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Resolving taxonomic and phylogenetic incongruence within species *Ceratocystiopsis minuta*

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Abstract: *Ceratocystiopsis minuta* (Siemaszko) H.P. Upadhyay & W.B. Kendr., originally isolated in Poland, is the type species of genus *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. Species in this genus are characterized by dark perithecia with short conical beaks, usually with convergent ostiolar hyphae and dark ascocarps, and by falcate or lunate ascospores. Work within the genus is complicated by historical inconsistencies, errors in strain identification and the absence of a holotype specimen. We used sequence data from the β-tubulin gene, internal transcribed spacer and large subunit regions of ribosomal DNA to phylogenetically characterize 23 putative strains of *Cop. minuta* from Europe, Japan and North America, as well as strains from other species in genus *Ceratocystiopsis*. Our results show that *Cop. minuta* strains from Europe and Japan are monophyletic, whereas those from North American are polyphyletic and likely misidentified. This suggests that prior research groups have used misidentified strains of *Cop. minuta* or fungal strains that were only distantly related to the *Cop. minuta* strain originally described from Poland. Further our multigene phylogenetic analysis also shows that *Cop. minuta* strains from Europe and Japan can be segregated into three clades. This suggests the presence of several phylogenetic species that are morphologically similar to *Cop. minuta*, and we anticipate that this species complex will challenge researchers until such relationships are resolved.

Key words: bark beetles, β-tubulin, phylogeny, rDNA, taxonomic confusion

INTRODUCTION

Ceratocystiopsis minuta (Siemaszko) H.P. Upadhyay & W.B. Kendr. (1975), the type species of fungal genus *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., is based on the minuta-spore group of Olchowecki and Reid (1974) and was erected by Upadhyay and Kendrick (1975). Its members are sensitive to cycloheximide, have dark ascocarps with short necks, and falcate, elongate ascospores; these usually have a hyaline sheath (Harrington 1981, Hausner et al 1993). While morphological and genetic inconsistencies within the genus led to it being synonymized with genus *Ophiostoma* H. & P. Sydow (Wingfield 1993, Hausner et al 1993), after extensive phylogenetic analyses this amalgamation was reversed by Zipfel et al (2006).

Strains identified as *Cop. minuta* have been found on six continents in association with at least five bark beetles and nine tree species (Siemaszko 1939; Davidson 1942; Mathiesen 1951; Hunt 1956; Mathiesen-Käärik 1960; Upadhyay 1981; Yamaoka et al 1997; Zhou et al 2001, 2004a, b, 2005). While in Europe and Japan they often are associated with species of *Ips* beetles and pine or spruce trees, in North America they also are common associates of the mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, and are found often in MPB-infested pines such as lodgepole (Mathiesen-Käärik 1960, Upadhyay 1981, Kim et al 2005). It has been reported as nonpathogenic (Yamaoka et al 1998), but its role in the MPB

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association and its effect on trees is not well characterized.

Cop. minuta first was isolated and described by Siemaszko (1939) in Bialowieza, Poland, from *Picea abies* (L.) H. Karst., which was infested with *Ips typographus* L., but no holotype specimen was deposited in a culture collection (Hunt 1956). This has resulted in confusion in the literature as to what actually constitutes *Cop. minuta*. Davidson (1942) in USA and Mathiesen (1951) in Sweden noted that the strains they identified as *Cop. minuta* had smaller perithecial measurements than those recorded by Siemaszko (1939). Hausner et al (2003) reported that there were sequence differences among their strains of *Cop. minuta* but because they did not fruit in culture their morphology could not be verified. They noted other incongruities in *Cop. minuta* strains from culture collections and suggested conducting comparative molecular analysis to resolve the taxonomic confusion surrounding this species.

The ''*minuta* complex'' of Olchowecki and Reid (1974) was phylogenetically delineated via the highly conserved large subunit (LSU) rDNA region (Hausner et al 1993, Hausner and Reid 2003), but because the partial sequences they used for their analyses were short (~250 base pairs) their neighbor-joining tree showed many polytomies. However since then ophiostomatoid phylogenies have been improved by using sequences of the internal transcribed spacer (ITS) regions and the β-tubulin (βT) gene in addition to sequences of the LSU (Lee et al 2003, Lim et al 2004, Zipfel et al 2006). We used the same regions (ITS and LSU regions from the nuclear rDNA operon and the protein coding gene βT) to re-evaluate the molecular taxonomy of *Cop. minuta*.

MATERIALS AND METHODS

Taxon sampling.—Strains in this study were selected based on results of Hausner et al (1993), Yamaoka et al (1997), Hsiau and Harrington (1997), Hausner and Reid (2003) and Zipfel et al 2006). Strains isolated recently from nature were selected based on morphological similarities to species within genus *Ceratocystiopsis*. In total 43 strains representing 12 species were studied (TABLE I), of which 41 were used in molecular analyses.

Molecular techniques.—Fungi were grown at 22 C on the surface of autoclaved cellophane that had been placed on 2% oxoid malt extract agar (OMEA) in the dark at 22 C for 2–3 wk. Mycelium was scraped from the surface of the cellophane and DNA was extracted according to Kim et al (1999). Purified DNA was amplified with PCR protocols described by Lee et al (2003). The LSU region was amplified with LR0R and LR3 (Vilgalys and Hester 1990), the β-tubulin region with T10 (O'Donnell and Cigelnik 1997) and BT12 (Kim et al 2003), and the ITS region with ITS1F (Gardes and Bruns 1993), ITS1, ITS3 and ITS4 (White et al 1990). Because of the difficulties in amplifying the ITS region multiple forward primers had to be used. PCR products were purified with a QIAQuick PCR Purification Kit (QIAGEN Inc.) and sequenced with an ABI 3700 automated sequencer (Perkin-Elmer Inc., USA) at the DNA synthesis and Sequencing Facility, Macrogen (Seoul, Korea).

Electropherograms were viewed with Chromas (McCarthy 2004) 1.43 (http://www.technelysium.com.au/chromas.html for latest version); files were edited in PHYDIT 3.2 (http://plaza.snu.aC.kr/~jchun/phydit/download.php, Chun 2001). Initial alignments were performed with Clustal ×1.83 (Thompson et al 1997) using default settings, and alignments were manually adjusted by eye in Se-Al 2.0 al carbon (Rambaut 2002). Finished alignments were exported into PAUP 4.0.B10 (Swofford 2003). Phylogenetic analysis of the three genetic sequences combined was conducted with maximum parsimony (MP), maximum likelihood (ML), neighbor joining (NJ) and Bayesian analyses (BA). MP, ML and NJ were performed in PAUP 4.0.B10, while BA was performed with MrBayes 3.12 (Ronquist and Huelsenbeck 2003). In all cases gaps were treated as missing data. Maximum parsimony was conducted with a heuristic search with TBR-branch swapping. Maximum likelihood, using a Rogers-Swofford approximation and TBR branch swapping, was conducted with a general time-reversible (GTR) model with rates of base pair substitutions and the proportion of invariable sites estimated by PAUP. For ML clade stability was evaluated with 100 bootstrap replicates; for MP clade stability was evaluated with 1000 bootstrap replicates and 1000 heuristic search replicates were used separately to evaluate stability. NJ analysis was conducted with a GTR model and 1000 bootstrap replicates were used to evaluate clade support. Bayesian analysis was conducted with a GTR + I + G model based on Modeltest 3.7 results (Posada and Crandall 1998). Posterior probability was conducted with 1 000 000 cycles using two runs of four chains (one hot, three cold) and discarding the first 500 000 trees as burn-in. Trees were sampled every 500 cycles. This analysis was duplicated and the latter 500 000 trees from both runs were combined to evaluate posterior probability, resulting in 2000 sampled trees used to evaluate posterior probability for BA. In all cases models of evolution were selected based on Modeltest 3.7 results (Posada and Crandall 1998). *Ophiostoma ips* was selected as outgroup (Hausner et al 1993, Hausner et al 2000).

RESULTS

Sequence alignments yielded 609 nucleotide positions from the LSU region and 700 and 928 from the ITS and βT segment respectively. While all positions from the LSU region were included, 46 and 24 positions from the ITS and βT regions respectively were excluded due to alignment ambiguities. The alignment matrix can be viewed in TreeBase with PIN 17305 along with the name Alex Plattner.

TABLE I. List of species used in phylogenetic analysis and their isolation source, geographic location and collector and accession number

Species name	Strain number[1]	Host[2]	Isolation source	Geographic region	Collector	28S rRNA*	ITS*	βT*
Ceratocystiopsis brevicomi Hsiau & T.C. Harr. (1997)	CBS 333.97 (UM 1452)	Unk	*Dendroctonus brevicomis*	California, USA	T. Harrington	EU913683	EU913722	EU913761
Ceratocystiopsis collifera Marm. & Butin (1990)	CBS126.89 (UM537)	*P t*	*Dendroctonus valens*	Santiago, Mexico	J. Marmolejo	EU913681	EU913721	EU913760
Ceratocystiopsis manitobensis	UM214	*P r*	Unk	Manitoba, Canada	J. Reid	EU913675	EU913715	EU913754
Ceratocystiopsis manitobensis (J. Reid & Hausner) RD Zipfel, Z.W. de Beer & M.J. Wingf. (2006)	UM237	*P r*	Galleries of bark beetle	Manitoba, Canada	J. Reid	EU913674	EU913714	EU913753
Ceratocystiopsis minima	CBS 182.86 (UM 1462)	*P b*	Unk	Wisconsin, USA	M.J. Wingfield	EU913663	EU913704	EU913743
Ceratocystiopsis minima	UM1501	*P b*	Galleries of bark beetles	Manitoba, Canada	J. Reid	EU913662	EU913703	EU913742
Ceratocystiopsis minima	UM235	*P b*	Galleries of bark beetles	Manitoba, Canada	J. Reid	EU913661	EU913702	EU913741
Ceratocystiopsis minima (Olchow. & J. Reid) H.P. Upadhyay (1981)	UM85	*P r*	Galleries of bark beetles	Manitoba, Canada	J. Reid	EU913660	EU913701	EU913740
Ceratocystiopsis minuta	C112	Unk	Unk	Louisiana, USA	J.R. Bridges	N/S	N/S	N/S
Ceratocystiopsis minuta	CBS 116796	*Pic a*	Sapwood of *Ips typographus*	Bialowieza National Park, Poland	T. Kirisits	EU913654	EU913695	EU913734
Ceratocystiopsis minuta	CBS 116963	*Pic a*	Perithecia in *Ips typographus* galleries	Bialowieza National Park, Poland	T. Kirisits	EU913655	EU913696	EU913735
Ceratocystiopsis minuta	CBS 441.94 (UM 1453)	*Pic a*	Wood inoculated with *Ips typographus*	Niederösterreich, Amstetten Austria	T. Kirisits	EU913649	EU913690	N/S
Ceratocystiopsis minuta	CBS116795	*Pic a*	Perithecia in galleries of *Ips typographus*	Bialowieza, Poland	T. Kirisits	EU913647	EU913688	EU913727
Ceratocystiopsis minuta	CBS117562	*L d*	Perithecia in galleries of *Ips cembrae*	Tyrol, Ehrwald, Austria	T. Kirisits	EU913648	EU913689	EU913728
Ceratocystiopsis minuta	CBS117566	*L d*	Perithecia in galleries of *Ips cembrae*	Scotland, UK	T. Kirisits	EU913653	EU913694	EU913733
Ceratocystiopsis minuta	CBS463.77 (UM846)	*Pic e*	Log	New Mexico, USA	Irez Ritos	EU913645	EU913686	EU913725
Ceratocystiopsis minuta	IMI 212115	*P s*	Log	Sweden	A. Käärik	N/S	N/S	N/S
Ceratocystiopsis minuta	RJ191 (UM 1535)	*Pic a*	*Ips typographus*	Limnowa Forest District, Poland	R. Jankowiak	EU913659	EU913700	EU913739
Ceratocystiopsis minuta	RJ5095 (UM 1533)	*Pic a*	*Ips typographus*	Krynki Forest District, Poland	R. Jankowiak	EU913657	EU913698	EU913737
Ceratocystiopsis minuta	RJ689 (UM 1534)	*Pic a*	*Ips typographus*	Biebrzanski National Park, Poland	R. Jankowiak	EU913658	EU913699	EU913738
Ceratocystiopsis minuta	RJ705 (UM 1532)	*Pic a*	*Ips typographus*	Biebrzanski National Park, Poland	R. Jankowiak	EU913656	EU913697	EU913736

TABLE I. Continued

Species name	Strain number[1]	Host[2]	Isolation source	Geographic region	Collector	28S rRNA*	ITS*	βT*
Ceratocystiopsis minuta	YCC139 (JCM9367)	*Pic j*	Adult beetle of *Ips typographus japonicus*	Hokkaido, Japan	Y. Yamaoka	EU913652	EU913693	EU913732
Ceratocystiopsis minuta	YCC251 (JCM 9368)	*Pic j*	Egg gallery of *Ips typographus japonicus*	Hokkaido, Japan	Y. Yamaoka	EU913651	EU913692	EU913731
Ceratocystiopsis minuta	YCC294 (JCM 9816)	*L k*	Gallery of Ips subelongatus	Yamanashi, Japan	Y. Yamaoka	EU913650	EU913691	EU913730
Ceratocystiopsis minuta (Siemaszko) H.P. Upadhyay & W.B. Kendr. (1975)	CBS145.59	Unk	Unk	USA	R.W. Davidson	EU913646	EU913687	EU913726
Ceratocystiopsis minuta-bicolor	UAMH9551 (UM479)	*P c*	Galleries of bark beetles	Alberta, Canada	J. Reid	EU913666	N/S	N/S
Ceratocystiopsis minuta-bicolor	UM480	*P c*	Galleries of bark beetles	Alberta, Canada	J. Reid	EU913664	EU913705	EU913744
Ceratocystiopsis minuta-bicolor (R.W. Davidson) H.P. Upadhyay (1975)	CBS635.66 (UM844)	*P c*	Galleries of *Ips* sp.	USA	R.W. Davidson	EU913665	EU913706	EU913745
Ceratocystiopsis pallidobrunnea (Olchow. & J. Reid) H.P. Upadhyay (1981)	UM51	*Pop t*	Wood and inner bark	Manitoba, Canada	J. Reid	EU913682	N/S	N/S
Ceratocystiopsis parva (Olchow. & J. Reid) H.P. Upadhyay (1981)	UM59	*A.b.*	Unk	Manitoba, Canada	J. Reid	N/S	N/S	N/S
Ceratocystiopsis ranaculosa J.R. Bridges & T.J. Perry (1987)	CBS216.88	*P t*	Tree infested with *Dendroctonus frontalis*	Louisiana, USA	J.R. Bridges	EU913673	EU913713	EU913752
Ceratocystiopsis rollhanseniana	UM110	*P s*	Beetle galleries of standing tree	Hedmark, Norway	J. Reid	EU913679	EU913719	EU913758
Ceratocystiopsis rollhanseniana (J. Reid, Eyjolfsdottir and Hausner) Zipfel, Z.W. de Beer and M.J. Wingfield (2006)	UM113	*P s*	Beetle galleries of standing tree	Akershus, Norway	J. Reid	EU913678	EU913718	EU913757
Ceratocystiopsis sp. 1 (*Cop. minuta*-like)	Cop. sp. 1i	*P c*	Log infested with *Dendroctonus ponderosae*	British Columbia, Canada	J.-J. Kim	EU913667	EU913707	EU913746
Ceratocystiopsis sp. 1 (*Cop. minuta*-like)	Cop. sp. 1ii	*P c*	Log infested with *Dendroctonus ponderosae*	British Columbia, Canada	J.-J. Kim	EU913668	EU913708	EU913747
Ceratocystiopsis sp. 1 (*Cop. minuta*-like)	Cop. sp. 1iii	*P c*	Log infested with *Dendroctonus ponderosae*	British Columbia, Canada	J.-J. Kim	EU913669	EU913709	EU913748

TABLE I. Continued

Species name	Strain number[1]	Host[2]	Isolation source	Geographic region	Collector	28S rRNA*	ITS*	βT*
Ceratocystiopsis sp. 2 (*Cop. minuta*-like)	YCC329	*L k*	Adult beetle of *Ips subelongatus*	Nagano, Japan	Y. Yamaoka	EU913671	EU913711	EU913750
Ceratocystiopsis sp. 2 (*Cop. minuta*-like)	YCC330	*L k*	Adult beetle of *Ips subelongatus*	Nagano, Japan	Y. Yamaoka	EU913670	EU913710	EU913749
Ceratocystiopsis sp. 2 (Cop. *minuta*-like)	YCC513	*L k*	Adult beetle of *Ips subelongatus*	Tochigi, Japan	Y. Yamaoka	EU913672	EU913712	EU913751
Ceratocystiopsis sp. 3 (*Cop. manitobense*-like)	Cop. sp3i (SWT1)	*Pic g*	Body of *Ips perturbatus*	British Columbia, Canada	S.M. Alamouti	EU913676	EU913716	EU913755
Ceratocystiopsis sp. 3 (*Cop. manitobense*-like)	Cop. sp3ii (SWT3)	*Pic g*	Body of *Ips perturbatus*	British Columbia, Canada	S.M. Alamouti	EU913677	EU913717	EU913756
Ophiostoma fasciatum (Olchow. & J. Reid) Hausner, J. Reid & Klassen (1993)	UM 56	*Ps m*	Unk	British Columbia, Canada	A. Olchowecki	EU913680	EU913720	EU913759
Ophiostoma ips (Rumbold) Nannf. (1934)	CBS 137.36	Unk	*Ips* beetle	Oregon, USA	C.T. Rumbold	EU913644	EU913685	EU913724
Ophiostoma longisporum (Olchow. & J. Reid) Hausner, J. Reid & Klassen (1993)	UM 48	*P b*	Unk	Manitoba, Canada	A. Olchowecki	EU913684	EU913723	EU913762

[1] CBS: Centraalbureau voor Schimmelcultures, Netherlands. YCC: Yamaoka's culture collection, Japan. UM: University of Manitoba, Reid's culture collection, Canada. UAMH: University of Alberta Microfungus Herbarium, Canada. *Cop.* sp. 1 and *Cop.* sp. 3 are maintained at UBC, Breuil culture collection, Canada. N/S: genetic sequence not submitted to GenBank.

[2] Hosts: P r = *Pinus resinosa*, Ps m = *Pseudotsuga menziesii*, P b = *Pinus banksiana*, P c = *Pinus contorta*, Pic e = *Picea engelmanii*, Pic a = *Picea abies*, L d = *Larix decidua*, Pic j = *Picea jezoensis*, L k = *Larix kaempferi*, P s = *Pinus sylverstris*, P t = *Pinus taeda*, Pic g = *Picea glauca*, Pop t = *Populus tremuloides*, P t = *Pinus teocote*, Unk = Unknown.

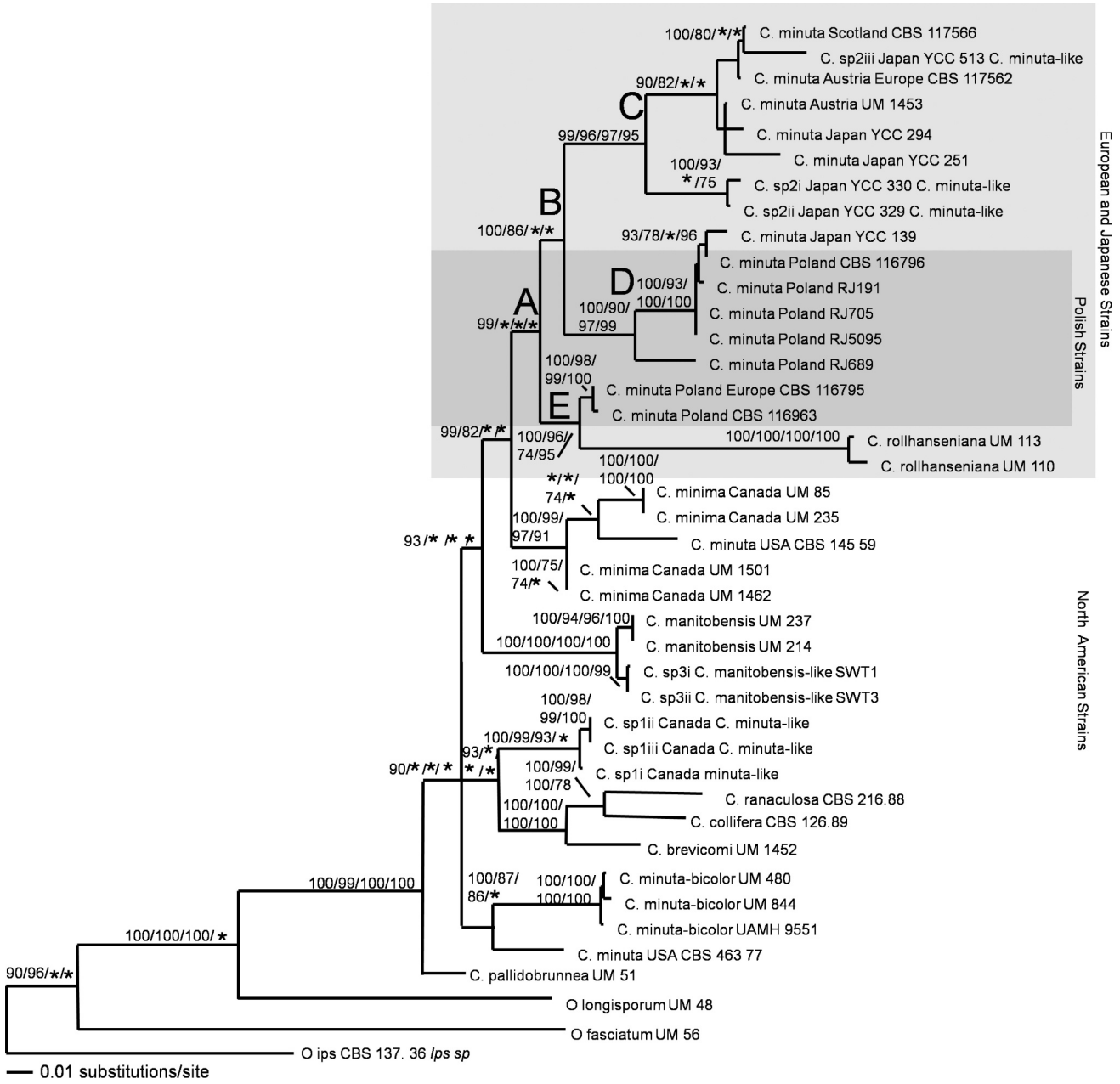

FIG. 1. Phylogram of *Ceratocystiopsis minuta* and related species. Support values are in this order: Bayesian analysis, maximum likelihood, maximum parsimony and neighbor joining. A* denotes less than 50% support. Three well supported clades (nodes B, C and D) contain strains from Europe and Japan. Four well supported clades (not shaded) contain strains from North America.

The combined dataset contained 2237 sites, of which 1309 were constant, 254 were parsimony uninformative and 674 were parsimony informative. This data produced 64 equally parsimonious trees requiring 2665 steps. The phylogram from the maximum likelihood analysis was used for the skeletal structure of the tree topology; support values from all analyses were overlaid onto this phylogram (FIG. 1).

Clustering of strains near end branches (terminal clades) was generally well supported by all four types of genetic analyses (FIG. 1). *Ceratocystiopsis* strains from Europe and Japan tended to form monophyletic clades (FIG. 1, shaded) that were distinct from those of North American clades of *Ceratocystiopsis* species (FIG. 1, nonshaded). Furthermore strains from Europe and Japan clustered together into two well supported monophyletic clades (FIG. 1, nodes C and D). The majority of the Polish strains were placed in clade D (FIG. 1, node D). Two other strains of *Cop. minuta* from Poland clustered with two strains of *Cop.*

TABLE II. Perithecia and ascospore measurements of *Ceratocystiopsis minuta* found in the literature or produced by the authors

Author	Year	Location	Perithecia (μm)				Ascospores (μm) length and width
			Base width	Base height	Neck length	Neck base	
Siemaszko	1939	Poland	84–140	74–125	98–140	14–20	8–10 × 1.5
Davidson	1942	USA	60–80		45–90	20–28[1]	10–15 × 1
Mathiesen	1951	Sweden	58–106		60–100	20	13.3 × 1.7
Yamaoka	2006	Japan YCC 251	56–75	56–75	119–183[2]	26–30	10.4–12.1 × 1.6–2.4
Plattner et al	2007	CBS 117562		45–90	50–175		5–11.2 × 0.7–1.6
	2007	CBS 116795	62.5–92.5	62.5–92.5	32.5–55	25–30	9–13 × 1
	2007	RJ705 (UM 1532)	75–112.5		82.5–137.5	30–47.5	Not produced

[1] Estimated from figure scale.
[2] Total height including base and neck.

rollhanseniana in a well supported third monophyletic clade. However *Cop. rollhanseniana* is considered morphologically distinct from *Cop. minuta* (Hausner et al 2003).

The putative strains of *Cop. minuta* from North America failed to group with the European/Japanese strains described above. Among the North American strains well supported monophyletic clades were observed for (i) four strains of *Cop. minima* from Canada and one strain of *Cop. minuta* from USA; (ii) two strains of *Cop. manitobensis* and two strains of *Cop.* sp. 3 that morphologically resemble *Cop. manitobensis*; (iii) three strains of *Cop.* sp. 1 from MPB in Canada, which was a sister clade of *Cop. ranaculosa*, *Cop. collifera* and *Cop. brevicomi* strains; and (iv) three strains of *Cop. minuta-bicolor* and one strain of *Cop. minuta*. *Cop. pallidobrunnea*, *Ophiostoma longisporum* and *O. fasciatum* did not form clades with other strains of *Ceratocystiopsis* species. However in the majority of cases the separation of these well supported monophyletic clades at earlier nodes (basal support) was weak (FIG. 1). BA and to a lesser extent ML tended to offer some basal support, while MP and NJ did not. When single gene trees were examined (data not shown) basal areas almost always consisted of polytomies between well supported terminal clades.

In a separate ML and BA analysis of LSU seqeunces *Cop. minuta* strains CBS 119682 and CBS 117566 from Scotland and CBS 117562 from Austria were closely grouped (data not shown). While CBS 119682 was used by Zipfel et al (2006), it was not used in the current analysis because of lack of available sequence data for βT.

The phylogenetic tree (FIG. 1, node A) suggests that among the taxa sampled, *Cop. rollhanseniana* followed by *Cop. minima* and finally *Cop. manitobensis* are the closest relatives to the clade of Japanese/European *Cop. minuta* strains. *Cop. pallidobrunnea* appears to be the most basal member in genus *Ceratocystiopsis*. Finally, as expected the two *Ophiostoma* species included, *O. longisporum* and *O. fasciatum* did not form clades with *Ceratocystiopsis* species.

Eight of the strains identified as *Cop. minuta* (CBS 116795, 117042, 117562, 117566; YCC 139, 251, 513; and RJ705) produced perithecia in varying degrees of abundance and maturity in artificial cultures (TABLE II). For example CBS 116795, a Polish strain phylogenetically related to *Cop. rollhanseniana*, produced mature perithecia with ascospores in culture after 4 mo. A second Polish strain, RJ705, which produced what appeared to be mature perithecia but no ascospores, showed an imperfect state almost identical to that described by Mathiesen (1951) and Davidson (1942). While RJ705 could be a candidate for selection as neotype, the description would be incomplete because of the absence of ascospores.

While we generated sequence data for two putative strains of *Cop. minuta*, C112 from Louisiana, USA (used as an outgroup by Hsiau and Harrington 1997), and IMI 212115 from Sweden (deposited by Mathiesen in 1952), we excluded these strains from our phylogenetic analysis; the sequence data suggested that these strains either were originally misidentified or that the current material available does not represent the original strains. While the LSU sequences were conserved and could be readily aligned with other *Ceratocystiopsis* sequences, this was not the case for the βT and ITS sequences. For strain C112, *Nectria* species were the closest BLASTN matches in GenBank for the LSU and ITS regions, while the βT and ITS sequences for strain IMI 212115 could not be aligned with available sequences of either strains of *Ophiostoma* or *Ceratocystiopsis*.

DISCUSSION

Identifying *Cop. minuta* with morphological structures is difficult because there is no holotype and no description of the anamorph. Researchers 1939–2007 have used many different strains to represent *Cop. minuta* when examining ophiostomatoid species. For example Hsiau and Harrington (1997) used two strains of *Cop. minuta* collected in Louisiana, USA, by Bridges and Perry (1987) while Hausner et al (1993) used CBS 145.59 and CBS 463.77 collected by Davidson during the 1940s in USA. Yamaoka et al (1997) included strains YCC 139 and YCC 251 from Japan when identifying new *Ophiostoma* species. Zipfel et al (2006) used strain CBS 119682 from Scotland, UK. Unfortunately none of these strains originated in Poland, the site of the original collection. It therefore is difficult to determine whether these strains properly represented *Cop. minuta*.

To resolve the confusion generated by the literature it would be desirable to propose a neotype for *Cop. minuta*. Initially we anticipated that CBS strains116796, 116795 and 116963 would be appropriate for this purpose; they originated from Bialowieza, Poland, where the original strain was isolated; they grouped phylogenetically with other *Cop. minuta* strains; and they are readily available from international culture collections. However selecting one of the above three strains is problematic because our results placed *Cop. minuta* strains from Poland into two clades. While strain 116795 produces a limited number of ascocarps, its morphology fits poorly within the original concept of *Cop. minuta*; furthermore its phylogenic placement is problematic. Strain 116796 did not fruit under our culture conditions.

Four other strains of *Cop. minuta* from Poland, RJ705, RJ5095, RJ689 and RJ191, were available to us; they produced what appeared to be fully mature perithecia but did not produce ascospores. In addition, because none of these strains were isolated from trees in Bialowieski National Park, they seem inappropriate as a source for a *Cop. minuta* neotype. Until additional fruiting strains with the appropriate morphological and genetic characteristics become available from the Bialowieza area, it will be difficult to select an appropriate neotype.

An unexpected finding was the placement of the *Cop. minuta* Japanese strains in two distinct monophyletic groups that shared a common phylogenetic ancestry. The first clade included strains from Japan, Austria and Scotland, while the second had strains from Japan and Poland. All above strains shared a more distant ancestry with two other putative strains of *Cop. minuta* from Poland and two strains of *Cop. rollhanseniania* from Norway. The absence of monophyletic grouping in Japanese *Cop. minuta* strains suggested either that the populations of *Cop. minuta* from Japan and Europe have been mixing for generations and no longer are genetically distinguishable or that different populations of *Cop. minuta* from Europe have been recently introduced in Japan via international trade. No Japanese strains of *Cop. minuta* genetically resembled North American strains of the species.

Five North American *Cop. minuta* strains or *Cop. minuta*-like strains did not form a monophyletic group with European and Japanese strains of *Cop. minuta*. Davidson collected two of the five strains. The first, CBS 145.59, grouped closely with four Canadian strains identified as *Cop. minima* (Olchowecki and Reid 1974); the second, CBS 463.77, grouped closely with three strains of *Cop. minuta-bicolor* from Canada. The latter species first was described by Davidson (1966) and studied by Upadhyay and Kendrick (1975) and Upadhyay (1981). The three remaining strains identified as *Cop.* sp.1 and isolated from MPB in British Columbia, Canada, did not produce perithecia in culture but closely resembled strains described as *Cop. minuta* in the vegetative state. When grown on 2% OMEA these three fungi formed white colonies, grew slowly and had *Hyalorhinocladiella* anamorphs with similar conidia. However they genetically grouped closely to but were distinct from *Cop. ranaculosa*, *Cop. collifera* and *Cop. brevicomi*. Robinson (1962) described *Cop. minuta* as being isolated occasionally from lodgepole pine associated with MPB in Canada. It is possible that her strains were misidentified and were *Cop.* sp. 1. We observed perithecia only once on MPB-infested lodgepole bark, and at the time because we were not aware that perithecia of *Cop.* sp. 1 were rare we took only one picture of the perithecia and ascospores; all our attempts to produce perithecia in culture have been unsuccessful. We also observed a few perithecia once in what might have been *Cop.* sp. 1 on white pine bark (*Pinus strobus* L.), which was infested by the mountain pine beetle. Although these perithecia resembled those of *Cop. minuta*, they were smaller than those from other *Cop. minuta* strains and yielded no ascospores. While *Cop.* sp. 1 grouped closely to other species in genus *Ceratocystiopsis* and likely represents a new species, a formal description cannot be made until a sufficient number of perithecia are found and measured.

Hsiau and Harrington (1997) noted that the two strains of *Cop. minuta* they used as outgroups had very different isozyme profiles; these were C112 (origin not given) and C332 from Bridges and Perry (1987). Because C332 is no longer viable we could not include

it in our analysis. A strain of *Cop. minuta* illustrated by Mathiesen (1951) did resemble the conidiophores observed in the RJ705 strain.

None of the Polish or Japanese strains of *Cop. minuta* are phylogenetically related to the putative strains of *Cop. minuta* from North America. This is consistent with our observation that all North American strains of *Cop. minuta* are either misidentified or represent new species. Some of the misidentified strains/species should be grouped either with one of the closely related species, such as *Cop. minutabicolor*, or with *Cop. minima*. These inconsistencies probably resulted from poor definitions of the morphological characteristics of this group.

Consistent with the results of Zipfel et al (2006) we found that *Cop. minuta*, *Cop. ranaculosa*, *Cop. minutabicolor*, *Cop. minima*, *Cop. rollhanseniana* and *Cop. manitobensis* grouped with other strains from genus *Ceratocystiopsis*. Strains of *Cop.* sp. 1 from mountain pine beetles grouped closely with *Cop. brevicomi*, *Cop. ranaculosa* and *Cop. collifera*. In our analyses *Cop. pallidobrunnea*, which was placed in the ''*minuta* group'' by Hausner and Reid (2003), was the most basal member of *Ceratocystiopsis*. Consistent with the literature neither *Ophiostoma longisporum* nor *O. fasciatum* grouped with other species *Ceratocystiopsis* (Hausner et al 1993, Hausner and Reid 2003, Zipfel et al 2006). We initially included *Ophiostoma retusum* and *O. carpenteri* in our analysis, but these failed to align with other taxa and did not form monophyletic groups with species in the ''*minuta* complex'' during early phylogram construction (results not shown). These findings agree with those of Hausner et al (1993) and Zipfel et al (2006); thus we excluded these taxa from the final analysis. While Zipfel et al (2006) placed *Cop. parva* and *Cop. concentrica* in genus *Ceratocystiopsis*, we were unable to amplify one of the genetic regions in these two species. We thus removed them from the analysis.

This study is the first attempt to examine the range of genetic diversity occurring within the species complex that is *Cop. minuta sensu lato*. To our surprise we found that this species name might refer to several phylogenetic species. A *Cop. minuta* neotype should be designated for future phylogenetic and taxonomic research to clarify species relationships within genus *Ceratocystiopsis*. At this stage we recommend that researchers focus on Polish strains from the region that includes Bialowieski Park Nardowy (Bialowieski National Park). While strains related to CBS 116796 are promising candidates, it is essential that a neotype produce abundant perithecia with mature ascospores and be closely related to other Polish *Cop. minuta* strains. Finally our results show that *Cop. minuta* does not seem to be present in North America, despite the numerous reports in the literature indicating its association with various beetles in USA.

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