Relationships among *Collybia* s. str. from Greenland, North America and Eurasia

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Abstract

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Collections of *Collybia* s. str. from Greenland were analyzed morphologically and by DNA sequencing of the nuclear ribosomal ITS1-5.8S-ITS2 region. Results confirmed that all three species of *Collybia* were present and sympatric in Greenland. *Collybia tuberosa* ITS sequences of collections from Greenland, Europe and North America were remarkably similar, with only one variable base and 3 apomorphies among ten geographically divergent collections. Two collections from Greenland differed with respect to the variable base. Nine collections of *C. cookei* were sequenced, including one from Greenland. Collections from North America and Greenland formed a well-supported clade, but two unique apomorphies were present in the Greenland collection. Twelve collections of *Collybia cirrata* from Europe and North America were also nearly identical but Greenland collections showed a number of unique apomorphies. Discovery of divergent genotypes in Greenland not detected in either North America or Eurasia may be a consequence of founder effects in Greenland populations – mutation and genetic drift in small isolated populations followed by range expansion.

Key words: biogeography, ITS, *Microcollybia*, ribosomal RNA

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Introduction

Collybia sensu Antonin and Noordeloos comprises four species, *C. racemosa* (Pers.:Fr) Quél, *C. tuberosa* (Bull.:Fr.) Kummer, *C. cirrata* (Pers.) Quél and *C. cookei* (Bres.) J. D. Arnold (Antonin and Noordeloos 1997). These are small (pileus up to 20 mm) grayish to white mushrooms which share an unique habitat, fruiting on the remains of dead fleshy mushrooms (Fig. 1). The host mushrooms include species of *Lactarius* and *Russula* and the polypore, *Meripilus giganteus* (Noordeloos 1995), some of which are known to be mycorrhizal. Hughes *et al.* (2001) showed that the four species were distinct and separable, based on ribosomal ITS1-5.8S-ITS2 (ITS) and large subunit (LSU) DNA sequences. These studies further showed that *C. racemosa* was highly divergent from a well-supported monophyletic clade consisting of *C. tuberosa, C. cirrata* and *C. cookei.* Based on observed sequence differences, and differences in morphology and habitat, a new genus was proposed for *C. racemosa*, *Dendrocollybia racemosa* R.H.Petersen & Redhead (Hughes *et al.* 2001)

Collybia tuberosa, C. cirrata and *C. cookei* are primarily distinguished from each other by the presence or absence, and the colour of sclerotia. *Collybia tuberosa* forms fusiform or ellipsoid reddish brown shiny sclerotia. *Collybia cookei* forms irregularly-shaped yellow to orange-yellow sclerotia. *Collybia cirrata* does not form sclerotia. *Collybia* s. str. morphological identifications may be confirmed using molecular tools. Species-specific ITS sequence synapomorphies were identified by Hughes *et al.* (2001) and allowed determination of simple diagnostic RFLP patterns for *Dendrocollybia racemosa* and the three *Collybia* species. These RFLP patterns were used to validate morphological designations and distributions.

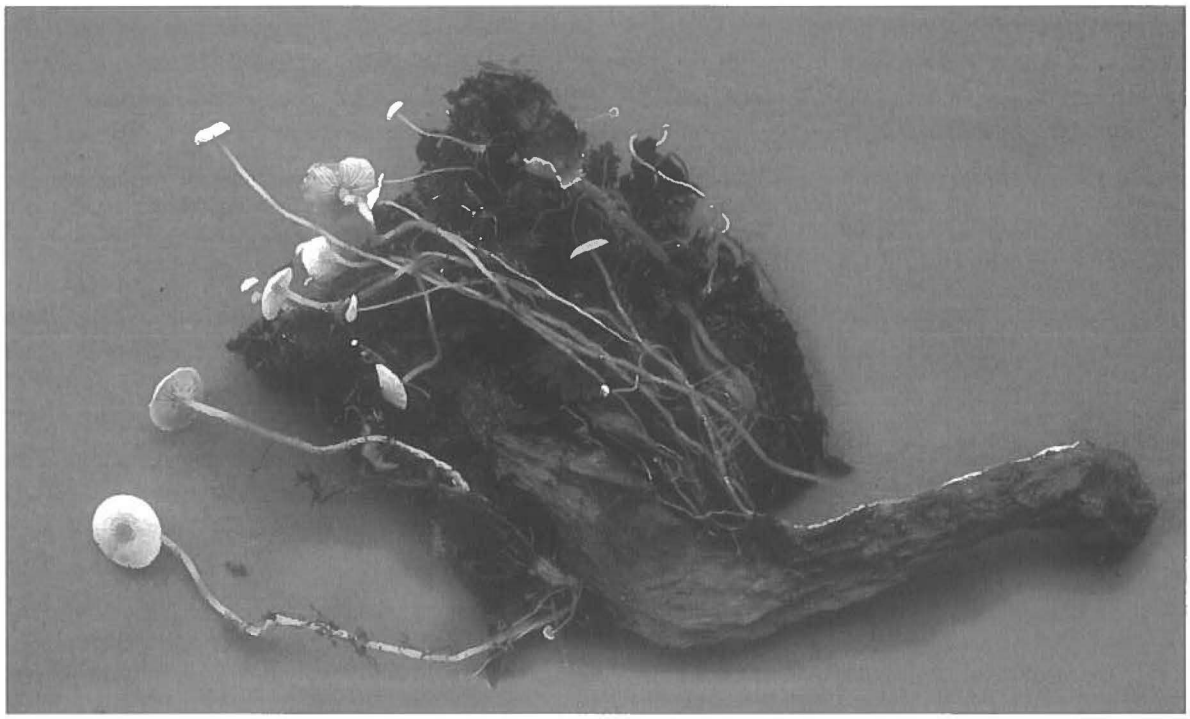

Fig. 1. *Collybia cirrata* collection 10721 growing on the mummified remains of another mushroom.

In this paper, we report ITS sequences and RFLP patterns for additional collections, and examine sequence variability among Greenland collections and putative relationships of collections from Greenland to collections from North America and Eurasia.

Methods and materials

Collections and cultures. Collections used for ITS1-5.8S-ITS2 sequencing and RFLP analysis are listed in Table 1. Morphological species determinations for Greenland specimens deposited at TENN were by R.H. Petersen, Henning Knudsen and Gro Gulden.

Cultures were established from spore prints on agar as described by Gordon and Petersen (1992). Spores were allowed to germinate and overgrow and the resultant polyspore (dikaryon) culture was subcultured for storage on malt agar slants (MA: 15g/L Difco malt extract; 20g/L Difco Agar) at 10° C and as disks of fungal mycelium on MA in cryovials (Nalgene) under sterile distilled water at room temperature (Burdsall and Dorworth 1994). For DNA, isolates were subcultured in 30 mL potato dextrose broth (PDB: 24 g/L Difco Potato Dextrose) at room temperature. When the mycelial mat reached approximately 2 cm diameter, tissue was recovered by filtering to remove the medium and blotting the mycelial mat dry with a paper towel.

DNA extractions. Major problems were encountered with mucoid substances produced by the cultures, especially cultures of *C. cirrata*. In previous studies, a number of different techniques specific for polysaccharide problems were tried with poor results. For this study, better results were obtained with a procedure using xanthogenate designed for extracting DNA from cyanobacteria (Tillett and Neilan 2000). Approximately 0.3 g fungal tissue was suspended in 50 µL weak TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) in a 1.5 mL microfuge tube and ground with a micro pestle. Seven-hundred-fifty µL of freshly made XS buffer was added [100 mM Tris-HCl, pH 7.4, 20 mM EDTA ph 8.0, 1% sodium dodeclysulfate, 800 mM ammonium acetate and 1% potassium ethyl xanthogenate (Fluka biochemicals)]. The solution was incubated at 70° C for 45 minutes. Every 10-15 minutes, the tube was briefly vortexed and vortexed again for ten seconds at the end of the incubation to release DNA. Tubes were placed on ice for 30 minutes to allow

Table 1. Collections and sequence accession numbers used in this study.

Collection (culture) Number1	TENN Number[2]	Species	Country	State or province	GenBank Accession Number
4171	50650	*Collybia cirrata*	UK	Scotland	AF3613123
4285	50621	*Collybia cirrata*	Switzerland	Maggia	AF3613133
7300	53540	*Collybia cirrata*	Sweden	Uppland	AF274380
7478	53599	*Collybia cirrata*	Finland	Etelä-Häme	AF274381
8107	53961	*Collybia cirrata*	USA	AK	AF3613183
8785	55213	*Collybia cirrata*	USA	NC	AF274382
9778ss1	58676	*Collybia cirrata*	Greenland	Kangerlussuaq	AF3613153
9779ss1	58677	*Collybia cirrata*	Greenland	Kangerlussuaq	AF3613143
9793ss1	58691	*Collybia cirrata*	Greenland	Kangerlussuaq	AF3613163
10721ss1	58805	*Collybia cirrata*	Greenland	Sisimuit	AF3613173
11628	59591	*Collybia cirrata*	Russia	Novgorod	DQ8308043
11632	59595	*Collybia cirrata*	Russia	Novgorod	DQ8308053
3235	49324	*Collybia cookei*	Russia	Primorsk	AF3613063
5696	52427	*Collybia cookei*	USA	WA	AF065120
7768	54154	*Collybia cookei*	Russia	Caucasus	AF3613053
8713	55143	*Collybia cookei*	Mexico	Tlaxcala	AFO65123
9780ss1	58678	*Collybia cookei*	Greenland	Kangerlussuaq	AF3613043
CBS 450.86	na	*Collybia cookei*	Belgium		AF274383
10815ss3	58224	*Collybia cookei*	Russia	vic. Leningrad	DQ8308033
10815ss5	58224	*Collybia cookei*	Russia	vic. Leningrad	DQ8308023
12045	60294	*Collybia cookei*	USA	NC, GSMNP	DQ8308063
3749	50486	*Collybia tuberosa*	UK	Scotland	AF274379
5613	52404	*Collybia tuberosa*	USA	ID	AF3613113
7265	53630	*Collybia tuberosa*	Sweden	Uppland	AFO65124
8784	55212	*Collybia tuberosa*	USA	NC	AFO65121
9781ss1	58679	*Collybia tuberosa*	Greenland	Kangerlussuaq	AF3613083
9838ss1	58817	*Collybia tuberosa*	Greenland	Sisimuit	AF3613093
12074	60322	*Collybia tuberosa*	USA	TN, GSMNP	DQ8308073
Duke 1424	na	*Collybia tuberosa*	USA	MT	AF274377
Duke 1809	na	*Collybia tuberosa*	USA	VA	AF274377
DAOM 191061	na	*Collybia tuberosa*	Canada	Newfoundland	AF274376

[1] Collection numbers with no proceeding letter are Tennessee field collection numbers and are also culture numbers. Cultures are currently maintained at the University of Tennessee, Department of Ecology and Evolutionary Biology. DED numbers are from Dennis E. Desjardin, San Francisco State University. DAOM numbers are from Scott Redhead, Agriculture Canada. CBS numbers are from the Centraalbureau voor Schimmelcultures, The Netherlands. Single spore isolates (monokaryons) are indicated as "ss." GSMNP is the Great Smoky Mountains National Park , USA which borders both Tennessee and North Carolina.

[2] TENN numbers are Tennessee herbarium accession numbers; na = not applicable

[3] Collections new to this study.

proteins and cell debris to aggregate (the solution became thick and viscous). Tubes were centrifuged at 14,000 RPM for 10 minutes. The supernatant containing DNA was removed and DNA was precipitated with 750 µL isopropanol for 15 minutes. DNA was pelleted by centrifugation at 10,000 RPM for 10 minutes. Alcohol was removed, the pellet washed with 70% ETOH and dried, then resuspended in 50 µL TE buffer and stored. If remaining polysaccharide was observed upon initial alcohol precipitation, the polysaccharide-DNA complex was re-suspended immediately in TE buffer and was purified utilizing the GENECLEAN® kit (BIO 101, Inc, Vista, CA) according to manufacturer's instructions.

PCR amplification and sequencing. The nuclear ribosomal ITS1-5.8S-ITS2 region was amplified with primers ITS1F and ITS4B (Bruns and Gardes 1993). The PCR product was purified using a Wizard PCR purification system (Promega) following manufacturer's directions. Both strands were sequenced with an ABI automated sequencing system using cycle sequencing primed in the forward direction (RNA strand) with ITS5 in the reverse direction with ITS4. Occasionally additional partial sequences were obtained using ITS3 and ITS2 (White *et al.* 1990). Sequences for each collection were aligned and spliced using the GCG Seqed program (Genetics Computer Group Sequence Analysis Software Package; GCG 2000), and corrected manually after examining electropherograms. Regions of homology to the yeast (*Saccharomyces cerevisiae*) 18S gene (Rubtsov *et al.* 1980), to the *Heterobasidion annosum* 5.8S gene (Kasuga and Mitchelson 1993), and to the yeast 25S gene (Georgiev *et al.* 1981) were determined by sequence comparison. Sequences were deposited in GenBank (Table 1).

Restriction Fragment Length Polymorphisms. Collections were digested with Hae III and Bsa HI following manufacturer's directions using 10 L of ITS DNA with 0.5 L (50U) of enzyme. Digestion products were electrophoresed in a 1.5% agarose gel to separate restriction fragments.

Phylogeny Estimation. Outgroup taxa were chosen based on blast searches in GenBank using *Collybia tuberosa*, *C. cirrata* and *C. cookei* ITS sequences and on a ribosomal LSU phylogeny (Moncalvo *et al.*, 2002). Both procedures suggested *Clitocybe nebularis* and *Lepista sordida* as the closest sequenced outgroup genera. For maximum parsimony, a heuristic search was performed using PAUP* version 10 (Swofford 2001) with the following parameters: characters were unordered and equally weighted; starting tree was obtained by stepwise addition; addition sequence was furthest; 'MaxTrees' setting was 100 with no increase; and the branch-swapping algorithm was tree-bisection-reconnection. Gaps were treated as a fifth base. Gaps were rare and informative and no gap coding was attempted. One-thousand bootstrap replicates were performed (Felsenstein 1985). Model test (Posada and Crandall 1998) was used to evaluate the data set and to select the best model for Bayesian analysis. Bayesian analysis was performed using MrBayes (Huelsenbeck and Ronquist 2000) with the following settings. The maximum likelihood model employed 6 substitution types ("nst = 6"), with base frequencies estimated from the data ("basefreq = estimate"). Rate variation across sites was modeled using a gamma distribution, with a proportion of sites being invariant (rates = "invgamma"). The Markov chain Monte Carlo search was run with 4 chains for 500,000 generations, with trees sampled every 100 generations (the first 1000 trees were discarded as "burn-in"). The posterior probabilities were estimated by sampling trees generated after likelihood values converged.

Results

Three monophyletic clades appeared in all analyses, *C. tuberosa*, *C. cookei* and *C. cirrata*. Support for a clade containing *C. tuberosa* and *C. cookei* was well-supported in Bayesian analysis but less so in parsimony analysis (Fig. 2). However, examination of the dataset showed there are few synapomorphies that could accurately indicate phylogenetic relationships between the three species.

Sequences from *Collybia tuberosa* collections from Europe, Greenland and Eastern North America were remarkably similar. These collections showed the least sequence divergence of the three *Collybia* species. Five of the ten collections examined had identical sequences. Only three base changes were observed among the remaining five collections. A single T/C transition in the ITS1 region at base 108 was shared by collections 8784 (USA, NC), 12074 (USA, TN) and 9781 (Greenland). The remaining sequence changes were: i) an A to C transversion in ITS 1 at base 139 which was apomorphic in collection 3749 from Scotland; and ii) an A to G transition in the first base of the ribosomal LSU gene present in collection 9838 from Greenland. The two Greenland collections differed from each other at two of the three variable sites.

Collybia cookei and *C. cirrata* collections were more variable; *C. cookei* had ten variable bases among nine collections. Collections of *C. cookei* from Greenland, Mexico, North Carolina (Great Smoky Mountains National Park) and Washington State grouped into a well-supported clade (putative "North American

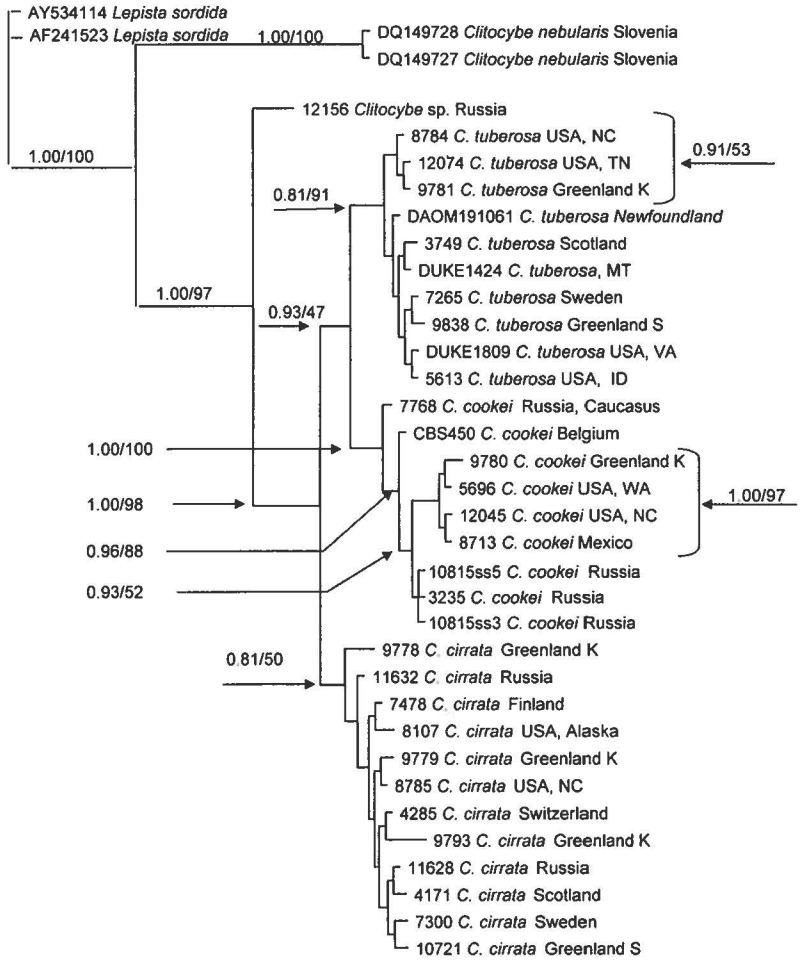

Fig. 2. Bayesian strict consensus tree of entire sequence data set based on 500,000 generations with trees sampled every 100 generations. Burn-in was set at 1000. Posterior probabilities greater than 0.70 are noted. Parsimony bootstrap support values are given to the right of the posterior probability where the same node is supported in both analyses. The number of parsimony-informative characters was 98. Tree length = 161 with seven trees recovered. Consistency index (CI) = 0.86. Homoplasy index (HI) = 0.16. Greenland K = collection from Kangerlussuak, Greenland S = Sisimiut.

clade"; Fig. 2). The "North American clade" shared three unique synapomorphies and was supported by 1.00 Bayesian posterior probability and 97% bootstrap support. The relative positions of remaining collections from Russia (including collection 3235 from Far Eastern Russia) and a collection from Belgium were not well-supported.

Collybia cirrata collections had eight apomorphies and two synapomorphies among 12 collections and was also the most commonly collected species in Greenland during mid-August, 2000. It grew more rapidly in culture that either *C. tuberosa* or *C. cookei*. Relationships among collections of *C. cirrata* were not well-supported in either Bayesian or parsimony analysis.

Previous studies noted that a variable area in the ITS 1 region contained species-specific sequences that were accessible by the restrictions enzymes Hae III (GGCC) and Bsa III (GGCGTC). *Collybia cookei* ITS sequences have a single Hae III site and no Bsa III site. *Collybia tuberosa* ITS sequences have a single Bsa III site and no Hae III site. *Collybia cirrata* ITS sequences do not have a restriction site for either restriction enzyme (Hughes *et al.* 2001). These synapomorphies were retained in Greenland collections.

Conclusions

Collybia s. str. has been reported from North Temperate forests of Europe and North America, and from high altitude, temperate forests of Arizona and Mexico (Noordeloos 1995; Antonin and Noordeloos 1997; Hughes *et al.* 2001). All three species of *Collybia* s. str. are present in Greenland. The more distantly related species, *D. racemosa* was not found by us and has not

been reported from Greenland (Borgen *et al.* 2000).

While collections of *Collybia* s. str. worldwide are remarkably similar in ITS sequence, unique apomorphies were observed in all three species in collections from Greenland and for *C. cirrata*, genetic divergence in Greenland was relatively high compared to divergence observed in North America and Europe. Morphological variation was also noted in this species in Greenland. An unusually dark fruitbody (collection 9793: "sayal brown" as opposed to "pale ochraceous buff", "pale ochraceus salmon" or "light buff"; (Ridgeway 1912) was collected and was shown by sequence analysis to be *C. cirrata*.

Within *C. cookei*, sequence variability was observed primarily among Eurasian collections. In contrast, ITS sequences of collections from North America (Mexico, North Carolina and Washington State) were very similar. The single *C. cookei* collection from Greenland had an ITS sequence which closely resembled that of the North American collections and did not resemble ITS sequences from European collections. In this case, we can suggest a possible North American origin for Greenland *C. cookei*, but more data are required to substantiate this hypothesis.

Collybia tuberosa collections worldwide were remarkably similar. Sequences differed at only three positions and two of these variants were observed in collections from Greenland. No biogeographical patterns were evident. A possible explanation for sequence similarity is that the ribosomal ITS sequence is tightly conserved in this species as opposed to other species where intercontinental species divergence has been observed (Lickey *et al.* 2002; Vilgalys and Sun 1994). Alternately, *C. tuberosa* could represent a relatively recent range expansion throughout the North Temperate zone with little accompanying sequence divergence.

Restriction fragment analysis showed that for all collections, diagnostic restriction sites were preserved. This allows for rapid identification of mixed collections or populations by restriction digestion prior to sequencing.

While variability overall in *Collybia* s. str. species is low, a slightly higher level of ITS base change was observed in collections from Greenland. There is a possible explanation for these observations. Melting of the Greenland icecap has left wet pockets and valleys which are separated from each other by ridges and bare rock. Many of these pockets and valleys became colonized with plants and their associated fungi relatively recently. Initial colonization was likely the result of a founder event in which one or few individuals colonized a new habitat. Resulting fungal populations in these valleys would be small and isolated. Under these conditions, random genetic drift would become a major factor, allowing the fixation of new mutations (Hartl 1988).

Greenland is a superb natural laboratory for examination of the relative roles of population size and isolation on establishment of variation in fungi. The sexual system for basidiomycete fungi such as *Collybia* strongly favors outbreeding and thus homogenization of alleles across broad geographical areas (see for example Methven *et al.* 2000) but in Greenland, gene flow may be limited by lack of contiguous substrate for plants and fungi and restriction of populations to wet isolated valleys. Further studies are needed with additional species to determine if the increased variation observed among Greenland *Collybia* collections is reflected in other Greenland species and to evaluate the roles of isolation and genetic drift.

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