

## Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi

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**Abstract:** The *Ophiostoma piceae* complex forms a monophyletic group of insect-dispersed pyrenomycetes with synnemata (*Pesotum*) and micronematous (*Sporothrix*) synanamorphs. Other species of *Ophiostoma* outside of the *O. piceae* complex that form synnemata lack the *Sporothrix* state. The nine recognized species within the *O. piceae* complex are delimited by synnema morphology, growth rate at 32 C, mating reactions and sequences of the internal transcribed spacer (ITS) region of the rDNA operon. Phylogenetic analysis of the ITS region suggests two major clades in the complex, one that causes bluestain in primarily coniferous hosts and the other on primarily hardwood hosts. In the coniferous group are *O. piceae*, *O. canum*, *O. floccosum* and the recently described *O. setosum* (anamorph *Pesotum cupulatum* sp. nov.). In the hardwood group are *O. querci*, *O. cationianum*, and the Dutch elm disease fungi: *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi*. Restriction fragment length polymorphisms of the ITS region are shown to be a convenient diagnostic tool for delimiting these species.

**Key Words:** Bluestain, Dutch elm disease, *Graphium*, *Leptographium*, *Pesotum*, *Phialographium*, *Sporothrix*

### INTRODUCTION

Adaptations for insect dispersal (ascomata and conidiomata with sticky spore drops) have arisen frequently in the evolution of the ascomycetes (Black-

well 1994), and convergence on similar morphologies has greatly confused generic and species delimitations of both teleomorphs and anamorphs (Spatafora and Blackwell 1994). One of the largest groups of insect dispersed pyrenomycetes is the genus *Ophiostoma*, which is now recognized as distinct from *Ceratocystis* based on anamorphs and biology (Hoog and Scheffer 1984, Harrington 1981, 1987). Considerable confusion remains with generic limits for anamorphic fungi dispersed by insects, many of which form droplets of conidia at the apex of compact conidiomata called synnemata (Okada et al 1998). Some synnema-forming species have affinities to *Ophiostoma* H. & P. Sydow, especially to the common sapwood-colonizing species *O. piceae* (Münch) H. & P. Sydow and the Dutch elm disease fungus *O. ulmi* (Buisman) Nannf. (Okada et al 1998).

Synnematous anamorphs of *Ophiostoma* species have been placed in the genus *Graphium* Corda, though it is now thought that *Graphium* species are anamorphs of the Microascales (Okada et al 1998), and the genus name *Pesotum* Crane & Schoknecht is available for the anamorphs of *Ophiostoma* species. *Pesotum* was based on the anamorph of *O. ulmi* and was characterized by the synanematous anamorph as well as a *Sporothrix* Hektoen & Perkins ex Nicot & Mariat synanamorph (Crane and Schoknecht 1973). *Sporothrix* conidiophores are micronematous, with conidiogenous cells that have prominent denticles at the point of conidium detachment (Hoog 1974, 1993). Secondary conidia are frequently formed from the primary *Sporothrix* conidia, and the name *Hyalodendron* Diddens has been used for such anamorphs. However, the name *Hyalodendron* should be reserved for basidiomycetous anamorphs, and there appears to be little need for distinguishing *Sporothrix* species with secondary conidium production (Hoog 1993). Species of *Hyalorhinoctadiella* Upadhyay & Kendrick form micronematous conidiophores similar to those of *Sporothrix* but without prominent denticles (Hoog 1993), and the conidiogenous cells of *Hyalorhinoctadiella* resemble those of *Pesotum* species. Synnema forming anamorphs with phialidic conidium production were placed in *Phialographium* Upadhyay & Kendrick, but Hoog and Scheffer (1984) and Wingfield et al (1991) do not distinguish *Phialographium* from *Graphium* (*Pesotum* sensu Okada et

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al 1998). *Pesotum*-type conidiophores with a single row of cells for the stipe (mononematous conidiophores) and a series of metulae at the stipe apex are placed in *Leptographium* Lagerb. & Melin (Harrington 1988).

Okada et al (1998) used *Pesotum* for all synnema forming species with affinities to *Ophiostoma*, even those species with no *Sporothrix* synanamorph. This paper focuses on those *Ophiostoma* species with *Pesotum* anamorphs sensu Crane and Schoknecht (1973), i.e., those that also have *Sporothrix* synanamorphs, such as *O. piceae*. *Ophiostoma piceae* was originally described by Münch (1907) from sapstain in pine and spruce but is now known as a complex of closely related *Ophiostoma* species with pigmented, synnematous anamorphs. Included in the complex are *O. querci* (Brasier and Kirk 1993) and the Dutch elm disease fungi, *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* (Brasier and Mehrotra 1995). We found four other species closely tied to the *O. piceae* complex. We used DNA sequences of the internal transcribed spacer (ITS) region of the nuclear rDNA and mating studies to identify lineages and potential intersterility groups, and morphological and physiological comparisons were used to delimit species in the complex.

#### MATERIALS AND METHODS

*Isolates.*—The isolates most intensively studied and considered as part of the *O. piceae* complex are listed in TABLE I. Isolates are maintained in the collection at Iowa State University. For morphological comparisons, isolates were grown at room temperature (21–24 C) and lighting for 10–14 d on malt extract agar (MEA, 1.5% Difco malt extract and 2.0% agar) or pine twig medium (MEA with a section of debarked *Pinus strobus* twig added to the molten agar medium, Harrington 1992). Isolates grown for DNA extraction were cultured on MYE broth (2% malt extract, 1% yeast extract).

*Temperature growth assay.*—Isolates were grown at 32 C on MEA because growth at this temperature was shown to distinguish *O. piceae* from *O. querci* (Brasier and Stephens 1993). Plates were inoculated using a plug of agar and mycelium (#1 cork borer, from the advancing margin of a colony) placed upside down in the middle of the plate. The plates were incubated upside down in the dark. After 7 d, the plates were removed from the incubator and two radial measurements taken for each plate. The assay was repeated twice at separate times, and the mean extent of radial growth determined from the three separate experiments.

*Pairings.*—Tests for sexual compatibility were conducted on MEA with pine twigs. Two methods of pairing were used. In most cases, a single isolate was grown on the agar medium for 1 wk and then spermatized with a conidial/mycelium slurry (Brasier and Kirk 1993, Harrington and McNew 1997). In other cases, two isolates were co-inoculat-

ed with colonized agar plugs placed ca 5 mm apart. Single ascospore strains were obtained from an ascospore mass at the tip of a perithecium by suspending the mass in a light oil and streaking onto MEA plates (Harrington and McNew 1997).

*PCR, DNA sequencing, and RFLP analysis.*—The primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns 1993, White et al 1990) were used to amplify the ITS region. Either extracted DNA (DeScenzo and Harrington 1994) at 10–100 ng per reaction or scraped mycelium with spores (Harrington and Wingfield 1995) were used as template for the polymerase chain reaction (PCR). The reaction mixture (100  $\mu$ L final volume) contained 2.5 units Taq DNA polymerase (Promega Inc., Madison, Wisconsin), 1X PCR reaction buffer, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 5% (V/V) DMSO, and 0.25 or 0.50  $\mu$ M of each primer. Reaction mixtures were placed into a precooled (4 C) thermocycler (MJ Research, Inc., Watertown, Massachusetts). Cycling conditions were an initial denaturation at 95 C for 95 s, followed by 35 cycles of 52 C for 40 s (annealing), 72 C for 2 min (extension), and 95 C for 35 s (denaturation). There was a final extension step of 10 min at 72 C.

Amplicons were either sequenced using the primers ITS1-F and ITS4 or were digested with restriction enzymes following the manufacturer's recommendations. Sequencing was performed on a ABI PRISM 377 Genetic Analyzer (Perkin-Elmer Inc., USA) at the DNA Synthesis and Sequencing Facility (Iowa State University, Ames, Iowa) after purification using QIAquick PCR purification Kits (Qiagen Inc., USA) or Microcon-100 Microconcentrators (Amicon, Inc., USA).

The ITS sequences of 58 isolates of taxa in the *O. piceae* complex and an outgroup taxon (isolate C327 of *Ophiostoma ips* from New York, USA) were aligned manually and analyzed using PAUP 4.0 (Swofford 1998). A total of 616 unordered characters, including gaps, were utilized, with gaps considered a "fifth base" and all characters with equal weight. Sixty-four of the characters were parsimony-informative, and 491 characters were constant. Heuristic searches used stepwise (simple) addition and tree-bisection-reconnection. Bootstrap support (Felsenstein 1985) was determined from 1000 replications.

Polymorphisms in restriction sites of the ITS region were identified among species in the *O. piceae* complex using the online tool Webcutter 2.0 (<http://www.ccsi.com/firstmarket/cutter/cut2.html>; Max Heiman, copyright 1997). The restriction enzyme *Hae*II (Gibco BRL, Inc., USA) produced the greatest number of polymorphisms among species; however, some species showed similar RFLP patterns with this enzyme. The restriction enzyme *Dde*I (Gibco BRL, Inc., USA) was used to differentiate some taxa. For either enzyme, the unpurified PCR product (17.5  $\mu$ L) was combined with 2.0  $\mu$ L of 10X buffer (supplied with the enzyme) and 0.5  $\mu$ L of restriction enzyme (5 units). Digestion was allowed to proceed for 1–16 h at the appropriate incubation temperature for the enzyme employed. Restriction fragments and a 100-bp ladder (Gibco BRL, Inc., USA) were separated by electrophoresis for 3 h at 75 volts in 2% agarose gels (Bio-Rad, Inc., USA) with a TBE

[89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8.0)] buffer system. Gels were stained with ethidium bromide and illuminated with ultraviolet light. Molecular weights of the DNA fragments were determined using digital images and GelReader 2.0.5 (National Center for Supercomputing Applications, University of Illinois, Urbana-Champaign).

## RESULTS

A wide range of synnema morphologies was seen among the isolates studied. All isolates forming both synnema and *Sporothrix* states were tolerant of cycloheximide (Harrington 1981) and had similar ITS sequences. The ITS sequence analysis grouped these isolates with bona fide isolates of *O. piceae* and *O. querci* (FIG. 1). Nine species in the *O. piceae* complex are recognized based on the ITS sequences (FIG. 1), mating reactions (TABLE I), cultural characteristics, and morphology (FIGS. 2–36, TABLE II). Other synnema-forming species, including *Ophiostoma* species excluded from the *O. piceae* complex (TABLE III) and isolates of *G. penicillioides*, the type species for *Graphium*, did not form a *Sporothrix* synanamorph (FIGS. 37–48). Their ITS sequences, where available, aligned poorly with sequences of *O. piceae* and *O. querci*.

*Culture characteristics.*—The distinguishing features of the species recognized in the *O. piceae* complex are listed in TABLE II. Morphological features are determined on MEA or MEA with pine twig medium, and a relatively weak medium is needed to discern these characteristics. Aroma is useful for distinguishing *O. querci* and *O. setosum* from the other species, and this feature is also best determined on MEA. Growth at 32 C is a distinguishing feature for species in the complex (TABLE I), but accurate temperature control is needed. Protoperithecia, which are distinctly light-brown in *O. querci*, are most reliably produced 1 wk after a conidial mass from a synnema is streaked over MEA or MEA with pine twig medium. The concentric rings of aerial mycelium that are diagnostic for the Dutch elm disease fungi (Brasier and Mehrotra 1995), *O. querci*, and *O. setosum* are also best seen on MEA.

*Phylogenetic analyses.*—Most of the sequences of the ITS region of other *Ophiostoma* species [e.g., *O. pluriannulatum* (Hedgcock) H. & P. Sydow, *O. stenoceras* (Robak) Melin & Nannf., and *O. europhioides* (Wright & Cain) Solheim] could not be unambiguously aligned with the ITS sequences of the *O. piceae* complex, so only members of the *O. piceae* complex were included in analyses. Of the species outside of the *O. piceae* complex, the ITS sequence of *O. ips* (Rum-

bold) Nannf. (AF198244) was reasonably similar, and *O. ips* was used as an outgroup taxon.

Two major clades in the *O. piceae* complex were inferred from parsimony analysis of the ITS sequences (FIG. 1). *Ophiostoma piceae* s.s., *O. canum*, *O. floccosum* and *O. setosum* formed a moderately supported group of species that are primarily found on conifer (Pinaceae) wood. *Ophiostoma querci*, *O. cationianum*, *O. himal-ulmi*, *O. ulmi* and *O. novo-ulmi* were found in a strongly supported branch of primarily hardwood (angiosperm) inhabiting fungi. The inferred hardwood-inhabiting clade was also seen in neighbor-joining analysis (Swofford 1998), as was the branch containing *O. himal-ulmi* and *O. querci*, but the conifer-inhabiting species did not resolve as a single clade.

In most cases, the well supported branches in the phylogenetic analysis (FIG. 1) were of isolates with unique cultural or morphological characteristics (TABLE II). The ITS sequences of *O. piceae* and *O. canum* were identical, though all of the examined *O. piceae* isolates had cylindrical to obovoid conidia, and the two examined isolates of *O. canum* had globose conidia at the tip of synnemata (FIG. 13). The synnema stipes of *O. piceae* isolates were consistently black, while some synnemata of *O. canum* had brown stipes. *Ophiostoma floccosum* formed red-brown synnemata, frequently with lateral knobs (FIG. 16). Another branch in the ITS tree contained isolates of the recently describe *O. setosum* (Uzunovic et al 2000), which is characterized by darkly pigmented synnemata with the vertical cells of the stipe extending into the conidial mass, forming a cup-shaped structure (FIGS. 21–23).

As expected, the Dutch elm disease fungi *O. ulmi* and *O. novo-ulmi* were grouped as closely-related sister species (FIG. 1). The *O. himal-ulmi* ITS sequence was closer to that of *O. querci* than to that of *O. ulmi*. The *O. querci* clade included the only available isolate of *O. cationianum* and anomalous isolates from New Zealand and Viet Nam. *Ophiostoma fagi* had an ITS sequence identical to *O. querci* and is considered a synonym.

*PCR-RFLP diagnostics.*—Based on the ITS sequences used in FIG. 1, we identified restriction enzymes that would differentiate among the species in the *O. piceae* complex, except that no attempt was made to differentiate among the three Dutch elm disease species. Most species could be differentiated based on *Hae*III digestions (TABLE IV, FIG. 49). The *Hae*III restriction pattern for *O. ulmi*, *O. novo-ulmi* and *O. himal-ulmi* is the same, but it is distinct from that of *O. querci* and the other species (TABLE IV, FIG. 50). *Ophiostoma piceae* and *O. setosum* have the same re-

TABLE I. Isolate numbers, accession numbers for ITS sequences, substrate, origin, mating type and growth at 32 C of isolates of the *Ophiostoma piceae* complex

Species	Isolate <sup>a</sup>	GenBank	Other no./collector or supplier	Substrate	Country of origin	Mating type <sup>b</sup>	Growth (mm) at 32 C <sup>c</sup>
<i>Ophiostoma piceae</i>	C82		A21	<i>Pseudotsuga</i>	Idaho, USA	A	0
	C967		H2181/Webber	<i>Picea</i>	United Kingdom	A	0
	C968		H2009/Gibbs	<i>Pinus</i>	United Kingdom	B	0
	C987		3NZ-S519	<i>Pinus</i>	New Zealand	B	0
	C992		3NZ-S501-5	<i>Pinus</i>	New Zealand	A	0
	C993		4NZ-125	<i>Pinus</i>	New Zealand	B	0
	C1087	AF198226	CBS 108.21/Munch (From the type)	Cat hair	Germany	B	0
	C1103		CBS 600.85 (as <i>O. perfectum</i> )		Germany	B	0
	C1201		CBS 102355	<i>Tsuga</i>	Washington, USA	B	0
	C1246		CBS 102356/Worrall	<i>Pseudotsuga</i>	New York, USA	A	0
C1209	AF198217	19NZ-1384	<i>Pinus</i>	New Zealand	B	0	
C114	AF198229	NFRI 60-165, NCC-60-16/Kaarik		Sweden	B	0	
C1088	AF198228	CBS 133.51/Kaarik		Sweden	B	0	
C424		CBS 102360, RS1		Sweden	B	0	
C425		RS2		Sweden	B	0	
C988		CBS 102361, 103		California, USA	mixed	3.7	
C989		CBS 102362, 2NZ-35		California, USA	?	2.8	
C1010		Gr4+/Webber		New Zealand	-	2.8	
C1011		Gr13+/Webber		New Zealand	+	3.0	
C1012		Gr36-/Webber		United Kingdom	+	4.9	
C1013		Gr10-/Webber		United Kingdom	+	2.8	
C1086	AF198231	CBS 799.73/Kaarik (Authentic for type)		United Kingdom	-	2.8	
				Sweden	+	2.1	
						4.9	
<i>Ophiostoma setosum</i>	C1160			<i>Pseudotsuga</i>	Washington, USA	mixed	3.0
	C1198		CBS 102363	<i>Tsuga</i>	Washington, USA	-	1.4
	C933		SSI202/Blanchette	<i>Pinus</i>	New Zealand	B	0
	C985		3NZ-S509	<i>Pinus</i>	New Zealand	B	0
	C986		CBS 102357, 2NZ-29	<i>Pinus</i>	New Zealand	B	0
	C1037		8NZ-211	<i>Pinus</i>	New Zealand	B	0
	C1194	AF198230	CBS 102358	<i>Pseudotsuga</i>	Washington, USA	B	0
	C1200		CBS 102359	<i>Tsuga</i>	Washington, USA	B	0

TABLE I. Continued

Species	Isolate <sup>a</sup>	GenBank	Other no./collector or supplier	Substrate	Country of origin	Mating type <sup>b</sup>	Growth (mm) at 32 C <sup>c</sup>
<i>Ophiostoma quercii</i>	C426		TAB103/Wingfield	?	South Africa	mixed	7.2
	C799		TAB191/Blanchette	<i>Abies</i> or <i>Pseudotsuga</i>	Washington, USA	A	7.2
	C934		103/Blanchette	<i>Pinus</i>	New Zealand	mixed	7.9
	C935		108/Blanchette	<i>Pinus</i>	New Zealand	B	8.2
	C936		109/Blanchette	<i>Pinus</i>	New Zealand	B	6.8
	C969	AF198238	CBS 102352, H1042/Scard and Webber	<i>Quercus</i>	United Kingdom	B	6.3
	C970	AF198239	CBS 102353, H1039/Scard and Webber	<i>Quercus</i>	United Kingdom	A	6.8
	C984		2NZ-26	<i>Pinus</i>	New Zealand	A	6.8
	CI085	AF198237	CBS 236.32/Loos (as <i>Ophiostoma fagi</i> , from the type)	<i>Fagus</i>	Germany	A	5.6
	CI159		CBS 102354	<i>Pseudotsuga</i>	Washington, USA	mixed	8.9
	CI283		CBS 277.54, ATCC 24586, JCM 9745/Käärík (as <i>Graphium aureum</i> )	<i>Pinus</i>	Sweden	B	6.3
<i>Ophiostoma catonianum</i>	CI084	AF198243	CBS 263.35/Goidanich (from the type)	<i>Pyrus</i>	Italy	?	<1
<i>Pesotum</i> sp., near <i>O. quercii</i>	CI203	AF198242	18NZ-1281	<i>Pinus</i>	New Zealand	?	4.4
<i>Pesotum</i> sp., near <i>O. quercii</i>	CI214	AF198241	144-E/Blanchette	<i>Aquilaria crassna</i>	Viet Nam	?	12.4
<i>Pesotum</i> sp., near <i>O. quercii</i>	CI215	AF198240	NZJum8/Blanchette	<i>Nothofagus</i>	New Zealand	?	0
<i>Ophiostoma ulmi</i>	C55		UM C-20/Blanchette	<i>Ulmus</i>	Minnesota, USA	?	17.0
	CI089		CBS 137.36 (as <i>O. ips</i> )	?	?	?	21.2
	CI182	AF198232	CBS 102.63, IMI 101223, JCM 9303/Holmes and Heybroek	<i>Ulmus</i>	Netherlands	?	13.3
	CI186		CBS 296.87, WCS 811/Elgersma	<i>Ulmus</i>	Netherlands	?	13.8
<i>Ophiostoma novo-ulmi</i>	C48		UM C56, Aggressive W-Z/Blanchette	<i>Ulmus</i>	United Kingdom	?	10.3
	C510	AF198236	Jacobs	<i>Ulmus</i>	Iowa, USA	?	<1.0
	C913		Jacobs	<i>Ulmus</i>	Illinois, USA	?	9.1
	C943		Jacobs	<i>Ulmus</i>	Illinois, USA	?	13.5
	CI184		CBS 377.79/Meulemans	<i>Ulmus</i>	Belgium	?	13.5
	CI185	AF198235	CBS 298.87, WCS 637/Heybroek	<i>Ulmus</i>	Russia	?	11.0
	CI187		CBS 297.87, WCS 802/Scheffer	<i>Ulmus</i>	Netherlands	?	<1.0
<i>Ophiostoma himal-ulmi</i>	CI183	AF198233	CBS 374.67, ATCC 36176, ATCC 36204/Heybroek	<i>Ulmus</i>	India	?	1.4
	CI306	AF198234	HP27/Brasier	<i>Ulmus</i>	India	?	3.5

<sup>a</sup> Isolate numbers from the collection of the senior author.<sup>b</sup> Designations of mating type as either A/B or ± were based on compatibilities with tester strains provided by C. Brasier, A. Uzunovic, or J. Webber, using their nomenclature. Isolates producing perithecia and ascospores without pairings ("mixed") are mixtures (heterokaryons or mixtures of mycelia) of both mating types.<sup>c</sup> Extent of radial growth on MEA after 7 d at 32 C.

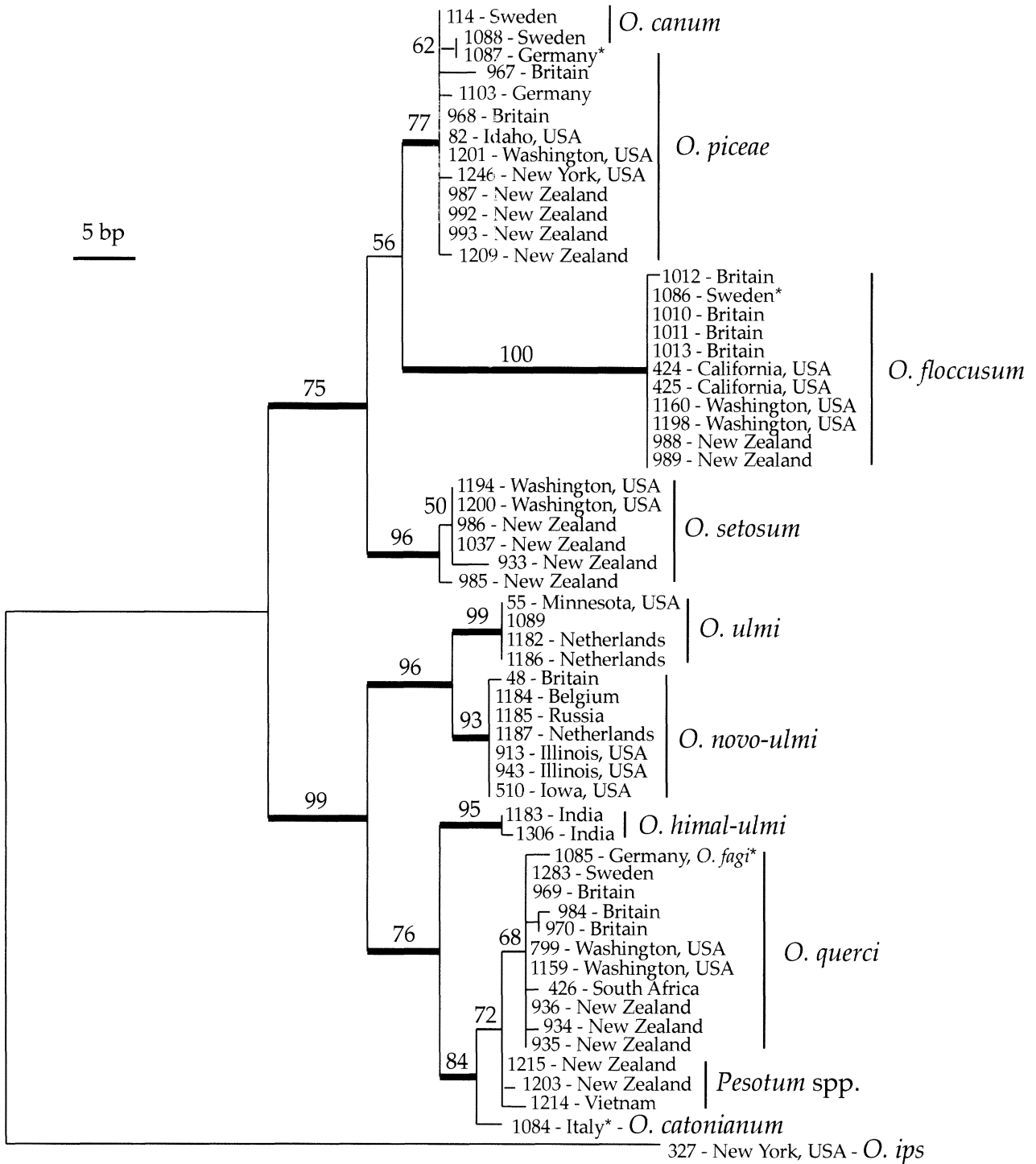


FIG. 1. One of four most parsimonious trees of the *Ophiostoma piceae* complex based on 616 characters, including gaps, of the ITS-1, 5.8S, and ITS-2 regions of the rDNA operon. The tree is rooted to *Ophiostoma ips*. Branches with strong support ( $\geq 75\%$ ) are bolder, with bootstrap values  $\geq 50\%$  indicated above the branches. Total tree length = 181 steps, consistency index = 0.8122, retention index = 0.9663. Asterisk indicates a culture from the holotype.

TABLE II. Distinguishing characteristics for species in the *Ophiostoma piceae* complex

Species	Color of synnema		Knobs on Cupulate synnema		Synnema conidial mass		Shape of synnema conidia	Color of protoperithecia	Mycelium with concentric rings <sup>a</sup>	Culture aroma <sup>b</sup>	Growth (mm) at 32 C <sup>c</sup>
	stipe	stipe	stipe	apex	condial	mass					
<i>O. piceae</i>	Dark-brown	Dark-brown	No	No	White	White	Cylindrical to Obovoid	Dark-brown	Rare	None	0
<i>O. canum</i>	Medium to dark-brown	Dark-brown	No	No	White	White	Globose	Not seen	No	None	0
<i>O. floccosum</i>	Red-brown	Dark-brown	Yes	No	Yellow	White	Cylindrical to Obovoid	Dark-brown	No	None	1-5
<i>O. setosum</i>	Dark-brown	Dark-brown	No	Yes	White	White	Cylindrical to Obovoid	Not seen	Most isolates	Sweet	0
<i>O. querci</i>	Dark-brown	Dark-brown	No	No	White	White	Cylindrical to Obovoid	Light-brown	Most isolates	Nutty	5-10
<i>O. catonianum</i>	Dark-brown	Dark-brown	No	No	White	White	Cylindrical to Obovoid	Not seen	No	None	<1
<i>O. ulmi</i>	Dark-brown	Dark-brown	No	No	White	White	Cylindrical to Obovoid	Not seen	Yes	None	10-25
<i>O. novo-ulmi</i>	Dark-brown	Dark-brown	No	No	White	White	Cylindrical to Obovoid	Dark-brown	Yes	None	<1-15
<i>O. himal-ulmi</i>	Dark-brown	Dark-brown	No	No	White	White	Cylindrical to Obovoid	Not seen	Yes	None	1-5

<sup>a</sup> Concentric rings of aerial mycelium, microconidiophores, and conidia on MEA.

<sup>b</sup> Smell on MEA.

<sup>c</sup> Extent of radial growth on MEA after 7 d at 32 C.

striction pattern with *HaeII*, but these two species can be differentiated based on *DdeI* restriction fragments (TABLE IV, FIG. 49).

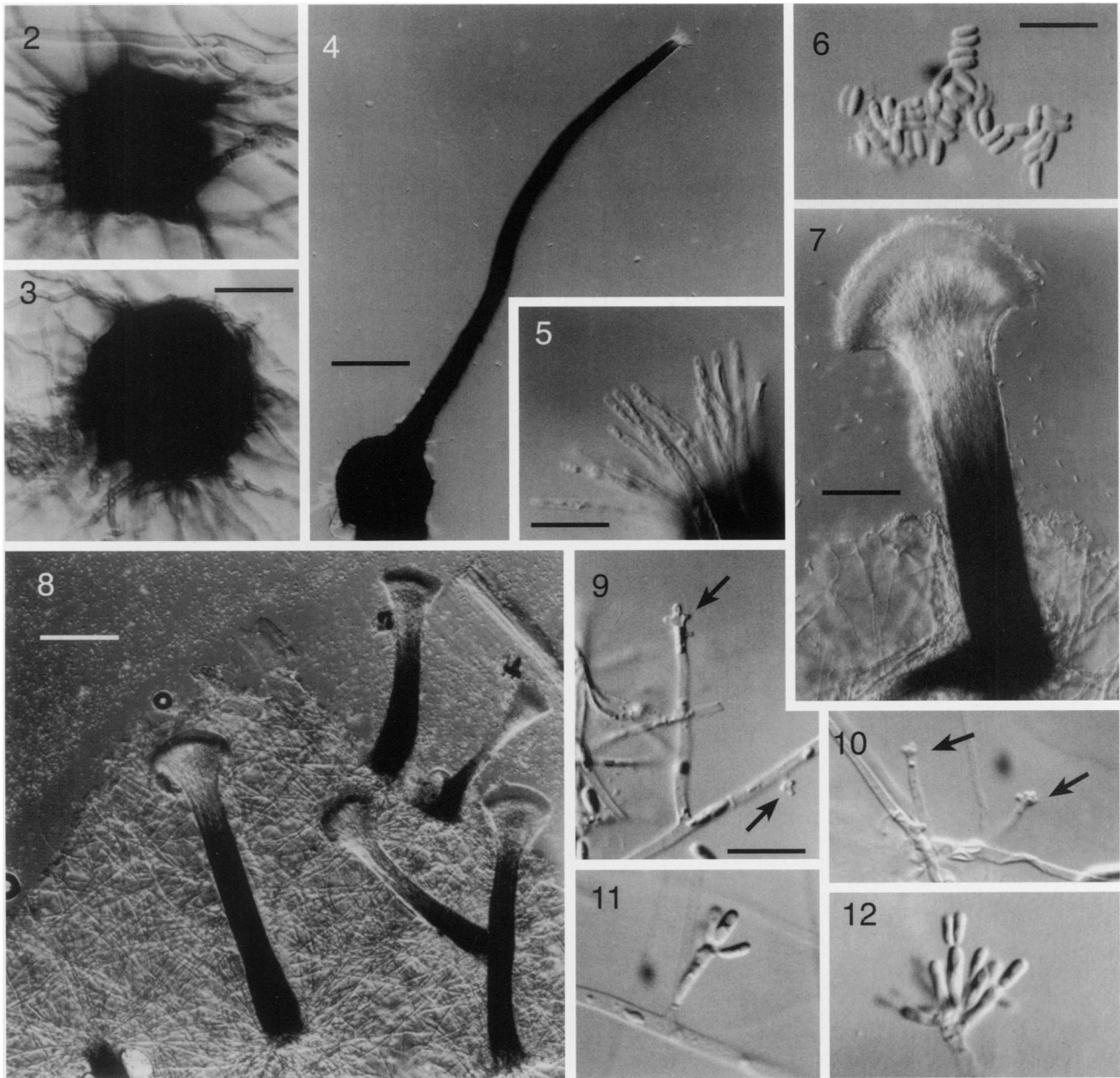
*Mating reactions.*—Tester isolates of each mating type of *O. piceae*, *O. querci* and an unidentified species found here to be *O. floccosum* were provided by J. Webber, and A. Uzunovic provided tester strains of *O. setosum*. Isolates of the Dutch elm disease fungi were not tested, but most other isolates of the *O. piceae* complex were identified as either of the two mating types based on pairings with these and other selected tester strains (TABLE I). Most species formed perithecia and ascospores within two weeks of pairing, but *O. floccosum* isolates, when paired with isolates of opposite mating type, formed perithecia only after 4-6 wk, if at all. Isolates of *O. piceae* generally formed perithecia and ascospores when paired with isolates of opposite mating type. The two isolates of *O. canum* tested did not produce perithecia when paired with each other but produced a few perithecia with aborted asci and misshapen ascospores with mating type A strains of *O. piceae* (FIG. 14). No protoperithecia or perithecia were seen in our isolates of *O. setosum* (TABLE I) when paired among themselves or with other isolates of the *O. piceae* complex. However, these isolates were interfertile with a mating type A strain of *O. setosum* when incubated at 20 C for 4-8 wk (Uzunovic et al 2000).

*Ophiostoma querci* isolates readily produced perithecia and ascospores when isolates of opposite mating type were paired. The isolate of *O. fagi* from the holotype also mated with *O. querci* testers. An isolate deposited in CBS as *G. aureum* (TABLE I) also paired with isolates of *O. querci*; however, we do not think this isolate represents *G. aureum*. An isolate from the holotype of *O. catonianum*, which is reportedly homothallic (Goidanich 1935), did not produce perithecia on its own and failed to mate with any other isolates.

#### TAXONOMY

*Species of the Ophiostoma piceae complex.*—Based on ITS sequence analysis, mating reactions, tests for growth at 32 C, and morphology, the following species are recognized in the complex.

- Ophiostoma piceae* (Münch) H. & P. Sydow, Ann. Mycol. 17: 43. 1919. FIGS. 2-12  
 ≡ *Ceratostomella piceae* Münch, Naturw. Land. Forstw. 5: 547. 1907.  
 ≡ *Ceratocystis piceae* (Münch) Bakshi, Trans. Br. Mycol. Soc. 33: 113. 1950.



FIGS. 2–12. *Ophiostoma piceae*. 2, 3. Protoperithecia. 4. Perithecium. 5. Ostiolar hyphae. 6. Ascospores. 7, 8. Synnemata. 9–12. Conidiophores and conidia of the *Sporothrix* synanamorph. Arrows in 9 and 10 indicate apex and side walls of conidiogenous cells, showing prominent denticles. 2, 3 from strain C967; 4–6 from a pairing of 967 and C1087; 7–12 from strain C1087, from the HOLOTYPE. Scale bars: 2, 3 = 25  $\mu\text{m}$ ; 4, 8 = 100  $\mu\text{m}$ ; 5, 6, 9–12 = 10  $\mu\text{m}$ ; 7 = 50  $\mu\text{m}$ .

*Anamorph.* *Pesotum piceae* Crane & Schoknecht, Am. J. Bot. 60: 348. 1973.

= *Graphium piceae* (Crane & Schoknecht) Wingfield & Kendrick, Mycol. Res. 95: 1331. 1991.

*Commentary.* Lectotype (BPI 595978 = FP 14395) and other material (BPI 595979, BPI 595977) from *Picea abies* sapwood in Germany, collected by Münch, was deposited in BPI, apparently by Hedgcock. Although there is some mold growth on these specimens, the lectotype has, in addition to synnemata and perithecia, the *Sporothrix* synanamorph (with

prominent denticles) typical of the *O. piceae* complex. The culture from the type (CBS 108.21 = C1087) forms synnemata on wood and is of the B mating type (TABLE I). This species is most commonly found on members of the Pinaceae throughout Europe, North America, and Japan (Brasier and Kirk 1993, Halmschlager et al 1994, Kim et al 1999, Morelet 1992, Pipe et al 1995). We have also identified isolates of *O. piceae* on the exotic *Pinus radiata* from New Zealand (TABLE I) and Chile.

Both the teleomorph and anamorphs of this spe-



cies lack distinctive features, which has led to the proposal of numerous synonyms, all of which we have excluded from *O. piceae*. Synnemata stipe lengths of *O. piceae* and *O. querci* overlap, but those of *O. piceae* are larger (Hamschlager et al 1994). The size of the ascospores ( $2.5\text{--}4.5 \times 1.5\text{--}2 \mu\text{m}$ ) and range in size of synnemata conidia ( $5\text{--}15 \times 1\text{--}3.5 \mu\text{m}$ ) (Upadhyay 1981) of *O. piceae* overlap with the sizes of most other species in the complex. However, ITS sequences (FIG. 1, Kim et al 1999), mating reactions (TABLE I, Brasier and Kirk 1993), dark protoperithecia, the inability to grow at 32 C (TABLE II, Brasier and Stephens 1993), and absence of a distinctive aroma or concentric rings on MEA (TABLE II) distinguish isolates of *O. piceae* from the more common and widespread *O. querci*. The synnemata of *O. piceae* are also similar to those of *O. setosum*, but the cup-like, conidial-bearing structure at the top of the synnemata of *O. setosum* distinguishes this species from the rest of the *O. piceae* complex. Red-brown synnemata distinguish *O. floccosum* from *O. piceae* and other members of this complex. Although *O. canum* has the same ITS sequence as *O. piceae*, the former is distinguished by its globose conidia and brown synnemata stipe.

*Ophiostoma canum* (Münch) H. & P. Sydow, Ann. Mycol. 17: 43. 1919. FIG. 13

≡ *Ceratostomella cana* Münch, Naturw. Z. Forst. Landw. 5: 558. 1907.

≡ *Ceratocystis cana* (Münch) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17:22. 1952.

Anamorph. *Pesotum canum* (Upadhyay & Kendrick) Okada & Seifert, Can. J. Bot. 76: 1503. 1998.

≡ *Pachnodium canum* Upadhyay & Kendrick, Mycologia, 67: 802. 1975.

**Commentary.** The lectotype of *O. canum* (BPI595767 = BPI 2107) and two other specimens (BPI 595765 = BPI 2110 and BPI 595766 = FP 14394) from Münch were deposited in BPI by Hedgcock. The lectotype has perithecia and ascospores, while the other two specimens have synnemata and a *Sporothrix* anamorph but no perithecium. In addition to the isolates listed in TABLE I, we have examined Norwegian isolates NFRI 1652/2 (C1478) and NFRI 97-33/47 (C1477), both from *Tomicus minor* Hartig or stained *Pinus sylvestris* sapwood around galleries of this beetle, and globose conidia are also formed by these isolates. Mathiesen (1950, Mathiesen-Kåarik 1960) noted the association of *O. canum* with *T. minor*; and this beetle association distinguishes *O. canum* ecologically from *O. piceae*. Mathiesen-Kåarik (1960) also noted physiological differences between *O. canum* and *O. piceae*. To date, *O. canum*

is known only from pine in Europe, from Scandinavia and Germany.

Mathiesen (1950) and Upadhyay (1981) noted ascospores  $5\text{--}6 \times 1.5\text{--}2.5 \mu\text{m}$  and  $4\text{--}6.5 \times 1.5\text{--}3 \mu\text{m}$ , respectively, in *O. canum*, somewhat larger than those in *O. piceae*. The globose conidia of *O. canum* are particularly distinctive (FIG. 13) and separate it from all other members of the complex. Although we found that the ITS sequence of *O. canum* is the same as that of *O. piceae*, Kim et al (1999) reported that PCR primers specific for the ITS region of *O. piceae* were unable to amplify this fragment in *O. canum*. Hausner et al (1993) found that 259 of 261 aligned bases of the 26S rDNA gene were identical between a culture from the holotype of *O. piceae* and a Norwegian isolate of *O. canum*.

We have not been able to produce perithecia or ascospores with the two isolates (TABLE I) of *O. canum* that we have studied closely, perhaps because these two isolates are of the same mating type. However, we were able to produce a few perithecia when either of these two isolates were paired with mating type A testers of *O. piceae*. Ascospores produced from these hybrid crosses were generally inviable, but viable ascospore progeny were recovered from one perithecium of the cross between isolate C967 of *O. piceae* and isolate C114 of *O. canum*. Ten viable progeny were recovered, with globose conidia of *O. canum* and the elliptical conidia of *O. piceae* seen among the progeny. The fact that the *O. canum* isolates are only partially interfertile with *O. piceae* strains suggests that there is an intersterility barrier between these two species and further supports recognition of *O. canum* as distinct (Brasier 1993, Harrington and McNew 1998).

*Ophiostoma floccosum* Mathiesen, Svenka Bot. Tidskr. 45: 219. 1951. FIGS. 15–20

≡ *Ceratocystis floccosa* (Mathiesen) Hunt, Lloydia, 19: 36. 1956.

Anamorph. *Pesotum aureum* (Hedgcock) McNew et Harrington, comb. nov.

≡ *Graphium aureum* Hedgcock, Mo. Bot. Gard. Rept. 17: 94. 1906.

**Commentary.** Although well described by Mathiesen (1951), this species has been rarely reported by others, probably because it was thought to be a morphological variant of *O. piceae*. Also, perithecia and ascospores are not commonly seen in cultures of *O. floccosum*, and perithecia often take many weeks to form in mating tests. We have examined specimens and cultures of this species from Europe, North America, Australia, and New Zealand (TABLE I), and also isolates from Korea. All of these isolates were from Pinaceae.

TABLE III. *Ophiostoma* species with *Pesotum*-like anamorphs but without a *Sporothrix* synanamorph

	Teleomorph	Anamorph	Reference
<i>O. ainoae</i>	Solheim	<i>Pesotum</i> -like	Solheim (1986), Okada et al (1998)
<i>O. araucariae</i>	(Butin) de Hoog & Scheffer	<i>Pesotum</i> -like	Butin (1968), Hausner et al (1993), Okada et al (1998)
<i>O. arborea</i>	(Olchow. & Reid) Yamaoka & Wingf.	<i>Pesotum</i> -like	Okada et al (1998), Olchowecki and Reid (1974), Upadhyay (1981)
<i>O. brunneo-ciliatum</i>	Mathiesen-Käärík	<i>Pesotum</i> -like	Mathiesen-Käärík (1953), Okada et al (1998), Upadhyay (1981)
<i>O. cainii</i>	(Olchow. & Reid) Harrington	<i>Pesotum</i> -like	Okada et al (1998), Olchowecki and Reid (1974), Upadhyay (1981)
<i>O. clavatum</i>	Mathiesen	<i>Pesotum</i> -like	Hunt (1956), Mathiesen (1951)
<i>O. clavigerum</i>	(Robinson-Jeffrey & Davidson) Harrington	<i>Graphiocladiella clavigerum</i> Upadhyay ≡ <i>Pesotum clavigerum</i> Okada & Seifert	Okada et al. (1998), Robinson-Jeffrey and Davidson (1968), Upadhyay (1981)
<i>O. columnaris</i>	(Olchow. & Reid) Seifert & Okada	<i>Pesotum</i> -like	Okada et al (1998), Olchowecki and Reid (1974), Upadhyay (1981)
<i>O. cucullatum</i>	Solheim	<i>Pesotum</i> -like	Okada et al (1998), Solheim (1986)
<i>O. davidsonii</i>	(Olchow. & Reid) Solheim	<i>Pesotum</i> -like	Okada et al (1998), Olchowecki and Reid (1974), Upadhyay (1981)
Teleomorph not known		<i>Graphium fragrans</i> Mathiesen-Käärík ≡ <i>Pesotum fragrans</i> Okada & Seifert	Mathiesen-Käärík (1953), Okada et al (1998)
<i>O. galeiformis</i>	(Bakshi) Mathiesen-Käärík	<i>Pesotum</i> -like	Bakshi (1951), Mathiesen-Käärík (1953), Okada et al (1998)
<i>O. introcitrina</i>	(Olchow. & Reid) Hausner, Reid & Klassen	<i>Hyalopesotum introcitrinum</i> Upadhyay & Kendrick ≡ <i>Pesotum introcitrinum</i> Okada & Seifert	Hausner et al (1993), Okada et al (1998), Olchowecki and Reid (1974), Upadhyay (1981), Upadhyay and Kendrick (1975)
<i>O. ips</i>	(Rumbold) Nannf.	<i>Pesotum</i> -like	Okada et al (1998), Rumbold (1931), Upadhyay (1981)
<i>O. japonicum</i>	Yamaoka & M.J. Wingf.	<i>Pesotum</i> -like	Yamaoka et al (1997)
<i>O. obscura</i>	(Davidson) von Arx	<i>Pesotum</i> -like	Davidson (1935), Hunt (1956)
<i>O. olivaceum</i>	Mathiesen	<i>Pesotum</i> -like	Hunt (1956), Mathiesen (1951), Okada et al (1998), Olchowecki and Reid (1974), Upadhyay (1981)
<i>O. olivaceapinii</i>	(Davidson) Seifert & Okada	<i>Pesotum</i> -like	Davidson (1971), Okada et al (1998), Upadhyay (1981)

TABLE III. Continued

Teleomorph	Anamorph	Reference
Teleomorph not known	<i>Hyalopesotum pini</i> Hutchinson & Reid ≡ <i>Pesotum pini</i> Okada & Seifert	Hutchinson and Reid 1988b
<i>O. sagnatospora</i> (Wright & Cain) Solheim	<i>Phialographium sagnatospora</i> Upadhyay & Kendrick ≡ <i>Graphium sagnatospora</i> Wingfield & Kendrick ≡ <i>Pesotum sagnatosporum</i> Okada & Seifert	Okada et al (1998), Upadhyay (1981), Upadhyay and Kendrick (1975), Wingfield et al (1991), Wright and Cain (1961)
<i>O. simplex</i> Jacobs & M.J. Wingf.	<i>Graphium simplex</i> Jacobs & M.J. Wingf.	Jacobs et al (1997)
<i>O. sparsum</i> (Davidson) de Hoog & Scheffer	<i>Graphilium sparsum</i> Upadhyay & Kendrick ≡ <i>Pesotum sparsum</i> (Upadhyay & Kendrick) Okada & Seifert	Davidson (1971), Okada et al (1998), Upadhyay (1981), Upadhyay and Kendrick (1975)
<i>O. torticiliata</i> (Olchow. & Reid) Seifert & Okada	<i>Pesotum-like</i>	Okada et al (1998), Olchowecki and Reid (1974), Upadhyay (1981)
<i>O. truncicolor</i> Davidson	<i>Pesotum-like</i>	Davidson (1955), Griffin (1966), Upadhyay (1981)
<i>O. vesca</i> (Davidson) Hausner, Reid & Klassen	<i>Pesotum-like</i>	Davidson (1958), Griffin (1966), Okada et al (1998)

No holotype for *O. floccosum* was designated by Mathiesen, but herbarium material is apparently available in Statens Skogsforskningsinstitut Experimentalfältet, Sweden (Hunt 1956). A culture from Kåårik deposited in CBS (799.73) is considered authentic for the type but does not produce perithecia and ascospores, unless mated with – (minus) mating type strains. Tester strains of an intersterility group identified by J. Webber proved to be *O. floccosum* in morphology and ITS sequence. We deposited a dried specimen (BPI 746439) with perithecia and ascospores that resulted from pairing CBS 799.73 (from the holotype) and C1013 (a tester strain from Britain provided by Webber).

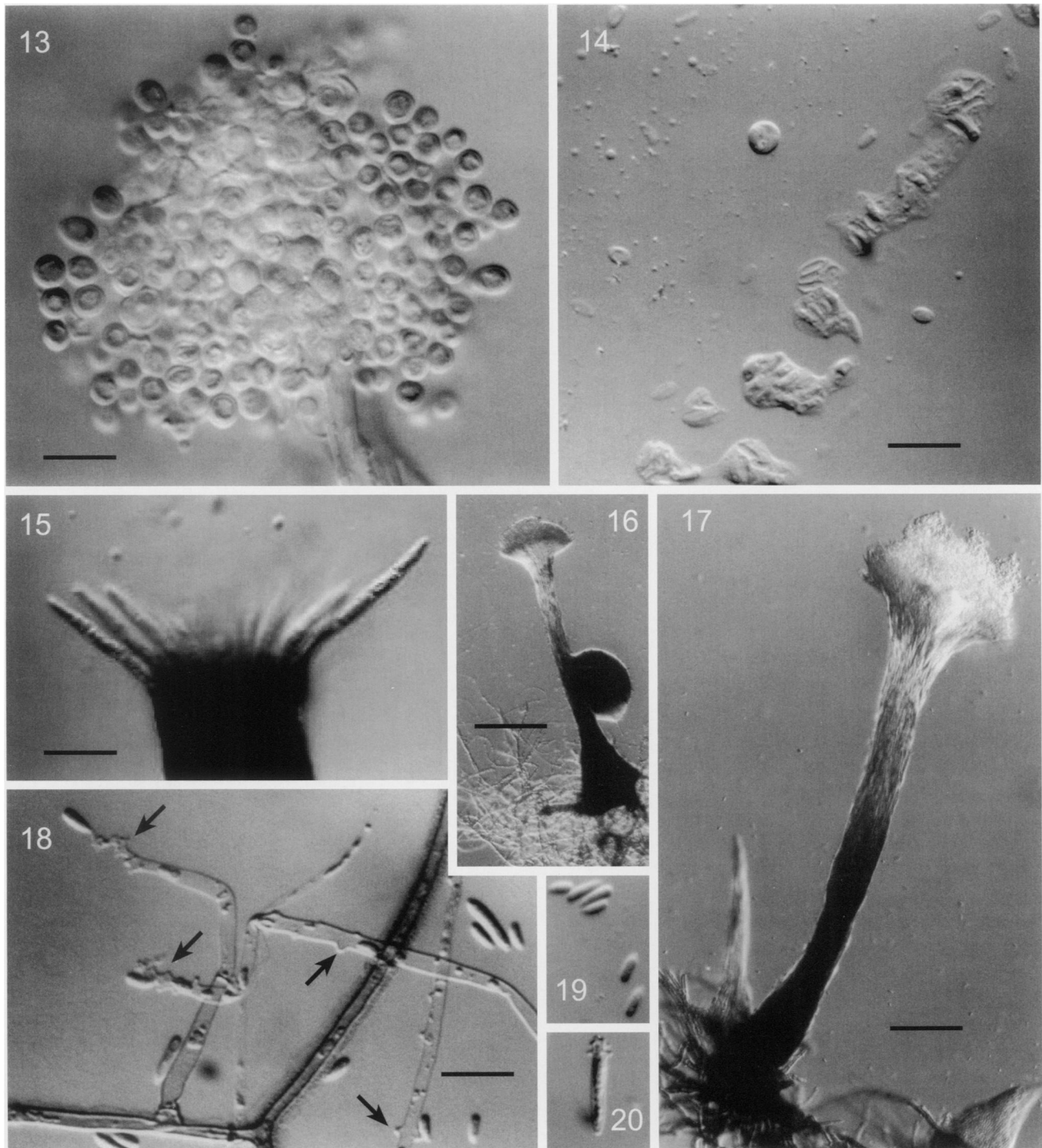
Mathiesen (1951) noted the relatively short ostiolar hyphae of this species when compared to *O. piceae*. We see little difference between the two species (FIGS. 5, 15), though ascospore masses at the top of perithecia are relatively small for *O. floccosum*. Synnemata of *O. floccosum* are distinctly red-brown, and lateral knobs on synnemata (FIG. 16) are common in all of the cultures of *O. floccosum* that we have examined. Also, secondary synnemata are frequently found emanating from the conidial mass on top of primary synnemata. Conidial masses on synnemata are yellow compared to white in fresh spore masses of other species in the complex.

Hedgcock (1906) described *Graphium aureum* from stained sapwood of *Pinus strobus* in Wisconsin. His description includes white to yellow conidial masses and stipes that are white at first, changing to yellow and often to dark brown at the base. The yellow conidial masses described by Hedgcock (1906) are typical for *O. floccosum*, though our cultures generally produce red-brown stipes instead of yellow to brown stipes. Hedgcock's type material is available (BPI 448701 and BPI 448702), and both specimens contain red-brown synnemata and black protoperithecia, which are also common for *O. floccosum*. A *Sporothrix*-like anamorph was noted by Hedgcock and was observed in the BPI specimens. Thus, we believe that Hedgcock's *Graphium* species is the anamorph of *O. floccosum*, and we have transferred his fungus to *Pesotum*. A culture deposited by Kåårik (CBS 277.54 = ATCC 24586 = JCM 9745 = C1283) as *G. aureum* mated with *O. querci* tester strains as discussed above and does not represent *G. aureum*.

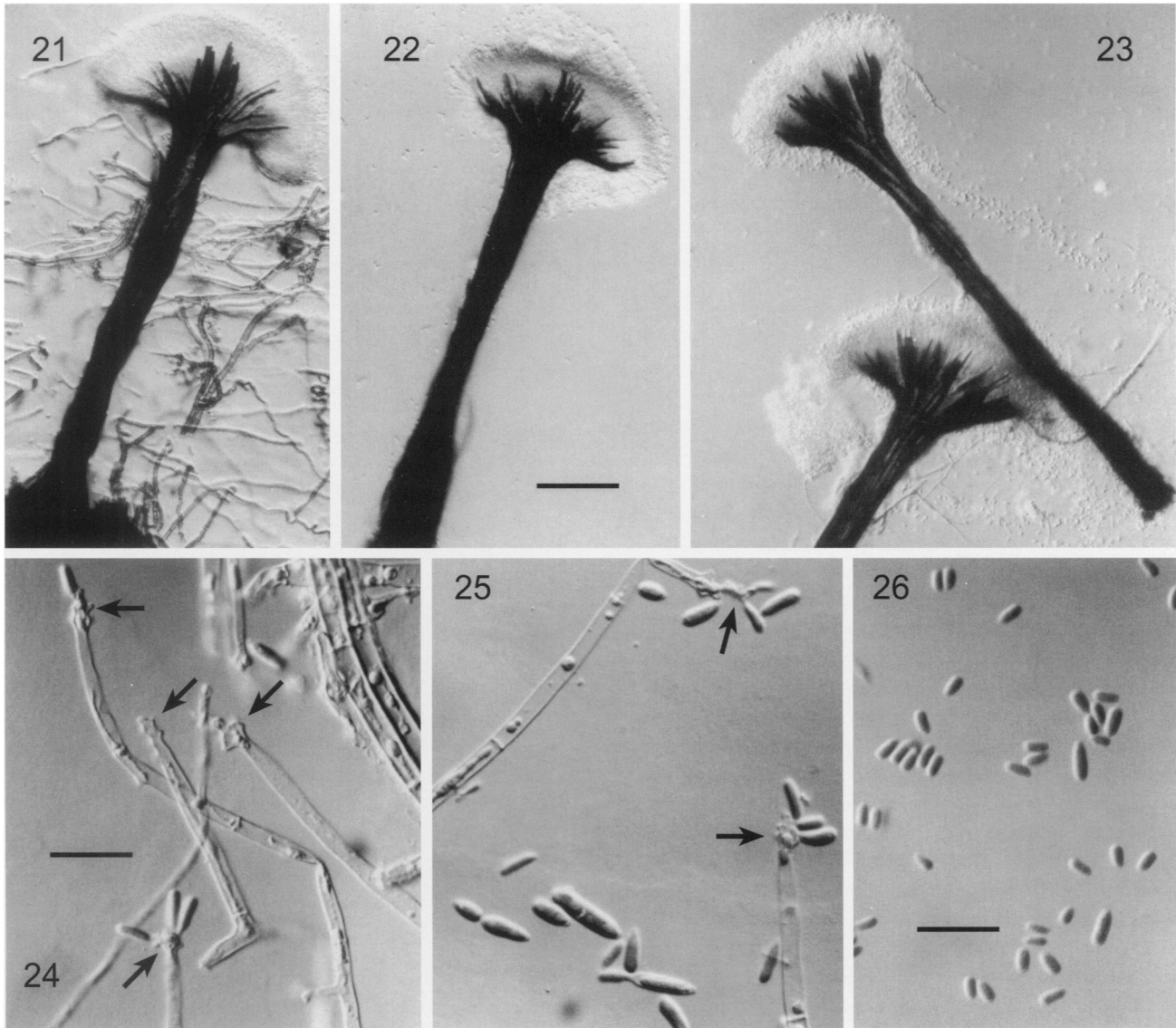
*Ophiostoma setosum* Uzunovic, Seifert, Kim & Breuil, Mycol. Res. 104:490. 2000.

Anamorph. ***Pesotum cupulatum*** McNew et Harrington sp. nov. FIGS. 21–26

Coloniae olivaceae-brunneae de infra, in agar extracti multi diam. 55 mm attingens post 11 dies ad 25 C, odore



FIGS. 13–20. *Ophiostoma canum* and *O. floccosum*. 13, 14. *O. canum*. 13. Synnema apex with globose conidia. 14. Aborted asci and ascospores from a crossing of *O. canum* and *O. piceae*. 13 from the LECTOTYPE (BPI 595765); 14 from a cross of strain C114 and strain C967. 15–20. *O. floccosum*. 15. Ostiolar hyphae. 16, 17. Synnemata. 18, 19. Conidiophores and conidia of *Sporothrix* synanamorph. Arrows in 18 indicate prominent denticles on apex and side walls of conidiogenous cells. 20. Primary conidium from *Sporothrix* synanamorph, with numerous denticles at apex where secondary conidia were attached. 15 from a cross of strain C1013 and C1086; 16–20 from strain 1086. Scale bars: 13–15 = 10  $\mu\text{m}$ ; 16 = 100  $\mu\text{m}$ ; 17 = 50  $\mu\text{m}$ ; 18–20 = 10  $\mu\text{m}$ .



FIGS. 21–26. *Pesotum cupulatum*, from the HOLOTYPE, anamorph of *Ophiostoma setosum*. 21–23. Synnemata. 24–25. Conidiophores and conidia of the *Sporothrix* synanamorph. Arrows in 24 and 25 indicate apex of conidiogenous cells, showing prominent denticles. 26. Conidia from synnema. Scale bars: 21–23 = 50  $\mu\text{m}$ ; 24–26 = 10  $\mu\text{m}$ .

dulci. Incremento nullo ad 32 C. Synanamorpha primaria stipite 80–820  $\mu\text{m}$  longo, cum cellulis fuscae protrudens in zonam conidiogenam, calyx formans. Conidia unicellularia hyalina, cylindrica vel obovoidea, 3.0–5.5  $\mu\text{m}$   $\times$  1.0–2.0  $\mu\text{m}$ . Synanamorpha altera, simili *Sporotrichi* in denticules conspicui ubi sporae portatae et quoque in catena acropeta conidia holoblastica efferens.

*Colonies* on malt extract agar at 25 C attaining a diameter of 55 mm in 11 d, appearing olive-green to gray from above, olive-green to brown from below, with concentric rings developing on the upper surface of the mycelium with age, with a distinctly sweet aroma. Cultures not inhibited by cycloheximide. No growth at 32 C on malt extract agar. *Hyphae* mostly submerged, aerial hyphae appressed, hyaline,

smooth-walled, 1.0–3.0  $\mu\text{m}$  wide, to brown, smooth or rough-walled, 2.5–4.0  $\mu\text{m}$  wide. Conidiophores of two types: *synnemata* (FIGS. 21–23) single or in groups of two to three, simple or rarely branched, macronematous. Stipe dark brown to black at base, becoming lighter towards apex, 80–820  $\mu\text{m}$  (mean 290  $\mu\text{m}$ ) long, base 10–40  $\mu\text{m}$  (mean 23  $\mu\text{m}$ ) wide, tapering to 6–32  $\mu\text{m}$  (mean 16  $\mu\text{m}$ ) near the apex and flaring to 14–120  $\mu\text{m}$  (mean 45  $\mu\text{m}$ ). Many of the synnema stipe cells extend into the mass of conidiogenous cells and conidia, forming a cup of brown to black, setae-like structures. Conidiogenous cells proliferating sympodially with inconspicuous conidial scars. *Synnematous conidia* (FIG. 26) hyaline, one-

TABLE IV. Restriction fragments of the amplified ITS product from species in the *Ophiostoma piceae* complex

Species	HaeIII fragments (bp)	DdeI fragments (bp)
<i>O. piceae/O. canum</i>	400, 200, 120	290, 270, 190
<i>O. floccosum</i>	280, 200, (120) <sup>a</sup>	290, 270, 190
<i>O. setosum</i>	400, 200, 120	270, (190) <sup>a</sup> , 100
<i>O. querci/O. cationianum</i>	520, 200	290, 270, 190
<i>O. ulmi/O. novo-ulmi/ O. himal-ulmi</i>	520, 170	290, 270, 190

<sup>a</sup> Band sizes in parentheses are for two co-migrating bands.

celled, mostly guttulate, cylindrical to obovoid with rounded apices and slightly tapering bases, which may be truncate to rounded, 3.0–5.5 µm (mean 4.0 µm) long and 1.0–2.0 µm (mean 1.6 µm) wide, accumulating in a white, gelatinous mass at the apex of the synnema. *Micronematous conidiophores* typical of the genus *Sporothrix* (FIGS. 24, 25), arising terminally or laterally from hyphae, 11–156 µm (mean 73 µm) long, 1.0–4.0 µm wide at the base, tapering slightly at the apex, sometimes septate. Conidia produced at the apex of the conidiogenous cell (FIGS. 24, 25), which has prominent denticles, 0.5–1.5 µm long, where conidia have abscised along a rachis 1.5–21 µm (mean 5.5 µm) long. Conidiogenous cells may proliferate at or below the rachis. *Sporothrix conidia* hyaline, smooth-walled, 6.5–24 µm (mean 12.0 µm) long, 2.0–3.5 µm (mean 2.5 µm) wide, with a rounded apex and tapering base, often giving rise to secondary conidia at the apex and having denticles at the point of dehiscence of the secondary conidia. *Secondary conidia* hyaline, smooth-walled, guttulate, cylindrical to obovoid, 3.0–8.0 µm (mean 5.5 µm) long, 1.0–2.5 µm (mean 2.0 µm) wide, with a rounded apex and tapering at the base. No protoperithecium or perithecium seen.

**HOLOTYPE.** USA. WASHINGTON: Aberdeen, from stained wood of *Pseudotsuga menziesii*, Dec 1997, *T. Harrington*, BPI 746441, from isolate C1194 (CBS 102358).

**Etymology.** Refers to the cup-like structure at the top of the synnemata.

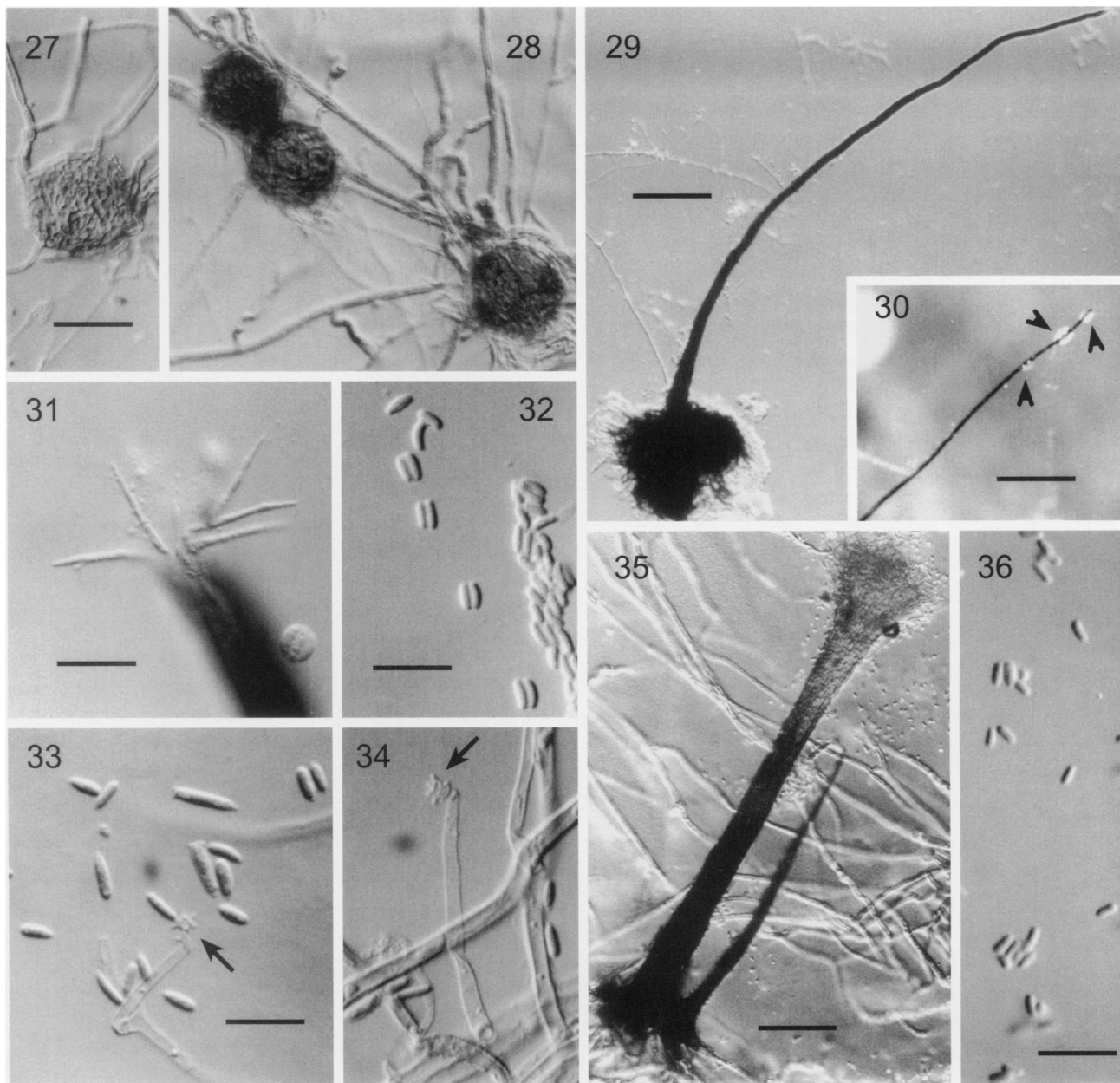
**Commentary.** Cultures representing the two mating types of a new *Ophiostoma* species (*O. setosum*) from Canada were kindly provided to us by A. Uzunovic (Uzunovic et al 2000) after our initial mating studies were completed. Pairings of our *P. cupulatum* isolates with the tester strains of *O. setosum* showed that all of our isolates were of the same mating type (B, TABLE I). After repeated pairings at 20 C, the isolates of *P. cupulatum* from the USA and New Zealand (TABLE I) were able to mate with the tester strains provided

by Uzunovic. Morphologically, our isolates and the Canadian isolates (Uzunovic et al 2000) are identical.

Synnemata of *O. setosum* are similar in size and pigmentation to those of *O. piceae* and *O. querci*, but the apex of the synnemata of *O. setosum* is unique in its cup-like structure formed by extension of the external stipe cells into the conidial mass (FIGS. 21–23). The concentric rings of aerial mycelium found in most isolates and the distinctively sweet aroma of cultures of *O. setosum* also distinguish this species from *O. piceae* (TABLE II). *Ophiostoma setosum* fails to grow at 32 C, as does *O. piceae*. In ITS sequence, *O. setosum* is close to *O. piceae*, and we have found *O. setosum* only on wood of conifers (Pinaceae), the most common substrate of *O. piceae*.

We originally isolated *O. setosum* from stained wood of *Pinus radiata* in New Zealand. Additional isolations were made from lumber of *Pseudotsuga menziesii* and *Tsuga* sp. from Washington, USA and from *Pinus radiata* wood from California. Uzunovic et al (2000) identified isolates from *Picea*, *Pinus*, and *Tsuga* in British Columbia, Canada and Oregon, USA. Thus, the species appears endemic to the Pacific Coast of North America on various Pinaceae and was likely introduced to New Zealand. Synnemata of this species are commonly found in association with *O. piceae*, *O. querci*, and *O. floccosum* on bluestained wood.

- Ophiostoma querci* (Georgévitch) Nannf., in Melin and Nannf., Sven. Skogsvardsforen Tidskr. 32: 408. 1934. FIGS. 27–36  
 ≡ *Ceratostomella querci* Georgévitch, C. R. Acad. Sci. Paris 183: 759. 1926.  
 ≡ *Ceratocystis querci* (Georgévitch) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22. 1952.  
 = *Ophiostoma fagi* (Loos) Nannf., in Melin & Nannf., Sven. Skogsvardsforen Tidskr. 32: 408. 1934.  
 ≡ *Ceratostomella fagi* Loos, Arch. Mikrobiol. 3: 376. 1932.  
 ≡ *Ceratocystis fagi* (Loos) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22. 1952.  
 ?= *Ophiostoma roboris* Georgescu & Teodoru, Anal. Inst. Cerc. Exp. For., Ser. 1, 11: 207. 1948.  
 ≡ *Ceratocystis roboris* (Georgescu & Teodoru) Potlajchuk, Nov. Sist. Niz. Rast. 22: 154. 1985.  
 ?= *Ophiostoma valachicum* Georgescu & Teodoru, Anal. Inst. Cerc. Exp. For., Ser. 1, 11: 198, 1948.  
 ≡ *Ceratocystis valachicum* (Georgescu & Teodoru) Potlajchuk, Novosti Sist. Niz. Rast. 22: 155. 1985.  
 ?= *Ophiostoma kubanicum* Sherbin-Parfenenko, Rak. Sos. Bol. List. Porod (Moscow) p. 49. 1953.  
 ≡ *Ceratocystis kubanicum* (Sherbin-Parfenenko) Potlajchuk, Novosti Sist. Niz. Rast. 22: 153. 1985.  
*Anamorph.* ?*Graphium roboris* Georgescu & Teodoru, Anal. Inst. Cerc. Exp. For., Ser. 1, 11: 213. 1948.



FIGS. 27–36. *Ophiostoma querci*. 27, 28. Protoperithecia. 29. Perithecium. 30. Perithecial neck with drops of ascospores (arrowheads). 31. Ostiolar hyphae. 32. Ascospores. 33, 34. Conidiophores and conidia of the *Sporothrix* synanamorph. Arrows in 33 and 34 indicate apex of conidiogenous cells, showing prominent denticles. 35. Synnema. 36. Conidia from synnema. 27, 28, 33–36 from strain C969; 29–32 from a pairing of 969 and C970. Scale bars: 27, 28 = 25  $\mu\text{m}$ ; 29 = 100  $\mu\text{m}$ ; 30 = 220  $\mu\text{m}$ ; 31–34, 36 = 10  $\mu\text{m}$ ; 35 = 50  $\mu\text{m}$ .

?= *Graphium kubanicum* Potlajchuk & Schekunova, Novosti Sist. Niz. Rast. 22: 153. 1985.

**Commentary.** Unfortunately, no type material or culture was preserved by Georgévitch, and no Latin description was made. However, this name has been commonly applied to the so-called “hardwood” form of the *O. piceae* complex, and we continue to apply the name in this sense. Morelet (1992) designated neotype material (PFN 1463) for *O. querci*, which was derived from

mating two French isolates (CBS 366.93 and CBS 367.93). We have deposited a dried specimen (BPI 746440) with perithecia and ascospores resulting from a cross of two British isolates, C969 and C970.

*Ophiostoma querci* is difficult to distinguish morphologically from *O. piceae*, though ITS sequencing shows it to be only distantly related (FIG. 1). It is common to find small, glistening drops of ascospores along the neck of *O. querci* perithecia (FIG. 30),

which are due to exudations of ascospores as the perithecial neck elongates. We have not seen this in perithecia of the other species in the *O. piceae* complex, but we have seen such ascospore droplets along the neck of *O. pluriannulatum* perithecia. Most isolates of *O. querci* and *O. piceae* form protoperithecia on MEA, but those of *O. querci* are a light, golden-brown color (FIGS. 27, 28), while those of *O. piceae* are black (FIGS. 2, 3). Most isolates of *O. querci* have a nut-like aroma when growing on MEA, in contrast to an indistinct aroma in cultures of *O. piceae* (TABLE II). Also, *O. querci* isolates grow at 32 C, while *O. piceae* isolates do not (Brasier and Stephens 1993, TABLE I). Many isolates of *O. querci* form concentric rings of aerial mycelium on MEA, similar to isolates of the Dutch elm disease fungi (TABLE II).

In pairings, *O. querci* produces perithecia and ascospores when isolates of opposite mating types are paired. An isolate (CBS 236.32) of *O. fagi* from the holotype had the ITS sequence of *O. querci* and mated with this species. It is, therefore, considered a synonym of *O. querci*, as was suggested by Brasier (1993). Three other *Ophiostoma* species (*O. roboris*, *O. kubanicum*, and *O. valachicum*) from *Quercus* in eastern Europe are likely synonyms of *O. querci* (Przybyl and de Hoog 1989), but there appears to be no authentic material available of these three species (Przybyl and de Hoog 1989). From descriptions by Potlajczuk and Schekunova (1985), *O. roboris*, *O. kubanicum*, and *O. valachicum* are indistinguishable from *O. querci*. Both *O. roboris* and *O. kubanicum* have been isolated from *Quercus* from central Europe to Azerbaijan (Brasier and Kirk 1993). An isolate of *O. roboris* from Azerbaijan by Guseinov paired with testers of *O. querci* to produce perithecia and ascospores (TABLE I), as was reported by Brasier and Kirk (1993).

Both Morelet (1992) and Okada et al (1998) list *Pesotum pirinum* as the anamorph of *O. querci*. We consider *O. catonianum* and its anamorph, *P. pirinum*, as distinct from *O. querci* and *O. piceae*, leaving no clear anamorph name for *O. querci*. An isolate deposited as *Graphium aureum* (CBS 277.54, C1283) had the ITS sequence of *O. querci*, and it paired with our tester strains of *O. querci*. However, examinations of the holotype specimen and description of *G. aureum* (Hedgcock 1906) show that it is the anamorph of *O. floccosum*, as noted above, and CBS 277.54 was misidentified as *G. aureum*. Should *O. roboris* prove to be a validly described synonym of *O. querci*, then the anamorph name *Graphium roboris* is available as a basionym for the anamorph of *O. querci*.

*Ophiostoma querci* appears to be a widespread species and is commonly found on conifers and hardwoods throughout Europe and North America (Brasier and Kirk 1993, Halmschlagler et al 1994, Kim et

al 1999, Morelet 1992, Pipe et al 1995). In addition to isolates from South Africa and New Zealand listed in TABLE I, we have identified isolates of *O. querci* from *P. radiata* in Australia, *Eucalyptus* sp. in Uruguay and *Pinus* sp. in Korea. In Europe, it apparently occurs more frequently on hardwoods, especially oak, than on conifers (Brasier and Kirk 1993, Halmschlagler et al 1994, Morelet 1992, Pipe et al 1995). In North America and the Southern Hemisphere, however, sapwood of Pinaceae is a common substrate.

*Ophiostoma catonianum* (Goid.) Goid., Boll. Stn. Patol. Veg. Roma, N. S., 15: 125. 1935.

≡ *Ceratostomella catonianum* Goid., Atti. Accad. Naz. Linc. Rc. Ser. 6, 21: 199. 1935.

≡ *Ceratocystis catonianum* (Goid.) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22. 1952.

Anamorph. *Pesotum pirinum* (Goid.) Okada & Seifert, Can. J. Bot. 76: 1504. 1998.

≡ *Graphium pirinum* Goid., Boll. Stn. Patol. Veg. Roma, N. S., 15: 132. 1935.

Synanamorph. *Sporothrix pirinum* (Goid.) Morelet, Extrait Annales S.S.N.A.T.V. 44: 110. 1992.

≡ *Hyalodendron pirinum* Goid., Boll. Stn. Patol. Veg. Roma, N. S., 15: 136. 1935.

*Commentary.* Although there appears to be no type specimen for *O. catonianum* (Hunt 1956), a culture of *O. catonianum* deposited by Goidanich is available from CBS (263.35). The ITS sequence of this isolate is unique but near that of *O. querci* (FIG. 1). The culture does not form protoperithecia, perithecia, or synnemata, and pairings with testers of the other species in the complex failed to result in perithecia. A *Sporothrix* anamorph was found in the culture, however. This culture grows very slowly at 32 C, in contrast to *O. querci* (TABLE I).

Goidanich (1935) described this species from *Pyrus* (pear) in Italy. His description of *O. catonianum* indicates that the perithecial necks are shorter and the ostiolar hyphae longer than those of *O. piceae*, *O. querci*, and *O. floccosum*. He also states that single ascospore and single conidial isolates produce perithecia and ascospores. Thus, *O. catonianum* is homothallic, in contrast to the heterothallic mating system of all other species in the complex.

*Ophiostoma ulmi* (Buisman) Nannf., in Melin and Nannfeldt, Svenska Skorgsfor. Tidskr. 32: 408. 1934.

≡ *Ceratostomella ulmi* Buisman, Tijdskr. Plantenziekt. 38: 1. 1932.

≡ *Ceratocystis ulmi* (Buisman) Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22. 1952.

Anamorph. *Pesotum ulmi* (Schwarz) Crane & Schoknecht, Am. J. Bot. 60: 348. 1973.



≡ *Graphium ulmi* Schwarz, Meded. Phytopathol. Lab. 5: 13. 1922.

**Commentary.** This species, and the following two species, are capable of causing Dutch elm disease, a vascular wilt disease, in inoculated elms (Brasier and Mehrotra 1995). Morphological and cultural characteristics of these three species are contrasted in detail by Brasier and Mehrotra (1995) and need not be reiterated here.

The natural geographic range of *O. ulmi* is still not clear (Brasier and Mehrotra 1995), but it was apparently introduced into Europe and North America, where dramatic losses to *Ulmus* species were found. In contrast to *O. quercii*, synnemata are generally not formed by isolates of *O. ulmi* on MEA or MYEA, but addition of twigs or linoleic acid to the medium stimulates synnemata production (Dalpé and Montant 1980). The typically pronounced concentric rings of the mycelium and relatively fast growth at 32 C distinguish this species from *O. novo-ulmi* (Brasier and Mehrotra 1995) and *O. quercii*. Only *O. ulmi* isolates grow at a relatively rapid rate at 32 C (TABLE I), and Brasier and Mehrotra (1995) have speculated that it may occur naturally in a tropical region of Asia.

*Ophiostoma novo-ulmi* Brasier, Mycopathologia 115: 155. 1991.

**Commentary.** The natural geographic range of *O. novo-ulmi* is also in question, though an Asian origin is suggested (Brasier and Mehrotra 1995). Like *O. ulmi*, *O. novo-ulmi* causes a devastating vascular wilt disease in *Ulmus* species in Europe and North America, and the fungus has been introduced to New Zealand. Isolates of *O. novo-ulmi* and *O. ulmi* are similar to each other, though differences in mycelium texture, temperature sensitivity, and growth rate (Brasier and Mehrotra 1995) separate these species, which are closely related based on ITS sequences (FIG. 1).

*Ophiostoma himal-ulmi* Brasier & M. D. Mehrotra, Mycol. Res. 99: 211. 1995.

**Commentary.** Thus far, *O. himal-ulmi* is known only from the Himalayas from branches of *Ulmus* species attacked by bark beetles (Brasier and Mehrotra 1995). Although thought to be closely related to *O. ulmi* and *O. novo-ulmi*, the ITS sequence data suggest that it is more closely related to *O. quercii*. Unlike the other Dutch-elm disease fungi, *O. himal-ulmi* produces synnemata on MEA, and morphologically it is difficult to distinguish from *O. quercii*. However, cultures of *O. himal-ulmi* do not produce the nutty aroma of *O. quercii* and grow slower than *O. quercii* isolates at 32 C (TABLE I).

**Synnematous *Ophiostoma* species without *Sporothrix* synanamorphs.**—A number of *Ophiostoma* species have been noted to have synnemata anamorphs but no *Sporothrix* synanamorph (TABLE III). *Ophiostoma novae-zelandiae* (Hutchison & Reid) Rulamort was said to rarely form synnemata (Hutchison and Reid 1988a), but we have not seen synnemata in a dried culture from the holotype [WIN (M) 869] or in living cultures (UAMH 9556-9) from New Zealand (Hutchison and Reid 1988a). The original description may have been from a culture of more than one species. Perithecia and ascospores as described by Hutchison and Reid (1988a) and as seen in the holotype look like those of *O. pluriannulatum*, which is not reported to have synnemata (Hunt 1956, Upadhyay 1981).

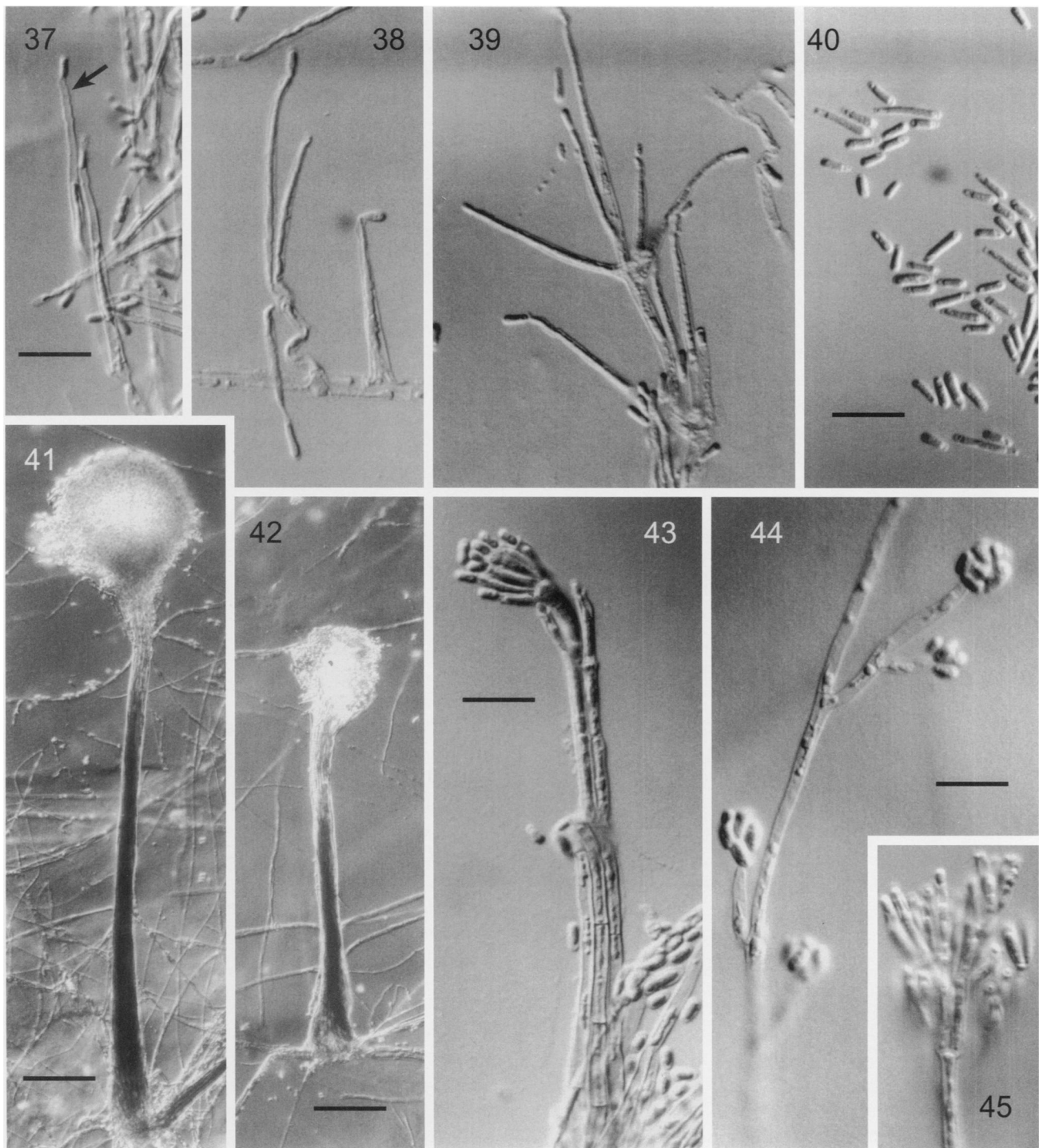
Reports of synnemata in *Ophiostoma* species other than those in the *O. piceae* complex have been of species without known *Sporothrix* synanamorphs, though many produce *Hyalorhinocladiella* synanamorphs. These species (TABLE III) are excluded from the *O. piceae* complex. Two such species are discussed below.

*Pesotum fragrans* (Mathiesen-Kåårik) Okada & Seifert, Can. J. Bot. 76: 1503. 1998. FIGS. 37–40  
≡ *Graphium fragrans* Mathiesen-Kåårik, Medd. Statens Skogsforskningsinst. 43: 59. 1953.

**Commentary.** An isolate deposited as *Graphium fragrans* (C1224 = CBS 279.54 = ATCC 24590) by Kåårik likely represents the species. However, this Swedish isolate no longer produces synnemata but does produce *Leptographium*-like or *Hyalorhinocladiella*-like conidiophores (FIGS. 37–39) that may represent degenerate synnemata. No denticles typical of *Sporothrix* are seen in the culture. The conidia (FIG. 40) of this species are longer than those of members of the *O. piceae* complex.

This species does appear to be an anamorph of an *Ophiostoma* species based on ITS sequence (AF198248), but it does not fall into the *O. piceae* complex (data not shown). Similar cultures with almost identical ITS sequences were isolated from pine wood in California, New Zealand and Australia (unpubl).

Okada et al (1998) list CBS 219.83 as authentic for the type of *G. fragrans*, but this isolate was collected by Solheim, not Kåårik, and CBS 279.54 should be authentic for the type. Okada et al (1998) found sympodial proliferation of the conidiogenous cells of synnemata and mononematous conidiophores of CBS 219.83, and they also found that the 18S rDNA sequence of this isolate was similar to that of other *Ophiostoma* species.



FIGS. 37–45. *Graphium fragrans* and *O. cucullatum*. 37–40. *G. fragrans* strain C1224, AUTHENTIC FOR TYPE. 37–39. Conidiophores and conidia. Arrow in 37 indicates abscission scars along the side of apex of conidiogenous cell. 40. Conidia. 41–45. *O. cucullatum*. 41–42. Synnemata. 43. Synnemata with metulae, phialides, and conidia. 44. Micronematous conidiophores and conidia. 45. Apex of *Leptographium*-type conidiophore with metulae, phialides and conidia. 41, 44 from strain 1216 as *O. cucullatum*; 42, 43, 45 from strain C1222, AUTHENTIC FOR TYPE of *Phialographium erubescens*. Scale bars: 37–40, 43–45 = 10  $\mu\text{m}$ ; 41, 42 = 50  $\mu\text{m}$ .

*Ophiostoma cucullatum* Solheim, Nord. J. Bot. 6: 202. 1986. FIGS. 41–44

Anamorph. **Phialographium erubescens** (Mathiesen-Kåårik) Harrington et McNew, comb. nov.

≡ *Pesotum erubescens* (Mathiesen-Kåårik) Okada, Studies in Mycology 45: 184. 2000.

≡ *Graphium erubescens* Mathiesen-Kåårik, Medd. Statens Skogsforskningsinst. 43: 62. 1953.

**Commentary.** The ITS sequence of *O. cucullatum* (AF198246, from isolate C1216 = NFRI 81–83/2, collected by Solheim) differed in only a single base substitution from that of *Graphium erubescens* (CBS 278.54 = JCM 9747 = C1222, from the holotype) and another culture deposited by Kåårik as *G. album* (CBS 276.54 = JCM 9744 = C1225, ITS sequence = AF198247). This isolate of *G. album* is morphologically identical to *G. erubescens* and is distinct from specimens representing *G. album* as emended by Hedgcock (1906). We believe that Kåårik's isolate of *G. album* was misidentified and that *G. erubescens* is the anamorph of *O. cucullatum*.

The ITS sequences of *O. cucullatum* and *G. erubescens* were not similar to those of other members of the *O. piceae* complex. Okada et al (1998) found the 18S rDNA sequence of *O. cucullatum* to be similar to that of *O. europhioides*, which has a *Leptographium* anamorph. Likewise, Hausner et al (1993) found the partial 28S rDNA sequence of *O. cucullatum* to be similar to those of other *Ophiostoma* species with *Phialographium* and *Leptographium* anamorphs.

All three cultures of *O. cucullatum* (C1216, C1222, C1225) failed to form *Sporothrix* conidiophores on MEA but did form synnemata with loosely- to tightly-fused outer stipe cells (FIGS. 41–43), with gradations to mononematous (*Leptographium*-type) conidiophores (FIG. 45). In general, the aerial synnemata are more tightly woven, and the synnemata submerged in the agar medium are a loose collection of *Leptographium*-like conidiophores. The conidiogenous cells on top of the synnemata and mononematous conidiophores are phialidic based on light microscopy (FIGS. 43, 45), as described for the synnemata of *O. cucullatum* (Solheim 1986). Okada et al (2000) reported that the conidiogenous cells of *P. erubescens* (isolate CBS 278.54) usually proliferate percurrently but also have an intermediate mode between sympodial proliferation and phialidic ontogeny based on scanning electron microscopy.

The anamorph of *O. sagmatosporae* also has conidiogenous cells that are phialidic in light microscopy (Seifert and Okada 1993), and this anamorph was the basis for the synnema-forming genus *Phialographium* Upadhyay & Kendrick. Okada et al (1998) consider the synnemata anamorphs of *O. sagmatosporae* and *O. cucullatum* to be *Pesotum* species, and

they (Okada et al 2000) formally transferred *G. erubescens* to *Pesotum*. However, the phialidic conidiogenous cells, the loose aggregation of *Leptographium*-like conidiophores, and the absence of a *Sporothrix* anamorph lead us to believe that these species are distinct from *Pesotum* as originally conceived by Crane and Schoknecht (1973). Further, *O. cucullatum* and *O. sagmatosporae*, like most other *Ophiostoma* species with *Leptographium* or *Phialographium* anamorphs, have ascospores with thickened outer walls (sheaths) that are cucullate, to rectangular, to triangular, depending on the view (Solheim 1986). In contrast, ascospores in the *O. piceae* complex are allantoid to orange section shaped, and are relatively thin-walled, with no visible sheath.

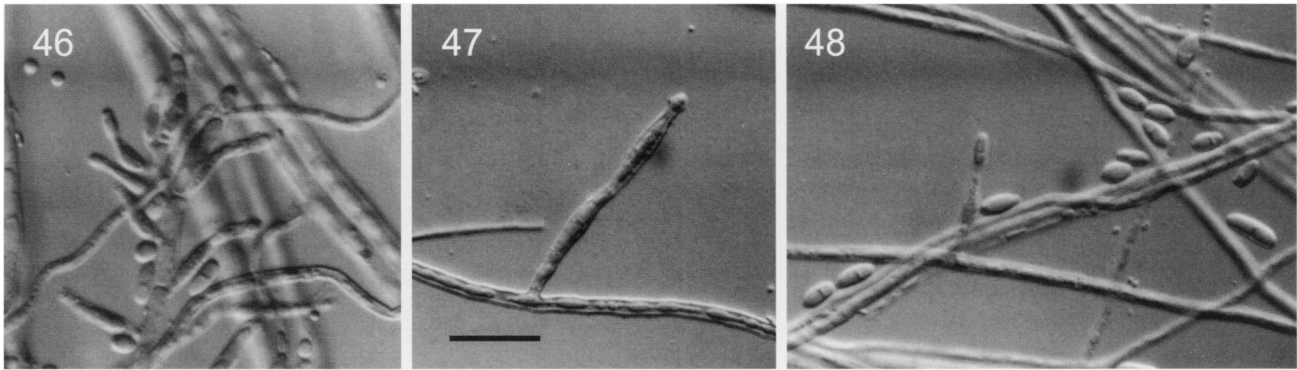
**Graphium species excluded from Ophiostoma.**—There are numerous *Graphium*-like species that are related to ascomycetes other than *Ophiostoma* (Okada et al 1998), and it is beyond the scope of the present paper to discuss these in detail. However, we did examine material of five *Graphium* species from Hedgcock's specimens in BPI and other CBS cultures, and these examinations suggest that they are not closely related to *Ophiostoma* species.

*Graphium album* (Corda) Sacc., Syll. Fung. 4: 618. 1886. emended Hedgcock, Mo. Bot. Gard. Rept. 17: 96. 1906.

≡ *Ceratopodium album* Corda, Ic. Fung. 1: 20. 1837.

**Commentary.** No type material of Corda's specimen is available (Seifert, pers comm), and his illustrations give little indication of the affinities of this species. It was found on *Betula* in Bohemia and described by Saccardo (1886) as having conidial masses that were white. Hedgcock (1906) emended the species description to include conidial masses white to creamy-yellow, or light brown when old and dry, and our examination his specimen (BPI 448665, from *Fagus* in Arkansas, USA) showed the conidial masses to be yellow-gold. The yellow-gold to light-brown conidial masses of the BPI specimen suggests that *G. album* as emended by Hedgcock is not an *Ophiostoma* species. However, Hedgcock's fungus may not be *G. album*, and the disposition of Corda's species remains in question.

A culture from *Pinus* in CBS (276.54, deposited by Kåårik) is listed as *G. album*, but the culture does not match the BPI specimen or Hedgcock's emended description of *G. album*. Rather, Kåårik's culture is conspecific with her *Graphium erubescens* (Mathiesen-Kåårik 1953). As discussed earlier, CBS 276.54 has the ITS sequence of *O. cucullatum* (anamorph = *Phialographium erubescens*). As Kåårik's culture is from



FIGS. 46–48. *Graphium rubrum* conidiophores and conidia. 46, 47 from strain C1223, AUTHENTIC FOR TYPE of *G. rubrum*; 48 from strain C1221, AUTHENTIC FOR TYPE of *G. silanum*. Scale bars = 10  $\mu\text{m}$ .

pine, it seems unlikely that it is *G. album* as described by Corda.

*Graphium ambrosiigerum* Hedgcock, Mo. Bot. Gard. Rept. 17: 85. 1906.

*Commentary.* The holotype specimen (BPI 448676) and co-type (BPI 448675) are in poor condition, but both have synnemata with dark stipes and black conidial masses. The species was described from galleries of ambrosia beetles in *Pinus arizonica* in Arizona, USA. It is possible that Hedgcock was working with a mixture of an *Ambrosiella* species with a *Graphium* species. The black conidial masses on the synnema of the *G. ambrosiigerum* specimens are not typical for anamorphs of *Ophiostoma* species.

*Graphium rigidum* (Pers.) Sacc., Syll. Fung. 4: 610. 1886. emended Hedgcock, Mo. Bot. Gard. Rep. 17: 92. 1906.

*Commentary.* Seifert (1985) suggested that the name *G. rigidum* had been frequently used for fungi with black synnemata, and he considered the species a *nomen dubium*. Siemaszko (1939) considered *G. rigidum* as a synonym of *G. penicillioides*, and *G. rigidum* may be a member of the *G. penicillioides* complex (Okada et al 1998). A specimen (BPI 448820) from *Quercus rubra* sapwood from Indiana, USA deposited by Hedgcock in BPI as the emended type of *G. rigidum*, may be of more than one fungus. The synnemata in the specimen have black stipes from the base up to the yellow-gold conidial masses.

*Graphium rubrum* Rumbold, Phytopathology 24: 300. 1934. FIGS. 46–47

*Commentary.* A specimen deposited by Rumbold (BPI 448830) can serve as the holotype of *G. rubrum*, and it shows black synnemata with pinkish-red to yellowish-red conidial masses, and a few syn-

nemata have gray conidial masses. A culture from the holotype (CBS 210.34) is no longer producing synnemata but is producing phialide-like structures on short, simple, hyaline conidiophores (FIGS. 46–47). Okada et al (2000) also observed phialides in CBS 210.34 using scanning electron microscopy.

Rumbold (1934) reported *G. rubrum* from *Quercus*, *Populus*, *Liquidambar* and *Pinus* in the USA. Goidanich (1936) described *G. silanum* from *Pinus* in Italy, and a culture (CBS 206.37) deposited by him produces no synnemata but does produce phialide-like structures on short, simple, hyaline conidiophores (FIG. 48), similar to those of *G. rubrum*. Also, CBS 206.37 has an ITS sequence identical to that of Rumbold's isolate of *G. rubrum*. It is possible, however, that CBS 206.37 is not *G. silanum*, as Goidanich described *G. silanum* as having a micronematous state with prominent denticles and ramoconidia, and

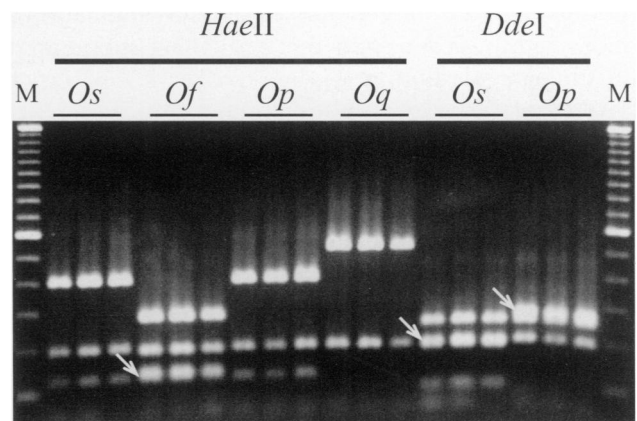


FIG. 49. Ethidium bromide stained agarose (2%) gel of *HaeII* and *DdeI* digestion products of the amplified ITS rDNA region from isolates of *Ophiostoma setosum* (*Os*), *Ophiostoma floccosum* (*Of*), *O. piceae* (*Op*), and *O. querci* (*Oq*). Co-migrating bands are indicated by arrows. One-hundred base pair ladders (M) are on either side of the gel.

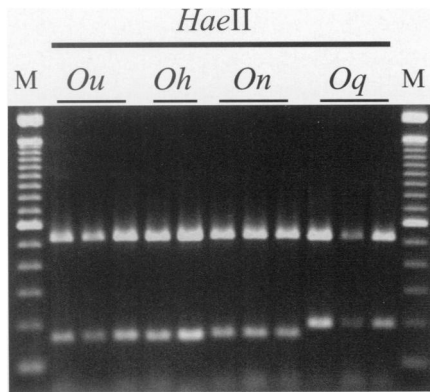


FIG. 50. Ethidium bromide stained agarose (2%) gel of *Hae*II digestion products of the amplified ITS rDNA region from isolates of *Ophiostoma ulmi* (*Ou*), *O. himal-ulmi* (*Oh*), *O. novo-ulmi* (*On*), and *O. querci* (*Oq*). One-hundred base pair ladders (*M*) are on either side of the gel.

CBS 206.37 does not form such a *Sporothrix*-like anamorph.

The ITS sequence (AF198245) of Rumbold's isolate CBS 210.34 did not match well with our sequences of *Ophiostoma* species. A BLAST 2.0 (NCBI) search showed this sequence to be closest to *Phialophora gregata* (Allington and Chamberlain) Gams, which may be an anamorph of an unknown discomycete (Paulin and Harrington 2000). The 18S rDNA sequence of Rumbold's culture also places it among the discomycetes (Okada et al 2000).

- Dendrostilbella smaragdina* (Alb. & Schw.) Seifert, Stud. Mycol. 45: 185. 2000.  
 = *Ceratopodium smaragdinum* A. & S., Consp. Fung., p. 335. 1805.  
 = *Stilbum smaragdinum* Alb. & Schw.:Fr., Sytem. Mycol. 3: 303. 1832.  
 = *Tubercularia smaragdinum* (Alb. & Schw.) Seifert, Stud. Mycol. 27: 127. 1985.  
 = *Graphium smaragdinum* (Alb. & Schw.) Sacc., Syll. Fung. 4: 618. 1886.

*Commentary.* Seifert (1985) examined what was considered authentic material of this species and described the green synnemata and acropleurogenous phialides. Hedgcock (1906) noted green synnemata stipes and gray or green conidial masses, and we also found very dark conidial masses on his specimen (BPI 448617). Based on Seifert's description and Hedgcock's specimen, *G. smaragdinum* is not a likely member of the *O. piceae* complex. This species may have an unnamed *Claussenomyces* (discomycete) teleomorph (Okada et al 2000).

#### DISCUSSION

Although there have been earlier studies distinguishing the so-called conifer form of *O. piceae* from the

so-called hardwood form, and the Dutch elm disease fungi have been clearly delimited from each other, this is the first comprehensive assessment of the entire complex. The ITS sequences of the nine recognized species were very similar to each other but quite distinct from the ITS sequences of *O. ips*, *O. simplex*, *O. galeiformis*, *O. cucullatum*, *O. europhioides*, *O. pluriannulatum*, *O. coronatum*, *O. perfectum*, *O. nigrocarpum*, *O. stenoceras* and other *Ophiostoma* species (data not shown). All members of the *O. piceae* complex form a *Sporothrix* synanamorph with prominent denticles in addition to the *Pesotum* synanamorph, and the presence of both synanamorphs sets the complex apart from the rest of *Ophiostoma*. Thus, this unique combination of anamorphs and the ITS sequence analysis suggest that *O. piceae* complex is a monophyletic group.

*Graphium-like fungi outside the O. piceae complex.*—Synnemata are efficient fruiting bodies for placing conidia in the path of small animals for acquisition and dispersal, and it is not surprising that many evolutionary lines of fungi have converged on such conidiomata. In a recent review of ascomycete-forming fungi with *Graphium*-like synnemata, Okada et al (1998) found that such synnemata are formed by species of the Microascales (pyrenomycetes) and Chaetothyriales (loculoascomycetes), and they recommend that the genus name *Graphium* be used only for the species with microascalean affinities, such as *G. penicillioides*. Further, we found that the ITS sequence of *G. rubrum* is closer to *Phialophora gregata* and discomycetes than to pyrenomycetes or loculoascomycetes, and *G. rubrum* produces a phialidic micronematous state, as does the synnemata *Dendrostilbella smaragdina*, which also appears to be a discomycete anamorph (Okada et al 2000). Species in *Ophiostoma* tolerate cycloheximide at high concentrations (Harrington 1981), and this can aid in separating synnemata-forming species with *Ophiostoma* affinities from those of other ascomycetes. However, there are other ascomycetes, including some true *Graphium* species, like *G. penicillioides*, that are not related to *Ophiostoma* but do tolerate cycloheximide.

Even within *Ophiostoma*, synnemata may have evolved more than once. *Ophiostoma* is a large genus with a wide variety of anamorphs, and many species have more than one synanamorph. In addition to the *Leptographium*-type (mononematous, with a stipe and a penicillately branched conidiogenous apparatus) and the *Graphium*-type (synnemata), some species of *Ophiostoma*, such as the anamorph of *O. clavigerum*, show both types of conidiomata. *Graphiocladiella* was proposed by Upadhyay (1981) to accommodate anamorphic fungi with both mononematous and syn-

nematous conidiomata. Harrington (1988) suggested that such intermediate types could be better accommodated in *Graphium* (*Pesotum*) than in *Leptographium*, but we now suspect that many such species have greater affinity with *Leptographium*.

Variability in conidiogenesis and stipe configuration among the anamorphs of *Ophiostoma* has led to a multitude of anamorph genera and continued controversy (Harrington 1988, Seifert and Okada 1993, Wingfield et al 1991). In some *Ophiostoma* species (TABLE III), the synnemata appear to be a loose aggregation of *Leptographium* conidiophores, without fused stipe cells. In our examination of *Ophiostoma* species, only members of the *O. piceae* complex have proven to produce both synnemata with fused stipe cells and a *Sporothrix* synanamorph (micronematous conidiophores with prominent denticles, Hoog 1974, 1993). Micronematous conidiophores are seen in many of the species listed in TABLE III, but these micronematous conidiophores lack prominent denticles at the conidiogenous cell apex and appear to be nearer to *Hyalorhinocladiella* (Hoog 1993) or to be degenerative *Leptographium*-type conidiophores. The conidiogenous cells of *Pesotum* and *Leptographium* resemble those of *Hyalorhinocladiella*, and it is possible that the macronematous conidiomata evolved from the *Hyalorhinocladiella*-like conidiophores.

Some of the synnemata-forming *Ophiostoma* species with ITS sequences differing greatly from those of *O. piceae* include *O. ips*, *O. simplex*, *O. galeiformis* and *O. cucullatum*. These species lack a *Sporothrix* anamorph, though *O. ips* forms micronematous, *Hyalorhinocladiella*-like conidiophores (Hoog 1993). Many of these and other synnemata-forming *Ophiostoma* species without *Sporothrix* synanamorphs form ascospores with sheath-like outer walls, extending to a cucullate brim around the spores (e.g., *O. clavigerum*, *O. simplex*, *O. galeiformis* and *O. cucullatum*) or in a box shape (*O. ips* and *O. ainoae*). In contrast, reniform ascospores without thickened outer cell walls are typical for species in the *O. piceae* complex.

The genus *Pesotum* was defined by the presence of both synnemata and micronematous conidiophores with denticles, with the synanamorphs of *Ophiostoma ulmi* as the type species (Crane and Schoknecht 1973). Accepting their definition, we would restrict *Pesotum* to those anamorphs with affinities to the *O. piceae* complex. Other synnematos anamorphs in *Ophiostoma* may have arisen through convergent evolution, perhaps from *Leptographium*-type conidiophores. The genus *Phialographium* is available for synnematos anamorphs of *Ophiostoma* species (e.g., *O. sagmatosporae* and *O. cucullatum*) with phialidic conidiogenous cells.

*Species delimitation and identification in the O. piceae complex.*—Use of a wide array of isolates from around the world and a holistic approach, including mating reactions, phylogenetic analysis, and phenotype (morphological and physiological), allows for a clearer delimitation of the species in the *O. piceae* complex. We emphasize phenotype in delimiting species (Harrington and Rizzo 1999), but mating compatibilities and phylogenetic analyses prove valuable in identifying lineages or mating populations where unique phenotypic characters may be found. It is noteworthy that ITS sequences were unable to distinguish *O. piceae* from *O. canum*, two species that are morphologically and biologically distinct. Other studies (Harrington and Rizzo 1999, Witthuhn et al 2000) have also found that ITS sequences of sympatric sibling species may be identical, and a strictly genealogical species concept cannot be applied if only nuclear rDNA sequences are utilized.

Production of the sexual state in culture is relatively easy for species in the *O. piceae* complex, and the heterothallic nature of most of the species allows for the identification of mating populations or biological species. Partial interfertility between species, as evidenced by poor ascospore production or aborted asci (Brasier 1993, Harrington and McNew 1998) was seen in some pairings between different species, and this strongly suggests that the species are distinct. In pairings between isolates of the same species, we generally had more success in producing perithecia and ascospores by spermatizing a recipient (female) strain with conidial suspensions of a donor (male) strain than by simply pairing two strains together on a agar medium. Two mating types are seen in *O. ulmi*, *O. novo-ulmi*, *O. himal-ulmi* (Brasier and Mehrotra 1995), *O. piceae*, *O. querci*, (Brasier 1993), *O. setosum* (Uzunovic et al 2000), and *O. floccosum*, but Goidanich (1935) reported *O. catonianum* to be homothallic.

A number of physiological and morphological characters were found useful in identifying cultures to species (TABLE II). Many of these characters have been used by others (Brasier and Kirk 1989, 1993, Brasier and Stephens 1991, 1993, Hamschlagler et al 1994, Pipe et al 1995, Webber and Brasier 1990) to distinguish *O. piceae* and *O. querci*. Some overlapping morphological characters have been used to distinguish these two species, and these characters, growth at 32 C, and mating reactions with testers are sufficient to identify species in the complex, but a more rapid and objective identification criterion is the RFLP patterns of the amplified ITS region.

We did not attempt to distinguish *O. ulmi*, *O. novo-ulmi*, and *O. himal-ulmi* by the PCR-RFLP technique, although ITS sequence divergence should allow de-

velopment of a similar protocol for distinguishing these three species. More importantly, the species most commonly found on conifer sapwood (*O. piceae*, *O. floccosum*, *O. querci* and *O. setosum*) can be identified by ITS-RFLP patterns. With experience, it is possible to amplify the ITS region using scrapes of mycelium for template DNA. Kim et al (1999) showed that the ITS region of species in the *O. piceae* complex can also be amplified directly from conidia at the apex of synnemata, and primers were designed to distinguish *O. piceae* and *O. querci*. With the RFLP diagnostic technique employed here, more species can be distinguished. Without the need for DNA extraction, the PCR-RFLP technique can be completed in a single day, as has been demonstrated for members of *Armillaria* (Harrington and Wingfield 1995).

Phylogenetic analysis of the ITS region supports the distinction between *O. piceae* and *O. querci*, with *O. piceae* grouping with other species (*O. canum*, *O. floccosum*, and *O. setosum*) found primarily on conifers. In contrast, *O. querci* has been reported primarily from hardwoods in Europe, as have its relatives, *O. catonianum* and the Dutch elm disease fungi. However, *O. querci* is apparently common on sapwood of *Pinus* and other Pinaceae in North America and the Southern Hemisphere.

*Conifer-inhabiting species of the O. piceae complex.*—*Ophiostoma canum*, *O. floccosum*, and *O. setosum* have only been isolated from conifers and are more closely related to *O. piceae* than to the species found primarily on hardwoods. The ITS sequence of *O. canum* is not distinguishable from that of *O. piceae*, but lack of interfertility with *O. piceae* mating testers and the distinctive conidia of *O. canum* set this species apart. Partial interfertility with *O. piceae* suggests that both of our isolates of *O. canum* are mating type B; thus, we do not appear to have the opposite mating type for production of perithecia and ascospores in culture. The other conifer-inhabiting species appear to be common sapwood staining fungi not associated with any particular insect vector, but *O. canum* may be an important symbiont with a specific insect (the bark beetle *Tomicus minor*, Mathiesen 1950). The collection of thick-walled conidia at the apex of the synnemata may be an adaptation for mycophagy by the bark beetle.

Our primary interest in the *O. piceae* complex has been in those species that cause bluestain in conifer wood. The most common synnemata-forming species we have encountered in New Zealand and western North America are *O. piceae*, *O. floccosum*, *O. setosum*, and *O. querci*. It is relatively common to see two or more of these species on a single chip of wood, and mixed cultures of these species are very fre-

quently seen, so these species apparently grow together well, with little inhibition of each other. Such mixed cultures have likely contributed to the confusion over the identification of these species. Unfortunately, the work done on *O. piceae* as a bluestain fungus (as reviewed by Seifert 1993) is difficult to interpret now that we realize that the studies could have utilized *O. querci*, *O. floccosum* or *O. setosum*.

*Hardwood-inhabiting species of the O. piceae complex.*—The taxonomy of *O. querci* remains unclear, and the basionym for the species is not ideal. No Latin description, dried specimen or culture was provided by Georgévitch (1926, 1927), but the fact that he described the fungus from oak in Europe (Croatia) makes it likely that his concept of the species matches the modern concept of the “hardwood form” of *O. piceae*. Accepting this, Loos’ (1932) species, *O. fagi*, is a clear synonym. We do not have authentic material for *O. roboris*, *O. kubanicum*, and *O. valachicum*, but these, too, are likely synonyms of *O. querci*. If *O. roboris* is a synonym, then *Graphium roboris* would be the earliest available name for the synnematus anamorph of *O. querci*. Okada et al (1998) proposed *Pesotum pirinum* as the anamorph of *O. querci*, but this is the anamorph name for *Ophiostoma catonianum*, which we consider distinct from *O. querci*. We also examined isolates from New Zealand and Vietnam that looked similar to *O. querci* but differed slightly in ITS sequence, and these cultures did not mate with *O. querci* testers. We suspect that more species closely related to *O. querci* will be recognized in the future.

The relatedness of *O. querci* to the Dutch elm disease fungi *O. ulmi* and *O. novo-ulmi* was demonstrated by similarity of ITS sequences. These Dutch elm disease fungi are very closely related but distinct species that have been introduced to North America and Europe. They may be native to Asia, though they have not been found there (Brasier 1990, Brasier and Mehrotra 1995). *Ophiostoma himal-ulmi* does appear to be native to the Himalayas, where it was found associated with bark beetle attacks in elm branches (Brasier and Mehrotra 1995). In inoculation studies, *O. himal-ulmi* was able to cause a vascular wilt disease in elm seedlings, and it was thought that this species was closely related to *O. ulmi* and *O. novo-ulmi* (Brasier and Mehrotra 1995). However, the ITS sequence analysis suggests that *O. himal-ulmi* is more closely related to *O. querci*.

The capacity of *O. querci* to cause disease in hardwood species has been debated, and fungi similar to *O. querci* have been frequently associated with declining oak trees in Europe (Brasier 1990, Brasier and Kirk 1989, Brasier and Mehrotra 1995, Harrington

1993, Morelet 1992, Oleksyn and Przybyl 1987). Its close relationship to *O. himal-ulmi*, *O. ulmi*, and *O. novo-ulmi* also suggests that it has the potential to cause a vascular disease, and attention should be given to introduction of this species to new environments.

We suspect that most species in the *O. piceae* complex are native to the Northern Hemisphere. Indeed, the common occurrence of *O. querci* on sapwood of Pinaceae in some regions (such as the Southern Hemisphere and North America) may be due to its not being native to these ecosystems. This species and others in the complex will produce synnemata and/or perithecia on logs, crating, dunnage and other materials commonly discarded from ships, and we believe that the wide distribution of some of these species is due to human activities.

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