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Position specificity in *Chitonomyces* (Ascomycota, Laboulbeniomycetes) on *Laccophilus* (Coleoptera, Dytiscidae): a molecular approach resolves a century-old debate

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Abstract: The occurrence of Laboulbeniomycete species consistently on a precise portion of beetle integument was investigated in 13 species of *Chitonomyces* ectoparasitic on the aquatic diving beetle *Laccophilus maculosus* (Coleoptera, Dytiscidae). The phenomenon was called ''position specificity'' by Roland Thaxter in 1896, yet the mechanism has remained unknown. By using molecular analysis of the nucSSU rRNA gene and the 5.8S and partial ITS1 rRNA regions, 13 species of *Chitonomyces* reported to exhibit position specificity on *Laccophilus maculosus* were placed neatly into pairs of morphotypes, resulting in synonomies and recognition of six phylogenetic species (one species is a triplet). Each phylogenetic species was located at corresponding positions on male and female beetles that make contact during mating. In addition, ecological data and video footage of the mating behaviors of *Laccophilus* confirmed that sexual transmission is the mechanism behind this enigmatic phenomenon.

Key words: Ascomycetes, Coleoptera, fungal parasitism, Laboulbeniales, molecular analysis, species delimitation, taxonomy

Introduction

The fungal order Laboulbeniales is a large, diverse, yet under explored lineage within the phylum Ascomycota (Weir and Blackwell 2001). They are microscopic, obligate ectoparasites of living arthropods and are unusual among fungi for their determinant growth, exclusively sexual mode of reproduction and intriguing patterns of specificity. In these fungi the ascospore gives rise to a multicellular thallus through repeated and precisely determined cell divisions, resulting in a much reduced hyphal system (Tavares 1985). The thallus typically includes development of an ascogonium and male antheridia, both of which are thought to function in sexual reproduction (Thaxter 1908, Tavares 1985).

The apparent restriction of the order to a life history totally dependent on living arthropods, mostly insects, has long attracted the attention of mycologists. The capacity for growing and reproducing only on a single species of host, or on closely related hosts, is of course characteristic of most obligate parasites. More intriguing perhaps are the documented occurrences of fungal thalli restricted to a given host body part (position specificity), or to a given sex of host (sex of host specificity) (Benjamin and Shanor 1952). Among the Laboulbeniales some of the most remarkable examples of position and sex of host specificity are found in the group *Chitonomyces*, most species of which occur on water beetles in the Dytiscidae, although several are known on Haliplidae and Gyrinidae (Benjamin 1971, Santamaria 2001).

Roland Thaxter, in his monumental work on Laboulbeniales, delineated 16 species of *Chitonomyces* from a population of the African Gyrinidae beetle, *Orectogyrus specularis* (Thaxter 1926). Each species was characterized by unique morphological features and strict occurrence at a given location on the body of the host. Thaxter found a similar pattern on the common North American water beetle *Laccophilus maculosus* (Thaxter 1896). While fewer than its African counterpart, the 12 species of *Chitonomyces* on *L. maculosus*, display an equally marked degree of position specificity and diversity of morphological structures. Thaxter delineated the 12 species based on variation in the overall shape of the thallus, pigmentation, position on the host and perithecial appendages. Often species were delineated by only one of these characters alone. For example, the hook-like perithecial appendage of *C. unciger* was noted as ''quite unlike that of any other species in form and position'' and *C. spiniger* was ''distinguished from all other species by the spur-like process from the base of the perithecium'' (Thaxter 1896). At this time Thaxter was unable to completely explain the unusual precision of each location or the mechanism behind this remarkable pattern. At this time nutritional differentiation of the host integument and sexual transmission both were proposed as possible mechanisms (Thaxter 1896, 1908).

Over the next century, other workers began to question the likelihood that the 16 species of

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Chitonomyces on *Orectogyrus* and the 12 species on *L. maculosus* were indeed separate species (Benjamin and Shanor 1952, Scheloske 1976). During the ensuing years as our concept of a species and our knowledge of the phenotypic plasticity of fungi developed, Thaxter's original species delineations based solely on morphology became more untenable. It was argued that the 12 or 16 species were likely to be one, or several, (Scheloske 1976) varying in morphology due to differences in the cuticular substrate (Richards and Smith 1954) or in the development of extra appendages acting as specialized spore dispersal structures adapted for a particular location (Benjamin and Shanor 1952, Scheloske 1976, Weir and Beakes 1996, Rossi and Kotrba 2004, Rossi and Proaño Castro 2009).

Santamaria (2001) re-examined the questions of sex of host and position specificity in a taxonomic review, comparing type material of *Chitonomyces* to his own collections of the genus in Spain. Fungi were found on multiple species of *Laccophilus* (Dytiscidae) and members of the Haliplidae family. No molecular analyses were undertaken for his study. Mating behavior was considered the mechanism to explain some observed patterns of position and sex of host specificity. For example *C. aculeifer* (synonymized with *C. bruchii* and *C. ceratomycetalis*) was reported to occur in two growth forms, one that was located on the claws of male beetles and the second form that was located on the elytra of female beetles (Santamaria 2001). However exceptions to the above pattern also occurred for other species and therefore not all positions could be explained by mating behavior. Santamaria observed two species of *Chitonomyces* only on male individuals, (notably, none exclusive to females) and cites Benjamin's work (Benjamin and Shanor 1952) with *Laboulbenia* and others, as conformation for both position and sex of host specificity and that mating behavior explains some examples of position specificity (Santamaria 2001).

As more species of Laboulbeniales are found all over the world, so do the records of this remarkable phenomenon. For example, eight growth forms of a single species of *Laboulbenia* were found on one species of South American fly, *Richardia teevani* (Diptera, Richardiidae) (Rossi and Kotrba 2004) and the new species, *Rhachomyces dimorphus*, from Ecuador (Rossi and Proaño Castro 2009) has two growth forms: one found only on the posterior, inferior margin of the left side of the prothorax, on female beetles, and the other positioned in the hollow of the ''antennal cleaner'' of the left protibia, of male beetles (Rossi and Proaño Castro 2009). These two positions contact each other while the beetles are mating, supporting the hypothesis that sexual transmission is the method of spore dispersal and the mechanism by which position specificity is maintained (Rossi and Proaño Castro 2009).

To date the Laboulbeniales remain intractable to culture, therefore mating tests to establish a biological species have not been possible. Molecular analysis is thought, by many to be the key to answering this century-old debate (Santamaria 2001, Rossi and Kotrba 2004, Weir and Blackwell 2005, Rossi and Proaño Castro 2009). The present study is the first published molecular analysis of position specificity. Our focus for this paper is on the genus *Chitonomyces* on *L. maculosus* for which all known North American morphotypes (13 in total) were found, and sequences of SSU, 5.8S and partial ITS1 rRNA regions were obtained.

MATERIALS AND METHODS

Collection of L. maculosus.—Individuals of *L. maculosus* were collected in a large freshwater pond at Heiberg Forest, New York, 4 May 2008–24 Oct 2008. Nine samples in total were taken two times a month. One collection date in August was excluded because so few beetles were present, due to a typical seasonal decrease in the population. A 15 cm diam wire mesh kitchen strainer was used to collect the beetles. *Laccophilus maculosus* were distinguished by sight and then removed from the strainer with an aspirator. Samples were taken within the first 1 m of the shallow, partly shaded pond edge, among aquatic and semi-aquatic vegetation. All specimens were transferred immediately to 95% ethyl alcohol for storage.

Scanning for infection and collection of ecological data.—Each beetle was scanned under an Olympus SZ40 40× dissecting microscope. All fungal species, their position on the host integument and sex of host were recorded for each collection date. The sex of *L. maculosus* was determined by sight. Many genera within Dytiscidae are recognized as having distinct male characters, such as highly modified setae, a more pronounced metacoxal file and the external shape of the genital area (Larson et al. 1999), which allows for easy differentiation between male and female individuals.

Fungal morphological analyses.—Individual thalli were removed from each separate position on the host integument by micropin. Slide mounts followed the techniques (Benjamin 1971) for the double cover slip method for archival microscope slides. All slides were viewed and photographed with a Nikon E800 equipped with differential interference contrast optics. Major components of the thallus for 20 individuals of *Chitonomyces* at each position were measured. All voucher slides are in the collection at SYRF (SUNY College of Environmental Science and Forestry, Syracuse New York).

DNA extraction and sequencing.—Three to 15 thalli were removed from each position with DNA extraction methods following those described in Weir and Blackwell (2001).

TABLE I. Morphotype, sex of host, position and corresponding phylogenetic species

	Morphotype	Sex of host		Position	Phylogenetic species
1a	*C. uncinatus*	♂		Left side, ventral abdomen, 4th, 5th sternites.	*C. simplex*
1b	*C. simplex*	♂	♀	Right elytron, distal half.	
2a	*C. lichanophorus*	♂		Left subgenital plate.	*C. affinis*
2b	*C. affinis*	♂	♀	Right elytron margin, distal quarter.	
3a	*C. hyalinus*	♂		Left posterior leg, 1st–4th tarsal lobes and hairs.	*C. hyalinus*
3b	*C. marginatus*	♂	♀	Left elytron margin among bristles, middle.	
4a	*C. appendiculatus*	♂		Left and right anterior and median legs.	*C. appendiculatus*
	C. distortus	♂		Left and right anterior and median legs.	
4b	*C. dentifer*	♂	♀	Prothorax and top of left elytron.	
5a	*C. spiniger*	♂		Right posterior leg 4th tarsal spine.	*C. rhyncostoma*
5b	*C. rhyncostoma*	♂	♀	Right elytron epipleuron	
6a	*C. unciger*	♂		Left posterior leg claw.	*C. paradoxus*
6b	*C. paradoxus*	♂	♀	Left elytron edge below epipleuron.	

The number of replicates varied from five for common species to 2–1 for rare species. The master mix concentrations for DNA amplification were 1.5 mM $MgCl_2$, 800 μM dNTPs, 0.5 μM each primer and 1.3 U DNA polymerase. For amplification an Applied Biosystems 2720 thermal-cycler was used with a single initial denaturation at 94 C for 3 min, then 35 cycles of 94 C for 35 s, 50 C for 55 s, 72 C for 1 : 10 + 5 min and a final extension at 72 C for 10 min (White et al. 1990). The primers were NS1 and NS4 (White et al. 1990) for the SSU rRNA gene and ITS1f and ITS4 (White et al. 1990) for the ITS1, and 5.8S rRNA region. Only partial ITS1 and the entire 5.8S were used in analyses; ITS2 was too variable to align. PCR product was cleaned with the QIAquick PCR Purification Kit (QIAGEN, Maryland), and an ABI 3730xl capillary DNA sequencer was used for sequencing.

Phylogenetic analyses.—Sequences were viewed and replicate sequences compared with 4 Peaks (Griekspoor and Groothuis 2005) and aligned in Clustal X (Larkin et al. 2007). Ambiguously aligned regions were excluded. Alignment matrices were deposited in TreeBASE (12024 for the ITS alignment and 12025 for SSU). Maximum parsimony (MP) (not shown), maximum likelihood (ML) analyses, generated with PAUP* 4.0b10 (Swofford 2003), and Bayesian Analysis (BA), using MrBayes 3.12 (Ronquist and Huelsenbeck 2003), were performed with these parameters. (i) MP was conducted with a heuristic search, all characters unordered and of equal weight and with gaps considered missing data. Multistate taxa interpreted as uncertain. Starting trees were obtained via stepwise addition, and the branch-swapping algorithm used was tree bisection reconnection (TBR). The steepest descent option was not in effect. The initial MAXTREES setting was 100 with the MULTREES option was in effect. Bootstrap analysis was performed with 1000 replicates. (ii) For ML analysis jModelTest 0.1.1 (Posada 2008, Guindon and Gascuel 2003) selected the GTR+G+I model with heuristic search settings the same as the above for MP including these additions: starting branch lengths were obtained with Rogers-Swofford approximation method, trees with approximate likelihoods 5% or further from target were rejected and branch-length optimization equaled a one-dimensional Newton-Raphson with pass limit = 20, delta = 1e-06. Bootstrap analysis was set at 1000 replicates with fast stepwise-addition. (iii) BA was performed under the GTR+G+I model with four Monte Carlo Markov chains run 1 000 000 generations for the ITS1 and 5.8S rRNA regions and 1 000 000 generations for the nucSSU rRNA gene and sampled every 100th generation with a burn-in of 25% for both.

Ecological analysis.—A chi-square test was used to determine significant differences between the proportion of infection of male and female beetles and to detect patterns of seasonal distribution of both morphological and phylogenetic species. The entire collection period was divided into three groups, the first three collection dates included May and June (spring) the second three collection dates include July and August (summer) and the last two occurred in October (fall).

Behavioral observations of Laccophilus.—Living *Laccophilus* were kept in a 10 gallon aquarium and observed periodically throughout July and August. Mating was recorded with a SONY Handycam equipped with an 800× digital zoom lens.

RESULTS

Collection and ecological analyses.—In total 308 individuals of *L. maculosus* were collected. Fifty-one percent were infected with at least one species of *Chitonomyces*. The infected population comprised 63% male and 37% female beetles. All 12 of the originally described species of *Chitonomyces* were found at their respective positions (TABLE I) as documented by Thaxter (1896) as well as thalli of *C. dentifer* (first described from *L. proximus* by Thaxter 1908). Two species, *C. appendiculatus* and *C. distortus*, were found to occupy the same position, the anterior legs of male beetles (and will be referred to as *C. appendiculatus* for the remainder of this paper). All 13 species were present on male individuals,

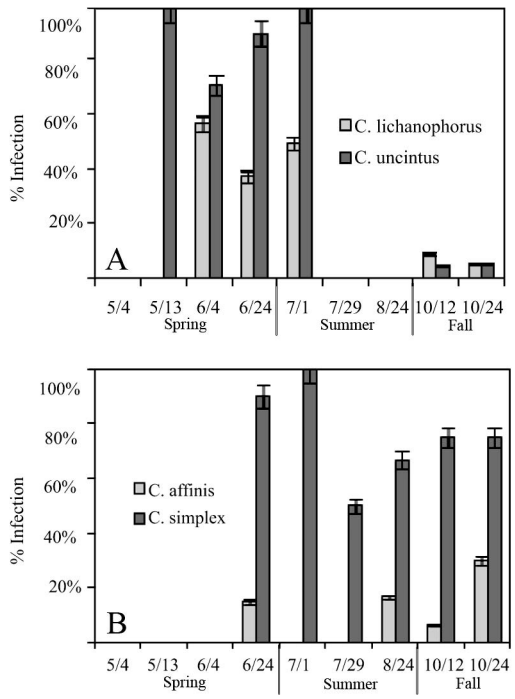

FIG. 1. A. Proportion of infection throughout spring, summer and fall seasons of two morphological species found only on male *L. maculosus*, *C. lichanophorus* and *C. uncinatus*. B. Proportion of infection throughout three seasons of the two morphological species *C. affinis* and *C. simplex* on female *L. maculosus*.

whereas only six of the species were found on female beetles. A seasonal pattern emerged ($P = 0.001$) in which a greater proportion of male *Laccophilus* were found with *C. uncinatus* and *C. lichanophorus* in spring and early summer (FIG. 1A) compared to a higher proportion ($P = 0.01$) of female *Laccophilus* infected with *C. simplex* in summer and fall (FIG. 1B). *Chitonomyces affinis* on female beetles also increased in the fall (FIG. 1B) but was not significant at $P < 0.05$.

Although individual beetles were found with as many as 6–8 species of *Chitonomyces* on their bodies, the most frequent number was two species (36% of infected hosts) followed by one species (31% of infected hosts) three (15% of infected hosts) four (9% of infected hosts) and five (7% of infected hosts). *Chitonomyces simplex* (TABLE II) accounted for 30% of all observed infection and occurred on both male and female beetles. All the other 13 species were much less abundant, ranging from 12% (*C. uncinatus* restricted to male beetles) to 0.5% occurrence (*C. appendiculatus* also restricted to male beetles).

Phylogenetic analyses.—The SSU alignment contained 19 taxa, and 744 characters of which 571 were constant and 127 were parsimony informative. The heuristic search produced one tree with a consistency index of 0.878 and a retention index of 0.9276. The ITS1 and 5.8S alignment contained 11 taxa and 290 characters of which 167 were constant and 51 were parsimony informative. Three trees were retained, all having a 0.885 consistency index and a 0.837 retention index.

The 13 species of *Chitonomyces* on *L. maculosus* for which we generated SSU rRNA gene sequences grouped neatly into six phylogenetic species (TABLE I, FIG. 2) with each phylogenetic species comprising two, (in one case three) previously recognized species. Nine of these taxa were grouped in four of the six phylogenetic species, in the 5.8S and partial ITS1 analysis (FIG. 3). Percent similarity values between phylogenetic species of 97.05–99.15% for the SSU gene were within the typical range of ≥ 1% difference found in other fungal groups (Katsuga et al. 2002). Percent similarity values within species were 100% for all but one pairing, *C. paradoxus* and *C. unciger* (99.84%). The similarity values for the 5.8S rRNA gene for nine of the 13 species were 95.86–98.82%, and for partial ITS1 and 5.8S sequences, for 9 of the 13, the interspecific values either fell well below the standard ≥ 5% difference for species delineation, in the range of 79.50–88.40% or for intraspecific values were 100% identical for species that now will be formally recognized as conspecifics.

Mating behavior as the mechanism for spore dispersal.—Detailed mating (n = 5) of our *L. maculosus* each lasted approximately 1 h and confirmed a specific progression of movements: mounting, lateral shake, swimming, copulation, female breathing and female swimming (Aiken 1992). In our observations the primary position was the male clasping the female's elytra with the posterior legs, while the anterior and median legs tapped the upper elytra and pronotum of the female (SUPPLEMENTARY DATA I). The six phylogenetic species each comprise a pair (in one case three) previously recognized morphological species (TABLE I) that correspond to points of contact between male and female beetles during copulation (FIGS. 4, 5). Based on video footage of five matings, we observed that all positions and their corresponding pairs could be explained through mating behaviors.

TAXONOMY

Chitonomyces Peyr. 1873: 250

The following is a generic summary of the 13 *Chitonomyces* on *Laccophilus maculosus* analyzed for this study.

Thalli are monoecious with a prominent perithecium containing ascospores, and a single, simple antheridium. The receptacle has six cells (I), (Ia), (II), (IIa), (III), (IIIa). Cell I is basal, typically large,

TABLE II. Percent abundance of each morphotype and the proportion of infection between the sexes

Species of fungi	Number of beetles infected	Number of beetles/ occurrences of infection	Males infected	Females infected
C. affinis	28	6.8%	61%	39%
C. appendiculatus	2	0.5%	100%	0%
C. dentifer	43	10.4%	67%	33%
C. distortus	5	1.2%	100%	0%
C. hyalinus	11	2.7%	100%	0%
C. lichanophorus	25	6.0%	100%	0%
C. marginatus	44	10.6%	52%	48%
C. paradoxus	30	7.3%	53%	47%
C. rhyncostoma	27	6.6%	33%	67%
C. simplex	124	30.0%	58%	42%
C. spiniger	7	1.7%	100%	0%
C. unciger	17	4.1%	100%	0%
C. uncinatus	51	12.3%	100%	0%

triangular, and connects to the foot. Cell I subtends the subbasal cell Ia, a rectangular cell that can vary from square shaped to a thin, flattened cell. Cell Ia subtends the receptacular cells II and IIa and the perithecial stalk cell VI. These three cells are similar in size, lay side by side and are triangular to rectangular. Cell IIa subtends cell III a long narrow cell adnate to and extending to approximately the midpoint of the perithecium. Cell III subtends the single, rounded antheridium typically tucked between the perithecium and cell IIIa, which is approximately the same size. The antheridium has a constricted black septum at the apex from which a filamentous and evanescent terminal cell extends. However these cells rarely remain after the thalli are removed from the host. Cell IIIa subtends the basal cell of the primary appendage and is slightly wider than cell III. The basal cell of the primary appendage is about the same length as the antheridium but wider, more prominent and begins at the apex of the antheridium. The primary appendage also subtends a filamentous and evanescent terminal cell from a constricted septum. Perithecial appendages and pigmentation variable as described below.

Specimens examined. UNITED STATES, NEW YORK: Onondaga County, Heiberg Forest. Collected in freshwater pond 3, May–Oct 2008 on *Laccophilus maculosus* (Coleoptera: Dytiscidae).

Chitonomyces simplex (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 292

≡ *Heimatomyces simplex* Thaxt. 1892. Proc. Am. Acad. Arts and Sci. 27, p. 30

= *Chitonomyces uncinatus* (Thaxt.) Thaxt. 1896. Thaxt. Mem. Am. Acad. Arts and Sci. 12, p. 291 syn. nov.

≡ *Heimatomyces uncinatus* Thaxt. 1892. Proc. Am. Acad. Arts and Sci. 27, p. 33 (FIG. 6)

Thalli positioned on the left side of the 3rd and 4th sternites on male *L. maculosus* are light amber with a perithecium that curves toward the primary appendage. Distinguishing morphology is a prominent, blunt cell IIIa that extends beyond the apex of the primary appendage. There are no perithecial appendages.

Dimensions. Total length of thallus: 112.5–150 μm. Perithecium: 80–100 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 55–80 μm. Height of cell I: 25–30 μm. Cell Ia: 5–10 μm. Cells II, IIa and VI: 7.5–12.5 μm.

Thalli positioned on the bottom half of the right elytron on both male and female *L. maculosus* are light amber to hyaline, with a perithecium that curves away from the primary appendage. Cell IIIa does not protrude beyond the basal cell of the appendage. The perithecial apex is blunt and rounded, with no perithecial appendages.

Dimensions. Total length of thallus: 75–87.5 μm. Perithecium: 62.5–65 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 50–55 μm. Height of cell I: 17.5–22.5 μm. Cell Ia: 5–7.5 μm. Cells II, IIa and VI: 7.5–12.5 μm.

Commentary. C. simplex positioned on the right elytron, in total length, are approximately 30% smaller than those on the left ventral abdomen.

Chitonomyces affinis (Thaxt.) Thaxt. 1896 Mem. Am. Acad. Arts and Sci. 12, p. 291

≡ *Heimatomyces affinis* Thaxt. 1892 Proc. Am. Acad. Arts and Sci. 27, p. 31

= *Chitonomyces lichanophorus* (Thaxt.) Thaxt. 1896 Mem. Am. Acad. Arts and Sci. 12, p. 290 syn. nov.

≡ *Heimatomyces lichanophorus* Thaxt. 1892 Proc. Am. Acad. Arts and Sci. 27, p. 32 (FIG. 6)

Thalli positioned on the distal third of the right elytron margin, typically outside the elytral hairs of

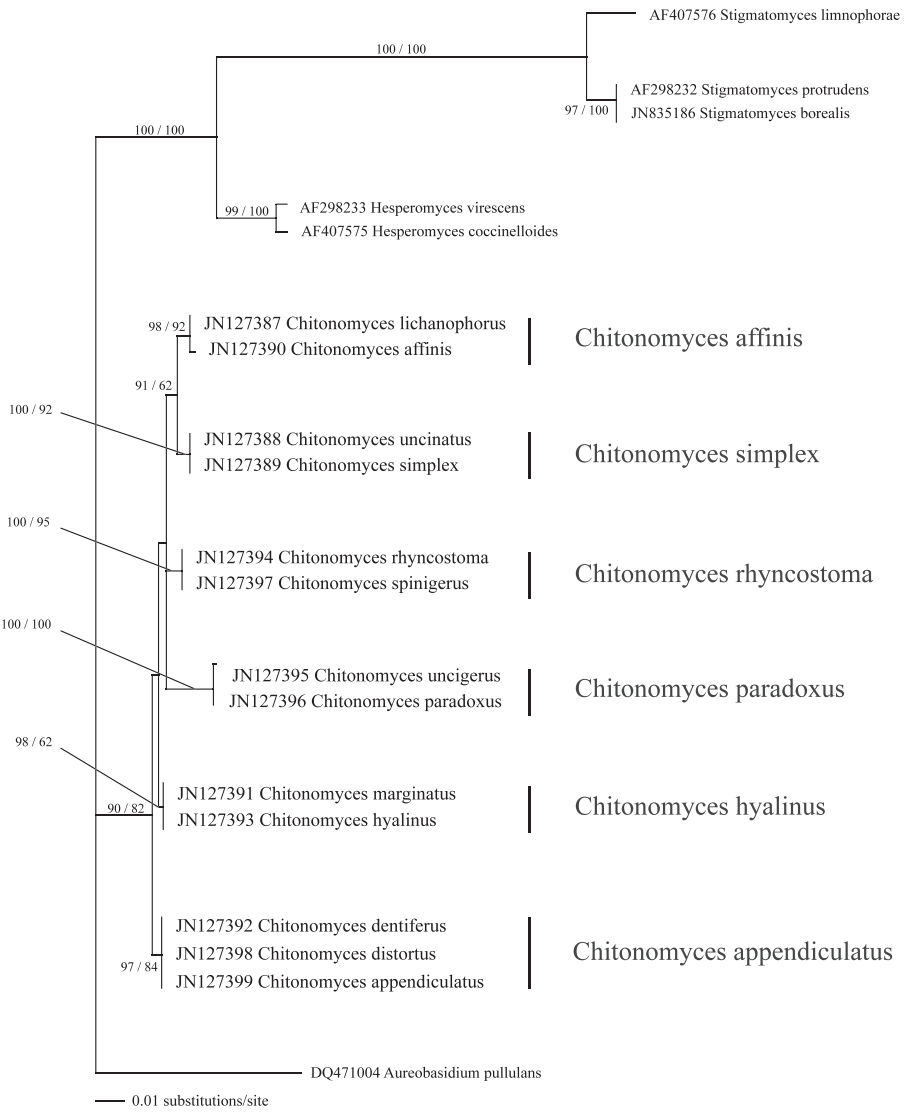

FIG. 2. Maximum likelihood tree based on nucSSU rRNA gene region. Values to the right are bootstrap support and to the left are Bayesian posterior probabilities.

male and female *L. maculosus*, have an amber brown, straight perithecium and are distinguished by a large, highly pigmented cell I and a highly flattened cell Ia and small thin cells II, IIa and VI. The perithecium is slightly curved at the apex with papillate lip cells.

Dimensions. Total length of thallus: 120–127.5 µm. Perithecium: 67.5–80 µm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 57.5–62.5 µm. Height of cell I: 42.5–52.5 µm. Cell Ia: 5 µm. Cells II, IIa and VI: 5–10 µm.

Thalli positioned on the left subgenital plate of male *L. maculosus* have a hyaline perithecium that curves away from the primary appendage and a large (approximately half the size of the thallus) and highly pigmented (blackened) cell I. Cell IIIa and the basal cell of the primary appendage are both remarkably long, extending well beyond the tip of the perithecium. Cells Ia, II, IIa and VI all are flattened and small. The perithecium has no appendages and a simple apex.

Dimensions. Total length of thallus: 137.5–170 µm. Perithecium: 57.5–100 µm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 105–112.5 µm. Height of cell I: 62.5–77.5 µm. Cell Ia: 5–7.5 µm. Cells II, IIa and VI: 2.5–7.5 µm.

Commentary. Thalli positioned on the edge of the right elytron have an amber-brown perithecium and are generally 20% smaller than those on the left subgenital plate that have a characteristically hyaline perithecium, black basal cell and an exceptionally long cell (IIIa) and primary appendage.

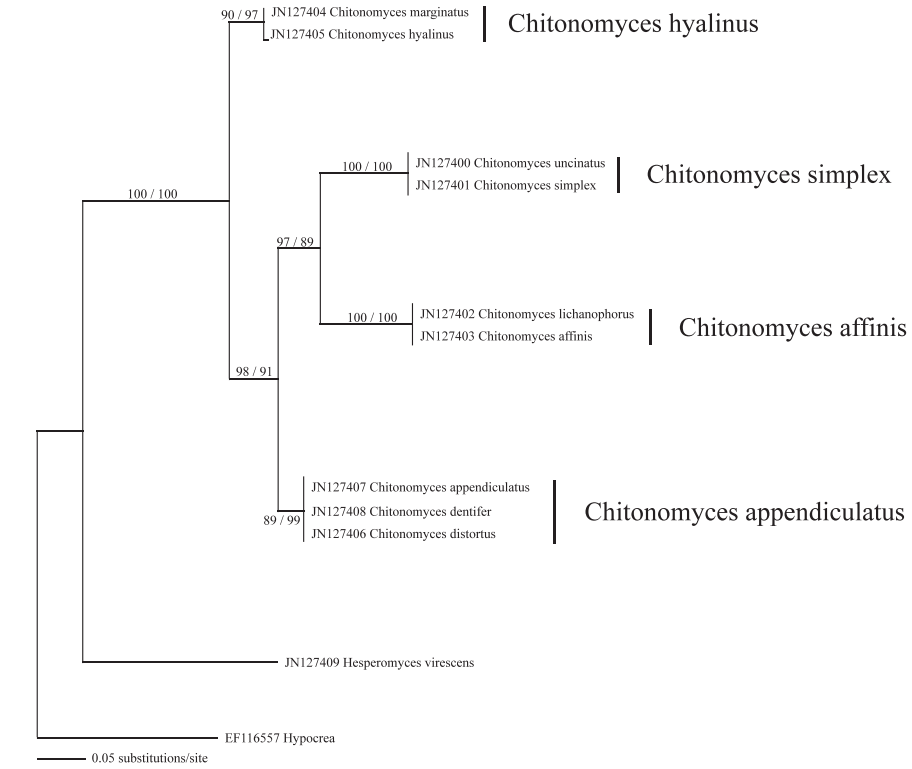

FIG. 3. Maximum likelihood tree based on partial ITS1 and the entire 5.8S rRNA gene regions. Values to the right are bootstrap support and to the left are Bayesian posterior probabilities.

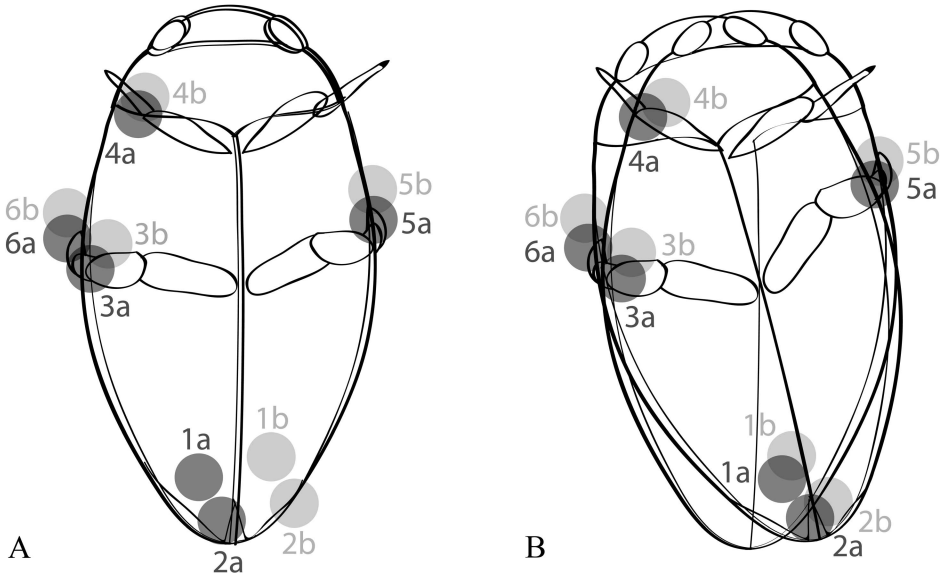

FIG. 4. Left. Male *Laccophilus* copulating with female in a parallel orientation, resulting in positions 1a to 1b and 2a to 2b to be misaligned. Right. Male oriented in a diagonal, as observed in video footage, resulting in the alignment of all positions. 1a. *C. uncinatus*, 1b. *C. simplex*. 2a. *C. lichanophorus*, 2b. *C. affinis*. 3a. *C. hyalinus*, 3b. *C. marginatus*. 4a. *C. appendiculatus*, 4b. *C. dentifer*. 5a. *C. spiniger*, 5b. *C. rhyncostoma*, 6a. *C. unciger*, 6b. *C. paradoxus*.

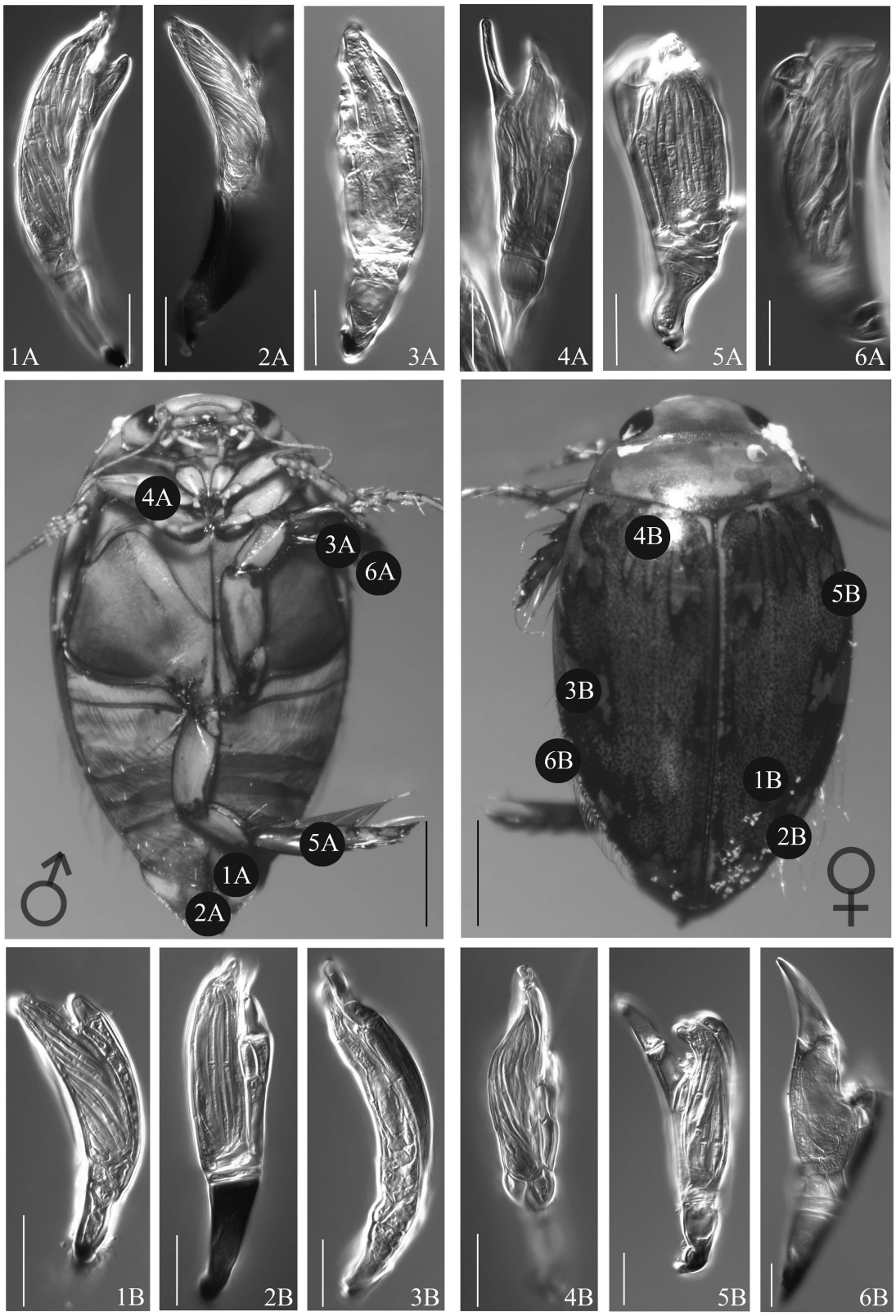

FIG. 5. 1a, b. *C. simplex*; 1a. The alternate form (previously known as *C. uncinatus*) found only on male beetles; 1b. *C. simplex* the typical form found on male and female beetles. 2a, b. *C. affinis*; 2a. The alternate form (previously known as *C. lichanophorus*) found only on male beetles; 2b. *C. affinis*, the typical form found on male and female beetles. 3a, b. *C. hyalinus*; 3a. the typical form found only on male beetles; 3b. *C. hyalinus*, the alternate form (previously known as *C. marginatus* found

Chitonomyces hyalinus (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 291

≡ *Heimatomyces hyalinus* Thaxt. 1892. Proc. Am. Acad. Arts and Sci. 27, p. 31

= *Chitonomyces marginatus* (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 289

≡ *Heimatomyces marginatus* (Thaxt.) Thaxt. 1892. Proc. Am. Acad. Arts and Sci. 27, p. 34

Thalli positioned on the tarsal segments of the left posterior leg of male *L. maculosus* are light amber-brown with a straight perithecium. Cell I is stout and sigmoid. The anterior side of the perithecium has a column of darker amber-brown cells that end just before the perithecial apex. The perithecial apex is slightly curved with a small ridge on the posterior side.

Dimensions. Total length of thallus: 112.5–137.5 μm. Perithecium: 75–92.5 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 55–62.5 μm. Height of cell I: 20–25 μm. Cell Ia: 10–15 μm. Cells II, IIa and VI: 5 μm.

Thalli positioned on the left elytron margin, frequently found among the line of elytral hairs of male and female *L. maculosus*, are amber-brown, narrow and curved, with a unique brown darkening of the appendage and the receptacle cells III and IIIa. The primary appendage cells are difficult to distinguish as a result of the dark pigmentation, however a narrow, tall antheridium is noticeable. A distinct, narrow, knob-like structure, the origin of which is difficult to determine, extends above the basal cell of the primary appendage and just beyond the tip of the perithecium, (this structure is possibly an extension of cell IIIa). The narrow perithecium is bent at the apex, creating a beak like appearance.

Dimensions. Total length of thallus: 125–150 μm. Perithecium: 85–95 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 85–95 μm. Height of cell I: 15–17.5 μm. Cell Ia: 7.5 μm. Cells II, IIa and VI: 12.5–20 μm.

Commentary. Thalli on the left posterior leg lack pigmentation are stout and have a pale strip of golden cells along the perithecium as their only distinction. Thalli positioned on the left elytron are distinctly narrow with a column of brown, pigmented receptacle cells that ends in a dark knob-like structure.

Chitonomyces appendiculatus (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 287

≡ *Heimatomyces appendiculatus* Thaxt. 1892. Proc. Am. Acad. Arts and Sci. 27, p. 31

= *Chitonomyces distortus* (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 287 syn. nov.

≡ *Heimatomyces distortus* (Thaxt.) Thaxt. 1895. Proc. Am. Acad. Arts and Sci. 30, p. 477

= *Chitonomyces dentifer* Thaxt. 1905. Proc. Am. Acad. Arts and Sci. 41, p. 306 syn. nov. (FIG. 7)

Thalli that are positioned on the right and left anterior and median legs of male *L. maculosus* are hyaline to pale amber-brown, with either a straight perithecium (*C. appendiculatus*) or a highly bent perithecium (*C. distortus*). Thalli with a straight perithecium typically have a prominent, thin, arm-like perithecial appendage that extends above the perithecium. The perithecial apex is straight, tapering through the neck to a pointed tip, which has a characteristic small node below the lip cells. Thalli with a perithecium the top third of which is bent away from the primary appendage at an approximately 90 degrees have a small, sigmoid appendage that often is obscured by the folded perithecial apex.

Dimensions. Total length of thallus: 112.5–132.5 μm. Perithecium: 62.5–75 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 35–52.5 μm. Height of cell I: 20–25 μm. Cell Ia: 10 μm. Cells II, IIa and VI: 7.5–12.5 μm.

Thalli positioned on the right and left upper portion of the elytra and prothorax of male and female *L. maculosus* are pale amber-brown, with a slightly sigmoid perithecium. The exceptions are somewhat inflated cells II, IIa and VI, a pointed perithecial apex that has pronounced lip cells, beneath which is a distinct protrusion, and level with the top of the basal primary appendage cell is a short, rounded, tooth-like perithecial appendage.

Dimensions. Total length of thallus: 112.5–120 μm. Perithecium: 75–85 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 45–50 μm. Height of cell I: 17.5–20 μm. Cell Ia: 7.5–10 μm. Cells II, IIa and VI: 15–17.5 μm.

Commentary. All three morphotypes, *C. appendiculatus*, *C. distortus* and *C. dentifer*, are identical except for variation in the perithecial appendages and the folded perithecium seen only in *C. distortus*.

←

on male and female beetles). 4a, b. *C. appendiculatus*; 4a. *C. appendiculatus*, the typical form found only on male beetles; 4b. *C. appendiculatus* the alternate form (previously known as *C. dentifer*) found on male and female beetles. 5a, b. *C. rhyncostoma*; 5a. the alternate form (previously known as *C. spiniger*) found only on male beetles; 5b. *C. rhyncostoma* the typical form found on male and female beetles. 6a, b. *C. paradoxus*; 6a. the alternate form (previously known as *C. unciger*) found only on male beetles; 6b. *C. paradoxus*, the typical form found on male and female beetles.

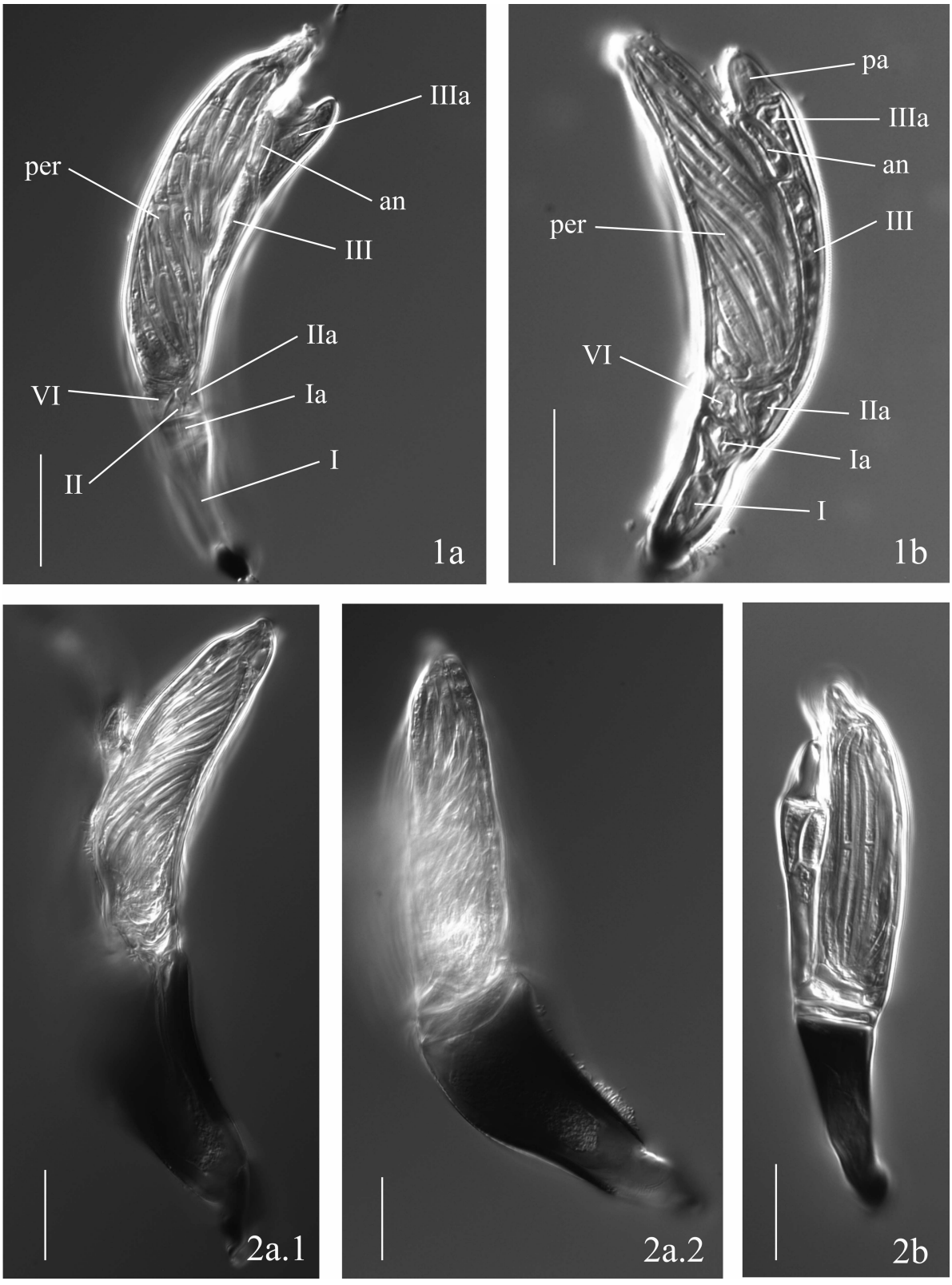

FIG. 6. 1a. *C. simplex* alternate form (I) basal cell of the receptacle, (Ia) subbasal of the receptacle, (II), (IIa), cells of the receptacle including (VI) the perithecial stalk cell, (per) is the perithecium. Receptacular cell III subtends the antheridim (an) and IIIa subtend the campanulate cell of the appendage). 1b. *C. simplex* typical form (the same as 1a, except that cell II is not clearly visible and the primary appendage (pa) is visible). 2a.1, 2a.2. *C. affinis* alternate form. 2b. *C. affinis* typical form. Bar = 25 μm.

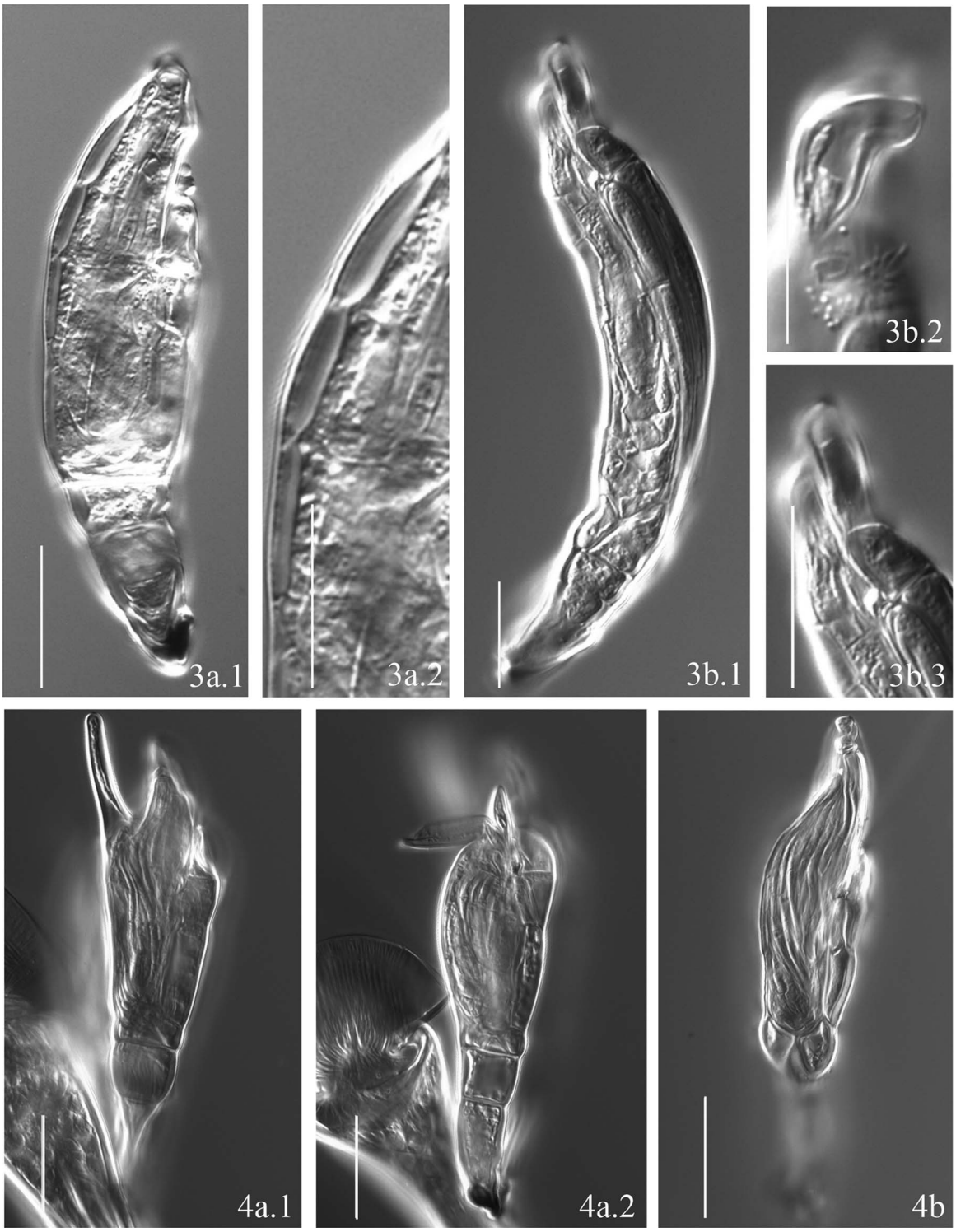

FIG. 7. 3a.1, 3a.2 *C. hyalinus*, typical form (3a.2 close up of outer wall cells along the edge of the perithecium). 3b.1–3b.3. *C.hyalinus*, alternate form (3b.2 tip of perithecium, 3b.3 protrusion at the tip of the primary appendage). 4a.1 *C. appendiculatus* typical form, attached to modified setae; 4a.2 alternate form (previously *C. distortus*) attached to the same setae; 4b alternate form (previously *C. dentifer*; tooth-like appendage not visible). Bar = 25 μm.

FIG. 8. 5a. *C. rhyncostoma* alternate form. 5b. *C. rhyncostoma* typical form. 6a. *C. paradoxus* alternate form. 6b. *C. paradoxus* typical form. Bar = 25 µm.

Chitonomyces rhyncostoma (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 290

≡ *Heimatomyces rhyncostoma* Thaxt. 1892. Proc. Am. Acad. Arts and Sci. 27, p. 33

= *Chitonomyces spiniger* (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 288 syn. nov.

≡ *Heimatomyces spiniger* Thaxt. 1895. Proc. Am. Acad. Arts and Sci. 30, p. 478 (FIG. 8)

Thalli positioned on the ridge above the epipluron of the right elytron of both male and female *L. maculosus* are orange-brown, with a straight perithecium that ends in a curved perithecial neck, and has a small hook-like perithecial appendage. Cell IIIa and the basal cell of the primary appendage are notably large, of similar length and extend above the perithecium. A ridge of cells extends down to the midpoint of the perithecium.

Dimensions. Total length of thallus: 100–122.5 μm. Perithecium: 62.5–75 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 72.5–77.5 μm. Height of cell I: 22.5–25 μm. Cell Ia: 5 μm. Cells II, IIa and VI: 7.5 μm.

Thalli positioned on the spine of the 4th tarsus on the right posterior leg of male *L. maculosus* are pale amber and stout. The distinguishing characters are two perithecial appendages, one at the base of the perithecium that is a blunt, thumb-like protuberance, and the second is an anvil-shaped appendage near the top of the perithecium, level with the tip of the antheridium, just below the perithecial apex. Cell IIIa protrudes slightly, reminiscent of *C. uncinatus*, that pushes the basal cell of the primary appendage toward the perithecial apex. The perithecial apex is curved orienting the mamillate lip cells horizontally.

Dimensions. Total length of thallus: 80–122.5 μm. Perithecium: 45–82.5 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 50–55 μm. Height of cell I: 17.5–22.5 μm. Cell Ia: 5–10 μm. Cells II, IIa and VI: 5–7.5 μm.

Commentary. *C. rhyncostoma* and *C. spiniger* are one of the more dissimilar pairs morphologically and also notably one of the most dissimilar phylogenetically, in addition to the two below.

Chitonomyces paradoxus (Peyr.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 287

≡ *Heimatomyces paradoxus* Peyritsch 1873. Sitzungsber. Kaiserl. Akad. Wiss., Math-Naturwiss. Cl., Abt. 1 [Wien] 64, p. 251

= *Chitonomyces unciger* (Thaxt.) Thaxt. 1896. Mem. Am. Acad. Arts and Sci. 12, p. 288 syn. nov.

≡ *Heimatomyces unciger* Thaxt. 1895. Proc. Am. Acad. Arts and Sci. 30, p. 478 (FIG. 8)

Thalli positioned on the edge of the left elytron of both male and female *L. maculosus* are large, deep orange-brown with a perithecial horn. At the midpoint of the perithecium, next to the antheridium, is a structure with two blunt protuberances, pointing in opposite directions. The perithecium is lumpy with a stout, rounded apex, and two lip cells that have the appearance of being opened. The perithecial horn is triangular, tapering distally to a rounded point.

Dimensions. Total length of thallus: 187.5–250 μm. Perithecium: 87.5–100 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 80–105 μm. Height of cell I: 40–47.5 μm. Cell Ia: 10–15 μm. Cells II, IIa and VI: 10–25 μm.

Thalli positioned on the claw of the left posterior leg of male *L. maculosus* are pale amber-brown, with a highly sigmoid receptacle and a slightly enlarged cell IIIa and primary appendage. The most distinct character is a large hook-like perithecial appendage that originates more or less at the tip of the antheridium and extends arm-like to the apex of the perithecium. The perithecium has a short curved neck resulting in a horizontal orientation of the perithecial lip cells.

Dimensions. Total length of thallus: 125–160 μm. Perithecium: 75–90 μm. Length from the bottom of cell III to the top of the basal cell of the primary appendage: 75–82.5 μm. Height of cell I: 37.5–42.5 μm. Cell Ia: 8.7–10 μm. Cells II, IIa and VI: 5–17.5 μm.

DISCUSSION

The majority of known Laboulbeniales species are capable of producing thalli on almost any body part and on either sex of their insect hosts. Ascospore transmission is achieved mainly by physical contact between hosts, with auto-infection and substrate-mediated infection being of minor importance (de Kesel 1993). The precise locations of some thalli on specific areas of the host body have been explained by the transmission of sticky ascospores between sexes during mating (Thaxter 1896, Benjamin and Shanor 1952, Scheloske 1976, Weir and Beakes 1996, Santamaria 2001, Rossi and Proaño Castro 2009). To date, however, this scenario has been used to explain only a few of the observed patterns of position and sex of host specificity found in Laboulbeniales, usually involving thalli showing similar morphological traits at different locations and on different sexes.

Species of *Chitonomyces* appear to display high levels of position and sex of host specificity, and in the past these patterns have been difficult to explain using the combination of position and morphological features of thalli. In this study we have brought aspects of ecology, behavior and molecular phylogenetics to bear on these problems.

Our results indicate that thalli of *Chitonomyces* are prevalent in natural populations of *Laccophilus maculosus* (51% collected beetles infected) and that males (63%) are more heavily infected than females (37%). Males also are susceptible to all 13 morphotypes (compared to the six morphotypes known on females), with peak infections occurring in the spring (compared to peak infections of females in the late

summer and fall). We think these trends are related to activity, with males showing territorial, aggressive or promiscuous behaviors, including males mounting other males, that facilitate increased infection (especially in the spring) and females showing depressed activity, no attempt at same-sex mountings, becoming infected through copulations in early summer and again in early fall.

Similar to accepted species delimitations within other genera of the Laboulbeniales, (FIG. 2), such as *Hesperomyces* and *Stigmatomyces* (Goldmann 2009), the six species of *Chitonomyces* on *L. maculosus* are resolved with a ≥ 1% difference (Katsuga 2002) between SSU rRNA sequences. In comparison, the ITS1 region produced homologies much lower than the typical 95% accepted for species delimitations. Of the nine morphotypes (*C. simplex*, *C. uncinatus*, *C. lichanophorus*, *C. affinis*, *C. appendiculatus*, *C. distortus*, *C. dentifer*, *C. hyalinus*, *C. marginatus*) for which partial sequences were generated, exceptionally low values of interspecific similarity of 79–88% were found while high values of 100% similarity were found for the same species. Notably the four missing morphotypes for ITS1 (*C. paradoxus*, *C. unciger*, *C. rhyncostoma*, *C. spiniger*) were the four most clearly delimited as two phylogenetic species (*C. paradoxus*, *C. rhyncostoma*) in the SSU analysis.

In combination the two regions, SSU and partial ITS1 and 5.8S rRNA, support speciation and substantiate, with modification, Thaxter's original description of the position specificity phenomenon. These data do not support recognition of all 13 morphological species originally described or the concept that all morphological variation indicates speciation. The pair of alternate morphotypes that comprise each species and that vary in conjunction with their position on the host also support the second hypothesis (Scheloske 1976, Rossi and Proaño Castro 2009) that there are two growth forms of the same species, possibly the result of different spore dispersal requirements at a specific location or substrate variation of the host integument. The mechanism for the latter premise is still undetermined. Phenotypic plasticity is thought to be particularly important to parasitic fungi that have the unique challenge of penetrating the host substrate and special dispersal requirements to maintain transmission from host to host (Nordbring-Hertz 2004). The six phylogenetic species and the positions they occupy, while still precise, are now expanded to include the corresponding locations on male and female beetles.

The likelihood that mating behavior could explain all positions in this system was called into question when two of the most commonly infected positions did not appear to align as expected (FIG. 4). The left lower abdominal segments of the male beetle that are infected with ''*C. uncinatus*'' did not align with the right lower region of the elytron of the female infected with *C. simplex*. The only possible explanation for this misalignment would be if the beetles did not copulate in a parallel orientation but rather at a diagonal. Our video footage confirmed this behavior and also explained two other ambiguously aligned positions. *Chitonomyces lichanophorus* is located only on the left subgenital plate of male beetles while its partner, *C. affinis*, is specific to the distal portion of the right elytron margin of both male and female beetles. If copulation was parallel how would fungi positioned on the left transfer spores to a position on the right? A diagonal orientation resolves this conflict by forming a contour of infection that aligns all positions. This asymmetrical shift also is evident in the elevated position of *C. rhyncostoma* on the right elytron and the lower location of *C. paradoxus* on the left elytron.

Species of *Laccophilus* in the family Dytiscidae are common, predaceous water beetles with worldwide distribution (Zimmerman 1960). Most North American Dytiscidae have a univoltine life history defined as: spring breeders that have summer larvae followed by pupation, adult emergence and adult overwintering (Nilsson 1986a). *Laccophilus maculosus* is unusual in that it is thought to be bivoltine, having two overlapping generations per year (Hilsenhoff 1992), thus enabling infected adults to provide inoculum for the next generation (Weir and Hammond 1997b). Dytiscidae also have been described as exhibiting antagonistic sexual behavior between males and females (Miller 2003, Miller and Bergsten 2007). It has been hypothesized (Miller and Bergsten 2007) that while the male is mounted on the female he controls when she is able to replenish her air supply, which she is continually fighting to retain. Evidence of this ''arms race between the sexes'' (Miller and Bergsten 2007) is found in structures on both male and female beetles that function to give one the advantage over the other. The male beetle has evolved modified setae with stalked suction-cups on the tarsomeres of the legs that stick to the elytra of the female with surprising force (Aiken and Khan 1992, Miller 2003). The female beetle in turn has evolved sculptured elytra with ridges and dimples to deflect the hairs suctioning capability. Therefore the patterns of specific movements during mating that have been described for *Dytiscus alaskansus*: mounting, lateral shake, swimming, copulation, female breathe and female swimming (Aiken 1992) each correlate to specific positions of the male body in relation to the female body, including the male re-orienting his position to let the female breathe. The behavior is

sustained at least 5 h or as long as 10. A single female may repeat this behavior several times in her lifetime (Aiken and Khan 1992).

A similar progression of movements was observed for *L. maculosus*. However mating in our system lasted only approximately 1 h and no plug or probing were recorded. In addition to the above observations, we also observed the anterior and median legs of the male to be loosely attached to the upper elytra of the female so that a tapping motion of these legs on the elytra and pronotum were visible, leaving the majority of the clasping to the posterior legs. If the female attempted to break free from the male hold, he compensated by assuming a diagonal position, and thereby likely increasing overall pressure. The key to this system is at this juncture when the female attempts escape or when the male transfers the spermatophore he counteracts the female's movement by tightening his hold in the diagonal position. It is this specific behavior that we think results in a type of pressure, exclusive to mating, that causes the release of spores from the perithecium and therefore is the only time at which spores are successfully dispersed at their specific locations.

Female beetles have no infection on their legs, lower abdomen or genital region. All six morphotypes found on females are located exclusively on the elytra. We have never observed same-sex mounting behavior in females, whereas we have several observations of the behavior for the male individuals, which explains why males are infected at all positions (Napela and Weir 2007) and why females are infected only dorsally. In addition, while females only have half the morphotypes present in this complex, each is one-half of a phylogenetic pair, meaning that the female, like the male, has all six phylogenetic species. Therefore no sex of host specificity is evident in this system, but there are six morphotypes found only on male beetles.

In summary, the number of species in this system and the maintenance of ''position specificity'' are driven by a tripartite system that confines spore dispersal and therefore limits gene flow, through the life-history of the fungus, sticky ascospores dependent on pressure for extrusion, the life-history of the host (aquatic, with overlapping generations) and host behavioral patterns, the longevity of sexual interaction and the recurring positions of the male and female body. Thus the host behavioral pattern providing the most significant and predictable host to host contact available for the fungus is sex. And therefore it is sex that the fungus exploits as its mode of spore dispersal. The above combination of interactions, allows for speciation, specificity of position and the evolution of an array of morphologically diverse dispersal structures, adapted to this unusual way of life.

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