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The Lichen Genus *Sticta* in the Great Smoky Mountains: A Phylogenetic Study of Morphological, Chemical, and Molecular Data

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Abstract. *In this paper we segregate specimens from the genus* Sticta *in the Great Smoky Mountains National Park into phenotypic groups corresponding to putative species using traditional taxonomic methods, paying particular attention to specimens from the* S. weigelii s. l. *group, then employ phylogenetic analyses and rigorous statistics to test the robustness of these species groups. In order to circumscribe putative species and to resolve the* S. weigelii *complex, morphological, chemical, and molecular characters from the nuclear ribosomal DNA sequences of the entire Internal Transcribed Spacer region are analyzed separately and simultaneously using maximum parsimony or maximum likelihood. In addition to the bootstrap method, Bayesian statistics with the Markov Chain Monte Carlo algorithm are used to estimate branch robustness on the resulting reconstructed trees. Five out of six analyses recover the same five monophyletic putative species from the genus* Sticta, *indicating the concordance of DNA-based and morphology-based species delimitation. The phylogenies show that lichens identified as* S. weigelii *represented* S. beauvoisii *and the two new species described here* – S. carolinensis *and* S. fragilinata. Sticta weigelii s. s. *does not occur in the park. Specimens from Oregon identified as* S. weigelii *belong to another unnamed* Sticta *taxon. The remaining two monophyletic groups represent two species well known from the park*–S. fuliginosa *and* S. limbata. *Characteristics of secondary compounds detected by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) in* S. fragilinata *thalli are provided. Detailed descriptions, including morphology and chemistry, are provided for four* Sticta *species found in the Smoky Mountains:* S. beauvoisii, S. carolinensis, S. fragilinata *and* S. fuliginosa.

The genus *Sticta* (Schreber) Ach. includes macrolichens that are found throughout the world (Galloway 1994, 1997, 1998; Joshi & Awasthi 1982). Though the genus is primarily tropical in distribution, its representatives are present as far north as Norway (Jørgensen 1969) and as far south as the southern tip of South America (Galloway 1994). Historically, there have been thought to be four frequently collected species of *Sticta* in the continental United States: *Sticta fuliginosa* (Hoffm.) Ach., a substipitate lichen with a thin thallus and laminal isidia; *Sticta limbata* (Sm.) Ach., a lichen with grayish marginal soralia; the large-lobed, laminally isidiate *Sticta sylvatica* (Huds.) Ach., purportedly found in the western United States; and *Sticta weigelii* (Ach.) Vainio, a species with coralloid marginal isidia (Hale 1979). However, according to Harris (1984), an additional *Sticta* species, *S. beauvoisii* Delise, which is geographically restricted mainly to the northeastern part of the U.S., is commonly misidentified as *S. weigelii*.

Dey (1976) recognized a lobulate, chemically distinct variant of *S. weigelii s. l.* in the southern Appalachians. A phyllidiate lichen lacking anthraquinones, also present in the region, is misclassified as *S. weigelii* (Brodo et al. 2001). A third unnamed species with pale, long, tufted tomentum is confused with *S. weigelii* in the southeastern United States (Harris 1984). In the northwest, a fuliginous lichen with anthraquinones in the medulla is confused with *S. weigelii*, as also noted by Brodo et al. (2001). Thus the distribution of *S. weigelii s. s.*

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in temperate North America is not clear, nor is the identity of temperate North American specimens identified as *S. weigelii*. As many as six distinct species may comprise *S. weigelii s. l.* in the United States alone, and more in the Caribbean (Harris 1984).

This paper focuses on the *Sticta* of the Great Smoky Mountains National Park located in North Carolina and Tennessee, U.S.A. The area is at the convergence of the projected ranges for several of the *S. weigelii s. l.* taxa, and therefore is a starting point for unraveling the *S. weigelii* problem in North America.

The main goals of this study are to: 1) recognize and delimit putative *Sticta* species occurring in the Great Smoky Mountains National Park with special emphasis on specimens and taxa from the *S. weigelii* complex, using phenotypic characters as grouping criteria; 2) test their monophyletic status based on separate and simultaneous phylogenetic analyses of morphology, chemistry, and molecular data obtained from specimens representing the phenotypic variation within each putative species; 3) propose *S. carolinensis* and *S. fragilinata* as new species, if the results from the phylogenetic analyses are in agreement with our conclusion based on morphological and chemical evidence; and 4) reconstruct relationships among these two new *Sticta* species and the existing taxa based on a combined data set, if no conflict is detected among data partitions.

To reach these goals, we conducted a detailed assessment of morphological, anatomical, and chemical features, and sequenced the entire Internal Transcribed Spacer region of the nuclear ribosomal DNA (ITS1, 5.8S and ITS2 nrDNA) for the five potential species of *Sticta* present in the Great Smoky Mountains, including reference material for *S. weigelii s. s.* from South America, for *Sticta* specimens recognized in the Pacific Northwest area of the U.S. as *S. weigelii*, and for three outgroup species from the Lobariaceae, closely related to the genus *Sticta*. This phylogenetic study of the genus provides a framework for future systematic work on *Sticta*, as well as a stable foundation for detailed study of the *weigelii* complex.

Materials and Methods

Taxon sampling.—Specimens were collected in the summer of 1999 at 43 localities in the Great Smoky Mountains National Park. At each locality, GPS position and habitat were recorded; substrate was recorded for each specimen. A total of 221 specimens of *Sticta* were collected. A small section from each specimen was removed for molecular work before the thallus was wetted and pressed. All specimens have been deposited at the University of Minnesota Herbarium (MIN). In addition to these collections, specimens of *S. limbata*, *S. weigelii s. l.*, *Lobaria oregana*, and *Pseudocyphellaria crocata*, all from the Pacific Northwest, and tropical specimens of *S. weigelii s. s.* were included.

Morphological, anatomical, and chemical studies were carried out on the *Sticta* collection from the Smoky Mountains, including the specimens listed in Table 1, and on selected material from various herbaria (see Taxonomy of the genus *Sticta* section; *Specimens examined*).

For molecular phylogenetic analyses based on ITS nrDNA, 24 specimens of *Sticta* were selected (Table 1). Twenty-one of them, collected in the Smoky Mountains, represent three recognized taxa (*S. beauvoisii*, *S. fuliginosa*, and *S. limbata*) and two potentially new species – *S. carolinensis* and *S. fragilinata*. One specimen of *S. limbata* and a single collection of *Sticta* representing a morphotype commonly identified as *S. weigelii* (named here *Sticta* sp.) come from the Pacific Northwest (Oregon). In addition, one tropical specimen of *S. weigelii s. s.* was included in the molecular data set. Except for *Sticta* sp., neotropical *S. weigelii*, and outgroup species, each *Sticta* taxon was represented by more than one collection from different localities in both types of data sets: morphology + chemistry and ITS nrDNA. To produce a stable root for the ingroup, three ITS sequences from GenBank were added, two from *Lobaria* (*L. oregana* and *L. pulmonaria*) and one from *Pseudocyphellaria crocata*, for a total of 27 specimens from 10 putative species.

Morphological data.—All 221 *Sticta* specimens were separated into putative species groups based on phenotypic characters derived from species descriptions and from literature on *S. weigelii s. l.* Five groups were recognized: *S. beauvoisii* groups I and II; *S. fuliginosa*; *S. carolinensis*; and *S. fragilinata*. Individuals from *S. beauvoisii* group II were chosen to be as different as possible from the first group of this taxon to assess the stringency of the subsequent analyses of the morphological characters. First, based on similarity to descriptions of the type for existing species, one representative specimen was chosen to exemplify the group. Then a survey was made of the remaining specimens in the group and the five morphologically most diverse lichens were chosen to represent the variation in that group. In addition to these thirty lichens, five tropical specimens of *S. weigelii* were included in the morphological data matrix. Five specimens each of *Lobaria oregana*, *L. pulmonaria*, and *Pseudocyphellaria crocata* were used as the outgroup representatives. All morphological data subjected to phylogenetic analyses were obtained from this set of 50 lichens. *Sticta* sp. was not included in the morphological data matrix due to the limited fresh material available.

A total of 26 morphological, anatomical, and ecological traits were scored for this 50-specimen set of lichens, including various measurements of the tomentum on the lower surface of the thallus, rhizines, cyphellae, vegetative propagules (isidia and soredia), and overall size, shape, and appearance of the thallus. All observations were made with a Heerbrugg Wild M20 compound microscope or Heerbrugg Wild M5-41053 dissecting microscope. Calculations of the density of cyphellae were made using a 0.5 by 0.5-cm grid. For anatomical studies, lichens were sectioned by hand or with a freezing microtome. The sections were mounted in distilled water.

This set of 26 characters consisted of 19 discrete and seven continuous characters. Discrete characters were scored and entered directly into a data matrix using MacClade 4.0 (Maddison & Maddison 2000). Continuous characters were converted to discrete characters before they were incorporated into the data matrix (Fig. 1). The

TABLE 1. Voucher specimen information and GenBank accession numbers for 27 ITS nrDNA sequences included in this study. The specimens from U.S.A. were collected in Smoky Mountains and deposited at MIN unless otherwise indicated. * = outgroup specimens/sequences obtained from GenBank; all other sequences were generated by this study.

Taxon	Voucher	GenBank accession no.
Sticta beauvoisii Delise 1	U.S.A., *McDonald 182*	AY173370
S. beauvoisii 2	U.S.A., *McDonald 300*	AY173371
S. beauvoisii 3	U.S.A., *McDonald 211*	AY173372
S. beauvoisii 4	U.S.A., *McDonald 301*	AY173373
S. beauvoisii 5	U.S.A., *McDonald 289*	AY173374
S. beauvoisii 6	U.S.A., *McDonald 535*	AY173375
S. beauvoisii 7	U.S.A., *McDonald 209*	AY173376
S. beauvoisii 8	U.S.A., *McDonald 433*	AY173377
S. beauvoisii 9	U.S.A., *McDonald 617*	AY173378
S. carolinensis 1	U.S.A., *McDonald 1076*	AY173379
S. carolinensis 2	U.S.A., *McDonald 558*	AY173380
S. carolinensis 3	U.S.A., *McDonald 192*	AY173381
S. fragilinata 1	U.S.A., *McDonald 464*	AY173382
S. fragilinata 2	U.S.A., *McDonald 551*	AY173383
S. fragilinata 3	U.S.A., *McDonald 562*	AY173384
S. fragilinata 4	U.S.A., *McDonald 463*	AY173385
S. fragilinata 5	U.S.A., *McDonald 949*	AY173386
S. fuliginosa (Hoffm.) Ach. 1	U.S.A., *McDonald 608*	AY173387
S. fuliginosa 2	U.S.A., *McDonald 364*	AY173388
S. fuliginosa 3	U.S.A., *McDonald 609*	AY173389
S. limbata (Sm.) Ach. 1	U.S.A., Oregon, *Wilson 4400a*	AY173390
S. limbata 2	U.S.A., *McDonald 1004*	AY173391
S. weigelii (Ach.) Vainio	Dominican Republic, *Zanoni & Pimentez 25829* (NY)	AY173392
Sticta sp.	U.S.A., Oregon, *McCune 23609* (OSU)	AY173393
**Lobaria oregana* (Tuck.) Müll. Arg.	Canada, *Goffinet 3141* (B. Goffinet Herb.)	*AF014111
**L. pulmonaria* (L.) Hoffm.	Switzerland, *Zoller* (K. Scheidegger Herb.)	*AF129286
**Pseudocyphellaria crocata* (L.) Vainio	U.S.A., *Oregon, McCune 25753A* (OSU)	*AF401980

conversion was done using a method developed by Lutzoni and Brodo (1995).

Details for some morphological characters in selected *Sticta* species are shown in Figs. 2–3. The following characters were included in the morphological data matrix (Table 2; characters 1–26):

1. Primary Photobiont: 0 = green alga; 1 = cyanobacterium.
2. Upper Surface Texture: 0 = smooth; 1 = ridged.
3. Lobe Shape: 0 = flat; 1 = undulating; 2 = contorted.
4. Margin Shape: 0 = entire [*S. beauvoisii* (Fig. 2D); *S. fuliginosa* (Fig. 2G)]; 1 = phyllidiate [*S. carolinensis* (Fig. 2F); *S. fragilinata* (Fig. 2C and E)]; 2 = lobate.
5. Reproductive Structure: 0 = soredium; 1 = isidium [*Sticta* spp. (Fig. 2A–C and E–G)]; 2 = apothecium.
6. Reproductive Structure Placement: 0 = laminal and scattered [*S. fuliginosa* (Fig. 2G)]; 1 = laminal along ridges; 2 = marginal [*S. weigelii* (Fig. 2A); *S. beauvoisii* (Fig. 2B); *S. fragilinata* (Fig. 2C and E)].
7. Isidium Shape: 0 = absent; 1 = simple [*S. fuliginosa* (Fig. 2G)]; 2 = coralloid [*S. weigelii* (Fig. 2A); *S. beauvoisii* (Fig. 2B)]; 3 = phyllidiate [*S. carolinensis* (Fig. 2F); *S. fragilinata* (Fig. 2C and E)].
8. Soredium Color: 0 = absent; 1 = white; 2 = gray; 3 = yellow.
9. Thallus Attachment Point: 0 = one; 1 = many.
10. Rhizine Arrangement: 0 = scattered; 1 = localized centrally and free; 2 = localized centrally and fused.
11. Tomentum Color: 0 = dark brown to black; 1 = brown; 2 = cream.
12. Tomentum Pattern: 0 = continuous; 1 = discontinuous.
13. Pseudocyphella: 0 = absent [*L. pulmonaria* (Fig. 3H)]; 1 = present [*P. crocata* (Fig. 3I)].
14. Cyphella: 0 = absent; 1 = present [*Sticta* spp. (Fig. 3A–G and J)].
15. Cyphella Shape: 0 = absent; 1 = round [*S. weigelii* (Fig. 3A and B); *S. beauvoisii* (Fig. 3D); *S. limbata* (Fig. 3F); *S. fuliginosa* (Fig. 3G and J)]; 2 = irregular [*S. fragilinata* (Fig. 3C and E)].
16. Cyphella Margin: 0 = absent; 1 = flat; 2 = raised.
17. Cyphella Arrangement: 0 = absent; 1 = single [*S. weigelii* (Fig. 3A and B); *S. beauvoisii* (Fig. 3D); *S. limbata* (Fig. 3F); *S. fuliginosa* (Fig. 3G and J)]; 2 = clustered [e.g., *S. fragilinata* (Fig. 3E)].
18. Cyphella Basal Membrane Color: 0 = absent; 1 = white; 2 = tan.
19. Medulla Color: 0 = white; 1 = tan.
20. Tomentum Length: 0 ≈ 0.00–0.13 mm; 1 ≈ 0.13–0.20 mm; 2 ≈ 0.20–0.30 mm; 3 ≈ more than 0.30 mm (Fig. 1A).
21. Cyphella Density: 0 = absent; 1 ≈ 1–18/0.25 square cm [*S. limbata* (Fig. 3F); *S. fuliginosa* (Fig. 3G and J)]; 2 ≈ 18–32/0.25 square cm; 3 ≈ more than 32/0.25 square cm [*S. beauvoisii* (Fig. 3D)] (Fig. 1B).
22. Cyphella Diameter: 0 = absent; 1 ≈ 0.0–0.2 mm; 2 ≈ 0.2–0.4 mm (Fig. 1C).
23. Isidium Length: 0 = absent; 1 ≈ 0.0–0.2 mm; 2 ≈ 0.2–0.5 mm; 3 ≈ more than 0.5 mm (Fig. 1D).
24. Lobe Width: 0 ≈ 0.0–0.5 cm; 1 ≈ 0.5–0.9 cm; 2 ≈ 0.9–1.4 cm; 3 ≈ more than 1.4 cm (Fig. 1E).
25. Lobe Length: 0 ≈ 0.0–1.8 cm; 1 ≈ 1.8–2.7 cm; 2 ≈ 2.7–4.0 cm; 3 ≈ more than 4.0 cm (Fig. 1F).
26. Elevation: 0 ≈ 0–1,000 m; 1 ≈ 1,000–1,500 m; 2 ≈ more than 1,500 m; ? = unknown (Fig. 1G).

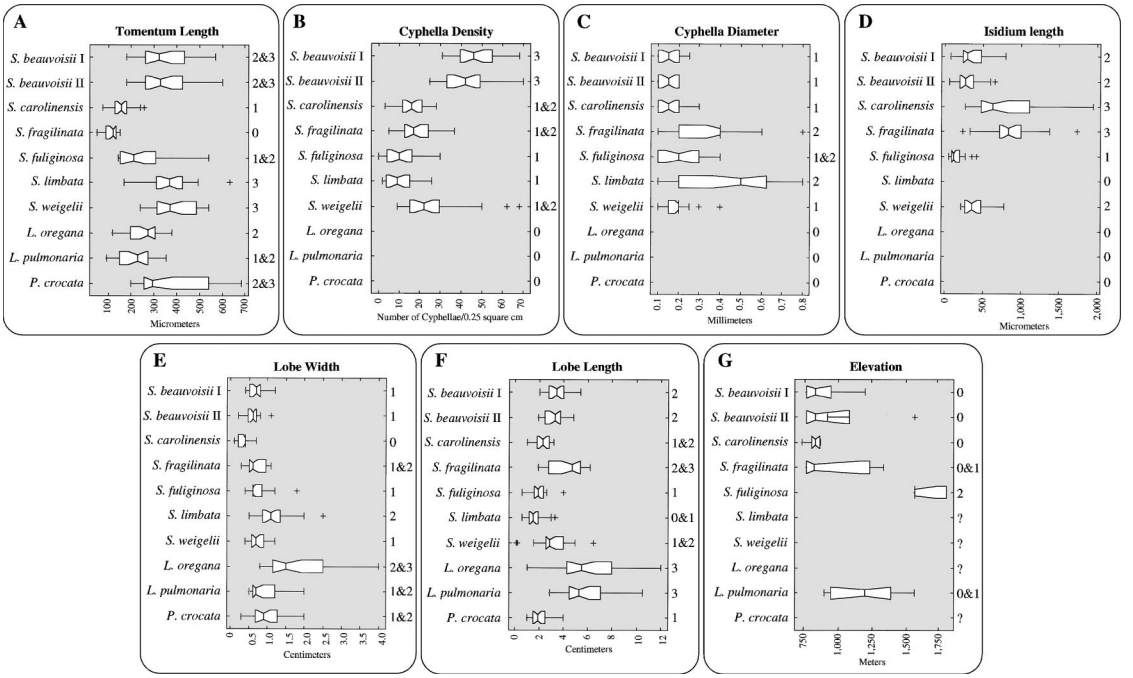

FIGURE 1. Conversion of continuous characters into discrete characters. Box plots representing seven continuous characters included in the morphological data set for six putative *Sticta* species and three outgroup taxa. For *S. beauvoisii*, two groups of specimens were subjected to statistical analyses according to information given in Materials and Methods section, *Morphological data*. The central vertical line is the median. The boxed area represents 50% of the sample. The terminal vertical lines represent the whole sample, except for outlying points denoted by a ''+''. Species whose distributions for a given trait overlapped significantly according to a Tukey test (data not shown) were given the same character state that is shown here on the right side of each plot. A, Tomentum Length (Table 2; char. 20); B, Cyphella Density (Table 2; char. 21); C, Cyphella Diameter (Table 2; char. 22); D, Isidium Length (Table 2, char. 23); E, Lobe Width (Table 2; char. 24); F, Lobe Length (Table 2; char. 25); G, Altitude (Table 2; char. 26).

Chemical data.—All 221 specimens of *Sticta* from the Great Smoky Mountains and 10 tropical specimens of *S. weigelii* were screened for positive chemical reaction with potassium hydroxide (K), calcium hypochlorite (C), and paraphenylenediamine (Pd). TLC was used to assay a selection of lichens from all morphological groups, plus two specimens each of *L. oregana*, *L. pulmonaria*, and *S. limbata*, and one specimen of *Pseudocyphellaria crocata*. One-dimensional TLC was performed using aluminum-backed sheets in solvent A, or on silica coated glass plates in solvents A, B, and C according to protocol presented in Culberson (1972) and Culberson and Johnson (1982). Two-dimentional TLC was also performed for selected specimens, using solvent C for the first direction and solvent B for the second direction.

The secondary compounds found in the putative new species *S. carolinensis* and *S. fragilinata* were further characterized by HPLC using a C-18 column. Additional analyses were performed using a C-8 column on a Shimadzu SPD-64 Spectrophotometric Detector set at 270 nm with a Perkin Elmer 410 pump and analyzed using the Maxima Chromatography Workstation program.

The chemical data matrix contains both the results of the spot tests (K and Pd reactions) of thalli medulla and the major group of secondary compounds resulting from TLC and HPLC studies. Supplementary data on the chemical compositions of the lichens from the outgroups were taken from the lichen literature (Culberson 1972; Kondratyuk & Galloway 1994; McCune & Geiser 1997). The following characters were included in chemical data matrix (Table 2; characters 27–32):

27. K Reaction: 0 = K−; 1 = K+ purple; 2 = K+ yellow.
28. Pd Reaction: 0 = Pd−; 1 = Pd+ orange.
29. Anthraquinones: 0 = absent; 1 = present.
30. Stictic Acid Aggregates: 0 = absent; 1 = present.
31. Terpenoids: 0 = absent; 1 = present.
32. Pulvinic Acids: 0 = absent; 1 = present.

Chemical delimitation of the neotropical *S. weigelii s. s.* remains unclear and was not specifically addressed in this paper. Although the type specimen (H-Ach, holotype) has anthraquinones in its medulla (spot reaction K+ red; pers. comm., R. Harris), some specimens considered to be typical lacked anthraquinones in the medulla and exhibited a negative K reaction. Therefore, to accommodate our concern about the potential chemotypical variation within *S. weigelii s. s.* and the possibility of decomposition of anthraquinones in aging specimens, the scores of these two characters for *S. weigelii s. s.* were treated as uncertain rather than polymorphic (Table 2; characters 27 and 29).

Molecular data.—DNA from lichen thalli was isolated using a CTAB/chloroform/ isopropanol mini-prep (Gardes & Bruns 1993). The ITS region was amplified by the Polymerase Chain Reaction method (PCR) using primer ITS1F on the coding strand and primer ITS4 on the non-coding strand (Gardes & Bruns 1993; White et al. 1990).

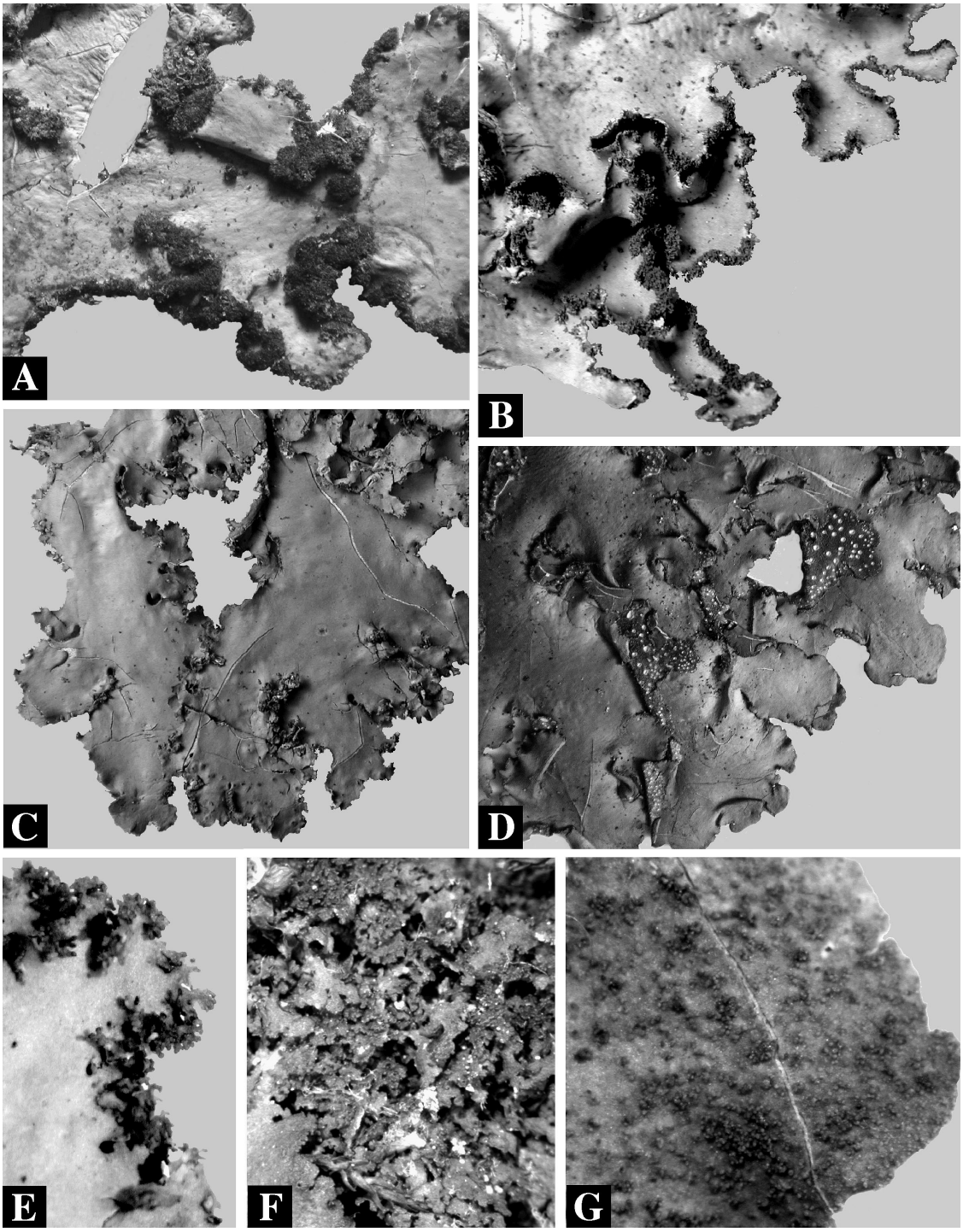

FIGURE 2. Thallus habit showing margin shape, isidium type, and its placement corresponding to the morphological character 4, 6, and 7 (Table 2) for selected *Sticta* species. Character states are presented in the Materials and Methods section. A, *Sticta weigelii*; B and D, *Sticta beauvoisii*; C and E, *Sticta fragilinata*; F, *Sticta carolinensis*; G, *Sticta fuliginosa*.

Attempts were made to amplify and sequence DNA from all 25 *Sticta* lichens used in the morphological analysis. After repeated attempts, if DNA from a particular specimen could not be amplified, DNA from another lichen in the group but not part of the morphological analysis was amplified in its place.

The PCR product was purified using either the QIAGEN QIAquick DNA Purification Kit, or low-binding regenerated cellulose 30,000 NMWL (nominal molecular weight limit) filter units (Millipore). Purified PCR products were sequenced using the following alternative pairs of primers: ITS5/IT1F and ITS3/5.8SR for the coding strand; ITS4/ITS2 and 5.8S for the non-coding strand (Vilgalys & Hester 1990; White et al. 1990). The sequencing reaction was performed using the Big Dye Terminator Cycle sequencing kit (ABI PRISM, Perkin-Elmer, Applied Biosystems) following the manufacturer's instructions. Polyacrylamide gel electrophoresis was conducted using Long Ranger Single gel packs (FMC BioProducts) and an ABI 377A automated DNA sequencer (Perkin-Elmer, Applied Biosystems). A few samples were sequenced, precipitated, and run using Amersham DYEnamic ET Terminator Cycle Sequencing Kit on a Long Ranger 5.75% acrylamide gel (BMA) following the manufacturer's instructions.

ITS nrDNA sequence fragments were assembled using Sequencher 3.0 and 4.1 and optimized by eye. Delimitation of the internal spacers and the 5.8S gene was obtained by comparison with complete sequences from closely related taxa within Peltigerineae (Goffinet & Goward 1998; Miadlikowska et al. 2003; Miadlikowska & Lutzoni 2002) and sequences available in GenBank.

Phylogenetic analysis.—Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) optimization criteria as implemented in PAUP*4.0b4a (Swofford 2001). For one specimen (*Sticta* sp.), we were not able to complete the morphological and chemical character matrix. Therefore, for the purpose of combinability we did phylogenetic analyses for two ITS data sets: 1) phylogenetic analyses for 26 ITS sequences (MP1 and ML1) without *Sticta* sp., and 2) phylogenetic analyses for 27 sequences (MP2 and ML2) including *Sticta* sp. Maximum parsimony searches were implemented on the combined morphological and chemical data set (MP3) and on the morphology + chemistry data set combined with the ITS sequences (MP4) for the same 26 individuals included in MP1 and ML1. For the separate analysis on morphology + chemistry (MP3), all changes among character states were weighted equally. To simultaneously accommodate taxa with multiple character states resulting from uncertainty or polymorphy, the ''variable'' option in PAUP* was used.

Constant sites were removed from all maximum parsimony analyses. The maximum parsimony analyses of the ITS data set (MP1, MP2, and MP4) involved, for unambiguously aligned portions of the alignment, three separate step matrices corresponding to the ITS1, 5.8S, and ITS2 regions; step matrices were constructed as outlined in Miadlikowska et al. (2002). Phylogenetic signal from ambiguously aligned portions of the alignment was integrated into maximum parsimony analyses without violating positional homology using the program INAASE 2.3b (Lutzoni et al. 2000). The weights for substitutions in INAASE were set as following: transitions = 0.5, transversions = 1.0, indels = 1.5. For maximum parsimony analyses, gaps from the unambiguous portions of the alignment were recognized as a fifth character state. All maximum parsimony analyses (MP1–4) were performed as heuristic searches with 1,000 random-addition-sequence replicates, TBR branch swapping, MulTrees option in effect, saving all trees, and collapsing branches with maximum branch length equal to zero.

Using the Hierarchical Likelihood Ratio Test as implemented in Modeltest 3.04 (Posada & Crandall 1998), the K80 two-parameter nucleotide substitution model (Kimura 1980) with equal base frequencies was selected for both the 26 OTUs (ML1) and the 27 OTUs (ML2) ITS nrDNA data sets (ML1: ti/tv ratio = 5.9726, gamma distribution shape parameter = 0.0103; ML2: ti/tv ratio = 6.8301, proportion of invariable sites I = 0.8357, number of rate categories = 4). Maximum likelihood analyses (ML1 and ML2) were implemented as heuristic searches with 100 random-addition-sequence replicates, TBR branch swapping, MulTrees option in effect, reconnection limit equal to eight, saving all trees and collapsing branches with maximum branch length equal to zero.

Branch support for MP and ML trees was estimated by bootstrap analyses (BS) (Felsenstein 1985) with full heuristic searches, 1,000 bootstrap replicates for MP and 100 for ML, two random-addition-sequence per bootstrap replicate and reconnection limit equal to eight for ML analyses. In addition, both ITS data sets (26 OTUs and 27 OTUs) were analyzed using Bayesian statistics with Markov chain Monte Carlo sampling (B/MCMC) method as implemented in MrBayes 2.01 (Huelsenbeck 2000). Four chains, each initiated with a random tree, were run simultaneously. One of every 100 trees was sampled for a total of 1,000,000 generations with DNA substitution parameters estimated during the search. The first 1,000 sampled trees were discarded before calculating the majority-rule consensus tree on the remaining 9,000 trees with PAUP*. Bipartitions were considered statistically significant when posterior probabilities were ≥95%. Congruence between the morphology + chemistry partition and the ITS partition for 26 OTUs was tested by inspecting bootstrap values ≥70% on topologies derived from each partition when analyzed separately as outlined in Miadlikowska and Lutzoni (2000).

RESULTS

MORPHOLOGY, CHEMISTRY, AND MOLECULAR CHARACTERS

Morphological and chemical characters.—Differences in morphological characters, especially for continuous traits, between two initially recognized groups of *S. beauvoisii* (I and II) were insignificant (Fig. 1); therefore both groups obtained identical scores for all morphological traits (Table 2; characters 1–26).

One-dimensional TLC detected no lichen chemicals in *Sticta beauvoisii* groups I and II, *S. carolinensis*, *S. fuliginosa*, *S. limbata*, *S. weigelii* 1, or *Sticta* sp. Both *S. fragilinata* and the other specimens of *S. weigelii*, however, exhibited rich chemistries. Analysis of *Sticta fragilinata* by one-dimensional TLC revealed nine spots in solvent A, seven spots in solvent B, and eight spots in solvent C (Fig. 4 and Table 3). Two-dimensional TLC in solvent C then solvent B further resolved the spots into 15 compounds. At least three of these compounds were anthraquinones, while the rest appeared to be fatty acids or terpenes.

The brightest anthraquinone spot (spots 1, 10, 17; Fig. 4 and Table 3) corresponded to the fragilin control. Another prominent anthraquinone spot (spots 4, 11, 21; Fig. 4 and Table 3) corresponded to the 2-chloroemodin control. The results of the two-dimensional TLC run corroborate the findings of the one-dimensional analyses.

Of the terpene-like chemicals, only one, Unknown 1, was present in large quantities. On the plate (spots 5, 13, 20; Fig. 4 and Table 3), this compound appeared pinkish-brown at first, then became brown and finally gray with age. When the spot was pre-treated with base before the sulfuric acid spray, the compound turned a dark purple gray color.

For *S. weigelii* 1, only one faint spot of an unidentified substance visible in daylight and ultraviolet light (350 nm) was found (Rf class = 5 in solvent A and B, Rf class = 3 in solvent C). A similar spot was already detected in another specimen of *S. cf weigelii* by C. Culberson (pers. comm.).

HPLC analysis using a C-18 column and standards identified four anthraquinones present in *S. fragilinata*. Fragilin and 2-chloroemodin were the major anthraquinones, while the two non-chlorinated versions of those chemicals, parietin and emodin respectively, were present in trace amounts. A separate analysis by C. Culberson using a control mixture of parietin, 2-chloroemodin, and emodin also demonstrated the presence of these anthraquinones. Two-chloroemodin was also demonstrated with TLC, while parietin and emodin were detected only with HPLC and then only in trace amounts.

ITS characters.—The final alignment for the 26 ITS and 27 ITS sequences consisted of 511 sites. A total of 18 ambiguous regions were delimited, resulting in the exclusion of 170 sites. Constant sites (298) were excluded from MP analyses, giving a total of 43 unambiguously aligned sites. The 18 ambiguous regions provided 18 additional INAASE coded characters for MP analyses for a total of 61 non-constant characters included in MP1, MP2, and MP4 analyses. Of these, 48 were parsimony informative. In the ML analyses, 172 characters were excluded, resulting in a total of 339 sites used in both ML1 and ML2 analyses.

For the final MP4 analysis on the 26 OTUs combined morphology + chemistry and ITS data set, 93 variable characters were included, of which 32 were derived from the morphological and chemical data set, and 61 were derived from ITS data set. In total 78 characters were parsimony informative; 30 of these were derived from morphological and chemical data set, while 48 were derived from ITS data set.

ITS sequences varied in total length from 469 to 491 nucleotides (ITS1: 162–170; 5.8S: 158; ITS2: 149–164). Sequences were identical in length within *S. carolinensis*, *S. fragilinata*, *S. beauvoisii* (except specimen 7), and *S. fuliginosa* (except specimen 3). Pair-wise sequence differences among taxa were distinctly higher than among specimens from the same taxon (Fig. 5). When comparing sequences between taxa, the least number of total differences was found between the following pairs of taxa: *Sticta* sp.-*S. fragilinata* (14 total / 2 within unambiguous regions), *Sticta* sp.-*S. beauvoisii* (19–21 total / 4–6 within unambiguous regions), and *S. fragilinata*-*S. beauvoisii* (20–22 total / 4–6 within unambiguous regions).

Phylogenetic Relationships

ITS sequence analyses (MP1 and ML1; MP2 and ML2).—Twelve equally most parsimonious trees were obtained from the MP1 search (Fig. 6A; tree length = 306.06 steps, single island hit 1,000 times) and a single most likely tree resulted from the ML1 analysis (results not shown; log likelihood = −790.56307, single island hit 100 times). Both topologies revealed identical taxa delimitations, congruent with our species circumscription based on morphological and chemical data. Relationships among these putative species are different between the MP1 and ML1 trees, but the differences are not significant due to the low support for at least one of the two analyses. Only one relationship among species (monophyly of all *Sticta* species except *S. fuliginosa*) received a bootstrap value higher than 50% (BS = 83%; Fig. 6A). Five monophyletic groups within *Sticta*, including three within the *weigelii* complex (*S. beauvoisii*, *S. fragilinata*, and *S. carolinensis*) are present on MP1 and ML1 trees. In the MP1 analysis, all putative species received high bootstrap values (BS ≥ 99%). None of the monophyletic groups within the *S. weigelii* complex obtained significant posterior probability support at a p level ≥95%. The only other *Sticta* species significantly supported by B/MCMC are *S. limbata* and *S. fuliginosa*. Similar structure at the population level was found on MP1 and ML1 trees; in the *S. beauvoisii* group, specimens 1–5 share a common ancestor, although this node has extremely low support (BS = 61% with MP1; Fig. 6A, and <50% with ML1; not shown). Twelve equally most parsimonious trees were obtained from the MP2 analyses [Fig. 6A; tree length = 312.40 steps, single island hit 1,000 times, CI (excluding uninformative characters) = 0.8728, RI = 0.9030] and a single most likely tree resulted from ML2 analysis (Fig. 6A; ln likelihood = −794.89370, single island hit 100 times). MP2 and ML2 trees resulted from analyses on 27 specimens (*Sticta* sp. included), and are

similar to the MP1 and ML1 results in term of species delimitation; however, bootstrap values are generally slightly lower, except for a distinct decrease of bootstrap support for *S. fragilinata* on ML2 (BS = 52% versus BS = 82% on ML1). Similar to ML1, none of the species from the *weigelii* complex received significant Bayesian support at $p \geq 0.95$; *S. limbata* is the only taxon outside of *S. weigelii* group significantly supported (B/MCMC = 100%). Relationships among species remain poorly supported; the only bootstrap value ≥50% supports a *Sticta* clade excluding *S. fuliginosa* (MP2; BS = 84%) as revealed by MP1 (Fig. 6A). Based on MP2 and ML2 analyses, the expected phylogenetic affiliation of *Sticta* sp. with *S. weigelii* was not established; but this sister relationship cannot be rejected based on the ITS data alone (Fig. 6A).

In general, the bootstrap values using MP as the optimization criterion were higher than bootstrap values using ML as the optimization criterion or B/MCMC. In the ML1 analysis, two out of the six putative *Sticta* species obtained distinctly lower bootstrap support: the putative *S. carolinensis* (BS = 76%) and *S. beauvoisii* (BS = < 50%). This is because MP allows the use of INNASE characters derived from ambiguously aligned regions (Lutzoni et al. 2000). Within an ML or a Bayesian framework, these characters have to be excluded.

Morphology + chemistry analyses (MP3).—Three equally most parsimonious trees were obtained from the MP3 search and are summarized here using a strict consensus tree (Fig. 6B). This topology is identical to topologies revealed by MP and ML analyses of the ITS data, in terms of putative species delimitation using monophyly as the grouping criterion. The morphology + chemistry tree differs from the ITS tree by the sister relationship of *S. weigelii* with *S. beauvoisii* (BS = 81%) and rooting of the ingroup. *Sticta limbata* is basal in the morphology + chemistry tree whereas *S. fuliginosa* is basal in the ITS tree. Because of the lack of morphological and chemical characters, relationships among species are mostly unresolved in the MP3 tree.

Using the 70% bootstrap support criterion, one conflict between the morphology + chemistry and ITS trees was detected (Fig. 6A and B; circled bootstrap values). Contrary to the morphological characters that support the basal placement of *S. limbata* (the only sorediate *Sticta* species in our ingroup) on the MP3 tree, the ITS data support *S. fuliginosa* at the base of the MP1 tree. Both reconstructions received bootstrap values higher than 70% but only slightly for MP3 analyses (71%). Bayesian posterior probability support for this conflicting bipartition based on the ITS data set was below 50%, suggesting that this conflict was not significant. Under these conditions we decided this conflict was not significant and we combined the morphological and chemical data set with the ITS nrDNA data set and analyzed them simultaneously using maximum parsimony (MP4).

Morphology + chemistry and ITS nrDNA combined analyses (MP4).—Twelve equally most parsimonious trees were obtained from the MP4 search and summarized using a strict consensus tree (Fig. 7). This final topology based on a combined data set revealed five *Sticta* species as being monophyletic and highly supported (BS = 100% for each). More sampling is needed from *Sticta weigelii* to establish its status. Population structure was found with combined ITS and morphology + chemistry within *S. beauvoisii*. Specimens 1–5 are grouped together as previously revealed from MP and ML analyses, except MP3 (on morphology + chemistry alone), but the bootstrap value remained very low (50%; Fig. 7). Relationships among species did not reflect any of the previous topologies; however, none of the bipartitions reflecting species relationships based on this combined data set was corroborated by high bootstrap support. The separation between *S. fuliginosa* and the remaining of the *Sticta* species included in this study is in accordance with separate maximum parsimony analyses on ITS (MP1 and MP2). However, by adding morphological and chemical characters to the ITS data, the bootstrap value for this relationship went down on the MP4 tree (from 84% on MP1 and 83% on MP3 to 67% on MP4 tree; Fig. 7).

Taxonomy of the Genus *Sticta*

High bootstrap values from our study support the monophyly of five putative *Sticta* species. Three of them (*S. beauvoisii*, *S. fuliginosa*, and *S. limbata*) were already recognized. Two newly delimited taxa are described here as new species from this genus – *S. carolinensis* and *S. fragilinata*. Each *Sticta* species is characterized by distinct and unique sets of unequivocal synapomorphies derived from the morphological and chemical data sets (Fig. 7). The same sets of synapomorphies were found for all 12 equally most parsimonious trees revealed from the combined data set (MP4). *Sticta fragilinata* received the greatest number of morphological/chemical synapomorphies (8), followed by *S. fuliginosa* with five synapomorphies. *Sticta beauvosii* and *S. limbata* were defined by one unequivocal morphological change, but were among species supported by the greatest number of nucleotide substitutions and indels (Fig. 6A).

Key to the Species of *Sticta* from the Great Smoky Mountains

1. Medulla K−; medulla and basal membranes of cyphellae white 2

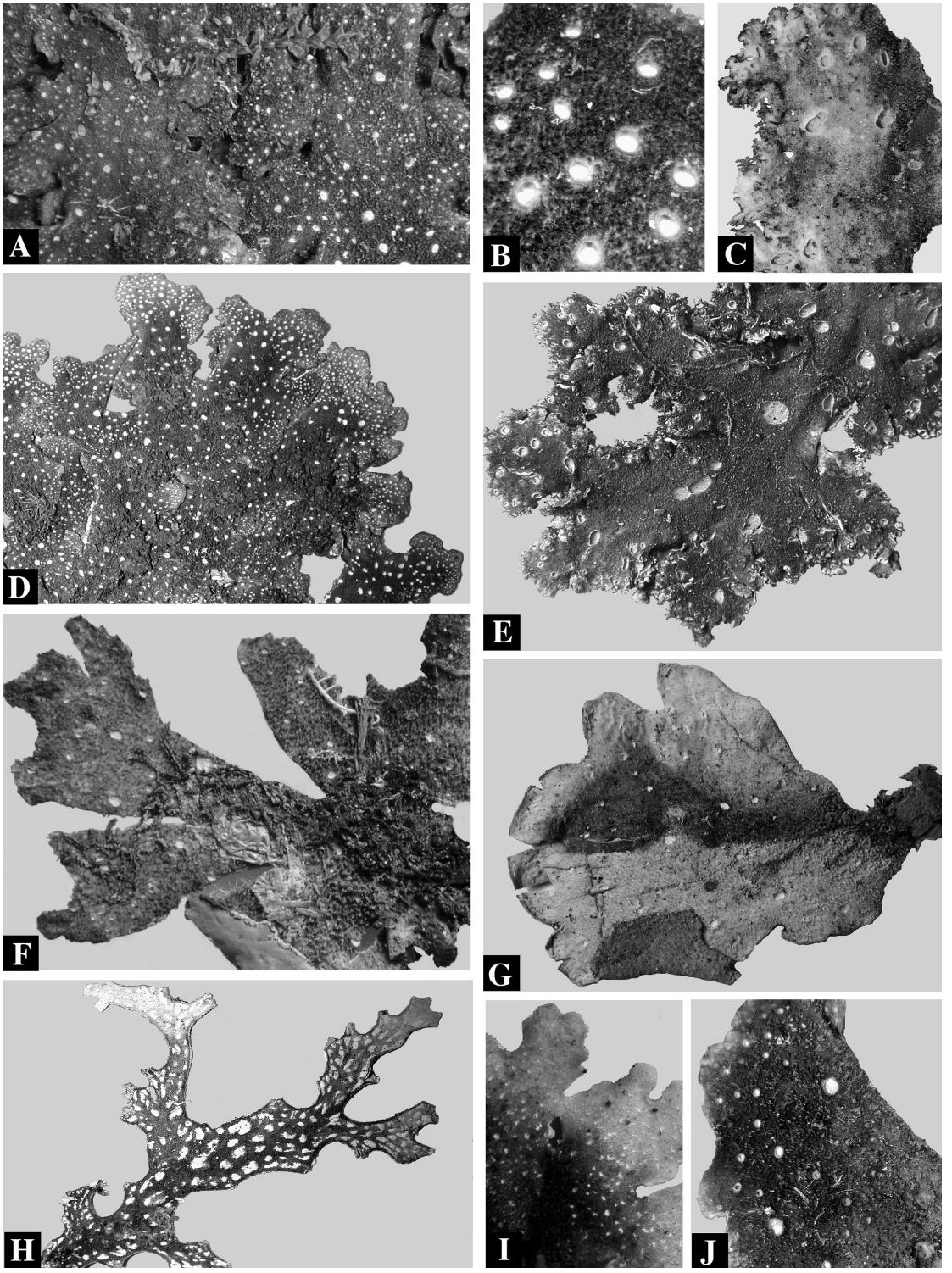

FIGURE 3. Lower side of thallus showing cyphella shape (char. 15), its arrangement (char. 17), its density (char. 21; Fig. 1B), and pseudocyphella (char. 13; Table 2) for selected *Sticta* and outgroup species. Character states are described in the Materials and Methods section. A and B, *Sticta weigelii*; C and E, *Sticta fragilinata*; D, *Sticta beauvoisii*; F, *Sticta limbata*; G and J, *Sticta fuliginosa*; H, *Lobaria pulmonaria*; I, *Pseudocyphellaria crocata*.

TABLE 2. Data matrix for morphological (1–26) and chemical (27–32) characters. Specimens within each taxon were scored identically. Characters and character states are described in the Materials and Methods section. ? = character state unknown. For certain characters, some taxa were assigned multiple character states (polymorphism) and were analyzed as such: a = 0 & 1; b = 0 & 2; c = 1 & 2; d = 2 & 3. For *S. weigelii*, two characters were scored as uncertain and analyzed as such: e = 0/1 and f = 0/1/2.

Taxon/ Character Number	*Sticta beauvoisii I & II*	*S. carolinensis*	*S. fragilinata*	*S. fuliginosa*	*S. limbata*	*S. weigelii s. s.*	*Lobaria oregana*	*L. pulmonaria*	*Pseudocyphellaria crocata*
Primary Photobiont 1	1	1	1	1	1	1	0	0	0
Upper Surface Texture 2	0	0	0	0	0	0	1	1	1
Lobe Shape 3	1	2	1	0	0	1	0	0	1
Margin Shape 4	0	1	1	0	0	0	2	0	0
Reproductive Structure 5	1	1	1	1	0	1	2	b	0
Reproductive Structure Placement 6	2	2	2	0	2	2	1	c	c
Isidium Shape 7	2	3	3	1	0	2	0	0	0
Soredium Color 8	0	0	0	0	2	0	0	1	3
Thallus Attachment Point 9	1	1	1	0	1	1	1	1	0
Rhizine Arrangement 10	0	0	0	2	0	0	0	0	1
Tomentum Color 11	0	2	1	2	1	0	2	1	1
Tomentum Pattern 12	0	0	0	0	0	0	1	1	0
Pseudocyphella 13	0	0	0	0	0	0	0	0	1
Cyphella 14	1	1	1	1	1	1	0	0	0
Cyphella Shape 15	1	2	2	1	1	1	0	0	0
Cyphella Margin 16	2	1	1	1	c	2	0	0	0
Cyphella Arrangement 17	1	c	2	1	1	1	0	0	0
Cyphella Basal Membrane Color 18	1	1	2	1	1	2	0	0	0
Medulla Color 19	0	0	1	0	0	1	0	0	0
Tomentum Length 20	3	1	0	c	3	3	2	c	d
Cyphella Density 21	3	c	c	1	1	c	0	0	0
Cyphella Diameter 22	1	1	2	c	2	1	0	0	0
Isidium Length 23	2	3	3	1	0	2	0	0	0
Lobe Width 24	1	0	c	1	2	1	d	c	c
Lobe Length 25	2	c	d	1	a	2	3	3	1
Elevation 26	0	0	1	2	?	?	?	a	?
K Reaction 27	0	0	1	0	0	f	2	2	2
Pd Reaction 28	0	0	1	0	0	0	1	1	1
Anthraquinones 29	0	0	1	0	0	e	0	0	0
Stictic Acid Aggregates 30	0	0	0	0	0	0	1	1	1
Terpenoids 31	0	0	1	0	0	0	0	0	1
Pulvinic Acids 32	0	0	0	0	0	0	0	0	1

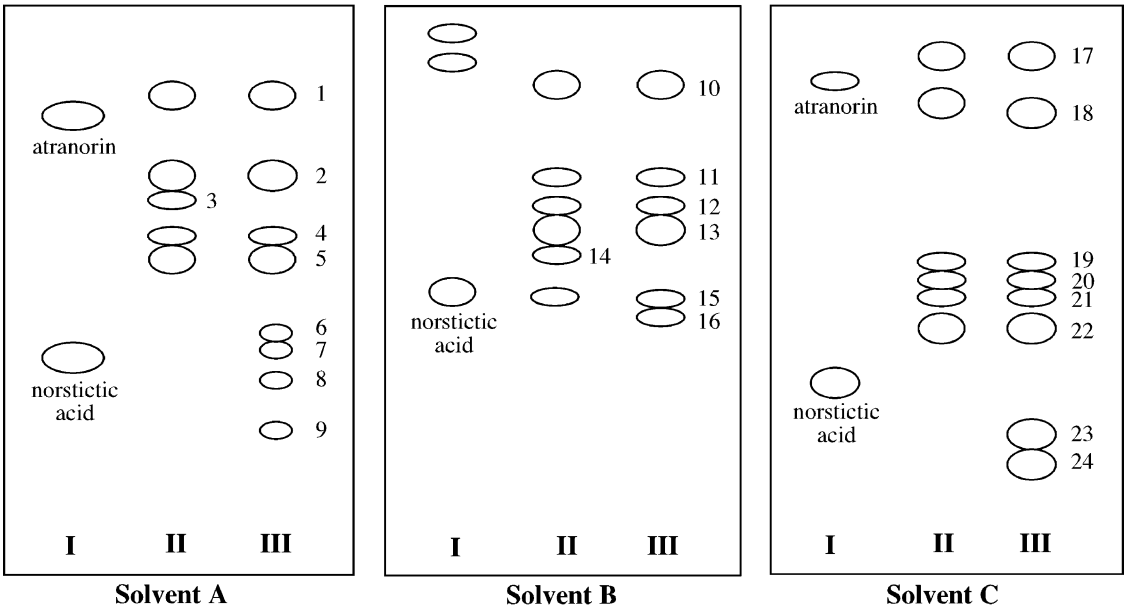

FIGURE 4. Schematic one-dimensional thin layer chromatography plates in three standard solvent systems (Culberson 1972) for *Sticta fragilinata*. The lichen secondary compounds were extracted with toluene and acetone and run simultaneously using atranorin and norstictic acid as the control substances. Ovals represent detected spots (secondary metabolites). First column on each plate represents control substances (I), the second one, the toluene extract (II) and the third one, the acetone extract (III). Each number indicates a single spot. Table 3 summarizes the colors, properties, and identities (if determined) of each numbered spot detected in *S. fragilinata* thalli.

1. Medulla K+ purple, red, or yellow; medulla and basal membranes of cyphellae tannish orange 5
   2. Soredia present ..... *S. limbata*
   2. Isidia present 3
3. Isidia laminal; rhizines in localized structure ..... *S. fuliginosa*
3. Isidia marginal; rhizines scattered 4
   4. Isidia coralloid; cyphellae with raised margins; tomentum dark brown ..... *S. beauvoisii*
   4. Isidia phyllidiate; cyphellae with flat margins; tomentum cream to tan ..... *S. carolinensis*
5. Isidia phyllidiate; cyphellae clustered, with flat margins; medulla Pd+ orange ..... *S. fragilinata*
5. Isidia coralloid; cyphellae single, with prominently raised margins (not found in the Great Smoky Mountains) ..... *S. weigelii s. s.*

STICTA BEAUVOISII Delise, Hist. Lich. Sticta 83: pl.7, fig. 25. 1822. FIGS. 2B, D; 3D

Thallus approximately 4–12 cm wide. *Lobes* imbricate, convolute, once, twice, or rarely three times divided, shallowly indented to deeply incised, with divisions slightly flabelliform, usually imbricate (Fig. 2B, D), 1.9–5.4 cm long, approximately 0.1–1.2 cm wide. *Margins* entire to lightly notched, generally raised with thin region lacking tomentum on underside. **Upper surface** chocolate brown to bluish gray, flat to slightly rugose at lobe divisions, not shiny, occasionally maculate. *Isidia* marginal and along wounds and cracks, coralloid (Fig. 2D), dark brown with smooth, shiny, rounded tips, 0.07–0.79 mm long. *Soredia* absent. *Apothecia* not seen. **Photobiont** a cyanobacterium. **Medulla** white. **Lower surface** brown, flat or slightly rugose toward edges. *Tomentum* dark brown to almost black, thick, shaggy, matted, arranged in tufts with spreading tops like shocks of wheat, 0.07–0.45 mm at edge and 0.18–0.57 mm in middle. *Rhizines* cream-colored to tan at edge of lobe, slender, 0.66–3.00 mm long; dark brown to black in middle of lobe, 1.32–9.48 mm long, thicker, branched along main axis, diverging at tip, treelike, mostly ropy to smooth. *Cyphellae* small (0.1–0.2 mm at edge, 0.5–1.2 mm in middle) rounded, single; more frequent at edge than in middle (Fig. 3D), margin slightly thickened, prominently raised, lacking tomentum, white basal membrane.

Chemistry.—Cortex and medulla K−, C−, and Pd−. No lichen secondary substances found by TLC.

Ecology and distribution.—*Sticta beauvoisii* commonly occurs near streams on rocks or on deciduous trees, often in moss. It is often found with *S. carolinensis* or *S. fragilinata* at or below 820 m, or less frequently with *S. fuliginosa* above 1,320 m. The lichen has been reported from southern Canada to northern Georgia in eastern North America. It has also been reported as far south as Mexico, New Mexico, Arizona, and Texas north to Oregon, Washington, and British Columbia, although further

TABLE 3. Characteristics of spots detected by one-dimensional Thin Layer Chromatography in three solvents (A, B, and C) for *Sticta fragilinata*. The numbers in the first column correspond to spot numbers shown in Fig. 4. Color before charring = color of spots before the application of sulphuric acid; Short-wave UV = presence or absence of fluorescence under UV light before charring; Long-wave UV = color of fluorescence under long-wave UV before charring; Color after charring = color of spots after application of sulfuric acid and 10 minute heat treatment in 110°C; lt. = light; unk. = unknown. Unknown terpenoids with numbers are recognizable in all solvent systems.

Solvent/ Spot	Color before charring	Short-wave UV	Long-wave UV	Color after charring	Identification
A					
1	lt. yellow	—	orange	faint yellow	fragilin
2	—	+	—	purplish-gray	unk. 2
3	—	—	—	lt. purplish-gray	—
4	yellow	—	red	—	2-chloroemodin
5	—	+	orange	gray	unk. 1
6	—	—	—	pinkish-gray	unk. terpenoid
7	—	—	—	pinkish-gray	unk. terpenoid
8	—	—	—	pinkish-gray	unk. terpenoid
9	—	—	—	pinkish-gray	unk. terpenoid
B					
10	yellow	—	yellow	faint yellow	fragilin
11	yellow	—	yellow	faint yellow	2-chloroemodin
12	—	—	red	purplish-gray	unk. 2
13	—	+	red	purplish-gray	unk. 1
14	—	—	—	faint gray	—
15	—	—	—	purplish-gray	unk. terpenoid 1&2
16	—	+	—	purplish-gray	unk. terpenoid 3
C					
17	lt. Yellow	—	orange	faint yellow	fragilin
18	—	—	—	lt. purplish-gray	unk. 2
19	—	—	red	—	anthraquinone
20	—	+	—	purplish-gray	unk. 1
21	yellow	—	faint orange	—	2-chloroemodin
22	—	—	—	lt. purplish-gray	unk. terpenoid 1
23	—	—	—	lt. purplish-gray	unk. terpenoid 2
24	—	—	—	lt. purplish-gray	unk. terpenoid 3

work needs to be done on the representatives from the west and from the north, which may represent a separate taxon (Brodo et al. 2001). The status of the Mexican specimens is also uncertain.

Taxonomic notes.—*Sticta beauvoisii* is the most common species of *Sticta* in the Great Smoky Mountains National Park. It may be recognized by its thick, dark tomentum and regular, rounded, small, white cyphellae. It may be distinguished from both *S. carolinensis* and *S. fragilinata* by its coralloid isidia (Fig. 2B versus C, E, & F). Infrequently, specimens of *S. beauvoisii* have well-developed phyllidia. In this case, *S. beauvoisii* may be distinguished from *S. carolinensis* and *S. fragilinata* by its dark brown tomentum and small, single cyphellae. Both *S. carolinensis* and *S. fragilinata* have cyphellae that are irregular and may cluster (Fig. 3D versus C & E). It may be further distinguished from *S. fragilinata* by a negative K or Pd reaction. This species may be distinguished from *S. weigelii s. s.* by the white basal membranes of its cyphellae and by its white medulla. *Sticta weigelii s. s.* has tan basal membranes and a tan medulla. *Sticta fuliginosa* has laminal isidia (Fig. 2G); a light tomentum; very large and simple lobes; and long, thin, brown rhizines compacted into a structure and it is not likely to be confused with *S. beauvoisii*. The unnamed species from the southeastern United States with long, tufted, pale tomentum is more likely to be confused with *S. beauvoisii* (Harris 1984).

Selected specimens examined.—MEXICO. CHIAPAS. Locality unknown, Hale 20544 (MIN). U.S.A. ARIZONA. Apache Co., *Ryan & Nash 26935* (MIN). NORTH CAROLINA. Jackson Co., *Trana 7938B* (MIN); Macon Co., *Trana 7586* (MIN); Swain Co., *McDonald 937* (MIN); Transylvania Co., *Harris 3311* (MSC); Haywood Co., *McDonald 795* (MIN). SOUTH CAROLINA. Oconee Co., *Hill 21989* (MIN). TENNESSEE. Blount Co., *McDonald 633* (MIN); Cocke Co., *McDonald 1032* (MIN); Sevier Co., *McDonald 606* (MIN); Unicoi Co., *Hodgdon & Trossbach 14578* (MIN). TEXAS. Brewster Co., *Anderson & Shushan 18772* (MIN). VIRGINIA. Madison Co., *Wetmore 15410* (MIN); Page Co., *Hermann 14979* (MIN).

STICTA CAROLINENSIS McDonald, *sp. nov.* FIG. 2F

Similis *S. beauvoisii* Del. sed differt isidiis phyllidiatis, margine plano cyphellarum, et tomento cremo (ad marginem thalli) ad dilute brunneo (in centro).

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *S. beauvoisii* 1, 2, 4, 5	-	1	1	2	31	27+	25+	21	51	50	36+	38	39	20	85	81	76
2. *S. beauvoisii* 3	1	-	2	3	32	28+	27+	22	52	51	37+	39	40	21	84	80	77
3. *S. beauvoisii* 6-8	1	2	-	1	30	26+	25+	20	50	49	35+	37	40	19	86	82	77
4. *S. beauvoisii* 9	1	2	0	-	30	26+	26+	20	50	49	35+	37	40	19	87	90	78
5. *S. carolinensis* 1	6	7	5	5	-	3+	2+	26	55	52	36+	41	44	28	83	90	83
6. *S. carolinensis* 2	5	6	4	4	1	-	0+	22+	48+	45+	33+	36+	40+	24+	78+	84+	77+
7. *S. carolinensis* 3	4	6	5	5	1	0	-	20+	48+	45+	35+	35+	35+	22+	76+	82+	71+
8. *S. fragilinata* 1-5	5	6	4	4	5	4	4	-	48	47	32+	35	35	14	87	89	77
9. *S. fuliginosa* 1	7	8	6	6	7	6	6	6	-	1	54+	54	55	47	83	94	85
10. *S. fuliginosa* 2, 3	7	8	6	6	7	6	6	6	0	-	53	43	63	46	83	94	85
11. *S. limbata* 1	8	9	7	7	10	9	9	7	6	6	-	1+	44+	33+	80+	86+	80+
12. *S. limbata* 2	8	9	7	7	10	9	9	7	7	7	0	-	51	36	86	91	90
13. *S. weigelii*	7	8	8	8	9	8	8	8	8	8	11	11	-	35	92	92	95
14. *Sticta* sp.	5	6	4	4	5	4	4	2	4	4	7	7	8	-	86	91	75
15. *L. oregana*	21	20	22	22	23	22	22	23	22	22	24	24	24	23	-	74	70
16. *L. pulmonaria*	18	17	19	19	19	18	18	19	21	21	21	21	21	19	18	-	67
17. *P. crocata*	17	18	18	18	18	17	17	18	20	20	19	19	20	18	18	15	-

FIGURE 5. Uncorrected pairwise differences among ITS sequences included in phylogenetic analyses (MP and ML). Above diagonal: total number of differences including optimal number of changes within ambiguously aligned regions as estimated with INAASE. Indels with multiple consecutive gaps were counted as one change. Ambiguously aligned regions for specimens with unknown sites were omitted; a cross indicates that the given number of differences can be higher due to the presence of unknown sites within ambiguous regions. Below diagonal: character differences among unambiguously aligned regions only. Boxes indicate pairwise sequence differences within recognized species, including two newly proposed species, *S. carolinensis* and *S. fragilinata*. Identical sequences were collapsed to one entry in the matrix. For example, ITS sequences for *S. beauvoisii* 1, 2, 4, and 5 were identical.

TYPE: U.S.A. ARKANSAS. Newton Co., Ozark National Forest, Upper Buffalo River Wilderness Area, Lower Fork Creek. At side ravine above waterfalls on rock ledges in woods with oak, hickory, and beech, elev. 585 m, Sec. 8, T14N, R23W. 35°53′37″ N, 93°26′48″ W. 26 May 2000. *Wetmore 84809* (holotype, MIN; isotypes, M, NY, S, US).

Thallus typically 3–12 cm broad, irregularly branching and spreading. *Lobes* 2–7 mm wide, tortuous, not clearly defined, imbricate or not, undulating to ascending, once, twice, or rarely three times shallowly to deeply divided or incised, becoming more so at apex, with divisions weakly flabelliform, often slightly imbricate. *Margins* much notched, rounded, becoming lobulate-looking as phyllidia are developed (Fig. 2F). **Upper surface** brown to brownish-gray, usually flat but occasionally slightly rugose, irregularly cracked, not shiny. *Isidia* phyllidiate, marginal or less frequently laminal (Fig. 2F), especially along wounds, with dark brown tips, not tomentose, 0.12–1.95 mm. *Soredia* absent. *Apothecia* not seen. **Photobiont** a cyanobacterium. **Medulla** white. **Lower surface** buff to reddish tan at edges, brown in middle, slightly rugose at edges, sparsely tomentose. *Tomentum* at edges cream-colored to light tan, simple, 0.12–0.24 mm long; dark brown and thicker in center, with terminal tufts, 0.07–0.23 mm. *Rhizines* at edge simple, brown, single or in clumps along margin or in center of lobe division, occasionally with tomentum decurrent on base, 0.60–4.38 mm, irregularly branching towards center of thallus, thickened at apex and spreading in plane along substrate, dark brown to black, 0.72–7.62 mm. *Cyphellae* very small, 0.1–0.3 mm at edge, diameter 0.3–0.9 mm in middle, deep, mostly irregularly shaped, infrequently merging, occasionally clustered, more frequent at edge of thallus (20 per 0.25 square cm) than in middle of thallus (10 per 0.25 square cm), margin thin, flat, lacking tomentum, white basal membrane.

Chemistry.—Cortex and medulla K−, C−, and Pd−. No lichen secondary substances found by TLC.

Ecology and distribution.—Sticta carolinensis occurs near streams on rocks and on deciduous trees, often in moss. It is often found growing with *Sticta beauvoisii* or, occasionally, with *Sticta fra-*

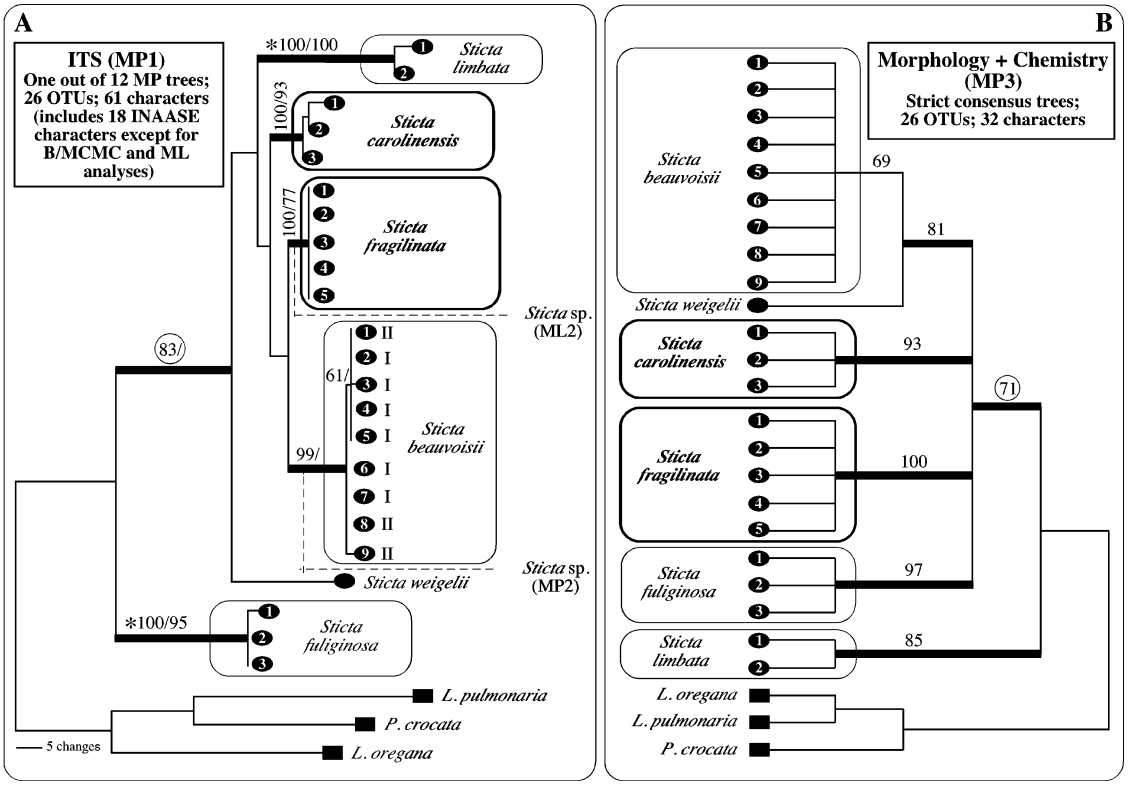

FIGURE 6. Phylogenetic relationships among 26 individuals from four known *Sticta* species, two putative species (*S. carolinensis* and *S. fragilinata*), and the outgroup taxa (*Lobaria oregana*, *L. pulmonaria*, and *Pseudocyphellaria crocata*) as revealed by maximum parsimony analyses of the ITS data set alone (A) and the Morphology and Chemistry data set alone (B). Monophyletic *Sticta* species recognized here are circled. Numbers corresponding to the specimens within each species included in Table 1 replace names on this figure. Bootstrap support >70% is represented by thicker branches. Asterisks indicate nodes that received significant Bayesian posterior probability ≥95%. Bootstrap values in circles indicate potential conflict between two partitions when using our ≥70% bootstrap support combinability criterion. A, MP1 analyses, one most parsimonious tree out of 12 equally most parsimonious trees representing one island, hit 1,000 times, tree length = 306.06 steps, CI (excluding uninformative characters) = 0.8832, RI = 0.9053. Numbers above internodes before and after back slash correspond to bootstrap from MP1 (≥50%) and B/MCMC posterior probability (≥50%) from ML1, respectively. Numbers for *S. beauvoisii* specimens correspond to group I and II as defined for analyses on morphological characters (Fig. 1; Table 2). Dashed lines indicate placement of *Sticta* sp. as revealed by ML2 and MP2 searches. B, MP3 analyses, strict consensus tree of three equally most parsimonious trees representing a single island, hit 1,000 times, tree length = 110.00 steps, CI (excluding uninformative characters) = 0.9074, RI = 0.9438. Numbers above internodes are bootstrap values.

gilinata at or below 820 m in elevation in the Smoky Mountains. This lichen has been found as far south as Mississippi, Georgia, and Alabama, as far west as Arkansas and as far north as North Carolina, although its full distribution is not yet clear.

Taxonomic notes.—Like *Sticta fragilinata*, this species has phyllidiate isidia (Fig. 2F) and irregularly shaped cyphellae. Unlike in the aforementioned species, however, cyphellae in *Sticta carolinensis* are much smaller (Fig. 1C) and their basal membranes are white. No secondary metabolites were found in this species; therefore a negative K or Pd test will distinguish *Sticta carolinensis* from *Sticta fragilinata*. It can be more difficult to distinguish *Sticta carolinensis* from specimens of *Sticta beauvoisii* with well-developed coralloid isidia or, more rarely, with phyllidiate isidia. The small lobe length and width, shorter and lighter tomentum (Fig. 1A, E & F), and lighter lower cortex all aid in the separation of *Sticta carolinensis* from *Sticta beauvoisii*.

Selected specimens examined.—U.S.A. ALABAMA. Dekalb Co., *Buck 34693* (NY); Jackson Co., *Harris 42362* (NY); Winston Co., *Harris 28427* (NY). ARKANSAS. Franklin Co., *Buck 5779* (NY); Newton Co., *Brako 2256* (NY); Polk Co., *Wetmore 84512* (MIN). GEORGIA. Treutlen Co., *Buck 27621* (NY). NORTH CAROLINA. Graham Co., *Harris 20919* (MIN); Haywood Co., *McDonald 793* (MIN); Macon Co., *Harris 41326* (NY); Swain Co., *McDonald 896* (MIN); Transylvania Co., *Buck 30393* (NY); Yancey Co., *Imshaug 22293* (MSC). SOUTH CAROLINA. Pichens Co., *Trana 8366* (MIN). TENNESSEE. Blount Co., *McDonald 692* (MIN);

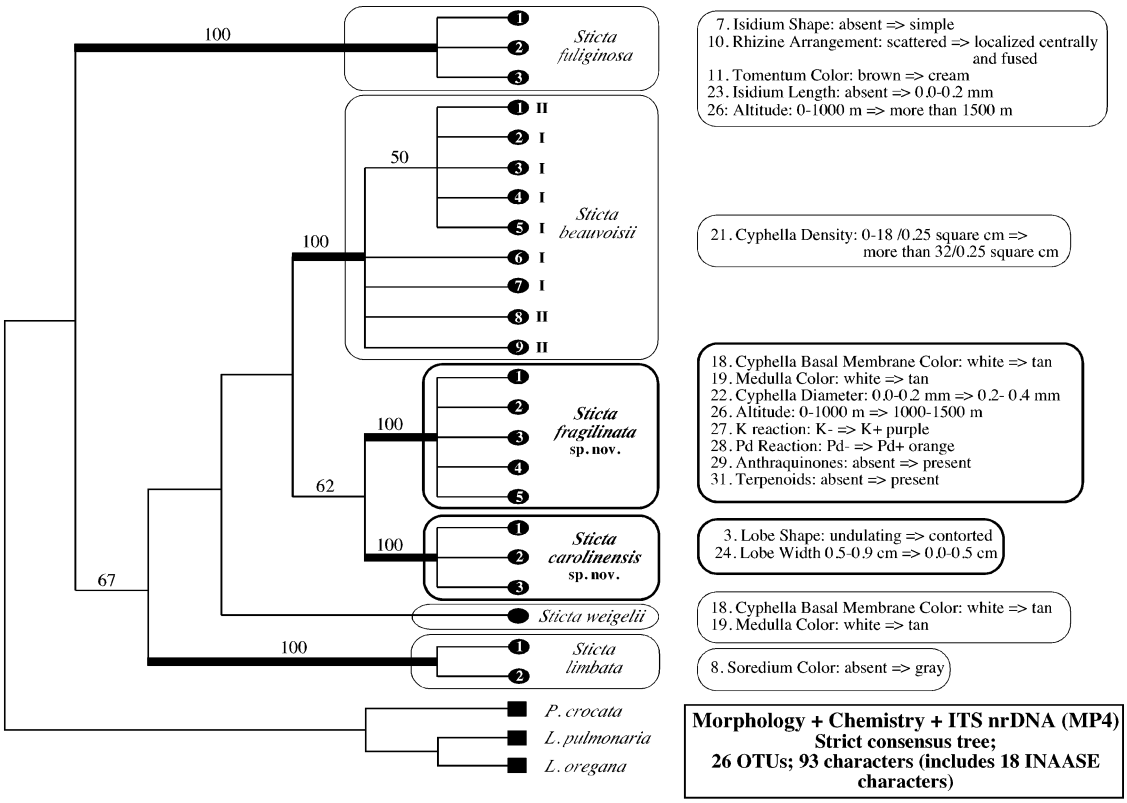

FIGURE 7. Phylogenetic relationships among 26 individuals from four known *Sticta* species, two putative species (*S. carolinensis* and *S. fragilinata*) and three outgroup taxa (*Lobaria oregana*, *L. pulmonaria*, and *Pseudocyphellaria crocata*) as revealed by a maximum parsimony search on the combined Morphology + Chemistry and ITS data set (MP4) and summarized by a strict consensus tree of 12 equally most parsimonious trees representing one island, hit 1,000 times [tree length = 422.76 steps, CI (excluding uninformative characters) = 0.8748, RI = 0.9127]. Bootstrap support >50% is shown above branches. Bootstrap support >70% is represented by thicker branches. Numbers for *S. beauvoisii* specimens correspond to group I and II as defined for analyses on morphological characters (Fig. 1; Table 2). Boxes delimit *Sticta* species recognized here. Boxes with arrows provide a set of unequivocal synapomorphies supporting each species.

Cocke Co., *McDonald 1006* (MIN); Polk Co., *Harris 42492* (NY); Sevier Co., *McDonald 192* (MIN).

STICTA FRAGILINATA McDonald, *sp. nov.*
FIGS. 2C, E; 3C, E

Similis *S. carolinensis* McDonald sed differt medulla pallide brunneo-aurantiaca et membranis basalibus cyphellarum concoloris; medulla K+ purpurea, Pd+ aurantiaca; fragilinum, 2-chloroemodinum, parietinum (minutissimum), emodinum (minutissimum), et substantia terpenoidea ignota adsunt.

TYPE: U.S.A. NORTH CAROLINA. Swain Co., Great Smoky Mountains National Park, Appalachian Trail from Newfound Gap Overlook (S of Gatlinburg). Open woods with sugar maple, beech, and other trees, elev. 1,646 m. 35°37′12″ N, 83°24′21″ W. 12 July 1999. On *Acer sacharum*, *McDonald 361* (holotype, MIN; isotype, US).

Thallus typically 3–14 cm broad, irregularly branching and spreading. *Lobes* 4–10 mm wide, undulating to ascending, once, twice, or rarely three times shallowly to deeply divided or incised, becoming more so at apex, divisions weakly flabelliform, often slightly imbricate. *Margins* much notched, rounded, becoming lobulate-looking as phyllidia develop (Fig. 2C, E). **Upper surface** brown to bluish-gray, usually flat but occasionally slightly rugose, irregularly cracked, not shiny. *Isidia* phyllidiate, marginal or less frequently laminal along wounds (Fig. 2C, E), with dark brown tips, not tomentose, 0.24–1.74 mm. *Soredia* absent. *Apothecia* not seen. **Photobiont** a cyanobacterium. **Medulla** white. **Lower surface** tan to brown at edges, brown in middle, slightly rugose at edges, sparsely tomentose. *Tomentum* tan to gray at edges, simple, 0.05–0.18 mm long, dark brown and thicker in center, with terminal tufts, 0.05–0.15 mm. *Rhizines* at edge simple, light colored, single or in clumps along margin or in center of lobe division, 0.36–5.28 mm, irregularly branching towards center of thallus, with much-branched tips, dark brown, 1.26–7.50 mm. *Cyphellae* diameter 0.1–0.6 mm at

edge, diameter 0.4–2.5 mm in middle, shallow, irregularly shaped, often merging, frequently clustered (Fig. 3C, E), more frequent at edge of thallus (15 per 0.25 square cm) than in middle of thallus (4 per 0.25 square cm), margins thin, flat, lacking tomentum, tannish basal membrane.

Chemistry.—Medulla K+ purple, Pd+ orange. Anthraquinones including fragilin, 2-chloroemodin, and trace amounts of parietin and emodin. Terpenes including one major and four or more minor ones (Table 3 and Fig. 4).

Ecology and distribution.—Sticta fragilinata occurs near streams on rocks and on deciduous trees, often in moss. It is often found growing with *Sticta beauvoisii* and, occasionally, with *Sticta carolinensis* in the Smoky Mountains. It has been found in Florida, Georgia, North Carolina, and Tennessee, although its precise range is not yet known.

Taxonomic notes.—This species is easily recognized by its tan-colored medulla; marginal phyllidia; and large, irregularly shaped, shallow cyphellae with tannish or orangish basal membranes (Figs. 2C, E; 3C, E). If the colors of the medulla and the basal membranes of the cyphellae are indeterminate, this species may be separated from others in the Great Smoky Mountains National Park using spot color tests. The medulla of *Sticta fragilinata* turns purple with potassium hydroxide (K) and orange with paraphenylenediamine (Pd), while the medullas of *Sticta carolinensis* and *Sticta beauvoisii* do not react. Separation of the species confused with *Sticta weigelii* in the Great Smoky Mountains using characteristics of the upper surface, including color and lobe shape, is dubious at best and should be avoided as these characteristics are highly variable. The unnamed lobulate *Sticta* species mentioned by Brodo et al. (2001) most likely refers to *S. fragilinata*.

Etymology.—The lichen derives its name from fragilin, a prominent anthraquinone in the medulla.

Selected specimens examined.—U.S.A. FLORIDA. Highlands Co., *Wetmore 13715* (MIN). GEORGIA. Rabun Co., *Harris 38903* (NY). NORTH CAROLINA. Graham Co., *Trana 8569* (MIN); Haywood Co., *McDonald 540* (MIN); Macon Co., *Harris 41316* (NY); Swain Co., *McDonald 362* (MIN); Yancey Co., *Imshaug 22305* (MSC). TENNESSEE. Blount Co., *McDonald 657* (MIN); Cooke Co., *McDonald 1104* (MIN); Monroe Co., *Buck 25182* (NY); Sevier Co., *McDonald 183* (MIN).

STICTA FULIGINOSA (Hoffm.) Ach., Meth. Lich. 280. 1803. FIGS. 2G; 3G, J

Lobaria fuliginosa Hoffm., Deutschl. Fl. 2: 109. 1796. TYPE: WALES. CADER IDRIS. August 1726 (Dillenius 1742, Tab XXVI, Fig. 100A–lectotype, OXF 100A–epitype; Laundon 1984; Galloway 1997).

Thallus very thin, brittle when dry, edges often curled downward. *Lobes* undulating, broad, rounded, usually only once divided, often whole, imbricate towards middle. *Margins* entire to slightly indented or torn (Fig. 2G). **Upper surface** black or dark gray to light gray, not shiny, flat to slightly rugose or wrinkled. *Isidia* laminal, not clustering around wounds, more dense towards edges, simple to diffuse coralloid (Fig. 2G), short, 0.05–0.42 mm, dark brown. *Soredia* absent. *Apothecia* not seen. **Photobiont** a cyanobacterium. **Medulla** white. **Lower surface** cream to tan, slightly wrinkled, densely tomentose. *Tomentum* light gray or tan, darker towards middle, sparse at edges, 0.12–0.45 mm at edge, slightly longer and more dense in middle, 0.14–0.54 mm. *Rhizines* localized, long, thin, brown, compacted into structure positioned towards edge of thallus, 0.5–1.0 cm long. *Cyphellae* flat, round to oval (Fig. 3G, J), rather large, 0.1–0.4 mm at edge, 0.3–1.0 mm in middle, rather sparse, 3–30 per square cm at edge, 0–9 per 0.25 square cm the middle, margins flat, lacking tomentum, basal membrane white or grayish, occasionally tan.

Chemistry.—Cortex and medulla K−, C−, and Pd−. No lichen secondary substances found by TLC.

Ecology and distribution.—Sticta fuliginosa is found on hardwoods at 1,485 m and higher in the Great Smoky Mountains National Park. It may occur with *S. beauvoisii*, or, less commonly, with *S. fragilinata*. Material has been examined from the West Coast of the U.S.A., the Great Lakes region, and the Appalachian region, confirming the distribution given in Brodo et al. (2001).

Taxonomic notes.—This distinctive lichen is the only *Sticta* in the Great Smoky Mountains that has laminal isidia (Fig. 2G). Its isidia are simple and short, unlike the marginal phyllidia of *S. carolinensis* and *S. fragilinata* or the marginal coralloid isidia of *S. beauvoisii*. *Sticta fuliginosa* may be further distinguished from the other Stictas by the structure made of compacted rhizines it has towards the edge of the thallus. In addition, the lobes of this lichen are rounder, smoother, and much wider than those of the other species. Most of the thalli found in the Great Smoky Mountains National Park were small and scrappy, very unlike the larger thalli found in South America and New Zealand.

Selected specimens examined.—AUSTRALIA. NEW SOUTH WALES, *Elix 22668* (MIN). CANADA. BRITISH COLUMBIA. Queen Charlotte Islands: Graham Island, Mayer Lake, *Brodo & Wong 18080* (MIN); Southern Cariboo Mountains: Wells Gray Provincial Park, Murtle Lake, *Ahti 13192* (MIN); Salt Spring Island, *Douglas 9537* (MIN). MEXICO. VERACRUZ. *Nash 35807* (MIN). NEW ZEALAND. NORTH ISLAND. Auckland, *Flockton 610* (MIN). U.S.A. CALIFORNIA. Santa Cruz Mts, *Herre 134* (MIN).

MINNESOTA. Koochiching Co., *Fink 998* (MIN); Lake Co., *Wetmore 74339* (MIN); St. Louis Co., *Wetmore 35508* (MIN), Voyageurs National Park, *Wetmore 32472* (MIN). NORTH CAROLINA. Macon Co., *Trana 8688A* (MIN); Swain Co., *McDonald 364* (MIN). OREGON. Benton Co., *Wilson 4703A* (MIN). TENNESSEE. Sevier Co., *McDonald 608* (MIN). WASHINGTON. Grays Harbor Co., *Wetmore 19193B* (MIN); Jefferson Co., *Wetmore 19244* (MIN); Snokomish Co., *Eyerdam 1100* (MIN).

STICTA WEIGELII (Ach.) Vainio, Acta Soc. Fauna Fl. Fenn. 4: 189. 1890. FIGS. 2A; 3A, B

Sticta damaecornis β *weigelii* Ach., Lich. Univ. 446. 1810. TYPE: MARTINIQUE. Sine coll. (H-Ach–holotype not seen).

Not found in the Great Smoky Mountains.

Selected specimens examined.—AUSTRALIA. N QUEENSLAND. *Weber L-51544* (MIN). BRAZIL. SAO PAULO. *Nital & Buck 12512* (NY). DOMINICAN REPUBLIC. PROV. LA VEGA. *Harris 14725* (NY). PROV. LA VEGA/SANTIAGO. *Harris 19707* (NY). PROV. INDEPENDENCIA. *Harris 20658* (NY). ECUADOR. GALAPAGOS ISLANDS. Isla Santa Cruz (Indefatigable Island), *Weber L-40881* (MIN). NAPO. *Luteyn & Boom 8350* (NY). GUATEMALA. DEPT. QUICHE. *Richards et al. 3003* (MIN); DEPT. BAJA VERAPAZ. *Richards et al. 2671* (MIN). PUERTO RICO. DISTR. PONCE. *Harris 22023* (NY). VENEZUELA. NYONAGAS. *Pursell 9198* (NY).

DISCUSSION AND CONCLUSIONS

Although the phylogenetic analyses say little conclusive about the relationships among the species under study, the analyses do confirm their distinctness and strongly support their designation as separate species (Figs. 6–7). The results also indicate that our taxonomic delimitation of species for *Sticta* based on morphology and chemistry is in agreement with molecular phylogenetic species recognition within the genus.

It has become clear that *Sticta weigelii* is absent in the Smoky Mountains; according to Brodo et al. (2001), is entirely absent from the United States. Morphologically, chemically, and molecularly, none of the species from the Great Smoky Mountains National Park called *S. weigelii* corresponds to *S. weigelii s. s.* The taxon from the Great Smoky Mountains that is most commonly misidentified as *Sticta weigelii* is *Sticta beauvoisii*, the most common *Sticta* in the Great Smoky Mountains National Park. This lichen has a chocolate brown to bluish-gray upper cortex, a white medulla, and prominently margined cyphellae with white basal membranes, whereas *Sticta weigelii s. s.* has a dark grayish upper cortex, a tannish medulla, and prominently margined cyphellae with tannish basal membranes (Table 2). Both species are clearly distinct based on phylogenetic analyses, however we cannot exclude their close relationship (Figs. 6A, 7).

For morphological study, *S. beauvoisii* was partitioned into two groups: one group with dark brown cortex, small isidia, and thick tomentum (II); the other group with bluish gray cortex, larger and much-branched isidia, and sparse tomentum (I). This partitioning was performed in order to determine whether the suite of analyses performed would separate members of species from each other into groups that might be falsely taken as distinct taxa. Our morphological study revealed high similarity of specimens from the two *S. beauvoisii* groups; they obtained identical scores for all morphological and chemical characters, including the seven continuous traits (Table 2 and Fig. 1). Phylogenetic analyses consistently recognized all potential members of *S. beauvoisii* as part of a single monophyletic entity. However, the molecular data suggest intraspecific variation and show that specimens 1–5 have a closer relationship to each other than to the remaining four individuals (6–9), although this internode was weakly supported (the highest BS = 61% for MP1; Figs. 6A, 7). This infraspecies relationship does not reflect the initial morphological division of *S. beauvoisii* specimens into two groups (groups I and II; Fig. 6A) as described above.

In all analyses of *S. weigelii s. l.* collected in the Great Smoky Mountains National Park, two groups of lichens, described here as new species, were consistently separated from *S. beauvoisii*. Morphologically and chemically, neither group matches *S. weigelii*, nor corresponds to other North American members of the genus *Sticta*, namely *S. fuliginosa*, *S. limbata*, and *S. sylvatica*. Furthermore, neither appears to match any described species of *Sticta*. These taxa both have phyllidiate marginal isidia, not coralloid isidia (Fig. 2E, F). One has anthraquinones in its medulla (*S. fragilinata*); the other has no detectable secondary compounds (*S. carolinensis*) (Table 2; characters 27–32).

A total of five *Sticta* species were found in the Great Smoky Mountains National Park: *S. beauvoisii, S. fuliginosa, S. limbata,* and the two new species–*S. carolinensis* and *S. fragilinata*. However, according to field notes and collection maps from the Park Headquarters in the Great Smoky Mountains National Park, *S. beauvoisii*, *S. dufourii*, *S. fuliginosa*, *S. limbata*, and *S. weigelii* all exist in the park. Our collections do not confirm the reports of *S. dufourii* (not included in phylogenetic analyses) or *S. weigelii*. It is probable that the specimens listed as *S. dufourii* were in fact representatives of the smaller *Sticta* lacking anthraquinones described here as *S. carolinensis*. One small, much-degraded specimen of *S. limbata* was found. Although the

specimen was missing almost all diagnostic morphological features, it was determined to be *S. limbata* (*S. limbata* 2) by matching its DNA to that of a herbarium specimen (*S. limbata* 1) included in phylogenetic analyses (Table 1; Figs. 5–7). Therefore, the report of *S. limbata* in the park can be tentatively confirmed.

Specimens from the Pacific Northwest (Oregon and Washington), commonly identified as *S. weigelii* and represented here by *Sticta* sp., belong to a different taxon. Depending on optimization criteria (parsimony versus likelihood), this taxon was placed sister to *S. beauvoisii* (MP2) or sister to *S. fragilinata* (ML2), always without bootstrap support, but never as a closer relative to *S. weigelii* (Fig. 6A). Its morphological and chemical characters (no secondary compounds detected by TLC), although poorly investigated, are most similar to the description of *S. beauvoisii* than to any other *Sticta* species from the *S. weigelii* complex. However, we cannot exclude the possibility that *Sticta* sp. represents a new species. To resolve its taxonomic placement, further studies including more extensive sampling are necessary.

In the course of the revision, a number of other taxa from the U.S. were consistently misclassified as *S. weigelii* in herbaria, including several specimens of a ''large-lobed variety of *S. beauvoisii*'' from the southwest; several specimens from the southeastern U.S. of a *Sticta* with small, rounded lobes and long, pale, tufted tomentum; and one gray *Sticta* with light tomentum from the Great Lakes region. These collections may represent up to three new species. Extensive taxonomic and phylogenetic studies of the species of *Sticta* in North America are needed to classify these lichens into appropriate groups.

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