Molecular and phenotypic study put eastern North American *Cetrelia* in a global context of biogeography and phylogeny

Jeremy W. Howland, James C. Lendemer
Author Affiliations +

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Abstract
Species of *Cetrelia* delimited based on chemical and morphological characters have been largely supported by subsequent phylogenetic analysis of molecular data. While a robust, taxonomically well sampled global phylogeny for *Cetrelia* exists, geographic sampling to date has focused on Europe and East Asia. Here we use extensive field, herbarium and laboratory study to examine the distributions and identities of the taxa occuring in eastern North America. The presence of three species in the region is confirmed with molecular data (*C. chicitae, C. monachorum, C. olivetorum*). While a fourth species (*C. cetrarioides*) also occurs based on phenotypic data; efforts to obtain sequences were unsuccessful. The subpopulations of *Cetrelia* species in the region have contrasting frequency and distribution patterns relative to disjunct European subpopulations. Quantification of frequency of *Cetrelia* occurrence compared to an index of habitat quality revealed that all species have a strong affinity to high quality habitats, reflecting broader connection between lichen species richness and disturbance found in numerous previous studies. The trends detected in *Cetrelia* are unlikely restricted to this genus and we suggest that large-scale detailed quantitative studies in local-scale occurrence over time are needed to address significant gaps in knowledge to advance lichen conservation.

Lichens have long been characterized as filamentous fungi that enter into obligately symbiotic relationships with one or more photosynthetic algae and or cyanobacteria (Ahmadjian 1993; Allen et al. 2021; Brodo et al. 2001; Hale 1967). Increasingly, though, lichens are conceptualized as complex ecosystems that include a broad range of bacteria, fungi and other microbes beyond the two dominant symbionts that have traditionally been recognized (Allen & Lendemer 2022; Hawksworth & Grube 2020; Spribille et al. 2016). The fungi that form lichens are also highly diverse, with approximately 20,000 described and thousands of species reported from nearly every continent, including more than 5,000 from North America north of Mexico (Esslinger 2021). Indeed, lichens are nearly ubiquitous mutualistic symbioses that occur on a multitude of living and non-living substrates (Brodo 1973; Pharo & Beattie 2002; Zúñiga et al. 2017) across elevational and climatic gradients (Bässler et al. 2016; Gauslaa et al. 2007).

As is the case for lichens generally, foliose macrolichens, characterized by their relatively large, leaf-like thalli, are diverse and abundant across numerous terrestrial ecosystems (Armstrong & Bradwell 2011; Dey 1978; Hill 1984). Foliose macrolichens have also been intensively studied compared to other subsets of lichen diversity, such as crustose microlichens, presumably in part because of the conspicuous appearance, ease of collection, and relative abundance (Lawrey 1984; McCune et al. 1997). The family Parmeliaceae includes the largest diversity of foliose macrolichens, with over 71 genera

and over 2,000 described species belonging to such iconic genera as *Hypotrachyna, Usnea* and *Xanthoparmelia* (Gómez-Serranillos et al. 2014; Thell et al. 2012).

The morphological diversity of this family has challenged efforts to formally recognize genera and species using integrated approaches that reconcile phenotypic characters and the results of molecular phylogenetic analysis (Grewe et al. 2020; Nelsen et al. 2011). One of the most well studied but difficult groups is the "parmelioid" group of Parmeliaceae (Crespo et al. 2010; Obermayer & Randlane 2012). This group has generally been characterized by its foliose to subfruticose thallus with marginal apothecia and pycnidia (Culberson & Culberson 1968). The many genera and evolutionary lineages that make up the "parmelioid" group have been, and continue to be, delimitated in a variety of ways (Kuznetsova et al. 2021). Molecular data have helped to delimit monophyletic genera with this group, but the process is incomplete and some genera remain polyphyletic depending on the taxonomy employed (Crespo et al. 2010; Nelsen et al. 2011; Thell et al. 2012). Within the "parmelioid" group, the genus *Cetrelia* W.L.Culb. & C.F.Culb. stands out for having been repeatedly shown to be monophyletic in molecular phylogenetic studies, reinforcing its original delimitation with phenotypic characters more than half a century ago (Mark et al. 2019).

Cetrelia was described by Culberson & Culberson (1968) who transferred a selection of species from *Cetraria* Ach. and *Parmelia* Ach. into a single genus. It was distinguished using a combination of secondary chemistry and morphological characters (e.g., broad lobes, pseudocyphellae on the upper surface, absence of marginal cilia, black lower surface, upper cortex with atranorin, marginal pycnidia, and various orcinol depsides in the medulla; Culberson & Culberson 1978). There are presently 18 accepted species of *Cetrelia*, including many with disjunct distribution patterns and a center of diversity in east Asia (Culberson 1972; Culberson & Culberson 1968, 1978). The species of *Cetrelia* delimited by Culberson & Culberson (1968, 1978) based on chemical and morphological characters have been largely supported by subsequent phylogenetic analysis of molecular data (Mark et al. 2019). While Mark et al. (2019) published a robust, taxonomically well sampled global phylogeny for *Cetrelia*, geographic sampling for that study focused on Europe and East Asia and lacked sampling from eastern North America, a region with disjunct subpopulations of at least three species where at least two species are common and widespread (Brodo et al. 2001; Lendemer et al. 2013). The present study aims to place the eastern North American subpopulations into the broader global *Cetrelia* phylogeny and examine their current taxonomic identities that have been based on morphological and chemical characteristics.

MATERIALS AND METHODS

Field and herbarium study. This study is based on specimens deposited in the herbarium of the New York Botanical Garden (NY), including many collected by the authors. Targeted fieldwork was conducted by the first author in the Appalachian Mountains of North Carolina, Virginia, and West Virginia in 2022–2023, with all specimens deposited in NY. Full georeferenced specimen data are available through the C.V. Starr Virtual Herbarium (https://sweetgum.nybg.org/science/vh/). Morphology of 1045 existing herbarium and newly field collected specimens were examined with dissecting microscopes to detect the distinguishing morphological characters of each

species following Obermayer & Mayrhofer (2007). Standard spot tests were used to detect the presence of olivetoric acid in *C. olivetorum* (i.e., K–, C+ red). Secondary chemistry was further studied with thin layer chromatography (TLC) following the methods of Culberson & Kristinsson (1970) using solvent C as modified by Lendemer (2011).

Specimens identified as *Cetrelia cetrarioides* and *C. monachorum* based on morphology and initial TLC were subjected to additional TLC following Obermayer & Mayrhofer (2007) using full size 20 × 20 cm aluminum backed TLC plates and run to a height of 15 cm. Both species produce similar substances that are categorized into two series described as the imbricaric (*C. monachroum*) and perlatolic acid (*C. cetrarioides*) "syndromes". The imbricaric syndrome is defined by the presence of two close spots (anziaic acid and 4-*O*-methyllimbricaric acid) and a small fatty acid like spot. After running in the solvent and drying the plate, imbricaric acid when treated with water appears as a homogeneous grey with a white ring on the outside. In contrast, perlatolic acid appears as a homogenous white spot without an outer ring. These substances were detected in this way for this study.

Molecular data generation and assembly. We generated 80 new sequences of the ITS barcoding region from a selection of *Cetrelia* specimens from throughout Eastern North America (See **Supplementary Table S1 (Howland & Lendemer Suppl Table S1.docx)** for GenBank Accession number and voucher metadata). This included 33 sequences of *C. monachorum*, 36 sequences of *C. chicitae*, and 11 of *C. olivetorum* all identified using the methods outlined above. Thallus fragments used for DNA extraction were those subjected to an acetone wash as part of TLC as outlined above. DNA extraction, and PCR amplification of the ITS region was performed following Muscavitch et al. (2017). Sanger sequencing of PCR products was conducted at Macrogen U.S.A. Sequencher 5.2.4 (GeneCodes, Ann Arbor, Michigan) was used to for sequence assembly and error checking. The identity of all newly generated sequences as representing *Cetrelia* was confirmed with NCBI BLASTn queries using max query cover and percent identity.

Taxon sampling and alignment construction. Taxon sampling followed Mark et al. (2019) who previously studied species delimitation in *Cetrelia* and inferred a phylogeny from extra-North American specimens across the genus. The sequences used by Mark et al. (2019) were downloaded from NCBI and compiled into an alignment in Mesquite v3.80 (Maddison & Maddison 2023) for each individual locus: ITS, IGS, MCM7, and RPB1. Newly generated ITS sequences were then added to the ITS alignment. All sequences from a given sample were then renamed to create a uniform specimen code system to facilitate comparison among single locus phylogenies and downstream concatenation (see Supplementary Table S1 (Howland & Lendemer Suppl Table S1.docx)). Sequences for each locus were aligned using the pairwise alignment in Mesquite, then manually checked and adjusted. Ambiguously aligned regions and terminal portions of the alignment were then defined as an exclusion set. Ambiguously aligned regions and ends were manually deleted, gaps at the terminal ends were then

converted to missing, and uncertainties/polymorphisms were converted to missing. Alignments were then exported in PHYLIP format.

Single-locus phylogenies were inferred from each individual alignment using maximum likelihood in both IQTree (Minh et al. 2020) and RAxML (Stamatakis 2006). In IQTree appropriate substitution models for each single-locus dataset were selected based on the minimum Bayesian Information Criterion (BIC) score using ModelFinder Plus (Kalyaanamoorthy et al. 2017). The substitution models selected were TNe+G4, TIM2+F+G4, K2P+I, and TNe+G4 for the ITS, IGS, MCM7 and RPB1, respectively. Support was estimated using 1000 "Ultrafast bootstrap" approximations (Hoang et al. 2018) and 1000 replicates of SH-like approximate likelihood ratio test (Guindon et al. 2010). In RAxML 8.2.10 a maximum likely topology search and bootstrapping analysis with 500 replicates implementing the most complex nucleotide substitution model available (GTRGAMMA) were performed.

The results of single each locus analyses were then visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/) to search for conflicts as defined by Mason-Gamer & Kellogg (1996). No conflict was observed, and the single locus alignments were concatenated in Mesquite. The same analysis outlined above, implementing the previously selected models, were then performed on the concatenated alignment with partitions defined for each of the four loci. The results of the analysis of the concatenated were also visualized in Fig Tree. The datasets used in this study are included here as **Supplementary Tables S1 (Howland & Lendemer Suppl Table S1.docx)** and **S2**.

Habitat quality assessment. Given that *Cetrelia cetrarioides* appears to be rare, and potentially in decline in eastern North America (see results section), we examined the broader connection between habitat quality and *Cetrelia* species in the region. For this we used a subset of 208 sampling locations within the southern Appalachian Mountains where total lichen inventories had been conducted (i.e., the "Southern Appalachian dataset" from Koch et al. 2023) and where habitat quality had been scored in the field using a modified ten-part score from Parkes et al. (2003) following Tripp et al. (2019). We created a presence-absence matrix of *Cetrelia* occurrences across all 208 locations crossreferenced by the scored habitat quality. Histograms were then built using number of occurrences of each species per habitat quality score binned in tens from 0–100.

RESULTS

Morphological identification of studied material. Specimens were morphologically identified following Obermayer & Mayrhofer (2007). Size, shape and frequency of pseudocyphellae on the upper surface were considered one of the most reliable ways to distinguish between species, and these can be broadly characterized into three main types: type-1 in *Cetrelia chicitae* (large, smooth and flat), type-2 in *C. monachorum* and *C. cetrarioides* (heterogenous in size, raised to convex, often cracked) and type-3 in *C. olivetorum* (very small, flat to concave) (Obermayer & Mayrhofer 2007). Size of soredia and shape of marginal soralia were secondary characteristics used

to support species determinations and were characterized into two main types: type-1 in *C. chicitae* and *C. monachorum* (coarse soredia), type-2 in *C. olivetorum* and *C. cetrarioides* (fine soredia). Within type-1, coarse soredia, formed in large, contorted soralia along with large, flat pseudocyphellae on the upper surface were used to distinguish *C. chicitae* (see Obermayer & Mayrhofer 2007: fig. 21). Specimens with coarse soredia and soralia that had a more regular, level margin, combined with raised pseudocyphellae on the upper surface were recognized as *C. monachorum* (see Obermayer & Mayrhofer 2007: fig. 22). Specimens with comparatively finer soredia were further distinguished by their upper surface pseudocyphellae; small and concave in *C. olivetorum* (see Obermayer & Mayrhofer 2007: fig. 23) versus large and flat for *C. cetrarioides* (see Obermayer & Mayrhofer 2007: fig. 24).

Obermayer & Mayrhofer (2007) also used the presence, abundance and morphology of pseudocyphellae on the lower surface of the thallus to distinguish the species with type-2 pseudocyphellae on the upper surface. Specifically, lower surface pseudocyphellae were considered typically present and abundant in *Cetrelia cetrarioides*, and to have a homogenous morphology of being larger in size, consistently circular in shape, and well-defined, clearly-delimited edges (see Obermayer & Mayrhofer 2007: fig. 19). Whereas in *C. monachorum* the lower surface pseudocyphellae were considered typically absent to infrequent, and to have a heterogeneous morphology of being smaller in size, varying in shape from circular to elongate and even branched with multiple individual pseudocyphellae apparently becoming interconnected, and poorly-defined and weakly-delimited edges (see Obermayer & Mayrhofer 2007, fig. 20). While all *Cetrelia* can produce pseudocyphellae on the lower surface, we confirmed the previously published characters outlined above and that *C. cetrarioides* can readily be separated from *C. monachorum* by the abundance and morphology of these structures.

Based on the morphological scheme outlined above, we assigned 456 specimens to *C. chicitae* (36 sequenced), seven to *C. cetrarioides* (none sequenced), 156 to *C. monachorum* (29 sequenced), and 426 to *C. olivetorum* (nine sequenced). These morphology-based identifications were then used as the basis for subsequent chemical studies and molecular phylogenetic analyses.

Chemical identification of studied material. All specimens identified morphologically above were also then subjected to chemical analyses with standard spot test reagents and, except for some specimens of *Cetrelia olivetorum* which were identified based on the strong C+ red reaction of the medulla, Thin Layer Chromatography (TLC) to confirm the presence of species specific secondary compounds as outlined by Obermayer & Mayrhofer (2007). The four species involved in this study produce atranorin in the upper cortex followed by unique suites of chemical substances in the medulla: perlatolic acid as the major substance with anziaic and imbricaric acids as minor substances in *C. cetrarioides*; α-collatolic and alectoronic acid as major substances together with 4-*O*-methylphysodic and physodic acid as minor accessories in *C. chicitae*; imbricaric acid as the major substance with 4-*O*-demethylimbricaric, anziaic and perlatolic acids as minor substances in *C.*

monachorum; and olivetoric acid as the major substance with anziaic acid as a minor accessory in *C. olivetorum*. Our TLC analyses of the specimens identified based on morphology consistently detected the suites of compounds that corresponded to those determinations, affirming the morphology-based identifications (see Obermayer & Mayrhofer 2007: figs. 1–3). In particular our TLC analyses produced results congruent with previous studies for the separation of *C. cetrarioides* and *C. monachorum* (see Obermayer & Mayrhofer 2007: fig. 1). This was contrary to the results of Lendemer et al. (2013) that led to the combined treatment of those taxa under *C. cetrarioides* as was implied by Brodo et al. (2001: 220). The identification of our studied material based on morphology and confirmed with chemistry was then used as the basis for naming newly generated molecular sequences, and for assigning names to occurrences for mapping and other analyses involving occurrence data.

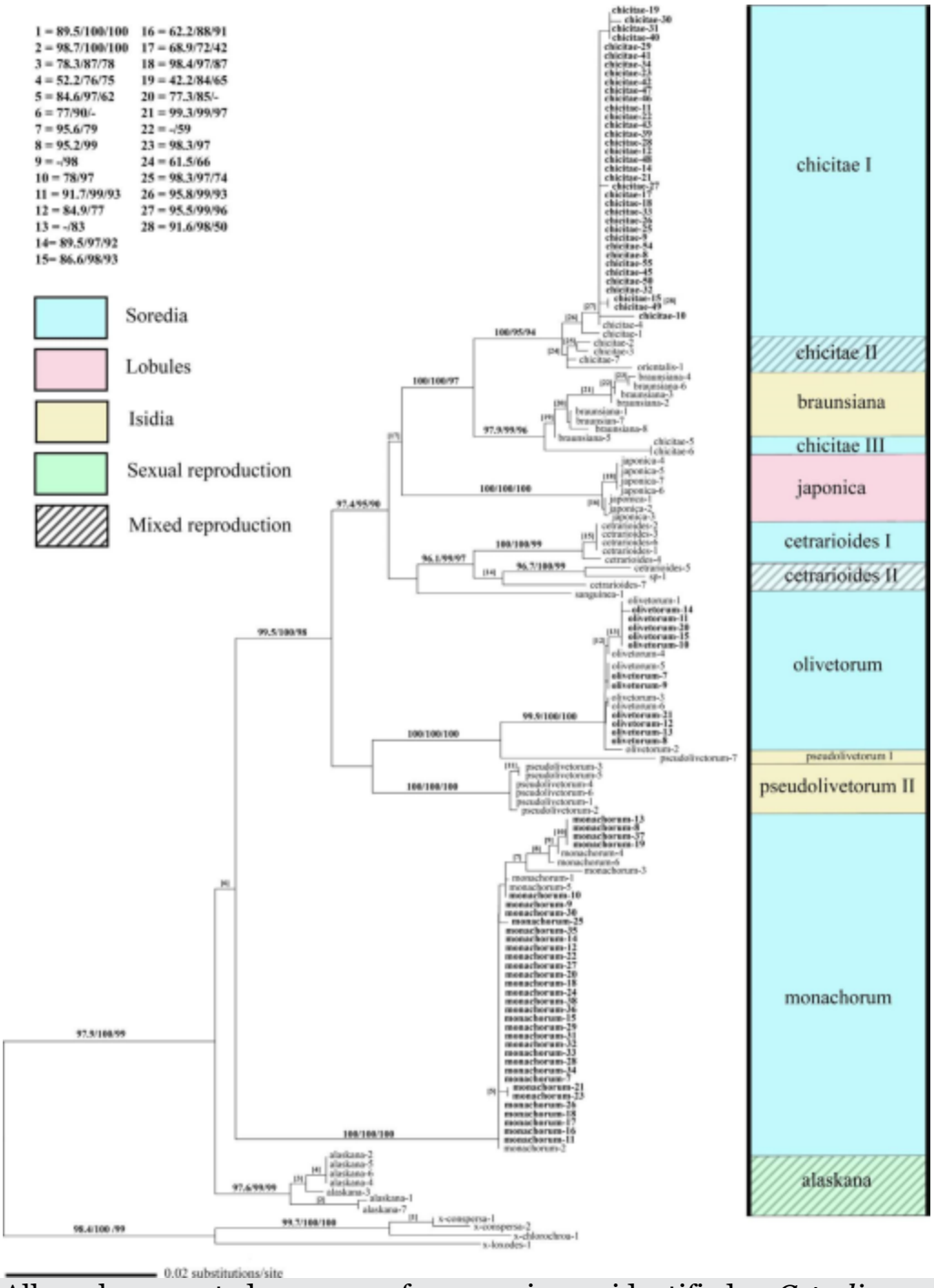
Attempted recollection of C. cetrarioides. We were unable to generate molecular data for *Cetrelia cetrarioides* from any of the existing vouchers that we examined and confirmed with the methods outlined above. As such we conducted targeted fieldwork for *C. cetrarioides* at two sites where confirmed specimens of the species had been found previously: Panther Knob, Pendelton County, West Virginia (reported by Culberson & Culberson 1978, digitial images of voucher seen, but not physically reviewed for this study) and Green Knob, Haywood County, North Carolina (located in 2019 during inventory work by JL and colleagues; vouchers reviewed for this study). At these two sites we collected replicates of what appeared to be morphologically distinct *Cetrelia* that either corresponded to the suite of characters for *C. cetrarioides*, or appeared otherwise aberrant and were not easily classified as any of the other three taxa in the field. In addition to targeting morphologically distinct material, we specifically searched and sampled across all available substrates at each site (i.e., all genera and species of woody plants, as well as rocks). This led to the collection of three specimens (*Howland 209b, 2010, and 211*, all NY) that were assigned to *C. cetrarioides* based on morphology of the soralia and soredia in the field. However, all three of these subsequently were determined to be *C. monachorum* based on morphology of the pseudocyphellae and chemistry.

Phylogenetic placement of morphologically and chemically determined material. The results of our molecular phylogenetic analysis were congruent with those of Mark et al. (2019) (**Fig. 1**, herein). As in previous studies, *Cetrelia* was recovered as monophyletic with strong support (IQTree/RAxML: 97.9/100/99). The following species were recovered as monophyletic with strong support as was the case in Mark et al. (2019): *C. alaskana* (97.6/99/99), *C. japonica* (100/100/100), *C. olivetorum* (99.9/100/100) *C. monachorum* (100/100/100), *C. cetrarioides* (96.1/99/97), and *C. braunsiana* (97.9/99/96). As was also the case with Mark et al. (2019) *C. chicitae* and *C. pseudolivetorum*, were found to be non-monophyletic. For *C. pseudolivetorum* all but one sequence was recovered in a strongly supported clade (100/100/100) while one sequence was recovered as sister to the otherwise monophyletic *C. olivetorum*. Sequences of *C. chicitae* were recovered in three clades, two of these were strongly supported as sister to each other (100/95/94), while the third clade was recovered as sister to the otherwise monophyletic *C. braunsiana* also with strong support (97.9/99/96). These two sets of sister clades (i.e., *C. chicitae* 1 + 2

and *C. braunsiana+C. chicitae* 3) were collectively recovered as a strongly supported monophyletic group (100/100/97).

Figure 1.

Phylogeny of *Cetrelia* based on sampling of Mark et al. (2019) and expanded for this study, displayed as the most likely tree resulting from RAxML. Support values are shown as: "Ultrafast bootstrap" IQTree/Approximate Likelihood Ratio Test IQTree/Maximum Likelihood bootstraps RAxML. Color coded boxes in right panel denote reproductive mode for all major supported clades with nomenclature following Mark et al. (2019), terminals represented by new sequences are bolded. Online pdf in color.



All newly generated sequences from specimens identified as *Cetrelia monachorum* were resolved in the same clade with the reference sequences of *C. monachorum* from different regions of Russia and Northern Japan. The newly generated sequences from specimens identified as *C. olivetorum* were resolved in the same clade with the reference sequences of *C. olivetorum* from Estonia, different regions of Russia, and Northern Japan. All our new sequences from specimens identified as *C. chicitae* were recovered in the clade defined as "chicitae 1" by Mark et al. (2019). This suggests that the eastern North American subpopulation of *C. chicitae* may belong entirely to that lineage, rather than to a mixture of the three lineages known from Asia.

Geographic distribution in eastern North America. As a whole, the species of *Cetrelia* in eastern North America have a range that is distributed across the Appalachian Mountains, spreading west in the Great Lakes Region of Canada and the U.S.A. (**Fig. 2A**). This "classic" Appalachian-Great Lakes distrubtion is associated with many other eastern North American lichen species, such as *Pseudevernia consocians* (Vain.) Hale & W.L. Culb. and *Usnocetraria oakesiana* (Tuck.) M.J. Lai & J.C. Wei (Brodo et al. 2001; Hale 1956). Within this broadly defined genus-level distribution, two species (*C. chicitae* and *C. olivetorum*) are widely distributed (**Fig. 2B**, **2D**) and found across a nearly complete elevational gradient, although they are primarily distributed at low and middle elevations (**Fig. 3**). Conversely, *C. cetrarioides* and *C. monachorum* occur only within the Appalachian Mountains and are absent from the Great Lakes Region (**Fig. 2C**, **2E**), and both species are restricted to higher elevations (**Fig. 3**) where they occur in northern hardwood or spruce-fir forests.

Figure 2.

Geographic distributions of *Cetrelia* in eastern North America based on specimens examined for this study. **A.** All *Cetrelia* species combined illustrating Appalachian-Great Lakes distribution pattern. **B.** *C. chicitae*. **C.** *C. monachorum*. **D.** *C. olivetorum*. **E.** *C. cetrarioides*.

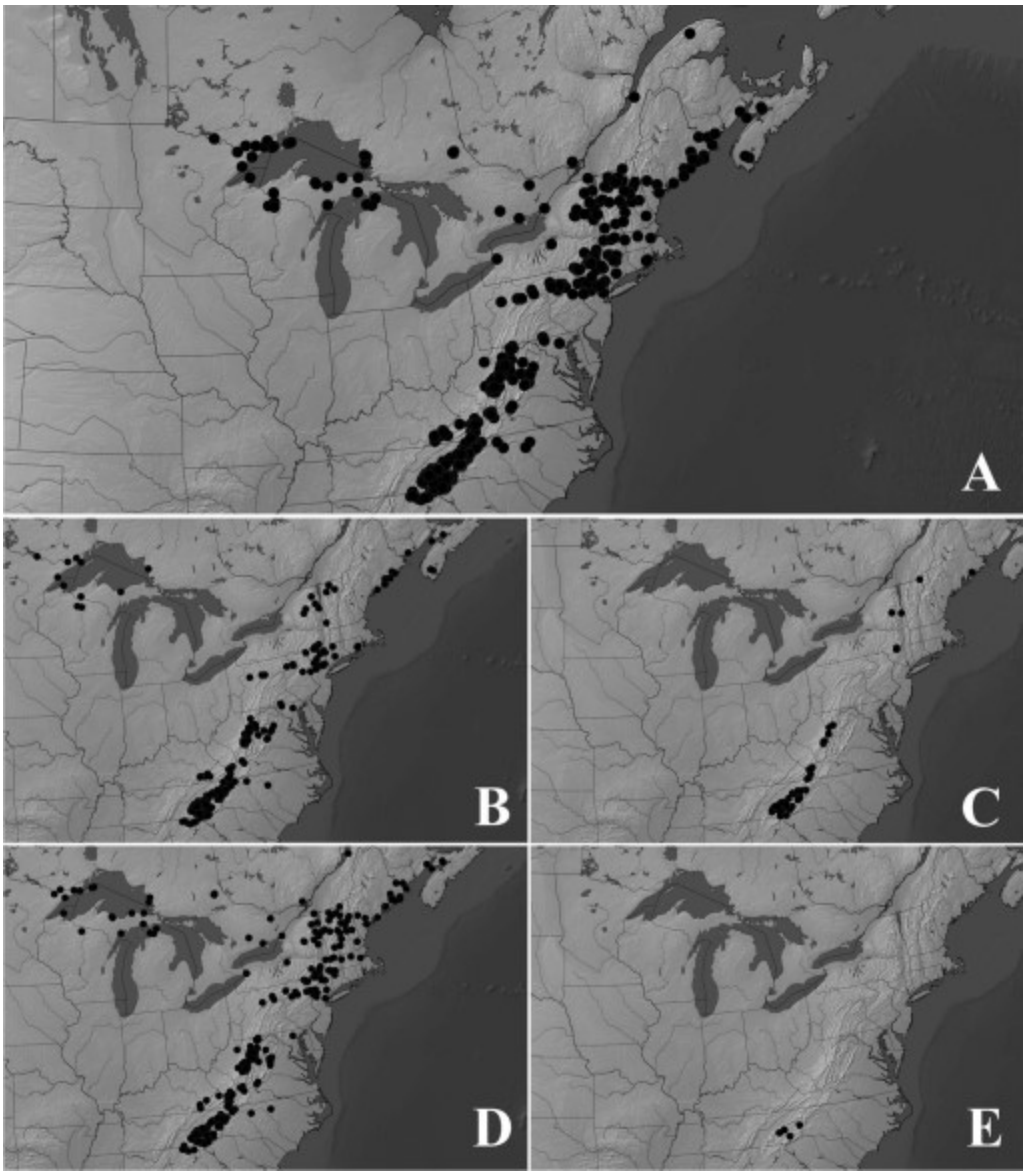
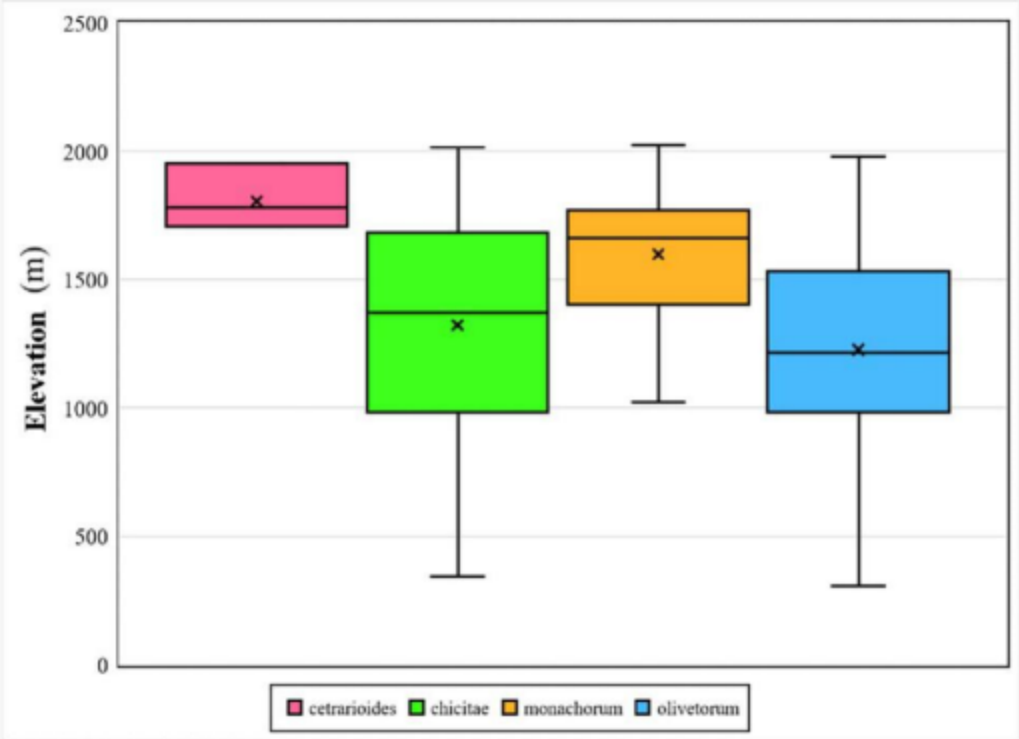


Figure 3.

Boxplot of elevations for eastern North American *Cetrelia* voucher specimens examined for this study. Online pdf in color.

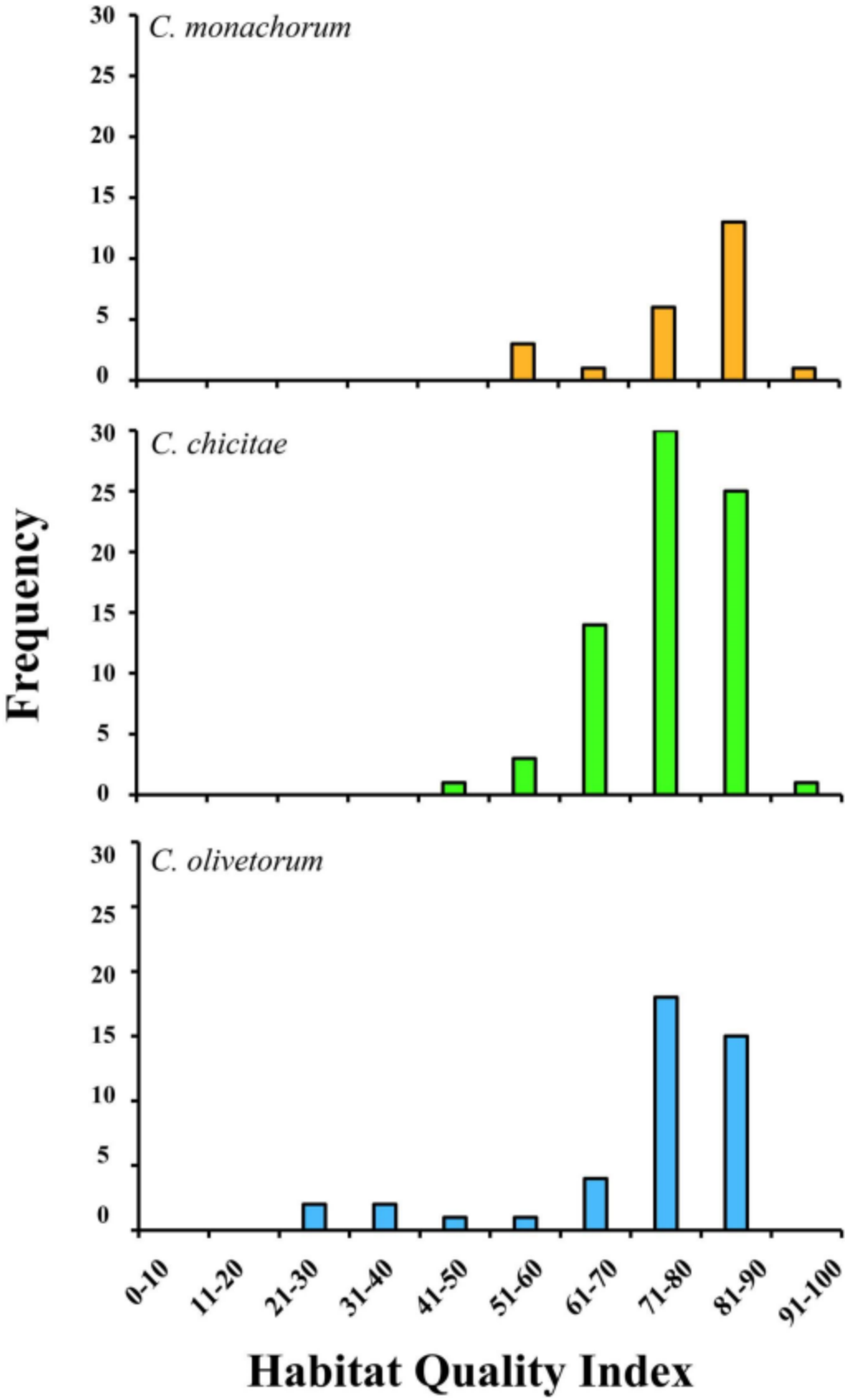


Contrasts between European and eastern North American occurrence. The subpopulations of *Cetrelia* species in eastern North America have contrasting frequency and distrubtion patterns relative to their disjunct European subpopulations. Notably, *C. chicitae* is common and widespread in eastern North America whereas in Europe it is extremely rare, and found mainly by targeted efforts (Farkas et al. 2021; Kukwa & Motiejūnaitė 2010; Obermayer & Mayrhofer 2007). Likewise, *C. monachorum* is restricted to high elevation sites in eastern North Amerrica, but typically cooccurs with *C. chicitae* in equal or less abundance (Howland & Lendemer, pers. obsv.) as is suggested by the differences in the number of vouchers examined for this study. In contrast, *C. monachorum* has been reported to be the most common species of *Cetrelia* in central Europe, where *C. chicitae* is otherwise rare (Obermayer & Mayrhofer 2007).

Habitat quality assessment. Quantifying the frequency of *Cetrelia* occurrence compared to habitat quality revealed that all species had a strong affinity to high quality habitats (**Fig. 4**). The majority of collections for all three species were found at locations with habitat quality scores of 70 or above. No specimens of *C. monachorum* or *C. chicitae* were collected at sites with habitat quality scores below 50.

Figure 4.

Histograms of eastern North American *Cetrelia* species occurance frequencies relative to site habitat quality based on data from 208 sampling locations within the southern Appalachian Mountains (see Koch et al. 2023). Online pdf in color.


C. monachorum
C. chicitae
C. olivetorum
Frequency
30
25
20
15
10
5
0
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
Habitat Quality Index

DISCUSSION

Our molecular phylogeny supports the presence of three morphologicaly and chemically distinct species of *Cetrelia* in eastern North America (ENA hereafter): *C. chicitae, C. monachorum* and *C. olivetorum*. While we examined specimens that were morphologically and chemically assigned to *C. cetrarioides*, which suggests this species is also present in ENA, we were unable to generate sequences from these collections to confirm their phylogenetic placement within the otherwise monophyletic *C. cetrarioides* recovered by Mark et al. (2019). Our targeted efforts to relocate this species at two sites where confirmed occurrences had been found previously were unsuccessful and led only to the collection of individuals of *C. monachorum* that had unsusally large, conspicuous, regularly delimited soralia with fine soredia. Hence we were unable to unambiguously determine whether *C. cetrarioides* is actually present in ENA.

It is noteworthy that we were unable to relocate this species at the two targeted sites, especially given that *Cetrelia cetrarioides* was collected at one of them in 2019 by one of the authors (JL). This suggests that *C. cetrarioides* is exceptionally rare at the sites in ENA where it occurs, and that if present at these sites, it is in low abundance below the level of routine detection by multiple individuals searching the same spatially constrained locality. It is also possible that the species has undergone past, and potentially ongoing, declines and may even be extirpated not just from sites of historical occurrence but also sites where it has been detected in the very recent past. In ENA, *C. cetrarioides* appears to be restrictedd to high elevation sites and spruce-fir ecosystems that are extremely spatially restricted and critically imperiled (Harris et al. 2022; Nicholas et al. 1992; White et al. 2012). The preferred substrate for *C. cetraroioides* in ENA also appears to be *Abies*, as five of the eight specimens we examined (**Supplementary Table S2 (Howland & Lendemer Suppl Table S2.docx)**) were found growing on *A. fraseri* in the southern Appalachian Mountains where that species is narrowly endemic and forms the cornerstone of spruce-fir ecosystems (Ellison et al. 2005). While Southern Appalachian spruce-fir forests have been greatly reducted in extent due to past land-use and resource extraction (Pyle & Schafale 1988; Silver 2003; White & Cogbill 1992), *A. fraseri* specifically has been significantly negatively impacted by historical pollution, coupled with subsequent infestation and dieback of the mature individuals most likely to host *C. cetrarioides*, due to the non-native pest *Adelges piceae* (McManamay et al. 2011). Further intensive targeted search efforts for *C. cetrarioides* at all of the known ENA sites where it has been previously located are urgently needed to determine its conservation status. Molecular data from this subpopulation are also required to confirm its conspecificty with other subpopulations of *C. cetrarioides* globally.

Quantifying the frequency of *Cetrelia* occurrence compared to habitat quality revealed that all species had a strong affinity to high quality habitats (**Fig. 4**). While the decline of the *C. cetrarioides* is clear, the historic and future stressors of *Cetraria* habitats and substrates have likely caused similar local declines of all *Cetrelia* species throughout ENA. High elevation spruce-fir ecosystems are also the primary habitat for *C. monachorum* (**Fig. 3**) and repeated surveys at historical sites for this species would likely show similar decline in abundance and local extripation as noted for *C.*

cetrarioides. The large spatial distribution (**Fig. 2**) and local abundance of *C. chictae* and *C. olivetorum* has likely also masked similar declines due to the same ecological stressors and land use changes, as has been inferred for habitat loss related declines in common species in coastal eastern North America (Allen & Lendemer 2016).

Beyond *Cetrelia*, long term studies of other lichens asssociated with high quality habitats, and mature or old-growth forests have revealed similar significant declines in population size driven by major disturbances like climate change and altered forest dynamics (Esseen et al. 2023). This almost certaintly reflects the broader connection between lichen species richness and forms of disturbance included in habitat quality (e.g., pollution, fragmentation and stand maturity) that has been found in numerous previous studies (Allen & Lendemer 2016; Ardelean et al. 2015; Benítez et al. 2018; Chuquimarca et al. 2019). While not all lichen species respond to disturbance and declines in habitat quality in uniform ways (Allen & Lendemer 2016), clearly *Cetrelia* are associated with higher quality habitats and could serve as reliable, conspicuous indicators of sites that host lichen species richness, and hence should be targeted for broader lichen inventories and conservation assessment.

CONCLUSIONS

Here we used a large-scale field, herbarium and laboratory study to examine the distributions and identities of a group of highly conspicuous and charismatic macrolichens in eastern North America (ENA). Our study confirms the presence of three species in the region with molecular data, and suggests that a fourth species (*Cetrelia cetrarioides*) also occurs but this requires additional study with molecular methods. All three ENA subpopulations of the species studied with molecular data, were found to be monophyletic and belong to an existing strongly supported previously sampled lineage from another region of the Northern Hemisphere, even in cases where the global population of the species as delimited was not monophyletic (e.g., all ENA *C. chicitae* belong to "Chicitae-1" of Mark et al. 2019). While the taxonomic and evolutionary affinities of ENA *Cetrelia* fit well within a global context, we found differences in frequency and distribution among ENA species and between disjunct European-ENA subspopulations of the same species. We also found that ENA *Cetrelia* are associated with high quality habitats, and that the one species for which we could not generate molecular data may be in decline and extirpated from at least a subset of historical locations.

Biodiversity conservation in the Appalachian-Great Lakes regions has generally targeted rare and narrowly distributed species, or abundant taxa that have suffered visibly striking declines, such as *Castanea dentata* and *Tsuga canadensis* (Elliott & Swank 2008; Krapfl et al. 2011; Ellison et al. 2018). Lichen conservation follows this same pattern in the region (Allen et al. 2019; Allen & Lendemer 2015). This focus on extreme rarity, range size and visibly evident declines has the potential to overlook broader declines in that individual taxa or communities that are more frequent and widespread, but nonetheless also impacted by the same threat categories. *Cetrelia* is an excellent example of a group of conspicuous macrolichens that are perceived as common and abundant, at least in ENA. Nonetheless they are are evidently associated with high quality habitats and have likely already undergone significant declines that have gone

unnoticed or undetected either because the rarity of the species was not fully appreciated (e.g., *C. cetrarioides*) or because current localized frequent or abundant occurrence has masked overall extirpations and declines. Given that lichens are broadly recognized as bioindicators it is unlikely that the trends detected in *Cetrelia* are restricted to this single genus of lichens. Large-scale detailed quantitative studies of demographic trends and changes in local-scale occurrence over time are urgently needed to address this significant gap in knowledge and could be transformative for lichen conservation globally.

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Supplementary documents online:

Supplementary Table S1 (Howland & Lendemer Suppl Table S1.docx). GenBank accession number, voucher metadata, and uniform specimen code system for all sequenced specimens.

Supplementary Table S2 (Howland & Lendemer Suppl Table S2.docx). Phylogenetic tree sequence alignment.

Supplementary Table S3 (Howland & Lendemer Suppl Table S3.docx). Specimens determined as *C. cetrarioides* with collection metadata including coordinates and substrate.

Supplementary Table S4 (Howland & Lendemer Suppl Table S4.docx). Habitat quality scores of *Cetrelia* occurrences from southern Appalachian lichen occurrence data set (McCain et al. in prep).

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