Mycologia, 97(3), 2005, pp. 680–694.

Systematics of the genus *Sphaerobolus* based on molecular and morphological data, with the description of *Sphaerobolus ingoldii* sp. nov.

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Abstract: Despite mycologists' interest in its unique spore-dispersal mechanism, systematic studies of the genus *Sphaerobolus* have received little attention. In our previous work multiple gene genealogies indicated the existence of three divergent lineages in the genus *Sphaerobolus,* each representing a phylogenetic species. Macro- and micromorphological analyses of colony and fruit-body characters presented here confirmed that these three phylogenetic species correspond to two known species, *S. iowensis* and *S. stellatus,* and a newly discovered species. In addition, an expanded gene genealogical analysis is presented for the three species. The new species, named *Sphaerobolus ingoldii* Geml, Davis et Geiser, is described based on both molecular and morphological data. In addition, while *S. iowensis* previously had been reported in only two localities, we found that it is as common as or more common than *S. stellatus* in North America. Despite the considerable amount of DNA polymorhism found in all species, nested clade analyses of *S. iowensis* and *S. stellatus* indicated little phylogeographic structure in either species, perhaps due to heavy movement mediated by human activities.

Key words: phylogenetics, *Sphaerobolus ingoldii,* systematics

Accepted for publication 15 Mar 2005.
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INTRODUCTION

Sphaerobolus Tode first was documented nearly 300 years ago under the name *Carpobolus* (Micheli 1729). Since then many mycologists have been fascinated by this unique fungus and several researchers have studied its growth and reproduction (Walker and Anderson 1925, Walker 1927, Buller 1933, Alasoadura 1963, Ingold and Peach 1970, Ingold 1971, 1972, Dykstra 1982, Fletcher and Cooke 1984). Its common name ''artillery fungus'' comes from its capability of ejecting a 1 mm diam gleba up to 6 m toward the brightest light in its environment (Walker 1927, Buller 1933). Each basidiocarp contains a single gleba consisting of spores embedded in an adhesive mass of decomposing hyphae. In recent years the artillery fungus has become a source of distress to homeowners, landscape mulch producers and insurance companies due to the strong adhesion of the discharged glebae to artificial surfaces including house siding, cars and windows (Lehman 1985, Brantley et al 2001a, b).

Because it produces its basidiospores in an enclosed structure, the genus *Sphaerobolus* has been classified as a member of the class Gasteromycetes along with fungi that undergo passive spore discharge, including bird's nest fungi (*Cyathus, Crucibulum* etc.), puffballs (*Lycoperdon, Langermannia* etc.), earth balls (*Scleroderma* etc.), stinkhorns (*Phallus, Mutinus, Pseudocolus* etc.), and earth stars (*Geastrum* etc.). Within this form class authors have placed the artillery fungus in family Sphaerobolaceae in these orders: Sphaerobolales (Ainsworth 1971); Nidulariales (Ulloa and Hanlin 2000); Sclerodermatales (Hawksworth et al 1996). However, molecular phylogenetic analyses of Hibbett et al (1997) revealed that the Gasteromycetes represents a polyphyletic, artificial taxon that does not represent a true evolutionary group within the Basidiomycota. Based on molecular data, the genus *Sphaerobolus* is best placed in the gomphoid-phalloid clade with these genera as closest relatives: *Geastrum, Phallus, Pseudocolus, Ramaria, Clavariadelphus, Gomphus, Gautieria* etc. (Hibbett et al 1997, Moncalvo et al 2002). These results are incorporated in the classification of Kirk et al (2001), who placed *Sphaerobolus* in the family Geastraceae, order Phallales, class Basidiomycetes, phylum Basidiomycota.

The artillery fungus produces spherical, whitish or orange basidiocarps (1–2 mm diam), in which a single spore mass (gleba or peridiole) is formed. The gleba contains numerous spores of two types, uninucleate, thick-walled basidiospores and thin-walled, elongated gemmae (asexual spores) with two or more nuclei (Walker 1927, Ingold 1972). These two types presumably play different adaptive roles in the dual lignicolous and coprophilous survival mechanism of this fungus. Basidiospores are stimulated to germinate when exposed to proteolytic enzymes (e.g. pepsin) and relatively high temperatures as expected in the digestive system of mammal herbivores after ingestion of a discharged gleba attached to plant material (Dykstra 1982). If no herbivore ingests the glebal mass, the gemmae can germinate directly on wood or other plant debris. Glebae have been reported to germinate after at least 11 y of storage (Walker 1927) and thus might serve as long-term survival structures. After the germination of gemmae, colonization of the substrate begins. The characteristic bleaching associated with this white rotting fungus can be attributed to the digestion of cellulose, hemicellulose and lignin in the substrate. After colonizing the substrate for some time the fungus produces phototropic fruiting bodies. At maturity these fruiting bodies orient themselves toward the brightest light source and shoot their glebae into air (Alasoadura 1963, Nawaz 1967). Similar active/passive mechanisms for dispersing spore masses can be observed in other dung-inhabiting fungi as well, including Pezizomycetes (e.g. *Ascobolus*) and Sordariomycetes (e.g. *Podospora*) of the Ascomycota and Mucorales of the Zygomycota (e.g. *Pilobolus*). This feature is likely an adaptation to the coprophilous habit and evolved many times independently during evolution.

The Greek origin of the name *Sphaerobolus* (''sphere thrower'') is related to the ability of the fungus to shoot its glebae a considerable distance. The genus contains two currently recognized species, *S. stellatus* (Tode) Pers. and *S. iowensis* Walker (Hawksworth et al 1996), that are distinguished by a number of micromophological characteristics. In the developing glebae of young *S. stellatus* basidiocarps, some of the binucleate hyphae at the center of the globular knots have enlarged, clavate basidial primordia (Walker 1927). New basidia proliferate in clusters, each eventually producing four (occasionally eight) basidiospores. The basidia decompose and disappear as the spores mature, making room for the enlargement and maturation of other basidia. In *S. iowensis* the development of primary basidia is followed by the formation of characteristic cavities or chambers in which basidiospores are produced (Walker 1927). In both species sterile hyphae undergo disintegration in maturing basidiocarps and break down by the time the glebal mass is discharged. Almost all studies in the genus have concentrated on isolates identified as *S. stellatus*. However, several other names of uncertain origin appear in culture collections, including *S. bombardioides, S. carpobolus, S. corii, S. crustacus, S. epigaeus, S. minimus, S. minutissimus, S. rubidus, S. sparsus* and *S. tubulosus*. The origin of these binomials cannot be found in the literature and thus cannot be considered scientifically valid. *Sphaerobolus* is a cosmopolitan genus reported from Alaska, British Columbia, most parts of the continental U.S. (it is particularly abundant in the Northeast), Europe (from Greece to Iceland), Asia (including Japan), Australia, New Zealand, Africa and Latin America (Aplin 1961, Dring 1964, Ingold 1972, Herrera and Perezsilva 1987, McKenzie and Foggo 1989, Halgrimsson et al 1992, Zervakis et al 1998, McGray pers comm 2002). Whether this is its natural range or whether it has been dispersed artificially is unknown.

In our studies (Geml et al 2005) we generated nucleotide sequences of three ribosomal DNA regions and one protein coding gene region (partial mitochondrial ribosomal RNA small subunit [mtSSU], the internal transcribed spacer regions of the nuclear ribosomal gene repeat [ITS], partial nuclear large ribosomal RNA subunit [LSU] and partial translation elongation factor 1-α gene [EF 1-α]). Multiple gene genealogies inferred from maximum likelihood, Bayesian and maximum parsimony analyses of sequence data from individual loci (mtSSU, ITS, EF 1-α, and LSU) and a combined dataset (mtSSU, ITS, EF 1-α) concordantly indicated the existence of three divergent lineages in the genus *Sphaerobolus,* each representing a phylogenetic species. In this paper we have investigated variation in morphological characteristics corresponding to the phylogenetic species revealed, associating them with new and known taxa. We have included additional isolates of the newly discovered species for phylogenetic analyses and described it based on both molecular and morphological data. We also have sampled various artillery fungus communities and identified them based on ITS sequences to reveal more information on the geographic distribution of the three species in the U.S. In addition we analyzed the nucleotide variation in ITS sequence in *S. iowensis* and *S. stellatus* to investigate their biogeography using nested clade analyses.

MATERIALS AND METHODS

Isolates and culture preparation.—Isolates of the artillery fungus either came from culture collections or were isolated from glebae collected in the field or received from

homeowners (TABLE I). To isolate pure cultures glebae were agitated in 20% bleach solution for 3 min, washed with distilled water, air dried, sprayed with 70% ethanol and air dried again on filter paper under aseptic conditions (Davis pers comm 2001). The surface-sterilized glebae were placed on 3% Difco® oatmeal agar (OA) (Becton Dickinson Microbiology Systems, Sparks, Maryland) to initiate cultures.

DNA extraction, PCR amplification and DNA sequencing.—Nucleic acids were extracted from collected isolates using DNeasy® Plant Mini Kits (QIAGEN Inc., Valencia, California) according to the manufacturer's protocol. Portions of the mtSSU, EF 1-α, and the entire ITS region were PCR amplified in reaction mixtures containing 37 μL PCR water, 5 μL 10× PCR buffer (0.5M KCl, 0.1M Tris HCl pH 8.3, 0.025M $MgCl_2$), 5 μL 10× dNTPs (2mM of each dNTP), 0.1 μL AmpliTaq® DNA polymerase (Perkin-Elmer, Foster City, California), 1 μL of 10 μM forward primer and reverse primer for the region of interest, and 1 μL template DNA (100-fold dilution of original DNA solution extracted). PCR reactions were performed in a 96-well thermocycler (PTC-100 Programmable Thermal Controller, MJ Research Inc.) using this temperature program for all ribosomal DNA regions: 94 C/5 min; 34 cycles of 94 C/1 min, 53 C/1 min, 72 C/1 min; and 72 C/5 min. For the single copy gene EF 1-α a ''touchdown'' PCR setting was used with an annealing temperature of 65 C in the first cycle, then successively reduced by 1 C per cycle to 56 C, after which the annealing temperature was maintained at 56 C for the remaining 30–36 cycles (Stephen Rehner pers comm). These primers were used for amplification: ITS5 and ITS4 for ITS (White et al 1990), MS1 and MS2 for mtSSU (White et al 1990), and EF1-983F (GCY CCY GGH CAY CGT GAY TTY AT) and EF1-1567R (ACH GTR CCR ATA CCA CCR ATCTT) for EF 1-α (Stephen Rehner pers comm). Amplification products were electrophoresed in 3.0% agarose gels and stained with ethidium bromide for visualization of the bands. PCR products were purified directly with the QIAquick® PCR Purification Kit (QIAGEN Inc., Valencia, California) and were sequenced with the Applied Biosystems (ABI) BigDye® v. 3.0 terminator kit and an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, California). Each sample was sequenced with the same primers used for PCR with the exception of the 1567R primer of the EF 1-α gene that was replaced by primer 1567Ra (ACH GTR CCR ATA CCA CC) in sequencing for better results (Stephen Rehner pers comm).

Phylogenetic analyses.—Sequence data obtained for both strands of each locus were edited and assembled for each isolate with Sequencher 3.1 (Gene Codes, Ann Arbor, Michigan). Sequence alignments were made by Clustal X (Thompson et al 1997). Analyses were conducted with maximum likelihood (ML) and maximum parsimony (MP) methods in PAUP* 4b10 (Swofford 2002) and Bayesian analysis in MrBayes 3.0 (Huelsenbeck and Ronquist 2001). Because methods follow different theories and algorithms, congruent features found in all three types of analyses were considered meaningful. At first we excluded ambiguously aligned regions, consisting of these positions: 47–63, 205–255, 296–308 in the mtSSU; 79–84, 160–221, 487–499, 623–666, 699–703 in the ITS; and 203–260, 408–460, 558–562 in the EF 1-α alignments. Alignments were deposited in the TreeBase database (http://www.treebase.org) under accession numbers SN2111-7316, SN2111-7317 and SN2111-7320, and sequences were deposited in GenBank (TABLES I and II). Different evolutionary models with varying values of base frequencies, substitution types, α-parameter of the γ-distribution of variable sites and proportion of invariable sites, among other parameters, were compared via the likelihood ratio test for each locus with PAUP* and Modeltest 3.06 (Posada and Crandall 1998) to determine the best-fit evolutionary model for both ML and Bayesian analyses. ML analyses were carried out with the heuristic search option using the tree bisection and reconnection (TBR) algorithm with 100 random sequence additions to find the overall optimum instead of local optima. To determine the statistical reliability of the trees and the stability of clades, the bootstrap test (Felsenstein 1985) was used with full heuristic search and 100 replicates. In Bayesian phylogenetic analyses, 100 000 generations were run in four chains. The chains were sampled every 100th generation. When the likelihood scores of trees sampled approached similar values, they were considered to have converged. In each run only trees after this convergence point were included in computing the consensus tree. In the MP analyses previously excluded ambiguous regions were included after being recoded with the program INAASE 2.3b (Lutzoni et al 2000). The code and step matrices were attached to the appropriate alignments. Gaps were treated as ''new state''. The bootstrap test was used with 500 replicates.

Morphological examinations.—We used revealed phylogenetic relationships to observe variation in morphological and/or biological characteristics corresponding to the separate lineages. Two isolates were selected from each phylogenetic species determined by the phylogenetic analyses for producing basidiocarps. Isolates were grown on OA and wheat straw agar (WSA) that were inoculated with a colonized piece of agar. WSA was included in the fruiting experiments because our preliminary studies showed that, while *S. stellatus* and *S. ingoldii* readily produces basidiocarp on OA, all our *S. iowensis* isolates consistently failed to fruit on this media. Wheat straw was adopted as a substrate based on the findings of Flegler (1984), prepared by placing approximately 20 g of dry, chopped, previously sterilized straw in 100 × 15 mm Petri plate and pouring 1.2% Difco® water agar on top of the straw. The cultures were incubated at 25 C with an initial dark period of 3 wk followed by 6 wk of intensive fluorescent lighting and lower temperature (16–18 C) to provide optimal fruiting conditions, based on Flegler (1984) and Ingold (1972) with slight modifications. High (95–100%) relative humidity (RH) was maintained during the period. We used three replicates per treatment that were arranged according to a randomized design. For morphological analyses, randomly chosen immature basidiocarps (before opening and expelling the glebae) were sampled from fruiting isolates of each species. Thin (10–20 μm) sections were sliced from the selected fruiting bodies with a cryotome (Model CTD International Harris Cryostat, International Equipment Co.) with TBS Tissue Freezing

TABLE I. *Sphaerobolus* isolates included in the phylogenetic analyses

Species	Isolate code	Geographic origin	GenBank accession number			
			mtSSU	ITS	EF 1-α	LSU
Sphaerobolus ingoldii Geml, Davis et Geiser sp. nov.	T-800	Kellogg Biological Station Long Term Ecological Research, Michigan	AY654739	AY654737	AY654734	AF139975*
	IFO 9597	Japan	AY488022	AY487971	AY487996	AY439013
	SS19	Atlanta, Georgia	AY488015	AY487965	AY487990	AY439012
	SS42	Hershey, Pennsylvania	AY654740	AY654738	AY654735	—
	SS93	Sandusky, Ohio	—	AY654736	—	—
Sphaerobolus iowensis Walker	ATCC 52850	East Lansing, Michigan	AY488008	AY487958	AY487984	AY439014
	SS1	Indiana	AY488000	AY487950	AY487976	—
	SS2	Elizabethtown, Pennsylvania	AY488001	AY487951	AY487977	—
	SS4	Langhorne, Pennsylvania	AY488003	AY487953	AY487979	—
	SS5	State College, Pennsylvania	AY488004	AY487954	AY487980	—
	SS9	Chapel Hill, North Carolina	AY488006	AY487956	AY487982	AY439010
	SS16	Olney, Maryland	AY488012	AY487962	AY487988	—
	SS17	Olney, Maryland	AY488013	AY487963	—	—
	SS18	Olney, Maryland	AY488014	AY487964	AY487989	—
	SS20	Olney, Maryland	AY488016	AY487966	AY487991	—
	SS21	Galion, Ohio	AY488017	AY487967	AY487992	—
	SS22	Ithaca, New York	AY488018	AY487968	AY487993	—
	SS23	Medina, Ohio	AY488019	AY487969	AY487994	—
Sphaerobolus stellatus (Tode) Pers.	ATCC 18339	Maryland	AY488007	AY487957	AY487983	AY439011
	CBS 321.32	The Netherlands	AY488026	AY487975	AY487999	—
	DSH 96-015	Great Brook State Park, Massachusetts	AY488009	AY487959	AY487985	—
	MIN 864513	Elm Creek Nature Reserve, Minnesota	AY488025	AY487974	AY487998	—
	SS3	State College, Pennsylvania	AY488002	AY487952	AY487978	—
	SS7	West Mifflin, Pennsylvania	AY488005	AY487955	AY487981	—
	SS13	Erie, Pennsylvania	AY488010	AY487960	AY487986	—
	SS14	Lucinda, Pennsylvania	AY488011	AY487961	AY487987	—
	SS24	Russell, Pennsylvania	AY488020	—	—	—
	SS25	Newton Centre, Massachusetts	AY488021	AY487970	AY487995	—
	SS27	Anchorage, Alaska	AY488023	AY487972	—	—
	SS28	Anchorage, Alaska	AY488024	AY487973	AY487997	—

* Submitted to Genbank by R.G. Thorn on 1 Apr 1999 under the name *Sphaerobolus stellatus*.

TABLE II. Geographic distribution, frequency, and GenBank accession numbers of ITS haplotype sequences

Species	Haplotype	Isolate code	Geographic origin	GenBank accession number
Sphaerobolus iowensis Walker	0	ATCC 52850	East Lansing, Michigan	AY487958
		SS1	Indiana	
		SS2	Elizabethtown, Pennsylvania	
		SS5	State College, Pennsylvania	
		SS22	Ithaca, New York	
		SS37	Elderton, Pennsylvania	
		SS75	State College, Pennsylvania	
		SS100	Blythewood, South Carolina	
		SS113	Berkeley Springs, West Virginia	
	1	SS4	Langhorne, Pennsylvania	AY487953
	2	SS9	Chapel Hill, North Carolina	AY487956
	3	SS16	Olney, Maryland	AY487962
		SS17	Olney, Maryland	
		SS18	Olney, Maryland	
	4	SS20	Olney, Maryland	AY650228
	5	SS21	Galion, Ohio	AY487967
	6	SS23	Medina, Ohio	AY487969
	7	SS110	Durham, North Carolina	AY650229
	8	SS11	Durham, North Carolina	AY650230
	9	SS116	Absecon, New Jersey	AY650231
	10	SS34	Centralia, WA	AY650232
	11	SS35	Branchville, NJ	AY650233
	12	SS38-2	Blairsville, Pennsylvania	AY650234
	13	SS39-1	Elderton, Pennsylvania	AY650235
	14	SS43	Ridgway, Pennsylvania	AY650236
	15	SS47-1	Hayward, Wisconsin	AY650237
	16	SS48	Ebensburg, Pennsylvania	AY650238
	17	SS49	Kresgeville, Pennsylvania	AY650239
	18	SS51	Indiana, Pennsylvania	AY650240
	19	SS56	Indiana, Pennsylvania	AY650241
	20	SS57	State College, Pennsylvania	AY650242
	21	SS65-2	Clyde, Pennsylvania	AY650243
	22	SS69	Indiana, Pennsylvania	AY650244
	23	SS73	Newport, Pennsylvania	AY650245
	24	SS80-1	Hancock co., West Virginia	AY650246
	25	SS82	Imperial, Pennsylvania	AY650247
	26	SS83-1	Steubenville, Ohio	AY650248
	27	SS83-3	Steubenville, Ohio	AY650249
	28	SS84	Moon, Pennsylvania	AY650250
	29	SS86-2	Belmont, Ohio	AY650251
	30	SS89-1	Plum Creek, Pennsylvania	AY650252
		SS94	Cleveland, Ohio	
	31	SS89	Plum Creek, Pennsylvania	AY650253
	32	SS90	Altoona, Pennsylvania	AY650254
	33	SS96	Morris, New Jersey	AY650255
	34	SS99	Lambsburg, Virginia	AY650256
Sphaerobolus stellatus (Tode) Pers.	0	ATCC 18339	Maryland	AY487957
		CBS 321.32	The Netherlands	
		MIN 864513	Elm Creek Nature Reserve, Minnesota	
		SS3	State College, Pennsylvania	
		SS25	Newton Centre, Massachusetts	
		SS28	Anchorage, Alaska	
		SS41	White River National Forest, Colorado	
		SS72	Shrewsbury, Pennsylvania	
		SS95	Poplar Bluff, Missouri	
		SS115	Harrisburg, Pennsylvania	

TABLE II. Continued

Species	Haplotype	Isolate code	Geographic origin	GenBank accession number
	1	DSH 96-015	Great Brook State Park, Massachusetts	AY487959
		SS13	Erie, Pennsylvania	
		SS14	Lucinda, Pennsylvania	
		SS92	Kennett Square, Pennsylvania	
	2	SS88	Martinsburg, Pennsylvania	AY650257
	3	SS62	Holidaysburg, Pennsylvania	AY650258
		SS66	Cresson, Pennsylvania	
	4	SS46	Mifflintown, Pennsylvania	AY650260
		SS63	Duncansville, Pennsylvania	
		SS65	Clyde, Pennsylvania	
	5	SS59	Corvallis, Oregon	AY650259
	6	SS7	West Mifflin, Pennsylvania	AY487955
	7	SS112-1	Winston-Salem, North Carolina	AY650261
	8	SS112-2	Winston-Salem, North Carolina	AY650262
	9	SS112-3	Winston-Salem, North Carolina	AY650263
	10	SS107	Durham, North Carolina	AY650264
	11	SS27	Anchorage, Alaska	AY487972

Medium® (Triangle Biomedical Sciences, Durham, North Carolina). Microscope slides were prepared with lactic acid and examined with a Nikon® compound microscope to detect micromorphological differences, including those described by Walker (1927) as distinctive characteristics between *S. stellatus* and *S. iowensis*. Growth-rate experiments were conducted with the same set of isolates used for the fruiting experiments. The cultures were grown on OA, WSA and Difco® potato-dextrose agar (PDA) at 25 C and 100% RH. Growth rate was recorded weekly by measuring two diameters of the colony at right angles to each other. We similarly measured the diameter of the expelled glebae for each fruiting isolate. Wet mounts were made from randomly selected gleba, and the sizes of the basidiospores (length and width) were recorded. The data were analyzed by one-way analysis of variance (ANOVA) with Minitab 13 (Minitab Inc.). Where the null-hypothesis was rejected that all means were the same, the Fisher's *LSD* (least significant difference) multiple range test (Ott 1993) was used to detect significant differences by testing each mean to each mean.

ITS-based identification and biogeography of species.—Eighty isolates were sampled in various parts of the U.S. or received from collections. The isolation and growth of pure cultures, DNA extraction, PCR amplification and sequencing of the ITS region were conducted in the same manner described above. Geographic distribution, frequency and GenBank accession numbers for ITS haplotype sequences are provided (TABLE II). Species identities of sampled isolates were made based on sequence similarity to various phylogenetic species determined by our earlier work. For this purpose we added newly generated sequences to an existing alignment (Geml et al 2005) and analyzed them with MP. Species identifications were inferred based on the phylogenetic placement of the sequences within one of the three species clades. Biogeographic patterns linked to the different phylogenetic species were investigated with nested clade analysis (NCA) (Templeton 1998). Maximum parsimony haplotype networks were generated by TCS v.1.13 (Clement et al 2000) and were used to define a series of nested clades that in turn were used to perform random, two-way contingency permutation analysis to detect any association between geographic distribution and genetic variation (Templeton 1998). The nested clade information, sample size for each haplotype and geographic location of each clade (latitude and longitude coordinates) were entered into the software package GeoDis v.2.0 (Posada et al 2000). GeoDis was used to calculate clade distance (D_c) and nested clade distance (D_n) and to test them for significance at the 5% level with a permutation technique with 1000 resampling replicates (Posada et al 2000). D_c was calculated as the average distance of all individuals in clade X from the geographic center of that clade, while D_n was the average distance of individuals in clade X from the geographic center of clades of the next highest nesting level. Where significant D_c and/or D_n values were detected, a set of criteria was used to discriminate between the effects of contemporary (e.g. gene flow) and historical (e.g. allopatric fragmentation, range expansion) processes (Posada et al 2000, Templeton 1998). Because only five *S. ingoldii* isolates were collected, this species was excluded from the nested clade analyses.

RESULTS

Phylogenetic analyses.—The intrageneric mtSSU, ITS, EF 1-α and the combined datasets respectively consisted of 583, 769, 628, and 1980 characters, including gaps. Of these 63, 78, 110 and 251 ambiguous positions were excluded in the intrageneric analyses. Of interest, we found that regions that could not be aligned unambiguously between the three different phylogenetic species were highly similar and some-

times identical within lineages, indicating the existence of fixed differences. Three types of long (>1 kb), independent insertions, each with a unique insertion site, length and nucleotide sequence, were detected in the mtSSU region of some of the isolates (Geml et al 2005). These insertions were excluded from the alignment. The Hasegawa-Kishino-Yano model (Hasegawa et al 1985) with no proportion of invariable sites (I = 0) and equal variation rates for all sites (HKY) was selected by hierarchical likelihood ratio tests as the best-fit evolutionary model for the mtSSU and ITS datasets. The base frequencies for the mtSSU and ITS alignments respectively were freqA = 0.3304, freqC = 0.1610, freqG = 0.2410, freqT = 0.2676, and freqA = 0.2617, freqC = 0.2256, freqG = 0.2012, freqT = 0.3115. The transition/transversion ratios were ti/tv = 1.4824 and ti/tv = 1.8035. The Tamura-Nei model (Tamura and Nei 1993) with no proportion of invariable sites (I = 0) and estimated α-parameter of γ-distribution (TN + G) was selected as the best-fit evolutionary model for the EF 1-α dataset. The base frequencies were freqA = 0.2349, freqC = 0.2869, freqG = 0.2492, freqT = 0.2290. The substitution rate matrix was: R(a) [A-C] = 1.0000, R(b) [A-G] = 2.7043, R(c) [A-T] = 1.0000, R(d) [C-G] = 1.0000, R(e) [C-T] = 11.3650, R(f) [G-T] = 1.0000. The among-site rate variation was characterized by variable sites following a γ-distribution with an estimated α-parameter = 0.3767. Because the HKY model was determined earlier as best-fit model for two of the three loci, that model was chosen for ML and Bayesian analyses of the combined dataset as well.

Likelihood values converged after about 16 000, 20 000, 19 000 and 16 000 generations in Bayesian analysis of the mtSSU, ITS, EF 1-α and combined datasets, respectively. The consensus trees were computed from 840, 800, 810 and 840 trees, after discarding the first 161, 201, 191 and 161 trees as burn-in. The ML phylograms and the Bayesian consensus trees generated with the models described earlier had similar topologies at each locus. After including the character matrices of the ambiguous regions recoded by INAASE, the final alignments of mtSSU, ITS, and EF 1-α for MP analyses consisted respectively of 522, 697, 520 and 1739 characters. One of the 26 most parsimonious trees of the combined dataset is shown with bootstrap values (FIG. 1).

In all analyses *Sphaerobolus* isolates formed three deeply divergent, highly supported clades in agreement with previous findings (Geml et al 2005). One of the clades corresponded to *S. stellatus,* as indicated by the presence of two isolates previously identified as *S. stellatus* from different culture collections: ATCC 18339, CBS 321.32. Isolates in the second

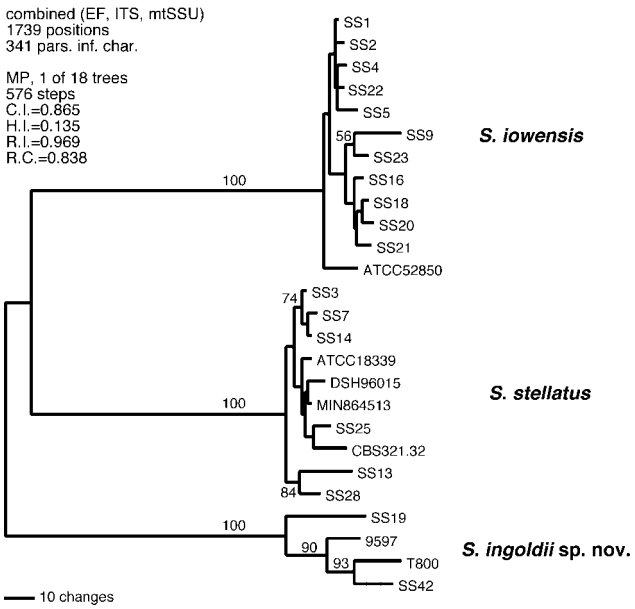


FIG. 1. Phylogram of *Sphaerobolus* species based on maximum parsimony analyses of the combined (mtSSU, ITS, EF 1-α) dataset. The tree is midpoint rooted. Bootstrap values are shown above the branches.

clade were considered *S. iowensis,* because this group included a specimen previously identified as *S. iowensis* (ATCC 52850). The third clade has no former taxonomic connection and represents a newly discovered taxon, described in this paper as *Sphaerobolus ingoldii* Geml, Davis et Geiser sp. nov. The species-level clades received 100% support in all analyses, and numerous subgroups receiving varying levels of support were found in *S. iowensis* and in *S. stellatus*.

Morphological examinations.—Morphological and growth characteristics of the three phylogenetic species of *Sphaerobolus* are summarized (TABLE III). Measurements were taken from 25 glebae per isolate (50 glebae per species). The only exception was *S. iowensis,* for which only a single fruiting culture (ATCC 52850) was obtained, producing only eight basidiocarps. All glebae recorded were produced on WSA. Although *S. ingoldii* and *S. stellatus* fruited abundantly on OA as well, *S. iowensis* failed to produce basidiocarps on OA, despite repeated efforts. Therefore this medium, as well as PDA that did not support any fruiting, was excluded from the gleba and basidiospore morphology evaluation. We found that significantly smaller ($P < 0.001$) glebae were produced by *S. ingoldii* (0.977 ± 0.198 mm) than produced by the other two species (*S. iowensis*: 1.549 ± 0.156, *S. stellatus*: 1.495 ± 0.289). Microscopic examinations revealed the existence of basidial chambers, a distinct character used in the species description by Walker (1927), in the premature glebae of *S. iowensis*. Nei-

TABLE III. Morphological characteristics of *Sphaerobolus* species. Mean value ± standard deviation is given for each character. Italicized small letters indicate significant differences ($P < 0.05$) among the species

Character	Sample size	*Sphaerobolus ingoldii* Geml, Davis et Geiser sp. nov.	*Sphaerobolus iowensis* Walker	*Sphaerobolus stellatus* (Tode) Pers.	*P*-value (ANOVA)
Basidial chambers		absent	present	absent	
Gemmae		absent	present	present	
Gleba diameter (mm)	N = 50*	0.977 ± 0.198 *a*	1.549 ± 0.156 *b*	1.495 ± 0.289 *b*	<0.001
Basidiospore length (μm)	N = 50	8.780 ± 0.761 *a*	7.215 ± 0.510 *b*	7.266 ± 0.498 *b*	<0.001
Basidiospore width (μm)	N = 50	5.874 ± 0.583 *a*	4.917 ± 0.217 *b*	4.571 ± 0.288 *b*	<0.001
Growth rate on OA (mm/day)	N = 12	2.095 ± 0.036 *a*	1.310 ± 0.241 *b*	1.381 ± 0.356 *b*	<0.001
Growth rate on WSA (mm/day)	N = 12	2.089 ± 0.029 *a*	1.221 ± 0.081 *b*	1.213 ± 0.288 *b*	<0.001
Growth rate on PDA (mm/day)	N = 12	1.345 ± 0.425 *a*	1.025 ± 0.131 *a*	1.101 ± 0.484 *a*	0.113

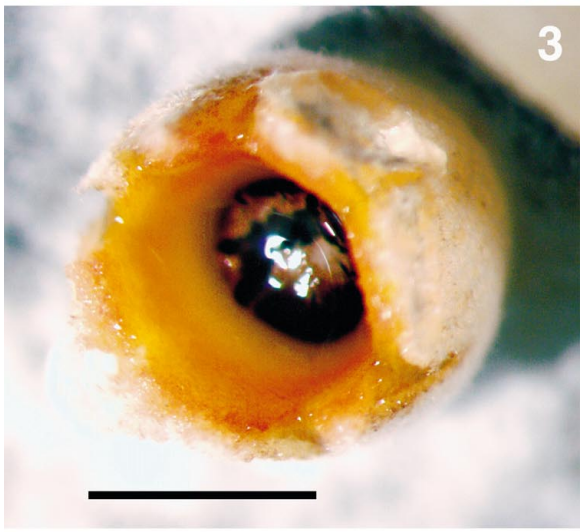
* Except in *S. iowensis,* where N = 8.

ther of the other species exhibited this trait. We found significant differences in the length and width of basidiospores produced (both $P < 0.001$). The largest spores were produced by *S. ingoldii* (8.780 ± 0.761 × 5.874 ± 0.583 μm), followed by *S. iowensis* (7.215 ± 0.510 × 4.917 ± 0.217 μm) and *S. stellatus* (7.266 ± 0.498 × 4.571 ± 0.288 μm) with nonsignificant difference between the latter two. Growth rates of the three species on OA and WSA were significantly different (both $P < 0.001$), while we failed to reject the null hypothesis (i.e. all rates are equal) when analyzing growth on PDA ($P = 0.113$). *Sphaerobolus ingoldii* showed the fastest growth on both OA and WSA, with daily linear growth of 2.095 ± 0.036 mm and 2.089 ± 0.029 mm, respectively. The growth rate of *S. iowensis* was 1.310 ± 0.241 mm (OA) and 1.221 ± 0.081 mm (WSA), while *S. stellatus* grew at a daily rate of 1.381 ± 0.356 mm (OA) and 1.213 ± 0.288 mm (WSA). Growth rates on PDA for *S. ingoldii, S. iowensis* and *S. stellatus* were respectively 1.345 ± 0.425 mm, 1.025 ± 0.131 mm and 1.101 ± 0.484 mm, (TABLE III). Morphological characteristics of *S. ingoldii, S. iowensis* and *S. stellatus* are shown (FIGS. 2–5, 6–9 and 10–12, respectively).

ITS-based identification and biogeography of species.— Of the 80 isolates included in the analyses, 48 were identified as *S. iowensis,* 27 as *S. stellatus* and five as *S. ingoldii* according to the ITS-based identification method. The geographic distribution of the samples is provided (FIG. 13). In *S. iowensis, S. stellatus,* and *S. ingoldii,* 35, 12, and five ITS sequence haplotypes were observed, respectively. The nested haplotype network of *S. iowensis* included 35 zero-step clades (haplotypes), 28 one-step clades, 15 two-step clades, six three-step clades, three four-step clades, and the entire network (FIG. 14A). The missing intermediate haplotypes were retained during the nesting procedure for consistency in nesting (Crandall 1996). Haplotype "0" was by far the most numerous and was found in nine individual samples from diverse locations and was considered the ancestral haplotype in the sample. Clade 4-1, showing the widest continuous distribution, contained the inferred ancestral haplotype. The null hypothesis of no association between genotype and geographic origin could not be rejected ($P > 0.05$) in any clade, except in the "tip" Clade 4-3, where significantly small D_c and D_n values were observed ($P = 0.007$ and $P = 0.006$, respectively). Based on the inference key (steps: 1. Yes; 2. Yes; 3. No; 4. No) of Templeton (1998), the significant statistical association between haplotype and geography was due to restricted gene flow with isolation by distance. The nested cladogram of *S. stellatus* included 12 zero-step clades (haplotypes), nine one-step clades, four two-step clades and the entire cladogram (FIG. 14B). The missing intermediate haplotypes were treated in the same manner detailed above. Similarly, haplotype "0" was the most numerous found in 10 individual samples and considered the ancestral haplotype in the sample. Clade 2-1, showing the widest overall geographic distribution, contained the ancestral haplotype. As in *S. iowensis,* the null hypothesis of no association between genotype and geographic origin could not be rejected ($P > 0.05$) in any of the clades, except in "tip" Clade 1-2 (tip), where significantly small D_c and D_n values were observed ($P = 0.032$ and $P = 0.014$, respectively). Based on Templeton's inference key (steps: 1. Yes; 2. Yes; 3. No; 4. No) the significant statistical association between haplotype and geography was due to restricted gene flow with isolation by distance, similar to that observed in *S. iowensis.*

TAXONOMY

Sphaerobolus ingoldii J. Geml, D.D. Davis et D.M. Geiser, sp. nov.



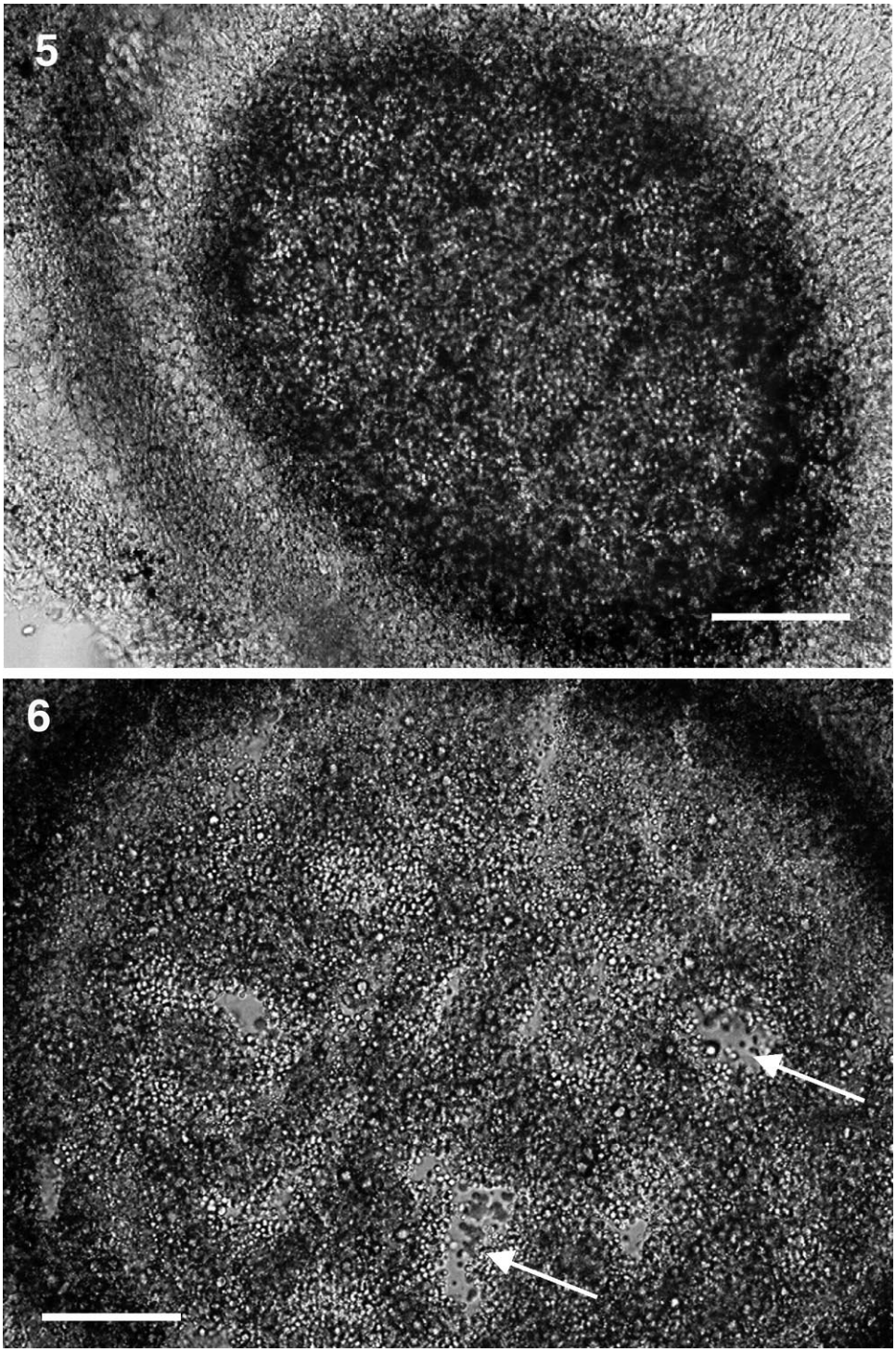
FIGS. 2–4. Mature basidiocarps of *Sphaerobolus* species. Bar = 1 mm. 2. *S. ingoldii* (T-800). 3. *S. iowensis* (ATCC 52850). 4. *S. stellatus* (SS13).

Peridium globosum, ≤1–1.5 mm diam. Basidiomata gregaria, aliquando solitaria. Superficies exoperidii saepe rimosa, subreticulata. Gleba brunnea aut nigrofusca, 0.6–1.3 mm diam. Basidiosporae hyalinae, glabrae, ellipsoideae, crassitunicatae, (7.2–)8–9.5(–10.2) × (5–)5.4–6.2(–7.1) µm, gemmae absentes. Coloniae in OA et WSA 2.0–2.2 mm, et PDA 0.9–1.8 mm per diem crescens. Color coloniarum in OA albus, eburneus aut alboluteus, mycelio saepe chordaceo.

Peridium globose, rarely exceeding 1–1.5 mm diam. Basidiocarps most often gregarious, occasionally solitary. The outer surface of the exoperidium is often rimose (cracked) in a reticulated manner. Expelled glebae dark brown to black, with a dull finish, 0.6–1.3 mm diam, with many basidiospores, but no gemmae. Even the ejected, hence slightly deformed (flattened) gleba rarely is larger than 1 mm. Basidiospores hyaline, smooth, elliptical, thick-walled, (7.2–)8–9.5(–10.2) × (5–)5.4–6.2(–7.1) µm, borne on basidia irregularly disposed in the gleba. Colonies on OA, WSA and PDA at 25 C exhibit respectively a daily linear growth of 2.0–2.2 mm, 2.0–2.2 mm, 0.9–1.8 mm. Mycelium off-white, sometimes with a yellow shade, often cord-like in appearance on OA.

Characteristic fixed DNA polymorphisms. Characteristic fixed DNA polymorphisms used in the description of *S. ingoldii* sp. nov. were determined based on the sequence alignments used in the phylogenetic analyses. Only those characters present in all *S. ingoldii* isolates and not present in other *Sphaerobolus* isolates were included. These fixed DNA polymorphisms are indicated with capital and italicized letters (nucleotides) or italicized numbers (gaps) with the alignment position given. **EF** 1-α: gca*A*ctc @ 161; acc/*T*tc*A*/*G*ac @ 304, 307; gcc*A*tggt*A*caa @ 471, 476; aca/*G*ag*T*ccggtgc/t*G*/*A*gt*G*aa @ 499, 502, 510, 513; a[-1-]g*C*cgtcc*TT*cc @ 566, 572–573. **ITS**: tg*C*tg @ 68; ggc*C*tta*C*ac*C*aa @ 76, 80, 83; tc*AA*atttc*T*aa @ 104–105, 111; tt*A*gacg*T*ag*A*t*TG*g*A*ctccc*G*g*T*/*C*t*T*ct-*AT*tgg/aagc*C*gtgag*CG*g @ 140, 145, 148, 150–151, 153, 159, 161, 163, 166–167, 175, 181–182; tg*A*ct*C*gt @ 220, 223; ac*A*a/gg @ 252; ta*A*t*G*gg @ 277, 279; ctt*A*ta @ 295; ac/ta*TT*aat @ 302–303; gcg*G*tt*GA*a*C*ta @ 538, 541–542, 544; a*TC*gc @ 553–554; tat*T*a*TA*[-1-]a[-1-]aa*CTCTA*tggt @ 598, 600–601, 607–611; aa*C*acaa*A*ttc[-*8*-]ttt @ 626, 631, 635–642; ct*A*t*G*/*A*gt*GA*ata @ 654, 656, 659–660; ga*T*gt @ 675; gtc*CA*ttgact*T*gga @ 695–696, 703; ta*TcACTT*tgt*T*atc @ 712, 714–717, 721. **mtSSU**: aa*T*g*C*a @ 74 and 76; ac*C*tt*G*cta*CGA*t*A*ag @ 98, 101, 105–107, 109; ca-*T*ctttt*G*a[-*1*-]a[-*3*-]tct[-1-]*C*tt[-*1*-]tt[-4-]g*G*[-*3*-]ga @ 200, 205, 207, 209–211, 216, 219, 227–230; aa*C*tct[-*4*-]g*TT*t*G*tt @ 292, 296–299, 301–302, 304; tac*AC*ag @ 329–330; gc*C*ga @ 399; tcg*C*tt @ 543. **LSU**: ggc*C*tt @ 407; tc[-1-]*T*tg @ 427; tt[-1-]*G*gac @ 435; ttt*C*gacca*C*tg @ 455, 461; gc*AAG*gg @ 473–475; gcc*C*ttg[-1-]t*T*gta @ 511, 517; agg*T*ctg @ 541.

Etymology. The name is a tribute to Charles T. Ingold, an outstanding British mycologist and former president of the British Mycological Society, who conducted fundamental research on the spore dispersal

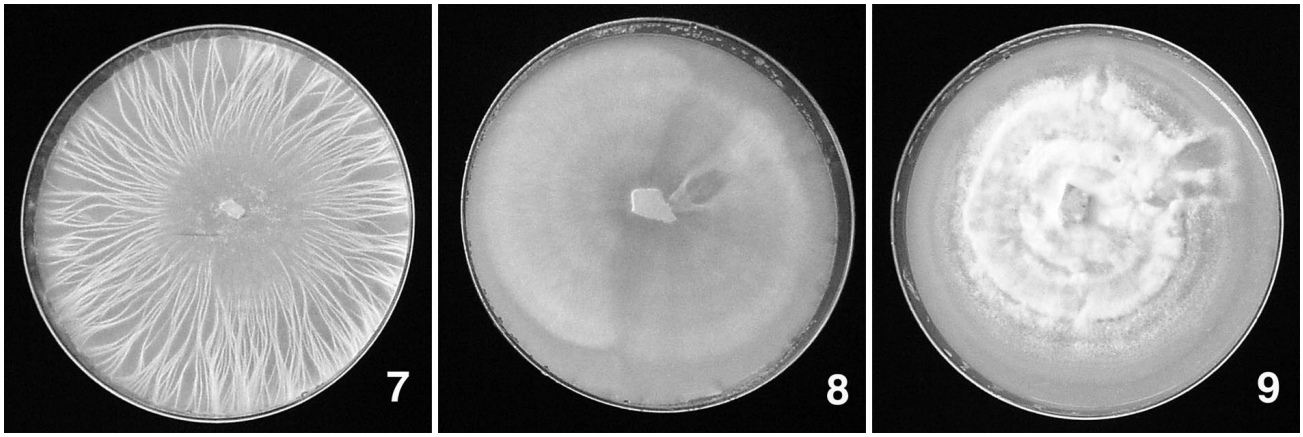


FIGS. 5–6. Cross section of unejected basidiocarp. Bar = 100 μm. 5. *S. ingoldii* (T-800). 6. *S. iowensis* with the characteristic basidial chambers indicated by arrows (ATCC 52850).

of Gasteromycetes, with a particular interest in *Sphaerobolus*.

Habitat. On rotten wood, forest litter, herbivore dung.

HOLOTYPE. USA: Michigan. Herbarium specimen (RGT 930825/04) at the USDA Forest Products Laboratory (Madison, Wisconsin) (CFMR). Originally collected and isolated by R.G. Thorn, 25 Aug 1993 as *Sphaerobolus stellatus* from Michigan, Kellogg Biological Station Long Term Ecological Research site (42°24′N latitude and 85°24′W longitude), near south edge of Treatment 8, on twig of *Quercus alba*.



FIGS. 7–9. Colony morphology on OA. 7. *S. ingoldii* (T-800). 8. *S. iowensis* (ATCC 52850). 9. *S. stellatus* (SS13).

Living culture ex holotype: T-800 CFMR. CBS MycoBank No. 500167.

Other specimens examined. Additional isolates were received as cultures from R. Hanlin (SS19, University of Georgia), and from K. Yokoyama (IFO 9597, Institute for Fermentation, Osaka, Japan) and were collected as gleba by D.D. Davis (SS42, Hershey, Pennsylvania, and SS93, Sandusky, Ohio). Cultures are available upon request.

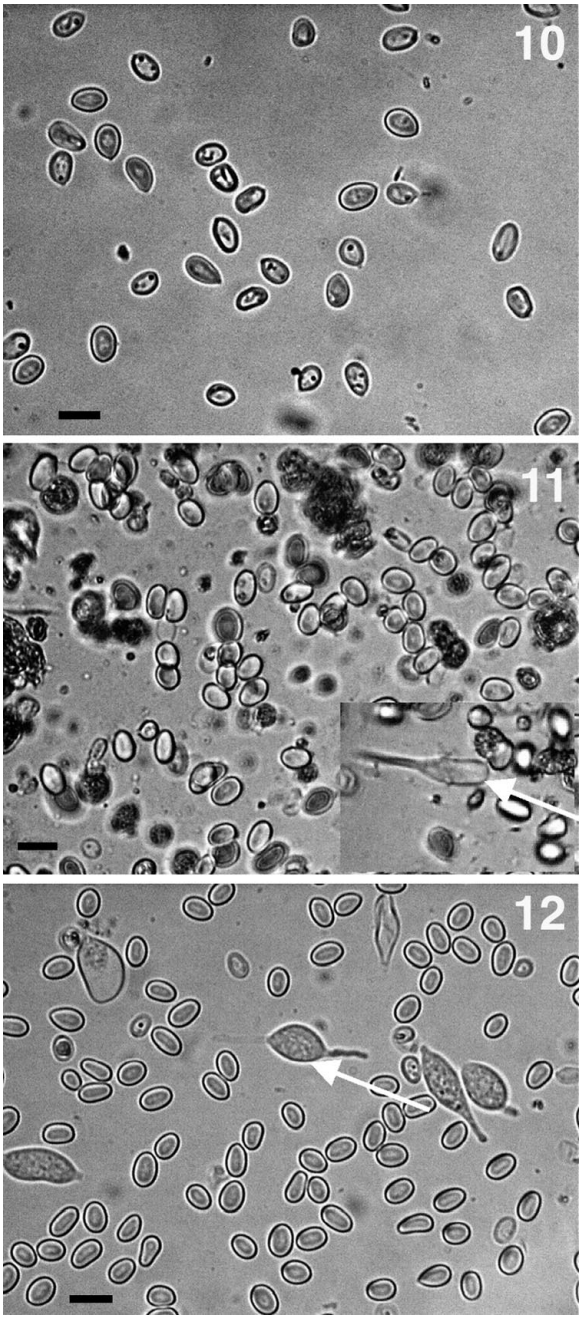
Known distribution. USA (Georgia, Michigan, Ohio, Pennsylvania), Japan.

DISCUSSION

Several distinctive macro- and micromorphological differences let us make connections between two of the three previously identified phylogenetic species in the genus *Sphaerobolus* and species defined previously based on morphology, *S. stellatus* and *S. iowensis*. The third species, *S. ingoldii,* is described here and differs from *S. iowensis* and *S. stellatus* in having smaller fruit bodies and glebae, slightly larger basidiospores, no gemmae, faster mycelial growth on OA and WSA, and the production of mycelial cords on OA and WSA. No statistically significant differences were found in the measured morphological characters between *S. iowensis* and *S. stellatus,* in agreement with Walker (1927). However the presence of basidial chambers in the immature gleba of *S. iowensis,* a distinct character in the species description by Walker (1927), let us make a connection between this species and one of the three phylogenetic species identified.

S. ingoldii was found to produce basidiospores but no gemmae in its glebae. Gemmae are assumed to play a crucial role in the lignicolous habit of *Sphaerobolus* because basidiospores were found not to germinate on wood (Ingold 1972). Because most of the isolates of *S. ingoldii* were found on wood, this raises the question whether the basidiospores of *S. ingoldii* in fact require the presence of proteolytic enzymes and high temperature to stimulate germination as had been reported in *S. stellatus* (Dykstra 1982) or are able to germinate on wood and other plant debris directly after discharge. It is possible that the in vitro fruiting conditions were inappropriate for stimulating gemmae production, although gemmae were produced in vitro in *S. iowensis* and *S. stellatus* under the same conditions. Another taxon that lacks gemmae in this group of fungi is *Nidulariopsis melanocarpa* Greis. The genus *Nidulariopsis* Greis is likely a synonym for *Sphaerobolus* as indicated by the name *Nidulariopsis iowensis* (Walker) Zeller, a synonym of *S. iowensis* and the morphological descriptions provided by Greis (1935). Although *N. melanocarpa*—characterized by basidiocarps 2.5–4 mm diam, yellow exterior, and a whitish basidiocarp interior—clearly is not conspecific with *S. ingoldii* described herein, both species lack gemmae. Future comparative investigations should address the systematic position and taxonomy of *N. melanocarpa,* as well as the ecological importance of the presence or absence of gemmae.

Although *S. ingoldii* has a wide range of geographic distribution, it seems to be more rarely encountered than the other two species. We found only five representatives of *S. ingoldii* in the 80 isolates analyzed. Somewhat to our surprise, we found that *S. iowensis* is quite common in North America, perhaps as or more common than *S. stellatus,* representing more than half the isolates we encountered. There are only two previous reports of *S. iowensis,* both from the Midwest (Walker 1927, Flegler 1984). While our geographic sampling admittedly is biased toward the eastern USA, we were able to obtain both *S. ingoldii* and *S. stellatus* from other continents, but not *S. iowensis*. It is worth noting that all three species are sympatric in the eastern USA and Midwest, often inhabiting the same microhabitat, apparently without interbreeding.



FIGS. 10–12. Basidiospores and gemmae. Arrows indicate germinating gemmae. Bar = 10 μm. 10. *S. ingoldii* (T-800). 11. *S. iowensis* (ATCC 52850). 12. *S. stellatus* (SS13).

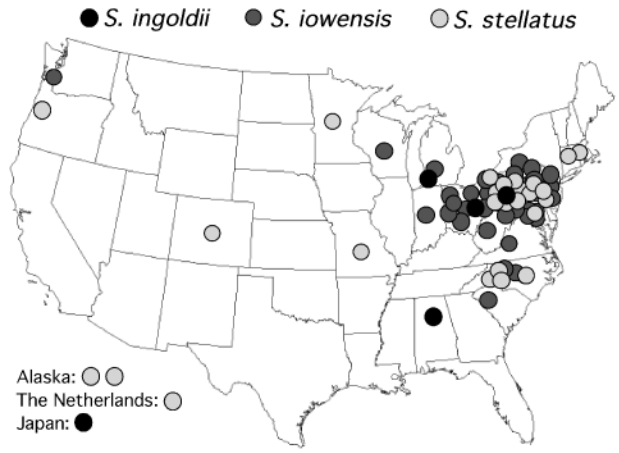


FIG. 13. Geographic distribution of *Sphaerobolus* isolates sampled.

While considerable DNA polymorhism was found in *S. iowensis* and *S. stellatus,* haplotype networks, in agreement with gene genealogies detailed earlier, little biogeographic structure was indicated in both species. In numerous instances isolates from the same region appeared in divergent portions of the networks, while isolates from distant localities often clustered together. Geographic distribution of the highest level nesting clades of *S. iowensis* and *S. stellatus* are shown (FIG. 14C, D, respectively). The null hypothesis of no geographic association of haplotypes could not be rejected in most clades, suggesting either panmixia and/or extensive dispersal or inadequate geographic sampling, possibilities that cannot be distinguished with this method. The dual coprophilous and lignicolous ecology of *Sphaerobolus* species provides many possible dispersal scenarios over great geographic distances. In prehistoric times animal migration might have been a considerable way of dispersal, occasionally supplemented with the natural movements of rotten wood and other plant debris. However, recent human activities might play a role in dispersal. Such activities include the transportation of commercial wood products and livestock between distant areas. The statistically significant results obtained in one clade of each species suggest restricted gene flow due to isolation by distance for some isolates. Because restricted gene flow implies only limited movement by individuals during any given generation, the geographic centers of all the clades nested together should be close, therefore the D_c and D_n values should show similar patterns under this scenario. We were interested in the fact that, even though most clades of both *S. iowensis* and *S. stellatus* did not show statistically significant genotype-geography association, the D_c and D_n values in fact were similar in most cases, supporting the hypothesis of restricted gene flow among at least some of the isolates.

In addition, while isolates of *S. stellatus* came from a much wider geographic area, nucleotide variation in *S. stellatus* was considerably smaller than that observed among *S. iowensis* isolates. This observation raises the question of whether *S. iowensis* represents a native species with genetically diverse populations and whether *S. stellatus* has been introduced to

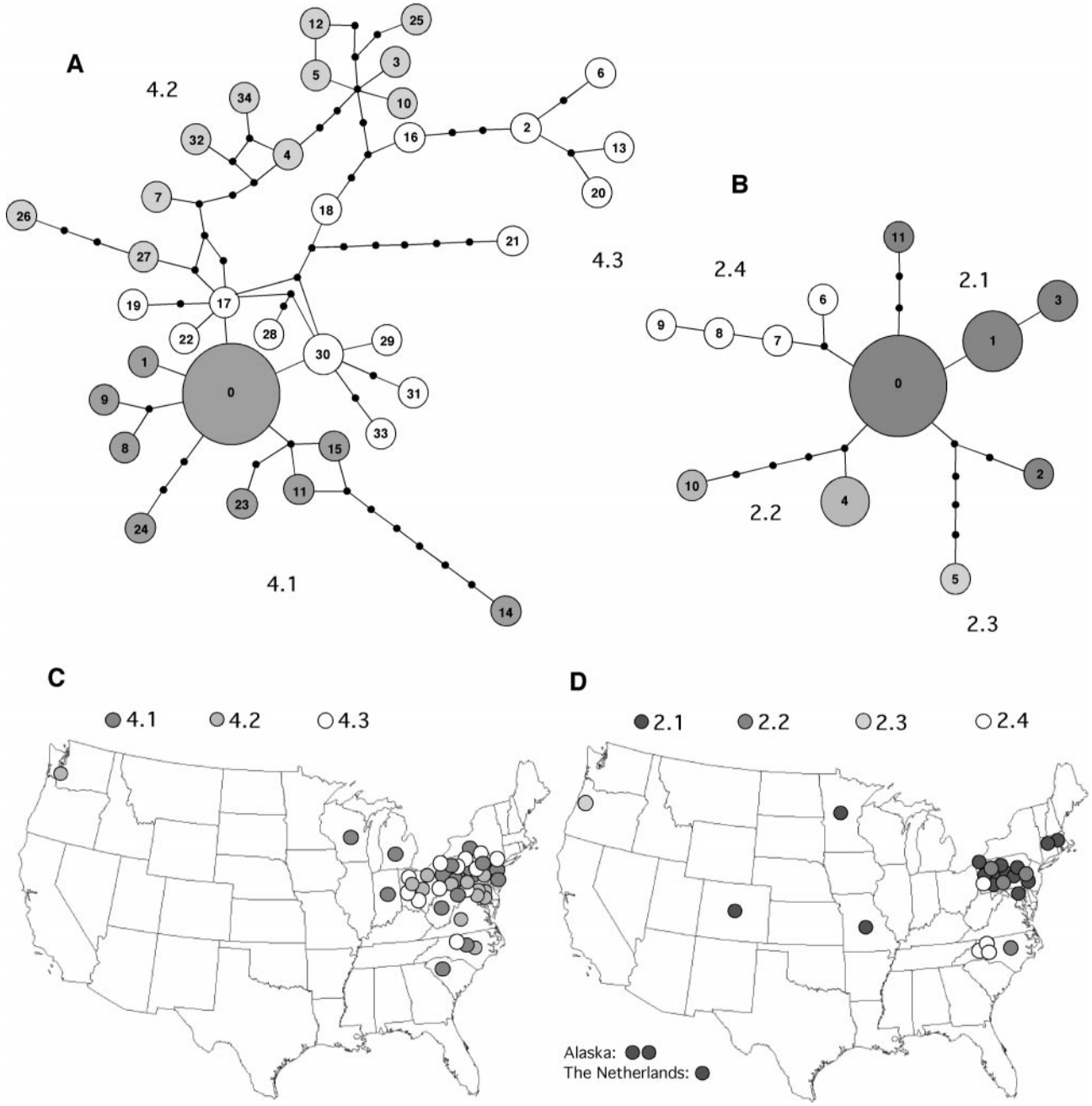


FIG. 14. ITS haplotype networks of *S. iowensis* (A) and *S. stellatus* (B) showing sampled haplotypes (white circles), unsampled, inferred haplotypes (black circles), and the highest level nesting clades (shaded groups). Size of circles refers to the observed haplotype frequency; haplotype "0" is considered the ancestral haplotype. Geographic distribution of the highest level nesting clades of *S. iowensis* (C) and *S. stellatus* (D) among sampled isolates.

North America. To answer this question is beyond the scope of this paper; however, the occurrence of isolates with identical ITS sequence (haplotype 0) in distant locations (Alaska, Colorado, Maryland, Massachusetts, Minnesota, Pennsylvania and The Netherlands) is consistent with the hypothesis of introduction and spread of *S. stellatus* to North America.

In this paper we provided the first systematic overview of the genus *Sphaerobolus* based on both molecular and morphological data that can serve as base for future research on the artillery fungi. Because our analyses included isolates predominantly from North America, future studies should include samples from other areas to further elucidate the genetic diversity and phylogenetic structure of the genus.

ACKNOWLEDGMENTS

This research was supported by the Pennsylvania Department of Agriculture, the Deep Hypha (NSF 0090301) and a doctoral research assistantship from the Department of Plant Pathology of Pennsylvania State University (PSU). The authors thank these people for providing cultures or herbarium specimens: Elizabeth Brantley, Richard Hanlin, David Hibbett, Kentaro Hosaka, David McLaughlin, Jean Lodge, Charikleia Perlerou, Rita Rentmeester, Amy Rossman, Greg Thorn, Lori Trummer and Kazumasa Yokoyama. We also thank Barrie Overton and Elwin Stewart for their help in micromorphological investigations, Jean Juba for her assistance in culturing and maintaining the isolates, Hanns Kreisel for reviewing the Latin diagnosis and the anonymous reviewers for their helpful comments on the manuscript. JG is grateful to the Mycological Society of America (MSA) and to the College of Agricultural Sciences of PSU for these awards: MSA Graduate Fellowship, the Howard E. Bigelow and the James M. Trappe Mentor Student Travel Awards (all MSA) and the Graduate Student Competitive Grant (PSU).

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