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Strangler unmasked

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Strangler unmasked: Parasitism of *Cystoderma amianthinum* by *Squamanita paradoxa* and *S. pearsonii*

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The mushroom-forming genus *Squamanita* comprises 10 described species, all parasitic on basidiomes of other members of the order Agaricales, including members of the genera *Cystoderma*, *Galerina*, *Inocybe* and *Hebeloma*. Here we report an anatomical investigation of the stipitate “mycocecidium' (=fungus gall) formed on the basidiome of *Cystoderma amianthinum* (“powdercap”) by *S. paradoxa* (“powdercap strangler”), alongside the development of taxon-specific-PCR primer to localise the presence of *S. paradoxa*/*C. amianthinum* mycelia within mycocecidia, in associated plant tissues and apparently healthy host basidiomes. Dissection of fungarium samples also confirmed these findings, whilst ITS barcode sequencing of all available samples held at the RBG Kew and Edinburgh fungaria did not reveal any variation in ITS sequences within UK populations of *S. paradoxa* or the closely related *S. pearsonii*. The absence of any ^{13}C or ^{15}N isotopic differences between *C. amianthinum* and *S. paradoxa* suggests that *S. paradoxa* is nutritionally dependent on its host. The status of *C. amianthinum* as host of *S. pearsonii* is also confirmed.

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1. Introduction

Mushrooms that grow on other mushrooms are evolutionarily rare, with only 18 of ~21,000 species of Agaricomycetes (Kirk et al., 2008) known (Carbó and Pérez-de-Gregorio; Buller, 1924; Weber and Webster, 1996; Lindner Czederpiltz et al., 2001; Machnicki et al., 2006). Whilst some species apparently colonize after their host dies (*Collybia* spp., *Asterophora* spp.), others occur on living, sometimes deformed, host tissues. The exact nature of these interactions is not clear, but in at least some cases a true (biotrophic) parasitism exists where the host is deformed or otherwise rendered infertile. Perhaps the most intriguing of these mycoparasites are the members of the genus *Squamanita* (Squamanitaceae). More than 20 names have been applied to *Squamanita* spp. but of these only 10 are fully described and accepted (Table 1; Supplementary data S1). However, all form basidiomes atop the deformed tissues (galls), derived from basidiomes of members of order Agaricales (Redhead et al., 1994; Matheny and Griffith, 2010). Putative hosts of *Squamanita* spp. include members of the genera *Amanita* (Amanitaceae), *Cystoderma* (Agaricaceae), *Galerina* (Hymenogastraceae), *Hebeloma* (Hymenogastraceae), *Inocybe* (Inocybaceae), *Pholiota* [formerly *Kuehneromyces*] *mutabilis* (Strophariaceae), and *Phaeolepiota* (Squamanitaceae) (Mondiet et al., 2007).

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Table 1
Global distribution of the 10 accepted *Squamanita* spp. (numbers of records per country for each species). Sources of the data for this table and details of non-accepted species are presented in Supplementary data 1.

Species	Host	Host family	Austria	British Isles	Canada	Costa Rica	Denmark	DR Congo	Ecuador	Finland	France	Germany	Italy	Japan	Korea	Mexico	Netherlands	Norway	N. Zealand	Poland	Sweden	Switzerland	USA	Total
1 *Squamanita citricolor*	Unknown	n/a						1																**1**
2 *Squamanita contortipes*	*Galerina (Mycena?)*	Strophariceae		2			3			1		2					3				46		1	**58**
3 *Squamanita fimbriata*	*Pholiota*	Strophariaceae			1													2						**3**
4 *Squamanita granulifera*	Unknown	n/a							1															**1**
5 *Squamanita odorata*	*Hebeloma*	Hymenogastraceae		2			54				1	28		7			24	6		1	3	1		**127**
6 *Squamanita paradoxa*	*Cystoderma*	Agaricaceae	2	50	4		5			2	2	8					4	5			97	1	2	**182**
7 *Squamanita pearsonii*	*Cystoderma*	Agaricaceae		3	2		2							1								1	1	**10**
8 *Squamanita schreieri*	*Amanita*	Amanitaceae	1								2	5										1		**9**
9 *Squamanita squarrulosa*	Unknown	n/a																	4					**4**
10 *Squamanita umbonata*	*Inocybe*	Inocybaceae				1						1	1	3	2	1							27	**36**
			3	**57**	**7**	**1**	**64**	**1**	**1**	**3**	**5**	**44**	**1**	**11**	**2**	**1**	**31**	**13**	**4**	**1**	**146**	**4**	**31**	**431**

Early authors referred to the galls as “protocarpic tubers” (Bas, 1965; Singer, 1986), a term that does not accurately describe the true nature of these structures as deformed basidiomes induced by infection with *Squamanita*. The parasitic nutritional status of *Squamanita* was demonstrated by Redhead et al. (1994) who favoured the term “galls” and also described the various types (amorphous, stipitate and pileate) formed during these interactions. Bas and Thoen (1998) later proposed the Latinized term “cecidiocarp” to emphasize their functional significance as sources of abundant chlamydospores and distinguish them from reproductively inert galls. However, this term, which roughly translates as “gall body”, is clumsily redundant and implies a reproductive function.

Given that there is no evidence that the primary purpose of the deformed host basidiome is for production of chlamydospores, rather than as a substrate for the development of the fertile *Squamanita* basidiome, we instead propose using the term “mycocecidium” (“fungus gall”) for these structures, following conventional use according to Kirk et al. (2001). Production of chlamydospores is, therefore, a characteristic of *Squamanita*-induced mycocecidia, but not a definition for mycocecidia as a whole, and it is possible that they are formed by the parasitised host. Since the basidiome of the host (and occasionally the parasite) lacks any pileus, identification of the host can be problematic (Redhead et al., 1994). Mondiet et al. (2007) conducted genetic analysis to confirm *Hebeloma mesophaeum* as the host of *S. odorata*, consistent with the raphanoid odour of the mycocecidia, as already suggested by Vesterholt (1991) based on morphological studies. Similarly, Matheny & Griffith (2010) confirmed the presence of *C. amianthinum* tissues within the stipitate mycocecidia of *S. paradoxa*, consistent with the distinctive odour and stipe morphology of the host (Fig. 1). However, for most other species the host is unknown or its identification only suspected (Table 1).

Records of *Squamanita* spp. are remarkably rare, with fewer than 450 observations/collections recorded globally on GBIF and other inventories (Table 1). Since it is only very rarely that fresh samples have been subject to detailed examination, knowledge of the biology of these fungi is very scant with no published details of the developmental biology of mycocecidia. Of the ca. 53 records of *Squamanita* spp. in the British Isles, 50 are of *S. paradoxa*, three of *S. pearsonii*, and two each of *S. contortipes* and *S. odorata* (Fig. 2; Supplementary data S2). *Squamanita paradoxa and S. pearsonii* are found at undisturbed grassland sites, and occasionally in woodland (Læssøe, 2012), in association with *Cystoderma amianthinum*. Two other species, *S. basii* and *S. umbilicata*, have been reported to be associated with *Cystoderma amianthinum* (Harmaja, 1988), but Lassøe (2008) considered these to be synonymous with *S. paradoxa*.

The aim of this study was to investigate the nature of this intriguing parasitic interaction by confirmation of the identity of host and parasite, morphological examination of fresh and dried samples, and also the use of species-specific PCR probes to localise *S. paradoxa*/*C. amianthinum* tissues within the chimeric basidiomes. The possibility of cryptic infection of *C. amianthinum* and distribution of *S. paradoxa* was also investigated.

2. Methods

2.1. Sampling and morphological analyses

Dried samples of *Squamanita* mycocecidia were obtained from the fungaria at RBG Edinburgh (7 samples; *Index Herbariorum* [*IH*] code E) and RBG Kew (11 samples; *IH* code K). Five additional samples from Wales are held at the Aberystwyth University Fungarium (*IH* code ABS<UK>) (Table 2; Supplementary data S2). Fresh samples of *S. paradoxa* (three basidiomes and associated

Fig. 1. Basidiomes of *Squamanita paradoxa* and associated mycocecidia at (A) Coity Wallia Commons, (B) Hay Common, (C) Broome, (D) ECN Yr Wyddfa and (E) Bronydd Mawr; *S. pearsonii* at (F) Haddo and (G) Moel y Ci. The distinctive morphology of the *Cystoderma amianthinum* host is readily apparent in the lower stipe regions for infections by *S. paradoxa*. For *S. pearsonii*, this feature is absent, however, specific PCR and ITS1 sequence data confirmed the lower stipe tissue of both *S. pearsonii* mycocecidia to contain host tissues. In many cases mycocecidia were caespitose (D,E,F), unusual for uninfected *C. amianthinum* basidiomes. Images courtesy of Richard Wright (A), David Mitchel(B), Liz Holden(F) and John Harold(G).

mycocecidia), and also apparently uninfected basidiomes of *C. amianthinum* from the same location (Broome, Worcestershire) and within 3 m of the *S. paradoxa* basidiomes, were kindly provided by John Bingham. Samples of apparently uninfected *C. amianthinum* basidiomes from a range of locations were sourced from the Aberystwyth University Fungarium (Figs. 1 and 2).

Dissection of the three fresh *S. paradoxa* basidiomes and associated mycocecidia (JB1,2,3; Table 2) was conducted using a scalpel, flamed between each incision to avoid DNA cross-contamination. Similar precautions were taken when excising portions of tissue from dried samples. Macroscopic features of basidiomes were recorded with a Nikon Coolpix 995 digital camera.

2.2. PCR and DNA sequencing

DNA was extracted from small (<2 mm^2) portions of dried or fresh mycocecidium tissues using the CTAB method, as described by Edwards et al. (2013), resuspended in 50 µl TE buffer and stored at −20 °C. A PCR master mix containing 1xGoTaqFlexi PCR buffer (Promega), 25 mM $MgCl_2$, 20 mg.ml-1 BSA (Promega), 200 µM dNTPs, 1U GoFlexi G2 Taq and 325 nM of each primer. Amplification of DNA (2 µl per 20 µl reaction) using the ITS1F primer (CTTGGTCATTTAGAGGAAGTAA), in combination with either ITS4 (GCATATCAATAAGCGGAGGA) or ITS2 (GCATCGATGAAGAACGCAGC)(White et al., 1990), was conducted as follows: Initial denaturation (95 °C/1 s; 94 °C

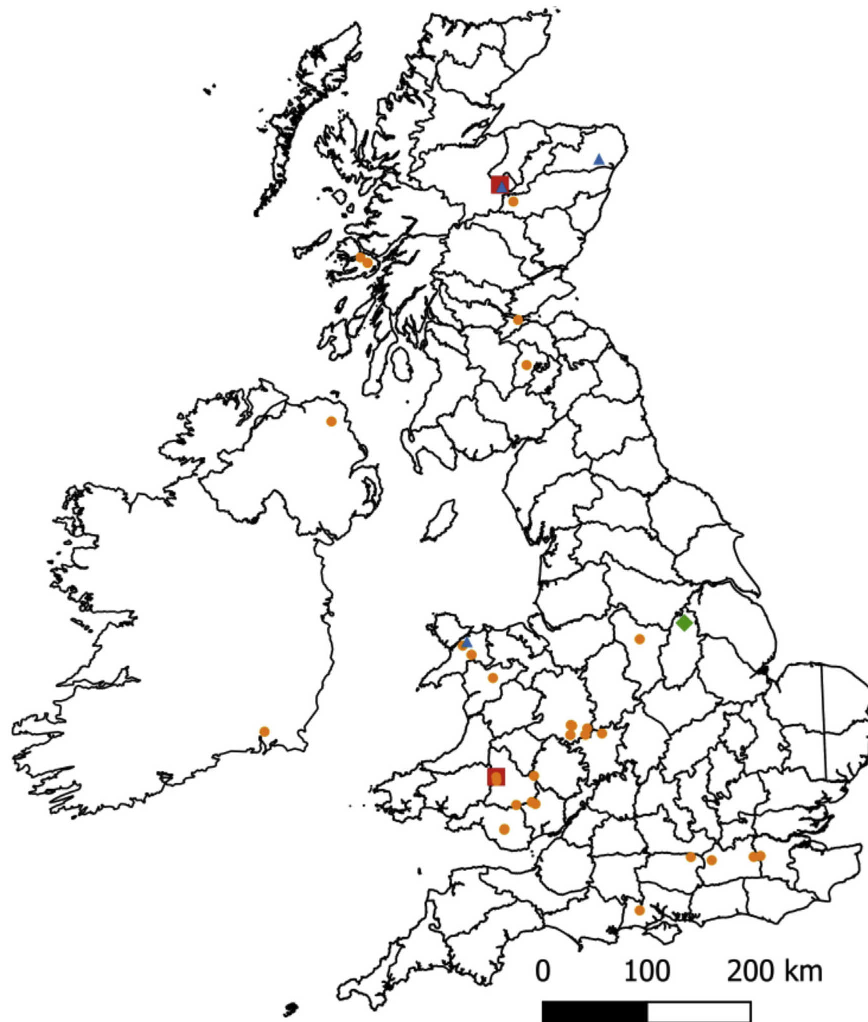

Fig. 2. Distribution map for *Squamanita* spp. in the British Isles, with *S. paradoxa* (orange circles), *S. pearsonii* (blue triangles), *S. contortipes* (red squares) and *S. odorata* (green diamond).

4 min, 1 cycle), amplification (95 °C/1 s; 94 °C/30 s; 53 °C/30s; 72 °C/60 s; 35 cycles) and final extension step (72 °C/5 min, one cycle). PCR products were visualised following gel electrophoresis (1% agarose gel, SYBRsafe staining) and following PCR product purification (Qiagen PCR Cleanup kit), samples were sequenced using unidirectional dye-terminated sequencing using the same primers used for PCR and analysed with an ABI3100 Genetic Analyzer (IBERS sequencing facility).

Inspection and editing of chromatograms was conducted using Geneious (v7.0; Biomatters Ltd.). The sequences generated from the present study were combined with additional *Squamanita* and *Cystoderma* ITS sequences (Mondiet et al., 2007; Matheny and Griffith, 2010; UNITE database) (Fig. 4). ITS sequences of *Crucibulum laeve*, *Mycocalia denudata*, *Nidula niveotomentosa*, and *Nidularia farcta* (Nidulariaceae) generated by the AFTOL project were downloaded from GenBank to use as an outgroup following previous studies (Matheny et al., 2006; Garnica et al., 2007; Matheny and Griffith, 2010). Multiple sequence alignment was achieved with the L-INS-i algorithm in MAFFT v7.392 (Katoh et al., 2017). The alignment was partitioned into the ITS1, 5.8S, and ITS2 regions according to the annotation of the *Nidularia farcta* sequence (GU296100). The best partitioning scheme and substitution model was determined using ModelFinder implemented in IQ-TREE v1.6.7, sharing the same set of branch lengths for all partitions and allowing each partition to have its own rate (*-spp* option) (Chernomor et al., 2016). Maximum likelihood analysis based on the best partitioning scheme was carried out in IQ-TREE with 1000 nonparametric bootstraps. Tree rendering was conducted using FigTree v1.4.4pre20171111 (http://tree.bio.ed.ac.uk/software/figtree/). ITS sequence data for representative samples studied here have been uploaded to GenBank (Accession numbers MK192929-MK192942.

2.3. Design of specific primers

Following of alignment of ITS sequences of all *Squamanita* spp. from GenBank (Table 2) combined with the newly generated sequences (above) and six *Cystoderma* spp. sequences from GenBank, reverse primers were designed within ITS1 where several consistent base-pair differences were observed between the two genera (Supplementary data S3). These new primers were then tested in combination with the fungal-specific forward primer ITS1F. The primers were designed so that the amplicon generated with the *C. amianthinum*-specific primer (CyamR1; GGGTATATGAAAAACG TAGACCTT) was longer (280 bp) than that generated with the *S. paradoxa*-specific primer (SqpaR3; TTTCCTCGAGAGTTGTTCAAGT;

Table 2
List of samples used in this study, including details of samples examined genetically (ITS1/ITS2/Host ITS) and using stable isotope analysis (^{15}N). A subset of the new sequences were submitted to GenBank (accession numbers given below).

Sample code	Species	ITS1	ITS2	Host ITS	^{15}N	Repository	Coll. Date	Accession	Location	Gridref		Collector
Spx01-Mull69	***Squamanita paradoxa***	MK192935				RBGE	7-Oct-1965	E:9303	UK Garbh Choire, Loch Ba, Mull	N56.4841	W5.9570	P. James
Spx02-Lui99	***Squamanita paradoxa***	MK192936				RBGE	11-Oct-1995	E:113464	UK Derry Lodge, Lui Flats, Braemar	N57.0219	W3.5808	E.M. Holden
Spx03-Daw01	***Squamanita paradoxa***			Y		RBGE	3-Oct-1997	E:152829	UK Dawyck Botanic Garden	N55.6047	W3.3198	R. Watling
Spx05-BM5	***Squamanita paradoxa***	GU29606		GU296098	**Y**	ABS	25-Oct-2001	ABS:BM5	UK Bronydd Mawr (BDD)	N51.9859	W3.6319	G.L. Easton
Spx06-ECN1	***Squamanita paradoxa***	MK192937		MK192930	**Y**	ABS	9-Sep-2004	ABS:ECN1	UK Yr Wyddfa/Snowdon (ECN)	N53.0755	W4.0351	D.A. Evans
Spx09-PIW07	***Squamanita paradoxa***			Y	**Y**	ABS	17-Nov-2003	ABS:PIW07	UK Mynwent Penisarwaun (PIW)	N53.1525	W4.1653	D.A. Evans
Spx10-JB1-26	***Squamanita paradoxa***	MK192938		MK192929	**Y**	ABS	14-Nov-2007	ABS:JB1-26	UK Broome, nr. Hagley (BME)	N52.4090	W2.1518	John Bingham
Spx11-MYG1	***Squamanita paradoxa***			Y	**Y**	ABS	13-Nov-2011	ABS:MYG1	UK Mynydd y Gaer (MYG)	N51.5623	W3.5108	Peter Sturgess
Spx11-WD82	***Squamanita paradoxa***	Y				KEW	10-Nov-1978	K(M)91714	UK West Kent Golf Course, Downe	N51.3261	E0.0474	J. Pitt
Spx12-BBC96	***Squamanita paradoxa***	Y				KEW	7-Oct-1992	K(M)42987	UK Railway, Cwm Clydach NNR, Brynmawr	N51.8056	W3.1282	S.E. Evans
Spx13-WF04	***Squamanita paradoxa***	Y				KEW	26-Sep-2000	K(M)125650	UK Bens Coppice, Wyre Forest	N52.3972	W2.3901	J. Bingham
Spx14-BCH04	***Squamanita paradoxa***	Y				KEW	6-Oct-2000	K(M)128905	UK Harewood, Brown Clee Hill	N52.4746	W2.5793	J. Bingham
Spx15-BRM05	***Squamanita paradoxa***	Y				KEW	28-Oct-2001	K(M)135781	UK Bramshill House, Hazeley	N51.3296	W0.9177	S. Moore
Spx16-BBA05	***Squamanita paradoxa***	Y				KEW	3-Nov-2001	K(M)135806	UK Keeper's Pond, The Blorenge	N51.7912	W3.0821	R.G. Betts
Spx17-MT06	***Squamanita paradoxa***	Y		Y		KEW	28-Oct-2002	K(M)142076	UK Merthyr Common (South)	N51.7794	W3.3497	P.J. Roberts
Spx18-WOK08	***Squamanita paradoxa***	Y				KEW	10-Oct-2004	K(M)159838	UK Brookwood Cemetery, Woking	N51.2988	W0.6296	B. Hughes
Spx19-EP3	***Squamanita paradoxa***	MK192939	MK192939			ABS	18-Oct-2010	ABS:SQP5	UK Epynt, subplot3 (4 separate cols)	N51.9854	W3.6403	Peter Roberts
Sps1-Had04	***Squamanita pearsonii***	MK192940		Y		RBGE	3-Oct-2000	E:204926	UK Haddo House	N57.40324	W2.22079	E.M. Holden
Sps2-Myc07	***Squamanita pearsonii***	MK192941		MK192931		RBGE	21-Sep-2003	E:282464	UK Moelyci, Tregarth	N53.18862	W4.10694	John Harold
Sps3-AV50	***Squamanita pearsonii***					KEW	23-Sep-1946	K(M)91715	UK Rothiemurchus, Aviemore	N57.15000	W3.77000	R.W.G. Dennis
Sps4-BG1	***Squamanita pearsonii***		MK192942			ABS	17-Oct-1993	ABS:SQE8_BG1	US Rockport State Park, WA	N48.49	W121.62	Helen Bassler

178 bp amplicon with ITS1F), permitting the respective PCR fragments to be clearly differentiated by gel electrophoresis.

DNA extracted from *C. amianthinum* basidiomes in areas where *S. paradoxa* has not been discovered, and with DNA from other grassland macrofungi, were used to test the specificity of the primers. A range of annealing temperatures (49–61 °C) were tested, with 55 °C empirically determined to provide optimal specificity and sensitivity. PCR master mix was formulated as described above (each primer at 325 nM). PCR reactions were set up using filter tips in a laminar flowhood and post-PCR procedures (agarose gel electrophoresis) were performed in a different laboratory in order to avoid cross-contamination.

2.4. Stable isotope analysis

Isotopic analyses ($\delta^{13}C$ and $\delta^{15}N$) were conducted on cap (pileus) tissues of basidiomes dried within 24 h of collection in a ventilated drying cabinet at 40 °C and stored desiccated at the Aberystwyth University Fungarium (Table 2). Subsamples of cap tissue were excised from these and ground for isotopic analysis. Isotope analyses were conducted by continuous flow-isotope ratio mass spectrometry (CF-IRMS), using an automated N/C analysis-mass spectrometry (ANCA-MS) system (Europa 20/20, Crewe, UK) at IBERS Aberystwyth by Conflo III Interface. Values were referenced against atmospheric nitrogen and VPDB limestone standards.

3. Results and discussion

3.1. Distribution of Squamanita in the UK

Upland grasslands are the dominant habitat in the UK for *Squamanita paradoxa* and its host *Cystoderma amianthinum*. Such grasslands are typically dominated by grasses belonging to the genera *Agrostis* and *Festuca* but also contain large amounts of moss biomass (mostly *Rhytidiadelphus squarrosus*) (Rodwell, 1992). *Cystoderma amianthinum* is particularly associated with mossy grasslands in northern Europe (Saar et al., 2009) and these hilly/mountainous areas are also known for their diverse populations of waxcap (*Hygrophoraceae*) and fairy club (Clavariaceae) fungi (Griffith et al., 2004), due to heavy sheep grazing alongside the absence of disturbance from ploughing and fertiliser addition.

Despite the abundance of *C. amianthinum*, macroscopically visible infection by *S. paradoxa* is remarkably rare. Fewer than 40 known locations for these species exist in the UK (Supplementary data S2), and even at these locations parasitised basidiomes are only rarely observed. For instance, >20 detailed macrofungal surveys at the Bronydd Mawr grassland experiment (Munro, 1994), over 5 y, recorded >3000 *C. amianthinum* basidiomes (the commonest macrofungal species present) but only one *S. paradoxa* basidiome (Roderick, 2009).

At another regularly surveyed field site in north Wales (ECN; Yr Wyddfa [Snowdon] Environmental Change Network site (Turner et al., 2009)), where fortnightly basidiome surveys were conducted for 10 y (2007–16), *S. paradoxa* has only been observed in four autumns, usually in very low numbers relative to its host (Fig. 3) but with no significant correlation between numbers of host and parasite basidiomes ($r = 0.59$; $P = 0.072$). Mondiet et al. (2007) reported the occurrence of *S. odorata* parasitizing *H. mesophaeum* in the same location over 13 y, suggesting that the host-parasite symbiosis may be quite stable, or at least strategies to ensure local persistence, potentially as chlamydospores, are highly effective.

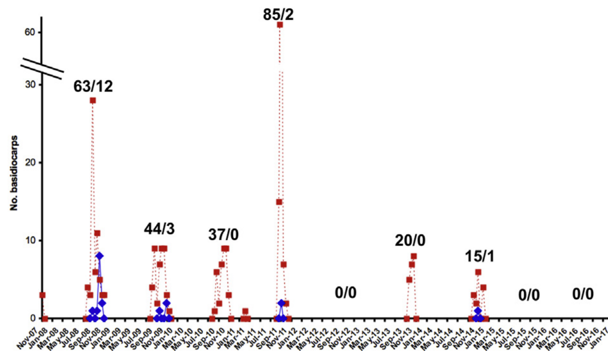

Fig. 3. Fruitbody data from Yr Wyddfa ECN survey site. Numbers of basidiomes of *Cystoderma amianthinum* (red squares) and *Squamanita paradoxa* (blue diamonds) at each fortnightly survey of the ca. 0.1 ha grassland area.

3.2. Phylogenetic analyses of UK Squamanita spp.

Due to the poor preservation of many of the fungarium samples, amplification of the whole ITS region failed for many samples, but it was possible to amplify and sequence the ITS1 region (using primers ITS1F/ITS2) in nearly all cases (Table 2). For *Squamanita*, DNA was successfully amplified from gill tissue for most samples, providing clean sequence (i.e. no contamination by host DNA). PCR amplification from the type specimen of *S. pearsonii*, collected in 1950, and noted to be in poor condition by Bas (1965), was unsuccessful despite repeated attempts.

Examination of ITS1 sequences from 11 *S. paradoxa* samples revealed a high level of homogeneity, with no polymorphisms present. For the three samples for which ITS2 sequence data were also obtained, there was also no variation in this region. The three *S. pearsonii* samples for which ITS1 sequences were obtained (including one from the USA) were also identical to each other, but clearly distinct from *S. paradoxa* (Fig. 4). The absence of variation in the ITS region was also reported for two *S. odorata* found at two sites 800 km apart (Mondiet et al., 2007), and the new *S. odorata* sequence (LOU12; K(M)178855; Table 2) differed from these at only a single position.

ModelFinder determined the best partitioning scheme was for combining the ITS1 and ITS2 regions (model TPM2+F + G4) and keeping the 5.8S region separate (model K2P). In the best ML tree, *Squamanita* is monophyletic, with *S. paradoxa* and *S. pearsonii* forming a clade and *S. odorata* in a sister position to these.

3.3. Mycocecidium anatomy

During the course of this study, we were fortunate to receive a fresh sample of three mycocecidia from a site in Broome, Worcestershire (following press attention on the BBC; see http://www.bbc.

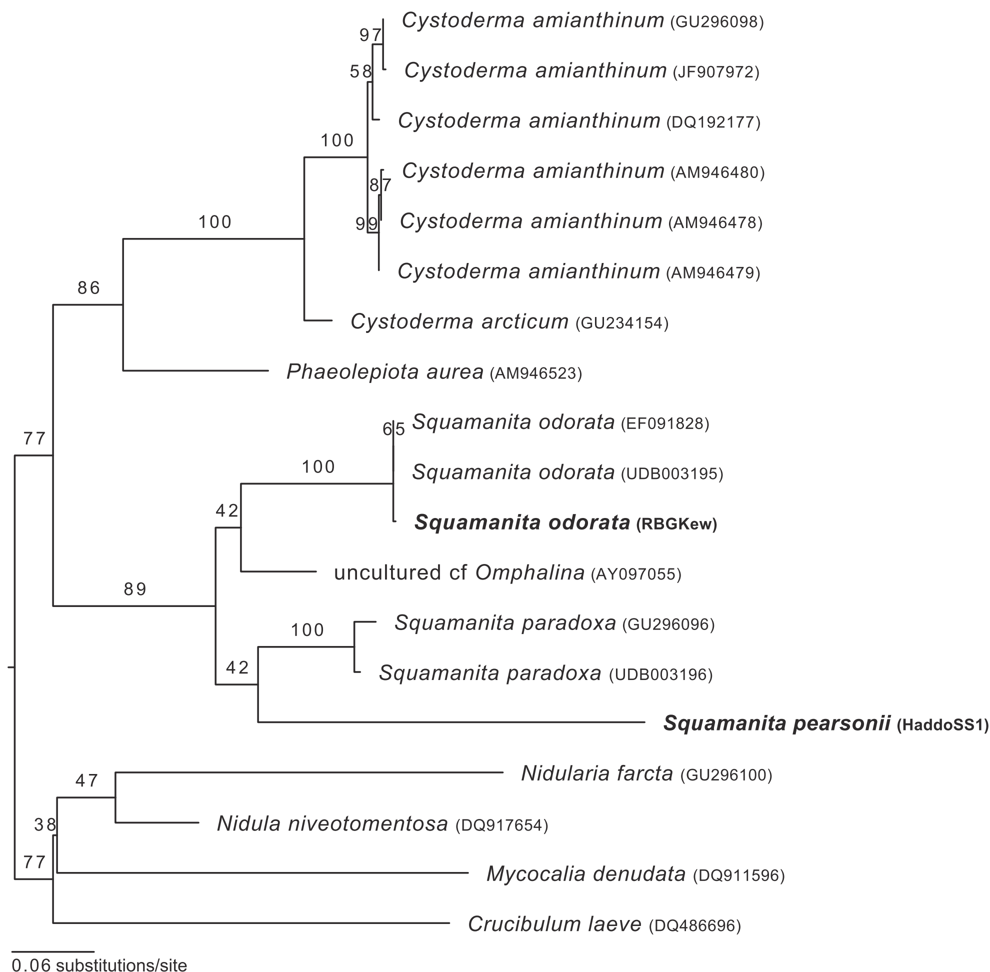

Fig. 4. Phylogentic reconstruction based on ITS 1 and 2 sequences for *Squamanita* spp. and *Cystoderma* spp. (RAxML tree with midpoint rooting). Scale bar indicates number of substitutions per site. Numbers at nodes indicate bootstrap percentages (1000 replicates).

co.uk/nature/16029977). These were dissected to elucidate the internal structure of mycocecidia, and also to obtain samples of tissues for genetic analysis with species-specific PCR primers (see below). The three mycocecidia emerged from a common base. Such caespitose (stipe bases touching but not fused) development is unusual for *C. amianthinum* but relatively common when this host is infected with *Squamanita* (Redhead et al., 1994) (Fig. 1). The distinctive musty-earthy odour and fibrillose stipe morphology of *C. amianthinum* was readily apparent in the basal regions of the mycocecidia with the morphology of the upper stipe region being quite different (Fig. 5A). It is usually supposed that the tissues above the graft between the lower and upper portions of the stipe (Fig. 5A arrowed) is *Squamanita*, whereas the tissues below, in the stipitate gall region, are mainly those of the host (Redhead et al., 1994).

When the stipe was dissected, the upper parts were hollow, with only the lower 2–3 cm from the stipe base being solid tissue. (Fig. 5C,H). Healthy basidiomes of *C. amianthinum* have solid stipes. Most curiously, inside the hollow stipe was a grey tubular projection held in position by glutinous fibres (Fig. 5C,F,G). This projection traversed the graft and was pointed at its upper extent but no connection to the main outer stipe was observed beyond the graft (Fig. 5D and E). Dissection of the lower solid parts of the stipitate galls revealed the presence of an inner zone of grey tissue, similar in colour to the pileus of *S. paradoxa*, surrounded by brown tissues similar in colour to *C. amianthinum* (Fig. 5H).

3.4. Localisation of host and parasite tissues using species-specific PCR amplification

Previous genetic studies of *Squamanita* (Mondiet et al., 2007; Matheny and Griffith, 2010) experienced difficulties with amplification of *Squamanita* DNA alone using generic ITS primers, and thus resorted to cloning of PCR products prior to sequencing. Mondiet et al. (2007) used the relative abundance of host vs parasite clones to show that *H. mesophaeum* DNA was more abundant in the gall tissues that characterise the parasitism of this species by *S. odorata*, an observation consistent with the odour of the gall when cut open. We developed specific PCR primers based on unique regions of the host and parasite ITS1 spacer regions that, when co-amplified with the ITS1F primer, yielded two amplicons of different size, allowing the host and parasite to be detected and partially quantified (Fig. 6; Supplementary data 2).

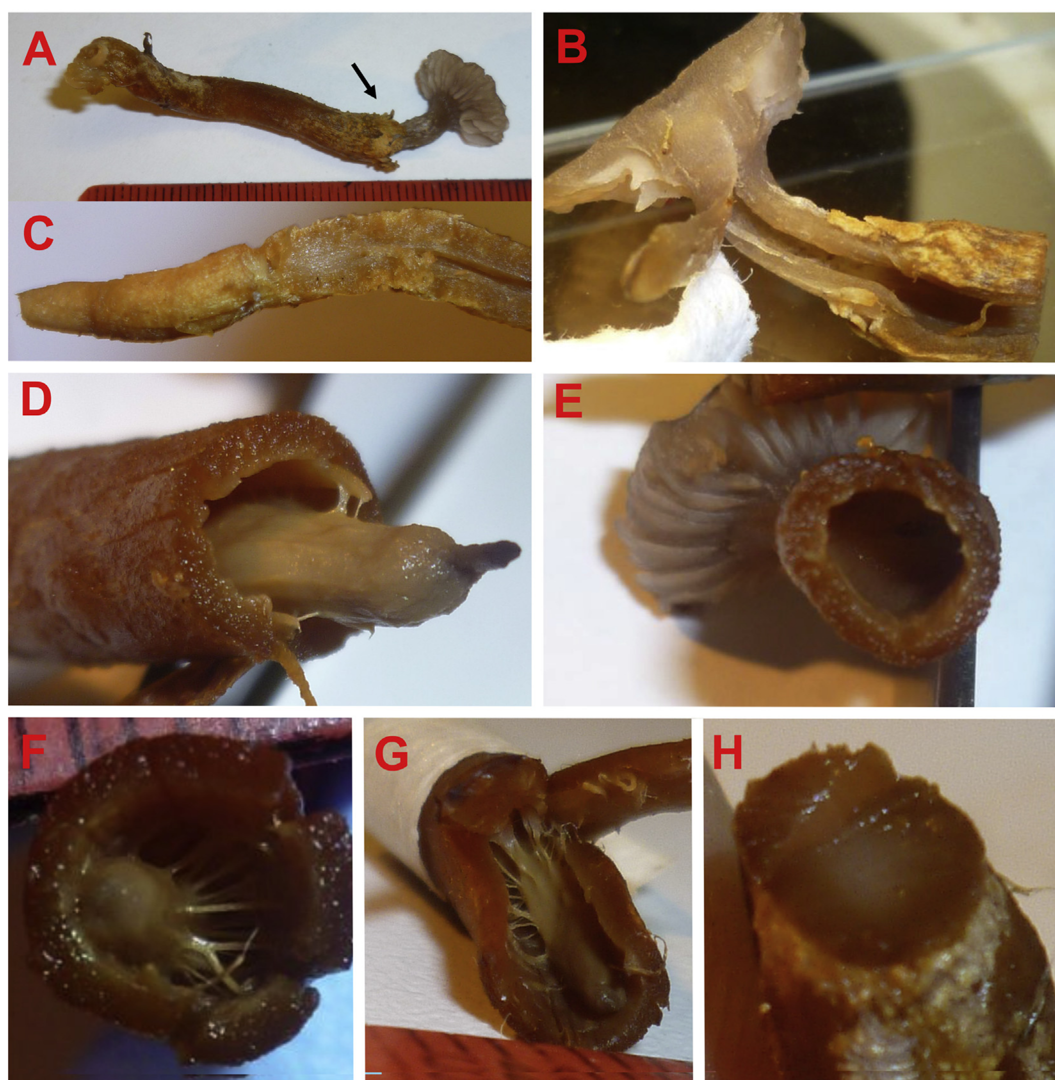

Fig. 5. Dissection of a mycocecidium of *S. paradoxa*. The junction (arrow) is readily visible, with the lower stipe region typical *C. amianthinum* and with the *S. paradoxa* basidiome above (A). The stipe is hollow both above (B) and partly below (C) the graft. Within the hollowed part of the stipe and projecting across the graft is a solid grey pointed tube (D,E) which below the graft is only connected to the outer stipe tissues by thin adhesions (F,G). Within the solid basal portion of the stipe, the central region is grey (H).

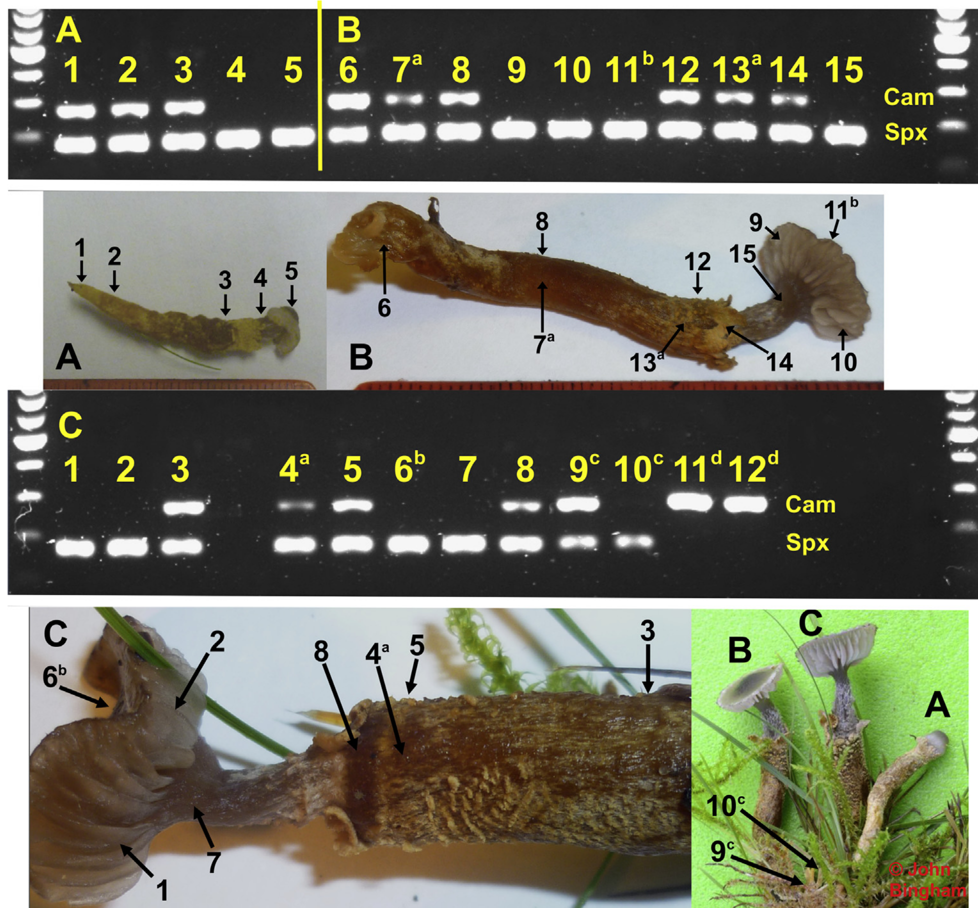

Fig. 6. Multiplexing of specific PCR primers to detect *S. paradoxa* (Spx) or *C. amianthinum* (Cam) DNA within the tissues of three mycocecidia (A,B,C). The larger band (280 bp) is specific to *C. amianthinum* and smaller band (178 bp) specific to *S. paradoxa.* Numbering indicates the tissues from which DNA (<2 mm^3 fragments) was extracted. a indicates where internal tissues were sampled; b indicates samples from top of pileus; c indicates samples taken from adherent moss tissues; d indicates two samples taken from the stipe bases of healthy *C. amianthinum* basidiomes (ECN Yr Wyddfa and Bronydd Mawr). Size marker on gel is 100 bp ladder. The 'despeckle' tool in Adobe Photoshop Elements was used to remove the high background in the original gel.

S. paradoxa DNA was detected in all mycocecidium samples including the outer tissues of the remnant *C. amianthinum* stipe (Fig. 6). However, *C. amianthinum* DNA was not detected above the graft in any of the samples. PCR analysis of cap tissues from dried fungarium samples yielded similar results, suggesting that the tissues above the graft are comprised solely of *S. paradoxa*. Use of the ITS1F-CyamR1 primers alone allowed amplification and sequencing of *C. amianthinum* DNA to confirm that this was indeed the host for six *S. paradoxa* samples (Table 2).

3.5. Presence of S. paradoxa within asymptomatic C. amianthinum basidiomes

Having demonstrated the ability of this PCR assay to determine the presence of host/parasite DNA within different mycocecidium tissues, we also used the same approach to test for the occurrence of *S. paradoxa* DNA within apparently healthy *C. amianthinum* basidiomes to identify cryptic infection. It was reasoned that DNA extracted from a transverse section of stipe base tissue would be most useful for detecting any cryptic infection, since to invade the host basidiome, *S. paradoxa* hyphae would have to traverse this area. The 178 bp *S. paradoxa*-specific band (with ITS1F-SqpaR3 primers) was never observed when tested with DNA from *C. amianthinum* stipe bases from locations where *S. paradoxa* was not known (AU campus, Braemar).

Analysis of *C. amianthinum* basidiomes collected from a site where *S. paradoxa* had been found (Fig. 6; lanes C11/12) did not yield any positive results. However, analysis of apparently uninfected *C. amianthinum* basidiomes, collected 16 d later (1st December 2011) from the same ca. 4.5 m diameter 'fairy ring' (at Broome) as the *S. paradoxa*-infected samples dissected above, did give positive results for the presence of *S. paradoxa* DNA. *S. paradoxa* DNA was, however, only detected in *C. amianthinum* basidiomes from the side of the ring opposite the mycocecidia (ca. 4 m away; 2/3 samples gave positive results) and not from three *C. amianthinum* basidiomes collected adjacent (within 1 m) to the mycocecidia (Supplementary dataS4). These data support the hypothesis that cryptic infection may occur, but the possibility of cross-contamination in the field, for example by basidiospores, cannot be excluded.

The question of where the mycelia of *Squamanita* spp. reside beyond mycocecidia, if at all, was raised by Henrici (2005). The parasitic interaction is limited to the exploitation of host basidiomes for the purposes of spore dispersal, but it is also possible that host hyphae are parasitised more extensively. In the case of *S. paradoxa*, its host (*C. amianthinum*) is known to occur predominantly in mossy habitats (mostly *Rhytidiadelphus squarrosus*). To test if either the host or parasite was present within moss tissues, unwashed pieces of moss present near the stipes of the *S. paradoxa* mycocecidia (visible in Fig. 6) were sampled and their DNA

extracted. In all cases, amplification of both *C. amianthinum* and *S. paradoxa* DNA was observed (Fig. 6; Supplementary data S4). Since the moss was not washed the fungal DNA present was likely due to contamination by *S. paradoxa* basidiospores adherent to the moss thalli and cells from the stipe of *C. amianthinum*, but this observation raises the possibility that both species may reside endophytically in the moss.

Diverse fungi are known to reside within the tissues of bryophytes, including several basidiomycetes, most interestingly *Entoloma conferendum*, a common grassland macrofungus (Kauserud et al., 2008; Griffith et al., 2013). Whilst *C. amianthinum* is clearly associated with mossy vegetation, recent ^{14}C ('bomb' radiocarbon) isotopic data suggest that it is saprotrophic (Halbwachs et al., 2018), as do $\delta^{13}C/\delta^{15}N$ stable isotope patterns ((Mayor et al., 2009) and below).

3.6. Stable isotope analysis

As noted above, it is unclear whether the parasitism of *Squamanita* is restricted to the destruction of host basidiomes or whether it also derives nutrition from its host, possibly extending to a more intricate dependence between the hyphae of the two species in the soil or other substrata (e.g. mosses). To test for an exclusive nutritional dependence on the *Cystoderma* hosts, we examined the $\delta^{13}C$ and $\delta^{15}N$ content of *Squamanita* gills and gill tissues from healthy *C. amianthinum* basidiomes (Fig. 7). No significant difference in either N or C content ($7.2 \pm 0.7\%$, $38.4 \pm 1.5\%$ respectively) or isotopic fractionation ($\delta^{13}C = -25.0 \pm 0.47‰$; $\delta^{15}N = 1.1 \pm 0.98‰$) between host and parasite was observed, within the range found for saprotrophic basidiomycetes in grassland habitats (Griffith, 2004; Griffith and Roderick, 2008). The similarity in N/C content and N/C isotopic signatures between host and parasite suggests that the symbiosis has a nutritional basis (i.e. data are consistent with the hypothesis that *S. paradoxa* derives all of its nutrition from *C. amianthinum*) and is not simply a physical association. This is perhaps not surprising since one of the main advantages of parasitizing another sporocarp would be to derive nutrition from these tissues.

3.7. Confirmation of C. amianthinum as the host of S. pearsonii

S. pearsonii is an exceptionally rare species with only ten records globally (Supplementary data S1). Unlike *S. paradoxa*, where the distinctive colour and farinose appearance of the remnant host stipe is visible on mycocecidia, this is not the case for *S. pearsonii* (Fig. 1). Three UK specimens of *S. pearsonii*, were analysed here using the *C. amianthinum*-specific PCR primers and for two of these, successful PCR amplification and sequencing of these amplicons showed *C. amianthinum* to be the host, confirming the earlier suspicions of Holden (2005).

3.8. Attempted inoculation of Cystoderma amianthinum with Squamanita paradoxa

Fresh basidiospores of *S. paradoxa* plated on potato dextrose or water agar did not germinate on their own after 8 weeks (in the dark at 25 °C). Attempts were made to inoculate cultures of *C. amianthinum* with basidiospores of *S. paradoxa* but despite the good condition in which the *S. paradoxa* basidiomes were received from Broome (<4 d since picking and kept fresh in moss), very few spores were shed. Pieces of gill tissue, presumably containing at least some mature basidiospores, were also placed amongst the hyphae of the growing *C. amianthinum*. Unfortunately, bacterial contamination rendered the trial inconclusive. Although very little is known about reproduction in *Squamanita*, infection of new host mycelia by the thick walled chlamydospores may be more efficient than by basidiospores, since the former may be able to remain dormant in soil and infect new hosts, as suggested by Redhead et al. (1994). However, it remains to be proven that chlamydospores are formed by *Squamanita* rather than as a response by the host to

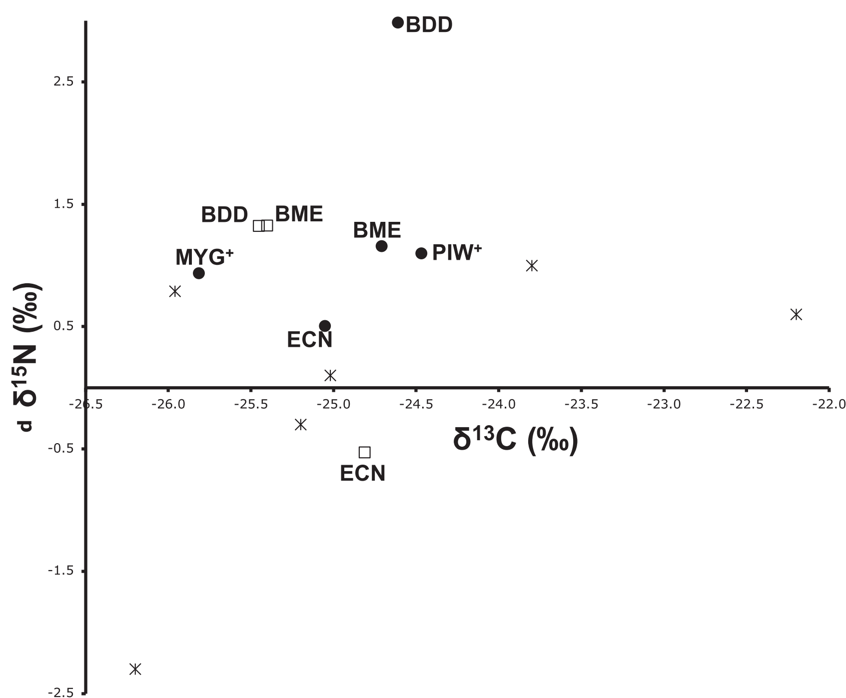

Fig. 7. $\delta^{13}C$ and $\delta^{15}N$ Stable isotope signatures of pileus tissues from five *S. paradoxa* (closed circles) and healthy *C. amianthinum* (open squares) samples from several sites. + indicates the two sites where only parasitised caps were collected. Location codes are Bronydd Mawr (BDD), Broome (BME), Mynydd Y Gaer (MYG), Penisarwaun (PIW) and Yr Wyddfa (ECN). ⁎ indicates published data for *C. amianthinum* basidiomes from woodland and grassland habitats (Halbwachs et al., 2018; Mayor et al., 2009).

infection, despite the assumptions that have been made in the past (Bas and Thoen, 1998). Further field trials are required to test these hypotheses and also whether *S. paradoxa* is only associated with *C. amianthinum* basidiomes or whether it also associates with the host mycelium in the soil (the latter being the more likely situation in our opinion).

3.9. Other potential host species

Next to *Cystoderma* spp. (known host to *S. paradoxa*, *S. pearsonii*, *S. basii*, and *S. umbilicata*), the most commonly occurring hosts of members of the genus *Squamanita* are *Galerina* spp. (*S. contortipes*). In UK grasslands, members of this genus (most frequently *G. vittiformis*) are strongly moss-associated. It may be possible that *S. paradoxa* is capable of parasitizing other hosts, such as *Galerina*, but that these less conspicuous infections are overlooked. Targeted surveys are needed to test this hypothesis.

3.10. Nature of mycoparasitisms in Agaricales

Other putative mycoparasites that deform host basidiomes include *Entoloma abortivum* on *Armillaria* (Lindner Czederpiltz et al., 2001) and *Psathyrella epimyces* on *Coprinus comatus* (Buller, 1924). In the former, the deformed basidiomes are independent of parasite basidiomes, so the nature of the parasitism appears to be purely nutritive. In the latter, the parasite forms basidiomes directly from the deformed host basidiomes, a situation that is very similar to *Squamanita* parasitism, although evidence that *Psathyrella* is using the host for nutrition is lacking. Other mushrooms that grow on mushrooms either colonize after the host is dead (*Collybia cirrhata*, *C. cookei*, *C. tuberosa*, *Dendrocollybia racemosa*, *Psathyrella globosivelata*, *Asterophora* spp.) or do not appear to affect the host (*Volvariella surrecta*, *Pseudoboletus parasiticus*) (Carbó and Pérez-de-Gregorio; Weber and Webster, 1996; Machnicki et al., 2006).

3.11. Coevolution

The fact that *Squamanita* basidiomes are rare suggests that the mycoparasitic lifestyle does not lead to ecological dominance. The low abundance yet extreme nature of the hostile takeover of a host basidiome presents a curious puzzle. One possible explanation for the low abundance is that host defences are highly effective in the majority of individuals. However, this situation is likely to lead to a coevolutionary arms race, which could lead to patchiness in infection incidence, where some populations of hosts are more susceptible than others, yet this is not a situation that has been observed in *Squamanita* where they are uniformly rare. However, the persistence of *Squamanita odorata* in a single location over several years (Mondiet et al., 2007) may be evidence of this phenomenon. At the same time, the apparent duplication pattern of distinct species of *Squamanita* infecting *Cystoderma amianthinum*, as well as extreme host-switching of *Squamanita* spp. correlated with cladogenesis, may be indicative of adaptive change in response to host defences. An alternative explanation for the low infection rate of the host is due to the difficulty of finding suitable hosts when they may be patchily distributed, ephemeral and unpredictable in their appearance. This may be especially true if the parasitism depends on the existence of basidiomes rather than on mycelia, as is the case here. As the probability of finding a host becomes low, coevolutionary theory predicts that strategies to better locate hosts, ensure successful colonization when hosts are located, and/or an increased ability to utlilize a broader spectrum of hosts should evolve in response. It is notable that the known hosts of *Squamanita* are also highly abundant, viz. *Cystoderma* and *Galerina* in European grasslands, perhaps indicating a pattern of host-switching that predicts abundance rather than host identity as a primary driver of adaptation in *Squamanita*.

4. Conclusion

We have demonstrated that *Squamanita* hyphae extend throughout the host tissue, but are compartmentalized with respect to the host stipe. Host hyphae are absent above the graft. Isotopic analysis suggests *Squamanita* derives its nutrition from the host, while *Squamanita* DNA has not been detected from samples far from hosts, supporting the possibility that they are obligate mycoparasites of mushrooms. High throughput sequencing datasets from environmental samples offer a promising resource for gaining a better understanding of abundance and distribution of rare taxa, such as *Squamanita* spp. Further experimental work is needed to clarify the nature of the relationship between *Squamanita* spp. and their hosts, and additional phylogenetic and genomic analyses will allow inferences of co-evolutionary patterns in this enigmatic host-parasite symbiosis.

Authors' contributions

GWG conceived and coordinated the study and wrote the manuscript with input from BTMD. Fieldwork was undertaken by GWG, JB, AT, VB, DAE, WGM. Molecular lab work was undertaken by KPG, APD, BTMD and BD. All authors gave final approval for publication.

Conflicts of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2018.11.012.

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