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The enigmatic *Squamanita odorata* (*Agaricales*, Basidiomycota) is parasitic on *Hebeloma mesophaeum*☆

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ABSTRACT

Squamanita odorata is an agaric which is parasitic on unrecognizable, previously unidentifiable sporophores of a fungal host that is transformed into galls at the bases of the parasite's sporophore. Amplification and sequencing portions of the nuclear (ITS) and mt rDNA from three samplings originating from two sites (from France and from Switzerland) demonstrate that the galls produce sequences that are identical to that of co-occurring *Hebeloma mesophaeum*. This demonstrates that *S. odorata* is a biotrophic parasite on *Hebeloma mesophaeum*.

Introduction

Squamanita is an enigmatic genus in the *Agaricales* whose species exhibit an unusual parasitism on sporophores of other basidiomycetes (Redhead *et al.* 1994). *Squamanita* spp. sporophores arise from enlarged bases, that have been labelled 'sclerotial bodies' or 'protocarpic tubers', and which sometimes give rise to multiple sporophores (Fig 1). This swollen basal structure displays highly variable microscopic features from one *Squamanita* species to another. These features fallaciously suggest affiliation of the genus to the families *Agaricaceae*, *Tricholomataceae* or *Amanitaceae*, depending upon the *Squamanita* sp. investigated. However, the sclerotial bodies were discovered to be deformed sporophores of other agaric species, i.e. they were galls in which *Squamanita* hyphae are growing (Redhead *et al.* 1994). Whereas previously suggested affiliations of the genus were based incorrectly on host (gall) features, recent molecular approaches have failed to place the genus *Squamanita* close to any other genus, although its monophyly was supported (Moncalvo *et al.* 2002). Parasitized hosts vary from one *Squamanita* sp. to another, and can sometimes be identified thanks to microscopy and rare parasitized specimens in which the host is still recognizable (Redhead *et al.* 1994).

Strikingly, the first *Squamanita* species historically described, *S. odorata* (Cool 1918, under the name *Lepiota odorata*), has a highly deformed and thus unknown host even at the generic level (Redhead *et al.* 1994). *S. odorata* sporophores have a strong, fragrant smell of grapes (amyl-acetate; Guény & Chiaffi 1994), but their galls have a distinct raphanoid smell and a taste reminiscent of *Hebeloma* species (Vesterholt 1991). Indeed, *H. mesophaeum* sporophores coincidentally fruit around *S. odorata* sporophores (Vesterholt 1991; Guény & Chiaffi 1994), suggesting that the host could be *Hebeloma*

☆ This paper is dedicated to Camille Divet, in memory of his passion for studying and teaching mycology.

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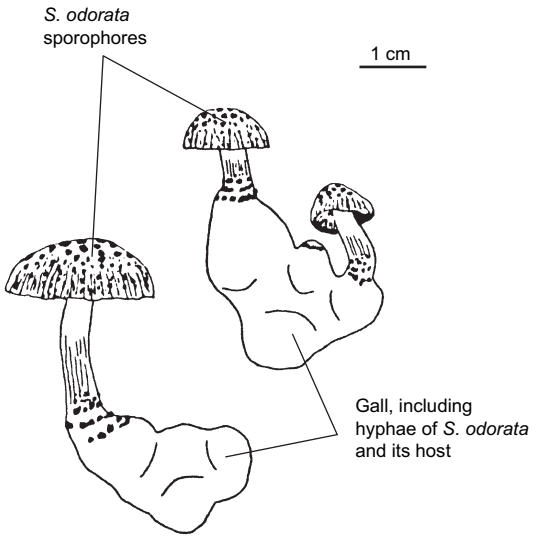

Fig 1 – Drawing of *Squamanita odorata*, after a Ruaudin sample (France, 1993). Modified with permission from Guény & Chiaffi 1994.

mesophaeum (R. Courtecuisse & M. Verbeken, pers. comm.). However, definitive evidence is lacking, and a sample from Japan convincingly pointed toward a parasitic relationship to *Phaeolepiota aurea* (Nagasawa *et al.* 1990). Although isoenzymes could be used, as in the case of a *Rhodocybe* sp. parasitic on an unknown host (Læssøe & Rosendahl 1994), direct DNA sequence comparisons are better suited to elucidate the identity of the hosts for *S. odorata*, given the extraordinary rise of these techniques for fungal identification (Selosse 2001). Here, we make use of relatively recent collections from France and Switzerland to investigate the identity of *S. odorata* hosts by PCR amplification and sequencing of mt-rDNA and nu-rDNA.

Materials and methods

Investigated samples

Site A is grassy and surrounded by young *Picea abies* on sandy soil at Ruaudin (Département de la Sarthe, East France, elevation 52 m. 47°56′50″N, 00°15′42″E), as described in Guény & Chiaffi (1994). We investigated a specimen from the initial October 1993 sampling provided by M. Chiaffi (sampling 1; voucher number: M. Chiaffi 931109) and five specimens, as well as two nearby *Hebeloma mesophaeum* sporophores, collected in October 1995 by C. Divet (voucher for *Squamanita odorata* sample 2: MAS95SQ02, M.-A. Selosse at CEFE-CNRS). Site B is situated at Villars-sur-Glâne (Canton de Fribourg, 2 km away from Fribourg, Switzerland, elevation 658 m, 46°47′25″N, 07°07′31″E) on molassic soil. Immediately after a disturbance in 1980, trees (*Salix caprea*, *Pinus sylvestris*, *Picea abies*, *Betula verrucosa* and *Sorbus aucuparia*) were planted on the site. *S. odorata* first fruited in 1990 and, in 2000, three specimens (voucher number: F. Ayer 91-012-2540) and a *H. mesophaeum* sporophore were collected by F. Ayer. All specimens were dried upon DNA extraction.

DNA amplification, cloning, and sequencing

DNA extraction and PCR amplification of the ITS using primers ITS1F and ITS4 were performed as described by Selosse *et al.* (2002). Swiss samples, and 1993 and 1995 Ruaudin samples were handled separately to avoid cross-contamination. Single PCR products were directly sequenced with the same primers on an ABI 3130xl sequencer (Applied Biosystems, Courtaboeuf) using the Big Dye Terminator kit. Whenever direct sequencing failed, PCR products were cloned using a pGEM-T easy vector systems kit (Promega, Charbonnières), followed by transformation into super-competent cells XL1-Blue (Stratagene, Amsterdam). Positive clones were sequenced using ITS1F, as above, to obtain a minimum of ten sequences; a reverse sequence using primer ITS4 was obtained from a single, randomly chosen clone for each different sequence found. For site A samples, the mitochondrial gene for LSU rDNA) was amplified and sequenced using primers ML5 (5′-CTCGGCAAA TTATCCTCATAAG-3′) and ML6 (5′-CAGTAGAAGCTGCATAGG GTC-3′) with PCR conditions identical to these used for ITS (Selosse *et al.* 2002). Sequences were edited and aligned using Sequencher 4.5 for MacOsX from Genes Codes (Ann Arbor) and a consensus was generated for cloned sequences. Data were deposited in GenBank (EF091825–EF091828). Searches for similar sequences allowing taxonomic identification were conducted using (1) the BLASTN algorithm available through the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/index.html) and the ectomycorrhizal fungal sequence database UNITE (Kõljalg *et al.* 2005; http://unite.zbi.ee/) as well as (2) the FASTA3 algorithm against the EMBL database (http://www.ebi.ac.uk/fasta33/nucleotide.html). Only BLAST analyses are reported below for *Hebeloma mesophaeum* sequences, as identical results were obtained by FASTA3 analysis.

Results and discussion

Hebeloma mesophaeum sporophores produced slightly different sequences from each site. Sequences A1 (at Ruaudin; GB accession number EF091826) and B1 (at Villars-sur-Glane; EF091827) shared 613 out of 619 bp (Table 1). Their closest relatives in GenBank by BLAST analysis were AY748854 (an uncultured ectomycorrhiza of *Hebeloma* sp.) and AB211272 (from *Hebeloma mesophaeum*). The latter only had seven differences out of 619 bp with the consensus of A1 and B1, a variation that is usual in *Hebeloma* spp. (Aanen *et al.* 2000; Boyle *et al.* 2006; H. Beker, pers. comm.). No better match was retrieved from UNITE.

All but two attempts to amplify the ITS from *Squamanita odorata* sporophores were successful (Table 1). Sequencing produced identical sequences from both sites (A2; GB accession number EF091828). The closest GenBank relatives by BLAST analysis matches were two *Bolbitiaceae*: AF325658 (*Descolea* sp.) and AF325623 (*Setchelliogaster tenuipes*). However, the affinity of *S. odorata* to the *Bolbitiaceae* is tenuous because (1) the expected value is quite high (10^{-97}; this value represents the likelihood of sequence matches expected by random chance), (2) the similarities are restricted to the 5.8S region of the sequence (not shown; note that GenBank contained no *Squamanita* ITS sequence before) and (3) FASTA3 research

Table 1 – Overview of the sequences retrieved from *Squamanita odorata*, its gall and co-occurring *Hebeloma mesophaeum* at Ruaudin and Villars-sur-Glâne

Sample Origin	Investigated part of sporophore	ITS (nu-rDNA) sequence[a]	Mitochondrial rDNA sequence
Site A (Ruaudin, France), 1993, sampling M. Chiaffy			
Squamanita. odorata sample 1	Gall	A1 + A2 (cloned, 9 + 1)	A3
	Sporophore	A2 (d. s.)	No PCR product
Site A (Ruaudin, France), 1995, sampling C. Divet			
S. odorata sample 1	Gall	A1 + A2 (cloned, 3 + 7)	A3
	Sporophore	No PCR product	No PCR product
S. odorata sample 2	Gall	A2 (cloned, 10 + 0)	A3
	Sporophore	A2 (d. s.)	No PCR product
S. odorata sample 3	Gall	A1 + A2 (cloned, 7 + 3)	No PCR product
	Sporophore	A2 (d. s.)	No PCR product
S odorata sample 4	Gall	A1 + A2 (cloned, 4 + 6)[b]	A3
	Sporophore	A2 (d. s.)	No PCR product
S. odorata sample 5	Sporophore	A2 (d. s.)	No PCR product
Hebeloma mesophaeum sample 1	Sporophore	A1 (d. s.)	A3
H. mesophaeum sample 2	Sporophore	A1 (d. s.)	A3
Site B (Villars-sur-Glâne, Switzerland), 2000, sampling F. Ayer			
S. odorata sample 1	Gall	A2 + B1 (cloned, 8 + 2)[b]	Not studied
	Sporophore	A2 (d. s.)	Not studied
S. odorata sample 2	Gall	B1 (d. s.)	Not studied
	Sporophore	A2 (d. s.)	Not studied
S. odorata sample 3	Gall	No PCR product	
	Sporophore	A2 (cloned, 10 + 0)	Not studied
H. mesophaeum sample 1	Sporophore	B1 (d. s.)	Not studied

a 'd. s.', direct sequencing was possible; otherwise, whenever PCR products were cloned, the cloning ratio of each sequence obtained is given ($n = 10$). GenBank accession numbers: A1, EF091826; A2, EF091828; B1, EF091827; A3, EF091825.
b Additionally, a chimaeric sequence involving the two obtained sequences was found in an 11th clone.

provided *Collybia cirrhata* (AF361318 and AF361316) as closest relatives. Although *Squamanita* appeared as a sister group to *Phaeocollybia* spp. in the phylogeny by Moncalvo *et al.* (2002), that placement lacked statistical support: the phylogenetic position of the genus *Squamanita* therefore deserves further work.

All but one investigated gall produced PCR products, and provided a single apparent band after electrophoresis on gel (not shown). After cloning, two divergent sequences were found in similar amounts, i.e. the ITS of *S. odorata* and the ITS of the *H. mesophaeum* growing on the same site (Table 1). This suggests that *H. mesophaeum* is the host for *S. odorata*. In only one occurrence, direct sequencing was possible and produced only ITS sequence of the host (sample 2 of site B; Table 1). A Chi square test with Yate's corrections showed that gall and sporophore differed significantly ($P < 0.05$) in their species composition. *H. mesophaeum* was significantly more frequent in the gall than in the *S. odorata* sporophore, supporting the hypothesis that it is the parasitized species. The fact that no *Hebeloma* ITS was amplified from *Squamanita* sporophores (even after cloning for sample 1 of site A, for which direct sequencing failed for unknown reasons) indicates that amplification of *Hebeloma* ITS from the gall is not the result of contamination by the other sampled sporophores, and that *S. odorata* sporophores are free of host hyphae.

Our attempts to amplify the LSU rDNA from site A samples unfortunately failed to produce any sequence for *S. odorata*. This could be explained by presence of introns in this gene that sometimes inhibit PCR amplifications. *H. mesophaeum* sporophores produced a unique sequence A3 (GB accession number EF091825) whose closest GenBank relatives by BLAST analysis are AJ920017 and AD001592 (sequences from *Hebeloma crustuliniforme*, expected value: 2×10^{-175} in both cases; note that neither GenBank nor UNITE contained any *H. mesophaeum* LSU rDNA sequences until now). This sequence was successfully obtained from four out of the five investigated galls from site A, further substantiating the idea that *H. mesophaeum* is the host to *S. odorata*. Therefore, the convincing report of *Phaeolepiota aurea* as a host based upon morphology by Nagasawa *et al.* (1990) suggests that these authors collected a different species (tentatively labelled '*S. phaeolepioticola*', an unpublished name, by Redhead *et al.* 1994). Indeed, Nagasawa *et al.* (1990) suspected this due to conidia produced by clampless hyphae, instead of clamped hyphae in true *S. odorata*.

Our data also demonstrate that some integrity of *H. mesophaeum* persists in the gall, so that DNA can be successfully recovered, as would be expected if *S. odorata* is a biotrophic parasite. Although the direction of the parasitism cannot be directly inferred from our observations, we favour the later explanation, rather than a parasitism of *H. mesophaeum* on *S. odorata*, as (1) *S. odorata* sporophores never grow alone, and (2) the biology of the *Squamanita* genus makes *Hebeloma* sequences unlikely to be adventitious in galls. Moreover, *S. odorata* often grows in anthropically disturbed sites (as A and B in this study, or sites described in Læssøe 1985); it could be hypothesized that such environments entail some weakness or maladaptation of *H. mesophaeum* favouring interaction with *S. odorata*. Alternatively, it could be that anthropically disturbed sites make *S. odorata* more obvious to mycologists.

Several features of *S. odorata* and other *Squamanita* spp. remain intriguing, such as the many host jumps in the evolution of the *Squamanita* genus, in spite of the high specificity of each *Squamanita* species. A second question is that of the persistence of the parasite populations over the years, e.g. from 1990 to 2003 at site B (F. Ayer, pers. comm.); do some propagules persist, such as the thick-walled chlamydospores, or do vegetative mycelia persist in soil, perhaps associated with that of *H. mesophaeum*? In the relationship between *Suillus bovinus* and *Gomphidius roseus*, hyphae of the latter associate with *S. bovinus* ectomycorrhizae (Olsson *et al.* 2000). A similar situation could occur with *H. mesophaeum* as it too is ectomycorrhizal, however, other *Squamanita* hosts are not (Redhead *et al.* 1994). Alternatively, repeated inoculation of sporophores by persistent propagules, as in *Hypomyces* spp. (Douhan & Rizzo 2003), could be involved. This also questions the exact nature of the interface between the two species, and the way in which *Squamanita* hyphae recover organic matter from their host, a biotrophic parasitism also reported in other *Squamanita* spp. and in *Entoloma abortivum* parasitic on *Armillaria* spp. (Czederpilz *et al.* 2001). Besides identification of *H. mesophaeum* as a host for *S. odorata*, this exquisite case of fungal parasitism thus deserves further study.

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