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Recent fieldwork and fungarium studies double known diversity of *Chlorosplenium* and improve understanding of species distributions

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ABSTRACT

Chlorosplenium is a small genus comprising five species of inoperculate discomycetes in the order Helotiales (Leotiomycetes) often recognizable by their bright yellowish-green colors and gregarious growth on wood. In this study, we describe five new species—*C. aotearoa, C. australiense, C. cusucoense, C. epimorsicum*, and *C. hawaiiense*—based on a combination of recent fieldwork and examination of previously collected fungarium specimens. We use an integrative taxonomic approach to support the distinction of new species, incorporating morphology and DNA sequence data with biogeography. Macro- and micromorphological features of apothecia for all species and culture characteristics for four of the five new species are documented. A multilocus phylogeny based on nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2, partial large subunit nuc ribosomal DNA (28S nuc rDNA), and A–B regions of the largest subunit of RNA polymerase II (*RPB1*) gene is presented. Additionally, we report *Chlorosplenium chlora* from Europe for the first time and expand our knowledge of the diversity and distributions of species in this genus in America, Australia, and New Zealand.

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INTRODUCTION

Helotiales (Ascomycota, Leotiomycetes) is an ecologically diverse order of primarily apothecial, nonlichenized ascomycetes with an estimated diversity of over 80 000 species, only 5–7% of which are described (Hawksworth 2001; Johnston et al. 2019). Recent reviews (Quandt and Haelewaters 2021) and large-scale phylogenetic studies (Haelewaters et al. 2021a; Johnston et al. 2019) of the order and broader class Leotiomycetes have highlighted deficiencies in our understanding of both the higher-level ordinal relationships in the class and the uncertain familial placement of up to 27% of genera in Helotiales (Jaklitsch et al. 2016). One of these genera, *Chlorosplenium*, was recently placed in a monotypic family, Chlorospleniaceae (Ekanayaka et al. 2019), in Helotiales, within a larger group that includes Loramycetaceae, Mollisiaceae, Vibrisseaceae, and the *Strossmayeria* lineage (Haelewaters et al. 2021a; Johnston et al. 2019; Quijada et al. 2022).

Chlorosplenium species are characterized macroscopically by small (<4 mm), sessile to subsessile, yellowish-green, discoid to cupulate apothecia that grow on dead wood (Dixon 1974; Zheng and Zhuang 2021). Microscopically, they have ±oblong ascospores, a medullary excipulum of lighter-colored textura intricata, an ectal excipulum of darker ±t. angularis-globulosa potentially with interspersed t. prismatica, and an exterior covering of cylindrical hyphae (Dixon 1974).

Throughout the 20th century, the genus *Chlorosplenium* grew speciose as mycologists added many greenish-blue species now considered to be in the genus *Chlorociboria*. Dixon (1974) developed the modern understanding of the genus, excluding all greenish-blue species (among others). This left only the type, *Chlorosplenium chlora*, and *Chlorosplenium hypochlorum* (syn. *Cenangium hypochlorum*), which Dixon (1974) combined into the genus as the orthographic variant '*hypochlora*' (Berk. & M.A. Curtis ex W. Phillips) J.R. Dixon. Dixon separated the species by the larger ascospore and ascus sizes in *C. hypochlorum*, and whether the medullary and hymenial tissues could be easily separated

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from the receptacle in dried material (yes in *C. hypochlorum*, no in *C. chlora*). Although only four specimens of *C. hypochlorum* were examined by Dixon (1974), these were all from tropical America, whereas the vast majority of *C. chlora* collections were from temperate regions, particularly the United States. Dixon did examine *Chlorosplenium* collections from Indonesia and Jamaica he identified as *C. chlora*, indicating a tropical distribution for this species, potentially overlapping with that of *C. hypochlorum* in tropical America. Although Dixon specifically noted that *C. chlora* appears absent from Europe, he considered it quite widespread: it has been reported from many other regions of the world, including temperate and tropical Asia, Australia, and Jamaica (Dixon 1974; Zheng and Zhuang 2021).

After Dixon's (1974) study, Müller (1977) combined *C. cenangium* (syn. *Dermatea cenangium*) into the genus and *C. fusisporum* (Liou and Chen 1977) and *C. hyperici-maculati* (Svrček 1992) were described as new. *Chlorosplenium cenangium* is only known from alpine environments in Europe where it grows on the wood of *Rhododendron ferrugineum*. Its larger, septate ascospores, short stipe, and downy/hairy receptacle differentiate it from *C. chlora* and *C. hypochlorum*. Most recently, Zheng and Zhuang (2021) excluded *C. fusisporum* and *C. hyperici-maculati* from *Chlorosplenium* based on morphological features that suggest an affinity with the genus *Chlorociboria*, such as deep green coloration and short hairs encrusted with green granules. They also described *C. sinicum* and *C. sinochlorum* (as the orthographic variant '*sinochlora*' H.D. Zheng & W.Y. Zhuang in Life 11:1167 (2021)) from China. *Chlorosplenium sinicum* is known from many collections and has a receptacle with long hairs at the margin, whereas *C. sinochlorum* was described from a single collection based on differing DNA sequence data in addition to slightly narrower asci and slightly wider ascospores compared with *C. chlora*, although the size ranges overlap. Zheng and Zhuang (2021) presented a key to species in the genus but noted that, with overlapping morphological features, molecular data may be needed to definitively delineate species. A phylogeny of *Chlorosplenium* using the internal transcribed spacer (ITS; consisting of ITS1, 5.8S, and ITS2) region of the nuclear ribosomal DNA (nuc rDNA) led to well-supported species-level clades in their study, but they also sequenced the partial large subunit (28S) nuc rDNA and A–B regions of the largest subunit of RNA polymerase II (*RPB1*) gene to confirm their results because variation was present within the ITS of *C. sinicum*. They stated that by incorporating DNA sequence data with morphological and ecological data, more "hidden" species may be discovered, potentially from collections previously identified as the putatively widespread *C. chlora*, such as many of their collections from China.

In ongoing fungal surveys in the Americas, Australia, and New Zealand, we collected *Chlorosplenium* species that could not be assigned to known taxa (Haelewaters et al. 2021b; Johnston 2020). Therefore, we undertook a revision of *Chlorosplenium* following an integrative taxonomy approach (Aime et al. 2021).

MATERIALS AND METHODS

Collections and morphology.—New specimens were primarily collected in Honduras (summers of 2019, 2022, and 2023), Panama (summer 2022), the United States (2014 to 2023), and Australia and New Zealand (1994 to 2023). In addition, specimens and cultures were requested on loan from the following fungaria and culture repositories: University of Arizona (ARIZ), Bridgewater College (BDWR), Cornell University (CUP), Harvard University (FH), International Collection of Microorganisms from Plants (ICMP), Royal Botanic Gardens (K), New York Botanical Garden (NY), and New Zealand Fungarium–Te Kohinga Hekaheka o Aotearoa (PDD). Documentation of macroscopic features occurred in the field, from field photographs, and/or from desiccated specimens in the laboratory. In the laboratory, macroscopic characteristics were documented with an SC30 camera mounted on an SZ61 stereo microscope (Olympus, Center Valley, Pennsylvania), using Olympus cellSens 1.18 software to take photographs. Microscopic structures were observed using an Eclipse E600 microscope (Nikon, Melville, New York) and photographed using the same camera and cellSens software. During fieldwork in Honduras, a RED233 microscope (Motic, Barcelona, Spain) and a cell phone camera were used to capture images of living cells, when possible. Cultures were made in the laboratory or field by placing fresh apothecia into an inverted Petri dish of potato dextrose agar (PDA) for 1–4 h so that spores would be ejected on to the agar medium (sensu Karakehian et al. 2021) and incubated at room temperature.

Descriptions of microscopic features were made from cultures and hand-sectioned apothecia rehydrated and/or stained with tap water (H_2O), Lugol's solution (IKI), ~5% potassium hydroxide (KOH), Melzer's reagent (MLZ), lactic acid (LA), and/or 1% Congo red (CR). Microscopic measurements are provided as (a–)b–c(–d), with b and c representing average minus/plus standard deviation and a and d representing extreme values. In addition, the following abbreviations and notations

were used: $\bar{X}$ = average, [n, m, p] = "n" structures measured from "m" ascomata of "p" collections, and * = living cells. Piximètre 5.10 (http://ach.log.free.fr/Piximetre/) was used to measure microscopic structures. Colors were determined from Kornerup and Wanscher (1978). Newly collected specimens are deposited at the Kriebel Herbarium at Purdue University (PUL), the Herbarium Universitatis Gandavensis in Belgium (GENT), and the Herbario de la Universidad Autónoma de Chiriquí (UCH) in Panama; cultures are deposited at the Canadian Collection of Fungal Cultures (DAOMC) or ICMP. Information on collections for new species is provided in the Taxonomy section. Information on collections discussed in this paper but not described as new species is provided in SUPPLEMENTARY FILE 1.

DNA extraction and Sanger sequencing.—DNA was extracted from apothecia using Qiagen's QIAamp DNA Micro Kit or DNeasy Plant Mini Kit (Qiagen, Stanford, California) or the E.Z.N.A. DNA/RNA Isolation Kit (Omega Bio-tek, Norcross, Georgia) following the manufacturer's instructions. During fieldwork in Honduras and Panama, fresh apothecia were placed in lysis buffer (buffers ATL and AP1 from Qiagen DNA Micro and Plant Mini kits) in the field; the remaining steps of DNA extraction were later completed in the laboratory as per manufacturer's instructions. For ARIZ-M-AN00721, a putative new species with only one collection, two DNA extractions were performed: one with the QIAamp DNA Micro Kit (ARIZ-M-AN00721Q) and a second one from a different apothecium with the E.Z.N.A DNA/RNA Isolate Kit (ARIZ-M-AN00721O). DNA from cultures <30 d in age was amplified via colony polymerase chain reaction (PCR; sensu Albu et al. 2015) in which a sterile toothpick was used to pick and suspend hyphae from the colony in 50 µL H_2O, microwaved (at 1100 watt power) for 2 min, then centrifuged for 2 min at 18 400 rcf (relative centrifugal force). Approximately 40 µL of the supernatant was retained, and 5 µL was diluted in 45 µL sterilized Millipore H_2O for subsequent use in PCR.

The ITS and 28S nuc rDNA were sequenced with a variety of primer pairs; in general, ITS1F or ITS5 forward primers were used in combination with ITS4A or ITS4 reverse primers to amplify the ITS region (Gardes and Bruns 1993; Larena et al. 1999; White et al. 1990). In cases where amplification could not be obtained for the entire region using these primers, shorter regions of the ITS were amplified using ITS1F/ITS2 (ITS1 spacer) and/or ITS3/ITS4A (ITS2 spacer) (Larena et al. 1999; White et al. 1990). Forward primers ITS3, LR0R, or LIC24R were combined with reverse primers LR3, LR5, LR6, or LR7 to amplify the 28S D1–D2 domains (Hopple 1994; Vilgalys and Hester 1990). The *RPB1* ~A–B region was amplified with forward primers RPB1-af or RPB1-afasc in combination with reverse primer RPB1cr (Hofstetter et al. 2007; Matheny et al. 2002; Stiller and Hall 1997). All PCRs were completed in 25-µL reactions with 12.5 µL of 2× MyTaq Mix (Bioline, Swedesboro, New Jersey), 9.5 µL of H_2O, 1.0 µL of each 10 mM primer, and 1.0 µL of DNA extract. Thermocycler conditions were as follows. For ITS: initial denaturation at 94 C for 5 min; then 40 cycles of denaturation at 94 C for 30s, annealing at 48 C for 45s, and extension at 72 C for 45s; and final extension at 72 C for 7 min. For 28S: initial denaturation at 94 C for 5 min; then 35 cycles of denaturation at 94 C for 30s, annealing at 50 C for 45s, and extension at 72 C for 1 min; and final extension at 72 C for 7 min. For *RPB1*: initial denaturation at 95 C for 5 min; then 5 cycles of denaturation at 94 C for 1 min, annealing at 37 C for 35s with 0.5 C increase per cycle, and extension at 72 C for 1 min; then 30 cycles of denaturation at 94 C for 15s, annealing at 45 C for 55s with 0.5 C increase per cycle, and extension at 72 C for 1 min; and final extension at 72 C for 10 min.

Gel electrophoresis was completed at 130 V for 30 min in a 1% TBE (Thermo Fisher Scientific, Waltham, Massachusetts) agarose gel using SYBR GelRed (Biotium, Fremont, California) to verify the presence of PCR product. PCR purification and Sanger sequencing were outsourced to GENEWIZ (South Plainfield, New Jersey). The same primers used for PCR were used for sequencing, and sequence reads were assembled and edited in Sequencher 5.4.6 (Gene Codes, Ann Arbor, Michigan) or Geneious 9.1.8 (https://www.geneious.com). Sanger sequences generated from this study were deposited in the National Center for Biotechnology Information (NCBI) GenBank sequence database (https://www.ncbi.nlm.nih.gov/genbank/) and are provided in TABLE 1 with other sequence data used in our phylogenetic analysis.

High-throughput sequencing.—Metagenomic sequencing of the ITS region was completed for some fungarium specimens that failed traditional Sanger sequencing, an approach that has been successful for older or historical samples (Forin et al. 2018; Miller et al. 2022; Olds et al. 2023) in which DNA fragmentation and environmental contaminations likely occurred. This was attempted for collections CUP-D-3882 (77-17) (isotype of *C. chlora*), K-M 1434235 (holotype of *C. hypochlorum*), CUP-MJ-000039 (*C. hypochlorum*), and ARIZ-M-AN00721Q and ARIZ-M-AN00721O (*C. hawaiiense*) because Sanger sequencing of the ITS region failed (CUP-D-3882 [77-17], K-M 1435235), only recovered the ITS2 region (both ARIZ-

Table 1. Metadata for isolates with DNA sequence data used in this study.

Voucher	Other identifiers (isolate, collection number, etc.)	Genus	Species	ITS	28S	*RPB1*	Notes	Family	Country	Reference
PDD 119471	ICMP 25104	*Chlorosplenium*	*aotearoa*	OR564033				Chlorospleniaceae	New Zealand	This study
PDD 64801	ICMP 23703	*Chlorosplenium*	*aotearoa*	MW191757				Chlorospleniaceae	New Zealand	Johnston (2020)
PDD 89942	ICMP 25103	*Chlorosplenium*	*aotearoa*	OR564034				Chlorospleniaceae	New Zealand	This study
PDD 93907	ICMP 25105	*Chlorosplenium*	*aotearoa*	OR564035				Chlorospleniaceae	New Zealand	This study
PDD 98717	ICMP 23734	*Chlorosplenium*	*aotearoa*	MW191758				Chlorospleniaceae	New Zealand	Johnston (2020)
PDD 98718	ICMP 23733	*Chlorosplenium*	*aotearoa*	MW191763	OR567427			Chlorospleniaceae	New Zealand	This study; Johnston (2020)
PDD 99090	ICMP 23731	*Chlorosplenium*	*aotearoa*	MW191761			Holotype	Chlorospleniaceae	New Zealand	Johnston (2020)
PDD 117588	ICMP 23737	*Chlorosplenium*	*australiense*	MW191764				Chlorospleniaceae	Australia	Johnston (2020)
PDD 117589	ICMP 23736	*Chlorosplenium*	*australiense*	MW191759	OR567429			Chlorospleniaceae	Australia	This study; Johnston (2020)
PDD 117590	ICMP 23735	*Chlorosplenium*	*australiense*	MW191760	OR567428		Holotype	Chlorospleniaceae	Australia	This study; Johnston (2020)
ARAN 00432	DH1448a	*Chlorosplenium*	*chlora*	OR724722	OR724723		File S1[a], type species	Chlorospleniaceae	Spain	This study
ARAN 03087	DH1449b	*Chlorosplenium*	*chlora*	OR724721	OR724724		File S1	Chlorospleniaceae	Spain	This study
BDWR F0116	F-0116	*Chlorosplenium*	*chlora*	OR653948	OR653948		File S1	Chlorospleniaceae	USA Virginia	This study
BDWR F0125	F-0125	*Chlorosplenium*	*chlora*	OR653949	OR653949		File S1	Chlorospleniaceae	USA Pennsylvania	This study
BDWR F0126	F-0126	*Chlorosplenium*	*chlora*	OR653950	OR653950		File S1	Chlorospleniaceae	USA North Carolina	This study
FH 00456429	D. Haelew. F-1452, JK14030204	*Chlorosplenium*	*chlora*	OR653947	OR602725	OR620540	File S1	Chlorospleniaceae	USA Massachusetts	This study
FH BHI-F736		*Chlorosplenium*	*chlora*	MG553993	OR602727		File S1	Chlorospleniaceae	USA Massachusetts	This study; Haelewaters et al. (2018)
FH BHI-F737		*Chlorosplenium*	*chlora*	MG553994			File S1	Chlorospleniaceae	USA Massachusetts	Haelewaters et al. (2018)
PUL F21304	20171013SDR018	*Chlorosplenium*	*chlora*	FF74-MM7659 (MycoMap.com)				Chlorospleniaceae	USA Indiana	S. D. Russell, unpubl.
PUL F27629		*Chlorosplenium*	*chlora*	MZ312166				Chlorospleniaceae	USA New York	M. C. Aime, unpubl.
PUL F29704	JKS375	*Chlorosplenium*	*chlora*	OR653951	OR602724	OR620542	File S1	Chlorospleniaceae	USA Michigan	This study
TUF 104917	KL605	*Chlorosplenium*	*chlora*	OR653952	OR602723		File S1	Chlorospleniaceae	Serbia	This study
GENT FG0001908, PUL F29706, UCH 15450	PANAMA22-F125	*Chlorosplenium*	*cusucoense*	OR653959				Chlorospleniaceae	Panama	This study
PUL F29693	HONDURAS19-F032a	*Chlorosplenium*	*cusucoense*	MT571528	OR602722	OR620541		Chlorospleniaceae	Honduras	This study; Haelewaters et al. (2021b)
PUL F29694	HONDURAS19-F050	*Chlorosplenium*	*cusucoense*	OR653953	OR602721	OR620543	Holotype	Chlorospleniaceae	Honduras	This study
PUL F29696	HONDURAS22-F019	*Chlorosplenium*	*cusucoense*	OR653954				Chlorospleniaceae	Honduras	This study
PUL F29697	HONDURAS22-F080	*Chlorosplenium*	*cusucoense*	OR653955				Chlorospleniaceae	Honduras	This study
PUL F29700	HONDURAS22-F242 2	*Chlorosplenium*	*cusucoense*	OR653956				Chlorospleniaceae	Honduras	This study
PUL F29702	HONDURAS22-F429	*Chlorosplenium*	*cusucoense*	OR653957				Chlorospleniaceae	Honduras	This study
PUL F29703	HONDURAS23-F467	*Chlorosplenium*	*cusucoense*	OR653958				Chlorospleniaceae	Honduras	This study
UCH 15449	PANAMA22-F017	*Chlorosplenium*	*cusucoense*	OR724720				Chlorospleniaceae	Panama	This study
	MushroomObserver 209090	*Chlorosplenium*	*cusucoense*	MG976227				Chlorospleniaceae	Mexico	A. Rockefeller, unpubl.
PDD 114266	JAC 17156	*Chlorosplenium*	*epimorsicum*	OR565284				Chlorospleniaceae	New Zealand	This study
PDD 114414	JAC 17304	*Chlorosplenium*	*epimorsicum*	OR565285				Chlorospleniaceae	New Zealand	This study
PDD 93100	ICMP 23732	*Chlorosplenium*	*epimorsicum*	MW191762	OR567430		Holotype	Chlorospleniaceae	New Zealand	This study; Johnston (2020)
ARIZ-M-AN00721		*Chlorosplenium*	*hawaiiense*	OR602793, OR653946	OR602726		Holotype	Chlorospleniaceae	USA Hawai'i	This study
CUP-052724		*Chlorosplenium*	*hypochlorum*	OR653960			File S1	Chlorospleniaceae	Mexico	This study
CUP-MJ-000039		*Chlorosplenium*	*hypochlorum*	OR653961	OR602720		File S1	Chlorospleniaceae	Jamaica	This study
GENT FG0001878	HONDURAS23-F235	*Chlorosplenium*	*hypochlorum*	OR653964			File S1	Chlorospleniaceae	Honduras	This study

(*Continued*)

Table 1. (Continued).

Voucher	Other identifiers (isolate, collection number, etc.)	Genus	Species	ITS	28S	*RPB1*	Notes	Family	Country	Reference
GENT FG0001880, PUL F29705, UCH 15451	PANAMA22-F005	*Chlorosplenium*	*hypochlorum*	OR653966			File S1	Chlorospleniaceae	Panama	This study
NY 3423615		*Chlorosplenium*	*hypochlorum*	OR653965	OR602719		File S1	Chlorospleniaceae	Venezuela	This study
PUL F29698	HONDURAS22-F240 2	*Chlorosplenium*	*hypochlorum*	OR653962			File S1	Chlorospleniaceae	Honduras	This study
PUL F29699	HONDURAS22-F242 1	*Chlorosplenium*	*hypochlorum*	OR653963			File S1	Chlorospleniaceae	Honduras	This study
HMAS 255820		*Chlorosplenium*	*sinicum*	MZ914623	MZ920114	MZ945728		Chlorospleniaceae	China	Zheng and Zhuang (2021)
HMAS 255822		*Chlorosplenium*	*sinicum*	NR_176163	MZ920115	MZ945729	Holotype	Chlorospleniaceae	China	Zheng and Zhuang (2021)
HMAS 255824		*Chlorosplenium*	*sinicum*	MZ914625	MZ920116	MZ945730		Chlorospleniaceae	China	Zheng and Zhuang (2021)
HMAS 255829		*Chlorosplenium*	*sinicum*	MZ914631	MZ920119	MZ945733		Chlorospleniaceae	China	Zheng and Zhuang (2021)
HMAS 266526		*Chlorosplenium*	*sinicum*	MZ914637				Chlorospleniaceae	China	Zheng and Zhuang (2021)
HMAS 275558		*Chlorosplenium*	*sinicum*	MZ914635	MH729335			Chlorospleniaceae	China	Zheng and Zhuang (2021); W. Y. Zhuang and Z. Q. Zeng, unpubl.
HMAS 290879		*Chlorosplenium*	*sinicum*	MZ914626	MZ920117	MZ945731		Chlorospleniaceae	China	Zheng and Zhuang (2021)
HMAS 290885		*Chlorosplenium*	*sinicum*	MZ914628	MZ920118	MZ945732		Chlorospleniaceae	China	Zheng and Zhuang (2021)
HMAS 290873		*Chlorosplenium*	*sinochlorum*	NR_176162	MZ920122	MZ945736	Holotype	Chlorospleniaceae	China	Zheng and Zhuang (2021)
TNS F17463	FC-1139, NBRC 112537	*Mollisia*	*fusca*	LC425049	LC429378	LC431675	Outgroup	Mollisiaceae	Japan	Johnston et al. (2019)
CBS 121003		*Vibrissea*	*flavovirens*	MT026430	MT026563	MT018435	Outgroup	Vibrisseaceae	Germany	Tanney and Seifert (2020)

[a]"File S1" indicates that complete collection information for this isolate is available in SUPPLEMENTARY FILE 1 instead of the text of the paper.

M-AN00721 isolates), or was successful but high-throughput sequencing was done as part of a test of the methodology (CUP-MJ-000039). Briefly, initial PCR was completed as described on the same DNA extractions but with the primer pair TS-ITS1-F/TS-ITS2-R (ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGTAA/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGCGTTCTTCATCGATGC) or TS-ITS3-F/TS-ITS4-R (ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATCGATGAAGAACGCAGC/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCCTCCGCTTATTGATATGC) (TruSeq, Illumina, San Diego, California). The initial PCR products were visualized using gel electrophoresis, and 5 µL of the PCR product was cleaned with 1 µL of 10 U/µL exonuclease and 1 µL of 2 U/µL shrimp alkaline phosphotase according to manufacturer's instructions (Thermo Fisher Scientific). Sample-specific and Illumina-adaptor barcodes were added in the second round of PCR in 25-µL reactions with 1 µL of cleaned PCR product, 12.5 µL of 2× MyTaq Mix, 9.5 µL of H_2O, and 1.0 µL of each adapter. Thermocycler conditions were as follows: denaturation at 95 C for 3 min; 8 cycles of 95 C, 55 C, and 72 C for 30s each; and final extension 72 C for 5 min. Final PCR product sizes from the first and second rounds were visually compared on a 1% TBE agarose gel run for 60 min at 60 volts to verify the addition of sample-specific and Illumina-adaptor barcodes. The PCR products from the second round of PCR were cleaned as above, and DNA concentration was checked on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Cleaned PCR products were pooled in equal concentration and submitted to the Bindley Core for Genomics at Purdue University and sequenced on an Illumina MiSeq v2 platform run using rapid 2 × 250 nucleotide (nt) paired-end reads. Resulting Illumina sequence data were processed with DADA2 1.29.0 (Callahan et al. 2016) with forward and reverse amplicons in R 4.1.2 (R Core Team 2021) using the methodology from Miller et al. (2022). Briefly, the filterAndTrim function was used with the options maxN = 0, maxEE = c(2,2), truncQ = 1, and rm.phix = True, and the learnErrors function was used on forward and reverse reads. Forward and reverse reads were then merged with the mergePairs function. The removeBimeraDenovo function with the method set to consensus was used to remove chimeric sequences. A FASTA file of the amplicon sequence variants (ASVs) derived from DADA2 was generated using SeaView (Galtier et al. 1996). The FASTA file was compared against the NCBI nucleotide database using NCBI BLASTn (Altschul et al. 1990), and the results were downloaded to XML files. The NCBI BLAST parser tool (Ream and Kiss 2013) was used to parse the NCBI XML files, and the putative taxonomic identity of each ASV was determined by the top NCBI BLASTn match. Raw Illumina reads were deposited into the NCBI Sequence Read Archive (SRA) database under BioProject PRJNA1018967.

For two collections, FH 00456429 and PUL F29693, *RPB1* sequences were derived from draft genome assemblies (C. A. Quandt and D. Haelewaters, unpublished) using the workflow at https://github.com/tinamelie/Geoglossomycetes-genomics-workflow.

Sequence alignment and phylogenetical analyses.— Newly derived sequences of *Chlorosplenium* were combined with *Chlorosplenium* sequences available via public repositories such as NCBI GenBank and UNITE (Abarenkov et al. 2010). We used all public sequences available for the genus in these repositories, except for *C. sinicum*, where we chose eight of the 17 available isolates, emphasizing those from different intraspecific clades in Zheng and Zhuang (2021; TABLE 1). *Mollisia fusca* and *Vibrissea flavovirens* from the broader clade including Chlorospleniaceae (Johnston et al. 2019) were used as outgroup taxa. Sequences were aligned using the MAFFT 7.308 (Katoh et al. 2002; Katoh and Standley 2013) plugin in Geneious with the settings Algorithm: Auto, Scoring matrix: 200PAM/k = 2, Gap open penalty: 1.53, and Offset value: 0.123. The ITS sequences were trimmed to begin after the 5′ starting motif 5′-ATCATTA-3′ and to end at the 5′ start of the 28S at 5′-TGACCT-3′. Similarly, 28S sequences were trimmed to begin at the 5′-TGACCT-3′ motif. After manual trimming, ITS, 28S, and *RPB1* alignments were further trimmed in the command-line version of trimAl 1.3 (Capella-Gutiérrez et al. 2009) using a gap threshold (-gt) of 0.60 and minimum coverage (-cons) of 0.50, with other settings on default. The ITS region was partitioned into the ITS1, 5.8S, and ITS2 regions using the conserved motifs 5′-AAACTTTCAACAAC-3′ for the start of the 5.8S and 5′-GACCAATCA-3′ for the start of the ITS2 region. Concatenation of loci was completed in SequenceMatrix (Vaidya et al. 2011).

Within the CIPRES Science Gateway (Miller et al. 2010), jModelTest2 (Darriba et al. 2012) was used with default settings to determine the best model at each partition under the Bayesian information criterion (BIC). Maximum likelihood (ML) trees were inferred in IQ-TREE 1.6.12 (Nguyen et al. 2015) with partitioned models (Chernomor et al. 2015) and 1000 nonparametric bootstrap (BS) replicates ("-b" option).

Bayesian trees were inferred using BEAST 1.10.4 (Suchard et al. 2018) and associated applications (BEAUTi, LogCombiner, and TreeAnnotater) and Tracer 1.7.2 (Rambaut et al. 2018). BEAST was run using the same models as the ML trees for each partition, starting from a random tree. An uncorrelated relaxed clock with a log-normal relaxed distribution and continuous quantile parameterization and a birth-death incomplete sampling speciation model (Stadler 2009) tree prior were selected. The length of the Markov chain Monte Carlo (MCMC) sample chain was 40 million, with sampling frequency of 4000. Viewing traces of log files in Tracer, effective sample sizes were all high (>200) and quickly reached a plateau; therefore, the default burn-in of 10% was used. Trees were combined in LogCombiner. TreeAnnotator was used to generate a consensus Maximum Clade Credibility tree with posterior probability (PP) values. Tree topologies of ML and Bayesian trees were visualized in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

High-throughput sequencing.—Metagenomic sequencing did not recover any sequences identified to the genus *Chlorosplenium* for CUP-D-3882 (77-17) or K-M 1434235, whereas the ITS2 region was recovered for both ARIZ-M-AN00721 isolates. The ITS1 region was recovered for ARIZ-M-AN00721Q and CUP-MJ-000039 (the only region attempted for this isolate), but not for ARIZ-M-AN00721O. In the ITS1 region of ARIZ-M-AN00721Q, two ASVs identified as *Chlorosplenium* sp. were recovered. The alignment showed an identical region 184 nucleotides long and a divergent region 67 nucleotides long between the two ASVs. Using BLASTn, it was determined that the divergent region of one ASV was chimeric. Therefore, only the ASV matching the target organism for the entire sequence length was kept for analysis. All resulting ASVs, counts, identifications, and final sequences retained after the DADA2 workflow are in SUPPLEMENTARY FILE 2, with associated BioSample accession numbers for the raw reads.

Phylogenetics.—We constructed four phylogenetic trees: ML and Bayesian trees of ITS1-5.8S-ITS2-28S-*RPB1* (FIG. 1, topology of ML tree shown) and ML and Bayesian trees of the ITS region only (SUPPLEMENTARY FIG. 1, topology of ML tree shown). Information on models selected, lengths of alignments, sites, and sequences available at each locus can be found in TABLE 2. Original, unedited alignments and trimmed alignments used for the ITS-28S-*RPB1* phylogeny can be found in SUPPLEMENTARY FILES 3 and 4, and those for the ITS phylogeny in SUPPLEMENTARY FILES 5 and 6.

All inferred trees recover the genus *Chlorosplenium* at maximum ML and PP support values and have identical topologies at supported nodes except for the positions of *C. sinochlorum* and *C. epimorsicum*. These species are recovered as sister taxa in the Bayesian ITS and multilocus trees with strong support (1.0 or 0.99 PP), but with no relationship in the ML trees. All individual species are recovered with high BS (>70) and PP (>0.90) support except for *C. epimorsicum*, which is only supported in our Bayesian analysis (67/1.0 in multilocus trees, 62/1.0 in ITS trees), and *C. sinochlorum*, for which only one isolate is available. *Chlorosplenium hypochlorum* is placed sister to *C. cusucoense*; other sister species are *C. hawaiiense* and *C. sinicum* and *C. australiense* and *C. aotearoa*. Deeper nodes are generally unsupported (BS < 70, PP < 0.90), besides the genus-level clade and a clade containing *C. aotearoa, C. australiense, C. chlora, C. hawaiiense*, and *C. sinicum* (76/0.95 in multilocus trees, 78/0.98 in ITS trees).

TAXONOMY

Chlorosplenium aotearoa P.R. Johnst. & Stallman, sp. nov. FIGS. 2 and 7

MycoBank MB851249

Typification: NEW ZEALAND. WAIKATO REGION: Taupō, vicinity of Kiko and Tiraki roads, 38.971486S, 176.074165E, on decorticated *Nothofagus* wood, 3 May 2001, *P.R. Johnston D1579, S. Whitton* (**holotype** PDD 99090), culture ICMP 23731. GenBank: ITS = MW191761.

Diagnosis: Differs from *Chlorosplenium sinochlorum* by its larger ascospores, from *C. sinochlorum* and *C. chlora* by its larger asci, and from all known cultured species by its white to cream-colored colony in age (>60 d) that produces crystals on PDA and by autapomorphies at positions 72, 73, 74, 82, 129, 374, and 384 in ITS nuc rDNA.

Etymology: Named for the traditional Māori language name for New Zealand, Aotearoa, the only country in which this taxon has been found. Used as a noun in apposition.

Description: Apothecia 2.0–5.0 mm wide, scattered to gregarious, noncespitose, sessile to subsessile; at first globose and closed, then opening and expanding to cupulate; margin entire to subcrenulate in desiccation,

Figure 1. Maximum likelihood multilocus (ITS1-5.8S-ITS2-28S-*RPB1*) phylogenetic reconstruction of the genus *Chlorosplenium*, with *Mollisia fusca* and *Vibrissea flavovirens* as outgroup. Bootstrap replicate values ≥70 and posterior probability values ≥0.90 are shown on branches. New species described in this study are in bold, and isolate location of origin, voucher number, and type status are provided.

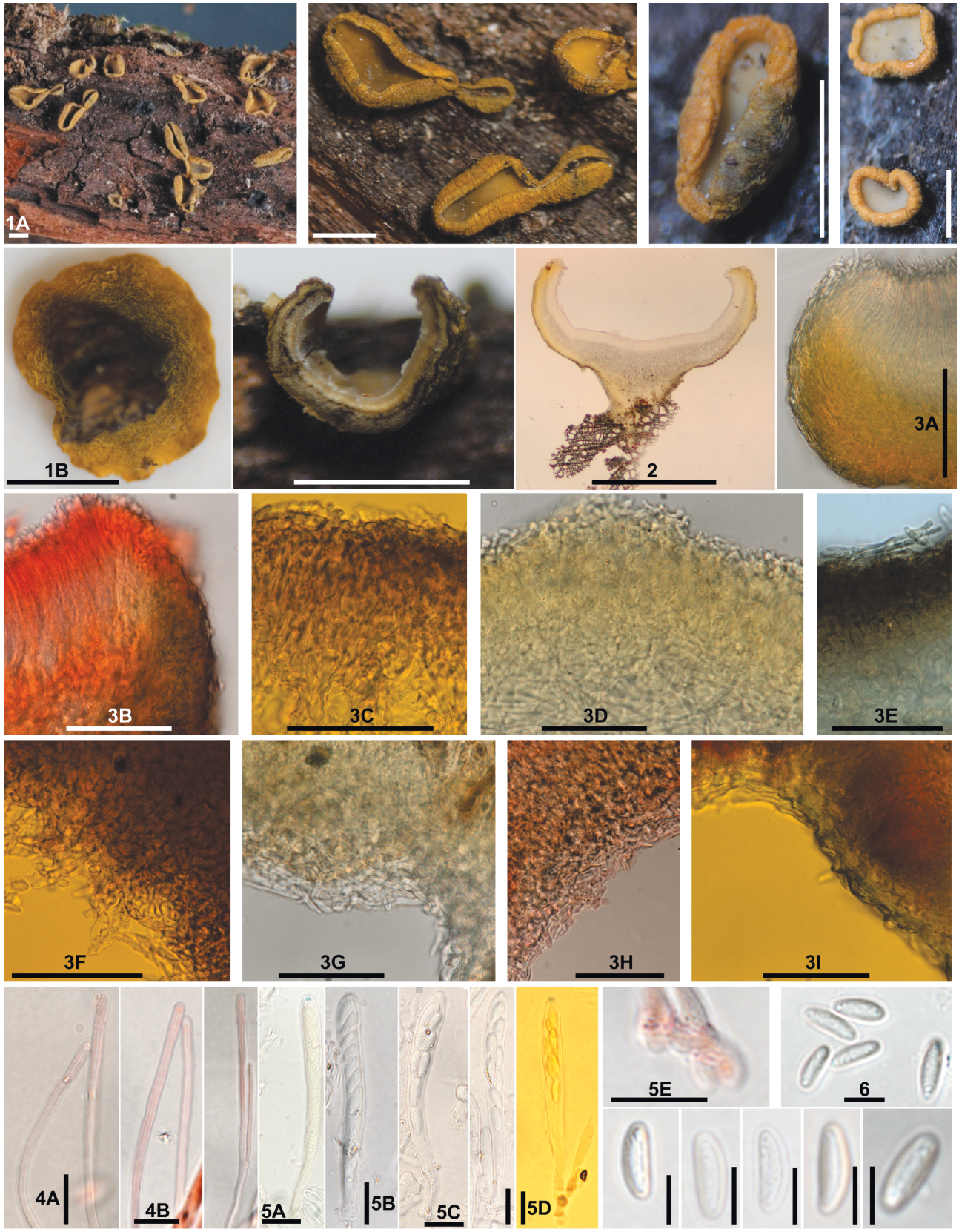

Figure 2. *Chlorosplenium aotearoa*. Images from PDD 99090 (holotype) unless otherwise stated. 1. Desiccated (A) and rehydrated (B) ascomata; bars = 1.0 mm. 2. Section in KOH; bar = 1.0 mm. 3. Ectal excipulum margin in H_2O (A) and CR+KOH (B), upper flanks in MLZ (C), upper and middle flanks and medullary excipulum in KOH (D), middle flanks in KOH (E), and lower flanks and base in MLZ (F, I), KOH (G), and CR+KOH (H); bars = 50 µm; A, D, G–I. PDD 89942. B. PDD 98718. 4. Paraphyses in CR+KOH (A–B); bars = 10 µm; B. PDD 89942. 5. Asci in IKI (A), KOH (B–C), MLZ (D), and CR+KOH (E); bars = 10 µm; C–E. PDD 98718. 6. Ascospores in KOH; bars = 5 µm.

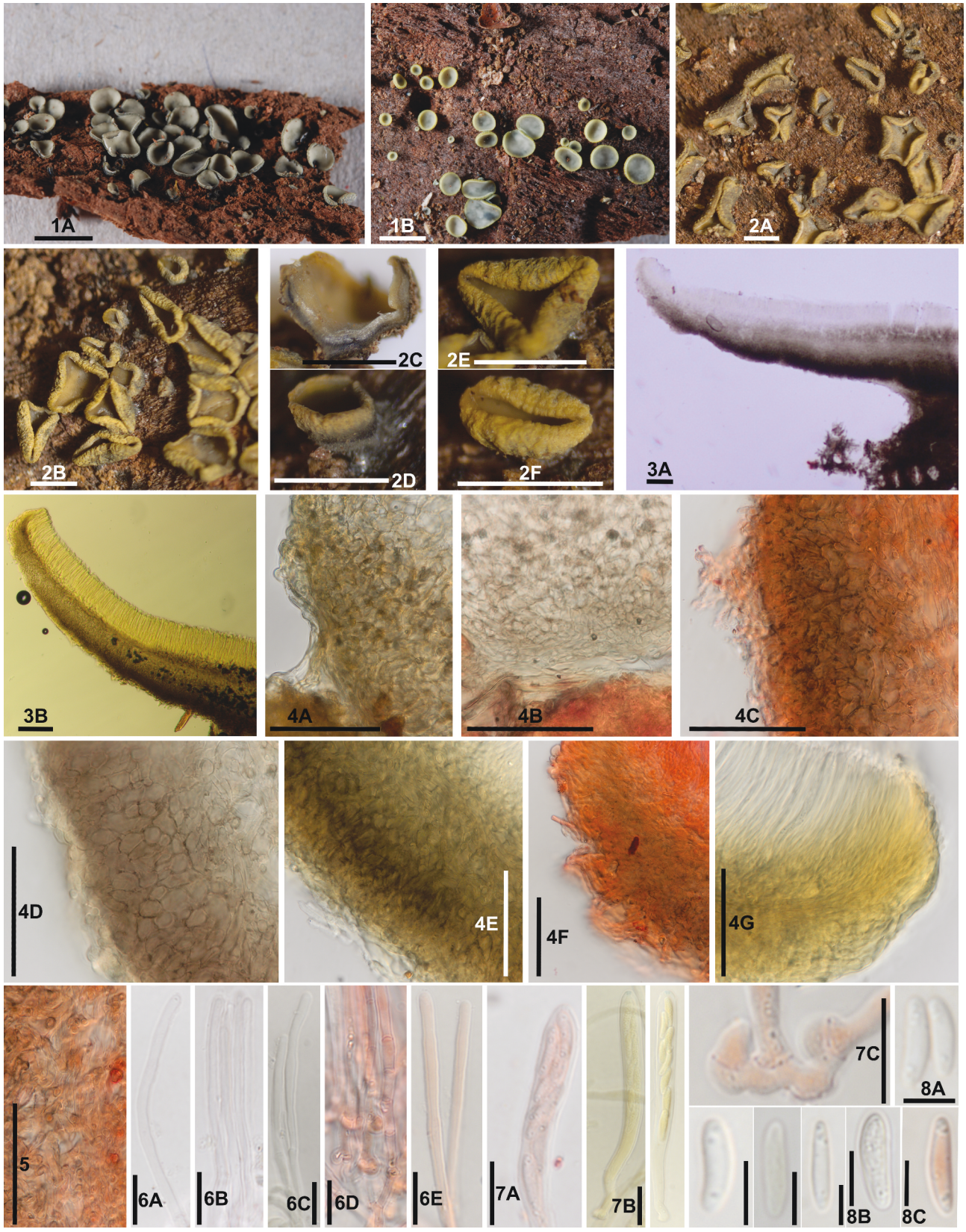

Figure 3. *Chlorosplenium australiense*. Images from PDD 117590 (holotype) unless otherwise stated. 1. Fresh ascomata in the field; bars = 5 mm; A. PDD 117589. 2. Desiccated ascomata; bars = 1 mm; A, C–D. PDD 117589. 3. Sections in IKI+KOH (A) and MLZ (B); bars = 100 µm; A–B. PDD 117589. 4. Ectal excipulum base in H_2O (A) and CR+KOH (B), lower flanks and medullary excipulum in CR+KOH (C) and MLZ (D), and upper flanks and margin in KOH (E, G) and CR+KOH (F); bars = 50 µm. 5. Medullary excipulum in CR+KOH; bar = 50 µm. 6. Paraphyses in KOH (A–C) and CR+KOH (D–E); bars = 10 µm; A–B, D. PDD 117589. 7. Asci in CR+KOH (A, C) and MLZ (B); bars = 10 µm; A. PDD 117589. 8. Spores in KOH (A), H_2O (B), and KOH+CR (C); bars = 5 µm.

Table 2. Models used for phylogenetic analysis, with information on number of sequences and number of variable and invariable sites at each locus.

Partition	Locus	Sequences	Sites	Parsimony-informative	Invariable	Model
1	ITS1	54	206	83	89	TrNef+G
2	5.8S	55	159	7	152	K80
3	ITS2	54	148	50	76	TPM2+I
4	28S	28	1065	92	918	TrN+I
5	RPB1	13	762	40	593	TrNef+G

inrolled; disc glabrous, pale creamy-green (~2A2), sometimes with grayish discolorations, similarly colored to bright or darker yellowish-orange (~4A6–4C6) in desiccation; receptacle yellowish-green, glabrous to subfibrillose, when desiccated varying from dark orange (~4C7) to greenish (3C6) to gray, in some places with a covering of lighter mustard yellow (4A7) to orangish fibrils, base dark, with or without grayish basal tomentum; sectioned apothecia releasing yellowish pigments into KOH medium. Asci (48.8–)56.2–65.9(–71.6) × (4.8–)5.2–6.1(–6.6) µm [34, 4, 3], cylindrical–subclavate, slightly tapering to a rounded to subtruncate base, with eight, uniseriate to biseriate ascospores, apical ring amyloid with or without KOH pretreatment, arising from croziers. Ascospores (5.2–)6.0–7.5(–9.0) × (1.6–)2.2–2.8 (–3.3) µm [207, 4, 3], hyaline, oblong to elliptic, slightly tapering to rounded ends, not to slightly curved, with (0–)2(–3) small guttules <1 µm wide, usually near poles. Paraphyses 1.4–3.0 µm wide, filiform, septate, and commonly branching near base. Medullary excipulum a t. intricata composed of loose hyaline hyphae 1.6–2.8 µm wide that become tighter packed and often yellowish to light brown in the subhymenium. Ectal excipulum a t. angularis-globulosa 20–55 µm thick, cells 2.3–9.7 µm wide, thinner and grading to t. prismatica at margins, interior cells hyaline to light brown, darkening toward exterior, in most areas with covering of hyaline to brown, refractive, cylindrical hyphae 1.7–3.0 µm wide, ±parallel to the ectal excipulum; aggregated hyphae extending up to 30 µm from ectal excipulum wall, generally thicker near base. Culture slow growing on PDA, 23 mm wide after 50 d, producing a white to cream to light brown zonate colony, darker colors more common in older tissues at center, yet almost completely obscured by whitish-cream hyphal covering, staining dark brown where bruised; hyphae growing upward into aggregated points from diffuse bases, giving the colony a fluffy appearance; guttation of small to large (<1–5(–10) mm wide) brownish drops frequent; colony with crystal depositions in agar and with fragile, crystalline projections up to 3 mm in length (after >30 d of growth); bottom of colony zonate, center brown then rings of brownish-yellow with hyaline leading edge; turning surrounding agar faintly yellowish-green (~3A2); composed of predominantly short-septate, hyaline to brown, cylindrical hyphae 1.6–3.6 µm wide; hyphae in upper areas generally lighter, sometimes aggregating into bundles up to 10 µm wide; lower areas generally darker; sterile on PDA and corn meal agar (CMA).

Habitat and distribution: New Zealand, growing on corticated or decorticated *Nothofagus* spp.

Additional specimens examined: NEW ZEALAND. BAY OF PLENTY REGION: Vicinity of Ruatahuna, Tarapounamu, 38.621125S, 176.863403E, on rotten wood, 7 Oct 2004, *P.R. Johnston D1877, B.C. Paulus* (PDD 98718), culture ICMP 23733; vicinity of Ruatahuna, Tarapounamu, 38.609779S, 176.878869E, on dead wood of *Nothofagus* sp., 8 Oct 2004, *P.R. Johnston D1885, B.C. Paulus TTT547* (PDD 98717), culture ICMP 23734; Whakamārama, Palmer Reserve, 37.698043S, 175.972931E, on fallen wood, 26 Jun 2006, *B.C. Paulus 3707, S. Tsai* (PDD 89942); HAWKE'S BAY REGION: Te Urewera, Tarapounamu UA2, on dead *Nothofagus* log, 8 Oct 2004, *B.C. Paulus 698* (PDD 119471), culture ICMP 25104; NELSON REGION: Lake Rotoiti, Loop Track, 41.822833S, 172.838193E, on dead wood of *Nothofagus* sp., 17 May 1994, *P.R. Johnston D1096* (PDD 64801), culture ICMP 23730; WAIKATO REGION: Vicinity of Awakino, Steuart Russell Awakino Beech Reserve, 38.576674S, 174.677604E, on decaying wood of *Nothofagus truncata*, 16 Apr 2007, *B.C. Paulus 4582, A.J. O'Donnell* (PDD 93907), culture ICMP 25105.

Notes: Chlorosplenium aotearoa is known from seven collections in New Zealand growing on *Nothofagus* spp. (Johnston 2020). It has been found on the central North Island and northern tip of the South Island and appears more common than *C. epimorsicum*, which has only been found further south on both *Metrosideros* and *Nothofagus* substrates. This species has overlapping ascospore and ascus sizes with several shorter-spored ($\bar{X}$ < 8.0 µm) species. It differs from *C. sinicum* by lacking long hairs on the ectal excipulum, from *C. cusucoense* by the color of its culture in age, from *C. epimorsicum* by not forming apothecia directly out of the tissue of older ascomata, and from *C. hawaiiense* by lacking well-developed t. prismatica in the ectal

excipulum and having a thinner hyphal covering of the receptacle. *Chlorosplenium aotearoa* generally has larger ascospores than *C. sinochlorum* and larger asci than both *C. sinochlorum* and *C. chlora* (TABLE 3). Additionally, *C. aotearoa* generally has a thinner ectal excipulum: 20–55 µm thick vs. 20–95 µm in *C. sinochlorum* (Zheng and Zhuang 2021) and 25–105 µm in *C. chlora* (Dixon 1974; this study). Small (1–2 µm wide), globose conidia were found attached to the apices of 5 out of 590 ascospores examined. Cultures of *C. aotearoa* produce crystalline apical projections or deposits in agar when grown for >30 d on PDA. The colony maintains a light, white to cream color in age (>60 d) and lacks chains of inflated cells, whereas the only other species known to produce crystals in culture, *C. cusucoense*, is brown in age and contains chains of inflated cells.

Chlorosplenium australiense P.R. Johnst. & Stallman, sp. nov. FIGS. 3 and 7

MycoBank MB851250

Typification: AUSTRALIA. NEW SOUTH WALES: Barrington Tops National Park, Gloucester Tops Road, Antarctic Beech Forest Walk, on decorticated wood of *Nothofagus moorei*, 16 May 2009, *P. R. Johnston AU09-10* (**holotype** PDD 117590), culture ICMP 23735. GenBank: ITS = MW191760; 28S = OR567428.

Diagnosis: Differs from other *Chlorosplenium* species by the gray color of its disc when fresh.

Etymology: Named after Australia, the only country in which this species has been found.

Description: Apothecia 1.5–4.0 mm wide, gregarious, sometimes cespitose, sessile to subsessile; at first globose and closed, then opening and expanding to discoid–cupulate–petaloid; margin entire, non-inrolled; disc glabrous, light translucent gray to gray (4A2–B2), dull or dark yellowish-orange, olive, or darker brown to black in desiccation; receptacle yellow (4A4–6) to gray-green to gray (~4C1–3), darkening toward base, glabrous or with fibrils similarly colored or lighter than underlying receptacle, in desiccation gray and partially covered by yellowish-orange fibrils (4A5–6) that often aggregate, giving a furrowed appearance, particularly near margin, with or without hyaline to gray basal tomentum; wood sometimes stained dark near base of apothecium; sectioned apothecia releasing yellowish pigments into KOH medium. Asci (53.9–)58.3–67.0(–70.7) × (4.0–)4.7–6.3(–7.5) µm [33, 5, 3], cylindrical–clavate, tapering to slightly enlarged, or not base, with eight, uniseriate to biseriate ascospores, apical ring amyloid with or without KOH pretreatment, arising from croziers. Ascospores (5.7–)7.4–9.2(–11.3) × (1.3–)1.9–2.6(–3.0) µm [231, 6, 3], hyaline, cylindrical to allantoid, usually with 2 (0–4) small (<1 µm) guttules near poles. Paraphyses 1.6–2.8 µm wide, filiform, septate, and commonly branching near base. Medullary excipulum a t. intricata composed of packed to loose, hyaline to light brown hyphae 1.3–3.3 µm wide, tighter packed and darker (light yellowish-brown) in the subhymenium. Ectal excipulum a t. angularis-globulosa 25–99 µm wide, thinning and grading to t. prismatica at margin; cells 2.8–13.0 µm wide, brown, interior cells generally larger than cortical cells, with an exterior covering of hyaline to golden, refractive, cylindrical hyphae 2.1–3.1 µm wide, ±parallel to the ectal excipulum, with aggregations of hyphae extending 7–31 µm from excipulum wall, with or without an additional layer of gelatinized t. intricata connecting base of ectal excipulum to substrate. Culture slow growing on PDA, 21 mm wide after 50 d, producing a zonate white to cream to light brown colony, colors almost completely obscured by white hyphal covering, staining dark brown where bruised; hyphae growing upward into aggregated points from diffuse bases, giving the colony a fluffy appearance; points often darker (brownish); guttation sometimes present at center of colony as small (<1 mm) brown droplets; bottom of colony zonate, center brown followed by rings of brownish-yellow with a hyaline leading edge, turning surrounding agar faintly yellowish-green (~3A2); composed of predominantly short-septate, hyaline to brown hyphae; in upper areas hyphae generally lighter and cylindrical, 1.5–2.5 µm wide, sometimes aggregating into bundles up to 30 µm wide; in lower areas hyphae darker, usually wider, 2.8–4.5 µm, with more ellipsoid elements interspersed; sterile on PDA and CMA.

Habitat and distribution: Tasmania and southeastern Australia, growing on corticated or decorticated *Nothofagus moorei*.

Additional specimens examined: AUSTRALIA. NEW SOUTH WALES: Barrington Tops National Park, Gloucester Tops Road, Antarctic Beech Forest Walk, on dead wood of *Nothofagus moorei*, 17 May 2009, *P.R. Johnston AU09-48* (PDD 117589), culture ICMP 23736; TASMANIA: Mount Field National Park, Lyrebird Nature Walk, on fallen wood of *Nothofagus cunninghamii*, 6 Oct 1997, *P.R. Johnston AU97-44* (PDD 117588), culture ICMP 23737.

Notes: Chlorosplenium australiense is known from two collections in New South Wales and one from Tasmania, Australia. This species is readily distinguished by its gray disc in fresh specimens and intermediate average ascospore length ($\bar{X}$ = 7.4–9.2 µm)

Table 3. Morphometric data, other distinguishing morphological features, and distributions for all *Chlorosplenium* species.

Species	Ascospore length × width (µm)	Ascus length × width (µm)	Known distribution	Other distinguishing characteristics	Reference(s)
C. aotearoa	(5.2–)6.0–7.5(–9.0) × (1.6–)2.2–2.8(–3.3)	(48.8–)56.2–65.9(–71.6) × (4.8–)5.2–6.1(–6.6)	New Zealand: North Island and northern South Island	Culture with crystals and light-colored colony in age	This study (PDD 99090, PDD 98718, PDD 89942); Johnston (2020)
C. australiense	(5.7–)7.4–9.2(–11.3) × (1.3–)1.9–2.6(–3.0)	(53.9–)58.3–67.0(–70.7) × (4.0–)4.7–6.3(–7.5)	Southeastern Australia: New South Wales and Tasmania	Fresh hymenium gray in color	This study (PDD 117588, PDD 117589, PDD 117590); Johnston (2020)
C. cenangium	15–18 × 4.0–5.0	80–100 × 10–12	Europe: high elevations on *Rhododendron ferrugineum*	Septate ascospores	Müller (1977)
C. chlora	(5.2–)5.7–7.5(–8.8) × (1.6–)1.8–2.5(–3.0)	(44.5–)47.2–54.2(–54.3) × (4.0–)4.4–5.4(–5.5)	Eastern USA, Europe (Serbia, Spain)		This study (PUL F29704)
C. chlora	(5.0–)6.0–7.0(–9.0) × 1.2–2.0(–3.0)	(40–)45–55(–60) × (4–)5–6(–7)	Eastern USA, Europe (Serbia, Spain)		Dixon (1974)
C. cusucoense	(4.9–)5.9–7.2(–8.8) × (1.8–)2.4–3.2(–3.7)	(48.7–)56.0–68.6(–73.5) × (4.6–)5.0–6.0(–7.0)	Central America, Mexico	Ascospores commonly with conidia; culture with crystals and dark-colored colony in age	This study (PUL F29694, PUL F29693, PUL F29706); Haelewaters et al. (2021b)
C. epimorsicum	(5.3–)6.4–7.9(–9.5) × (1.7–)2.0–2.6(–3.3)	(48.1–)54.5–62.2(–68.5) × (4.1–)4.5–5.9(–7.5)	New Zealand: South Island and Stewart Island	Apothecia growing directly from dead/decaying apothecia	This study (PDD 93100); Johnston (2020)
C. hawaiiense	(5.6–)6.3–7.5(–8.5) × (1.7–)2.0–2.6(–3.2)	(49.1–)51.8–61.3(–67.8) × (3.6–)4.5–5.6(–6.0)	Hawai'i Island	Stipe up to 0.5 mm long; ectal excipulum covering up to 60 µm wide	This study (ARIZ-M-AN00721); Wong and Korf (2009)
C. hypochlorum	(6.5–)8.0–10.1(–12.5) × (2.0–)2.4–3.0(–3.7)	(56.4–)59.9–78.9(–88.7) × (4.3–)5.0–7.0(–7.9)	Central America, Caribbean, and northern South America	Ascospores may have conidia	This study (PUL F29698, PUL F29699, PUL F29705, NY 03423615)
C. hypochlorum	(8–)9–14(–15) × 2.0–4.0	70–95 × (5.0–)6.0–8.0(–9.0)	Central America, Caribbean, and northern South America	Hymenium easily separable from receptacle (not found in this study)	Dixon (1974)
C. sinicum	5.5–7.7 × 1.5–2.2	45–66 × 4.0–6.0	China: particularly in southern and eastern provinces	Long hyphae/hairs on apothecial margin (up to 90 µm)	Zheng and Zhuang (2021)
C. sinochlorum	5–6.5 × 1.8–2.2	45–53 × 3.5–4.5	China: Hunan Province		Zheng and Zhuang (2021)

between the smaller-spored species such as *C. chlora, C. sinicum*, and *C. sinochlorum* ($\bar{X}$ < 8.0 µm) and the larger-spored *C. cenangium* ($\bar{X}$ = 15–18 µm; Müller 1977). The spores are smaller than Dixon's (1974) length measurements for *C. hypochlorum* ($\bar{X}$ = 9.0–14 µm) but overlap with our average length measurements ($\bar{X}$ = 8.0–10.1, n = 193, collections NY 03423615, PUL F29705, PUL F29699, and PUL F29698).

Chlorosplenium cusucoense Stallman & Haelew., sp. nov. FIGS. 4 and 7

MycoBank MB851251

Typification: HONDURAS. DEPARTAMENTO DE CORTÉS: Parque Nacional Cusuco, Base Camp, site 6 of transect 1, 15.506543N, 88.215003W, 1694 m above sea level (a.s.l.), in mixed broadleaf forest on decomposed fallen log among bryophytes, 30 Jun 2019, *D. Haelewaters, A. Ward HONDURAS19-F050* (**holotype** PUL F29694). GenBank: ITS = OR653953; 28S = OR602721; *RPB1* = OR620543.

Diagnosis: Differs from all known cultured *Chlorosplenium* species by its brown-colored colony in age (>60 d) that produces crystals on PDA, from other short-spored ($\bar{X}$ < 8.0 µm long) species by its ascospores that frequently produce conidia, and by autapomorphies at positions 25, 31, 45, 56, 66, 394, 457, and 483 of the ITS nuc rDNA.

Etymology: Referring to Cusuco National Park, an intensely studied cloud forest ecosystem in northwestern Honduras where this species was discovered.

Description: Apothecia 1.0–3.0(–4.0) mm wide, scattered to gregarious, noncespitose, sessile to subsessile; at first globose and closed, then opening and expanding to turbinate–cupulate to discoid, often appressed to substrate or applanate with central depression in disc; margin entire, non-inrolled (inrolled in desiccation); disc glabrous, creamy/translucent yellowish-green (2A6–3A5), sometimes discoloring greenish-gray (1C7), darkening to dull yellowish-orange (4B5–6) when desiccated; receptacle glabrous and concolorous to, or paler than, disc, in desiccation with a sparse to well-developed fibril covering varying from cream to orangish-brown (~5C7) to gray, generally thicker at base, with or without whitish-gray basal tomentum; wood often stained dark near base of apothecium; sectioned apothecia releasing yellowish-green pigments into KOH medium. Asci (48.7–)56.0–68.6(–73.5) × (4.6-)5.0–6.0(–7.0) µm [38, 4, 2], cylindrical–clavate, tapering to a rounded to truncate base, with eight, biseriate ascospores, apical ring amyloid with or without KOH pretreatment, arising from croziers. Ascospores (4.9–)5.9–7.2(–8.8) × (1.8–)2.4–3.2(–3.7) µm [139, 4, 3], hyaline, oblong to elliptic, slightly tapering toward one or both ends, not to slightly curved in side view, with (0–)2(–3) small (<1 µm) guttules near poles; conidia commonly forming singly from ascospore ends, globose to ellipsoid, (1.4–)1.8–2.6(–3.2) × (1.2–)1.5–1.9(–2.2) µm, rarely multiple conidia forming on same end or simultaneously budding from both ends of ascospore. Paraphyses 1.1–2.7 µm wide, filiform, septate, and commonly branching near base. Medullary excipulum a t. intricata composed of hyaline hyphae 1.5–3.5 µm wide. Ectal excipulum a t. angularis-globulosa 15–81 µm thick, cells 4.8–16.1 × 2.9–9.5 µm wide, grading to t. prismatica at margin; interior region hyaline to light brown, cortical cells golden to darker brown and usually smaller, with hyphal covering (6–)10–25(–40) µm thick of hyaline to golden or brown, refractive, cylindrical, short-septate hyphae 2.5–3.5 µm wide, ±parallel to the ectal excipulum, with or without an additional layer of gelatinized t. intricata connecting base of ectal excipulum to substrate. Culture slow growing on PDA, ~10 mm wide after 50 d, producing a white to gray/brown (6C3–6E6) colony, dark brown in age; colony humped, fluffy, guttation of dark orangish-brown drops up to 2 mm wide common; colony with crystal depositions in agar and with fragile, crystalline apical projections up to 8 mm in length (after <30 d of growth); bottom of colony mostly dark brown (5F5–7) with areas of lighter browns and a lighter leading edge, staining surrounding agar light yellowish-green (~4A2); composed of short-septate, hyaline to brown hyphae; in upper areas hyphae generally cylindrical, 1.9–3.6 µm wide, sometimes aggregating into bundles; in lower areas hyphae often darker including chains of ellipsoid to globose–truncate cells 3.8–7.8 µm wide; apical crystalline projections 105–192 µm wide; sterile on PDA and CMA.

Habitat and distribution: Honduras, Mexico, and Panama, growing on corticated and decorticated wood in humid, montane forests containing *Quercus* spp. Potentially also present in Jamaica.

Additional specimens examined: HONDURAS. DEPARTAMENTO DE CORTÉS: Parque Nacional Cusuco, Cantiles, transect 2, 15.516472N, 88.229635W, 2193 m a.s.l., on rotting wet log, 24 Jun 2019, *D. Haelewaters, A. Ward HONDURAS19-F032* (PUL F29693); Base Camp, transect 4, 15.49464N, 88.21620W, 1628 m a.s.l., on well-decayed log, 19 Jun 2022, *J.K. Stallman HONDURAS22-F019* (PUL F29696); Base Camp, transect 4, 15.49464N, 88.21620W, 1628 m a.s.l., on decorticated wood, 22 Jun 2022, *J.K. Stallman HONDURAS22-F080* (PUL F29697); Base Camp, transect

3, on wood, 21 Jul 2022, *J.K. Stallman HONDURAS22-F429* (PUL F29702), culture DAOMC 252832; Base Camp, transect 3, 15.48579N, 88.21064W, 1417 m a.s.l., in broadleaf forest on decorticated wood among moss, 30 Jul 2022, *J.K. Stallman HONDURAS22-F497* (GENT, PUL F29692); Base Camp, El Danto trail, 15.49783N, 88.21515W, 1662 m a.s.l., on decorticated wood, 27 Jul 2022, *J.K. Stallman HONDURAS22-F480* (PUL F29695); Base Camp, transect 4, 15.49464N, 88.21620W, 1622 m a.s.l., on well-decayed log, 20 Jul 2023, *J.K. Stallman HONDURAS23-F467* (PUL F29703); Cantiles, transect 4 at ~600 m, on wood, 19 Jul 2022, *J.K. Stallman HONDURAS22-F416* (PUL F29701); Guanales, site 3 of transect 4, 15.48475N, 88.23454W, 1213 m a.s.l., on corticated wood, 2 Jul 2022, *J.K. Stallman, Z. Benefer, L. Brown, M. Gadaloff, M. Shahscott HONDURAS22-F242.2* (PUL F29700). JAMAICA. SAINT ANDREW PARISH: Cinchona Botanical Garden, 18.07066N, 76.655704W, 1153 m a.s.l., Sep 1906, *Underwood 3205* (CUP-D-8044 [77-29]); PROVINCIA DE CHIRIQUÍ: Boquete, Jaramillo Arriba, El Musgo trail, 8.791313N, 82.409557W, 1669 m a.s.l., in mixed montane forest with *Quercus* sp. and *Oreomunnea mexicana* on leaf litter and decomposing (decorticated) wood, 25 Jul 2022, *M. Cuevas, A.C. Grupe II, D. Haelewaters, T. A. Hofmann, J. Nuytinck, C.C. Perrotta, C.A. Quandt PANAMA22-F017* (GENT, UCH 15449); Reserva Forestal de Fortuna, in humid mixed montane rain forest with *Quercus* sp. on heavily decayed decorticated tree trunk, 29 Jul 2022, *M. Cuevas, A.C. Grupe II, D. Haelewaters, T.A. Hofmann, J. Nuytinck, C.C. Perrotta, C.A. Quandt PANAMA22-F125* (PUL F29706, UCH 15450).

Notes: Chlorosplenium cusucoense is known from twelve collections in Honduras, Mexico, and Panama at elevations 1100 to 2200 m a.s.l. This species has wider ascospores on average ($\bar{X}$ = 2.4–3.2 µm) than other short-spored ($\bar{X}$ < 8.0 µm) *Chlorosplenium* species, but values overlap with all except *C. sinicum* ($\bar{X}$ = 1.5–2.2) and *C. sinochlorum* ($\bar{X}$ = 1.8–2.2). It differs from *C. epimorsicum* by not growing out of old ascomata and from *C. hawaiiense* by its ectal excipulum covering up to 25(–40) µm wide (vs. 30(–60)). It differs from *C. chlora* by its culture that produces crystals, a feature not reported in a detailed culture description by Dixon (1974). Conidia attached to the apices of ascospores were found in 13.4% of all ascospores examined (n = 1382). One collection (PUL F29706) had amyloid hyphae connecting the base of the ectal excipulum to the substrate (Fig. 4; 2B, 4B), but this was not found in other apothecia or collections.

The northern range limit of *C. cusucoense* and southern range limit of *C. chlora* are unknown. Although *C. cusucoense* generally has wider spores and larger asci than *C. chlora*, these measurements and other features of the ascomata overlap. Currently, all collections of *C. chlora* confirmed by DNA sequence data occur above 35° latitude and all collections of *C. cusucoense* confirmed by DNA sequence data occur below 20° latitude. Dixon (1974) reported a collection of *C. chlora* from Jamaica (CUP-D-8044 [77-29]). Molecular sampling of this collection was not permitted, and an attempt to liberate spores from an apothecium resulted in only two spores in the mount, one with a conidium attached. Conidia have not been reported from *C. chlora* in the literature, nor were they found in specimens examined here (BDWR F0116, BDWR F0123, BDWR F0125, BDWR F0126, BDWR F0376, PUL F29704, PUL F29760, n = 230). The presence of conidia from ascospores and growth in a tropical, montane setting suggests this collection from Jamaica is *C. cusucoense. Chlorosplenium chlora* has also been reported from the state of Hidalgo, Mexico (Valenzuela et al. 2021). No DNA sequence data, the presence of conidia, nor a culture was reported for this collection. No Mexican material was examined morphologically in this study, but the ITS sequence from a collection from Veracruz (MG976227) shares 100% identity with the holotype sequence of *C. cusucoense* and was included in our phylogenetic reconstructions (FIG. 1; SUPPLEMENTARY FIG. 1).

Chlorosplenium epimorsicum P.R. Johnst. & Stallman, sp. nov. FIGS. 5 and 7

MycoBank MB851252

Typification: NEW ZEALAND. SOUTHLAND REGION: Stewart Island, Pryse Peak Track, 46.9374S, 168.0151E, on fallen wood of *Metrosideros umbellata*, 26 Apr 2002, *P.R. Johnston PRJ D1683, R. Leschen, S.R. Whitton, et al.* (**holotype** PDD 93100), culture ICMP 23732. GenBank: ITS = MW191762; 28S = OR567430.

Diagnosis: Differs from all other *Chlorosplenium* species by its propensity to develop ascomata directly on older ascomata, and by autapomorphies at positions 173, 366, 486, and 494 of the ITS nuc rDNA.

Etymology: Named for the Greek "epi" (on, upon) and Latin "mors" (death) because this species often grows on older, dead apothecia.

Description: Apothecia 0.7–2.0 mm wide (desiccated), gregarious, sometimes cespitose, sessile; growing from woody substrate or directly from the disc of older apothecia; at first globose and closed, then opening and expanding to turbinate then discoid–cupulate; margin

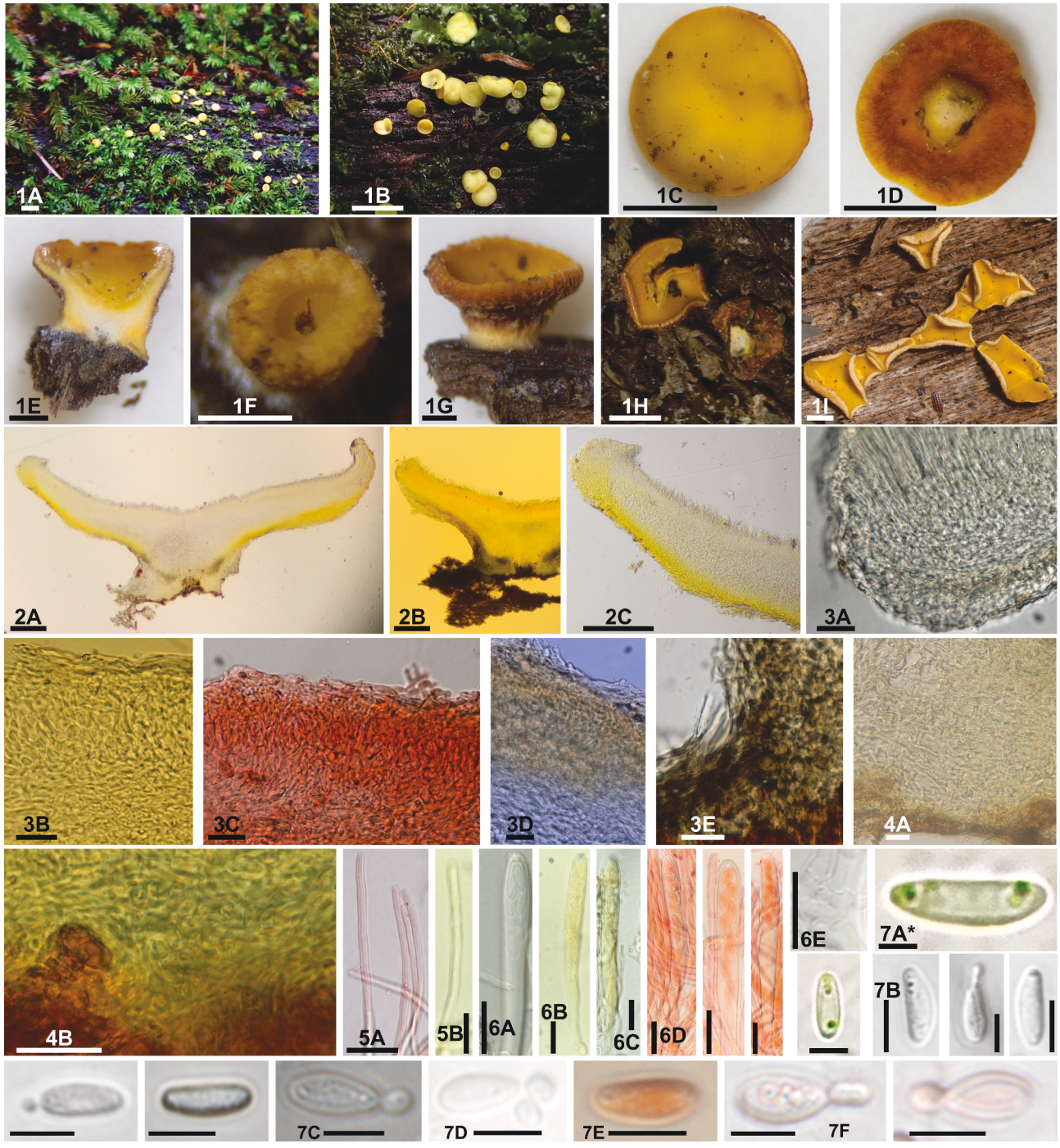

Figure 4. *Chlorosplenium cusucoense*. Images from PUL F29694 (holotype) unless otherwise stated. 1. Fresh (A–B), rehydrated (C–D), and desiccated (E–I) ascomata; bars = 5.0 mm (A–B), 1.0 mm (C–D, H–I), and 250 µm (E–G); B. PUL F29703. I. PUL F29693. 2. Sections in KOH (A, C) and MLZ (B); bars = 250 µm; A–C. PUL F29706. 3. Ectal excipulum margin in KOH (A), middle to upper flanks and medullary excipulum in MLZ (B), middle to lower flanks in CR+KOH (C), and lower flanks and medullary excipulum (D) and base (E) in KOH; bars = 20 µm; A, E. PUL F29706. 4. Hyphae connecting ectal excipulum base to substrate in KOH (A) and MLZ (B); bars = 20 µm; A–B. PUL F29706. 5. Paraphyses in CR+KOH (A) and MLZ (B); bars = 10 µm; A. PUL F29706. 6. Asci in KOH (A, E), MLZ (B, C), and CR+KOH (D); bars = 10 µm; C. PUL F29693. 7. Ascospores in H_2O (A*, B), KOH (C, D), and KOH+CR (E, F); bars = 5 µm; A. PUL F29692. C. F PUL F29693.

entire, inrolled; disc glabrous, yellow, in desiccation yellow (4A5) to yellowish-greenish-gray (~4B3), uncommonly with gray discolorations; receptacle greenish, darker at base, in desiccation upper flanks concolorous to slightly darker than disc, then grayish-yellow (4C2–3) to gray, continuing to darken toward base, with matted fibrils with colors varying as in the receptacle (yellowish to dark gray), with or without hyaline to gray basal tomentum; wood often stained dark near base of apothecium; sectioned apothecia releasing yellowish

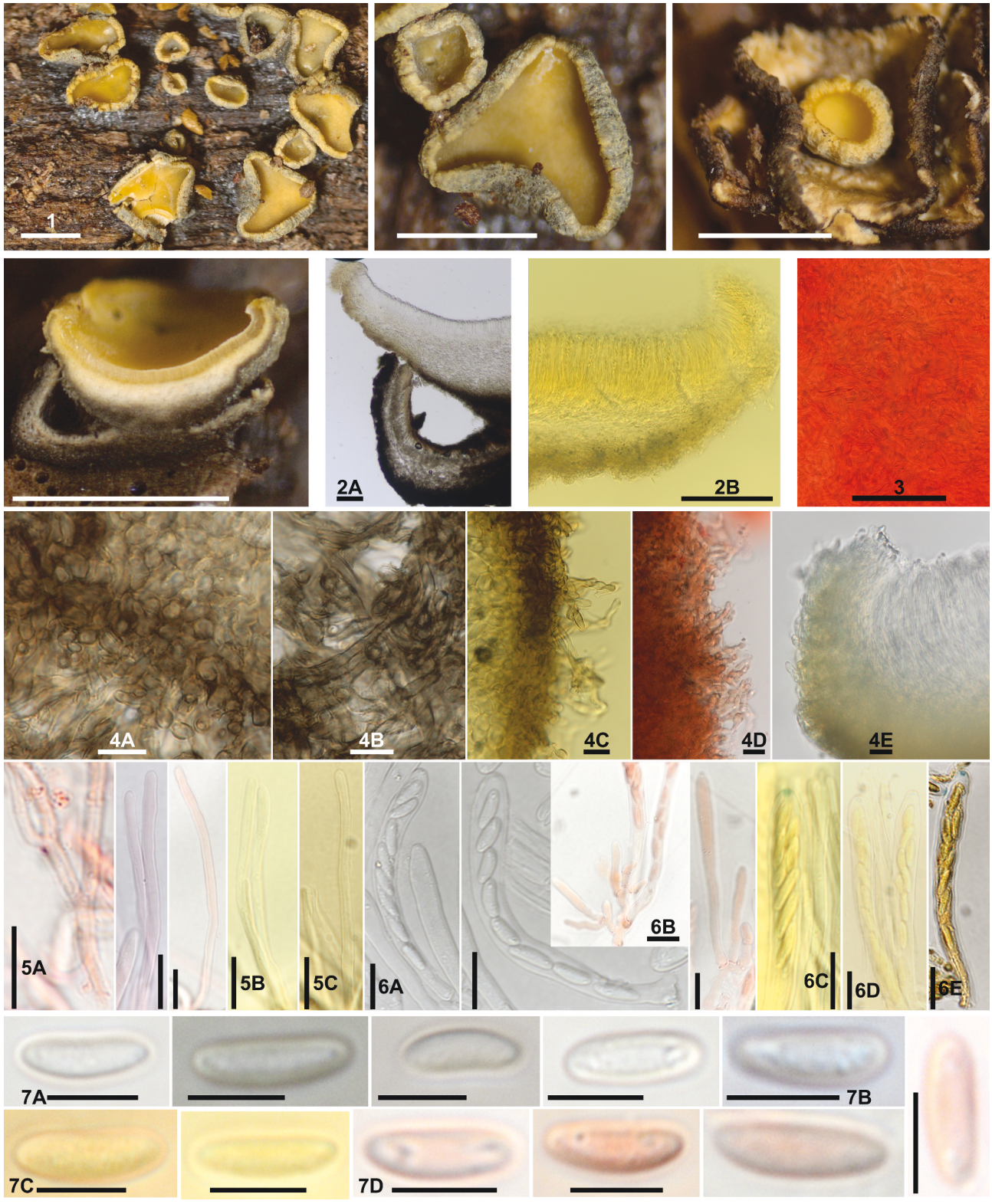

Figure 5. *Chlorosplenium epimorsicum*. Images from PDD 93100 (holotype). 1. Dried ascomata on substrate; bars = 1 mm. 2. Sections in KOH (A) and MLZ (B); bars = 100 µm. 3. Medullary excipulum in CR+KOH; bar = 50 µm. 4. Ectal excipulum base (A) and hyphae connecting base to substrate (B) in KOH, lower flanks (C) in MLZ, upper flanks in CR+KOH (D), and margin in H_2O (E); bars = 10 µm. 5. Paraphyses in CR+KOH (A), MLZ (B), and IKI+KOH (C); bars = 10 µm. 6. Asci in KOH (A), CR+KOH (B), MLZ (C), IKI+KOH (D), and IKI (E); bars = 10 µm. 7. Ascospores in KOH (A) H2O (B), MLZ (C), and CR+KOH (D); bars = 5 µm.

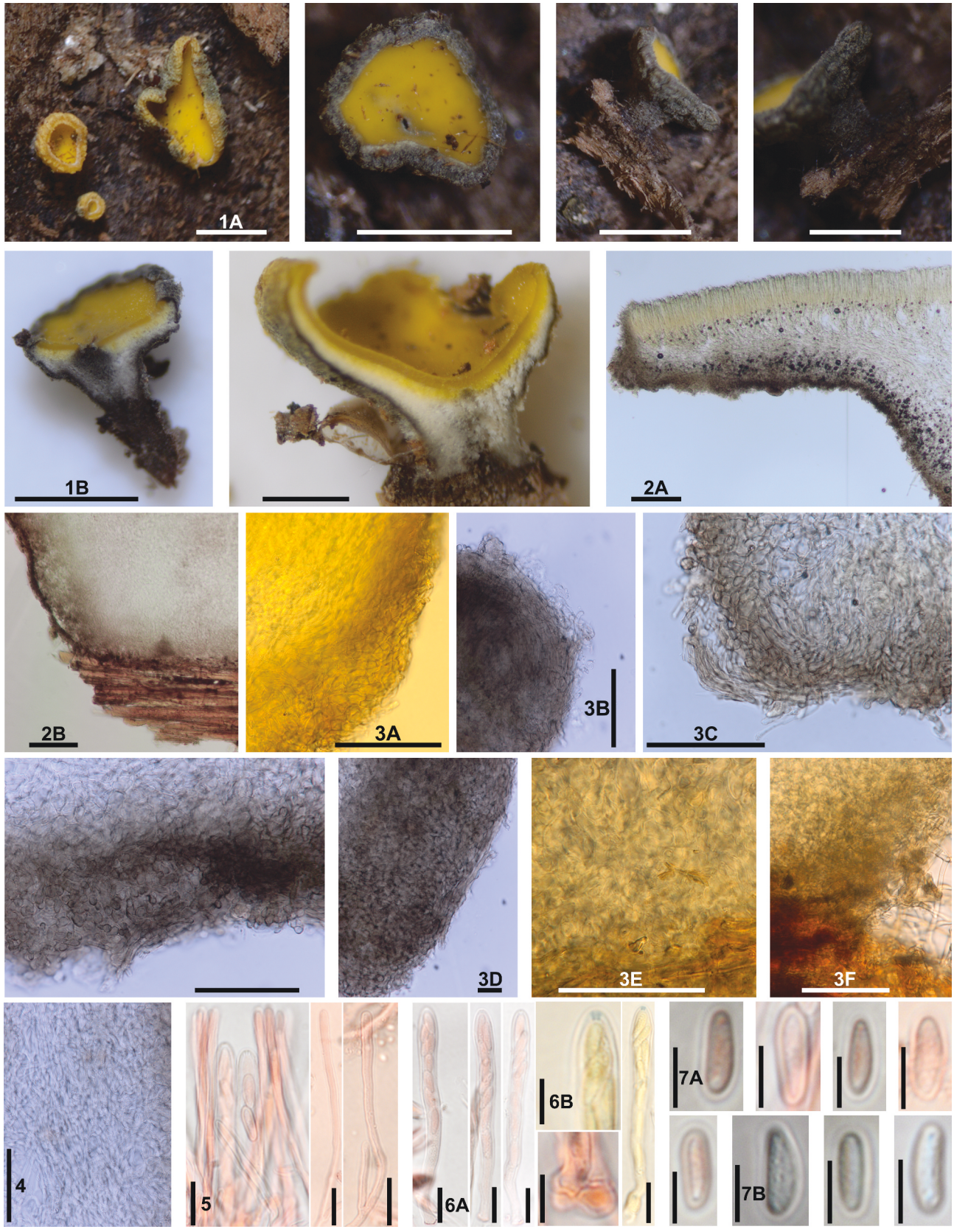

Figure 6. *Chlorosplenium hawaiiense*. Images from ARIZ-M-AN00721 (holotype). 1. Desiccated (A) and rehydrated (B) ascomata; bars = 1 mm. 2. Sections in MLZ (A) and KOH (B); bars = 100 µm. 3. Ectal excipulum upper flanks and margin in MLZ (A) and KOH (B), middle flanks (C) and lower flanks (D) in KOH, and base (E) and hyphae connecting base and substrate (F) in KOH; bars = 50 µm. 4. Medullary excipulum in KOH; bar = 50 µm. 5. Paraphyses in KOH+CR; bars = 10 µm. 6. Asci in KOH+CR (A) and MLZ (B); bars = 10 µm. 7. Ascospores in KOH+CR (A) and KOH (B); bars = 5 µm.

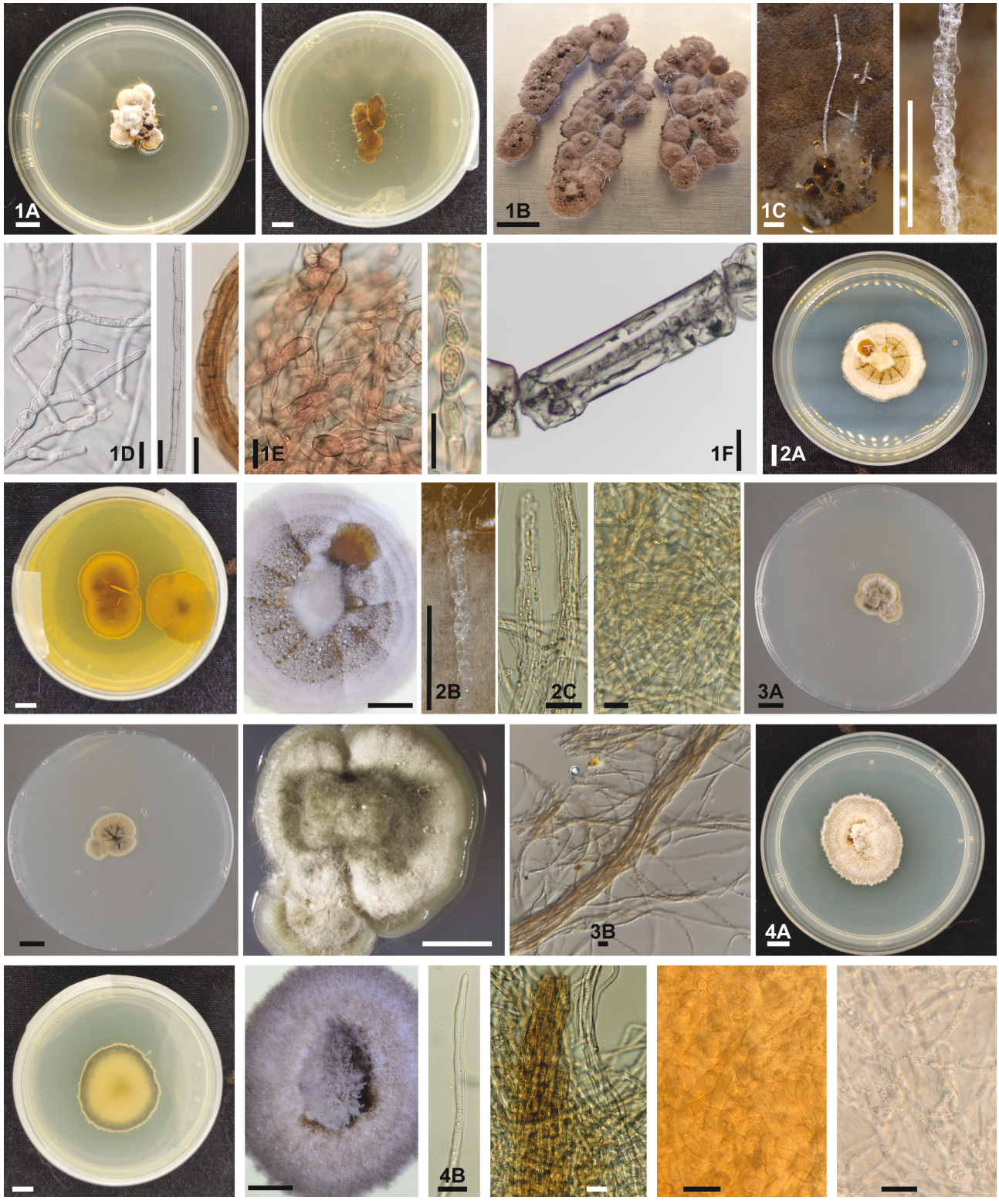

Figure 7. Morphology of cultures. 1. *Chlorosplenium cusucoense* (DAOMC 252832) morphology after 50 d (A, D–F), 110 d (different culture; B), 60 d (different culture; C); microscopy in H_2O (D, F) and MLZ (E); bars = 10 mm (A–B), 1 mm (C), 10 µm (D–E), and 100 µm (F). 2. *Chlorosplenium aotearoa* (ICMP 23733), morphology after 50 d (A, C) and 130 d (different culture; B); microscopy in H_2O; bars = 10 mm (A), 1 mm (b), and 10 µm (C). 3. *Chlorosplenium epimorsicum* (ICMP 23732, ex-holotype), morphology after 40 d; microscopy in LA; bars = 10 mm (A) and 10 µm (B). 4. *Chlorosplenium australiense* (ICMP 23736) morphology after 50 d; microscopy in H_2O; bars = 10 mm (A) and 10 µm (B).

pigments into KOH medium. Asci (48.1–)54.5–62.2(–68.5) × (4.1–)4.5–5.9(–7.5) µm [63, 4, 1], cylindrical–clavate, tapering gradually to a usually blunt, subtruncate, base, with eight, uniseriate to biseriate ascospores, apical ring amyloid with or without KOH pretreatment, arising from croziers. Ascospores (5.3–)6.4–7.9(–9.5) × (1.7–)2.0–2.6(–3.3) µm [98, 3, 1], hyaline, oblong, slightly tapering at ends and curved in side view, with 2 (0–3) small (<1 µm) guttules near poles. Paraphyses 1.5–2.5 µm wide, filiform, equal to slightly tapering to base, septate, and commonly branching near base. Medullary excipulum a t. intricata composed of hyaline to light brown hyphae 1.5–3.8 µm wide. Ectal excipulum composed of t. angularis-globulosa 34–96 µm wide through base and flanks, thinning and sometimes grading to t. prismatica at margin; cells 4.4–12.5 × 2.9–10.3 µm, hyaline to light brown in inner region, darkening to brownish-green in outer layer, with a covering of hyaline to dark brownish-green, refractive, cylindrical hyphae 1.8–3.1 µm wide, sometimes with ellipsoid elements interspersed and subcapitate or clavate terminal elements, extending 10–30(–45) µm from ectal excipulum wall, with or without an additional layer of gelatinized t. intricata connecting base of ectal excipulum to substrate. Culture slow growing on PDA, ~7–12 mm wide after 4 wk; aerial mycelium sparse, fine, grayish, some aggregated into fine, synnemata-like upright strands; surface of colony convoluted, dark gray with hyaline to yellowish margin; bottom of colony dull creamy brown, cracking in places to reveal the darker pigments of the colony upper surface; hyphae 2.5–3.5 µm wide, septate, thin-walled, in places with small, crystal-like encrustations; some hyphae with brown vacuolar pigment, especially within synnemata-like strands, strands aggregating up to 80 µm thick, tapering narrower toward apex; sterile.

Habitat and distribution: New Zealand, growing on corticated or decorticated *Metrosideros umbellata* and *Nothofagus cliffortioides*.

Additional specimens examined: NEW ZEALAND. CANTERBURY REGION: Kaikōura, Mt. Lyford Avenue, 42.485S, 173.151E, on rotten bark of *Nothofagus cliffortioides*, 3 Jul 2022, *J.A. Cooper 17304* (PDD 114414); ibid., 9 Mar 2022, *J.A. Cooper 17156* (PDD 114266).

Notes: Chlorosplenium epimorsicum is known from three collections in New Zealand. It differs from other species in the genus in that apothecia commonly develop from the center of dead ascomata of what are presumed to be the same thallus. Beyond this aspect, other morphological features overlap with those of the other small-spored *Chlorosplenium* species such as *C. aotearoa, C. chlora*, and *C. sinochlorum*. In comparison with *C. aotearoa*, which also occurs in New Zealand, *C. epimorsicum* appears to be less common, has a more southern distribution, grows from *Metrosideros* wood in addition to *Nothofagus*, and does not produce crystals in culture.

Chlorosplenium hawaiiense Stallman, sp. nov. FIG. 6
MycoBank MB851253

Typification: USA. HAWAIʻI: Hawaiʻi Island, Hawaiʻi Volcanoes National Park, Kīpuka Kī, 1300 m a.s.l., on *Acacia koa*, 24 Oct 1991, *R.L. Gilbertson 18723* (**holotype** ARIZ-M-AN00721). GenBank (isolate ARIZ-M-AN00721Q): ITS = OR602793; 28S = OR602726. GenBank (isolate ARIZ-M-AN00721O): ITS = OR653946.

Diagnosis: Differs from other *Chlorosplenium* species by the hyphal covering extending up to 60 µm from the flanks of the ectal excipulum, from other short-spored ($\bar{X}$ < 8.0 µm) species by its stipe up to 0.5 mm long, and by an autapomorphy at position 469 of the ITS nuc rDNA.

Etymology: Named for the Hawaiian Islands, the only known locality of this taxon.

Description: Apothecia 0.8–3.1 mm wide (desiccated), scattered to gregarious, noncespitose, sessile to short-stipitate; at first globose and closed, then opening and expanding to turbinate then cupulate; margin entire, inrolled; central stipe up to 0.5 mm long; disc glabrous, bright orangish-yellowish-green (5A7–B7), sometimes with light grayish discoloration in older examples; receptacle slightly duller and darker than disc, with sparse hyaline to yellowish fibrils when young, darkening in age from dull oranges to warm grays (14C2–F2) to dark gray or black, particularly toward base, developing a fibrillose appearance from hyaline to grayish-brown covering, often with hyaline to dark gray basal tomentum; wood often stained dark near base of apothecium; sectioned apothecia releasing yellowish pigments into KOH medium. Asci (49.1–)51.8–61.3(–67.8) × (3.6–)4.5–5.6(–6.0) µm [33, 3, 1], cylindrical–clavate, tapering to slightly enlarged, or not, rounded to subtruncate base, with eight, uniseriate to biseriate ascospores, apical ring amyloid with or without KOH pretreatment, arising from croziers. Ascospores (5.6–)6.3–7.5(–8.5) × (1.7–)2.0–2.6(–3.2) µm [63, 3, 1], hyaline, oblong, slightly tapering to one or both ends, sometimes slightly curved in side view, generally aguttulate, sometimes with two small (<1 µm) guttules visible near poles. Paraphyses 1.0–2.5 µm wide, filiform, septate, and commonly branching near base. Medullary excipulum a t. intricata composed of loose, hyaline to light brown hyphae, sometimes with darker discolorations, 1.1–3.3 µm wide, becoming tighter packed and yellowish in the subhymenium. Ectal excipulum a t.

angularis-globulosa-prismatica 35–123 µm thick, upper flanks and margin usually of t. prismatica cells 5.6–15.0 × 2.4–5.4 µm, lower flanks and base of t. angularis-globulosa cells 3.4–11.2 × 3.0–9.5 µm, generally larger and hyaline to light brown in interior region, darker brown and smaller in exterior region, with an exterior covering of hyaline to dark brown, short-septate, refractive, cylindrical hyphae ±parallel to the ectal excipulum, with groups of hyphae extending 15–30(–60) µm from ectal excipulum from base to upper flanks; individual hyphae 1.6–2.6 (–3.0) µm wide, uncommonly with ellipsoid–clavate elements interspersed; with or without an additional layer of gelatinized t. intricata connecting base of ectal excipulum to substrate. Culture unknown.

Habitat and distribution: Hawai'i Island, Hawai'i, USA, growing on corticated or decorticated *Acacia koa*.

Notes: Chlorosplenium hawaiiense is known from a single collection. Compared with other short-spored species ($\bar{X}$ < 8.0 µm), *C. hawaiiense* lacks long hairs at the margin as in *C. sinicum* and does not grow from decaying apothecia like *C. epimorsicum*. It differs from *C. sinochlorum, C. aotearoa*, and *C. cusucoense* by its well-developed t. prismatica in the ectal excipulum, and from all these species by its hyphal covering extending up to 60 µm from the receptacle wall (14 µm in *C. sinochlorum*, 30 µm in *C. chlora* and *C. aotearoa*, and 45 µm in *C. sinicum*). It also differs from all other species, except *C. cenangium*, by having a stipe up to 0.5 mm long. *Chlorosplenium hawaiiense* was collected on the wood of *Acacia koa*, a tree only known from the Hawaiian archipelago and Réunion (Le Roux et al. 2014).

We attempted to examine other collections identified as *Chlorosplenium* spp. in Hawai'i. Unfortunately, BISH 741599 and CUP-063505, both collected in Waimea Canyon State Park on the island of Kaua'i associated with *Pinus elliottii* and identified as *C. chlora*, either could not be found (pers. comm., B. Kennedy, BISH, 12 Sep 2022) or were not permitted for loan due to insufficient material (pers. comm., K. T. Hodge, CUP, 13 Apr 2022). More collections of *Chlorosplenium* species in the Hawaiian Islands, including from different islands and different host trees, will help clarify whether one or multiple species are present, and whether these species are widespread generalists or specific to certain islands or substrates.

DISCUSSION

***Species and molecular diversity in the genus* Chlorosplenium.—**Through a combination of fieldwork, study of material from fungaria, and integrative taxonomy, we formally describe five new species of *Chlorosplenium*, doubling the number of known taxa. All new species were noted in prior publications, either recorded as the putatively widespread *C. chlora* (Wong and Korf 2009) or as undescribed taxa (Haelewaters et al. 2021b; Johnston 2020).

Zheng and Zhuang (2021) reported that species of *Chlorosplenium* have overlapping morphological features, and we agree. Two of the five species described here are distinct due to disc color (*C. australiense*) or growth from old apothecia (*C. epimorsicum*). However, many morphological features of *C. aotearoa, C. cusucoense*, and *C. hawaiiense* overlap with each other and with *C. chlora* and *C. sinochlorum*. Despite this, no more than two *Chlorosplenium* species are currently known from a single, discrete location, and co-occurring species have distinct morphological features of ascomata or cultures (TABLE 3). Therefore, *Chlorosplenium* species with overlapping morphologies can be considered semicryptic species (sensu Mann and Evans 2008) in that they can be identified by morphology when their collection locality is known. The term semicryptic species is commonly used to characterize lichenized fungi (e.g., Coca et al. 2018), although in these cases the definition is expanded to include differing ecologies in addition to distinct distributions (Vondrák et al. 2009).

Metagenomic sequencing recovered target reads from two of four taxa attempted (50%). Recent collections (1971, 1991) were more successful than older collections (before 1857) in recovering ASVs from the target organism. Our success rate falls between the 64% from Forin et al. (2018) for 36 specimens collected in the late 1800s identified as *Peziza* and the 25% from Miller et al. (2022) for both newer and older specimens across Ascomycota. It is difficult to draw conclusions based on such small samples sizes except that it is possible to obtain sequences from fungarium specimens with variable success rates. For example, although we could not generate an ITS1 sequence for one *C. hawaiiense* isolate (ARIZ-M-AN00721O), collected in 1991, with Sanger or Illumina sequencing, we obtained a full-length ITS sequence for *C. hypochlorum* (CUP-52724), collected in 1909, via Sanger sequencing without deviations from our standard protocol. Although Zheng and Zhuang (2021) found high diversity in the ITS of *C. sinicum*, our phylogenetic analysis shows short branch lengths and therefore limited intraspecific diversity in isolates of *C. chlora, C. hypochlorum*, and all of our new species except *C. hawaiiense*, for which only one collection is known (FIG. 1; SUPPLEMENTARY FIG. 1).

Chlorosplenium chlora *and* C. hypochlorum.—We report *C. chlora* from Europe with evidence from ITS and 28S sequences, confirming several unpublished

reports of this taxon occurring in the region from citizen science repositories and forums (e.g., ASCOfrance.fr, iNaturalist.org). Sequences of the 28S from the Serbian collection (TUF 104917) and two Spanish collections (ARAN 00432, ARAN 03087) share 100% similarity with each other and with six isolates from the midwestern and eastern United States. Sequences of the ITS from these collections share 100% sequence similarity with most *C. chlora* isolates from North America, although all European collections contain an insertion of eight nt (5′-CTGCGGAA-3′) in the ITS2 not present in any other isolates in our dataset. There is little diversity within the nuc rDNA and *RPB1* regions of *C. chlora* isolates across seven American states in the midwestern and eastern USA. All 28S and *RPB1* sequences available have 100% identity, whereas minor divergences in the ITS region only occur in three isolates and these are all in locations with at least four mononucleotide repeats and likely represent sequencing or editing errors.

We were unable to recover regions of the ITS from one of the isotype specimens of *C. chlora* (CUP-D-3882 [77-17]) via Sanger or Illumina sequencing. Dixon (1974) considered the type locality of *C. chlora* to be Bethlehem in Northampton County, Pennsylvania, but a biography of Schweinitz (Shear and Stevens 1917) states that he was living (and collecting) around Salem, North Carolina, from 1812 to 1817 and 1819 to November 1821, then moved to Bethlehem, Pennsylvania. *Peziza chlora* (the basionym of *C. chlora*) was described in 1822, and a reprint of his book from the same year is entitled "Synopsis fungorum Carolinae superiori" (van Schweinitz et al. 1822). Fries' Systema Mycologicum from the same year states the taxon "Frequens ad latera interiora truncorum Carolinae" (Fries 1822). Both Fries' statement and Schweinitz's title refer to a Carolina location. Our sampling of *C. chlora* includes both collections from Northampton County, Pennsylvania (BDWR F0125), and Lincoln County, North Carolina (BDWR F0126), ~100 km from Salem.

A field guide to North American ascomycetes (Beug et al. 2014) lists *Chlorosplenium olivaceum* Seaver occurring in Georgia and North Carolina with more green coloration and longer spores (9–10 µm) than *C. chlora*. Dixon (1974) synonymized this species with *C. chlora* after examining the isotype (CUP-051722), stating that it was similar in all respects to *C. chlora* besides its somewhat darker coloration. Although Dixon reported *C. chlora* from southeastern Asia and Japan, several new species have been described since that time that closely resemble *C. chlora* and have closer geographic affinities to these regions, such as *C. sinochlora*. Therefore, collections from these localities should be examined considering the totality of evidence from morphology of ascomata, morphology of cultures, and DNA sequence data for identification.

We were unable to recover regions of the ITS from the type specimen of *C. hypochlorum* (K-M 1434235). In addition to previous reports from Cuba, Jamaica, and Mexico, *C. hypochlorum* is here confirmed from Honduras, Panama, and Venezuela. In notations attached to the type specimen of *C. hypochlorum* (K-M 1434235), Dixon describes the ascospore length as 14–18(–20) µm, but later states ascospores are (8–)9–14(–15) µm long (Dixon 1974). In the three collections we examined, we found smaller spores, on average 8.0–10.1 µm in length.

The ectal excipulum of* Chlorosplenium *species.— The color and overall appearance of the receptacle and the underlying ectal excipulum of *Chlorosplenium* species is useful in recognizing this genus. The majority of the ectal excipulum is composed of t. angularis-globulosa that grades to t. prismatica at the margin; t. prismatica may also occur in the flanks, particularly in *C. hawaiiense*. The inner region of the ectal excipulum, closer to the medullary excipulum, is lighter in color than the outer region. The ectal excipulum is covered by ±cylindrical hyphae up to ~30 µm wide, although some species have potentially thicker coverings, such as *C. sinicum* (up to 90 µm at margin, 45 µm at flanks), *C. hawaiiense* (up to 60 µm), and *C. epimorsicum* (up to 45 µm). Although *Chlorosplenium* species usually appear glabrous when fresh, this changes in age and with desiccation. When desiccated, the underlying receptacle and cryptic hyphal covering both change colors, usually darkening to grays or deeper oranges. The hyphal covering, which usually adheres to the ectal excipulum when fresh, often pulls away from the cortical cells in an irregular manner, so some areas remain inconspicuous and appressed whereas other regions appear fibrillose, particularly under magnification with a hand lens or dissecting microscope (~10×). This is likely what leads to descriptions of "glabrous" specimens when fresh that may appear fibrillose when desiccated. For example, *C. chlora* is described as "slightly furfuraceous" (Seaver 1951, as *C. olivaceum*), whereas Dixon (1974) describes the receptacle as glabrous.

One of Dixon's (1974) differentiating features between *C. chlora* and *C. hypochlorum* besides spore size is the easy separation of the hymenium from the receptacle in *C. hypochlorum*. We did not observe this

separation in recent collections of *C. hypochlorum* (PUL F29705, PUL F29699, and PUL F29698), nor in older material examined (NY 03423615).

Conidia and cultural characters.—*Chlorosplenium cusucoense* frequently produces conidia from its ascospores (incidence rate of 13.4%, n = 1382), as is known from species in other genera in Leotiomycetes, such as *Ascocoryne* (Quijada et al. 2017) and *Micraspis* (Quijada et al. 2020). Additionally, one collection of *C. hypochlorum* (PUL F29705) had 12.2% of ascospores with conidia attached (n = 279), whereas others (PUL F29698, PUL F29699) had none (n = 165). *Chlorosplenium aotearoa* had an incidence rate of 0.8% of ascospores with conidia attached (n = 590). Conidia were not found in collections of *C. australiense, C. chlora, C. epimorsicum*, or *C. hawaiiense*, nor have they been reported from any other *Chlorosplenium* species. Conidia are always found on ejected ascospores, never within asci, and we did not find an increased incidence of conidia in overmature (vs. mature) ascomata of *C. cusucoense*. Although most species examined here are consistently with or without conidia, the variability observed between collections of *C. hypochlorum* shows that this detail, on its own, should not be used to identify species.

In culture, most *Chlorosplenium* species appear as slow-growing and zonate, with fluffy, lighter hyphae over a brownish colony that may darken in age and may or may not have yellow to brown guttation. Microscopically, all species have examples of hyaline to brown, cylindrical hyphae that are diffuse or can be bundled. Some species (*C. australiense, C. chlora, C. cusucoense*) produce ellipsoid and inflated cells in chains, particularly in the brown-pigmented region of the colony, whereas others (*C. aotearoa, C. epimorsicum*) do not. Both *C. aotearoa* and *C. cusucoense* excrete macroscopic crystals from their colonies in culture, but *C. cusucoense* produces them sooner (<30 d vs. >30 d), has darker (brown vs. white to cream) colonies in age, and has chains of inflated ellipsoid cells that *C. aotearoa* lacks. Some hyphae of *C. epimorsicum* in culture appear encrusted with what may be tiny (<1 μm) crystals. Cultural characteristics for *C. cenangium, C. hawaiiense, C. sinicum*, and *C. sinochlorum* are unknown, except that *C. cenangium* is sterile (Müller 1977).

CONCLUSION

The genus *Chlorosplenium* is more diverse than previously understood. As Zheng and Zhuang (2021) predicted, more in-depth studies incorporating molecular phylogenetic data have uncovered additional species, including specimens that had previously been identified as *C. chlora*. Although all species are readily identifiable by a mixture of morphology and biogeography, our knowledge of species ranges is incomplete, and the presence of additional species is possible. Therefore, incorporating DNA sequence data, when possible, will be important in clarifying species ranges to verify the utility of biogeography in assisting with species identifications.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

DATA AVAILABILITY STATEMENT

Additional specimen metadata, figures, and final alignments can be found in SUPPLEMENTARY FILES 1–6 and SUPPLEMENTARY FIG. 1. Newly generated sequences

were submitted to the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers indicated in TABLE 1.

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