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Phylogeny of *Cyttaria* inferred from nuclear and mitochondrial sequence and morphological data

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Abstract: *Cyttaria* species (Leotiomycetes, Cyttariales) are obligate, biotrophic associates of *Nothofagus* (Hamamelididae, Nothofagaceae), the southern beech. As such *Cyttaria* species are restricted to the southern hemisphere, inhabiting southern South America (Argentina and Chile) and southeastern Australasia (southeastern Australia including Tasmania, and New Zealand). The relationship of *Cyttaria* to other Leotiomycetes and the relationships among species of *Cyttaria* were investigated with newly generated sequences of partial nucSSU, nucLSU and mitSSU rRNA, as well as *TEF1* sequence data and morphological data. Results found *Cyttaria* to be defined as a strongly supported clade. There is evidence for a close relationship between *Cyttaria* and these members of the Helotiales: *Cordierites*, certain *Encoelia* spp., *Ionomidotis* and to a lesser extent *Chlorociboria*. Order Cyttariales is supported by molecular data, as well as by the unique endostromatic apothecia, lack of chitin and highly specific habit of *Cyttaria* species. Twelve *Cyttaria* species are hypothesized, including all 11 currently accepted species plus an undescribed species that accommodates specimens known in New Zealand by the misapplied name *C. gunnii*, as revealed by molecular data. Thus the name *C. gunnii* sensu stricto is reserved for specimens occurring on *N. cunninghamii* in Australia, including Tasmania. Morphological data now support the continued recognition of *C. septentrionalis* as a species separate from *C. gunnii*. Three major clades are identified within *Cyttaria*: one in South America hosted by subgenus *Nothofagus*, another in South America hosted by subgenera *Nothofagus* and *Lophozonia*, and a third in South America and Australasia hosted by subgenus *Lophozonia*, thus producing a non-monophyletic grade of South American species and a monophyletic clade of Australasian species, including monophyletic Australian and New Zealand clades. *Cyttaria* species do not sort into clades according to their associations with subgenera *Lophozonia* and *Nothofagus*.

Key words: Encoelioideae, Leotiomycetes, *Nothofagus*, southern hemisphere

INTRODUCTION

Species belonging to *Cyttaria* (Leotiomycetes, Cyttariales) have interested evolutionary biologists since Darwin (1839), who collected on his *Beagle* voyage their spherical, honeycombed fruit bodies in southern South America (FIG. 1). His collections of these obligate, biotrophic associates of tree species belonging to genus *Nothofagus* (Hamamelididae, Nothofagaceae) became the first two *Cyttaria* species to be described (Berkeley 1842, Darwin 1839). Hooker reported to Darwin a third species from *Nothofagus* trees in Tasmania (Berkeley 1847, 1848; Darwin 1846). Over time *Cyttaria* species have been shown to be restricted to *Nothofagus* trees in southern South America (Argentina and Chile) and southeastern Australasia (southeastern Australia, including Tasmania, and New Zealand).

Cyttaria species are presumed to be weak parasites (Gamundí and Lederkremer 1989) that produce trunk and branch cankers on *Nothofagus* trees. Two types of cankers generally are produced (Gamundí 1971, Rawlings 1956): globose ones that arise from growth mainly in the transverse axis of the branch and longitudinal ones that arise from growth mainly along the long axis.

A typical mature fruit body of a *Cyttaria* species consists of what may appear to be an orange, pitted ascoma, somewhat similar to a morel or a deeply dimpled golf ball. However each fruit body is actually composed of sterile fungal tissue, the stroma, in which apothecia are immersed. The stromata typically have a fleshy-gelatinous consistency, but those of some species are gummy or slimy. As the stromata develop, apothecia form beneath a membrane that envelopes the fruit body. At maturity this membranous ectostroma peels away to reveal, depending on the species and the stroma, 1–200 apothecia, each lined with asci. The eight-spored asci are inoperculate with *Bulgaria inquinans*-type ascus apices (Mengoni 1986) that possess an annulus that stains blue in iodine. Ascospores are uninucleate (Mengoni 1986), subglobose to ovoid, smooth to rugulose, at first hyaline to yellowish but later becoming pigmented,

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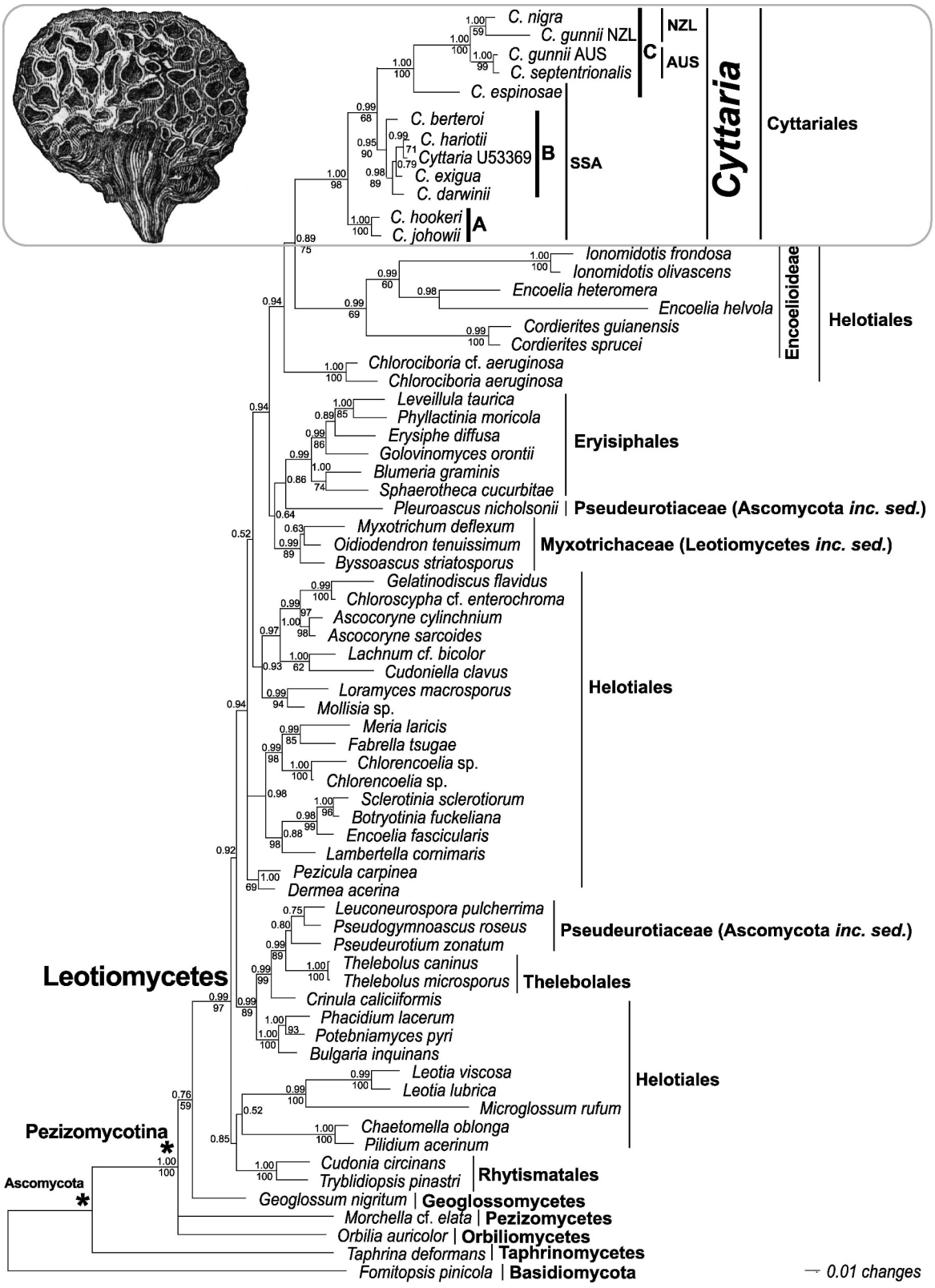

FIG. 1. Bayesian tree from the combined molecular dataset showing the monophyly of *Cyttaria* and its relationship to members of Leotiomycetes. Numbers associated with nodes represent posterior probabilities from BI analyses (above branches) and >50% bootstrap support from P analyses (below branches). The illustration from Darwin (1846) is reproduced courtesy of the library of the Gray Herbarium, Harvard University, Cambridge, Massachusetts. Asterisks are discussed in Peterson et al. (2010). AUS = Australia, NZL = New Zealand, SSA = southern South America.

are actively discharged, producing a dark gray to black spore print. In some species, before forming apothecia, the stromata produce pycnidia in which monoblastic, uninucleate (Mengoni 1989), haploid mitospores, or conidia, are produced in basipetal succession from conidiophores. The function of these mitospores has not been confirmed, but they have been proposed to be involved in sexual reproduction (Gamundí 1971, Minter et al. 1987).

The 11 currently accepted species of *Cyttaria* (Gamundí 1971, 1991; Gamundí et al. 2004; Rawlings 1956) are obligate, biotrophic associates of all 11 species of *Nothofagus* subgenera *Lophozonia* and *Nothofagus*. The relationship between *Cyttaria* species and *Nothofagus* hosts often is cited as a classic example of cospeciation, and because of this well known association it is one of the few cases where the biogeography of a fungus is commonly mentioned. This is despite the fact that the associations between species of *Cyttaria* and *Nothofagus* usually do not correspond in a simple one to one relationship; several *Cyttaria* species may infect the same *Nothofagus* species and a single *Cyttaria* species may infect several *Nothofagus* species (TABLE I).

Relationships within Nothofagus.—*Nothofagus* is one of the few southern hemisphere taxa for which a robust fossil record and well studied phylogeny exist (Jordan and Hill 1999) and often is included in biogeographic studies (e.g. Cook and Crisp 2005, Heads 2006, Knapp et al. 2005, Swenson et al. 2001). It comprises 35 extant species divided into four subgenera (Dettmann et al. 1990, Hill and Jordan 1993, Hill and Read 1991): subgenus *Brassospora* with 19 species in New Caledonia and New Guinea, from which no *Cyttaria* species have been recorded; subgenus *Fuscospora* with five species in South America and Australasia, from which no *Cyttaria* species have been recorded; subgenus *Lophozonia* with six species in South America and Australasia, all which host *Cyttaria* species; and subgenus *Nothofagus* with five species in South America, all which host *Cyttaria* species. Seven *Cyttaria* species are endemic to southern South America (Chile and Argentina) on subgenera *Lophozonia* and *Nothofagus*, and the other five are endemic to southeastern Australasia (southeastern Australia and New Zealand) on subgenus *Lophozonia*.

Relationship of Cyttaria *to other Leotiomycetes.*—The nature of the phylogenetic relationship of *Cyttaria* to its closest relatives remains relatively unclear, which, along with its unusual compound fruit bodies, specialized habit and lack of cell-wall chitin (Oliva et al. 1986), further obscure its phylogenetic affinities. Although generally regarded to be so distinct as to justify placement in its own order (Carpenter 1976, Eriksson and Hawksworth 1986, Gamundí 1971, Gernandt et al. 2001, Kimbrough 1970, Korf 1973, Luttrell 1951, Rifai 1968), from the description of the first *Cyttaria* species (Berkeley 1842), taxonomists often have hypothesized relationships of *Cyttaria* with taxa belonging to Helotiales (Pezizomycotina, Leotiomycetes). In early molecular studies *Cyttaria*, represented by a single published sequence (Landvik and Eriksson 1994), grouped with other Leotiomycetes, including members of Erysiphales, Helotiales, Rhytismatales, Thelebolales and Myxotrichaceae (Leotiomycetes incertae sedis), as well as members of Pseudeurotiaceae (Ascomycota incertae sedis) (Döring and Triebel 1998, Gernandt et al. 2001, Landvik and Eriksson 1994, Landvik et al. 1998, Marvanová et al. 2002, Mori et al. 2000, Paulin and Harrington 2000, Sugiyama et al. 1999, Winka 2000). In none of these phylogenies is *Cyttaria* monophyletic with the Helotiales as a whole. Using unpublished *Cyttaria* sequences generated in this study, other phylogenetic studies of the Helotiales and Leotiomycetes by Wang et al. (2006a, b) and (Schoch et al. 2009), hypothesized a close relationship among *Cyttaria*, *Chlorociboria* (Helotiales, Helotiaceae) and Erysiphales; these studies again identified Cyttariales as members of Leotiomycetes and acknowledged Helotiales to be an unnatural group. Hibbett et al. (2007), placed Cyttariales in Leotiomycetes in their revised higher-level phylogenetic classification of the fungi based on molecular data.

Relationships within Cyttaria.—Relationships among species belonging to *Cyttaria* have been considered by Kobayasi (1966), Korf (1983), Humphries et al. (1986) and Crisci et al. (1988), the latter two using cladistic analyses of morphological characters. In general these hypotheses infer a non-monophyletic grade of South American *Cyttaria* species on subgenus *Nothofagus* basal to a non-monophyletic grade of South American species on subgenus *Lophozonia* that is itself basal to a monophyletic clade of Australasian species on subgenus *Lophozonia*. Korf's (1983) hypothesis however delimits monophyletic Australasian and South American lineages, with South American *Cyttaria* species on subgenus *Lophozonia* basal to the remaining South American species, specialists on subgenus *Nothofagus*. The main difference between these hypotheses and perhaps the crux to understanding the phylogenetic history of *Cyttaria* is the relationship of the two South American species associated with subgenus *Lophozonia*: Are they more closely related to the other South American species, which are associated with subgenus *Nothofagus*, or are they more closely related to the other species that

TABLE I. *Cyttaria* species, hosts and geographical occurrence (from Calvelo and Gamundí 1999, Gamundí 1971, Rawlings 1956). AUS = Australia, NZL = New Zealand, SSA = southern South America

Cyttaria taxon	Host(s) (*Nothofagus* species)	Host subgenus	Geographical occurrence
Cyttaria berteroi Berk. 1842. Trans Linn Soc London 19:41.	*N. glauca* (Phil.) Krasser *N. obliqua* (Mirb.) Oerst.	*Lophozonia*	SSA
Cyttaria darwinii Berk. 1842. Trans Linn Soc London 19:40.	*N. antarctica* (Forst) Oerst. *N. betuloides* (Mirb.) Oerst. *N. dombeyi* (Mirb.) Oerst. *N. pumilio* (Poeppl. & Endl.) Krasser	*Nothofagus*	SSA
Cyttaria espinosae Lloyd. 1917. Mycol Notes Lloyd Libr Mus 48:673, FIGS. 995, 998.	*N. alpina* (Poeppl. & Endl.) Oerst *N. glauca* (Phil.) Krasser. *N. obliqua* (Mirb.) Oerst. [*N. dombeyi* (Mirb.) Oerst.(?)][a]	*Lophozonia*	SSA
Cyttaria exigua Gamundí. 1971. Darwiniana 16:495.	*N. betuloides* (Mirb.) Oerst. *N. dombeyi* (Mirb.) Oerst.	*Nothofagus*	SSA
Cyttaria gunnii Berk. in Hooker. 1847. The botany of the Antarctic voyage of HM discovery ships Erebus and Terror, in the years 1839–1843, part 2:453.			
See also Berk. 1848. Lond J Bot 7:576.	*N. cunninghamii* (Hook.) Oerst.	*Lophozonia*	AUS
Cyttaria gunnii in the sense of New Zealand authors (misapplication of *Cyttaria gunnii* Berk.)	*N. menziesii* (Hook.) Oerst.	*Lophozonia*	NZL
Cyttaria hariotii E. Fisch. 1888. Bot Zeitung Berlin 46:816.	*N. antarctica* (Forst) Oerst. *N. betuloides* (Mirb.) Oerst. *N. dombeyi* (Mirb.) Oerst. *N. nitida* (Phil.) Krasser. *N. pumilio* (Poeppl. & Endl.) Krasser.	*Nothofagus*	SSA
Cyttaria hookeri Berk. in Hooker. 1847. The botany of the Antarctic voyage of HM discovery ships Erebus and Terror, in the years 1839–1843, part 2:452, plate 162.	*N. antarctica* (Forst) Oerst. *N. pumilio* (Poeppl. & Endl.) Krasser. [*N. obliqua* (Mirb.) Oerst.(?)][b]	*Nothofagus*	SSA
Cyttaria johowii Espinosa. 1940. Bol Mus Nac Hist Nat Santiago de Chile 18:23.	*N. betuloides* (Mirb.) Oerst. *N. dombeyi* (Mirb.) Oerst.	*Nothofagus*	SSA
Cyttaria nigra Rawlings. 1956. Trans R Soc NZ 84:26.	*N. menziesii* (Hook.) Oerst.	*Lophozonia*	NZL
Cyttaria pallida Rawlings. 1956. Trans R Soc NZ 84:27.	*N. menziesii* (Hook.) Oerst.	*Lophozonia*	NZL
Cyttaria septentrionalis Herbert. 1930. Proc R Soc Queensland 41:158.	*N. moorei* (Muell.) Krasser.	*Lophozonia*	AUS

[a] See Gamundí and Minter (2004c) and http://194.203.77.76/herbIMI/. This host record apparently was based on a single collection, IMI 314589, which was examined by KRP; the fungus did seem to be *C. espinosae* but no host material was included for verification.

[b] See Gamundí and Minter (2004f), who listed this as a possible host but could not verify reports of the association.

associate with subgenus *Lophozonia*, half a world away in Australia and New Zealand?

The current study.—We used partial nuclear small subunit (nucSSU), nuclear large subunit (nucLSU) and mitochondrial small subunit (mitSSU) ribosomal RNA (rRNA), as well as translation elongation factor 1-alpha (*TEF1*), sequences to elucidate the relationship of *Cyttaria* to other Leotiomycetes and the relationships among *Cyttaria* species. Morphological data are included in phylogenetic analyses to assess the latter relationships. Furthermore two opposing hypotheses are investigated: that *Cyttaria* species found on subgenus *Lophozonia* are more closely related to each other than they are to species on subgenus *Nothofagus* (Crisci et al. 1988, Humphries et al. 1986, Kobayasi 1966) versus the idea that South American *Cyttaria* species are more closely related to each other than they are to the Australasian species (Korf 1983). In other words, Are *Cyttaria* species

TABLE II. Voucher information and GenBank numbers for *Cyttaria* taxa included in molecular analyses. An asterisk indicates that the sequence was provided to the AFToL project. Note: *C. gunnii* AUS = *C. gunnii* sensu stricto and *C. gunnii* NZL = *C. gunnii* sensu auctorum NZ

Species	Voucher	nucSSU	nucLSU	mitSSU	EF1-alpha	ITS
C. berteroi	CHILE. Región de la Araucanía. On *N. obliqua*, 1985, *Cannon*, *Peredo*, *IMI 314598* (IMI).	EU107178	EU107205	EU107234	—	—
C. darwinii	ARGENTINA. RÍO NEGRO: Parque Nacional Nahuel Huapi, Laguna Verde Trail, Cumbre Chall-Huaco. On *N. pumilio*, 21 Jan 2000, *Peterson*, *Gamundí*, *Ruffini*, *KRP 00-01-21-9* (BCRU, FH).	EU107179*	EU107206*	EU107235*	—	—
C. darwinii	ARGENTINA. TIERRA DEL FUEGO: Parque Nacional Tierra del Fuego, Río Pipo, camino a las cascadas. On *N. betuloides*, 8 Nov 1999, *Greslebin s. n.* (FH).	EU107180	EU107207	EU107236	—	—
C. darwinii	ARGENTINA. TIERRA DEL FUEGO: Parque Nacional Tierra del Fuego. On *Nothofagus*, 22 Feb 1988, *Lincoff 88-Arg-1* (NY).	EU107181	EU107208	—	—	EU107253
C. darwinii	ARGENTINA. TIERRA DEL FUEGO: Dpto. Río Grande, Ea. Ushuaia. On *N. antarctica*, 10 Nov 1999, *Greslebin* s. n. (FH).	—	EU107209	—	EU107250	—
C. darwinii	ARGENTINA. RÍO NEGRO: Parque Nacional Nahuel Huapi, Mirador Ñirihuau. On *N. pumilio*, 21 Jan 2000, *Peterson*, *Gamundí*, *Ruffini*, *KRP 00-01-21-7* (BCRU, FH).	—	EU107210	—	—	—
C. darwinii	ARGENTINA. RÍO NEGRO: Parque Nacional Nahuel Huapi, Mirador Ñirihuau. On *N. pumilio*, 21 Jan 2000, *Peterson*, *Gamundí*, *Ruffini*, *KRP 00-01-21-8* (BCRU, FH).	—	EU107211	—	—	—
C. espinosae	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, Lago Lácar, Yuco. On *N. obliqua*, 25 Oct 1995, *Gamundí*, *Amos*, *BCRU 848* (BCRU).	EU107182	EU107212	EU107237	—	—
C. espinosae	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, Lago Lácar, Yuco. On *N. obliqua*, 25 Oct 1995, *Gamundí*, *BCRU 868* (BCRU).	EU107183	—	EU107238	—	—
C. exigua	ARGENTINA. RÍO NEGRO: Parque Nacional Nahuel Huapi, Villa Tacul. On *N. dombeyi*, 7 Oct 1993, *Gamundí*, *BCRU 802* (BCRU).	EU107184	EU107213	EU107239	—	—
C. exigua	ARGENTINA. TIERRA DEL FUEGO: Parque Nacional Tierra del Fuego, Camino Lago Roca al Hito 24. On *N. betuloides*, 5 Dec 1997, *Calvelo*, *BCRU 01814* (BCRU).	EU107185	EU107214	EU107240	—	—
C. gunnii AUS	AUSTRALIA. VICTORIA: Yarra Ranges National Park, Cambarville, Cumberland Memorial Scenic Reserve, Cumberland Walk. On *N. cunninghamii*, 15 Dec 01, *Peterson 01-12-15-8* (MEL, FH).	EU107186	EU107215	EU107241	—	—

TABLE II. Continued

Species	Voucher	nucSSU	nucLSU	mitSSU	EF1-alpha	ITS
C. gunnii AUS	AUSTRALIA. VICTORIA: Yarra Ranges National Park, Cambarville, Cumberland Memorial Scenic Reserve, Cumberland Walk. On *N. cunninghamii*, 15 Dec 01, *Peterson 01-12-15-12* (MEL, FH).	EU107187	—	—	—	—
C. gunnii AUS	AUSTRALIA. VICTORIA: Yarra Ranges National Park, Cambarville, Cumberland Memorial Scenic Reserve, Cumberland Walk. On *N. cunninghamii*, 15 Dec 01, *Peterson 01-12-15-13* (MEL, FH).	EU107188	—	—	—	—
C. gunnii AUS	AUSTRALIA. VICTORIA: Yarra Ranges National Park, Acheron Way, ca. 2 km south of Acheron Gap. On *N. cunninghamii*, 16 Dec 01, *Peterson 01-12-16-1* (MEL, FH).	EU107189	—	EU107242	—	—
C. gunnii NZL	NEW ZEALAND. Mt. Aspiring National Park, Cannan's Creek, between Davis Flat and Haast Pass. On *N. menziesii*, 5 Dec 01, *Peterson 01-12-5-1* (PDD, FH).	EU107190	EU107216	—	—	—
C. gunnii NZL	NEW ZEALAND. Lewis Pass National Reserve, Marble Hill parking lot, trailhead of Lake Daniels Track. On *N. menziesii*, 24 Nov 01, *Peterson 01-11-24-5* (PDD, FH).	EU107191	—	EU107243	—	—
C. gunnii NZL	NEW ZEALAND. Fiordland National Park, Kiosk Creek DOC Campground. On *N. menziesii*, 30 Nov 01, *Peterson 01-11-30-1* (PDD, FH).	EU107192	—	EU107244	—	—
C. gunnii NZL	NEW ZEALAND. Fiordland National Park, Kiosk Creek DOC Campground. On *N. menziesii*, 30 Nov 01, *Peterson 01-11-30-2* (PDD, FH).	EU107193	—	—	—	—
C. hariotii	ARGENTINA. TIERRA DEL FUEGO: Dpto. Ushuaia, Ea. Moat, Río Chico. On *N. betuloides*, 9 Nov 1999, *Greslebin* s. n. (FH).	EU107194	EU107217	EU107245	EU107251	EU107254
C. hariotii	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, near Lago Huechulafquen, Pto. Canoa guard station trail to Volcan Lanín. On *N. antarctica*, 30 Jan 2000, *Peterson 00-01-30-2* (BCRU, FH).	EU107195	EU107218	EU107246	EU107252	—
C. hariotii	ARGENTINA. RÍO NEGRO: Parque Nacional Nahuel Huapi, Trail Mirador Ñirhuau, near Refugio J. J. Neumeyer, 1320 m. On *N. pumilio*, 21 Jan 2000, *Peterson*, *Gamundí*, *Ruffini*, *KRP 00-01-21-3* (BCRU, FH).	—	EU107220	—	—	—
C. hariotii	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, near Lago Huechulafquen, Pto. Canoa guard station trail to Volcan Lanín. On *N. antarctica*, 30 Jan 2000, *Peterson 00-01-30-3* (BCRU, FH).	—	EU107221	—	—	—

TABLE II. Continued

Species	Voucher	nucSSU	nucLSU	mitSSU	EF1-alpha	ITS
C. hariotii	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, near Lago Huechulafquen, Pto. Canoa guard station trail to Volcan Lanín. On *N. antarctica*, 30 Jan 2000, *Peterson 00-01-30-4* (BCRU, FH).	—	EU107222	—	—	—
C. hariotii	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, near Lago Huechulafquen, Pto. Canoa guard station trail to Volcan Lanín. On *N. antarctica*, 30 Jan 2000, *Peterson 00-01-30-6* (BCRU, FH).	—	EU107223	—	—	—
C. hookeri	ARGENTINA. RÍO NEGRO: Parque Nacional Nahuel Huapi, Trail Mirador Ñirhuau, 1450 m. On *N. antarctica*, 21 Jan 2000, *Peterson, Gamundí, Ruffini, KRP 00-01-21-5* (BCRU, FH).	EU107196	EU107224	—	—	—
C. hookeri	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, near Lago Huechulafquen, Pto. Canoa guard station trail to Volcan Lanín. On *N. antarctica*, 30 Jan 2000, *Peterson 00-01-30-1* (BCRU, FH).	EU107197	EU107225	—	—	—
C. hookeri	ARGENTINA. CHUBUT: Parque Nacional Los Alerces. On *N. antarctica*, 28 Jan 2000, *Peterson 00-01-28-4* (BCRU, FH).	—	EU107226	—	—	EU107255
C. hookeri	ARGENTINA. CHUBUT: Parque Nacional Los Alerces. On *N. antarctica*, 28 Jan 2000, *Peterson 00-01-28-4* (BCRU, FH).	—	EU107226	—	—	EU107255
C. hookeri	ARGENTINA. CHUBUT: Parque Nacional Los Alerces. On *N. antarctica*, 28 Jan 2000, *Peterson 00-01-28-4* (BCRU, FH).	—	EU107227	—	—	EU107256
C. hookeri	CHILE. MAGALLANES: Parque Nacional Torres del Paine, Río Serrano picnic area. On *N. antarctica*, 11 Mar 1988, *Halling 5840* (NY).	—	EU107228	—	—	—
C. johowii	ARGENTINA. NEUQUÉN: Parque Nacional Lanín, Lago Tromen. On *N. dombeyi*, 1996, *Haurylenbo, BCRU 1480* (BCRU).	EU107198	EU107229	—	—	EU107257
C. johowii	ARGENTINA. RÍO NEGRO: Dpto. Bariloche, Reserva Municipal Llao-llao, Lago Escondido. On *N. dombeyi, Baez, BCRU 1039* (BCRU).	EU107199	EU107230	—	—	—
C. nigra	NEW ZEALAND. Lewis Pass Area, St James Walkway, Subalpine Track. On *N. menziesii*, 24 Nov 2001, *Peterson 01-11-24-3* (PDD, FH).	EU107200	EU107231	EU107247	—	—
C nigra	NEW ZEALAND. Fiordland National Park, Te Anau area. On *N. menziesii*, 28 Nov 2001, *Peterson 01-11-28-1* (PDD, FH).	EU107201	EU107232	EU107248	—	—
C. septentrionalis	AUSTRALIA. NEW SOUTH WALES: Near Styx River Forest Reserve. On *N. moorei*, 24 Sep 1992, *Priest, DAR 69357* (DAR).	EU107202	—	—	—	—
C. septentrionalis	AUSTRALIA. NEW SOUTH WALES: New England National Park, near Tom's Hut, 30d25m00s, 152d25m00s. On *N. moorei*, 4 Oct. 2002, *Guymer, BRI AQ772796* (BRI).	EU107203	—	EU107249	—	—

''sufficiently accurate as taxonomists,'' as Korf (1983) proposes, or are they better geographers?

MATERIALS AND METHODS

Taxonomic sampling.—Unless otherwise specified, the taxonomic arrangement for supraspecific meiosporic taxa follows (Lumbsch and Huhndorf 2007) and Hibbett et al. (2007), where applicable, and specific names follow www.indexfungorum.org. Taxonomy of *Cyttaria* species follows the treatments of Gamundí (1971) and Rawlings (1956). Representatives from all currently accepted species of *Cyttaria* (TABLES I and II) were sampled. For analyses to find the closest relatives of *Cyttaria*, representative ingroup taxa were chosen from each order belonging to Leotiomycetes, the Cyttariales, Erysiphales, Helotiales, Rhytismatales and Thelebolales, as well as one family of uncertain placement, the Myxotrichaceae (Lumbsch and Huhndorf 2007, Schoch et al. 2009). Because several authors have proposed a close relationship between *Cyttaria* and the likely non-monophyletic Helotiales representatives were chosen from as many families from Helotiales as possible, which included 10 out of 11 families, Bulgariaceae (note that *Potebniamyces pyri* is a member of the Rhytismatales, according to Lumbsch and Huhndorf [2007], but a member of the Bulgariaceae, according to www.indexfungorum.org; we follow the latter hypothesis), Dermateaceae, Helotiaceae, Hemiphacidiaceae, Hyaloscyphaceae, Leotiaceae, Loramycetaceae, Phacidiaceae, Rutstroemiaceae, and Sclerotiniaceae. Some possible relatives of Leotiomycetes also were added, such as members of Pseudeurotiaceae (Ascomycota incertae sedis) (Gernandt et al. 2001, Landvik et al. 1998, Marvanová et al. 2002, Mori et al. 2000, Paulin and Harrington 2000, Winka 2000) (note that *Pseudogymnoascus roseus* is a member of Myxotrichaceae, according to Lumbsch and Huhndorf [2007], but a member of Pseudeurotiaceae, according to www.indexfungorum.org; we follow the latter hypothesis) and mitosporic species belonging to *Chaetomella* and *Pilidium* (Lutzoni et al. 2004, Rossman et al. 2004, Shear and Dodge 1921, Wang et al. 2006a). Ascomycetous outgroup taxa from the Geoglossomycetes, Orbiliomycetes, Pezizomycetes and (Pezizomycotina) also were included as was the basidiomycetous *Fomitopsis pinicola* (Agaricomycotina) (TABLE III).

Sequence determination.—DNA was extracted from dried, buffer- and ethanol-preserved specimens, as well as cultures from taxa other than *Cyttaria* species, which themselves are difficult to culture (Gamundí 1971). The general DNA extraction protocol involved grinding approximately 2–20 mg hymenial or other tissue in 500 µL extraction buffer (1% sodium dodecyl sulfate, 0.15 M NaCl, 50 mM Tris, 50 mM ethylenediaminetetraacetic acid) with liquid nitrogen, heated at 70 C for 1 h, purified twice with 600 µL phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and once with 600 µL chloroform-isoamyl alcohol (24 : 1). DNA was precipitated from solution on ice for 30 min with 0.1 solution volume of 3 M sodium acetate and 1.8 solution volume of 95% (v/v) ethanol, centrifuged 10 min, washed with 1 mL 70% (v/v) ethanol, centrifuged 3 min, air-dried and resuspended in 50 µL double-distilled water. The GENECLEAN II (Qbiogene, Irvine, California) or Elu-Quik DNA purification (Whatman, Florham Park, New Jersey) kits often were used to further purify the released DNA after extraction.

Double-stranded copies of partial nucSSU, nucLSU and mitSSU rRNA, as well as nuclear internal transcribed spacer (nucITS) rRNA and *TEF1*, were amplified with the following primer pairs. Primers PNS1/NS41 and NS51/NS8 (Hibbett 1996, White et al. 1990) were used for partial nucSSU rRNA, as were newly designed primers NRC3 (sequence 5′-GGA TCG GGC GAT GTT MTC-3′; in combination with NS8), NRC3R (the reverse complement of NRC3; in combination with PNS1), NRC4 (sequence 5′-CGA ACG AGA CCT TAA CCT GC-3′; in combination with NS8), and NRC4R (the reverse complement of NRC4; in combination with PNS1). Primer pairs LR0R/LR5, LR0R/LR7, and JS-1/JS-8 (Landvik 1996, Vilgalys and Hester 1990, Vilgalys http://www.botany.duke.edu/fungi/mycolab) were used for partial nucLSU rRNA, as well as newly designed primers LRC3 (sequence 5′-CTC ACC TCC GTT CAC TTT CAT TCC-3′; in combination with LR0R), LRC3R (the reverse complement of LRC3; in combination with LR7 or LRC7) and LRC7 (sequence 5′-CTC ACG CCC AGG GCT TCG-3′; in combination with LR0R or LRC3R). Primer pair ITS1/ITS4 (White et al. 1990) were used for complete nucITS rRNA. MS1/MS2 or NMS1/NMS2 (Li et al. 1994, White et al. 1990) (also MS1/NMS2 and NMS1/MS2) were used for partial mitSSU rRNA. No data were obtained from mitLSU rRNA with primer pairs ML3/ML4 and ML7/ML8 (Bruns http://plantbio.berkeley.edu/%Ebruns/primers.html). Primer pairs EF1-526F/EF1-1567R, EF-df/EF1-2218R, EF1-1577F/EF1-2218R (Rehner and Buckley 2005, Rehner http://www.aftol.org) were used for partial *TEF1*. No data were obtained from the second largest subunit of the nuclear RNA polymerase II gene (*RPB2*) either from published primers (Liu et al. 1999) or from newly designed primers. With the polymerase chain reaction, in MJ Research PTC 100, MJ Research PTC 200, or Perkin-Elmer 480 thermo-cyclers, reactions were heated at 94 C for 3 min, then subjected to 34 cycles of 1.5 min at 94 C, 2 min at 48 C, and 3 min at 72 C. In some cases DMSO was added. These products were cleaned before sequencing with polyethylene glycol precipitation, with the QIAquick Spin Kit (QIAGEN, Valencia, California), or QIAquick Gel Extraction Kit (QIAGEN, Valencia, California). Cloning was performed in many cases to retrieve individual PCR products with the protocol specified by the pGEM-T Easy Vector System (Promega, Madison, Wisconsin) and purified with the protocol of the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, California).

Sequencing was done with dye terminator cycle sequencing following the protocol specified by the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). Cycle sequence reactions were cleaned and then run on ABI 377 or ABI 3100 automated DNA sequencers. Primers used for amplification served as sequencing primers.

TABLE III. GenBank accession numbers for taxa included in molecular analyses, excluding *Cyttaria* spp., which are listed in TABLE II. An asterisk indicates that the sequence was generated in this study

Taxon	nucSSU	nucLSU	mitSSU	*TEF1*
Ascocoryne cylichnium (Tul.) Korf	EU107258*	EU107266*	—	—
Ascocoryne sarcoides (Jacq.) J.W. Groves & D.E. Wilson	FJ176830	FJ176886	—	—
Blumeria graminis (DC.) Speer	AB033476	AB022362	—	—
Botryotinia fuckeliana (de Bary) Whetzel	AY544695	AY544651	AY544732	DQ471045
Bulgaria inquinans (Pers.) Fr. (1822)	EU107259*	EU107267*	—	DQ471079
Bulgaria inquinans	EU107260*	EU107268*	—	—
Byssoascus striatisporus (G.L. Barron & C. Booth) Arx	AJ315170	AB040688	—	—
Chaetomella oblonga Fuckel	AY487081	AY487080	—	—
Chlorencoelia sp.	EU107261*	EU107269*	—	—
Chlorociboria aeruginosa (Oeder) Seaver ex C.S. Ramamurthi, Korf & L.R. Batra	AF292087	Z81402	—	—
Chlorociboria cf. *aeruginosa*	AY544713	AY544669	AY544734	AY544734
Chloroscypha cf. *enterochroma* (Peck) Petrini	AY544700	AY544656	AY544735	—
Cordierites guianensis Mont.	EU107262*	EU107270*	—	—
Cordierites sprucei Berk.	AF292089	—	—	—
Crinula caliciiformis Fr.	AY544729	AY544680	AY544738	
Cudonia circinans (Pers.) Fr.	AF107343	AF279379	AY584700	
Cudoniella clavus (Alb. & Schwein.) Dennis	DQ470992	DQ470944	FJ713604	DQ471056
Dermea acerina (Peck) Rehm	DQ247809	DQ247801	DQ976373	DQ471091
Encoelia fascicularis (Alb. & Schwein.) P. Karst.	Z81379	AJ226080	—	—
Encoelia heteromera (Mont.) Nannf.	EU107204*	EU107233*	—	—
Encoelia helvola (Jungh.) Overeem	AF292090	—	—	—
Erysiphe diffusa (Cooke & Peck) U. Braun & S. Takam.	AB120748	AB022397	—	—
Fabrella tsugae (Farl.) Kirschst.	AF106015	AF356694	—	—
Fomitopsis pinicola (Sw.) P. Karst.	AY705967	AY684164	FJ436112	AY885152
Gelatinodiscus flavidus Kanouse & A.H. Sm.	—	EU652381	—	—
Geoglossum nigritum (Fr.) Cooke	AY544694	AY544650	AY544740	
Golovinomyces orontii (Castagne) V.P. Heluta	AB033483	AB077697	—	—
Ionomidotis olivascens E.J. Durand	EU107263*	EU107271*	—	—
Ionomidotis frondosa (Kobayasi) Kobayasi & Korf	AY789353	AY789354	—	—
Lachnum bicolor (Bull.) P. Karst.	AY544690	AY544674	AY544744	—
Lambertella corni-maris Höhn.	EU107264*	EU107272*	—	—
Leotia lubrica (Scop.) Pers.	AY544687	AY544644	AY544746	DQ471041
Leotia viscosa Fr.	AF113715	AF113737	—	—
Leuconeurospora pulcherrima (G. Winter) Malloch & Cain	AF096178	AF096193	FJ190639	FJ238409
Leveillula taurica (Lév.) G. Arnaud	AB033479	AB022387	—	—
Loramyces macrosporus Ingold & B. Chapm.	DQ471005	DQ470957	FJ190599	DQ471076
Meria laricis Vuill.	DQ471002	DQ470954	FJ190598	DQ842026
Microglossum rufum (Schwein.) Underw.	DQ471033	DQ470981	—	DQ471104
Mollisia sp.	EU107265*	EU107273*	—	—
Morchella cf. *elata* Fr.	AY544709	AY544665	AY54474	—
Myxotrichum deflexum Berk.	AB015777	AB040689	AY575096	—
Oidiodendron tenuissimum (Peck) S. Hughes	AB015787	AB040706	—	—
Orbilia auricolor (A. Bloxam ex Berk.) Sacc.	U72598	AY261125	—	DQ471072
Pezicula carpinea (Pers.) Tul. ex Fuckel	DQ471016	DQ470967	FJ190608	DQ479932
Phacidium lacerum Fr.	DQ471028	DQ470976	FJ190623	FJ238396
Phyllactinia moricola (Henn.) Homma	AB033481	AB022401	—	—
Pilidium acerinum (Alb. & Schwein.) Kunze	AY487093	AY487092	—	—
Pleuroascus nicholsonii Massee & E.S. Salmon	AF096182	AF096196	—	—
Potebniamyces pyri (Berk. & Broome) Dennis	DQ470997	DQ470949	—	DQ471068
Pseudeurotium zonatum J.F.H. Beyma	AF096184	AF096198	FJ90655	DQ471112
Pseudogymnoascus roseus Raillo	AB015778	AB040690	—	—
Sclerotinia sclerotiorum (Lib.) de Bary	L37541	AB040689	AY575096	—

TABLE III. Continued

Taxon	nucSSU	nucLSU	mitSSU	*TEF1*
Sphaerotheca cucurbitae = *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff	AB033482	AB022410	—	—
Taphrina deformans (Berk.) Tul.	DQ471024	DQ470973	FJ713610	DQ471097
Thelebolus caninus (Auersw.) Jeng & J.C. Krug	FJ176840	FJ176895	FJ190657	—
Thelebolus microsporus (Berk. & Broome) Kimbr.	FJ176851	FJ176905	FJ190662	FJ238418
Tryblidiopsis pinastri (Pers.) P. Karst.	AF106013	AY004335	AF431963	DQ471106

Sequence alignment.—Consensus sequences were built from chromatograms with Sequencher 3 (Gene Codes Corp., Ann Arbor, Michigan), aligned with the default parameters of Clustal X (Thompson et al. 1997) and edited manually in MacClade 4.07 (Maddison and Maddison 1996). Ambiguously aligned regions were excluded from further analysis. Sequences were deposited at GenBank (TABLES II and III).

Morphological character coding.—Morphological data were obtained for *Cyttaria* primarily from the literature and supplemented with personal observations (see online SUPPLEMENT I). Most morphological characters originally were generated by Crisci et al. (1988), but many of those characters were reinterpreted and recoded for this study. In addition to that study, important sources for character information were Rawlings (1956), Gamundí (1971), Gamundí and Minter (2004a, b, c, d, e, f, g, h) and Minter and Gamundí (2004a, b). Because most of these characters were unique to *Cyttaria*, relating to their stromatal characteristics and habit, outgroup taxa were chosen from within *Cyttaria*, as determined by analyses of molecular data.

Phylogenetic analysis.—Only one *Cyttaria* representative was included for each species, in part due to the presence of identical or nearly identical sequence data. Because many nucleotide sites potentially informative within *Cyttaria* had to be excluded due to ambiguous alignment in the analyses to find the closest relatives of *Cyttaria*, two datasets were assembled—one to analyze the relationship of *Cyttaria* to other Leotiomycetes and the other to analyze the relationships within *Cyttaria*. Analyses to assess the relationship of *Cyttaria* to other Leotiomycetes included four data partitions, nucSSU rRNA, nucLSU rRNA, mitSSU rRNA and *TEF1* sequence data. This combined data matrix of 69 taxa consisted of 6762 total characters; 4429 included characters, of which 1833 were variable and 1128 were parsimony informative. Analyses to assess the relationships within *Cyttaria* included five partitions, nucSSU rRNA, nucLSU rRNA, mitSSU rRNA, *TEF1* sequence data and morphological data. This combined data matrix consisted of 4521 total characters, 4491 included characters, of which 297 were variable and 175 were parsimony informative. Chosen taxa with data for at least one partition were included in all analyses regardless of whether they contained data for all partitions (see simulation studies by Wiens 1998, 2003). Except *Cyttaria pallida* and *Gelatinodiscus flavidus*, all taxa included in both datasets were represented by nucSSU sequences; except *Cordierites sprucei*, *Cy. pallida*, *Cy. septentrionalis* and *Encoelia helvola*, all taxa were represented by nucLSU sequences; substantially fewer taxa were represented by mitSSU and *TEF1* sequences (TABLES II and III). Only morphological data were available for *C. pallida*.

Phylogenetic analyses were conducted with two methods, Bayesian inference (BI) and parsimony (P). These methods were used due to the different ways that they allow molecular and morphological data to be treated. MrBayes (Huelsenbeck and Ronquist 2001) was used in BI analyses because it allows data partitions to be analyzed separately, each with its own model, and can analyze molecular and discrete morphological data simultaneously. In addition P analyses were conducted with PAUP* 4.0b10 (Swofford 2002) because it allows continuous characters to be treated quantitatively. Continuous morphological characters were coded with the step-matrix gap-weighting method of Wiens (2001), which allows continuous characters to be treated quantitatively by applying small weights to small differences between taxa and large weights to large differences. (See online SUPPLEMENT I for step matrices for continuous and other morphological characters, in which the values rise to 999; when morphological characters, including the continuous characters, were included in analyses, all other, discrete, characters were given a weight of 999. Note that the three continuous characters were necessarily excluded from BI analyses.)

Bayesian analyses were performed with Metropolis-coupled Markov chain Monte Carlo (MCMCMC) methods in MrBayes (Huelsenbeck and Ronquist 2001). Default settings were used for the incremental heating scheme as well as the priors on the topology (uniform), branch lengths (exponential with parameter 10), gamma shape parameter (0.1–50), proportion of invariable sites (0–1) and the four stationary frequencies of the nucleotides and six different nucleotide substitution rates (Dirichlet; with all values = 1). Each partition was allowed to possess its own evolutionary model, parameters and rates under the general time reversible (GTR) model. For each dataset four independent runs starting from randomly chosen trees were run 2 000 000 generations. Each run was sampled every 100 generations for a total of 20 000 trees per chain sampled from the posterior distribution of trees and used to calculate posterior probabilities of clades. Burn-in samples were discarded from each run, and the remaining samples from each run were pooled and summarized as 50% majority rule consensus trees, with the percentages representing posterior probabilities for each node.

Parsimony analyses were conducted with heuristic search methods in PAUP* 4.0b10 (Swofford 2002) with multiple Wagner trees, tree bisection reconnection (TBR) branch

swapping, collapse of zero-length branches and equal weighting of all characters. Searches were repeated 100 times with starting trees obtained by the random addition Wagner algorithm option. To assess nodal support in resulting tree topologies, nonparametric bootstrap tests (Felsenstein1985, Hillis and Bull 1993) were performed with 300 replicates with search parameters as outlined above. In analyses to assess relationships within *Cyttaria* searches for most parsimonious trees and bootstrap values were found with the branch and bound method.

Morphological characters were traced onto phylogenies depicting relationships within *Cyttaria* in MacClade 4.07 (Maddison and Maddison 1996). For both BI and P analyses, two sets of analyses were performed, in which (i) all molecular and morphological data partitions were included and (ii) only molecular data partitions were included. The combined datasets and resulting phylogenies from BI analyses were deposited at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S10431).

Hypothesis testing.—Constraint trees, branch and bound search parameters, and nonparametric Templeton (Wilcoxon signed ranks) and winning-sites (sign) tests were used under the P criterion in PAUP* 4.0b10 (Swofford 2002) to test phylogenetic hypotheses.

RESULTS

Relationship of Cyttaria *to other Leotiomycetes.*—BI and P searches resulted in single trees with identical topologies in which the monophyly of *Cyttaria* was supported by a posterior probability of 1.0 and a P bootstrap value of 98% (FIG. 1). A clade formed by *Ionomidotis frondosa*, *I. olivascens*, *Encoelia helvola*, *E. heteromera*, *Cordierites guianensis* and *Co. sprucei* was found to be the closest sister group of *Cyttaria* (0.89 posterior probability, 75% bootstrap support). Sister of that group was a clade consisting of *Chlorociboria aeruginosa* and *Ch.* cf. *aeruginosa* (0.94 posterior probability). Sister of this larger group was a clade formed by striate-spored members of Myxotrichaceae (Leotiomycetes incertae sedis), Erysiphales and *Pleuroascus nicholsonii* (Pseudeurotiaceae, Ascomycota incertae sedis) (0.94 posterior probability).

Relationships within Cyttaria.—Analyses of the relationships among *Cyttaria* species recovered these notable clades (FIG. 2; numbers in figure and in text before and after slashes represent values obtained when morphological data are included or excluded respectively): one composed of the South American species *C. hookeri* and *C. johowii* (clade A; 1.00/1.00 posterior probability, 100%/100% bootstrap support), which forms a clade with the remaining species; one composed of the South American species *C. berteroi*, *C. darwinii*, *C. exigua* and *C. hariotii* (clade B; 0.99/0.99 posterior probability, 72%/100% bootstrap support), which forms a clade with the remaining species; one composed of the South American species *C. espinosae* plus the Australasian species (clade C; 0.97/1.00 posterior probability, 73%/100% bootstrap support); a monophyletic Australian lineage; and a monophyletic New Zealand lineage. In summary these data indicate that South American species are not monophyletic while Australasian species are. Furthermore as currently used the name *C. gunnii* refers to two entities, *C. gunnii* sensu stricto in Australia (including Tasmania) and an unrelated species in New Zealand. Analyses in which morphological data were excluded (results not shown) recovered trees similar to our Bayesian tree from the combined molecular and morphological datasets (FIG. 2), the differences being that (i) *C. pallida* was necessarily excluded, (ii) the relationships among *C. darwinii*, *C. exigua* and *C. hariotii* were unresolved (P) or resolved with *C. exigua* and *C. hariotii* more closely related with 0.92 posterior probability (BI), and (iii) bootstrap support and posterior probability values were higher in many cases (FIG. 2).

Morphological tracing of discrete characters (or when continuous, using coding of Crisci et al. 1988) as well as host leaf type (deciduous or evergreen) and host habitat type (some data, results not shown) provided no interesting trends for discussion. Certain characters and combinations characteristic of clades however are discussed below.

Hypothesis testing.—We tested our phylogenetic proposals against certain alternatives. The first set, that the taxon known as *C. gunnii* in New Zealand is a species distinct from the true *C. gunnii* in Australia vs. a single species were significantly different (L = 391 vs. L = 402, N = 25: $P < 0.03$, Templeton test; $P < 0.04$, winning-sites test). The second set, that *C. berteroi* forms a monophyletic group with clade B vs. with the other species hosted by subgenus *Lophozonia* (clade C) were not significantly different (L = 391 vs. L = 398 N = 15: $P < 0.07$, Templeton test; $P < 0.12$, winning-sites test). The third set, that *C. espinosae* forms a monophyletic group with the Australasia species vs. the other South American species were significantly different (L = 391 vs. L = 408, N = 19: $P < 0.0001$, Templeton test; $P < 0.0001$, winning-sites test).

DISCUSSION

We used partial nucSSU rRNA, nucLSU rRNA, mitSSU rRNA and *TEF1* sequence data and morphological data to infer relationships among species of *Cyttaria* and the relationship of *Cyttaria* to other Leotiomycetes.

Relationship of Cyttaria *to other Leotiomycetes.*—Phylogenetic hypotheses identify *Cyttaria* as a strongly supported clade (FIG. 1) and provide evidence for a relatively close relationship between *Cyttaria* species and a clade consisting of *Cordierites guianensis*, *Co. sprucei*, *Encoelia helvola*, *E. heteromera*, and *Ionomidotis frondosa* and *I. olivascens* of the Encoelioideae (Helotiaceae, Helotiales). Sister of this larger clade is one consisting of *Chlorociboria aeruginosa* and *Ch.* cf. *aeruginosa*. Sister of this is a clade consisting of members of Myxotrichaceae (Leotiomycetes incertae sedis), *Pleuroascus nicholsonii* of Pseudeurotiaceae (Ascomycota incertae sedis) and Eryisphales (FIG. 1).

When he described *Cyttaria* Berkeley (1842) suggested a relationship with *Bulgaria* (Helotiales, Bulgariaceae). Mengoni (1986) provided transmission electron micrographs of *Cyttaria* ascus apices, in which she demonstrated the apices to be inoperculate and concluded they were of the *Bulgaria inquinans*-type as described by Bellèmere (1969). To date *Bulgaria* and *Cyttaria* are the only taxa reported to have the *B. inquinans*-type of ascus apex (Döring and Triebel 1998, Gamundí 1991). In our phylogenetic analyses (FIG. 1) and the analyses of others (Döring and Triebel 1998; Schoch et al. 2009; Wang et al. 2006a, b) *Cyttaria* species and *Bulgaria inquinans* are not particularly closely related.

Carpenter (1976), who hypothesized a close relationship between *Cyttaria* species and *Gelatinodiscus flavidus* Kanouse & A.H. Sm. (Helotiales, Helotiaceae), compared their ascus apices with light microscopy, noting that they are inoperculate, broad and stain blue in iodine. He also mentioned that the ascospores of both have the unusual property of becoming pigmented after discharge. According to our results based on a single nucLSU sequence for *G. flavidus*, it is not particularly closely related to *Cyttaria* but instead shows a greater affinity for fellow Helotiaceae members *Chloroscypha* and *Ascocoryne* (FIG. 1).

Our analysis provides evidence for a close relationship between *Cyttaria* and *Cordierites*, a hypothesis that is suggested in the older taxonomic literature as well by our results (FIG. 1). Montagne (1840) erected *Cordierites* to accommodate *Co. guianensis*, which had a fruit body composed of numerous apothecia supported by branches that he interpreted to be stroma. Schröter and Lindau (1897) placed Cordieritaceae and Cyttariaceae close to each other in their taxonomic arrangement. Noting that they did not consider it to be a natural family, Clements and Shear (1931) placed *Cordierites* in Cyttariaceae. Boedijn (1936) in response said it was ''useless to say that the latter procedure [was] wholly unfounded.'' The *Cordierites-Cyttaria* connection apparently was discarded after that. Ciferri (1957) suggested that *Cordierites* should be in Helotiaceae. Korf (1973), Rifai (1977), Dennis (1978) and Zhuang (1988) placed *Cordierites* in Encoelioideae of what is now known as Helotiaceae (Pezizomycetes, Helotiales). In a molecular phylogeny of Encoelioideae by Zhuang et al. (2000), *Cordierites sprucei* and *Encoelia helvola* were found to form a clade and were related to *Chlorociboria aeruginosa* (Hymenoscyphoideae) but Encoelioideae as a whole was not monophyletic. Wang et al. (2006a) hypothesized a close relationship between *Cyttaria* and *Cordierites frondosa* (Kobayasi) Korf, accepted as reversionary work by Zhuang (1988) as *Ionomidotis frondosa*. In our analysis *I. frondosa* and *I. olivascens* together formed a clade (FIG. 1) that also includes *Co. guianensis*, *Co. sprucei*, *Encoelia helvola* and *E. heteromera*.

Encoelia species generally possess a stromatic base from which apothecia arise (Spooner and Trigaux 1985). In our analysis *Encoelia* does not form a clade (FIG. 1); *E. fascicularis* is closely related to the *Lambertella corni-maris* of Rutstroemiaceae and *Sclerotinia sclerotiorum* and *Botryotinia fuckeliana* of Sclerotiniaceae (Helotiales), in agreement with Holst-Jensen et al. (1997), while *E. heteromera* and *E. helvola* are more closely related to *Cordierites* and *Inonomidotis* (Helotiales, Helotiaceae), which together form a monophyletic group with *Cyttaria*. Zhuang et al. (2000) found a close relationship between *E. helvola* and *Co. sprucei*. Although *Encoelia* is currently placed in Sclerotiniaceae (Lumbsch and Huhndorf 2007), it has been treated also in the Encoelioideae of the Helotiaceae.

Some have compared *Chlorociboria* to members of the Sclerotiniaceae, but most studies (e.g. Holst-Jensen et al. 1997) exclude it from that family and consider it to be part of what is currently called Helotiaceae (Dixon 1975, Lumbsch and Huhndorf 2007). Results of this study indicate that *Chlorociboria* is potentially one of the closest living relatives of *Cyttaria* (FIG. 1), a finding shared by Platt (2000), Wang et al. (2006a, b) and Schoch et al. (2009); the latter three studies used unpublished *Cyttaria* sequences generated by the current study. The apothecia produced by species of *Chlorociboria* arise singly from irregularly shaped, as in *Ch. aeruginosa*, or multiply from scarcely differentiated, as *in Ch. aeruginascens*, fundaments or stromatic masses (Dixon 1975). Furthermore *Ch. aeruginascens* is associated with a mitosporic state; *Dothiorina tulasnei* (Sacc.) v. Hohn. *Dothiorina*, like *Chlorociboria*, occurs on decayed wood (Dixon 1975). It produces gelatinous, subspherical to moriform stromata that contain numerous pycnidial chambers in which mitospores are produced. *Ch. aeruginosa* and *Ch. aeruginascens*

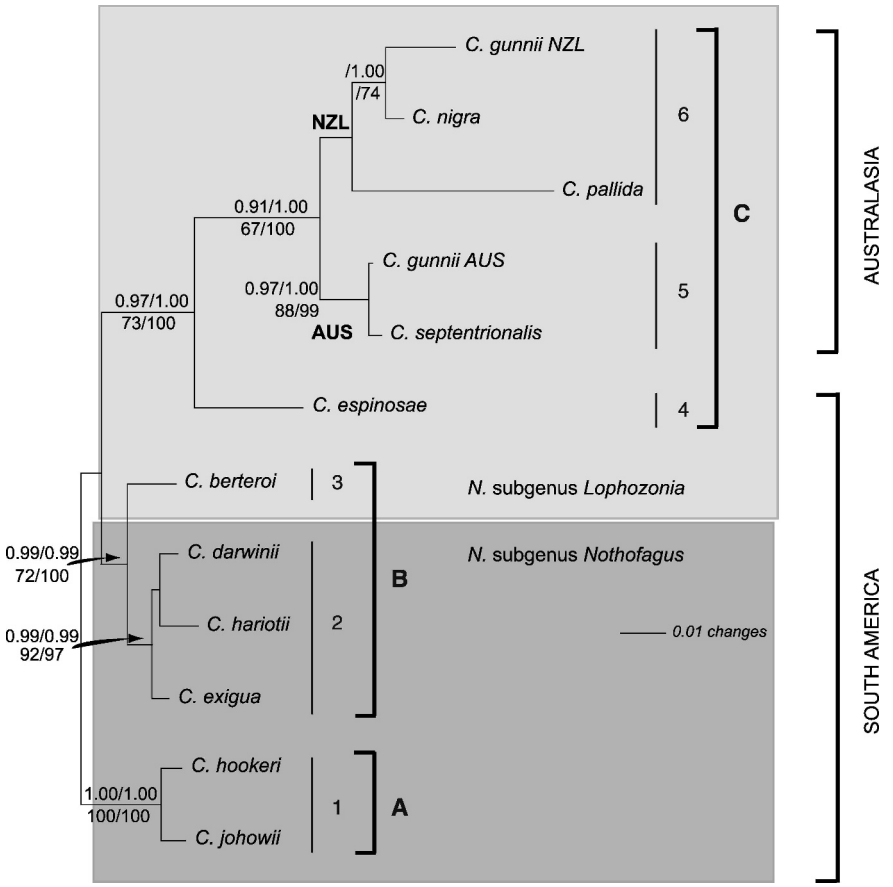

FIG. 2. Bayesian tree from the combined molecular and morphological datasets showing the relationships among species of *Cyttaria*. Numbers associated with nodes represent posterior probabilities from BI analyses (above branches) and >50% bootstrap support from P analyses (below branches); numbers after slashes represent values obtained when morphological data are excluded. Clades A–C and subclades 1–6 are discussed in text. AUS = Australia, NZL = New Zealand.

are each other's closest relative, according to Dixon (1975), which might indicate the facility with which the stroma can evolve from producing single to multiple apothecia arising from organized to scant stromata or vice versa. Perhaps retention of the pycnidial, meiosporic stage on the mitosporic stroma is another change that could indicate a phylogenetic affinity between *Cyttaria* and *Chlorociboria*. Of interest, Johnston and Park (2005) hypothesize a possible Asian/Australasian center of diversity for *Chlorociboria*. Note that Pfister and LoBuglio (2009) inferred a close relationship between *Chlorociboria* and *Medeolaria farlowii* Thaxt. (Pezizomycotina incertae sedis, Medeolariales). When *Medeolaria* nucSSU and nucLSU sequences (GenBank accession numbers GQ406808 and GQ406807) are included in our analyses, *Medeolaria* forms a monophyletic group with *Pleuroascus nicholsonii* (results not shown).

The remaining, non-helotialean, taxa possibly closely related to *Cyttaria* are represented by a monophyletic group composed of members of Myxotrichaceae (Leotiomycetes incertae sedis) with longitudinally striate ascospores, Erysiphales and *Pleuroascus nicholsonii* of Pseudeurotiaceae (Ascomycota incertae sedis) (FIG. 1). It is difficult to propose well supported hypotheses regarding close relationships of *Cyttaria* to many of these.

Most of the potential relatives of *Cyttaria* live on woody substrates as either biotrophs or saprotrophs; they have adaptations for protecting ascospore development or prolonging ascospore dispersal, such as angiocarpy or gelatinous tissues; many of their apothecia arise from stromata, and many possess anamorphs. This suite of features unfortunately is common to many members of the Ascomycota and cannot be used to provide evidence for the monophyly of these taxa with *Cyttaria*. Nevertheless our results suggest *Cyttaria* is related to a group of Helotiales that produces stromata, or stromata-like structures, from which one or more apothecia and/or perhaps pycnidial anamorphs arise, the main members belonging to certain members of Encoelioideae.

Current morphological and molecular evidence support the continued recognition of Cyttariales. As

stated above, some of the older literature includes *Cordierites* in the Cyttariaceae (see Clements and Shear 1931, Boedijn 1936), a finding supported by our analysis, but then certain members of *Encoelia*, *Ionomidotis*, and possibly *Chlorociboria*, would need to be included as well. These taxa are so morphologically and ecologically dissimilar that it is difficult to propose synapomorphies with which to unite them. We therefore recommend maintaining the Cyttariales as is in recognition of their unique endostromatic apothecia, lack of cell-wall chitin and highly specialized habit. In-depth studies of the Encoelioideae are needed because its possible status as an equally ranked taxon also might be warranted.

Relationships within Cyttaria.—Phylogenetic hypotheses are compatible with the existence of 12 (FIG. 2) instead of 11 (Gamundí 1971, 1991; Rawlings 1956) *Cyttaria* species.

C. gunnii specimens from Australia do not form a clade with specimens known as *C. gunnii* from New Zealand (FIG. 2), according to molecular sequence data. This hypothesis is significantly different from the alternative, that specimens known as *C. gunnii* in New Zealand and Australia represent a single species. The holotype of *C. gunnii* is from Australia (Tasmania); therefore that name has been misapplied to specimens from New Zealand. *Cyttaria purdiei*, a name that has not been used since its original description, could be the valid name for this species. Although the author, Buchanan (1886), furnished an illustration and a few comments, these comments do not effectively distinguish the species from any other. Rawlings (1956) considers *C. purdiei* to be nomen nudum, although we think that it is validly published. In the early literature many considered *C. purdiei* to be synonymous with, or indistinguishable from, *C. gunnii* (Herbert 1930, Lloyd 1917, Saccardo 1889, Santesson 1945), the only other name applied to *Cyttaria* specimens from New Zealand until Rawlings (Rawlings 1956) described *C. nigra* and *C. pallida* in his monograph on Australasian *Cyttaria*. Others considered *C. purdiei* nomen dubium (Palm 1932), but most simply disregarded it, probably due to the following: Little information was given about the collection on which the name is based, and none of it was diagnostic; the accompanying illustration was highly stylized and, although immature and mature fruit bodies in part are characteristic of *C. gunnii*, with a wide conical base, smooth membranous sheath surrounding immature fruit bodies, and numerous, crowded apothecia in mature fruit bodies, mature fruit bodies were depicted in grayscale as black, like in another New Zealand species, *C. nigra*; the fruit bodies were shown growing on *N. fusca*, when only *N. menziezsii* hosts *Cyttaria* in New Zealand (McKenzie et al. 2000); no canker or swelling was shown, when all New Zealand species produce either longitudinal or globose cankers (Rawlings 1956); and specimens known as *C. gunnii* in New Zealand are seemingly identical to specimens in Australia (Rawlings 1956, KRP pers obs). Furthermore we were unable to locate the holotype or any other collection of *C. purdiei*, despite extensive searches of all relevant herbaria. Due to these shortcomings, we think the original description of *C. purdiei* was inadequate because we cannot be sure what Buchanan had in mind when he described this species. Hints in the illustration pointed toward *C. purdiei* being the valid name for specimens known by the misapplied name *C. gunnii* in New Zealand, those hints included the lack of the pronounced papillae characteristic of immature fruit bodies of *C. nigra* and a wider base than the long, narrow conical base of *C. nigra*. The species known in New Zealand by the misapplied name *C. gunnii* also possesses papillae, however they are relatively inconspicuous. The other New Zealand species, *C. pallida*, has far fewer, more widely spaced, apothecia per stroma than depicted in the illustration (up to 50 vs. up to 200 in the species known in New Zealand by the misapplied name *C. gunnii*) as well as a ''short, hidden, undifferentiated'' base (Rawlings 1956). These hints unfortunately were negated by the fact that what little information is given includes two likely inaccuracies and rendered the stylized illustration in the protolog unreliable. A dedicated study of fresh fruiting bodies of all developmental stages of the undescribed species known in New Zealand by the misapplied name *C. gunnii* is necessary before a new species can be described to accommodate it because many important macro- and microscopic characters are lost in dried specimens. Even though KRP launched a collecting expedition to New Zealand and Australia, she obtained inadequate material for this purpose.

Although *C. gunnii* sensu stricto from Australia and the species known in New Zealand by the misapplied name *C. gunnii* are almost identical, we were able to find a character that might be used to distinguish them morphologically. *Cyttaria gunnii* sensu stricto from Australia sometimes has highly deciduous, black, pycnidia-like incrustations on immature fruit bodies early in their development (KRP pers obs), while the equivalent, undescribed species from New Zealand, known by the misapplied name *C. gunnii*, lacks pycnidia-like incrustations (Rawlings 1956, KRP pers obs). Further in-depth morphological studies of these two species might reveal additional characters.

Across the Tasman Sea in Australia is another taxonomic problem involving *C. gunnii*. Even though

molecular sequence data fail to resolve *C. septentrionalis* as a species separate from *C. gunnii* (results not shown), we think that the considerably larger fruit bodies and spores of *C. septentrionalis* (Rawlings 1956, KRP pers obs) do for now. *Cyttaria septentrionalis* occurs on a host species (*N. moorei*) that occurs much farther north than the host of *C. gunnii*, *N. cunninghamii*. There is no doubt that *C. gunnii* and *C. septentrionalis* are closely related. Even though samples of *C. septentrionalis* and *C. gunnii* are not resolved into species clades, they do exhibit nucSSU and mitSSU rRNA sequence differences. Other markers, such as nucITS rRNA, nucLSU rRNA or *RPB2* between motifs 6 and 7, which often are used in fungal phylogeny studies at this level, might be able to distinguish between *C. gunnii* and *C. septentrionalis*. Despite attempts to do so, we were unable to obtain nucITS rRNA, nucLSU rRNA or *RPB2* data. Also Rawlings (1956) suggested that two different species of *Cyttaria* might be growing on *N. moorei* because Wilson (1937) describes both globose and longitudinal cankers from *C. septentrionalis* (KRP pers obs), an unknown phenomenon in other species. Therefore we propose the continued recognition of *C. septentrionalis* as separate from *C. gunnii* based on morphological and habit data until this matter can be investigated further. Thus for the time being the name *C. gunnii* sensu stricto is reserved for *Cyttaria* specimens occurring on *N. cunninghamii* in Australia (including Tasmania).

Relationships between clades within Cyttaria.—Phylogenetic analyses resolve and support the existence of three major clades within *Cyttaria* (FIGS. 1, 2): the South American species *C. hookeri* and *C. johowii* (clade A); the South American species *C. berteroi*, *C. darwinii*, *C. exigua*, and *C. hariotii* (clade B); and the South American species *C. espinosae* with the Australasian species, *C. gunnii* and *C. septentrionalis* from Australia, and the species known in New Zealand by the misapplied name *C. gunnii*, *C. nigra* and *C. pallida* from New Zealand (clade C). Clades B and C appear to be more closely related to each other than either is to clade A (FIG. 1). Clade A occurs in South America exclusively on *Nothofagus* subgenus *Nothofagus*, clade B occurs on both subgenera *Nothofagus* and *Lophozonia* exclusively in South America and clade C occurs in both South America and Australasia exclusively on subgenus *Lophozonia*, thus producing a grade of South American species and a clade of Australasian species, including monophyletic Australian and New Zealand clades. *Cyttaria* species do not sort into clades according to their associations with *Nothofagus* subgenera *Lophozonia* and *Nothofagus*. Therefore six clades are restricted to a single region and single host subgenus (FIG. 2), *C. hookeri* and *C. johowii* in South America on subgenus *Nothofagus* (subclade 1), *C. darwinii*, *C. exigua* and *C. hariotii* in South America on subgenus *Nothofagus* (subclade 2), *C. berteroi* in South America on subgenus *Lophozonia* (subclade 3), *C. espinosae* in South America on subgenus *Lophozonia* (subclade 4), *C. gunnii* and *C. septentrionalis* in Australia on subgenus *Lophozonia* (subclade 5), and the species known in New Zealand by the misapplied name *C. gunnii*, *C. nigra* and *C. pallida* on subgenus *Lophozonia* (subclade 6). Sublade 1 is synonymous with clade A; subclades 2 and 3 comprise clade B; and subclades 4, 5 and 6 comprise clade C.

Two critical pieces of literature on *Cyttaria* systematics are Gamundí's (1971) monograph on the South American species and Rawlings' (1956) monograph on the Australasian species. Both rely primarily on macromorphological characters of the immature and mature stromata as well as canker morphology to differentiate between species.

Most *Cyttaria* species produce stromata that are yellow to orange, fleshy-gelatinous, subglobose to globose with a cylindrical to conical base, around 2–3 cm diam, containing up to at least 50 yellow to orange apothecia. Half of the species produce mitospores within pycnidia, another three produce similar pycnidia-like, black incrustations in which no mitospores have been observed and the remaining three produce no such structures. Cankers are usually globose or longitudinal.

Producing fruit bodies not representative of other *Cyttaria* species, *C. hookeri* and *C. johowii*, which occur on subgenus *Nothofagus* in South America, form a well supported clade (A and subclade 1, FIG. 2). Gamundí (1971) considered *C. hookeri* and *C. johowii* to share an affinity based on the gummy and resinous consistency of stromata that have totally immersed pycnidia. She suggested that the remaining species in the genus, with their fleshy-gelatinous consistency, represent another group, a hypothesis that is congruent with the results of this study and of Crisci et al. (1988).

Phylogenetic analyses identify all but one of the remaining South American *Cyttaria* species as part of a second clade, which includes *C. berteroi*, *C. darwinii*, *C. exigua* and *C. hariotii* (clade B, FIG. 2). *Cyttaria berteroi* (subclade 3) occurs on *Nothofagus* subgenus *Lophozonia*, while the remaining species in this group, *C. darwinii*, *C. exigua* and *C. hariotii* (subclade 2), occur on subgenus *Nothofagus*. Gamundí (1971) considered *C. darwinii* and *C. exigua* to share an affinity due to thick, membranous ectostroma, well separated apothecia and basal spermogonia. She noted that mature *C. darwinii* and *C. hariotii* are

almost identical in appearance. She compared *C. hariotii* and *C. espinosae* based on color, superficial spermogonia and form of cankers. She also compared *C. berteroi* to the latter two with respect to the consistency, flavor and color of stromata.

The third major clade of *Cyttaria* species (C, FIG. 2), which occurs on *Nothofagus* subgenus *Lophozonia*, is composed of the South American *C. espinosae* (subclade 4) as well as all Australasian species, *C. gunnii* and *C. septentrionalis* (subclade 5) from Australia and species known in New Zealand by the misapplied name *C. gunnii*, *C. nigra* and *C. pallida* (subclade 6) from New Zealand.

Evolution of Cyttaria.—Kobayasi (1966), Korf (1983), Humphries et al. (1986), Crisci et al. (1988) and Setoguchi (2005) present hypotheses regarding the evolution of *Cyttaria*. In short Kobayasi (1966), Humphries et al. (1986), and Crisci et al. (1988) inferred a grade of South American *Cyttaria* species on subgenus *Nothofagus* basal to a grade of South American species on subgenus *Lophozonia* that is monophyletic with a clade of Australasian species on subgenus *Lophozonia*, including monophyletic Australian and New Zealand clades. Korf's (1983) hypothesis however delimited monophyletic Australasian and South American lineages, with the South American *Cyttaria* species on subgenus *Lophozonia* basal to the remaining species, which are specialists on subgenus *Nothofagus*. The main discrepancy in these hypotheses regards the positions of *C. berteroi* and *C. espinosae*, the only two South American species associated with subgenus *Lophozonia*. In one hypothesis they are more closely related to other South American species, which are associated with subgenus *Nothofagus*. In the other they are more closely related to other *Cyttaria* species on subgenus *Lophozonia*, which occur in Australasia. Our phylogenetic analyses identify a non-monophyletic grade of South American *Cyttaria* species and a monophyletic clade of Australasian species (FIG. 2), in agreement with those of Kobayasi (1966), Humphries et al. (1986) and Crisci et al. (1988). As predicted by those hypotheses, South American *C. espinosae* forms a clade with Australasian species, all associates of subgenus *Lophozonia*, which is statistically significant from the alternative, that *C. espinosae* forms a clade with other South American species. However the South American *C. berteroi*, also an associate of subgenus *Lophozonia*, fails to group with that clade. Instead it groups with a clade of South American species on subgenus *Nothofagus*. Although our hypothesis is well supported (FIG. 2), the difference between these opposing hypotheses is not significant. That *C. berteroi* groups with other South American species in our hypothesis is a finding in agreement with Korf's (1983) hypothesis that predicts monophyletic South American and Australasian clades. In short the phylogenetic history of *Cyttaria* cannot be explained solely by geographical location or host association.

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