ISSN (print) 0093-4666 ISSN (online) 2154-8889

MYCOTAXON

Volume 115, pp. 311–322 *January–March 2011*

DOI: 10.5248/115.311

Mycosphaerella nyssicola revisited: a species distinct from *M. punctiformis*

Andrew M. Minnis[1*], Amy Y. Rossman[1] & Richard T. Olsen[2]

[1]*Systematic Mycology & Microbiology Laboratory, USDA-ARS &*
[2]*Floral and Nursery Plants Research Unit, US National Arboretum, USDA-ARS:*
Rm. 128, B010A, 10300 Baltimore Avenue, Beltsville, MD 20705, USA
* Correspondence to: *Drew.Minnis@ars.usda.gov*

Abstract —*Nyssa* trees involved in a breeding program for ornamentals were found to be affected deleteriously by a leaf spot disease. The causative agent was identified as a species of *Mycosphaerella* that had been classified as morphologically indistinguishable from *M. punctiformis*. A subsequent taxonomic investigation using morphological data from new and existing herbarium collections, cultural data, and ITS region rDNA sequences suggested that this was a distinct species on *Nyssa* that represents the previously described *M. nyssicola*. The species is lectotypified and epitypified to promote nomenclatural and taxonomic stability.

Key words — *Ascomycota*, *Asteromella nyssae*, *Dothideomycetes*, lectotype, *Phyllosticta nyssae*

Introduction

Nyssa (*Cornaceae*) is a small genus of trees disjunct between eastern Asia and eastern North America and Central America (Wen & Stuessy 1993). Four North American species (*N. aquatica* L., *N. biflora* Walter, *N. ogeche* W. Bartram ex Marshall, and *N. sylvatica* Marshall), collectively known as gums or tupelos, are found in swamps and alluvial soils as well as uplands. The black gum, *N. sylvatica*, is the most commonly cultivated species for ornamental and urban tree use due to its beautiful red fall color. The USDA-ARS National Arboretum began a *Nyssa* breeding program in the early 1990's directed at improving urban adaptability and transplantability for the nursery industry. Increased use of *Nyssa* has been tempered by a disfiguring leaf spot disease that results in reduced aesthetic appeal and premature defoliation (Dirr 1998). In 2006, the Arboretum refocused the *Nyssa* program towards developing resistance to this organism.

Recent attempts at identifying the causal organism failed or resulted in isolation of secondary pathogens (Dirr 1998, Charles Hodges pers. comm.). It was assumed to be a widely distributed species of *Mycosphaerella* Johanson (*Dothideomycetes*, *Ascomycota*) that has been classified as morphologically indistinguishable from and probably conspecific with *M. punctiformis* (Pers.) Starbäck, the type species of the genus (Aptroot 2006). The genus *Mycosphaerella* includes a vast diversity of species on multitudinous hosts, and it is successful in several ecological roles, including most importantly as a plant pathogen (Verkley et al. 2004, Crous 2009). Species of *Mycosphaerella* are in general host specific, but several species have broader host ranges (Crous 2009). A recent paper serving to stabilize the taxonomic application of *M. punctiformis* suggested that this species, in a strict sense, was specific to *Quercus* (Verkley et al. 2004).

Knowledge of the identity and host range of the leaf spot fungus from *Nyssa* is important in the development of disease control strategies and resistant cultivars. New isolates were obtained from *Nyssa* in field plots of the USDA breeding program and DNA sequence data of the ITS region were generated for comparison with other *Mycosphaerella* species. New specimens were compared with herbarium collections including type specimens, which are designated herein to promote nomenclatural and taxonomic stability.

Materials & methods

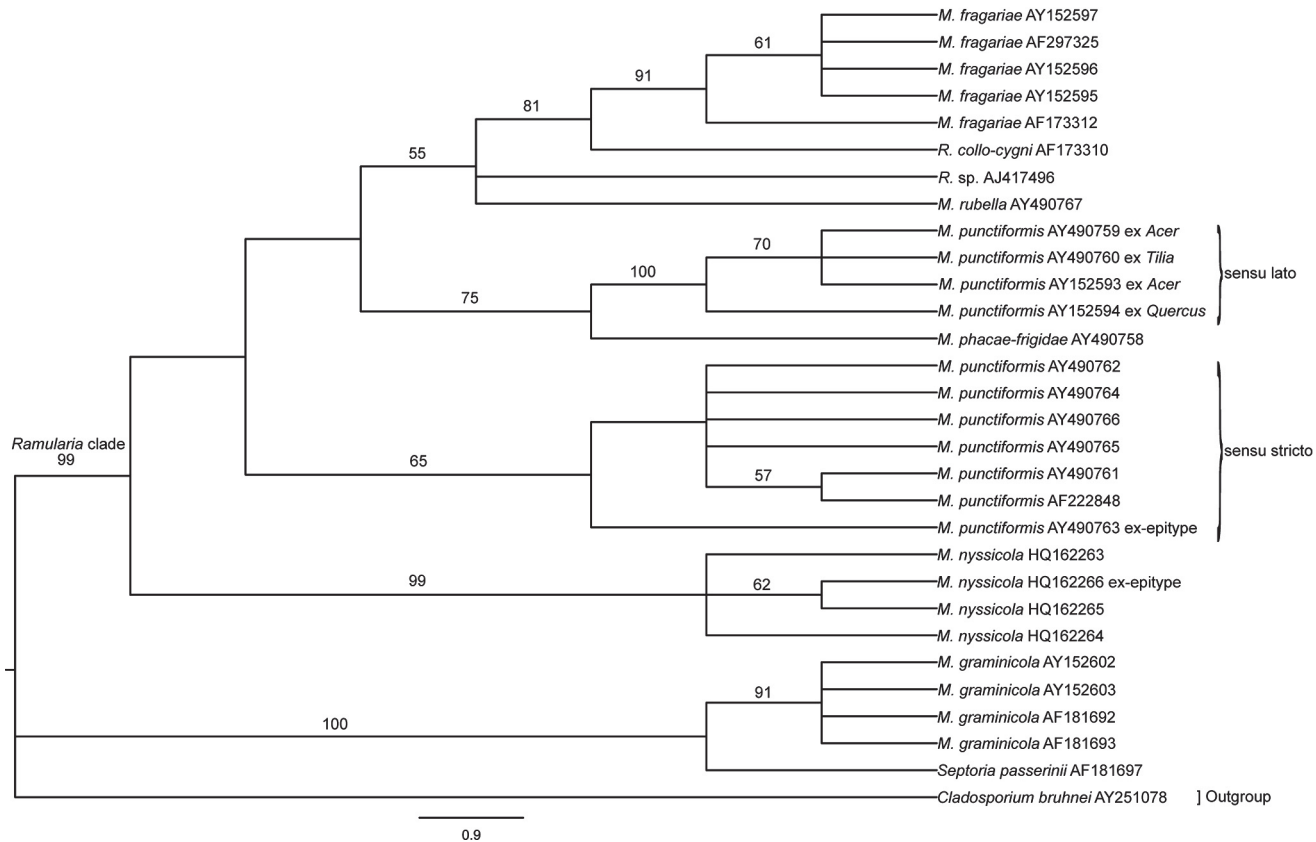
Isolation from fruiting bodies

Overwintered, dead, and fallen leaves were collected in spring. Cultures on cornmeal dextrose agar (CMD) were obtained variously by growth at room temperature from ascospores ejected from bits of leaves containing pseudothecia glued to the top lid of Petri dishes that were incubated with either the top lid oriented upwards or downwards or ascospores, usually with associated asci, from crushed pseudothecia isolated with a pipette. Cultures were deposited in the Centraalbureau voor Schimmelcultures (CBS).

Morphological examination

Microscopic observations and measurements of sections and crush mounts of fresh material were made in water. For viewing of dried material, material was wetted with 95% ethanol and subsequently rehydrated and viewed in 3% KOH. Phloxine solution (Largent et al. 1977) was also used to aid in microscopic examination. In the descriptions, an ^{m} indicates average or mean and Q is the length to width ratio. For phenotypic observations, cultures were grown on potato dextrose agar (PDA). See Farr & Rossman (2010) for complete collection data of examined historical herbarium collections. New collections were deposited in the US National Fungus Collections (BPI).

Fig. 1. Strict consensus of the 600 equally most parsimonious trees resulting from the phylogenetic analysis. Percent bootstrap support values from the 1000 replicates are indicated above branches. GenBank accession numbers are shown to the right of the names.


M. fragariae AY152597
M. fragariae AF297325
M. fragariae AY152596
M. fragariae AY152595
M. fragariae AF173312
R. collo-cygni AF173310
R. sp. AJ417496
M. rubella AY490767
M. punctiformis AY490759 ex *Acer*
M. punctiformis AY490760 ex *Tilia*
M. punctiformis AY152593 ex *Acer*
M. punctiformis AY152594 ex *Quercus*
M. phacae-frigidae AY490758
M. punctiformis AY490762
M. punctiformis AY490764
M. punctiformis AY490766
M. punctiformis AY490765
M. punctiformis AY490761
M. punctiformis AF222848
M. punctiformis AY490763 ex-epitype
M. nyssicola HQ162263
M. nyssicola HQ162266 ex-epitype
M. nyssicola HQ162265
M. nyssicola HQ162264
M. graminicola AY152602
M. graminicola AY152603
M. graminicola AF181692
M. graminicola AF181693
Septoria passerinii AF181697
Cladosporium bruhnei AY251078
] Outgroup
sensu lato
sensu stricto
Ramularia clade
99
61
91
81
55
70
100
75
65
57
99
62
91
100
0.9

DNA isolation, amplification, sequencing

DNA was isolated from cultures on CMD and the ITS region rDNA was amplified and sequenced following the methods of Bao et al. (2010). Sequences were also assembled and edited from chromatographs according to Bao et al. (2010).

Phylogenetic analyses

An initial BLAST search on GenBank indicated that the *Nyssa* isolates were closely related to *M. punctiformis* with 96% maximum identity. The ITS region sequence data from these isolates were subsequently aligned with that of the taxa included in a prior taxonomic study of *M. punctiformis* (Verkley et al. 2004) using Clustal X version 2.0.10 (Larkin et al. 2007). A preliminary analysis employed PAUP* 4.0b10 (Swofford 2002) using the same settings as Verkley et al. (2004). A reduced dataset was constructed using the *Nyssa* isolates and selected members of the *Ramularia* clade and closely related species from the Verkley et al. (2004) study with *Cladosporium bruhnei* Linder as the outgroup [= "*Davidiella tassiana*" (De Not.) Crous & U. Braun of Verkley et al. 2004] and aligned with Clustal X using the default settings (Larkin et al. 2007) with the ingroup aligned in the first step and the outgroup added in a subsequent second step alignment. The resulting alignment was visually inspected and the unaligned ends were omitted from later analyses. GenBank accession numbers of included sequences are indicated in Fig. 1. A maximum parsimony analysis was performed using the default parameters of the heuristic search of PAUP* 4.0b10 (Swofford 2002) that included characters 50-560 of the dataset. A parsimony bootstrap analysis was performed using the default settings of PAUP* 4.0b10 (Swofford 2002) with 1000 replicates and maxtrees set at 1000.

Results

The preliminary analysis showed the *Nyssa* isolates were part of the *Ramularia* clade (Verkley et al. 2004, Crous et al. 2009), also known as *Mycosphaerella* sensu stricto (Crous et al. 2009). Analyses of the reduced ITS region dataset contained thirty taxa with *Cladosporium bruhnei* as outgroup and included 501 characters, of which 83 were parsimony informative. The heuristic search of the maximum parsimony analysis produced 600 equally most parsimonious trees of length 225. The consistency (CI), homoplasy (HI), retention (RI), and rescaled consistency (RC) indices were 0.831, 0.169, 0.912, and 0.758, respectively. A strict consensus tree is presented in Fig. 1. The *Ramularia* clade (*Mycosphaerella* sensu stricto) was recovered with 99% bootstrap support (BS). The isolates from *Nyssa* formed a well-supported clade (99% BS). A larger clade, one lacking significant statistical support but composed of several moderately supported subclades, was sister to the *Nyssa* isolate clade. One of these subclades is *M. punctiformis* sensu stricto with 65% BS, which is distinct from the isolates from *Nyssa* that are determined to represent *M. nyssicola*. Isolates of *M. punctiformis* sensu lato and *M. phacae-frigidae* grouped in a clade (75% BS) with *M. phacae-frigidae* sister to a strongly supported (100% BS) *M. punctiformis* sensu lato clade.

Taxonomy

Mycosphaerella nyssicola (Cooke) F.A. Wolf, Mycologia 32: 333. 1940, as '*nyssaecola*'. Figs. 2–5

≡ *Sphaerella nyssicola* Cooke, Hedwigia 17: 40. 1878, as '*nyssoecola*'.

Lectotypus of *Sphaerella nyssicola* (**hic designatus**): USA. Florida: Gainesville, on leaves of *Nyssa*, leg. Ravenel, Fungi Americani Exsiccati no. 96 (BPI *608979*). Note: BPI has more than one of these exsiccatae. This one is mounted on a separate sheet and given a specific BPI number.

Epitypus of *Sphaerella nyssicola* (**hic designatus**): USA. Maryland: Prince George's Co., Glenn Dale, U.S. Plant Introduction Station, 11601 Old Pond Dr. (38°58'00.49"N 76°48'12.78"W), on overwintered, dead, and fallen leaves of *Nyssa* spp., V.2009, leg. RT Olsen (BPI *880897*, AR 4656 ex-epitype culture deposited at CBS 127665, ITS GenBank Accession No. HQ162266).

Pseudothecia developing overwinter on fallen dead leaves; chiefly hypophyllous; scattered to gregarious, sometimes touching but not confluent; erumpent; semi-immersed; globose; black; 65–125 µm diam. (74–97 µm diam. in lectotype); ostiole apical, 10–23 µm diam. Pseudothecial wall with 2–3 layers of brown textura angularis. Paraphyses absent. Asci bitunicate, 34–48 × 6.5–8 µm, fasciculate, cylindrical, straight to slightly curved, with a short pedicel, octosporous. Ascospores 6.4–8.3 × 1.9–3.2 µm, Q = 2–3.3 µm (^{m} = 7.1 × 2.6 µm, Q ^{m} = 2.7 µm), fusoid-ellipsoid, tapering towards ends, tapering more prominently towards lower end, ends obtuse, widest above septum, with 1 septum, median or slightly supramedian, slightly constricted at septum, thin-walled, hyaline, uniseriate to biseriate, sometimes oblique within and among asci. Germination not observed.

In Culture: Colony after 31 days on PDA at approx. 22°C and exposed to ambient lighting, up to 29 mm diam., low without abundant aerial mycelium, raised in a mound at center, texture somewhat slimy but not yeast-like, pinkish, or with a pinkish base covered by a whitish tomentum, paler at margin, reverse pinkish to rosette, not sporulating, older cultures sometimes developing fruiting structures more or less globose, black, presumably immature, with walls textura angularis. Mycelium with hyphae branching, septate, hyaline or faintly pigmented at times when viewed in mass, walls smooth, width 1.9–3.2 µm.

Specimens examined: **USA. Florida:** (BPI *608979*, designated lectotype); idem (3 additional exsiccatae duplicates of lectotype without BPI numbers); **Georgia: Richmond Co.** Augusta, Phinizy Swamp Nature Park, on overwintered, dead and fallen leaves of *Nyssa aquatica*, 19.III.2010, leg. J.E. Gordon (BPI *880908*, asci and ascospores not observed); **Maryland: Prince George's Co.** Glenn Dale, U.S. Plant Introduction Station, 11601 Old Pond Dr., 38°58'00.49"N 76°48'12.78"W, on overwintered, dead and fallen leaves of *Nyssa* spp., V.2009, leg. RT Olsen (BPI *880897*, designated epitype, ex-epitype culture deposited at CBS 127665 = AR 4656, ITS GenBank Accession No.

Fig. 2. *Mycosphaerella nyssicola*. Upper: leaf discoloration and defoliation at U.S. National Arboretum research plots, Glenn Dale, MD. Lower: leaf spot symptoms on *Nyssa ogeche* in fall, USNA, Washington, DC.

HQ162266; AR 4655 additional culture not preserved, ITS GenBank Accession No. HQ162265); idem (BPI 880800, culture deposited at CBS 127664 = AR 4629, ITS GenBank Accession No. HQ162264; AR 4626 additional culture not preserved, ITS GenBank Accession No. HQ162263); **North Carolina:** (BPI *608981*, *876718*); **West Virginia:** (BPI *601980*, immature; BPI *608980*, immature).

Fig. 3. *Mycosphaerella nyssicola*. Upper left: leaf spot symptoms on *Nyssa sylvatica* × *N. ogeche* hybrid in USNA research plots, Beltsville, MD. Upper right: leaf spot symptoms on *N. ogeche* and *N. sylvatica* growing at USNA research plots, Washington, DC. Lower left: leaf spot symptoms on *N. sinensis* growing in the Asian collections at the USNA, Washington, DC. Lower right: overwintering *Nyssa* leaves at USNA research plot, Glenn Dale, MD.

Anamorph (spermatial state): ***Asteromella nyssae*** (Cooke) Aa, A revision of the species described in *Phyllosticta*: 336. 2002.

≡ *Phyllosticta nyssae* Cooke, Grevillea 12: 26. 1883.

Lectotypus of *Phyllosticta nyssae* (**hic designatus**): USA. Georgia: Darien, on leaves of *Nyssa capitata*, leg. Ravenel, Fungi Americani Exsiccati no. 798 (BPI *353790*). Note: BPI has more than one of these exsiccatae. This one is mounted on a separate sheet and given a specific BPI number.

Leaf spots epiphyllous, but discoloring beneath, irregular, frequently coalescing, large, pallid with margin purplish or purplish. Spermogonia chiefly hypophyllous; frequently but not always associated with spots; scattered to

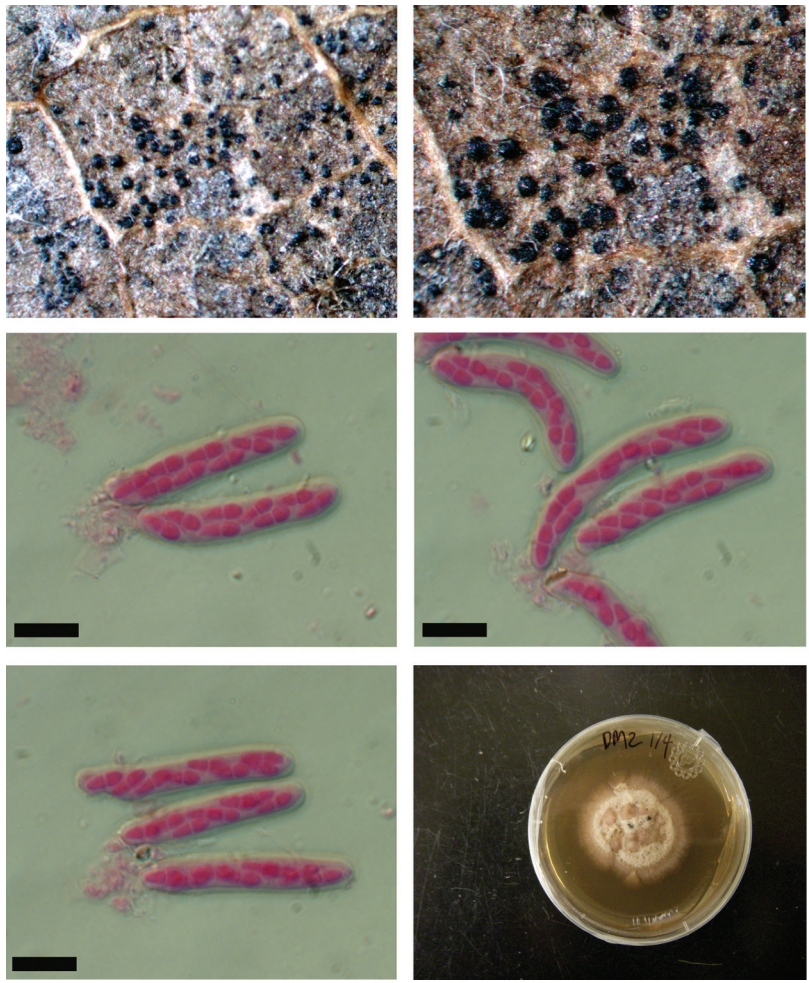


Fig. 4. *Mycosphaerella nyssicola*. Upper row: pseudothecia of BPI *608981*. Middle row and lower left: asci and ascospores of BPI *608981*. Lower right: ex-epitype culture on PDA (CBS 127666). Scale bars = 10 µm.

gregarious, sometimes touching but not confluent; erumpent; semi-immersed; pseudopycnidial; globose; black; 61–103 µm diam. (65–103 µm diam. in lectotype); ostiole apical, 16–29 µm diam. (16–23 µm diam. in lectotype); unilocular. Spermagonial walls composed of an outer layer of brown textura angularis and a hyaline inner layer that produces the spermatogenous cells. Spermatogenous cells determinate; phialidic; hyaline; walls smooth; discrete or more typically integrated on spermatiophores; spermatiophores up to three

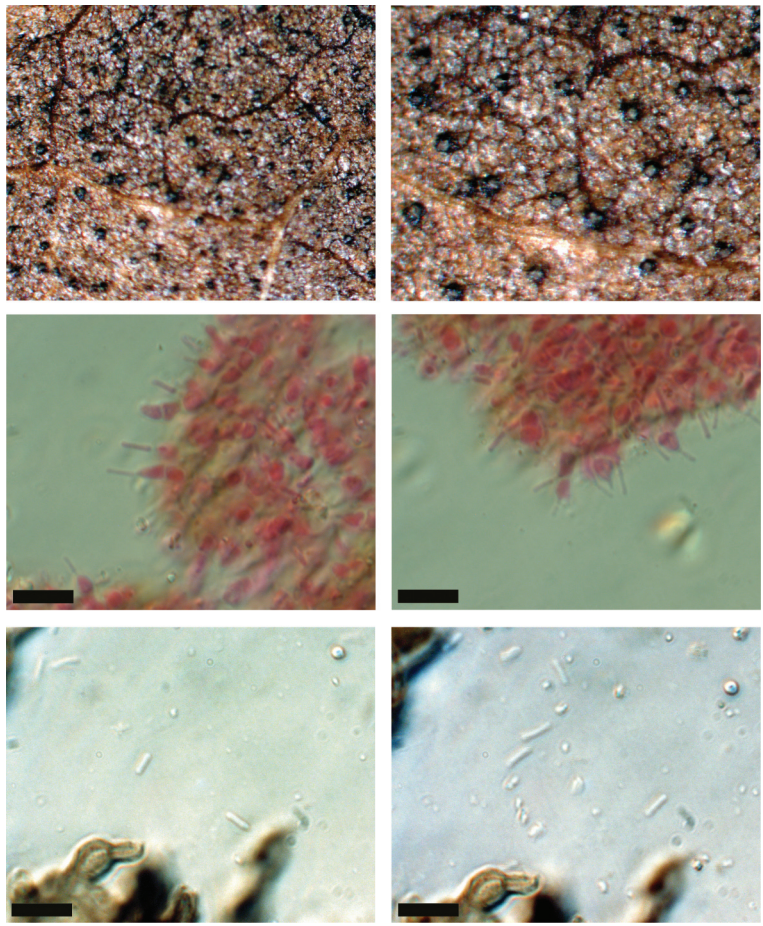


Fig. 5. *Asteromella nyssae* (spermatial state of *Mycosphaerella nyssicola*). Upper row: spermogonia of BPI *353792*. Middle row: conidiogenous cells of BPI *353799*. Lower row: spermatia of BPI *353792*. Scale bars = 10 μm.

septate, occasionally branching at base; loci apical or on short side branches formed one per cell immediately below septa of spermatiophores. Spermatia 3.2–5.8 × 0.6–1.6 μm, Q = 2.4–5.3 μm (^{m} = 4.1 × 1.2 μm, Q ^{m} = 3.4 μm) (3.8–4.5 × 1–1.3 μm in lectotype), at times copious and adhering in large numbers, bacilliform, aseptate, walls smooth, hyaline, eguttulate.

Specimens examined: **USA. Georgia:** (BPI *353789*, duplicate of lectotype; BPI *353790*, designated lectotype); **Michigan:** (BPI *353793*); **North Carolina:** (BPI *353792*); **South Carolina:** (BPI *353796*, *353797*); **Tennessee:** (BPI *860755*); **Virginia:** (BPI *353788*, *353791*, *353795*, *353799*); **West Virginia:** (BPI *353798*, *521392*, *521393*).

Habitat & Distribution — Based on Farr & Rossman (2010) and examined specimens, *Mycosphaerella nyssicola* is known to occur on leaves of *Nyssa aquatica*, *N. biflora* (≡ *N. sylvatica* var. *biflora*), *N. ogeche*, and *N. sylvatica*, and it is widely distributed in the eastern USA (AL, FL, GA, MD, MI, NC, NY, PA, SC, TN, VA, WV), especially in the southeastern states. Observations of disease symptoms at the USDA field plot also confirmed as hosts: *N. sinensis* and two F1 hybrids, *N. sylvatica* × *N. ogeche* and *N. sylvatica* × *N. sinensis*. It is possible that the distribution of *M. nyssicola* is greater than that reported in the literature, and the fungus may occur throughout the range of *Nyssa* in the USA.

Comments — Analyses of the DNA sequence data from the ITS region suggest that isolates from *Nyssa* are distinct from isolates of *M. punctiformis* both sensu stricto and sensu lato as presented by Verkley et al. (2004). Morphological examination of new collections and historical herbarium collections indicates that the *Nyssa* isolates are identical with *Mycosphaerella nyssicola*. Although *M. nyssicola* is more or less indistinguishable from *M. punctiformis*, as was noted by Aptroot (2006), DNA evidence and host specificity support *M. nyssicola* as a distinct species on *Nyssa.*

With respect to types, Cooke (1878) described *Sphaerella nyssicola* as a new species occurring on *Nyssa*, and the original material was distributed as exsiccatae in Ravenel's Fungi Americani Exsiccati. Confusingly, the labels of the exsiccatae cited in the protologue by Cooke (1878) indicate specimens from Florida on *Nyssa*, while the protologue lists South Carolina and *Nyssa multiflora* (= *N. sylvatica*) for distribution and host. These exsiccatae are syntypes (McNeill et al. 2006: Art. 9.4). Aptroot (2006), who listed a specimen from South Carolina at K as holotype and exsiccata no. 96 at BPI from Florida as isotype, did not use the phrase 'hic designatus,' which would have caused an accidental lectotypification of the species (McNeill et al. 2006: Arts. 7.11, 9.8). However, to assume that a collection housed in the original author's herbarium represents the holotype when no holotype has been designated is not the correct course of action. Additional confusion results from whether original material exists from Florida, from South Carolina, or from both regions. To resolve the confusion, we designate one syntype as lectotype (McNeill et al. 2006: Art. 9.2). The original material (as noted by Cooke, 1878) is immature. Despite repeated attempts, we were unable to find asci or ascospores in any of the four exsiccatae (including the designated lectotype) housed at BPI.

Similarly, Cooke (1883) described *Phyllosticta nyssae* as a new species on *Nyssa capitata* (= *N. ogeche*), and the original material was distributed as exsiccatae in Ravenel's Fungi Americani Exsiccati. Seaver (1922), Wolf (1940), and Aa & Vanev (2002), all of whom provided morphological data on this species and cited the exsiccatae, did not designate a type. Hence, we here also designate one syntype as lectotype.

Lastly, because the type material is immature, we epitypify *S. nyssicola* based on a recent collection of *Mycosphaerella nyssicola* from Maryland. The living culture and DNA data obtained from the epitype aid in the application of the name (McNeill et al. 2006: Art. 9.7), although we are somewhat reluctant to epitypify the name based on a specimen that was not collected in the type locality (i.e., either Florida or South Carolina). The epitype is otherwise identical in all regards to material observed by Wolf (1940) and so maintains current usage of the name.

Wolf (1940) provided an excellent symptomatological account of this leaf spot disease and the life history of *M. nyssicola*. We have nothing to add except that Koch's postulates have not yet been completed, linkage of the teleomorphic and spermatial states is circumstantial, and no attempts have been made to germinate spermatia that would confirm they are not conidial in nature. We also have not studied fresh material of the spermatial state. As with Wolf (1940), we found no *Ramularia* or other conidial state for this fungus. *Mycosphaerella nyssicola* is typical of members of the *Ramularia* clade (also known as section *Mycosphaerella* or *Mycosphaerella* sensu stricto) and it is quite similar to *M. punctiformis* (Verkley et al. 2004, Crous 2009, Crous et al. 2009). At this time, we do not propose a new combination in *Ramularia* based on the teleomorphic state as advocated by Crous et al. (2009) for species of *Mycosphaerella* sensu stricto, as the latter generic name correctly adheres to ICBN Art. 59.1 (McNeill et al. 2006).

Acknowledgments

We thank Tunesha Phipps and Sasha Allen of the Systematic Mycology and Microbiology Laboratory for assistance with DNA sequencing. Judith Gordon of Augusta State University is warmly acknowledged for sending fungal material. We extend our gratitude to Uwe Braun of Martin-Luther-Universität and Charles Hodges of North Carolina State University for their expert reviews of this manuscript.

Literature cited

Aa HA van der, Vanev S. 2002. A revision of the species described in *Phyllosticta*. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Aptroot A. 2006. *Mycosphaerella* and its anamorphs: 2. Conspectus of *Mycosphaerella*. CBS Biodiversity Ser. 5: 1–231.

Bao X, Carris LM, Huang G, Luo J, Liu Y, Castlebury LA. 2010. *Tilletia puccinelliae*, a new species of reticulate-spored bunt fungus infecting *Puccinellia distans*. Mycologia 102: 613–623. doi:10.3852/09-135

Cooke MC. 1878. North American fungi. Hedwigia 17: 37–40.

Cooke MC. 1883. New American fungi. Grevillea 12: 22–33.

Crous PW. 2009. Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs. Fungal Diversity 38: 1–24.

Crous PW, Summerell BA, Carnegie AJ, Wingfield MJ, Hunter GC, Burgess TI, Andjic V, Barber PA, Groenewald JZ. 2009. Unraveling *Mycosphaerella*: do you believe in genera? Persoonia: 23: 99–118. doi:10.3767/003158509X479487

Dirr MA. 1998. Manual of Woody Landscape Plants: Their Identification, Ornamental Characteristics, Culture, Propagation, and Uses. Stipes, Champaign, IL.

Farr DF, Rossman AY. 2010. Fungal databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Retrieved June 14, 2010, from http://nt.ars-grin.gov/fungaldatabases/.

Largent D, Johnson D, Watling R. 1977. How to identify mushrooms to genus III: Microscopic Features. Mad River Press, Eureka, CA.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948. doi:10.1093/bioinformatics/btm404

McNeill J, Barrie FR, Burdet HM, Demoulin V, Hawksworth DL, Marhold K, Nicolson DH, Prado J, Silva PC, Skog JE, Wiersema JH, Turland NJ (eds.). 2006. International Code of Botanical Nomenclature (Vienna Code): Adopted by the Seventeenth International Botanical Congress, Vienna, Austria, July 2005. ARG Gantner, Ruggell, Liechtenstein.

Seaver FJ. 1922. *Phyllostictales Phyllostictaceae* (pars). N. Amer. Fl. 6: 1–84.

Swofford DL. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland, MA.

Verkley GJM, Crous PW, Groenewald JZ (Ewald), Braun U, Aptroot A. 2004. *Mycosphaerella punctiformis* revisited: morphology, phylogeny, and epitypification of the type species of the genus *Mycosphaerella* (*Dothideales*, *Ascomycota*). Mycol. Res. 108: 1271–1282. doi:10.1017/S0953756204001054

Wen J, Stuessy TF. 1993. The phylogeny and biogeography of *Nyssa* (*Cornaceae*). Syst. Bot. 18: 68-79. doi:10.2307/2419789

Wolf FA. 1940. A leafspot fungus on *Nyssa*. Mycologia 32: 331–335. doi:10.2307/3754315