A new conifer-inhabiting species of *Ceratocystis* from Norway

J. Reid, M. Iranpour, S.M. Rudski, P.C. Loewen, and G. Hausner

Abstract: A new species, ***Ceratocystis norvegica*** J. Reid & Hausner sp. nov., is described from Norway. Based on morphological criteria and analyses of rDNA internal transcribed spacer and small subunit rDNA sequences, strains collected from galleries of the bark beetle *Ips typographus* on *Picea abies* (L.) H. Karst, were shown to be distinct both from members of the *Ceratocystis coerulescens* complex and from other species described previously from conifers. *Ceratocystis norvegica* has the following defining characteristics: convergent ostiolar hyphae; a sharply defined temperature optimum at 20 °C; an apparent lack of a conidial state; and ascospores that on germination produce either self-fertile or self-sterile strains.

Key words: Ceratcystis, new species, coerulescens complex, *Ips typographus*.

Résumé : Les auteurs décrivent une nouvelle espèce, le ***Ceratocystis norvegica*** J. Reid & Hausner sp. nov., provenant de Norvège. À partir de critères morphologiques et d'analyses d'ITS du rADN et de séquences rSSU, on démontre que des souches récoltées à partir de galeries du scolyte *Ips typographus* sur *Picea abies* se distinguent à la fois des membres du complexe *Ceratocystis coerulescens* et d'autres espèces déjà décrites à partir d'autres conifères. Le *Ceratocystis norvegica* possède les caractéristiques suivantes : hyphes ostiolaires convergents; une température optimale de 20 °C nettement déterminée; une absence apparente de stade conidien; et des ascospores qui produisent après germination, des souches soit auto fertiles ou soit auto stériles.

Mots-clés : Ceratocystis, nouvelle espèce, complexe coerulescens, *Ips typographus*.

[Traduit par la Rédaction]

Introduction

The fungal genus *Ceratocystis* Ellis & Halst. sensu stricto consists of plant pathogens or sapwood-staining species found in association with both angiosperms and gymnosperms; many are adapted for insect dispersal. Their various attributes were reviewed recently by Montoya and Wingfield (2006), who noted that those occurring on host members of the Pinaceae (conifers) may be blue-staining fungi, virulent pathogens, secondary colonizers of wounds, mutualistic symbionts of their vectors, or combinations thereof.

Harrington and Wingfield (1998) recognized seven distinct *Ceratocystis* species occurring on conifers: *Ceratocystis coerulescens* (Münch) Bakshi (in Bakshi 1950), *Ceratocystis douglasii* (R.W. Davidson) M.J. Wingf. & T.C. Harr (in Wingfield et al. 1997), *Ceratocystis laricicola* Redfern & Minter, *Ceratocystis pinicola* T.C. Harr. & M. J. Wingf., *Ceratocystis polonica* (Siemaszko) C. Moreau, *Ceratocystis resinifera* T.C. Harr. & M.J. Wingf., and *Ceratocystis rufipenni* M.J. Wingf. et al. These, along with four other nonconifer-inhabiting members of the genus, form the *C. coerulescens* complex (Wingfield et al. 1997; Montoya and Wingfield 2006). Marin et al. (2005) noted that problems existed regarding species relationships within this complex, particularly with respect to *C. polonica* and *C. laricicola*, but some of these were resolved by their recognition of another new species, *Ceratocystis fujiensis* M.J. Wingf. et al. (in Marin et al. (2005)) within the *C. polonica* subgroup.

During a morphological reassessment of several strains in our culture collection, isolated in 1974 in eastern Norway from galleries of the bark beetle *Ips typographus* on specimens of *Picea abies* (L.) H. Karst. or from stained wood samples of the latter, we concluded that we had probably found representatives of a new *Ceratocystis* species. Therefore, to determine whether this was so, we compared nuclear small subunit rDNA (rSSU) gene and rDNA internal transcribed spacer (ITS) region (ITS1, 5.8S gene, and ITS2) sequences from our isolates with those of other *Ceratocystis* species. We also assessed the growth rates and cultural characteristics of three of our strains at different temperatures, and determined whether isolates derived from single ascospores formed perithecia.

Collectively, the results show that our Norwegian isolates do represent a discrete taxon within the genus *Ceratocystis* that is distinct both from members of the *C. polonica* subgroup and from other morphologically similar conifer-inhabiting species; it is described herein as *Ceratocystis norvegica* sp. nov.

Received 2 February 2010. Accepted 28 September 2010. Published on the NRC Research Press Web site at botany.nrc.ca on 19 November 2010.

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Table 1. Sources and GenBank accession Nos. for the isolates examined in this study.

Taxon	Source[a]	Substrate, locality, and notes	GenBank accession No.	
			SSU	ITS
Ceratocystis adiposa (E.J. Butler) C. Moreau	CBS 127.27	*Saccharum officinarum* L., India	—	DQ318195
	CBS 138.34	Isolated from air, as *Ceratocystis major* (von Beyma) C. Moreau	—	= DQ318195
Ceratocystis fagacearum (Bretz.) Hunt	ATCC 24790 = WIN(M) 892	*Quercus rubra* L., USA	—	DQ318193
Ceratocystis fimbriata Ellis & Halst	DAOM 195 303 = WIN(M) 931	*Picea abies* (L.) H. Karst, France	—	DQ318204
Ceratocystis moniliformis (Hedgc.) C. Moreau	CBS 773.73	*Quercus ellipsoidalis* E.J. Hill, USA	—	DQ318207
Ceratocystis norvegica J. Reid & Hausner	UAMH 11187 = WIN(M) 87	*Picea abies*, Norway, ex-holotype	DQ318194	DQ318209
	UAMH 11188 = WIN(M) 196	*Picea abies* (L.) H. Karst., Norway	= DQ318194	= DQ318209
	UAMH 11189 = WIN(M) 197	*Picea abies*, Norway	= DQ318194	= DQ318209
	UAMH 11190 = WIN(M) 224	*Picea abies*, Norway	—	= DQ318209
	UAMH 11191 = WIN(M) 225	*Picea abies*, Norway	—	= DQ318209
	UAMH 11192 = WIN(M) 226	*Picea abies*, Norway	—	= DQ318209
	UAMH 11193 = WIN(M) 227	*Picea abies*, Norway	—	= DQ318209
Ceratocystis paradoxa (Dade) C. Moreau	CBS 107.22 = WIN(M) 925	*Cocus nucifera* L.	—	DQ318203
Ceratocystis pinicola T.C. Harr. & M.J. Wingf.	CBS 100200 = WIN(M) 1437	*Pinus* sp., England, ex-holotype	—	DQ318198
	UAMH 9550 = WIN(M) 98	*Picea abies*, Norway, as *Ceratocystis coerulescens* (Münch) B.K. Bakshi	—	DQ318196
Ceratocystis polonica (Siemaszko) C. Moreau	WIN(M) 132	Norway	—	DQ318201
	WIN(M) 143	Norway	—	—
	UAMH 9779 = WIN(M) 153	*Picea abies*, Norway	—	—
	WIN(M) 178 (= Käärik B-100)	Sweden	—	—
	WIN(M) 195	Norway	—	—
	WIN(M) 198	Norway	—	DQ318202
	WIN(M) 199	Norway	—	—
	UAMH 9783 = WIN(M) 325	*Picea abies*, Norway	—	DQ318200
	NFRI 80–53/7 = UAMH 9586 = WIN(M) 450	*Picea abies*, Norway	—	DQ318199
	CBS 133.38 = WIN(M) 452	Gallery of *Ips typographus* L. in *Picea abies*, Poland, ex-holotype	—	—
Ceratocystis resinifera T.C. Harr. & M.J. Wingf.	WIN(M) 1409	British Columbia, Canada	—	DQ318205
	WIN(M) 1410	British Columbia, Canada	—	= DQ318205
	WIN(M) 1411	British Columbia, Canada	—	= DQ318205
	UAMH 9644 = WIN(M) 79	*Picea abies*, Norway, as *Ceratocystis coerulescens*	—	DQ318197
	UAMH 9645 = WIN(M) 81 = NFRI 1750/2	*Picea abies*, Norway	—	GU084160
Kernia pachypleura Malloch & Cain	UAHM 9790 = WIN(M) 253	Isolated from soil, Canada	—	DQ318208

[a]Cultures are deposited in one of the following culture collections: ATCC, American Type Culture Collection, Manassas, Virginia, USA; CBS, CBS-KNAW Fungal Biodiversity Center, Utrecht, Netherlands; DAOM, Canadian Collection of Fungus Cultures, Ottawa, Ontario, Canada; NFRI, Norwegian Forest Research Institute, Ås, Norway; UAMH, University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Edmonton, Alberta, Canada; WIN(M), culture collection of J. Reid, Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada.

Materials and methods

Strains studied and culturing procedure

Strains examined directly and (or) sequenced are detailed in Table 1. Living cultures and dried specimens of the newly described species have been deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH). Additional GenBank sequences incorporated in our analyses are listed in Table 2.

Unless stated otherwise, strains were grown on malt extract agar amended with yeast extract (MEA YE : 20 g/L malt extract; Difco, Mich., USA), 20 g/L agar (GIBCO BRL, Paisley, UK), and 1 g/L yeast extract (Gibco)) in 100 mm Petri plates (FisherBrand, Thermo Fisher Scientific, Ottawa, Ont., Canada). Cultures were grown and characterized according to Hausner et al. (2003), and colour designations are based on Rayner (1970). Microscopic preparations were mounted in 85% lactic acid.

We selected three of our isolates, UAMH 11187, 11188, and 11189, and determined their growth rates at different temperatures using the protocols of van Wyk et al. (2006), which were modified as follows: using a sterile 5 mm cork borer, mycelial inoculation plugs were cut from the margins of 2-week-old cultures grown at 20 °C on MEA.YE and inoculated centrally onto fresh medium. Six plates of each isolate were incubated at 10, 15, 20, 25, 30, and 35 °C for a period of 5 days, and the daily incremental growth increases were recorded within a fixed 2 h time interval on each successive day following Petri plate inoculation. Each set of measurements was taken and recorded in 2 h.

All plates incubated at 20 °C were retained for a further 16 days (thus 21 days in total from inoculation) to determine whether any significant differences between the test strains would become apparent.

Mycelium generation for DNA extraction and protocols for whole-cell DNA isolation followed Hausner et al. (1992).

Mating type determination

To determine the mating nature of our isolates, 20 single ascospore lines were obtained from 15-day-old sporulating cultures of UAMH 11187 and 11188. Depending on the size of the spore drops at the neck apices of individual perithecia, one to three drops were removed using a sterile inoculating needle and dispensed aseptically into 1 mL aliquots of a sterile aqueous solution of 10% dimethylsulfoxide (DMSO; Sigma–Aldrich Canada Ltd., Oakville, Ont., Canada) (Chung et al. 2006) in sterile, capped test tubes; eight tubes were prepared for each isolate. This was done using a variable magnification dissecting microscope fitted with both substage and incident illumination (Wild MB, Wild Leitz, Willowdale, Ont., Canada) positioned in an inoculation chamber.

Next, 2 mL of sterile distilled water was added to each tube, and these were kept upright for 2 h with periodic agitation. The contents of each were then poured aseptically into individual 2% water agar Petri plates and spread evenly over the entire surface of the medium with a sterile bent glass rod. Sixteen plates in all were kept upright for 2 h at room temperature to allow spores to settle, then inverted and placed in overlapping rows in a sterile tray. Thus, each plate was positioned at 20°–35° to the vertical, and excess moisture was drained into the lid of individual plates. The tray was then placed for 36–48 h in a darkened incubator at 20 °C. Next, single germinating ascospores were removed from these plates, placed centrally on fresh MEA.YE in 60 mm × 15 mm Petri plates (FisherBrand), and incubated in the dark at 20 °C.

Five and 8 days after the single-spore isolates were made, eight fresh plates were inoculated centrally with a single ascospore droplet from individual perithecial tips of a freshly sporulating culture of UAMH 11188. Sporulation development on these was compared with that on the single-ascospore derived cultures. The delayed inoculation times allowed the single ascospore cultures to develop more fully before the comparison trial began.

DNA extraction and amplification protocols

Primers SSUZ and LSU4 (Hausner et al. 2005) were used to amplify ITS regions, and primers SSJ and SST (Hausner et al. 1993*a*) were used for amplifying the nuclear rSSU gene. From the resulting PCR products, DNA sequencing templates were prepared using the Promega Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, Wis., USA). The purified PCR products were sequenced with primers previously described in Hausner and Reid (2004) and Hausner and Wang (2005) for the rSSU and ITS region, respectively. Sequences were generated using the Big Dye Version 3.1 cycle-sequencing kit according to the manufacturer's protocols (Applied Biosystems, Foster City, Calif., USA); the sequencing products were analyzed by automated fluorescent DNA sequence analysis as performed at the University of Calgary DNA Sequencing Facility (Calgary, Alta., Canada) using an ABI Prism 310 Genetic Analyzer System (Applied Biosystems).

Analyses of DNA sequence data

The sequences were aligned with ClustalX (Thompson et al. 1997) and, when appropriate, modified with the alignment editor program GeneDoc (version 2.5.010; Nicholas et al. 1997), but regions that could not be aligned unambiguously were deleted. The rSSU data set was analyzed using PHYLIP (Felsenstein 2006); both DNAPARS (maximum parsimony) and DNADIST (F84 setting), and the distance matrix generated from the latter, was utilized in the NEIGHBOR program (neighbor-joining (NJ) setting) for inferring a phylogenetic tree. The phylogenetic estimates were evaluated using the bootstrap procedure (Felsenstein 1985) (SEQBOOT 1000 replicates, and CONSENSE) in PHYLIP. The Tree-Puzzle (TP) program, a maximum likelihood phylogenetic analysis using quartets and parallel computing (Schmidt et al. 2002), was also used to infer phylogenetic relationships among the rSSU sequences. The settings for the quartet puzzling algorithm were 10 000 puzzling steps, transition–transversion parameter estimated from data set, and HKY evolutionary model (Hasegawa et al. 1985).

The ITS data set was analyzed with FASTME (Desper and Gascuel 2002) as implemented in DAMBE (Xia and Xie 2001) and with NJ and DNAPARS in combination with bootstrap analysis.

Phylogenetic trees were drawn with the TreeView program (Page 1996) using the PHYLIP tree outfiles and anno-

Table 2. List of sequences obtained from GenBank.

Taxon	Source[a]	GenBank accession No. SSU	GenBank accession No. ITS	References
Beauveria bassiana (Bals.-Criv.) Vuill.	ARSEF 2427	AF280633	—	Suh et al. 2001
Calcarisporium arbuscula Preuss	CBS 221.73	AY271796	—	Hausner and Reid 2004
Ceratocystis coerulescens	CBS 140.37	—	U75615	Witthuhn et al. 1998
	CL13-12	—	AY214000	Massoumi Alamouti et al. 2009
Ceratocystis douglasii (R.W. Davidson) M.J. Wingf. & T.C. Harr.	C324	—	U75626	Witthuhn et al. 1998
Ceratocystis fimbriata	CBS 146.53 = ATCC 14503	U43777	—	Issakainen et al. 1997
Ceratocystis fujiensis M.J. Wingf. et al.	CMW 1952	—	AY233924	Marin et al. 2005
Ceratocystis laricicola Redfern & Minter	CMW 7759	—	AY233919	Marin et al. 2005
	CMW 1016	—	AF043600	Witthuhn et al. 1999
Ceratocystis moniliformis	ATCC 12861	AY271799	—	Hausner and Reid 2004
Ceratocystis polonica	CMW 1164	—	AY233901	Marin et al. 2005
	CMW 5026	—	AY233907	Marin et al. 2005
Ceratocystis rufipenni M.J. Wingf. et al.	C612	—	U75621	Witthuhn et al. 1999
Chaetomium elatum J.C. Schmidt & Kunze:Fr.	UCB 81-063	M83257	—	Berbee and Taylor 1992*a*
Colletotrichum truncatum (Schwein.) Andrus & W.D. Moore	CBS 710.70	AJ301945	—	Nirenberg et al. 2002
Cornuvesica falcata (E.F. Wright & Cain) C.D. Viljoen et al.	UAMH 9702 = WIN(M) 792	AY271797	—	Hausner and Reid 2004
Chrysoporthe cubensis (Bruner) Gryzenhout & M.J.Wingf. as *Cryphonectria cubensis* (Bruner) C.S. Hodges	ATCC 64159	L42439	—	Chen et al. 1996
Eurotium rubrum Konig et al.	UCB 88.016	U00970	—	Berbee and Taylor 1993
Fomes fomentarius (L.:Fr.) J. Kickx fil.	DAOM 12903	U59069	—	Hibbett 1996
Galactomyces geotrichum (E.E. Butler & L.J. Petersen) Redhead & Malloch	CBS 772.71 = ATCC 22600 = IFO 9541	AB000647	—	Ueda-Nishimura and Mikata 2000
Gondwanamyces proteae (M.J. Wingf. et al.) G.J. Marais & M.J. Wingf.	CBS 486.88	AY271804	—	Hausner and Reid 2004
Graphium penicillioides Corda	CBS 506.86	AB007652	—	Ueda-Nishimura and Mikata 2000
Graphium tectonae C. Booth	CBS 127.84	U43907	—	Issakainen et al. 1997
Grosmannia cucullata (H. Solheim) Zipfel et al.	JCM 8815	AB007664	—	Okada et al. 1998
Halosarpheia retorquens Shearer & J.L. Crane	A68-1D	AF050486	—	Chen et al. 1999
Hypomyces chrysospermus Tul. & C. Tul.	na	M89993	—	Berbee and Taylor 1992*b*
Leucostoma persoonii (Nitschke) Höhn.	LP8	M83259	—	Berbee and Taylor 1992*a*
Lomentospora prolificans Hennebert & B.G. Desai	CBS 467.74	U43909	—	Issakainen et al. 1997
Microascus cirrosus Curzi	CBS 301.61 = UAMH 963	M89994	—	Berbee and Taylor 1992*b*
Monascus purpureus Went	CBS 109.07 = ATCC 16365	M83260	—	Berbee and Taylor 1992*a*
Morchella elata Fr.	na	L37537	—	Gargas and Taylor 1995
Myrothecium roridum Tode	BBA 63372	AJ301994	—	Unpublished
Naïs inornata Kohlm.	J115-5C	AF050482	—	Chen et al. 1999
Neurospora crassa Shear & B.O. Dodge	na	X04971	—	Sogin et al. 1986
Ophiostoma stenoceras (Robak) Nannf.	UCB 57.013	M85054	—	Berbee and Taylor 1992*c*
Ophiostoma ulmi (Buisman) Nannf.	ATCC 32437	M83261	—	Berbee and Taylor 1992*a*
Paecilomyces fumosoroseus (Wize) A.H.S. Br. & G. Sm.	IFO 7072	AB086629	—	Unpublished

Table 2 (*concluded*).

Taxon	Source[a]	GenBank accession No. SSU	GenBank accession No. ITS	References
Petriella setifera (Alf. Schmidt) Curzi	CBS 385.87	U43908	—	Issakainen et al. 1997
Phlebia radiata Fr.	FPL 6140	AF026606	—	Hibbett et al. 1997
Pseudallescheria boydii (Shear) McGinnis et al.	UAMH 4304	M89782	—	Berbee and Taylor 1992*b*
Saccharomyces cerevisiae Meyen ex E.C. Hansen	na	J01353	—	Rubtsov et al. 1980; Mankin et al. 1986
Talaromyces flavus Stolk & Samson	FRR 2386	M83262	—	Berbee and Taylor 1992*a*

[a]Source information taken from GenBank records. Acronyms for culture collections are as folllows: ARSEF, ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, New York, USA; ATCC, American Type Culture Collection, Manassas, Virginia, USA; BBA, culture collection of the Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Mikrobiologie Berlin, Germany; C, culture collection of T.C. Harrington, Plant Pathology, Iowa State University, Ames, Iowa USA; CBS, CBS-KNAW Fungal Biodiversity Center, Utrecht, Netherlands; CMW, culture collection of the Forestry and Agricultural Biotechnology Institute, FABI, Pretoria, South Africa; DAOM, Canadian Collection of Fungus Cultures, Ottawa, Ontario, Canada; FPL, USDA Forest Products Laboratory, Madison, Wisconsin, USA; FRR, culture collection of the CISRO Food Research Laboratory, North Ryde, New South Wales, Australia; IFO, culture collection of the Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama, Japan; LP, culture collection of G.C. Adams, Plant Pathology, Michigan State University, East Lansing, Michigan, USA; UAMH, University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Edmonton, Alberta, Canada; UCB or UCBH, culture collection of the University of California, Berkeley, California, USA; WIN(M), culture collection of J. Reid, Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada; na, no data available.

tated using Corel Draw (Corel Corporation, Ottawa, Ont., Canada).

Results

Cultural and morphological characteristics

The three isolates tested showed very similar morphological growth–temperature responses under each test condition. Therefore, only the response of UAMH 11187 is shown in Fig. 1A. However, the experiment was terminated on day 5 because by then, at 20 °C, the mycelium had completely covered at least one of the test plates.

The very similar growth–temperature profiles of the three isolates are depicted graphically in Fig. 1B, with optimum growth peaking sharply at 20° C for all strains, decreasing markedly at 25 °C, and none occurring at 30 °C . By day 21, no significant differences were noted between the retained cultures of the three strains tested; therefore, the description of cultural characteristics is based on UAMH 11187, and the representative temperature-related colour change is shown in Fig. 1(A).

The mycelium was appressed initially, with only relatively sparse aerial hyphae being produced, but perithecial initials were visible by day 5 at 10, 15, and 20 °C. By day 3 at 20 °C, small knots of mycelium were forming randomly over the mycelium surface. Initially, these knots were pale mouse grey in colour, becoming mouse grey as they continued to enlarge. They never coalesced fully as the cultures aged except at the culture margins. Within these knots and mounds of hyphal elements, depending on their size, one to several perithecia developed, and the mycelium between the mounds became buff coloured; a similar central mound developed at the inoculation point in each plate.

When the mycelium reached the margins of the Petri plates it piled up uniformly like a narrow dam, or ring, surrounding the more central area; this ring gradually broadened inwardly. At first this mycelium was pale mouse grey in colour, but darkened to mouse grey as it aged, and it was in this mycelium that the majority of the perithecia produced in a plate eventually developed (Fig. 1C, arrow). By day 15 the appressed mycelium within this mycelial ring had darkened because of pigment excretion into the medium, and the culture appeared fuscous black centrally, but with scattered mounds of mouse grey hyphae in some of which perithecia had formed. By day 21, when observations were terminated, the peripheral hyphal ring had expanded further inwardly, although the mycelium of this fresh growth was slightly less dense.

Mating type determination

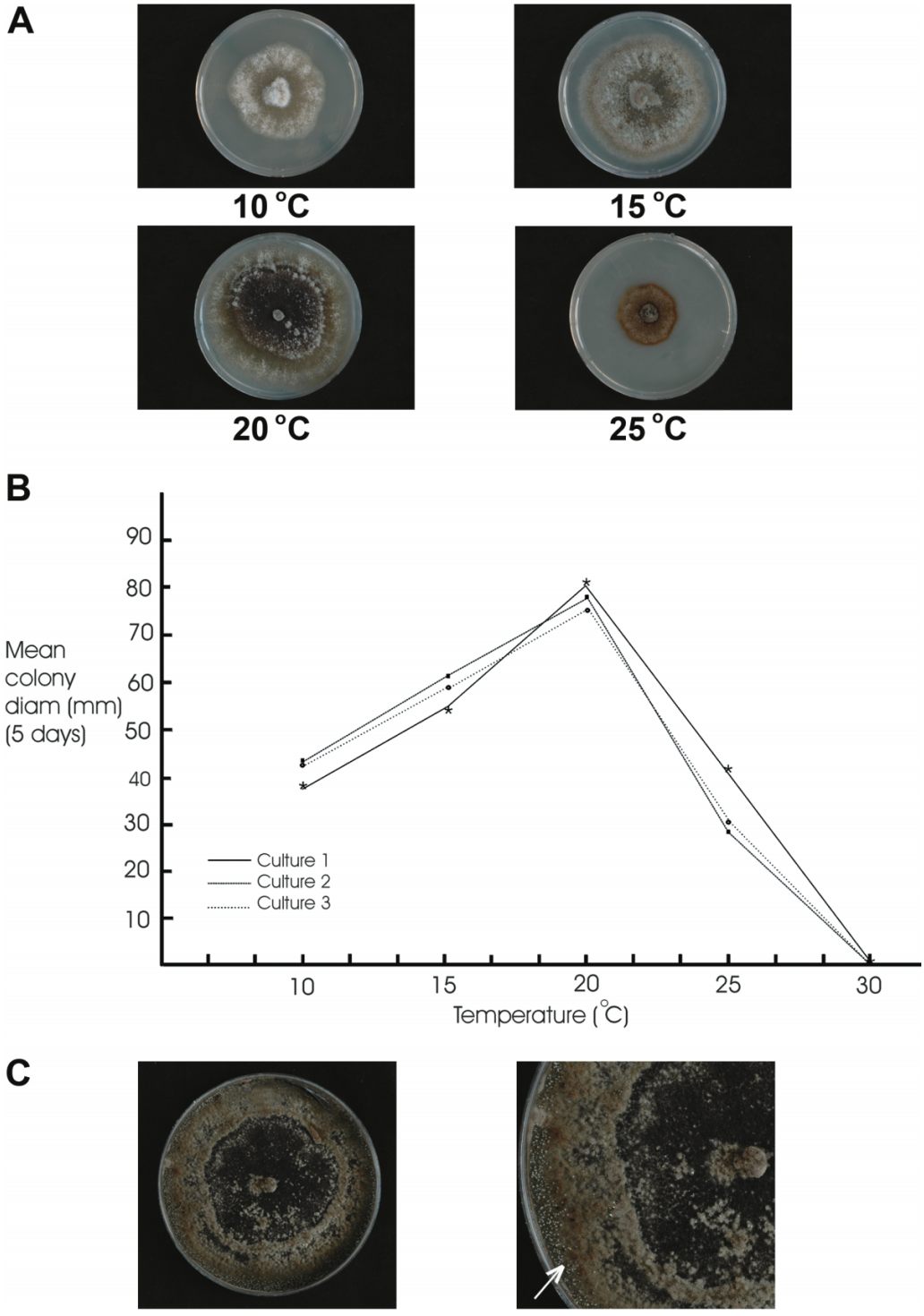
Having noted that when the mycelial mats reached plate edges they became extensively mounded and barrier-like, and here perithecia developed most rapidly and abundantly, we established the single ascospore cultures on the smaller Petri plates, hoping to shorten the time required to induce the barrier formation and subsequent development of mature perithecia; it did.

These culture plates were examined 20 days after inoculation; this was 12 and 15 days after inoculation of the UAMH 11188 spore droplet plates, and at that time 18 single-ascospore plates of UAMH 11187 and 17 plates of UAMH 11188 had vigorous, normal-appearing colonies filling the plates. Microscopic examination using incident illumination (plate lids removed) revealed that while the majority of cultures from single ascospores had produced abundant perithecia, a few were sterile; perithecia were abundant on all plates of both check sets. This suggests that ascospores of *C. norvegica* may produce either self-fertile or self-sterile strains.

DNA sequence analysis

Analysis of our combined rSSU sequence data set helped to confirm placement of our new species. The aligned data set contained 34 unique rSSU sequences covering 1693 positions and included appropriate strains of *Ceratocystis*, *Cornuvesica* C.D. Viljoen et al., *Gondwanamyces* G.J. Marais & M.J. Wingf., and *Ophiostoma* H. & P. Sydow (see Tables 1 and 2). Phylogenetic analysis based on NJ, maximum parsimony, and maximum likelihood analyses yielded phylogenetic trees with nearly identical topologies and bootstrap

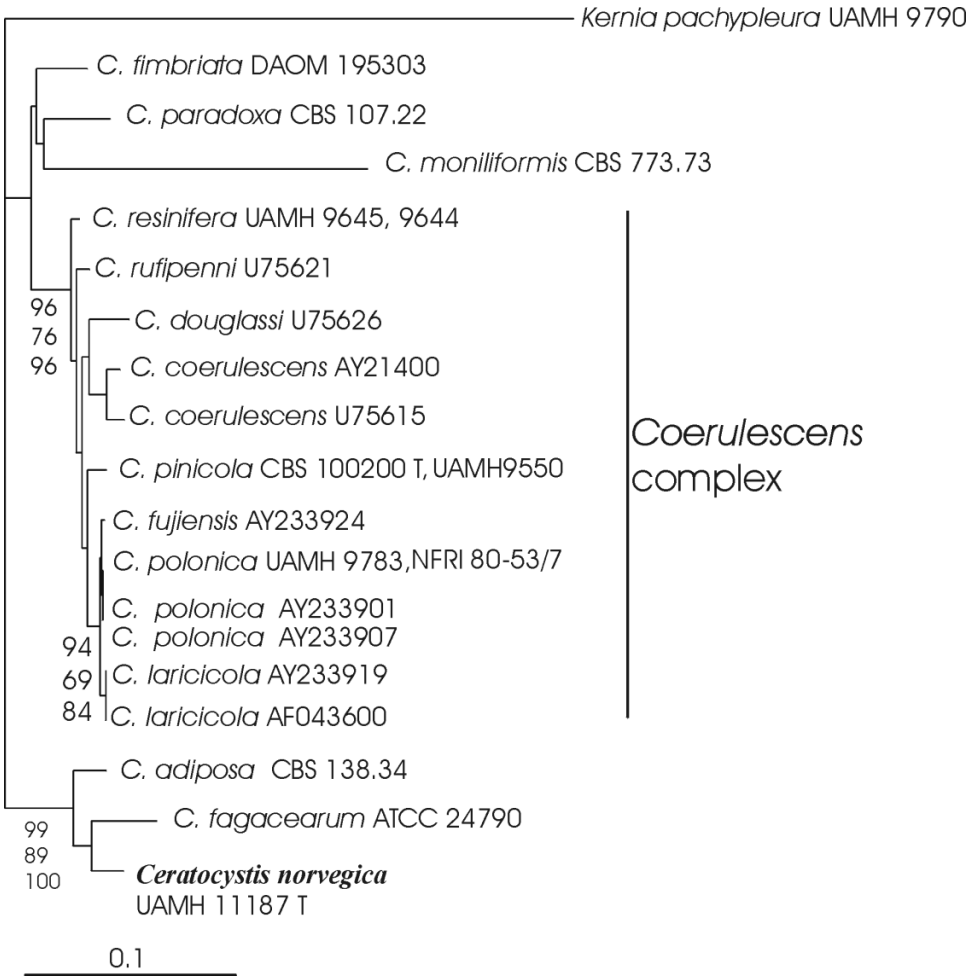
Fig. 1. (A) Morphology of UAMH 11187 grown at 10, 15, 20, and 25 °C for 5 days (no growth occurred at 30 °C). (B) Temperature–growth curves for UAMH 11187 (culture 1), 11188 (culture 2), and 11189 (culture 3) incubated at 10, 15, 20, and 25 °C for 5 days. (C) A typical culture of UAMH 11187 grown at 20 °C for 21 days (left). Note the dam-like mycelial margin at the edge of the Petri plate and, at right, an enlarged section thereof, in which abundant perithecia, with spore droplets at their neck tips, can been seen in the mycelial dam (arrow).



support for the major nodes (Fig. 2). Isolates of *Ceratocystis norvegica* grouped with *Ceratocystis fimbriata* and *Ceratocystis moniliformis*, and these three species formed a clade that was sister to *Cornuvesica falcata*, the only species in the monotypic genus *Cornuvesica*. *Gondwanamyces proteae*, the three species of *Ceratocystis*, and *Cornuvesica falcata* formed a well-supported clade that was, in turn, sister to members of the Microascales.

The ITS data set contained 25 sequences covering 573 positions (see Table 1) in which *Kernia pachypleura* served as the outgroup. Based on the four different methodologies (NJ, DNAPARS, and ML-Quartet puzzling), the ITS se-

Fig. 2. Phylogenetic tree based on rSSU sequence data. The strains of *Ceratocystis norvegica* represented by DQ318194 include the exholotype UAMH 11187. The levels of confidence for the major nodes in the phylogenetic tree are based on bootstrap analysis in NEIGHBOR (first value), DNAPARS (second value), and TREE-Puzzle (third value).



quence analysis yielded phylogenetic trees with similar topologies and node support values (Fig. 3). In this phylogeny, isolates representing *C. coerulescens*, *C. polonica*, *C. resinifera,* and *C. pinicola* formed a well-supported group corresponding to part of the *C. coerulescens* complex sensu Harrington and Wingfield (1998). *Ceratocystis norvegica* J. Reid & Hausner sp. nov. did not belong to this complex, but was positioned in a separate lineage with strains representing *Ceratocystis fagacearum* (1956) and *Ceratocystis adiposa* [≡*Ceratocystis major fide* (Upadhyay 1981)].

Taxonomy

As both our molecular analyses and morphological data show that our isolates are distinct from other tested *Ceratocystis* species, we now describe a new species first reported from Norway.

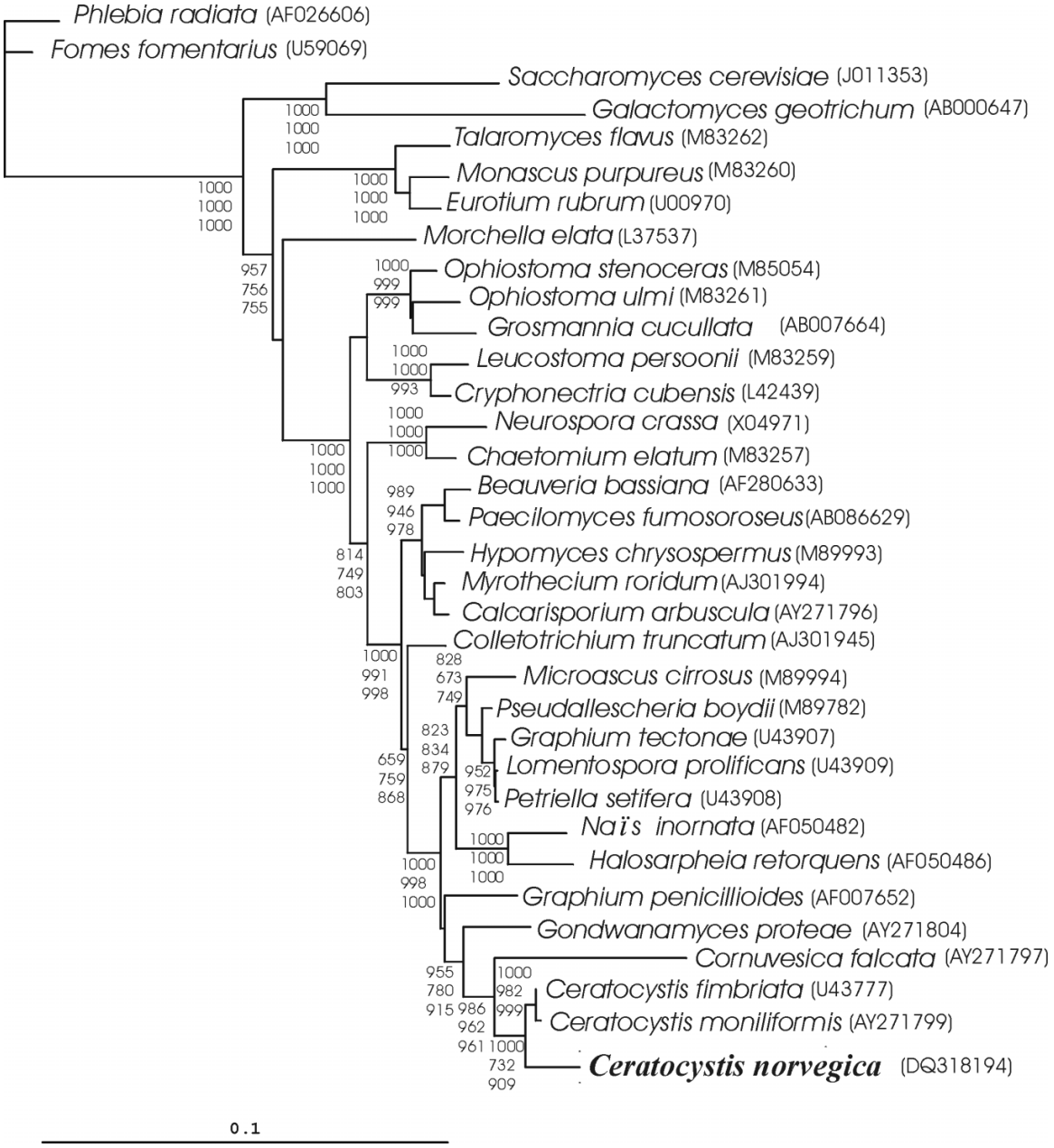
Ceratocystis norvegica J. Reid & Hausner sp. nov. (Figs. 1 and 4). ETYMOLOGY: Latin. Referring to the country of origin of the isolates.

DESCRIPTION: *Coloniae (conidiomata) celeriter crescentes, in agaro cum extracto malti (2%) fermentique post 4 dies ad 25 °C, 34.08 mm diametro; sine auctu supra 30 °C. Mycelium ad 20 °C ab initio hyalinum adpressum faciens ex hyphis compactis tumulos accrescentes murinescentesque; hyphae prostratae interjacentes primo hyalinae demum bubalinae postremo nigrae. Perithecia abunda ad 20 °C, aliquot in singulis tumulis et multa in peripheria aggeracea coloniae inclusa; bases nigrae sub luce incidenti obpyriformes ad ampulliformes raro globosae, multis fuscis hyphis ornatae, 125–275* μm *latae. Colla fusca elongata angustataque pallescentia versus apicem, basi lata 37.5–87.5* μm *apice lata 17.5–25* μm *longa sine hyphis ostiolaribus 605–945* μm. *Hyphae ostiolares 25–105* μm *longae convergentes hyalinae in apices obtusatas rotundatasque parum contractae. Ascosporae unicellulares hyalinae vagina translucida oblongae vel aliquando leviter curvatae; cum vagina longae 6.0–9.0* μm *latae 2.0–5.0* μm, *sine vagina longae 4.0–7.0* μm *latae 1.5–3.0* μm. *Conidia non visa.*

TYPE MATERIAL EXAMINED:

Holotype: Dried culture prepared from UAMH 11187 [=WIN(M) 87] obtained from a perithecial spore drop produced at the neck apex of a perithecium found in a gallery of the beetle *Ips typographus* L. on slash of *Picea abies* (L.) Karst., September 1973, Highway 21, near Sandem, Ostfold, Norway. Collected and isolated by J. Reid. Also cultured on wood, specimen retained (ISOTYPE).

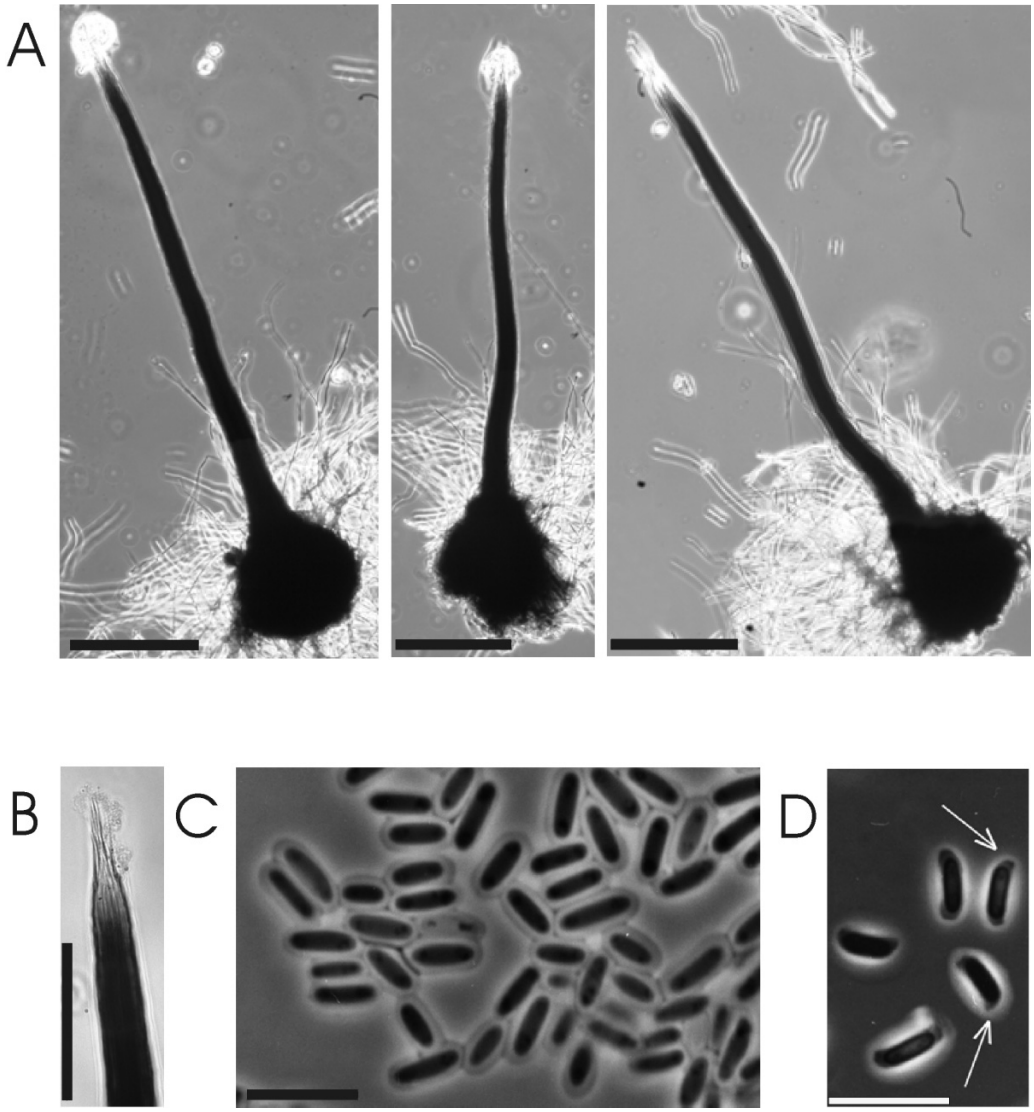
Fig. 3. Phylogenetic tree based on ITS sequence data. The levels of confidence for the major nodes in the phylogenetic tree are based on bootstrap analysis in NEIGHBOR (first value), DNAPARS (second value), and FASTME (third value).



Colonies fast growing on 2% malt extract agar amended with yeast extract; optimum growth at 20 °C, and maximum growth between 25 and 30 °C (Fig. 1B); after 4 days reaching a mean diameter of 34 mm at 25 °C, but no growth at or above 30 °C. At 20 °C, mycelium initially hyaline and appressed, then forming small pale mouse grey mounds of compact hyphae that gradually enlarge and become mouse grey in colour; though some mounds fuse, they never all fully coalesce, and the cultures retain an irregular surface. Intermound prostrate hyphae initially hyaline, gradually become buff coloured and, on further aging, black. Mycelium at the margin of the Petri plate forming a dense, floccose band of aerial mycelium that eventually becomes mouse grey in colour.

Perithecia form abundantly at 20 °C, embedded one to several in individual hyphal mounds, particularly abundant in the dam-like periphery of the thallus; bases appearing black by incident light; obpyriform to ampulliform, rarely globose in outline, and ornamented with numerous dark, hyphal elements; height 125–275 μm (194.54 ± 42.54 μm; mean ± SD) ; width 125– 238 μm (185.71 ± 50.74 μm). Necks dark and flared at their bases, elongate, tapering, and becoming slightly paler where ostiolar hyphae are produced; bases 37.5–87.5 μm wide (60.08 ± 11.82 μm); apices 17.5–25.0 μm wide (23.08 ± 2.47 μm); neck length (excluding ostiolar hyphae) 605–945 μm (849.63 ± 148.5 μm). Ostiolar hyphae 25–105μm long (78.4 ± 17.76 μm), convergent, hyaline, and tapered slightly to blunted, rounded tips (Fig. 4B).

Fig. 4. Morphological features of *Ceratocystis norvegica* UAMH 11187. (A) Three perithecia depicting base shape and ornamentation, and neck shape and apex. Scale bar = 200 μm. (B) Convergent ostiolar hyphae at the apex of the perithecial neck. Scale bar = 100 μm. (C) Ascospores with mostly uniform sheaths in plan view. Scale bar = 10 μm. (D). Ascospores, some curved, in side view showing variations in sheath thickness. Scale bar = 10 μm.



Ascospores unicellular, hyaline, oblong-cylindrical in plan view (Olchowecki and Reid 1974) with a uniform translucent sheath (Fig. 4C), but occasionally appearing slightly curved in side view, and then the sheath often appears thicker at the spore ends (Fig. 4D), with sheath 6.0–9.0 μm long (6.98 ± 0.95 μm) and 2.0– 5.0 μm wide (3.46 ± 0.58 μm), without sheath 4.0–7.0 μm long (5.38 ± 0.75 μm) and 1.5–3.0 μm wide (1.79 ± 0.41 μm). Neither a conidial anamorph nor perithecial spines were observed.

ADDITIONAL MATERIAL EXAMINED: Both geographical and collection data were as cited above. (1) A dried culture obtained from UAMH 11188 (=WIN(M) 196) prepared, as above, from a second perithecium found in a gallery in a second piece of slash. (2) A dried culture of an isolate UAMH 11189 (=WIN(M) 197), obtained from stained sapwood adjacent to a beetle gallery in a third piece of slash. Four additional isolates (UAMH 11190, =WIN(M) 224; UAMH 11191, =WIN(M) 225; UAMH 11192, =WIN(M) 226; UAMH 11193, =WIN(M) 227) were also obtained from the same piece of slash as UAMH 11187, but from various other spore ball producing perithecia.

Discussion

As the comparative data presented in Table 3 show, only four cultural characteristics separate our proposed new *Ceratocystis* species from all others described previously on conifers.

First, *C. norvegica* has convergent ostiolar hyphae, while in all other species they are divergent, except for *C. polonica*; in the latter they are parallel to divergent. Second, it has a sharply defined temperature optimum at 20 °C, and such a result is, for example, in sharp contrast to those reported for *C. polonica*, *C. laricicola*, and *C. fujiensis* (Marin et al. 2005); the latter each has a rather broad optimum temperature range, from 20 to 25 °C, with growth only slightly less at 20 °C than at 25 °C, and they all pro-

Table 3. Morphological and cultural features of *Ceratocystis norvegica* and members of the "*coerulescens*" species on conifers that lack hat-shaped ascospores.

Ascomata and culture characteristics	*Ceratocyst coerulescens* (1)	*Ceratocystis douglasii* (2)	*Ceratocystis fujiensis* (3)	*Ceratocystis laricicola* (4)	*Ceratocystis norvegica* (5)	*Ceratocystis pinicola* (6)	*Ceratocystis polonica* (7)	*Ceratocystis resinifera* (8)	*Ceratocystis rufipenni* (9)
Base ornamentation	White-tipped brown hyphae	Short pigmented hyphae	Dark hyphae	Brown-walled hyphae	Dark multicellular hyphae	Long distinct spines	Dark multicellular hyphae	Distinct spines	Short pigmented hyphae
Base form	Round to ovoid	Globose	Globose	Globose	Opyriform to ampulliform	Not stated	Subglobose	Subglobose	Globose
Ostiolar hyphae	Divergent	Divergent	Divergent	Divergent	Convergent	Divergent	Parallel to divergent	Divergent	Divergent
Ascospre shape with sheaths	Elongate with rounded ends	Elongate to slightly curved rounded ends	Oblong sheaths widest at middle	Ellipsoid sheaths widest at sides	Oblong to occasionally slightly curved	Ellipsoid to elongate, sometimes curved	Ellipsoid to broadly ellipsoid, widest at middle	Elongate to ellipsoid	Elongate to slightly curved rounded ends
Temp. max. (°C)	Not noted	>30	33	33	>25	Not noted	32	Not noted	>25
Temp. min. (°C)	Not noted	Not noted	10	Not noted	<5	Not noted	<10	Not noted	Not noted
Optimum temp. (°C)	22	20–25	25	25	20	25	25	20 to 25	20
Mycelium	Appressed sparse aerial mycelium	Virtually no aerial mycelium	Abundant aerial mycelium	Abundant aerial mycelium	Appressed with aerial hyphal mounds	Appressed little aerial mycelium	Abundant aerial mycelium	Appressed little or no aerial mycelium	Appressed little or no aerial mycelium
Mating type	Homothallic and (?) heterothallic	Not noted	Heterothallic	Heterothallic	Self-fertile and self-sterile	Homothallic and heterothallic	Homothallic and heterothallic	Homothallic and heterothallic	Homothallic and heterothallic

Note: Data derived from the following: (1) Bakshi 1951; (2) Harrington and Wingfield 1998; (3) Harrington et al. 2002, Marin et al. 2005; (4) Redfern et al.1987, Harrington et al. 2002, Marin et al. 2005; (5) this paper; (6) Harrington and Wingfield 1998; (7) Siemaszko 1939, Harrington and Wingfield 1998, Harrington and McNew 1998, Marin et al. 2005; (8) Harrington and Wingfield 1998; (9) Wingfield et al. 1997.

duce measurable growth at 30 °C. Third, to date we have not seen a conidial state in our fungus. Other *Ceratocystis* spp., including those listed in Table 2, have anamorphs assigned to *Thielaviopsis* Went emend. Paulin-Mahady et al. (2002), wherein conidia are produced at the base of the deep, cylindrical phialide collarette (Minter et al. 1983; Minter 1987); in some species, dark, thick-walled aleurioconidia are also produced (Paulin and Harrington 2000; Paulin-Mahady et al. 2002). Finally, *C. norvegica* has a somewhat different pattern of mycelial production in Petri plate cultures (Fig. 1C) from that of previously described species.

Although the above characters alone might not justify erection of a new species, we feel that when they are combined with our molecular data (rSSU and ITS sequences), and when this total data set is contrasted with that published for other *Ceratocystis* spp. (Table 3), these results confirm that our isolates do represent a new species.

Van Wyk et al. (2004, p. 276) reported that *C. bhutanensis*, *C. moniliformis*, and *C. moniliformopsis* cultures tended to degenerate on their formulation of MEA, stating, "Degenerated cultures became white in colour, displayed reduced growth and ceased to produce ascomata in culture." Not infrequently one of our isolates, UAMH 11189, also showed abnormal development when transfers were made. In these cases, however, while the mycelium was more uniform in appearance over the surface of the plate and the colour variation was greatly reduced, perithecia could still be seen. We cannot explain what underlies this variation, but this is observed in the one isolate originally derived from stained, infected wood.

There were also other minor variations between our isolates in culture. UAMH 11189 produced both smaller and fewer perithecial initials during the growth culture experiment than did UAMH 11187 and 11188, and although all strains became pigmented, with its intensity increasing with temperature rise, at all temperatures colour was slightly more intense in UAMH 11189.

That our rSSU data demonstrated a relationship of *C. norvegica* to both *C. fimbriata* and *C. moniliformis*, and then a relationship of these three to *Cornuvesica falcata* and members of the Microascales, was not surprising; similar findings have been reported previously (Hausner et al. 1993*b*; Spatafora and Blackwell 1994; Hausner and Reid 2004). These data confirmed that *C. norvegica* is a member of the genus *Ceratocystis*, but it provided no information as to its relationship to the *Ceratocystis* species on conifers sensu Harrington and Wingfield (1998).

While neither our ITS data, nor those of Witthuhn et al. (2000), are strongly supported, our phylogram shows that relationships amongst the species within the "coerulescens" clade are more complex than does theirs. For example, in their subclade (Witthuhn et al. 2000, Fig. 2), *C. douglasii* is linked with both *C. pinicola* and *C. coerulescens*, but our results set the former apart from all other species in that clade. In so doing, our findings are similar to those of Harrington and Wingfield (1998, p. 1456), who stated that, "Of all the species treated here, *C. douglasii* has the most distinctive isozyme profile (Harrington et al.1996) and ITS sequence (Witthuhn et al. 1998)." Although our ITS data does demonstrate the monophyly of the "coerulescens" clade as defined by the species we studied, the monophyly of the then-known

Ceratocystis species on conifers had been demonstrated previously by Witthuhn et al. (1998) and Paulin-Mahady et al. (2002). In contrast though, Witthuhn et al. (2000) have also shown that, at times, ITS data might not resolve relationships between closely related sympatric species, whereas physiological characters (isozyme analysis) would.

It had been suggested that conifer-inhabiting species of *Ceratocystis* are monophyletic, but some species were impossible to distinguish morphologically. Therefore, species relationships had to be resolved using techniques such as enzyme pattern variation; analysis of sequence differences in the ITS of their rDNA operons and other genomic regions; sexual compatibility; and restriction enzyme polymorphism analysis of selected genome regions (Harrington et al. 1996, 2002; Witthuhn et al. 1998, 1999; Loppnau and Breuil 2003; Marin et al. 2005). These studies generally confirmed that there were indeed nine monophyletic *Ceratocystis* spp. inhabiting coniferous tree species, but the taxonomy of such conifer-inhabiting fungi had not actually been resolved. Later, two additional species from conifers were isolated and described, *Ceratocystis bhutanensis* M . van Wyk et al. (in van Wyk et al. 2004) and *Ceratocystis tribiliformis* M. van Wyk & M.J. Wingfield (in van Wyk et al. 2006), but these two were readily distinguishable from the others noted earlier, as they have hat-shaped ascospores. Furthermore, using comparison of polymorphic simple sequence repeat content, Marin et al. (2009) have shown that even within the well-known species *C. polonica*, previously unrecognized genetic diversity exists. Thus, the presumed implication from many studies that all *Ceratocystis* species similarly adapted to conifers will be closely related is, as has been shown by van Wyk et al. (2004, 2006) and our results, not the case.

Acknowledgments

We gratefully acknowledge funding for this project from operating grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to G. Hausner and P.C. Loewen and a Canada Research Chair to P.C. Loewen. We are also grateful to Dr. David Punter for providing the Latin diagnoses.

References

Bakshi, B.K. 1950. Fungi associated with Ambrosia beetles. Trans. Br. Mycol. Soc. **33**(1–2): 111–120.

Bakshi, B.K. 1951. Studies on four species of *Ceratocystis*, with a discussion on fungi causing sapstain in Britain. Commonwealth Mycol. Inst. Mycol. Pap. **35**: 1–16.

Berbee, M.L., and Taylor, J.W. 1992*a*. Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. Mol. Biol. Evol. **9**(2): 278–284. PMID:1560763.

Berbee, M.L., and Taylor, J.W. 1992*b*. Convergence in ascospore discharge mechanism among pyrenomycete fungi based on 18S ribosomal RNA gene sequence. Mol. Phylogenet. Evol. **1**(1): 59–71. doi:10.1016/1055-7903(92)90036-G. PMID:1342925.

Berbee, M.L., and Taylor, J.W. 1992*c*. 18S ribosomal RNA gene sequence characters place the human pathogen *Sporothrix schenckii* in the genus *Ophiostoma*. Exp. Mycol. **16**: 87–91. doi:10.1016/0147-5975(92)90044-R.

Berbee, M.L., and Taylor, J.W. 1993. Dating the evolutionary radiations of the true fungi. Can. J. Bot. **71**: 1114–1127.

Chen, B.S., Chen, C.H., Bowman, B.H., and Nuss, D.L. 1996. Phenotypic changes associated with wild-type and mutant hypovirus RNA transfection of plant pathogenic fungi phylogenetically related to *Cryphonectria parasitica*. Phytopathology, **86**(3): 301–310. doi:10.1094/Phyto-86-301.

Chen, W., Shearer, C.A., and Crane, J.L. 1999. Phylogeny of *Ophioceras* spp. based on morphological and molecular data. Mycologia, **91**(1): 84–94. doi:10.2307/3761195.

Chung, W.-H., Kim, J.-J., Yamaoka, Y., Uzunovic, A., Masuya, H., and Breuil, C. 2006. *Ophiostoma breviusculum* sp. nov. (Ophiostomatales, Ascomycota) is a new species in the *Ophiostoma piceae* complex associated with bark beetles infesting larch in Japan. Mycologia, **98**(5): 801–814. doi:10.3852/mycologia.98.5.801. PMID:17256583.

Desper, R., and Gascuel, O. 2002. Fast and accurate phylogeny reconstruction algorithms based on the minimum-evolution principle. J. Comput. Biol. **9**(5): 687–705. doi:10.1089/106652702761034136. PMID:12487758.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, **39**(4): 783–791. doi:10.2307/2408678.

Felsenstein, J. 2006. PHYLIP (Phylogeny Inference Package) version 3.6c [computer program]. Distributed by the author. Department of Genetics, University of Washington, Seattle, Wash.

Gargas, A., and Taylor, J.W. 1995. Phylogeny of Discomycetes and early radiations of the apothecial Ascomycotina inferred from SSU rDNA sequence data. Exp. Mycol. **19**(1): 7–15. doi:10.1006/emyc.1995.1002. PMID:7614369.

Harrington, T.C., and McNew, D.L. 1998. Partial interfertility among the ceratocystis species on conifers. Fungal Genet. Biol. **25**(1): 44–53. doi:10.1006/fgbi.1998.1083. PMID:9806805.

Harrington, T.C., and Wingfield, M.J. 1998. The *Ceratocystis* species on conifers. Can. J. Bot. **76**(8): 1446–1457. doi:10.1139/cjb-76-8-1446.

Harrington, T.C., Steimel, J., Wingfield, M.J., and Kile, G.A. 1996. Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. Mycologia, **88**(1): 104–113. doi:10.2307/3760789.

Harrington, T.C., Pashenova, N.V., McNew, D.L., Steimel, J., and Konstantinov, M.Y. 2002. Species delimitation and host specialization of *Ceratocystis laricicola* and *C. polonica* to larch and spruce. Plant Dis. **86**(4): 418–422. doi:10.1094/PDIS.2002.86.4.418.

Hasegawa, M., Kishino, H., and Yano, T.-A. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22**(2): 160–174. doi:10.1007/BF02101694. PMID:3934395.

Hausner, G., and Reid, J. 2004. The nuclear small subunit ribosomal genes of *Sphaeronaemella helvellae*, *Sphaeronaemella fimicola*, *Gabarnaudia betae*, and *Cornuvesica falcata*: phylogenetic implications. Can. J. Bot. **82**(6): 752–762. doi:10.1139/b04-046.

Hausner, G., and Wang, X. 2005. Unusual compact rDNA gene arrangements within some members of the Ascomycota: evidence for molecular co-evolution between ITS1 and ITS2. Genome, **48**(4): 648–660. doi:10.1139/g05-037. PMID:16094433.

Hausner, G., Reid, J., and Klassen, G.R. 1992. Do galeate-ascospore members of the Cephaloascaceae, Endomycetaceae and Ophiostomataceae share a common phylogeny? Mycologia, **84**(6): 870–881. doi:10.2307/3760285.

Hausner, G., Reid, J., and Klassen, G.R. 1993*a*. On the phylogeny of *Ophiostoma*, *Ceratocystis* s.s., and *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. Can. J. Bot. **71**: 1249–1265.

Hausner, G., Reid, J., and Klassen, G.R. 1993*b*. *Ceratocystiopsis*: a

reappraisal based on molecular criteria. Mycol. Res. **97**(5): 625–633. doi:10.1016/S0953-7562(09)81188-X.

Hausner, G., Eyjólfsdóttir, G.G., and Reid, J. 2003. Three new species of *Ophiostoma* and notes on *Cornuvesica falcata*. Can. J. Bot. **81**(1): 40–48. doi:10.1139/b03-009.

Hausner, G., Iranpour, M., Kim, J.-J., Breuil, C., Davis, C.N., Gibb, E.A., Reid, J., Loewen, P.C., and Hopkin, A.A. 2005. Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada, and comments on the taxonomy of *Leptographium lundbergii*, *Leptographium terebrantis*, *Leptographium truncatum*, and *Leptographium wingfieldii*. Can. J. Bot. **83**: 1222–1237. doi:10.1139/b05-095.

Hibbett, D.S. 1996. Phylogenetic evidence for horizontal transmission of group I introns in the nuclear ribosomal DNA of mushroom-forming fungi. Mol. Biol. Evol. **13**(7): 903–917. PMID: 8751999.

Hibbett, D.S., Pine, E.M., Langer, E., Langer, G., and Donoghue, M.J. 1997. Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. Proc. Natl. Acad. Sci. U.S.A. **94**(22): 12002–12006. doi:10.1073/pnas.94.22.12002. PMID: 9342352.

Issakainen, J., Jalava, J., Eerola, E., and Campbell, C.K. 1997. Relatedness of *Pseudallescheria*, *Scedosporium* and *Graphium pro parte* based on SSU rDNA sequences. J. Med. Vet. Mycol. **35**: 389–398.

Loppnau, P.A., and Breuil, C. 2003. Species level identification of conifer associated *Ceratocystis* sapstain fungi by PCR–RFLP on a β-tubulin gene fragment. FEMS Microbiol. Lett. **222**(1): 143–147. doi:10.1016/S0378-1097(03)00256-8. PMID:12757958.

Mankin, A.S., Skryabin, K.G., and Rubtsov, P.M. 1986. Identification of ten additional nucleotides in the primary structure of yeast 18S rRNA. Gene, **44**(1): 143–145. doi:10.1016/0378-1119(86)90054-5. PMID:3021584.

Marin, M., Preisig, O., Wingfield, B.D., Kirisits, T., Yamaoka, Y., and Wingfield, M.J. 2005. Phenotypic and DNA sequence data comparisons reveal three discrete species in the *Ceratocystis polonica* species complex. Mycol. Res. **109**(10): 1137–1148. doi:10.1017/S095375620500362X. PMID:16279408.

Marin, M.M., Preisig, O., Wingfield, B.D., Kirisits, T., and Wingfield, M.J. 2009. Single sequence repeat markers reflect diversity and geographic barriers in Eurasian populations of the conifer pathogen *Ceratocystis polonica*. For. Pathol. **39**(4): 249–265. doi:10.1111/j.1439-0329.2009.00585.x.

Massoumi Alamouti, S., Tsui, C.K., and Breuil, C. 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. Mycol. Res. **113**(8): 822–835. doi:10.1016/j.mycres.2009.03.003. PMID:19348942.

Minter, D.W. 1987. The significance of conidiogenesis in pleoanamorphy. *In* Pleomorphic fungi. *Edited by* J. Sugiyama. Kodansha, Tokyo and Elsevier, Amsterdam. pp. 241–262.

Minter, D.W., Sutton, B.C., and Brady, B.L. 1983. What are phialides anyway? Trans. Br. Mycol. Soc. **81**(1): 109–120. doi:10.1016/S0007-1536(83)80210-1.

Montoya, M.M., and Wingfield, M.J. 2006. A Review of *Ceratocystis sensu* stricto with special reference to the species complexes *C. coerulescens* and *C. fimbriata*. Rev. Fac. Nac. Agron. Medellín, **59**: 3045–3075.

Moreau, C. 1952. Coexistence des formes *Thielaviopsis* et *Graphium* chez une souche de *Ceratocystis major* (van Beyma) nov. comb. Rev. Mycol. (Paris) Suppl. Col. **17**(17): 17–25.

Nicholas, K.B., Nicholas, H.B., Jr., and Deerfield, D.W., II. 1997. GeneDoc: analysis and visualization of genetic variation. EMBNEW. NEWS, **4**: 14.

Nirenberg, H.I., Feiler, U., and Hagedorn, G. 2002. Description of *Colletotrichum lupini* comb. nov. in modern terms. Mycologia, **94**(2): 307–320. doi:10.2307/3761809.

Okada, G., Seifert, K.A., Takematsu, A., Yamaoka, Y., Miyazaki, S., and Tubaki, K. 1998. A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. Can. J. Bot. **76**(9): 1495–1506. doi:10.1139/cjb-76-9-1495.

Olchowecki, A., and Reid, J. 1974. Taxonomy of the genus *Ceratocystis* in Manitoba. Can. J. Bot. **52**(7): 1675–1711. doi:10.1139/b74-222.

Page, R.D. 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12**(4): 357–358. PMID:8902363.

Paulin, A.E., and Harrington, T.C. 2000. Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* and other ascomycetes. Stud. Mycol. **45**: 169–186.

Paulin-Mahady, A.E., Harrington, T.C., and McNew, D. 2002. Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis* anamorphs associated with *Ceratocystis*. Mycologia, **94**(1): 62–72. doi:10.2307/3761846.

Rayner, R.W. 1970. A mycological colour chart. Commonwealth Mycological Institute, Kew, UK.

Redfern, D.B., Stoakley, J.T., Steele, H., and Minter, D.W. 1987. Dieback and death of larch caused by *Ceratocystis laricicola* sp.nov. following attack by *Ips cembrae.* Plant Pathol. **36**(4): 467–480. doi:10.1111/j.1365-3059.1987.tb02264.x.

Rubtsov, P.M., Musakhanov, M.M., Zakharyev, V.M., Krayev, A.S., Skryabin, K.G., and Bayev, A.A. 1980. The structure of the yeast ribosomal RNA genes. I. The complete nucleotide sequence of the 18S ribosomal RNA gene from *Saccharomyces cerevisiae*. Nucleic Acids Res. **8**(23): 5779–5794. doi:10.1093/nar/8.23.5779. PMID:7008030.

Schmidt, H.A., Strimmer, K., Vingron, M., and von Haeseler, A. 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics, **18**(3): 502–504. doi:10.1093/bioinformatics/18.3.502. PMID: 11934758.

Siemaszko, W. 1939. Fungi associated with bark-beetles in Poland [Zespoly gryzbów fowarzyszacych kornikom polskim]. Planta Pol. **7**: 1–54.

Sogin, M.L., Miotto, K., and Miller, L. 1986. Primary structure of the *Neurospora crassa* small subunit ribosomal RNA coding region. Nucleic Acids Res. **14**(23): 9540. doi:10.1093/nar/14.23.9540. PMID:2948156.

Spatafora, J.W., and Blackwell, M. 1994. The polyphyletic origins of ophiostomatoid fungi. Mycol. Res. **98**(1): 1–9. doi:10.1016/S0953-7562(09)80327-4.

Suh, S.O., Noda, H., and Blackwell, M. 2001. Insect symbiosis: derivation of yeast-like endosymbionts within an entomopathogenic filamentous lineage. Mol. Biol. Evol. **18**(6): 995–1000. PMID:11371588.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25**(24): 4876–4882. doi:10.1093/nar/25.24.4876. PMID:9396791.

Ueda-Nishimura, K., and Mikata, K. 2000. Two distinct 18S rRNA secondary structures in *Dipodascus* (Hemiascomycetes). Microbiology, **146**(5): 1045–1051. PMID:10832631.

Upadhyay, H.P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, Ga.

van Wyk, M., Roux, J., Barnes, I., Wingfield, B.D., Chhetri, H.H., and Wingfield, M.J. 2004. *Ceratocystis bhutanensis* sp. nov., associated with the bark beetle *Ips schmutzenhoferi* on *Picea spinulosa* in Bhutan. Stud. Mycol. **50**: 365–379.

van Wyk, M., Roux, J., Barnes, I., Wingfield, B.D., and Wingfield, M.J. 2006. Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis* sp. nov. Fungal Divers. **21**: 181–201.

Wingfield, M.J., Harrington, T.C., and Solheim, H. 1997. Two species in the *Ceratocystis coerulescens* complex from conifers in western North America. Can. J. Bot. **75**: 827–834.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M., and Harrington, T.C. 1998. Monophyly of the conifer species in the *Ceratocystis coerulescens* complex using DNA sequence data. Mycologia, **90**(1): 96–100. doi:10.2307/3761017.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., and Harrington, T.C. 1999. PCR based identification and phylogeny of species of *Ceratocystis sensu stricto.* Mycol. Res. **103**(6): 743–749. doi:10.1017/S0953756298007679.

Witthuhn, R.C., Harrington, T.C., Steimel, J.P., Wingfield, B.D., and Wingfield, M.J. 2000. Comparison of isozymes, rDNA spacer regions, and MAT-2 DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. Mycologia, **92**(3): 447–452. doi:10.2307/3761503.

Xia, X., and Xie, Z. 2001. DAMBE: software package for data analysis in molecular biology and evolution. J. Hered. **92**(4): 371–373. doi:10.1093/jhered/92.4.371. PMID:11535656.