

## Mycoparasitism between *Squamanita paradoxa* and *Cystoderma amianthinum* (Cystodermateae, Agaricales)

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**Abstract** Circumstantial evidence, mostly morphological and ecological, points to ten different mushroom host species for up to fifteen species of the mycoparasitic genus *Squamanita*. Here, molecular evidence confirms *Cystoderma amianthinum* as the host for *S. paradoxa*, a sporadically occurring and rarely collected mycoparasite with extreme host specificity. This is only the second study to use molecular techniques to reveal or confirm the identity of a cecidiocarp of *Squamanita* species. Phylogenetic analysis of combined nuclear ribosomal RNA genes suggests the monophyly of *Squamanita*, *Cystoderma*, and *Phaeolepiota*, a clade referred to as the tribe Cystodermateae. If true, *S. paradoxa* and *C. amianthinum* would represent a relatively closely related species pair involved in a mycoparasitic symbiosis.

**Keywords** Basidiomycota · Cecidiocarp · Herbaria · Phylogeny · Squamanitaceae

The genus *Squamanita* Imbach is a remarkable genus of Agaricales, fifteen species of which parasitize at least ten different species of Agaricales (Redhead et al. 1994; Bas and Læssøe 1999) (Table 1). For some time it was accepted that *Squamanita* species emerged from a protocarpic tuber (Bas 1965; Singer 1986) until detailed studies by Redhead et al. (1994) demonstrated that *Squamanita* fruitbodies arise

from basidiocarps or parasitized galls or tissue of other agarics. On occasion, chimeric fruitbodies appear obvious, as in *S. paradoxa* (A.H. Sm. & Singer) Bas (Fig. 1), but for other species, the hosts are unknown (Table 1). It appears that in all cases, galls induced by *Squamanita* mycelium contain chlamydospores, and the term protocarpic tuber has been replaced by the term cecidiocarp (Bas and Thoen 1998). Hosts of *Squamanita* include distantly related species of Agaricales, such as *Galerina* Earle, *Inocybe* (Fr.) Fr., *Hebeloma* (Fr.) P. Kumm., *Kuehneromyces* Singer & A.H. Sm., and *Amanita* Pers. However, *Squamanita* also parasitizes species of *Phaeolepiota* Maire ex Konrad & Maubl. and *Cystoderma* Fayod (Singer 1986), which are believed to be closely related to *Squamanita*, although little molecular evidence and sparse taxon sampling to date have failed to resolve the phylogenetic position of *Squamanita* with confidence (Moncalvo et al. 2002; Saar et al. 2009). Only one study (Mondiet et al. 2007) has confirmed the host species of a *Squamanita* by molecular methods.

Fruitings of *Squamanita* are extraordinarily rare and sporadic (Bingham and Bingham 2005; Holden 2005; Evans 2009; Kibby 2009). The Global Biodiversity Information Facility (GBIF) portal (<http://www.gbif.net>), for example, lists six records of *S. paradoxa* from Europe. Other occurrences are also reported (Bas 1991). Though we have not conducted an exhaustive survey of the distribution of *Squamanita* species in Europe, these fungi are recorded with greater frequency than in North America. The Fungal Records Database of Britain and Ireland (FRDBI) (<http://www.fieldmycology.net>) reports, for example, 23 records of *S. paradoxa* in the United Kingdom alone. Detailed surveys of macrofungi at a low nutrient grassland Bronydd Mawr restoration experiment in Powys, Wales, (Fothergill et al. 2001) over 6 years reported >3100 fruitbodies of *Cystoderma amianthinum* (Scop.: Fr.) Fayod but

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**Table 1** List of *Squamanita* species and known and suspected hosts

<i>Squamanita</i> spp. (parasites)	Known and suspected hosts	Evidence for host–parasite relationship	Source
<i>S. basii</i> Harmaja	<i>Cystoderma</i> sp.	Morphology	Harmaja (1988); Redhead et al. (1994)
<i>S. cettoiana</i> M.M. Moser, nom. inval., Art. 36.1	Unknown		Index fungorum
<i>S. citricolor</i> Thoen	Unknown		Bas and Thoen (1998)
<i>S. contortipes</i> (A.H. Sm. & D.E. Stuntz) Heinem. & Thoen (as <i>S. scotica</i> nom. prov. Bas (1965))	<i>Galerina</i> sp.	Morphology	Redhead et al. (1994)
	<i>G. punilla</i> ?		Bas and Læssøe (1999)
	<i>Mycena aetipes</i> ?		Stridvall and Stridvall (1994)
<i>S. fimbriata</i> Gulden, Bendiksen and Brandrud	<i>Kuehneromyces mutabilis</i>	Ecology	Gulden et al. (1977); Cervini (2008)
<i>S. granulifera</i> Bas and Læssøe	Unknown		Bas and Læssøe (1999)
<i>S. odorata</i> (Cool) Imbach	<i>Hebeloma mesophaeum</i>	Morphology	Vesterholt (1991)
		Molecular	Bas and Læssøe (1999) Mondiet et al. (2007)
<i>S. paradoxa</i> (A.H. Sm. & Singer) Bas	<i>Cystoderma amianthinum</i>	Morphology, ecology	Reid (1983)
	<i>Cystoderma carcharias</i>	Molecular	Redhead et al. (1994)
		Morphology	This study Eriksen (1997)
<i>S. pearsonii</i> Bas	Unknown	Morphology	Redhead et al. (1994)
	<i>Cystoderma amianthinum</i>		Holden (2005) Charles Aron (pers. comm.)
<i>S. phaelepiotica</i> nom. prov.	<i>Phaeolepiota aurea</i>	Morphology	Nagasawa et al. (1990) (as <i>S. odorata</i> ) Redhead et al. (1994)
<i>S. schreieri</i> Imbach	<i>Amanita echinocephala</i>	Ecology	Bas (1965)
	<i>Amanita strobiliformis</i>		Redhead et al. (1994)
<i>S. squarrolosa</i> G.S. Ridl.	Unknown		Ridley (1988) Redhead et al. (1994)
<i>S. tropica</i> nom. prov.	Unknown		Redhead et al. (1994)
<i>S. umbilicata</i> Harmaja	<i>Cystoderma</i> sp.	Morphology	Harmaja (1988)
			Redhead et al. (1994)
<i>S. umbonata</i> (Sumst.) Bas	<i>Inocybe oblectabilis</i>	Morphology	Vizzini and Girlanda (1997)
	Unknown gall		Wang and Yang (2004)

only one visibly parasitized by *S. paradoxa* (Roderick 2009). Whereas most UK records comprise a very small number of fruitbodies on a single survey occasion, the most prolific site known is the UK Environmental Change Network site at Snowdon, North Wales (<http://www.ecn.ac.uk>), where fifteen *S. paradoxa* fruitbodies were recorded in the autumns of 2008 and 2009 during six different surveys compared with 110 visibly uninfected *C. amianthinum* fruitbodies (Evans 2009; Alex Turner personal communication). It is not clear whether this unusually high prevalence will continue or whether this is related to high rainfall levels during recent autumn periods.

Only two collections of *Squamanita* are preserved in the New York Botanical Garden and the University of Tennessee Fungal Herbarium combined, an indication of the rarity of the genus in North America. Unfortunately, sightings of species of *Squamanita*, such as *S. umbonata*,

may be reported [as on Mushroom Observer (<http://www.mushroomobserver.org>)], but materials are not deposited in herbaria. Indeed, *S. paradoxa* is known only from six collections from Oregon (the type locality) and Idaho, USA, which are located at the University of Michigan Herbarium based on literature reports (Smith and Singer 1948; Redhead et al. 1994). The seventh find of this species occurred during autumn 2009 by Oluna and Adolf Ceska on Vancouver Island, British Columbia (material referenced as 2091127-001, splits of which are deposited at the University of British Columbia and the University of Tennessee).

Material of *S. paradoxa* from Bronydd Mawr (26 October 2005) was sent by G. W. Griffith to P. B. Matheny for sequencing and inclusion in the Assembling the Fungal Tree of Life (AFTOL) project. This material is now accessioned at the University of Tennessee (TENN063549),



**Fig. 1** *Squamanita paradoxa* fruitbodies emerging from cecidiocarps of *Cystoderma amianthinum*. **a** Material from Wales (ECN, Snowdon; courtesy of Victoria Bowmaker, CCW). **b** Material from British Columbia (2091127-001, TENN). Bars 1 cm

with DNA stored at Clark University (AFTOL-DNA 662, box 99, row 4). Between 10 and 20 mg of dried material was excised from a cecidiocarp but without record of the precise area of removal. Direct sequencing results of nuclear ribosomal RNA (rRNA) regions produced poor sequence quality (heterogeneous peaks), which delayed inclusion of *Squamanita* in any AFTOL publications. Assuming the source genomic DNA included heterogeneous templates, purified polymerase chain reaction (PCR) products of the three rRNA gene regions were cloned using a TOPO-TA cloning kit following the DNA extraction protocol, PCR, and sequencing procedures described in Matheny et al. (2007). Three clones of *S. paradoxa* and one of *C. amianthinum* were recovered after PCR amplification of genomic DNA using primers ITS1F/ITS4. Three clones of *S. paradoxa* and one of *C. amianthinum* were recovered from a LR0R/LR7 PCR product [large subunit (LSU) region]. The small subunit (SSU) region was

amplified in two steps using primer pairs PNS1/NS41 and NS51/NS8, after which each product was cloned. Five and two clones, respectively, were used to recover the SSU of *S. paradoxa* and *C. amianthinum*. Amplification and cloning of the SSU region obtained by PNS1/NS41 also recovered an unknown Leotiomycetes sequence, with 94% sequence similarity to *Bulgaria inquinans* (Pers.: Fr.) Fr. and *Geomyces destructans* Blehert & Gargas, the latter implicated in white nose syndrome of bats. It is unclear whether the ascomycete sequence is a contaminant.

Three data sets were assembled, all of which are available online at [http://www.bio.utk.edu/matheny/Site/Alignments\\_%26\\_Data\\_Sets.html](http://www.bio.utk.edu/matheny/Site/Alignments_%26_Data_Sets.html). Each was analyzed by a maximum likelihood (ML) method using RAXML version 7.2.3 (Stamatakis et al. 2008). All free model parameters were estimated by RAXML using a general time-reversible (GTR) substitution matrix and a proportion of invariable sites estimate. One hundred rapid ML bootstraps were performed for each data set calling a GTRCATI nucleotide model of substitution. The first data set included a comprehensive combined nuclear rRNA matrix of LSU, SSU, and 5.8S rRNA sequences previously published by Matheny et al. (2006) (GenBank accession numbers are provided in Supplementary Table 1 of this 2006 study). To this were added LSU (AF261508) and 5.8S (EF184305) sequences of *S. umbonata* and LSU (AF261507) and 5.8S (EF091828) sequences of *S. odorata*. This data set was analyzed to assess the phylogenetic placement of *Squamanita* in relation to the tribe Cystodermateae Singer (Singer 1986). This inclusive data set represented 257 species of Agaricales and 3545 sites, including a small sample of Boletales, and *Fibulorhizoctonia* sp. (Atheliales) used as an outgroup. The cloned sequences of *S. paradoxa* (EF535266, GU296095, GU296096) and *C. amianthinum* (EF535265, GU296097, GU296098) were included, in addition to new sequences of diverse taxa of the Nidulariaceae: *Mycocalia denudata* (Fr. & Nordholm) J.T. Palmer (DQ911596-98), *Nidularia farcta* (Roth) Fr. (EF535276, GU296100), and *Nidula niveotomentosa* (Henn.) Lloyd (DQ986295, GU296099, DQ917654). Prior studies (Garnica et al. 2007; Matheny et al. 2006) indicate the Cystodermateae as the sister group to the Nidulariaceae Dumort., with significant measures of support (based on Bayesian posterior probabilities), although neither study included representatives of *Squamanita*, which was considered *incertae sedis* in the Agaricales by Moncalvo et al. (2002). ML analyses of unpartitioned and partitioned combined rRNA data sets produced a sister group arrangement between the three *Squamanita* species and a clade of *C. amianthinum* but with weak bootstrap support (23–27%, data not shown).

To bring more power to bear on evolutionary relationships in the Cystodermateae, we reduced the comprehensive

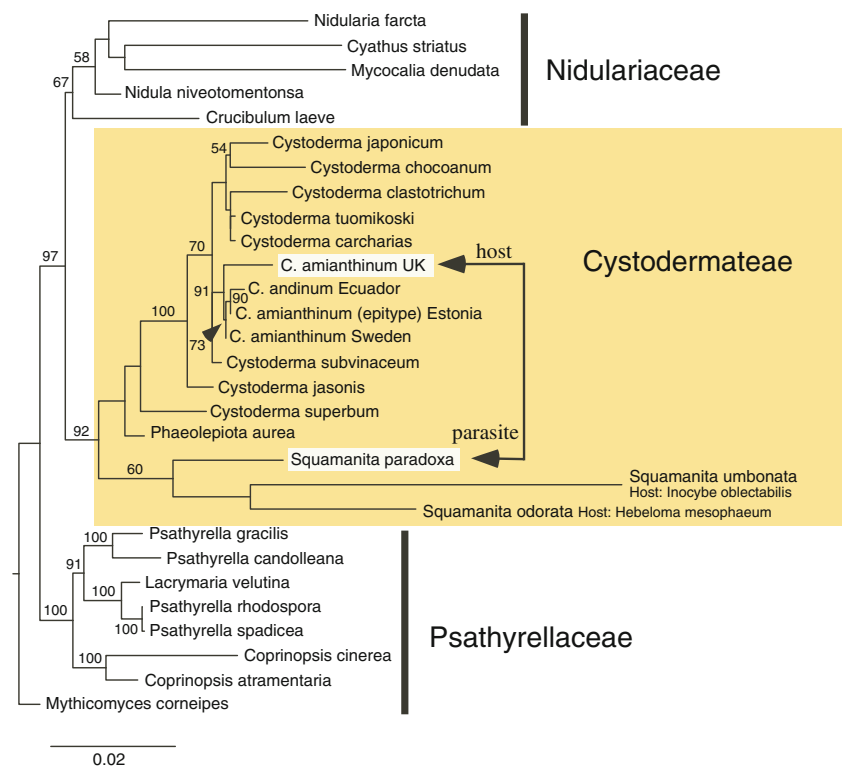
data set above to taxa of the Cystodermateae, Psathyrellaceae Vilgalys, Moncalvo & Redhead, and Nidulariaceae and added LSU and 5.8S sequences of *Phaeolepiota aurea* (Matt.) Maire (DQ071704, AM946523) produced by Garnica et al. (2007) and Saar et al. (2009), in addition to LSU and 5.8S sequences representing *S. umbonata* (Sumst.) Bas and *S. odorata* (Cool) Imbach (Hughes, unpublished; Moncalvo et al. 2002; Mondiet et al. 2007). To this we supplemented the supermatrix with LSU and 5.8S sequences of ten additional *Cystoderma* species from Saar et al. (2009) (AM946443 + AM946503, AM946436 + AM946492, AM946441 + AM946501, AM946423 + AM946479, AM946444 + AM946505, AM946435 + AM946491, AM946434 + AM946490, AM946428 + AM946483, AM946425 + AM946481, U85302). The reduced but combined rRNA data set included 29 taxa and 3404 sites. Using *Mythicomycetes corneipes* (Fr.) Redhead & A.H. Sm. as outgroup, the ML analysis (Fig. 2) reinforced the Nidulariaceae and Cystodermateae as a monophyletic group and also supported a sister arrangement between *Squamanita* and *Phaeolepiota* + *Cystoderma*, hereafter referred to as the tribe Cystodermateae. This tribe has also been referred to as Cystodermataceae (Singer) Locq., an invalid name per the Centre for Agriculture and Biosciences International (CABI) Bioscience Family Names Databases (ICBN, Art. 36.1, lacking a Latin diagnosis or reference to a previously published Latin diagnosis) or Squamanitaceae Jülich, a valid family name if not equated with Tricholomataceae Pouzar

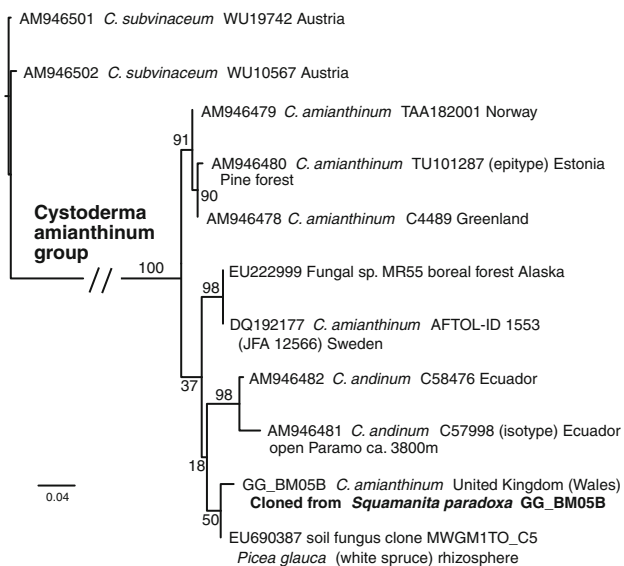
(Knudsen and Vesterholt 2008). The family name Squamanitaceae should likely be applied to what we refer to here as the Cystodermateae clade once the phylogenetic affiliation of the type of *Squamanita* (*S. schreieri* Imbach) is known with confidence.

We found the three sequences of *Squamanita* to be monophyletic. These results suggest that *S. paradoxa* parasitizes a closely related relative, *C. amianthinum*, within its own tribe (or family). Indeed, of the twelve accepted species of *Squamanita*, four are suspected to parasitize *C. amianthinum* or possibly other species of *Cystoderma* (Table 1). However, multiple accessions of *C. amianthinum* exhibit some genetic variation, and the species may be paraphyletic, in which the neotropical species *C. andinum* I. Saar & Læssøe is derived. Coincidentally, a new species of *Squamanita* (*S. granulifera* Bas & Læssøe) was described recently from Ecuador (Bas & Læssøe 1999), a source locality for *C. andinum* sequences. A second species of *Squamanita*, that has yet to be formally described from Japan (*S. phaeolepiotocola* nom. prov. in Redhead et al. 1994), fruits from cecidiocarps that probably represent parasitized *Phaeolepiota aurea*. Thus, up to five species of *Squamanita* may parasitize closely related species within their own clade, if *Squamanita* and *Phaeolepiota* + *Cystoderma* are indeed sister groups, but bootstrap support is weak for this association.

The third data set included eleven internal transcribed spacer (ITS) sequences of the *Cystoderma amianthinum*

**Fig. 2** Nuclear ribosomal RNA (rRNA) phylogeny [supermatrix of large subunit (LSU), small subunit (SSU), 5.8S rRNA sequences) of the Cystodermateae (colored box) inferred by maximum likelihood (ML) analysis. Numbers at internodes refer to confidence estimates based on 100 rapid ML bootstraps. Both *Cystoderma amianthinum* (host) and *Squamanita paradoxa* (mycoparasite) are shaded to indicate the phylogenetic proximity of the mycoparasitic symbiosis





**Fig. 3** Maximum likelihood (ML) phylogeny of internal transcribed spacer (ITS) data for the *Cystoderma amianthinum* group. Numbers at internodes refer to confidence estimates based on 100 rapid ML bootstraps. *Cystoderma subvinaceum* is used for rooting purposes based on Saar et al. (2009)

group and two outgroup sequences of *C. subvinaceum* A.H. Sm. (outgroup chosen based on Saar et al. 2009). ML bootstrapping results are consistent with the inclusive rRNA results above in that *C. amianthinum* is recovered as a paraphyletic group in which the neotropical *C. andinum* is derived (Fig. 3). *Cystoderma amianthinum* thus probably represents a complex of species possibly parasitized by four *Squamanita* species: *S. basii* Harmaja, *S. paradoxa*, *S. pearsonii* Bas, and *S. umbilicata* Harmaja. Our results of cloned nuclear rRNA products from the cecidiocarp of an herbarium specimen of *S. paradoxa* suggests that molecular tools can be applied to identify the mushroom hosts of *Squamanita* and clarify the phylogeny of this rare biotrophic mycoparasitic genus. Herbarium specimens of *Squamanita* should prove valuable to test patterns of potential coevolution with closely related hosts and shifts to more distantly related mushroom species of Agaricales.

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