. Arussi Province, north shore, Lake Langano, Ethiopia
69	63243	stevenii	C	D.Viney F.426	4 June 1997	Ht. above ground, 20 cm, cap felty, covered with spores. Specimen collected as unbroken 'egg'. Bekoy, n. Cyprus

Table 6. *Battarrea phalloides* specimens from the English Nature/Suffolk Wildlife Trust/Suffolk County Council protected site at Blyford, Suffolk and specimens donated by the Suffolk Wildlife Trust

UKC Project Number	K(M) Kew	Species	Packet or carp.	Collector number	Date collected	Site Country
70 sp (spores)	English Nature	*phalloides*	-	L.P. McLain	Nov. 2002	Specimens found on roadside hedgerow habitat, a protected site, growing amongst leaf litter. To the east of Blyford Church, Blyford, Suffolk.
70 st (stipe)	English Nature	*phalloides*	-	L.P. McLain	Nov. 2002	Specimens found on roadside hedgerow habitat, a protected site, growing amongst leaf litter. To the east of Blyford Church, Blyford, Suffolk.
70 c (cap)	English Nature	*phalloides*	-	L.P. McLain	Nov. 2002	Specimens found on roadside hedgerow habitat, a protected site, growing amongst leaf litter. To the east of Blyford Church, Blyford, Suffolk.
71	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1998	Marlsford, Suffolk

UKC Project Number	K(M) Kew	Species	Packet or carp.	Collector number	Date collected	Site Country
72	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1999	Reydon, Suffolk
73	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1998	Roadside hedgerow, Blyford Church, Suffolk
74	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1998	Roadside hedgerow, Blyford Church, Suffolk
75	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1998	Roadside hedgerow, Blyford Church, Suffolk
76	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1998	Roadside hedgerow, Blyford Church, Suffolk
77	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1998	Roadside hedgerow, Blyford Church, Suffolk
78	Suffolk Wildlife Trust	*phalloides*	-	Suffolk Wildlife Trust	1998	Roadside hedgerow, Blyford Church, Suffolk

Appendix 2. Statistical analyses

One-way ANOVA: length of spores *vs.* species

1 = *Battarrea phalloides* 2 = *Battarrea stevenii*

```
Analysis of Variance for Length
Source     DF        SS        MS        F        P
Species     1     2.245     2.245    12.15    0.002
Error      30     5.544     0.185
Total      31     7.789

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -+---------+---------+---------+-----
1          16    5.5925    0.4661  (------*-------)
2          16    6.1223    0.3904                    (------*------)
                                  -+---------+---------+---------+-----
Pooled StDev =   0.4299           5.40      5.70      6.00      6.30
```

As the P-value is smaller than our significance level (5 per cent), we can reject the null hypothesis that there is no difference between the means.

One-way ANOVA: breadth of spores *vs.* species

1 = *Battarrea phalloides* 2 = *Battarrea stevenii*

```
Analysis of Variance for Breadth
Source     DF        SS        MS        F        P
Species     1     1.698     1.698     9.55    0.004
Error      30     5.333     0.178
Total      31     7.031

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
1          16    5.0766    0.4437  (--------*--------)
2          16    5.5373    0.3984                     (-------*--------)
                                  ------+---------+---------+---------+
Pooled StDev =   0.4216              5.00      5.25      5.50      5.75
```

As the P-value is smaller than our significance level (5 per cent), we can reject the null hypothesis that there is no difference between the means.

One-way ANOVA: cap diameter *vs.* species

1 = *Battarrea phalloides* 2 = *Battarrea stevenii*

```
Analysis of Variance for Cap diam
Source     DF        SS        MS        F        P
Species     1      1748      1748    10.96    0.002
Error      50      7976       160
Total      51      9724

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
1          26     29.27      7.97  (--------*-------)
2          26     40.87     15.99                     (-------*-------)
                                  ----------+---------+---------+------
Pooled StDev =    12.63                    30.0      36.0      42.0
```

As the P-value is smaller than our significance level (5 per cent), we can reject the null hypothesis that there is no difference between the means.

One-way ANOVA: cap height *vs.* species

1 = *Battarrea phalloides* 2 = *Battarrea stevenii*

```
Analysis of Variance for Cap heig
Source     DF        SS        MS        F        P
Species     1     816.1     816.1    16.09    0.000
Error      50    2535.8      50.7
Total      51    3351.8
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
1          26    17.096     5.008   (------*------)
2          26    25.019     8.738                        (------*------)
                                  -----+---------+---------+---------+-
Pooled StDev =    7.121              16.0      20.0      24.0      28.0
```

As the P-value is smaller than our significance level (5 per cent), we can reject the null hypothesis that there is no difference between the means.

One-way ANOVA: stipe length *vs.* species

1 = *Battarrea phalloides* 2 = *Battarrea stevenii*

```
Analysis of Variance for Stipe length
Source     DF        SS        MS        F        P
Species     1      7779      7779     2.31    0.135
Error      50    168285      3366
Total      51    176063
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
1          26    207.75     57.23  (-----------*----------)
2          26    232.21     58.79               (----------*-----------)
                                  --------+---------+---------+--------
Pooled StDev =    58.01                200       220       240
```

The P-value is 0.135 so we cannot reject the null hypothesis that there is no difference between the means.

ENGLISH NATURE

English Nature is the Government agency that champions the conservation of wildlife and geology throughout England.

This is one of a range of publications published by:
External Relations Team
English Nature
Northminster House
Peterborough PE1 1UA

www.english-nature.org.uk

Cover printed on Character Express, post consumer waste paper, ECF.

ISSN 0967-876X

Cover designed and printed by Status Design & Advertising, 2M, 5M, 5M.

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