

**Genetical and molecular systematic study on the genus *Montagnea* Fr.,
a desert adapted Gasteromycete**

by

Chang Chen

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APPROVED:

Orson K. Miller, Jr.

Khidir W. Hilu

Duncan M. Porter

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(ABSTRACT)

Montagnea arenaria [Hymenogastreales, Basidiomycota] , adapted to desert and xeric habitats, is morphologically and phenotypically variable. Species have been described on the basis of macromorphology and spore shape and size. This study was initiated to investigate populations of *M. arenaria* from Namibia in Africa and the Southwestern United States. It was hypothesized that biological species would exist in the widely separated populations. Spores from single sporocarps were germinated, single spore isolates were obtained and selfed to obtain mating types. On transfer, clamp connections were not maintained and mating patterns could not be achieved. Nuclear staining revealed multinuclei in the hyphae of both single spore isolates and compatible crosses. Spores were stained and found to have either 1 or 2 nuclei, but only four sterigmate basidia were observed. Limited partial compatibility was achieved and in some cases clamp connections formed within and between crosses from the two continents. Genomic DNA was extracted from old herbarium specimens. The ITS1, 5.8S, and ITS4 regions of nuclear ribosomal DNA were amplified and sequenced directly. Phylogenetic analysis using PAUP was performed. The hypothesis that *Montagnea* would form different

biological species based on continental separation was rejected. In fact, the complex of isolates from widely varying locations not only had partial compatibility, but the variation in ITS sequences among widely distributed collections was relatively low. Lastly, no correlation between sporocarp size and gene flow among specimens from a wide variety of habitats was found. It appears that *M. arenaria* is a single, highly variable, widely distributed species.

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INTRODUCTION

Montagnea Fr. is a secotioid higher fungus adapted to arid and desert regions and is distributed worldwide. It closely resembles most of the characteristics of the species *Coprinus comatus* in the Agaricales (cap, stem, volva, dark spores with apical germ pore, etc.). Traditionally, however, the genus has been placed in the Hymenogastrales in the Gasteromycetes (Miller & Miller, 1988). The basidiospores in *Montagnea*, like in many other secotioid agarics, develop orthotropically and are no longer forcibly discharged. In this type of development, the spores are symmetrically attached to the sterigmata, a plug develops in the sterigmata at maturity and the spores are passively detached from the basidium. In the Agaricales, species have been included that have heterotropically developed basidiospores that are asymmetrically attached to the sterigmata. A small liquid filled drop forms below the spore just prior to the forcible discharge of the spore. Since this does not occur in *Montagnea*, dissemination of the spores depends on wind or wind blown sand. The fruiting body of *Montagnea*, fleshy at first, becomes very hard and woody, adapting to the dryness in the desert environment, and the gills do not deliquesce at maturity. Instead, the brittle remains of the gills with spores, called gussets, are blown about.

The close relationship between *Montagnea arenaria* and *Coprinus comatus* has been confirmed by restriction site mapping of nuclear ribosomal DNA by Hopple and Vilgalys (1994). They studied the phylogenetic relationships among coprinoid and closely related taxa by analyzing the large subunit and ITS region of rDNA. The grouping of *C. comatus*

and *M. arenaria* is the most strongly supported clade in their phylogenetic trees. It appears that *Montagnea* represents a transition between two large groups of fungi, the Gasteromycetes and the Agaricales (Morse, 1948). There are quite a few distinct and fairly convincing connecting series between Hymenogastrales and fleshy Hymenomycetes. Singer and Smith (1960) suggested that Hymenomycetes have been derived from gasteromycete ancestors with secotioid taxa as intermediates. If this is the case, several separate lines must have been involved and, therefore, the basidium as a “spore-gun” must have developed again and again from “non-explosive” basidia (Ingold, 1971). This seems extremely difficult in the natural history. It is much easier to imagine that the types of basidia found in Gasteromycetes are evolutionary novelties and have arisen by degeneration from distinct hymenomycete ancestors (Ingold, 1971). Generally speaking, Hymenomycetes are little adapted to xeric or alpine conditions; the great majority of fleshy fungi are very sensitive to dryness and coldness. On the other hand, Gasteromycetes are modified and reach their fullest development in extreme environments including both desert and mountain regions of the world.

NOMENCLATURE AND TAXONOMY

Fries first published *Montagnea* in his “Genera Hymenomycetum” in April 1836 in honor of the French mycologist C. Montagne. He later changed the name to *Montagnites* in the “Epicristis” to make way for DeCandolle’s *Montagnea*, which was a renaming of a composite family, and not published until October 1836. So *Montagnea* Fr. is the older

name according to the International Code of Botanical Nomenclature, which recognizes his 1836 name (Zeller, 1943).

There is extraordinary variation in the size and shape of the fruiting bodies and spores of *Montagnea*. Cleland accepts wide variations in spore size in a single species (1934). Zeller (1943) and Morse (1948) include a number of different species, which have been published, as synonyms under *M. arenaria*. On the contrary, some mycologists recognize different species based largely on spore size and the number of spores produced per basidium (Reid and Eicker, 1991). Reid and Eicker have described additional species of *Montagnea* including *M. haussknechtii* Rab. with small elliptic spores (6.0-10.0 x 3.0-6.0 μm) and *M. arenaria* var. *macrospora* Reid & Eicker with much larger spores (14.0-27.0 x 9.0-16.0 μm) and basidia with 2 and 3 sterigmata. Biological species concepts have not been developed in this group of fungi, nor in other secotioid desert agarics, such as *Podaxis*, *Longula*, or *Endoptychum*. The number of biological species in *Montagnea* has not been determined. However, from morphological studies, the following synonyms have been accepted.

The type species of *Montagnea* is *M. arenaria*.

Montagnea arenaria (DC.) Zeller, Mycologia 35:418, 1943.

Basionym *Agaricus arenarius* DC. Flore Francaise 6:45. 1815.

Synonym *Agaricus radiosus* Pallas, Reise. 2:744. 1777.

= *Montagnites candollei* Fr. Epicrisis, p 241. 1838.

= *Montagnites pallasii* Fr. Epicrisis, p 241. 1838.

= *Montagnea candollei* var. *texensis* Berk. & Curt. Ann. Mag. Nat. Hist. 2:422.

1853.

= *Montagnea candollei* Fr. ap. Corda, Icones Fung. 6 (edit. Zobel): 85. 1854.

= *Montagnites radiosus* (Pall.) P. Henn. Hedw. Beibl. 40:98. 1901.

= *Montagnites radiosus* (Pall.) Hollos Gasterom. Ungarns, p 30. 1904. (invalid)

= *Montagnites arenarius* (DC.) Morse, Mycologia 40:256. 1948.

SPOROCARP DESCRIPTION

Fruiting body oval at first, deeply buried and enclosed in a tough universal veil (volva), then expanding from the universal veil, which remains as a persistent volva at the base of stalk. Mature fruiting body epigeous with a stalk and disc-like cap, 4-30 cm tall, dry, and grayish brown. **Pileus** 1-5 cm broad, convex becoming plane to depressed with a fragile exoperidium that breaks down very early. **Gleba** black, recurving to form gussets (black plates of gleba), which become exposed and hang from the margin of cap and are entirely free from the stalk at maturity. The gussets, when mature, become hardened and tough with a hymenium of collapsed basidia and abundant spores. Eventually, the gussets are eroded or fall off the cap as they disintegrate. Some deliquescence may occur in wet weather but this is rare. **Stalk** 2.5-20.0 cm long, 2.0-15.0 mm thick, equal or often tapering downward, hollow, tough to woody with scattered loose fibrils. Sometimes forming a series of ragged rings over the middle to lower part of the stipe. **Volva** at base of stalk saclike, thick and double layered, embedded in sand, often missing in age. (Arora, 1986; Miller and Miller, 1988). **Pileipellis** a cystoderm with a filamentous **pileus trama**. **Basidia** club shaped or elongate, 4-spored, 26.0-50.0 x 10.0-13.0 um, thin-walled,

light brown in 3% KOH. **Basidiospores** 7.0-22.0 x 4.5-14.0 um, ovoid, oblong, broadly elliptical to elliptical (Em values from 1.3 to 1.9), smooth, thick walled with a hyaline apical germ pore, dark brown in Meltzer's solution and 3% KOH.

Habit, Habitat and distribution: Found solitary, scattered, or gregarious in deserts, on sandy soil, rocky soil, granite soil, dry river beds, old fields, and other waste places; widely distributed through out the world and fairly common in arid and semi-arid areas. It is a decomposer on organic matter, dead desert plants and grass, sometimes associated with juniper or shrubs (Arora, 1986; Miller and Miller, 1988).

Observations: Immature fruiting bodies are rarely found among specimens preserved in herbaria, and only occasionally is the well developed volva present. Basidia are very difficult to observe in dried material as they collapse in age, but they can be found in some crushed mounts of the few young specimens which were examined. Basidiospores are highly variable in both shape and size. They are not forcibly discharged; desert wind-blown sand erodes away pieces of the gussets, distributing the spores.

GENETICS OF THE BASIDIOMYCOTINA

In the Basidiomycotina, biological species have been termed intersterility groups (ISGs) (Korhonen, 1978), which are used to better identify species when morphological characters of the basidioma are too subtle or too variable (Petersen and Ridley, 1996). Two strains are thought to belong to the same biological species if they can interbreed and fruit. Many systematic studies employ mating criteria for delimiting biological species and establishing stable taxonomic concepts (Boidin, 1986).

There are no sexual organs in the Hymenomycetes and Gasteromycetes, and interaction between two individuals occurs exclusively by fusion of undifferentiated vegetative cells. “The solution to the problem offered by this group of organisms by the need to regulate the breeding mechanism in the absence of sexual differentiation can be seen as a classical solution to a problem in genetic organization. The incompatibility system in its most complex form is probably the most flexible in the regulation of both the inbreeding and outbreeding potential of a species” (Koltin, 1978). The outbreeding potential is the major factor that will determine the degree of diversification of the population, whereas the degree of inbreeding determines the short range adaptation to prevailing conditions (Koltin, 1978).

Mating within ISGs is regulated by mating type incompatibility (also termed homogenic incompatibility) (Burnett, 1975). Mycelium germinated from a single basidiospore usually has uninucleate cells and is called a homokaryon (or monokaryon). When paired, homokaryons with different mating type alleles are compatible and form binucleate heterokaryons (or dikaryons) with the potential for developing fruiting bodies. Homokaryons carrying identical alleles are incompatible and cannot mate to form dikaryons. A newly formed dikaryotic mycelium can be easily identified microscopically in many species by the presence of clamp connections, which is considered indicative of mating compatibility (Vilgalys and Miller, 1983). However, many Basidiomycetes do not form clamp connections (Raper, 1976), and one must use a vital stain to observe the nuclear condition of the mycelium. In contrast, homokaryons from different ISGs usually fail to form dikaryons when paired with each other despite having different

incompatibility alleles. This phenomenon has been termed heterokaryon incompatibility (Burnett, 1975) or intersterility (Korhonen, 1978).

Most heterothallic basidiomycetes have a bipolar (unifactorial) or tetrapolar (bifactorial) mating system (Esser and Raper, 1965). The mating system determines the intercompatibility of conspecific monokaryons. Bipolar species contain a single locus, the A incompatibility factor. The locus is multiallelic and mating occurs only when differing alleles are present (i.e., A1 and A2). In tetrapolar forms there are two incompatibility factors, A and B, each consisting of two closely linked, multiallelic loci, α and β . A difference at either the α or β locus, or any $\alpha\beta$ combination, generates a unique A or B factor specificity. Mating occurs when different alleles are present in both of the two factors (i.e., A1B1 and A2B2) (Ullrich, 1977). It is possible that the bifactorial system originated by splitting or duplication of the one factor in a unifactorial system into two, and the subsequent processes came to be regulated differently by the two factors (Elliott, 1994). These mating systems with a large number of different alleles give a high probability of compatibility between random pairs of the same genotype, while restricting fertility among close relatives. In other words, multiple-allelic systems promote outbreeding and limit self-fertilization (Fincham, 1979).

MOLECULAR SYSTEMATICS AT THE SPECIES LEVEL

Information on species boundaries from molecular data is often invaluable for separating intraspecific morphological polymorphisms from diagnostic characters (Hillis *et al.*, 1996). Polymerase chain reaction (PCR) is a molecular technique widely used

recently in fungal systematics. It allows amplification of a specific DNA fragment from a heterogeneous mixture of sequences, and the amount of DNA needed for PCR is minute. It also makes it possible to routinely obtain sequence data from a large number of samples, including herbarium specimens (Bruns *et al.*, 1990).

The sequences coding for nuclear ribosomal RNA (rDNA) have been chosen in many studies of phylogenetic systematics and evolutionary patterns of fungi (Ko *et al.*, 1997). Previous study showed that much of the variability in the rDNA repeat is phylogenetically informative or potentially diagnostic for individual species (Anderson and Stasovski, 1992). And with its multiple copies, even less starting material is required (0.1 to 10 ng of total DNA) for analysis. Moreover, the separation of nuclear and organelle genomes is unnecessary because target amplification can be directed with eukaryotic-specific primers to amplify specific genomic fractions (Lee and Taylor, 1990). In eukaryotes, rDNA genes are repeated up to several hundreds of times in a clustered manner. These tandem copies are considered to have been homogenized by concerted evolution via unequal crossing over or gene conversion, thus they are almost always treated as a single locus that can be compared among species (Dover, 1982).

The fungal rDNA genes are separated by nontranscribed spacer regions (NTS), which contain the signals for rDNA expression. Each of the rDNA genes codes for one copy of 18S, 28S and 5.8S rRNA. The regions that lie between these RNAs are the two noncoding internal transcribed spacers (ITS) (Gardes and Bruns, 1993). The ITS region, as well as the intergenic NTS repeat, shows much evolutionary change, and differences in these regions occur between species within a genus (Goosen and Debets, 1996). Several

studies have demonstrated that the ITS region is often highly variable among morphologically distinct fungal species, but the intraspecific variation is low in most cases (Gardes and Bruns, 1991; Chen *et al.*, 1992; Lee and Taylor, 1992). The nucleotide sequences of the rDNA repeat unit have been determined from a large number of eukaryotes. This allows the design of primers according to the highly conserved 18S and 28S regions. The entire ITS and NTS regions can be reliably amplified from most basidiomycetes with universal primers (White *et al.*, 1990). The most detailed information can be obtained by direct sequencing of the PCR products, which will detect every single base-pair difference of the amplified fragment between samples. In fungi, the entire ITS region is often between 600 and 800 base pairs in length and can be sequenced directly using the double-strand PCR products. Phylogenetic analysis using sequence data combined with mating compatibility studies has shown much promise for resolving phylogenetic relationships and understanding speciation for many problematic species complexes in basidiomycetes (Vilgalys and Sun, 1994).

PHYLOGENY CONSTRUCTION

Many methods have been used in the phylogenetic analysis of molecular data including sequencing data. Nei (1987) and Felsenstein (1988) placed the methods for constructing phylogenetic trees into several categories, including the distance matrix, parsimony and maximum likelihood method. The parsimony method seems to represent the most popular analytical approach by far (Crawford, 1990). The parsimony method constructs phylogenetic trees by assuming that the observed characters evolved by the minimum

number of changes, and the tree that requires the fewest total changes is the one with maximum parsimony, or the most parsimonious tree. Use of parsimony assumes that evolution occurs in the simplest manner or by the most direct route. While this is not always the case, there is a lack of data to the contrary (Crawford, 1990). The heuristic search performed in PAUP (Swofford, 1993) is an estimation of the shortest tree, obtained by starting with one individual and adding the others such that the tree length is minimized, then switching branches on the starting tree until all of the shortest trees are found.

Within-tree measures of robustness on a branch-by-branch basis often employ bootstrap analysis. Bootstrap analysis is a statistical method that involves resampling of data in phylogenies. The original data matrix is sampled with replacements until a new data set of the same size is achieved, and this new data set is then used to construct a tree. The process of sampling and tree construction is repeated many times to give a majority consensus tree. Those groups that occur in over half of the trees are considered to be supported by this method (Felsenstein, 1988). On the other hand, the consistency index has been broadly applied in parsimony analysis in global measures of robustness of an entire topology (Archie, 1989).

Often different parsimony trees may be produced even from the same data set and construction of a strict consensus tree is one approach for dealing with different topologies. A strict consensus tree includes only groups of taxa that occur in all of the trees. This method is the most conservative approach for reconciling differences in phylogenetic studies (Hillis, 1987). The value of consensus trees for resolving conflicting

phylogenetic hypotheses is debatable. It does not really solve the problem of why there are conflicting trees and a great deal of resolving power is often lost with consensus trees. But they do allow one to see the similarities among trees from different data sets (Hillis, 1987).

OBJECTIVES

1. Determine the mating system of *Montagnea* Fr.
2. Determine if populations of *Montagnea* from Africa and North America are interfertile.
3. Determine the nuclear contents of basidiospores and mycelia in crossing studies.
4. Determine the phylogenetic relationships of *Montagnea* populations.

In addition, the collections from which genetic material has been derived will be examined both macroscopically and microscopically to see what important differences and similarities exist between intercompatible and intersterile populations.

HYPOTHESES

1. The variations in spore size and sporocarp size are useful in distinguishing species of *Montagnea*.
2. The mating system in *Montagnea* is tetrapolar.
3. Populations from different continents are intersterile.
4. Additional sequence data from populations of two continents are closely related to *Coprinus*.

MATERIALS AND METHODS

Reagents and media:

The following reagents were used for microscopic examinations of the fruiting bodies: Melzer's solution (KI 1.5 g, Iodine 0.5 g, H₂O 20 g, Chloralhydrate 22 g); 3% KOH in water for reviving dried tissue; 95% ETOH as a wetting agent; Congo red (1% solution in H₂O) staining hyphae for more distinct viewing of structures such as clamp connections.

The following reagents and media were used for cultural studies: Gum guaiac (0.5 g in 30 ml of 95% ETOH) and Syringaldazine (0.1% solution in ETOH) for laccase and tyrosinase testing; Sudan IV (0.09% in 95% ETOH) staining lipid material; Sudan Black B (Saturated solution in 70% ETOH) staining lipid material. Nobel's medium (Malt extract 15 g, Agar 15 g, Water 1 L, sterilize for 20 minutes) was used as growth medium for crosses; Antibiotic medium (Penicillin G 150 mg, Streptomycin 150 mg, in 1 L Nobel's medium) was used to make single spore isolates (SSIs).

The following nuclear stains were used to demonstrate the nuclear situation of spores and hyphal cells both monokaryotic and dikaryotic: DAPI solution (4,6-diamidino-2-phenylindole) (5 µg/ml in McIlvaine's buffer (105 ml of 0.1 M anhydrous citric acid and 45 ml of 0.4 M anhydrous sodium phosphate dibasic, pH was adjusted to 4.4); Safranin-O solution (1 g sodium acetate and 1 g Safranin-O dissolved in 50 ml of 95% ethanol); Iron-hematoxylin solution (4% hematoxylin and 1% iron alum (ferric ammonium sulfate) in 45% acetic acid). In hematoxylin stain, Newcomer's solution (Isopropyl alcohol 6 : propionic acid 3 : petroleum ether 1 : acetone 1 (v/v)) was used as fixation solution

(Newcomer, 1953), and 1N HCl containing 2% aluminum alum (aluminum ammonium sulfate), 2% chrome alum (chromium potassium sulfate) and 2% iodic acid was used as a hydrolysis solution.

The following reagents were used in genomic DNA purification: CTAB buffer (1% (w/v) CTAB (hexadecyltrimethylammonium bromide), 50 mM Tris, pH 8.0, 10 mM Na₂EDTA, and 0.7 M NaCl) was used as DNA extraction buffer (Hillis *et al*, 1996); Chloroform-Isoamyl alcohol (24:1 (v/v)) as purifying solution; and Isopropyl alcohol was used as DNA precipitating solution. Agarose gel of 1% was used to detect DNA samples by electrophoresis.

The following reagents were used in PCR reactions: Bovine serum albumin at 10 mg/ml; 10xPCR buffer (commercial); dNTPs (dATP, dCTP, dGTP, dTTP) at 1.25 mM (commercial); ITS1 and ITS4 (White *et al*, 1990), or ITS1-F and ITS4-B (Gardes and Bruns, 1993) at 10 µM were used as primer pairs; Amplitaq *Taq* polymerase as the reacting enzyme.

The QIAquick PCR purification kit was used in PCR product purification. A commercial sequencing kit was used for the sequencing reaction. ITS1 or ITS4 at 1 µM was used as the sequencing primer in each sequencing reaction. Sephadex column was used in sequencing product purification.

Specimens examined:

***Montagnea arenaria* (DC) Zeller:**

Egypt: Gebel Abraç, Bir Abu Saa'fa, Feb. 28, 1985, Coll. R. W. Storer and R.L.Shaffer. RWS850228 (MICH). **Mexico:** Baja California, Bahia de Los Angeles, Nov. 1987, Coll. Joanne Turner. JT 871100 (VPI); Sonora, Black Mountain, Choya, March 1968, Coll. H. T. White and A.H.Smith. HTW680300 (MICH). **Namibia:** Kuiseb River, near Homeb, May 8, 1991, Coll. Nad Brain. KJ91050801. (VPI); Naukluft, Far East Dune, April 27, 1991, Coll. Kathy Jacobson. KJ 91042706 (VPI). **United States: Arizona:** Coconino County, Jacob Lake, House Rock Valley, July 26, 1955, Coll. H. E. Ahles. HEA9515 (MICH); as *Montagnites candollei* Fr., Navajo County, Hopi Res., Oraibi town, Aug. 24, 1936, Coll. A. F. Whiting and A.H.Smith. AFW360824 (MICH); Yuma County, Yuma desert, Feb. 14, 1973, Coll. E. Lee and Tom Tang. EL73003 (MICH). **California:** Imperial County, Anza Barrego Desert, New Tamarisk Campground, March 8, 1993, Coll. O.K. and H.H. Miller. VTMH 2466, VTMH 2505 (VPI); Imperial County, Colorado Desert, April 6, 1980, Coll. Mona Bowell. VTMH 2932 (VPI); Kern County, north of Mojave, Sept. 1983, Coll. Barbara Butera and O.K. Miller. OKM 20923 (VPI); as *Montagnites radiosus* (Pall.) Hollos, Los Angeles County, Mint Canyon, Oct. 10, 1941, Coll. Rea. Rea1009 (MICH); as *Montagnites radiosus* (Pall.) Hollos, Los Angeles County, Palmdale, Nov. 13, 1941, Coll. Rea. Rea1031 (MICH); as *Montagnites candollei var texensis* Berk. & Curt., Riverside County, Mecca, July 3, 1935, Coll. D.E. Bliss. DEB350703 (VPI); as *Montagnites radiosus var. tenuis* Pat., Ventura County, upper Cuyama Valley, June 1, 1939, Coll. Rea. Rea200. (MICH). **Idaho:** Owyhee County, southwest of Grandview, May 8, 1978, Coll. Roger Rosentreter. VTMH 2931 (VPI); Owyhee County, Rabbit Creek, Oct. 29, 1971, Coll. E. Trueblood. ET4918

(MICH). **Montana:** Lake County, June 25, 1981, Coll. ECM. VT 3750 (VPI). **New Mexico:** Hidalgo County, north of Lordsburg, Yucca elata-Ephedra-Prosopis. July 1969, Coll. M. Vinegar and A.H.Smith. MV690700 (MICH). **Oregon:** Harrey County, northwest of Venator, June 15, 1980, Coll. Leeds and Marie Bailey. VT 2933 (VPI); Malheur County, Owyhee Reservoir, June 14, 1970, Coll. E. Trueblood and A.H. Smith. ET3833 (MICH); as *Montagnites candollei* Fr., Morrow County, Boardman Bombing Range, July 1962, Coll. G. M. Baker and A.H.Smith. GMB620700 (MICH). **Texas:** as *Montagnites candollei* Fr., Eastland County, Oct. 21, 1935, Coll. E. A. Smith and A.H.Smith. EAS351021 (MICH); Stove wall County, Old Glory, June 1981, Coll. Eustaguio Castro Mendoza and O.K. Miller. VT4514 (VPI). **Utah:** Juab County, north of Lynndyl, July 2, 1955, Coll. K. H. McKnight. F247 (MICH). **Washington:** Benton County, north of Horn Rapids Road, Richland, April 15, 1972, Coll. H. A. Treibs and A.H.Smith. HAT720415 (MICH). **Wyoming:** Lincoln County, northwest of Granger, 6500 ft alt., August 21, 1973, Coll. S. Shushan. SF925 (MICH).

***Gyrophragmium* Fr.:**

Namibia: Oranjernund, Auchas Mine Area, COM, June 18, 1993, Coll. Kathy Jacobson. KJ 93061801. (VPI).

Methods:

Sporocarp examination

For each collection, macroscopic characters were observed and recorded. In addition, microscopic characters including pileipellis (type), pileus trama, gusset trama, basidia

and basidiospores were examined. Spore size was recorded for 30 spores for each collection and the mean and standard deviation were calculated.

Single spore isolates and culture observations

Small pieces of spore-bearing gleba from dried specimen were suspended in 10 ml of sterile distilled water and shaken vigorously then left to soak over night. Dilutions of the original suspension were made at 1/10, giving concentrations of 1, 10%, 1%, 0.1%. From the third and fourth dilutions 1 ml was plated on each of two antibiotic plates and the liquid was spread out with a sterile glass rod “hockey stick”. Plates were then incubated at 28°C and checked for signs of germination. Spores apparently germinating alone were examined with the light microscope, and colonies without clamp connections were transferred onto separate culture plates as single spore isolates and incubated continuously. For each collection, from which single spore isolates were obtained, a dikaryon with clamps was selected as wild type.

The cultural characters were observed and the cultures were tested for the laccase enzyme system with Gum guaiac and syringaldehyde. Sudan IV and Sudan Black B were used to test the lipid inclusions. The mycelia were studied microscopically to record conidia, unusual swellings, clamp connection morphology and any other morphological characters.

Genetic study

Selfcrosses

Ten single spore isolates of a chosen specimen were paired among themselves in all combinations. Small cubes of agar containing mycelium were cut with a cork borer, and two cubes from different single spore isolates were placed 1.5 cm apart on one culture plate. Crosses were then incubated at 28° C. After 5 to 10 days of growing, the hyphae at the confluent zone were stained with 3% KOH and Congo red and then examined for clamp connections. The mating type was determined according to the mating patterns derived from the self crosses. Results that yield 50% compatibility indicate a bipolar mating type while 25% indicates a tetrapolar system. Representatives of each mating type were saved and labeled for future crosses.

Outcrosses

Single spore isolates from different collections were used to mate with each other to test the compatibility among populations of *Montagnea* from different geographical locations.

Nuclear staining of hyphae and spores

The nuclear condition of the mycelia for both SSIs and crosses was examined by staining hyphae with DAPI, which specifically binds to AT-rich regions of DNA making nuclei fluorescent under epifluorescence microscopy (Williamson and Fennel, 1975; Schnedl *et al.*, 1977).

A modification of the method described by Lowry (1963) was used for nuclear staining of spores (Treu and Miller, 1993) as well as mycelia to confirm the results of DAPI staining. Small bits of hymenium from dried specimens or fresh mycelium from cultures

were fixed over night in Newcomer's (1953) solution. The fixed material was hydrolysed for 5 minutes at room temperature and then at 60° C for 30 minutes in the hydrolysis solution. The hydrolysed tissue was washed with distilled water 3 times to remove the excess mordanting salts and HCl, both of which interfere with subsequent staining. The material was then stained for 2 hours in iron-hematoxylin solution (Whittmann, 1962). Slides were made using small pieces of the stained hymenium. The preparation was heated in flame before observation to intensify the stain in the chromosomes and clear the cytoplasm.

Molecular analysis

Procedures were performed at Duke University according to the methods used by the Duke mycology laboratory (unpublished method).

Fungal genomic DNA extraction:

Small pieces of stipe tissue were cut off from dried herbarium specimens. The tissue was ground into fine powder in a 1.5 ml eppendorf microfuge tube with a small grinder. The tissue powder was suspended in 400 µl CTAB buffer. For fresh tissue, mycelia from cultures were freeze-dried before grinding and suspended in 2X CTAB buffer. The suspension was vortexed to make uniform slurry and incubated at 65° C for 30 minutes in a water bath. CTAB is a cationic detergent that solubilizes membranes and forms a complex with DNA.

An equal volume (400 ml) of chloroform-isoamyl alcohol was added to the above sample to extract and remove proteins. The tube was capped tightly and shaken

vigorously to form an emulsion. Continued extraction was performed for several minutes by periodic shaking to maintain the emulsion. The tube was centrifuged at 12,000 g for 5-10 minutes to produce a clear supernatant. The upper aqueous layer in the tube was removed carefully to a clean tube, avoiding any of the proteinaceous interface material. It is better to leave some interface behind if necessary to avoid protein contamination. Re-extraction was performed the same way for a second time but using slightly less chloroform-isoamyl alcohol (300 μ l). An equal volume of isopropyl alcohol was added to the final supernatant and mixed by inverting the tube gently. The sample was placed on ice for several minutes, and the DNA precipitate was collected by centrifuging the sample for 10 minutes at high speed. The alcohol supernatant was discarded, and the DNA pellet was washed briefly with 500 μ l of cold 80% ETOH. The ETOH was removed and the pellet was dried completely in a vacuum centrifuge. The dried DNA pellet was resuspended in 55 μ l double distilled water and stored in a freezer.

Total DNA dilution:

The DNA sample was checked on 1% agarose gel by using 5 μ l of the sample solution, and visualized by ethidium bromide staining. The amount of DNA was estimated by eye compared to the molecular marker with a known concentration. Small portions of the sample were diluted from 1/30 to 1/300 as the DNA template in the following PCR reaction.

PCR amplification of the Internal Transcript Spacer (ITS) region of ribosome DNA:

Each PCR reaction contained: Bovine serum albumin 2.5 μ l, H₂O 4.75 μ l, 10xPCR buffer 2.5 μ l, dNTPs 4 μ l, primer 1 (ITS1 or ITS1-F) 0.5 μ l, primer 2 (ITS4 or ITS4-B)

0.5 μl , Amplitaq 0.25 μl , and DNA template 10 μl at 0.1-1 $\text{ng}/\mu\text{l}$. The albumin is useful in overcoming an inhibitory activity of unknown origin that is present in many old extracts (Paabo, 1990). The reaction mixture was covered by a drop of mineral oil to prevent water vaporizing and concentration changing. A positive control with a DNA template that worked previously as well as a negative control with no DNA were performed to detect the efficiency and contamination in the reacting system.

The PCR conditions were as the follows: one cycle of 94° C for 3 minutes to denature the DNA double strands, followed by 35 cycles of 3 steps: 94° C for 1 minute, 50° C for 30 seconds and 72° C for 1 minute. Then one cycle of further extension was carried out at 72° C for an extra 7 minutes, the samples were then soaked at 4° C until being taken out of the PCR machine (DNA Thermal Cycler Model 480).

PCR products purification:

The results of PCR amplification were checked on 1% agarose gel before purification. The QIAquick PCR purification kit was used to purify the PCR products from primers, nucleotides, polymerases and salts. The purified PCR products were suspended in water and the amount was detected by agarose gel electrophoresis. The necessity for dilution or concentration of the products was estimated by eye to give a DNA concentration of 15-35 $\text{ng}/\mu\text{l}$.

PCR cycle sequencing of the ITS region:

Each sequencing reaction contained: 4 μl FS juice (containing Taq DNA polymerase, dNTPs and fluorescently labeled ddNTPs, supplied and pre-mixed by the manufacturer), 2 μl primer (ITS1 or ITS4) at 1 μM , 4 μl DNA template in water ranging from 50 to 150

ng. Thus, two reactions (one for each primer) were set up for each DNA sample. The reaction mixture was covered with mineral oil as in the PCR reaction. Standard Autocycle was used for the sequencing reaction.

Column purification of sequencing products:

Sephadex was mixed with water at 10 g/200 ml, and decanted until water was 1/6 of the volume. Columns were rinsed with 400 µl water before using, and the washed columns were packed with 690 µl of the above Sephadex, and spun 2 minutes at 2800 rpm to remove extra water.

The sequencing products were withdrawn from below oil and put onto the Sephadex, spun 2 minutes at 2800 rpm to filter the DNA to a clean 1.5 ml eppendorf tube. The cleaned DNA samples were thoroughly dried in a vacuum centrifuge and kept frozen. Before running on the sequencing gel, the DNA sample was suspended in 3 µl sequencing buffer, heated 2 minutes at 93° C to denature the double strands, and loaded on 8% polyacrylamide gel on the sequencing machine (ABI Model 373 DNA Sequencer).

Phylogenetic analysis:

Both DNA stands of the PCR sequencing products were sequenced. The sequences were double checked to reduce errors and contiguous sequences were assembled using Sequencher 3.1. The sequence data were aligned by hand in Phylogenetic Analysis Using Parsimony (PAUP 3.1) (Swofford, 1993). The ITS sequence of *Coprinus comatus* was chosen as an outgroup in the analysis based on the unpublished result of Vilgalys *et al.* A heuristic search for the most parsimonious trees was conducted in PAUP with standard

variable regions excluded. Bootstrap value was calculated from the search. Consensus trees derived from all of the most parsimonious trees were generated.

RESULTS

Sporocarp examination

There are wide variations in the size and shape of the fruiting bodies. The diameter of the caps range from about 5 to 50 mm, and the stipes vary from 3 to 15 cm long, and 2 to 15 mm thick (Fig. 1).

The pileipellis is a cystoderm with a filamentous pileus trama (Fig. 2). These characters and the elliptical spores with an apical pore are all very similar to those of *Coprinus comatus*. No cystidia were observed in any of the collections. Most basidia are fully mature and collapsed in age; one seldom finds young fresh specimens. In all the collections studied, including those which collapsed, only four-spored basidia were observed (Fig. 3).

The spore size is extremely variable among collections as well as within a given specimen. The spores ranged from about 7 to 22 μm long, and the standard deviation of spore length for one collection could be more than 2. The shape of the spore is also highly variable, and the Em value (length/width) was found to range from 1.3 to 1.9 (Fig. 4). The fruiting body and spore measurements for all specimens examined are recorded in Table 1.

Single spore isolates and culture observations

The original attempt to transfer single spore isolates that were stored at -20°C to either agar or liquid malt was not successful. Pure cultures were obtained from dried gussets of

herbarium specimens, and spores germinated within two days depending on the age of the collection and how well it was preserved. The oldest specimen which spores could germinate was collected in 1978 (VT2931), but some collections younger than that, the spores could not germinate (Table 6). The appearance of the young mycelia was different among SSIs, from flat to fluffy, but no macro-morphological difference could be discovered by comparing dikaryons with colonies derived from single spore isolates.

The enzyme test with Gum guaiac was blue and Syringaldezine was pink, which indicated that laccase enzymes are present and the fungus is a white rot. Sudan IV and Sudan Black B tests were both negative and indicated that metabolic reserves were not lipids. No conidia were observed in any of the cultures, and the clamp connections in the wild type were buckle-like. Swellings, also called hyphal-bodies, were common in the hyphae of many cultures and these may function as storage organs.

Genetic study

Two sets of selfcrosses were performed for two specimens, both from Namibia, KJ91042706 and KJ91050801. Single colonies without clamp connections were used as SSIs and ten SSIs were paired among themselves in all combinations for each selfcross. The results are recorded in Table 2 and 3. Clamps did form in some crosses, but some SSIs did not form clamps with any others, such as SSI 7 and SSI 10 of KJ91042706, and SSI 27 of KJ91050801. Single spore isolate 1 of KJ91050801 was determined to be a dikaryon that is self-fertile. Repulsion zones were formed between some SSIs, which indicated an incompatible confrontation. Normal bipolar or tetrapolar mating systems

were not obtained from the two selfcrosses. The fact that some SSIs were not compatible with others indicated that a bifactorial system might exist. Attempts were made to find a fourth mating type by testing more selfcrosses. Five more SSIs were tried for each of the two collections, but neither a fourth mating type nor a clear mating pattern was obtained.

Clamps were present but infrequent to rare in compatible crosses and only found at the contact zones. No clamps were observed at the colony edge of either donor SSIs when they were compatible. Clamps were even fewer in some of the crosses and very difficult to find, which made the negative results less reliable. Once clamp connections were observed in a given culture, subsequent examination revealed no clamps as the culture continued to grow. In contrast, the usual dikaryon formation results in new growth of almost completely clamped hyphae. Transfer of the crosses and continued incubation was carried out on new plates but no clamps were observed. These mating abnormalities prevented a routine genetic analyses of SSIs from different collections. However, compatibility among collections was established and in few case were repulsion zones observed. Since no tester strains were available, two SSIs of the specimens that had previously yielded viable spore isolates were picked up randomly, and crosses were made among them. Results are recorded in Table 4 and 5. Only a few compatible crosses were obtained from the out crosses, but as before, scattered clamp connections were present and few repulsion zones were observed.

Nuclear condition

The unusual results obtained in the mating studies were followed up by a study of the nuclear condition during the early stages of spore germination and growth. With DAPI staining, the germ tubes from single spores were at first (24 hours after plating on malt) coenocytic, having more than 5 nuclei in the cell. Subsequent hyphal cells were multinucleate before the septa were formed. Extensive multinucleate mycelia were produced within 36 hours (Fig.5). The septa were not easily observed with DAPI, but it was obvious that the majority of hyphal cells had more than three nuclei. After growing for several days on new plates, the colonies from single spores reached 3 to 4 cm in diameter. A reexamination using DAPI revealed that most mycelia had no more than 4 nuclei in one cell. The hyphae of crosses with or without clamps were both multinucleate, and the number of nuclei could be from 0 to 5, but most were 2 to 4 (Fig. 6 and 7). The mycelia from the fruiting body (ie. volva or stipe) were also multinucleate (Fig. 8). According to these results, the nuclear number could not be used to separate the dikaryon from a monokaryon.

Some spores germinated with one or more non-germinating spores nearby. In these cases, the second spore was not observed to germinate. When this occurs, however, there is a chance that germination may take place later on (Fig. 9). In that way, colonies which appear to arise from single spores may be from multiple spores. A microscope view of the SSIs was, therefore, necessary to insure that a single spore was present.

Safranin-O staining of hyphae was not successful, and too many materials inside cells were stained by this method, giving very high background. Hematoxylin staining was then employed and was successful. Most of the basidiospores have one nucleus, while a

small number have two in one spore. But spores with two nuclei were not bigger than those with one nucleus per spore (Fig. 10). There is also no way to determine if the second nucleus resulted from one mitotic division or was a product of meiosis. It is possible that some cultures derived from a SSI could be functional dikaryons.

Molecular analysis

Genomic DNA was first extracted from gussets as usual, no DNA band was visible when checked on agarose gel, and RNA was abundant. PCR reactions using these DNA samples as templates all failed. Several methods were tried, including dilution of the samples up to 1000-fold, washing of the DNA with salt and Ethanol to precipitate and resuspend the DNA in water, and lastly adding small amounts of albumin to the PCR reactions. None of these methods made amplification work. Positive control that was successful became negative when the above DNA samples were added into the reaction mixtures. The obvious inhibition showed that too many impurities and inhibitors were present in the gusset extraction, possibly due to the dark pigments.

New DNA extraction was performed from the stipe of the fruiting bodies as well as from pure culture. DNA band was visible on agarose gel in some of the samples with the presence of a large amount of RNA. PCR reactions were successful with most of these DNA samples at different dilutions, including those which were not visible on the gel. A 600 to 700 bp fragment was amplified in each of these reactions.

Some DNA samples were contaminated by other organisms, and there was more than one kind of DNA template present in the DNA extraction. PCR reactions using these

DNA templates would amplify two products with similar molecular weights. The two bands were close to each other on the gel and were not separated unless the gel was run for a long time. When these PCR products were first thought to be one wider band and used in sequencing analysis, the sequence data were not readable and were highly polymorphic. According to molecular weight, attempts were made to use the upper band (longer fragment) that recovered from the gel as the template for PCR, but the amplified products had tails at both ends of the band and were not clean enough for sequencing. Furthermore, the sequence results of some specimens (i.e. VT4514) were just from contaminations and turned out to belong to other taxon, such as *Fusarium*, an Ascomycetes.

Taxon-selective primer pair ITS1-F and ITS4-B were then used in PCR reactions for the above specimens and solved most of the problems. ITS1-F is specific for higher fungi, and ITS4-B is designed with mismatches between Ascomycetes and Basidiomycetes at its 3' end to be specific for the latter. This primer pair is outside the region of ITS1 and ITS4, and PCR amplifies a longer DNA fragment. The PCR product can then still be sequenced using ITS1 and ITS4 primers. There were very few specimens that failed to yield results in either PCR or the sequencing reaction. Some of the specimens were simply too badly damaged (HEA9515 and Rea1009) or contaminated (VT4514), and others were thought to be polygenetic due to interallelic variation in the multiple copies of rDNA. Too many chemical impurities could also cause failure in the PCR reaction. Specimen RWS850228 has a variable morphology, with both large and small spores (Fig. 11); the PCR amplifications using both sets of primers were strong and

clean, but the sequence data were polymorphic and not interpretable. Cloning of the PCR products before sequencing might solve the problem and would be worth trying in future work. The spores of RWS850228 would not germinate in spite of the relative young age of the specimen. Detailed results of the molecular work carried out for each specimen are recorded in Table 6.

Phylogenetic analysis using PAUP

Sequence data were double checked according to the chromatograph, and contiguous sequences were aligned by eye in PAUP using the ITS sequence of yeast as a reference. A total of 614 bases of the sequence were included in the analysis, and 529 of those characters were constant. There were 62 variable positions that had characters that occur uniquely in only one genotype and were parsimony-uninformative. Only 23 positions had states shared by two or more genotypes and were thus inferred to be phylogenetically informative. In the heuristic search, 72 most parsimonious trees (unrooted) were found with the 62 uninformative variable regions being excluded, and the tree length was 95. Bootstrap values were relatively low for quite a few branches. But the consistency index (CI) and retention index (RI) were both relatively high and above 0.85. A strict consensus tree of all the 72 most parsimonious trees was constructed with the same tree length, CI and RI values (Fig. 12 and 13).

From the consensus tree, no clear biogeographic isolation was obtained, nor was any correlation between spore size and relatedness found. Specimens from California were widely distributed within the tree and closely related to those from the northern United

States. Specimens from Namibia were also closely related to those from North America. Spore size was also distributed randomly in the tree, with small-spored (about 10 μm) specimens closely related to large spored-ones (about 17 μm). Neither were other distinctive morphological characters found among different clades.

An interesting find from the sequence data was that Specimen KJ93061801, which was identified as *Montagnea*, has a similar ITS sequence to *Gyrophragmium*, and quite different from that of *Montagnea*. This specimen has ovoid almost roundish basidiospores without an apical germ pore (about 5 μm in diameter, $E_m=1.1$), which resembles the characters of *Agaricus* (Fig. 14). Unlike *Montagnea*, the pileipellis of this specimen is a mixocutis, and the spores took almost one month to germinate. In addition, no clamp connections were found in the wild type nor in any of the crosses. Recent phylogenetic work on the Agarics and their allies based on the large subunit ribosomal DNA sequence confirmed the close relationship between *Gyrophragmium* and *Agaricus*, as well as *Montagnea* and *Coprinus* (Rytas Vilgaleys, unpublished results). *Gyrophragmium* has been traditionally thought to be closely related to *Montagnea* and *Coprinus* based on comparative morphology (Heim, 1971).



KJ91042706 (Namibia)

KJ91050801 (Namibia)



DEB350703 (CA)



HAT720415 (WA)

Fig. 1. Variation in the size and shape of the fruiting body of *Montagnea*.

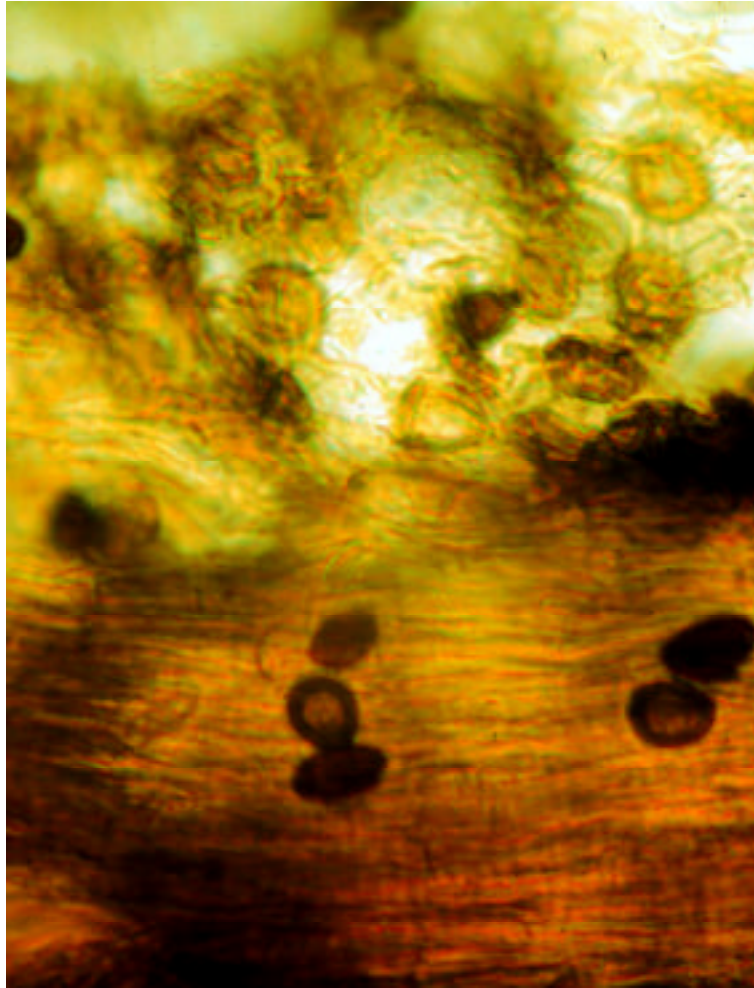


Fig. 2. Cystoderm pileipellis and filamentous pileus trama of *Montagnea arenaria* (KJ9105080). 10x40.

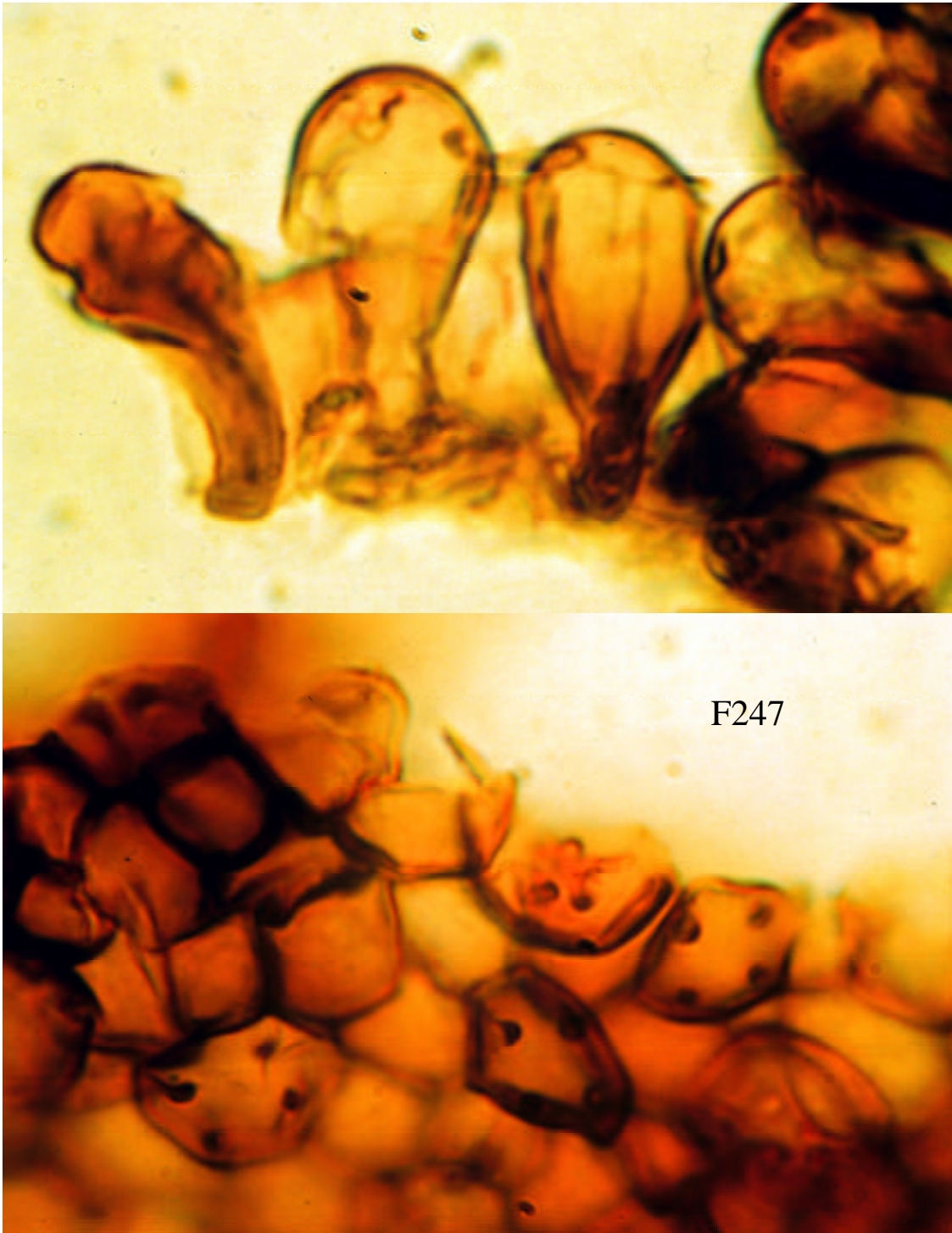


Fig. 3. Basidia and collapsed basidia of *Montagnea* showing 4 sterigamata per basidium. 10x100.

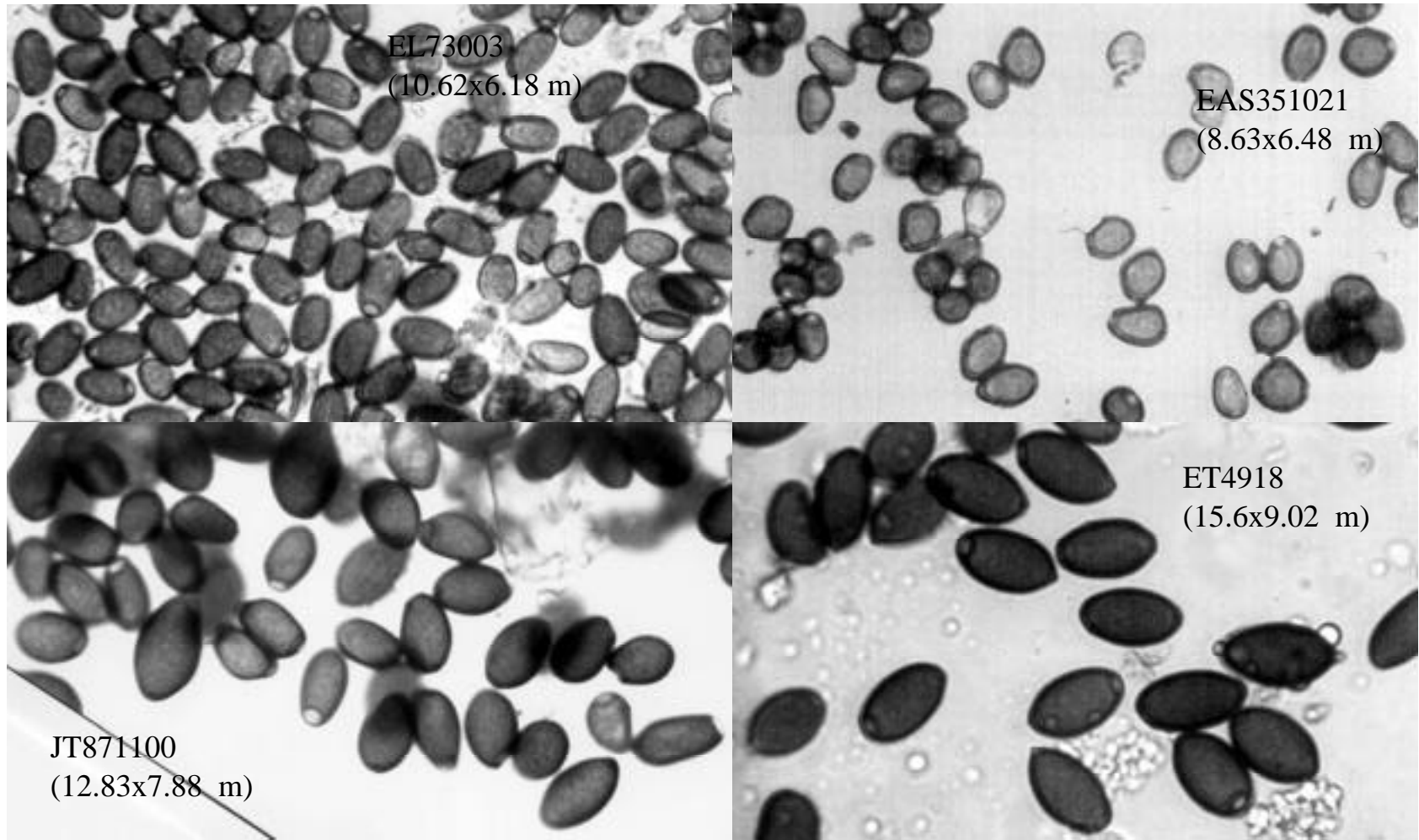


Fig. 4. Variation in spore size and shape of *Montagnea*. 10x100.

Specimens examined	Pileus diameter	Stipe length	Stipe width	Spore Length um	Average	Standard Deviation	Spore Width um	Average	Standard Deviation	Spore Em=L/W
VT4514	11-18mm	3-9cm	2.5-4mm	7.0 - 10.0	8.42	0.708	5.0 - 8.0	6.03	0.629	1.396
EAS351021	15-25mm	>6cm	2-3mm	7.5 - 10.0	8.63	0.669	5.5 - 9.0	6.48	0.701	1.332
KJ91042706	11-18mm	5-6cm	2.5-3.5mm	7.5 - 13	9.92	1.153	5 - 6.5	5.7	0.428	1.74
VT2466	18-23mm	2.5-6cm	3-5mm	8.0 - 13.0	10.35	1.026	6.0 - 9.0	7.6	0.768	1.336
EL73003	22mm	13.5cm	7mm	7.0 - 15.0	10.62	1.946	5.0 - 8.0	6.18	0.924	1.718
VT2505	11-18mm	4-7cm	2-3mm	8.0 - 13.0	10.65	1.211	7.0 - 8.0	7.48	0.425	1.424
VT3750	31mm	10.5cm	10mm	7.2 - 13.2	10.92	1.456	4.8 - 9.6	7.76	1.168	1.407
OKM20923	28-38mm	7.5-15cm	4-8mm	8.0 - 14.0	11.27	1.57	4.5 - 7.5	6.45	0.746	1.747
HAT720415	11-16mm	5.5-11.5cm	2-3mm	9.0 -14.0	11.7	1.08	6.0 - 8.0	6.9	0.687	1.696
AFW360824	20mm	7-9cm	4mm	9.0 -14.0	11.77	1.569	6.0 - 9.0	7.27	0.868	1.619
Rea200	20-30mm	6.5-8cm	4-5mm	9.0 -14.0	11.8	1.356	6.0 - 9.0	7.77	0.74	1.519
VT2933	19mm	7.5cm	8mm	9.6 - 15.6	12.36	1.58	4.8 - 9.6	7.6	1.421	1.626
JT871100	15-27mm	>6cm	2-3mm	11.0 -15.0	12.83	1.262	6.0 - 10.0	7.88	0.916	1.628
KJ91050801	5- 7mm	7-8.5cm	3-5mm	10.0 - 15.0	13	1.358	6.5 - 9.5	8.05	0.735	1.615
VT2932	14-18mm	4.5-7cm	3-4mm	10.0 -15.0	13.18	1.316	6.0 -9.5	7.87	0.991	1.675
ET3833	15mm	?	3mm	11.0 -16.0	13.55	1.255	7.0 - 9.0	8.05	0.514	1.683
SF925	8-10mm	7-10cm	2-3mm	11.0 -17.0	13.85	1.378	7.0 - 9.0	8.23	0.583	1.683
F247	15-22mm	6.5-8.5cm	3-5mm	11.0 -20.0	13.9	1.863	7.0 - 9.0	8	0.63	1.738
DEB350703	25-42mm	>12cm	7-9mm	11.5 -17.0	14.37	1.42	7.5 - 11.0	8.52	0.793	1.687
GMB620700	15-20mm	7-10cm	3-4mm	11.5 17.0	14.5	1.196	7.5 - 10.5	9.22	0.784	1.573
VT2931	20mm	6-10.5cm	5-7mm	10.0 - 17.0	14.65	1.372	7.5 - 9.0	8.42	0.872	1.741
HTW680300	>23mm	8.5-10.5cm	4-7mm	11.0 -18.0	14.95	1.615	6.5 - 9.0	7.78	0.597	1.922
HEA9515	30mm	>15cm	7.5mm	12.5 -18.0	15.55	1.14	8.0 - 11.0	9.42	0.644	1.651
ET4918	13-21mm	7-8cm	3-4mm	12.5 -18.0	15.6	1.329	7.0 - 10.5	9.02	0.815	1.729
MV690700	25mm	>8cm	5mm	13.0 -18.0	16.33	1.162	8.0 - 10.0	8.93	0.521	1.829
Rea1009	40-50mm	13cm	7-10mm	12.0 -20.0	17.8	1.896	6.5 - 12.0	9.98	1.141	1.784
Rea1031	>30mm	>10cm	10-15mm	11.0 - 22.0	17.87	2.623	7.0 - 14.0	10.03	1.871	1.782
RWS850228s	25-50mm	9-12cm	3-5mm	6.0 - 7.5	6.83	0.479	4.0 - 7.0	4.9	0.7	1.394
RWS850228l				12.5 -20.0	15.13	2.46	6.5 - 13.0	9.38	2.184	1.613
KJ93061801	10-16mm	6cm	3-5mm	4.0 - 7.2	5.16	0.852	4.0 - 6.0	4.64	0.583	1.114

Table 1. Specimens examined of *Montagnea* including fruiting body size and spore measurements for each collection.

	1	2	3	4	5	6	7	8	9	10
1	--	--	--r	--	--r	+	--r	+	--	--
2		--	+	+	--	F	--	--	--	--
3			--	--	--	+	--	+	+	--
4				--	--	+	--	+	--	--
5					--	+	--r	--	--	--
6						--	--	--	--	--
7							--	F	--	--
8								--	--	--
9									--	--
10										--

Table 2. Selfcrosses of *Montagnea arenaria* (KJ 91042706).
+: clamps; --: no clamps; r: repulsion zone; F: false clamps

SSIs	1	2	6	9	10	14	15	17	27	29
1	+	+	+	+	+	+	+	+	+	+
2		--r	--r	+	--r	--	--r	+	Fr	--
6			--	+	--	--	--	+	--	--
9				--	+	+	+	--	--	+
10					--	--	--r	+	--	--
14						--	--r	--	--r	--r
15							--	+	--r	--r
17								--r	--	+
27									--	--r
29										--

Table 3. Selfcrosses of *Montagnea arenaria* (KJ 91050801).
+: clamps; --: no clamps; r: repulsion zone; F: false clamps

		KJ91050801		VT2466		VT2505		VT2931		VT2932	
		3	8	1	2	1	3	1	2	1	4
KJ91042706	1	--	--	--	--	--	--	--	--	--	--
	2	--	--	--	--	--	--	--	--	--	--
KJ91050801	3			--	--	--	--	+	--	--	--
	8			--r	--r	--	+	+	--	--r	+
VT2466	1					+	+	+	--	+	+
	2					+	+	+	--	+	+
VT2505	1							+	--	+	--
	3							+	+	+	--
VT2931	1									+	+
	2									+	+

Table4. Outcrosses of *Montagnea*. SSIs were obtained from colonies apparently single.
 +: clamps; --: no clamps; r: repulsion zone

		KJ91042796		KJ91050801		JT871100		VT2466	
		1	5	3	5	1	3	1	3
KJ91042706	1	--	+	--	--	--	--	--	--
	5		--r	--	--	--	--	--	--
KJ91050801	3			--	+	+	--	--	--
	5				--	--	--	--	--
JT871100	1					--	+r	--	--
	3						--r	--	--
Vt2466	1							--	+
	3								--

Table 5. Outcrosses of *Montagnea*. SSIs were examined under the microscope.
+: clamps; --: no clamps; r: repulsion zone.

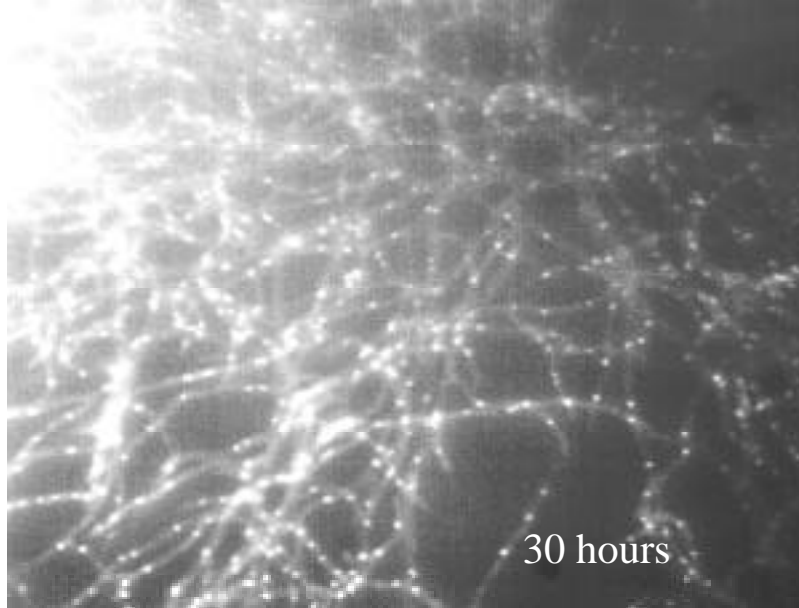
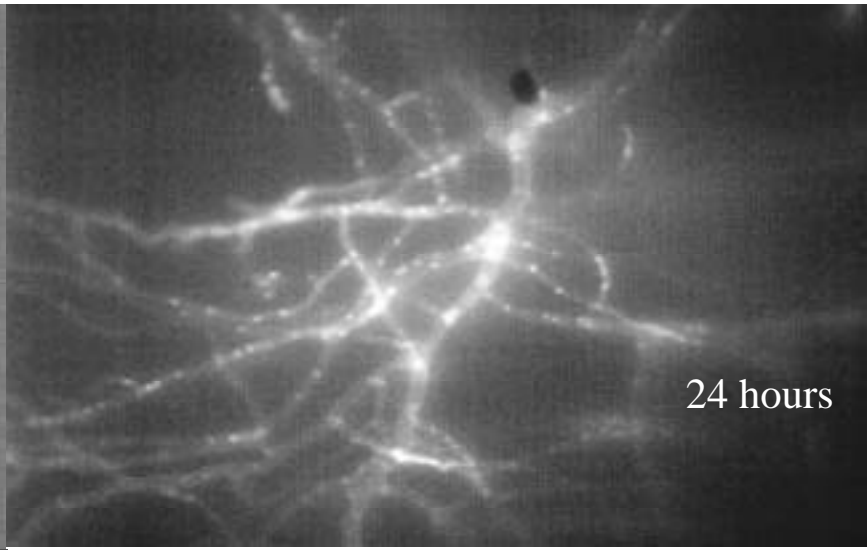
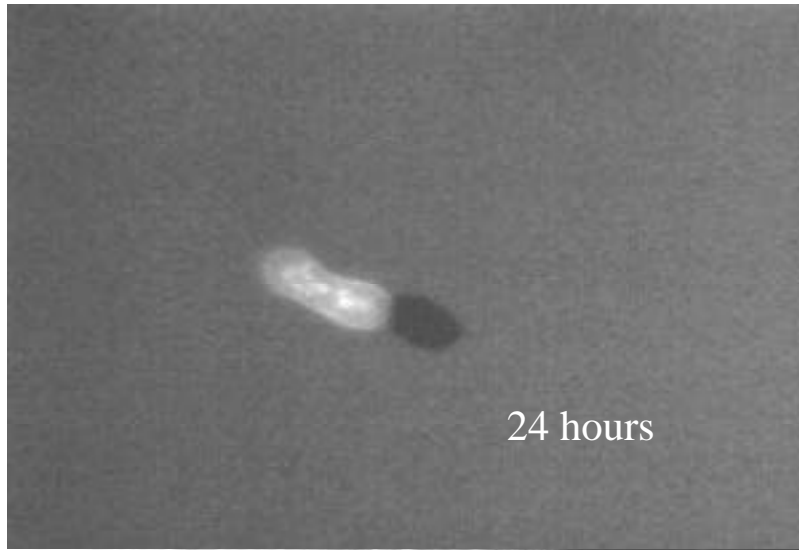


Fig. 5. Single spore germination of *Montagnea arenaria* (KJ91050801) with DAPI staining. Germ tube at first coenocytic, subsequent hyphal cells multinucleate, and no clamps present from the single spore.

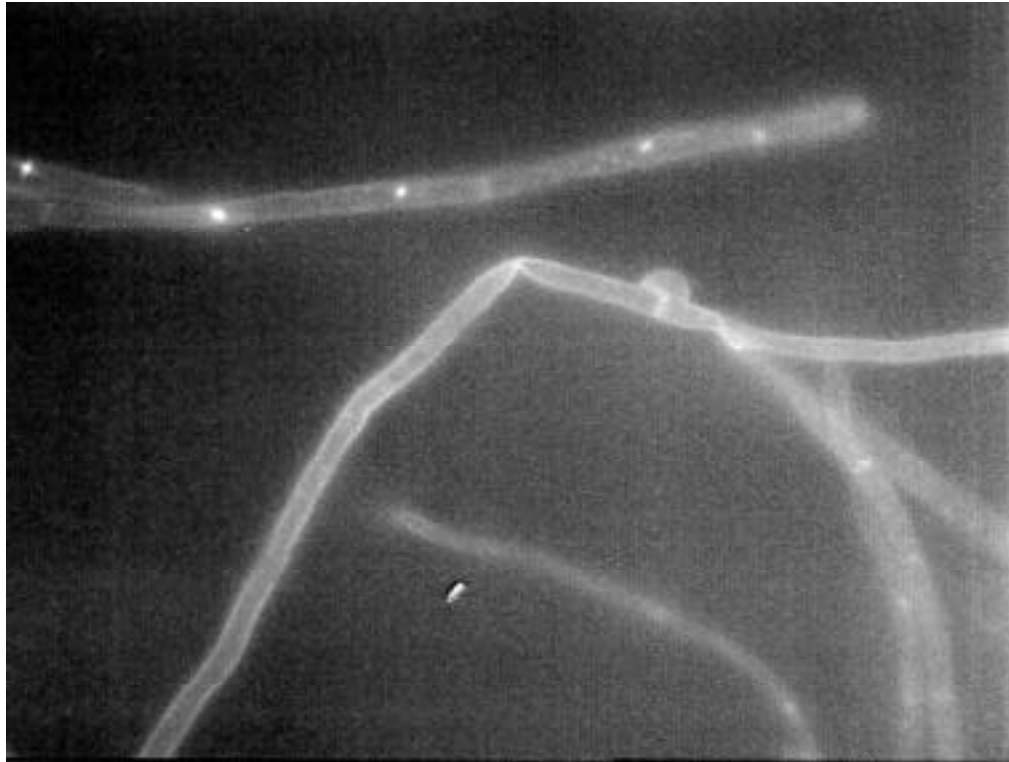


Fig. 6. Compatible cross of *Montagnea arenaria* (KJ91050801). Clamp connections were formed but were not frequent in the culture. Nuclear number was variable in different cells with DAPI staining.

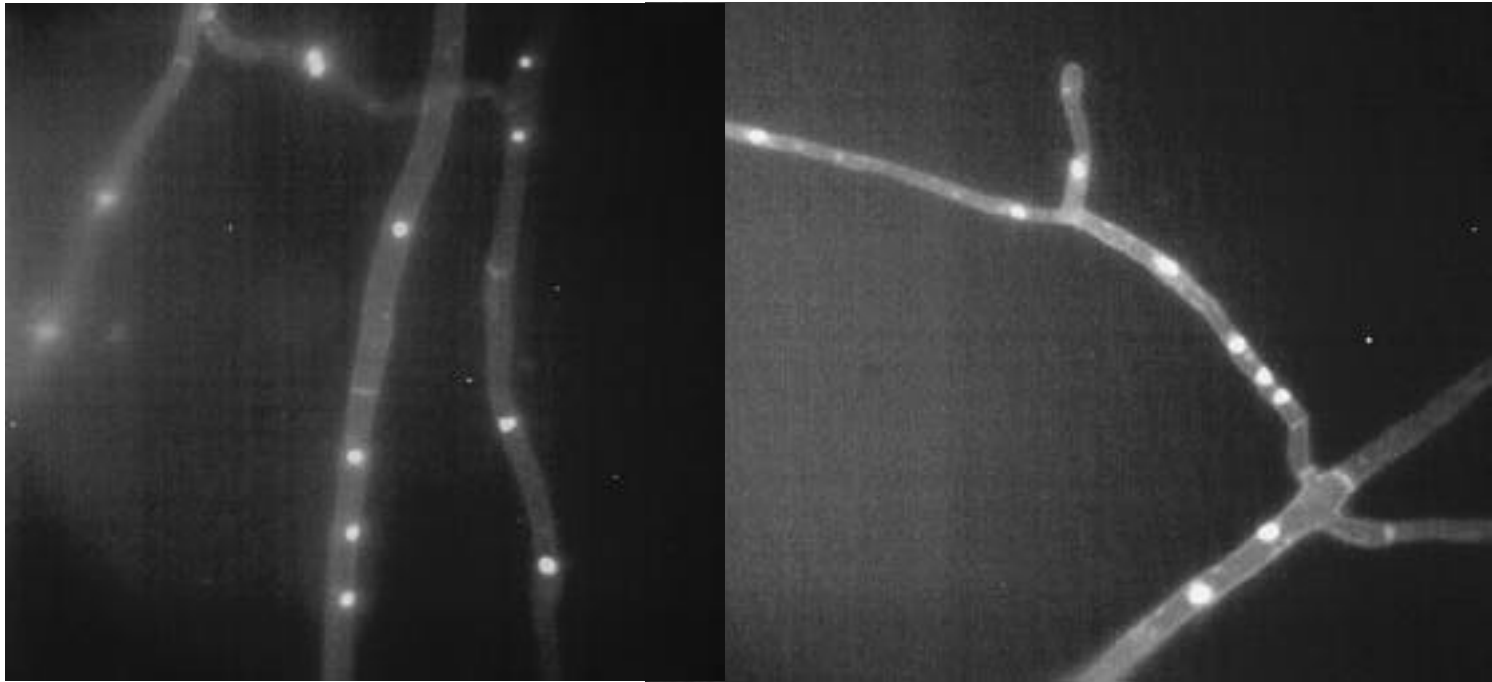


Fig. 7. Nuclear staining with DAPI of crosses with and without clamps of *Montagnea arenaria* (KJ91050801). Nuclear number of hyphal cells was variable in both cases.

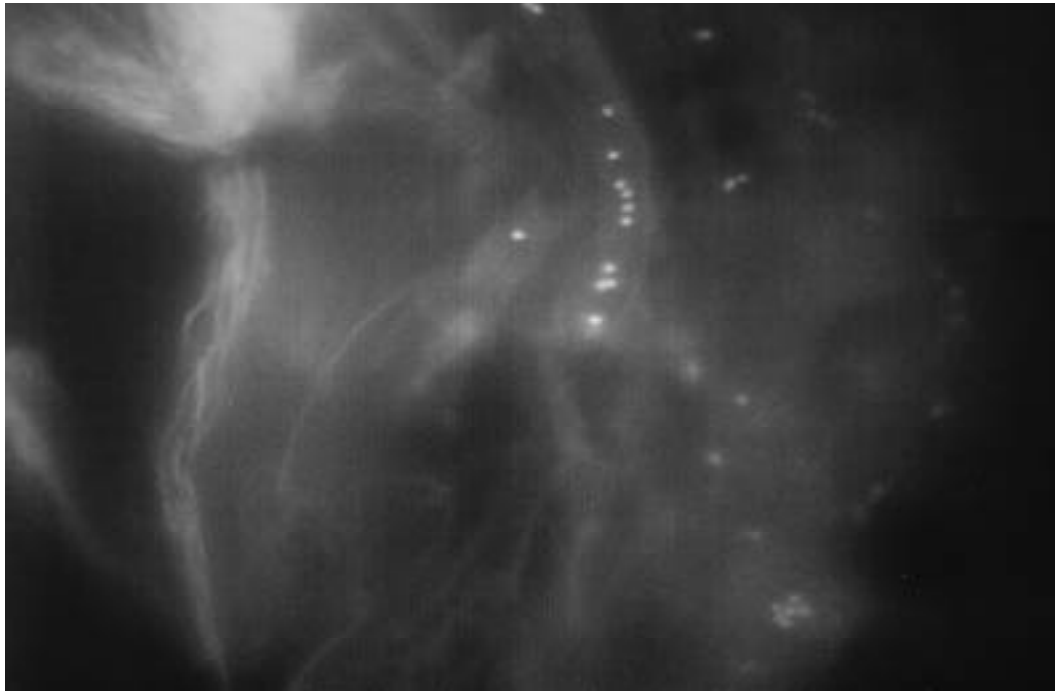


Fig. 8. DAPI staining of hyphae from volva tissue of fruiting body of *Montangea arenaria* (KJ91050801) showing multinucleate hyphal cells.

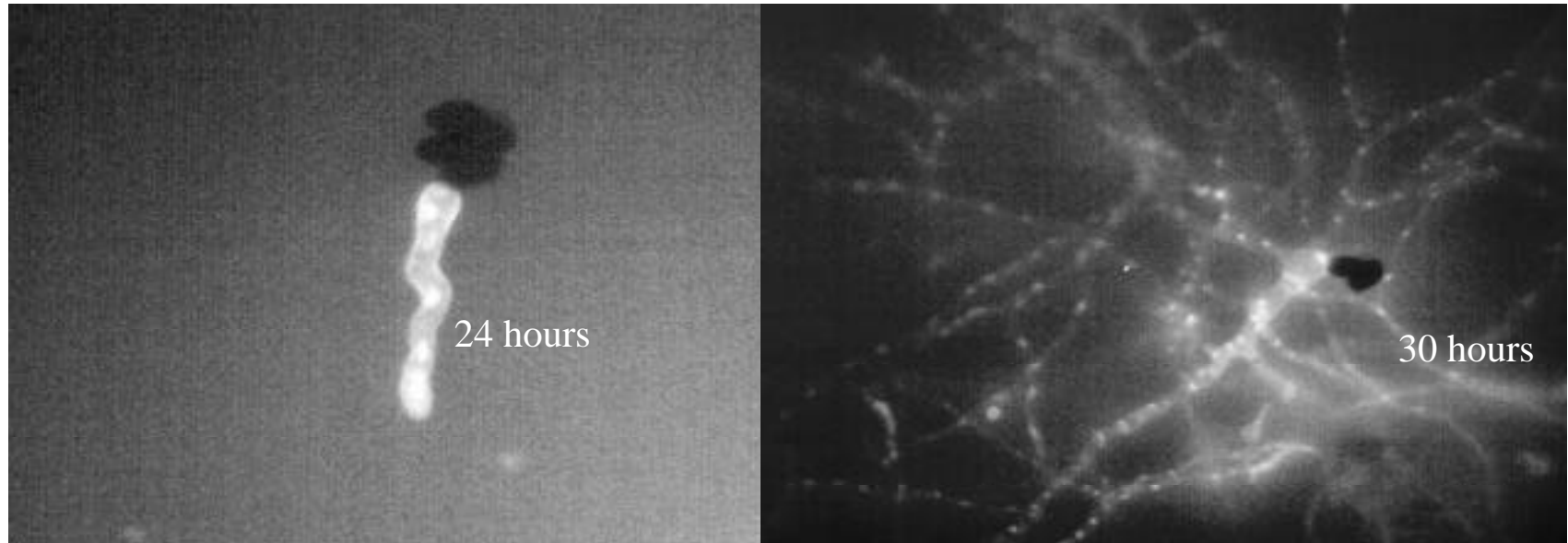


Fig. 9. Spore germination of *Montagnea arenaria* (KJ91050801). Single spore germinated with one or more ungerminated spores next to it.

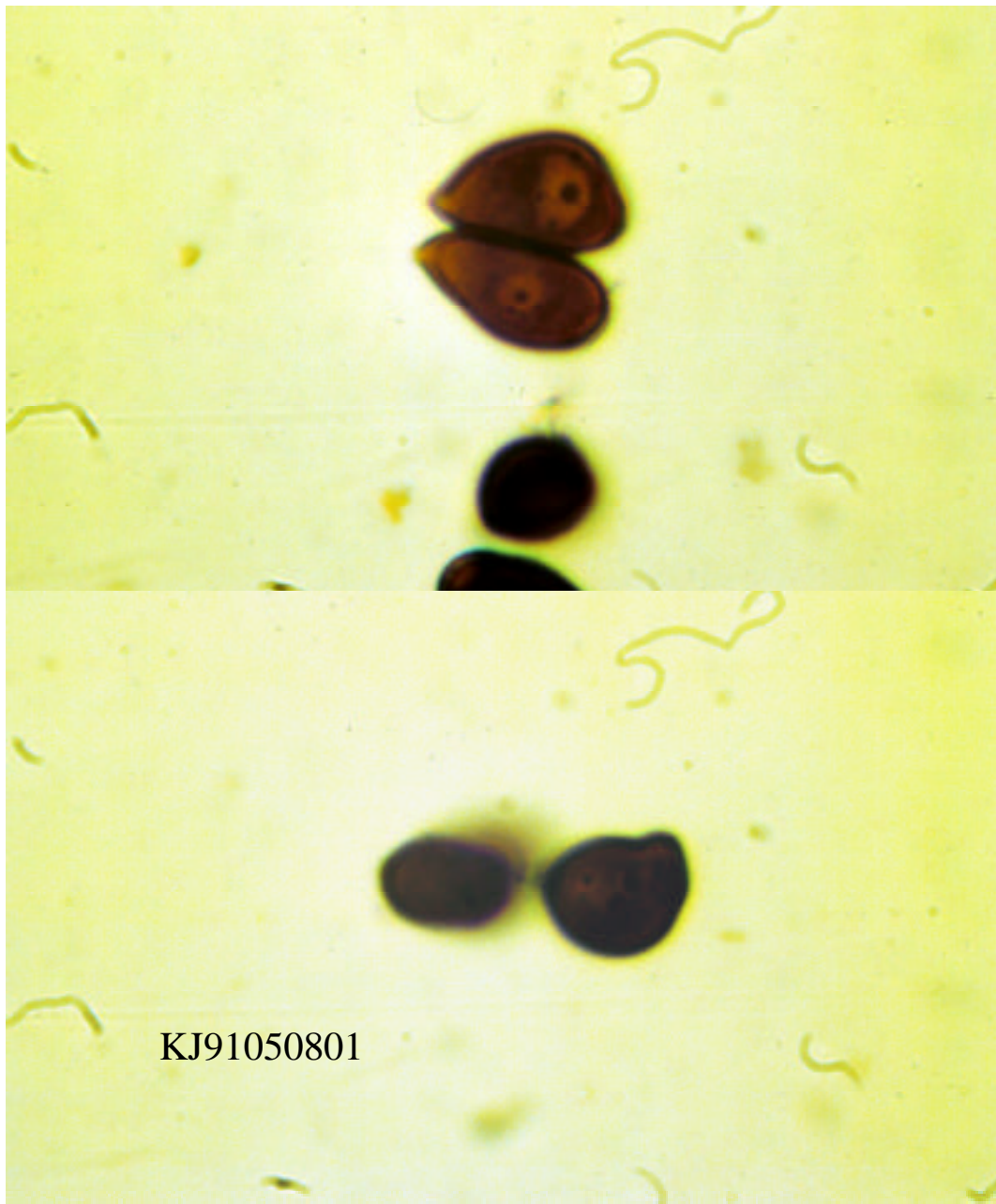


Fig. 10. Hematoxylin stain of the basidiospores of *Montagnea arenaria*. Most spores have one nucleus (upper microphotograph), very few spores (lower one) show two nuclei in one spore. 10x100.

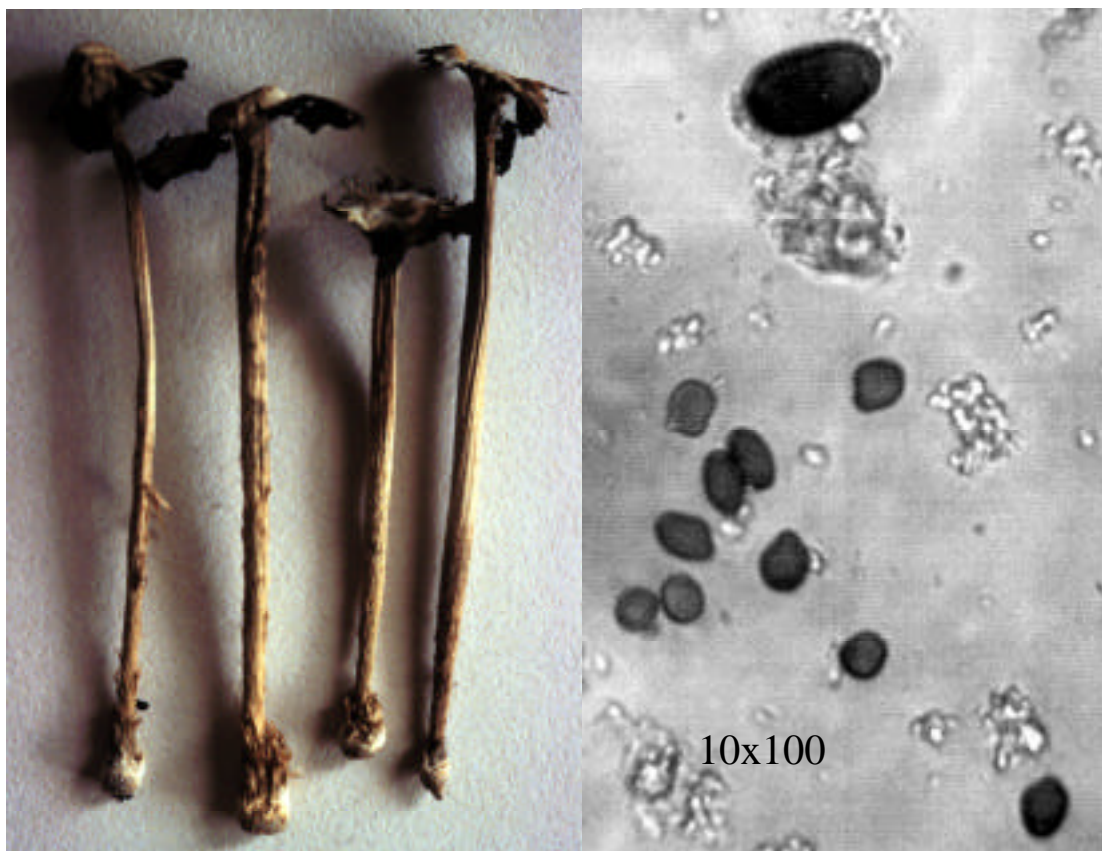


Fig. 11. Fruiting body and basidiospore of *Montagnea arenaria* RWS850228.

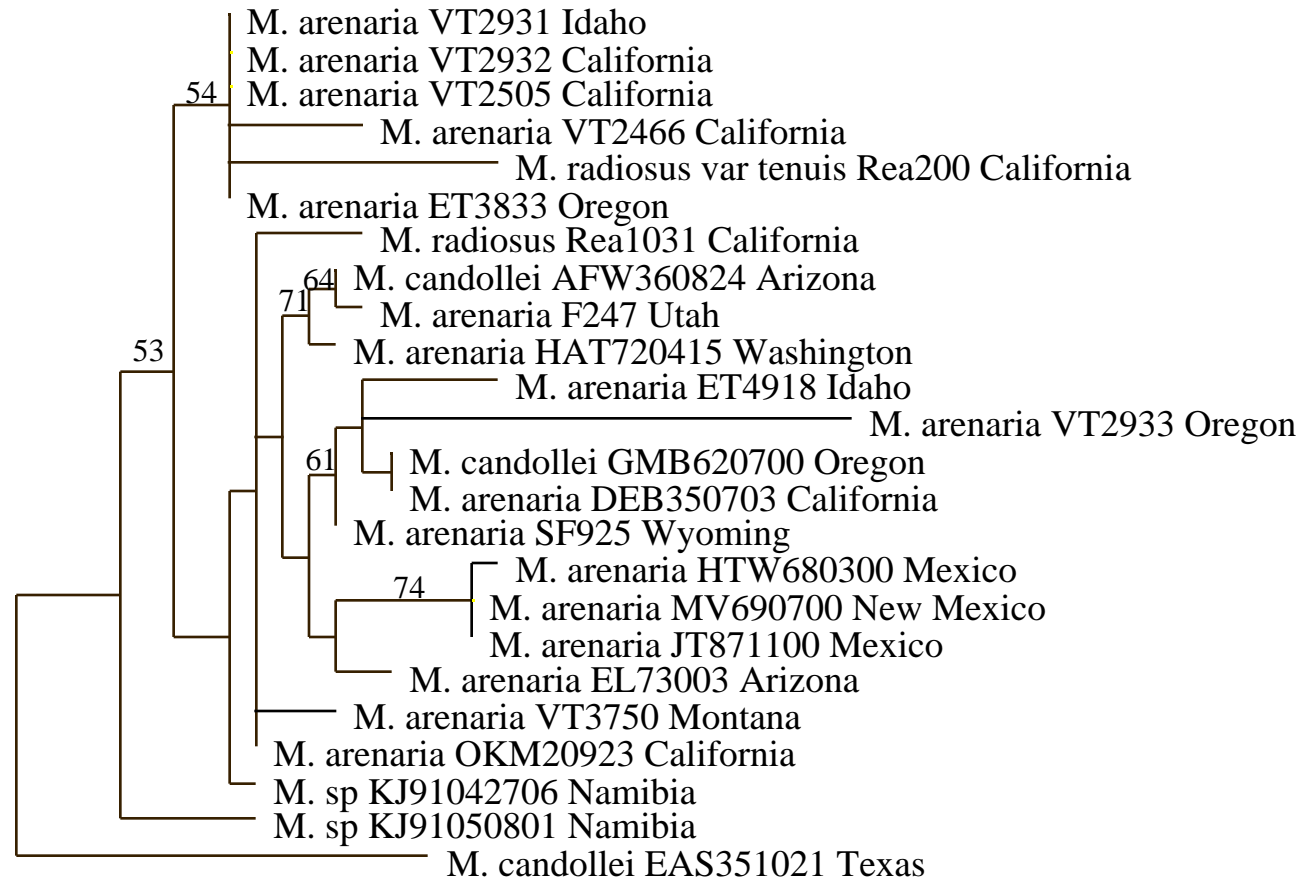


Fig. 12. One of 72 maximum parsimony trees of *Montagnea* based on ITS sequence data. Tree length = 95. Consistency index (CI) = 0.8947. Retention index (RI) = 0.8571., 23 parsimony-informative characters.

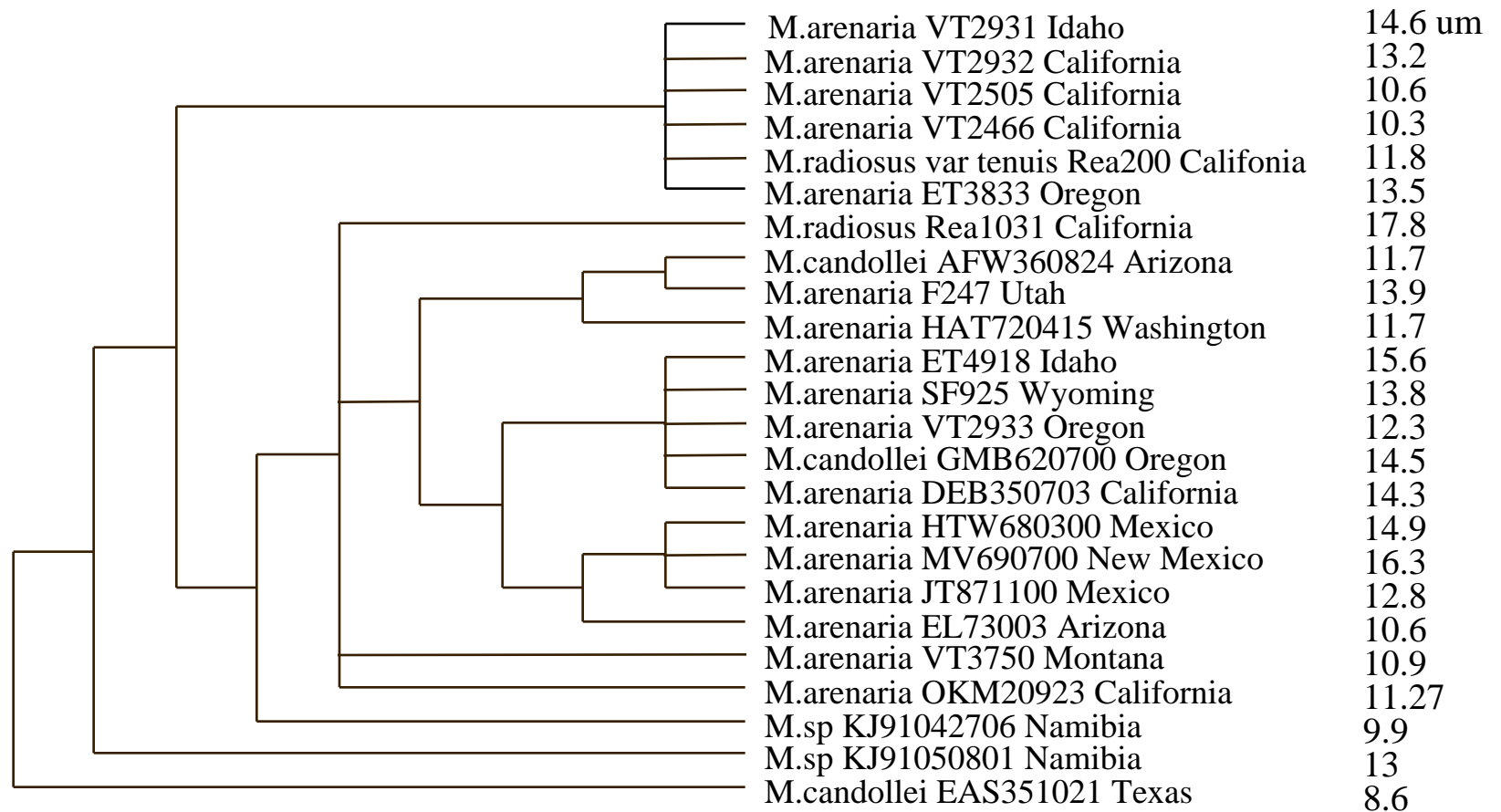


Fig. 13. Strict consensus of 72 mp trees of *Montagnea*. Tree length = 95. Consistency index (CI) = 0.8947. Retention index (RI) = 0.8571. Spore size is shown beside each specimen.

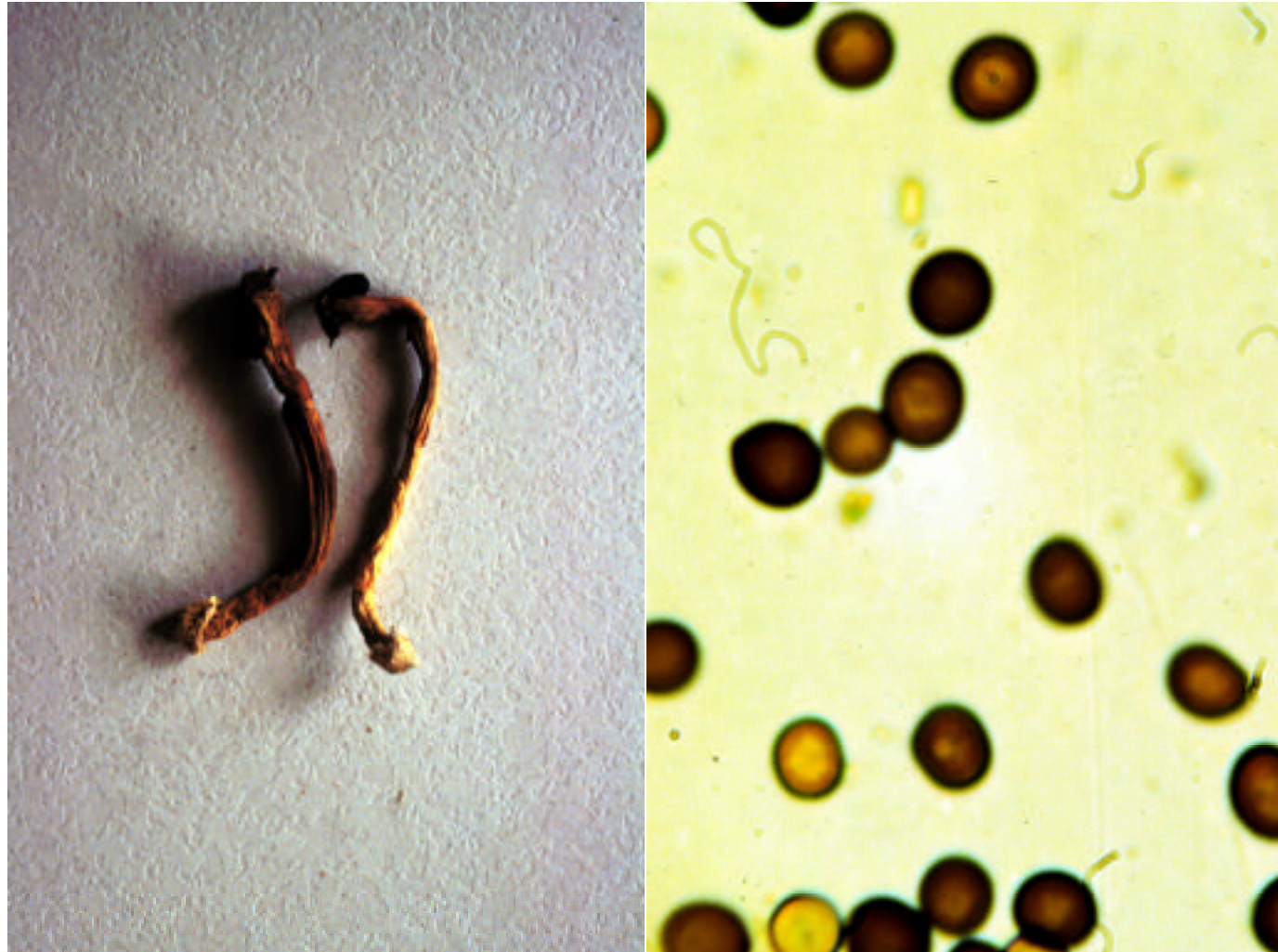


Fig. 14. Fruiting body and basidiospore of *Gyrophragmium*. Sporocarp resembles *Montagnea* but with annulus on stipe and oval spores with no germ pores, closely related to *Agaricus*.

Specimens examined	Age	DNA extraction	PCR w/ Primers 1	PCR w/ Primers 2	ITS Sequence	Damaging	Poly-geneity	Spore germination
VT4514	1981	stipe	2 bands	yes	no	yes		N/A
EAS351021	1935	stipe	2 bands	yes	yes			N/A
KJ91042706	1991	culture	yes	N/A	yes			yes
VT2466	1993	stipe	yes	N/A	yes			yes
EL73003	1973	stipe	2 bands	yes	yes			N/A
VT2505	1993	culture	yes	N/A	yes			yes
VT3750	1981	stipe	yes	N/A	yes			yes
OKM20923	1983	stipe	N/A	yes	yes			no
HAT720415	1972	stipe	yes	N/A	yes			no
AFW360824	1936	stipe	yes	N/A	yes			N/A
Rea200	1939	stipe	weak	no	partial	yes		N/A
VT2933	1980	stipe	weak	N/A	yes			no
JT871100	1987	stipe	N/A	yes	yes			yes
KJ91050801	1991	culture	yes	N/A	yes			yes
VT2932	1980	culture	yes	N/A	yes			yes
ET3833	1970	stipe	2 bands	yes	yes			N/A
SF925	1973	stipe	weak	yes	yes			no
F247	1955	stipe	weak	yes	yes			N/A
DEB350703	1935	stipe	N/A	yes	yes			N/A
GMB620700	1962	stipe	2 bands	yes	yes			N/A
VT2931	1978	culture	yes	N/A	yes			yes
HTW680300	1968	stipe	yes	N/A	yes			N/A
HEA9515	1955	stipe	no	no	no	yes		N/A
ET4918	1971	stipe	yes	N/A	yes			no
MV690700	1969	stipe	weak	yes	yes			no
Rea1009	1941	stipe	weak	weak	no	yes		N/A
Rea1031	1941	stipe	weak	yes	yes			N/A
RWS850228	1985	stipe	yes	yes	no		yes	no
KJ93061801	1993	stipe	yes	N/A	yes			yes

Table 6. The results of molecular work and spore germination of *Montagnea* for all specimens examined

DISCUSSION

Culture longevity and spore germination

The unsuccessful transfer of pure cultures of *Montagnea* from strains stored in the freezer indicate that the hyphae could may not survive freezing and should not be kept under 0° C. The reason could be that this species typically grows in warmer habitats. This situation made it difficult to keep the single spore isolates for a long period of time. Furthermore, cultures stored at 16° C could also die in a short period and needed to be transferred every two months during the study. Single spore isolates that did not form clamps with any others (i.e. SSIs 7 and 10 of KJ91042706) were more likely to die.

Those basidiospores of *Montagnea* that germinated did so very quickly (in 24 to 48 hours) after they were first soaked in water and then spread on media. The rehydration indicated an adaptive mechanism of this fungus to the dry environment in deserts. The thick-walled spores are resistant to dryness and can survive over a period of 10 to 20 years. In the very short rainy season, when water and nutrients are available, the spores germinate immediately. It was observed, however, that some spores that are younger (collected within the past 20 years) could not germinate (i.e. RWS850228, OKM20923). The storage condition after collection, as well as the age and situation of the specimens in the field before they were collected, could affect spore germination.

Clamp stability

The loss of clamp connections when compatible crosses were transferred indicated the

instability of dikaryons. Because of the variable number of nuclei observed in both monokaryons and dikaryons, it was difficult to determine if the dikaryons were didikaryotized when the clamps were lost. Also, clamps only formed at the contact zones of crosses, which indicated that there was most likely no nuclear migration taking place. Compatibility could not be confirmed unless fruiting actually occurs. It is worth trying to fruit the crosses that had clamp connections in future work. Kemp, a classical fungal genetist who has done a large amount of work on the genetics of *Coprinus*, pointed out that if there is no nuclear migration and dikaryons are growing slower than monokaryons, the dikaryons could be lost. Kemp also indicated that in this case, it is hard to know if negative results in matings really mean anything (personal communication). In *Pleurotus ostreatus*, clamp formation depends not only on the proper incompatibility factors, but also on environmental conditions (Eger, *et al.*, 1979). Kemp related that in one of his studies, and as he suggested, different temperatures including 16°C and 28°C were tried, but clamps were not maintained at either temperature.

Nuclear situation

Hyphae of both single spore isolates and crosses with clamps were shown to be multinucleate in *Montagnea*, which made it difficult to distinguish monokaryons from dikaryons by observing the nuclear situation. Butler (1972) reported that the cells of homokaryons are not always uninucleate, nor those of dikaryons always binucleate. In *Coprinus disseminatus*, for example, the tip cell of homokaryons can have from 4 to 14 nuclei, and in dikaryons trinucleate cells are quite frequent (Butler, 1972). In *Agaricus*

bitorquis, the homokaryons had variable numbers of nuclei per cell, from 1 to 14, but mostly 2 to 5. The heterokaryons had mostly 2 nuclei per cell but clamps were absent (Raper, 1976). In *Agaricus nivescens*, the heterokaryons have multinucleate cells, binucleate cells are rarely found (Elliott, 1978). Moreover, in *Coprinus narcoticus*, homokaryotic mycelia may possess clamp connections (Raper, 1966). Boidin (1971) indicated that in holocoenocytic fungi composed of multinucleate hyphae, diagnostic markers (i.e. clamps, dikaryotic cells) are often shared or absent in both primary and secondary mycelia, which complicates analysis of mating systems and life history. A similar aberrant nuclear situation is also encountered in *Montagnea*.

Mating system

The mating system of *Montagnea* was not clearly determined due to the instability of clamps, the shared nuclear situation, and crosses that should have been compatible. It was observed that species of some genera, such as *Agaricus*, seem disposed to producing a mixture of mating types (Raper, 1976). In the genus *Stereum*, two breeding strategies both occur: outcrossing and non-outcrossing (Ainsworth, 1987). In these situations, the determination of the mating system could be very complicated.

It has been estimated that some 90% of higher fungi (Aphyllophorales and Agaricales with their Gasteromycete relatives) are heterothallic (some 65% are bifactorials) and need cross-mating between homokaryons to complete the sexual cycle. The remaining 10% of species are either homothallic (self-fertile and lack an incompatibility system) or secondarily homothallic (Whitehouse, 1949). It is supposed that the homothallism was

derived from a bipolar heterothallic system by a relatively simple loss or inactivation of the incompatibility locus (Elliott, 1994).

In *Montagnea*, spores with two nuclei were found in a very small percentage of the time and the monokaryotic spores were the most common spores. It is unknown whether or not spores with two nuclei were the only ones that would germinate. There are *Coprinus* species with two-spored basidia, and the spores may have two nuclei yielding a compatible mating type. When they germinate, they give rise to fertile dikaryons and the process is called secondary homothallism (Kemp, 1980). A post-meiotic mitosis is a regular occurrence in the homobasidiomycetes, but usually half of the eight daughter nuclei will degenerate (Duncan and Galbraith, 1972). Tommerup, *et al.*, (1991) found in *Hydnangium carneum* that the post-meiotic division of nuclei happened in the basidium and all daughter nuclei remained and migrated into the spores, two nuclei into each of the four spores. The result is that some of the spores are secondarily homothallic. On the other hand, those spores with the same mating type nuclei are therefore homokaryotic and function as monokaryons. Even a small percentage of heterokaryotic spores would affect the ecology of a species by making it possible for those heterokaryotic spores to utilize ephemeral substrates, colonize new habitats, fruit and disperse spores in a very effective way, whereas a great majority of uninucleate spores maintain the potential for outbreeding (Treu and Miller, 1993). Petersen (1995) also reported that secondary homothallism seemed more common in tropical than in temperate ecosystems.

Whether *Montagnea* has both homothallic and heterothallic breeding strategies is not clear. But some single spore isolates were found to be able to form clamps in all pairings

(ie. SSI 1 of KJ91050801). This was first thought to be a mistake when deriving SSIs, but the same situation was found with single spore isolates chosen after careful observation with the microscope. If a heterokaryotic spore isolate is used in the selfcross test, it will cause the “di-mon” phenomenon. In “di-mon” mating (a dikaryon paired with a monokaryon), it simply requires a different factor to be present in the monokaryon for successful dikaryotization, the common factor does not prevent it (ie. A1B1 could be dikaryotized by A1B1+A2B2) (Casselton and Economou, 1985). Buller (1931) suggested that the advantage of a mycelium being dikaryotic rather than diploid was that the dikaryon could donate nuclei to any monokaryon it happened to meet. But in the cases where clamps are neither frequent nor constant, some secondarily homothallic spores and “di-mon” matings might not be detected.

Compatibility among geographically different collections

Limited partial compatibility was found among collections of *Montagnea* from different geographic locations. It is not uncommon that geographically widely separated individuals are found to retain their compatibility recognition mechanisms in the in vitro experiments, but an in vitro situation might not reflect reality in nature. Factors, such as, continental drift, pre- and post-glaciation plant migration, and prevailing ocean currents, for instance, could bring scattered populations to the point where they could encounter each other in nature, enabling interspecific gene flow to occur (Petersen, 1995). Boidin (1986) theorized that partially compatible collections producing a mixture of non-

patterned pairings are thought to be genetically more distant than those which exhibit universal compatibility.

Other genes outside the incompatibility factors can also influence mating behavior. Kemp (1980) has discovered an additional cause of incompatibility due to the interaction of particular alleles of different loci in *Coprinus bisporus*. Chase and Ullrich (1990 a, b) described another genetic system causing incompatibility, superimposed on the A or A and B factors (heterogenic incompatibility). These systems result in the species comprising a number of intersterile groups, each isolated from the other. Heterogenic incompatibility functioning as an isolating mechanism may be observed when races of the same species derived from different geographic sources are intercrossed and prove to be incompatible. It may exist between races of a compatible species as well as between races of a homogenically incompatible species (Esser and Kuenen, 1967).

In general, homogenic incompatibility (heterothallic incompatibility) prevents mating between strains having the same factors, and favors recombination and evolution of the species. While homothallic (both primary and secondary), heterokaryon and heterogenic incompatibility (vegetative incompatibility) restrict mating between strains having different factors, in which inbreeding is favored and outbreeding is discouraged (intersterility).

It was found that intersterility groups (ISG or biological species) of fungi may or may not be coupled to morphological variation. In fact, statistically significant morphological differences may occur within an ISG (Boidin, 1986). Significant differences in mean basidiospore size were found within the *Collybia subnuda* intersterility groups, and there

is a high degree of macromorphological variation as well (Murphy, 1997). On the other hand, morphologically indistinguishable fungi may belong to distinct incompatibility groups (Petersen, 1995). Boidin (1986) interpreted these situations as indicative of taxa in the process of speciation, particularly if the intersterility barrier is incomplete.

PCR amplification and sequencing of ITS rDNA

The application of the PCR technique opened up the possibility of isolating DNA sequences from a few copies of “intact” DNA present in the genome where the majority of the molecules are damaged and degraded, which is difficult to analyze by other molecular techniques (Paabo, 1990). Also, in the absence of molecules that are long enough to serve as templates, PCR can amplify fragmented DNA by “reassembling” the target through successive cycles, with each partially extended sequence acting as a primer for the next fragment. This process of “jumping PCR” allows for the amplification of DNA segments that are actually longer than the longest intact template present in the extract (Paabo *et al.*, 1989). This made DNA amplification possible from herbarium specimens in which the DNA was highly degraded due to drying and storage, which is the case in this study. However, the great sensibility of PCR makes it capable of picking up even very minor contamination, and the modifications present in old template DNA as well as other components of old tissues may inhibit the DNA polymerase and influence the results obtained (Paabo, 1990). In the former case, more than one DNA band will be amplified, while in the latter one no primer dimers will be seen, but they may be visible in the positive control. The DNA extraction should in this case be progressively diluted to

dilute away the contaminant while still having enough DNA templates left. Also, the amount of DNA polymerase can be increased.

The focus of this thesis is to study a desert-adapted fungus, with no fresh specimens but all the samples examined being from herbarium. By using the PCR technique, 24 sequences were obtained out of 28 samples, only 4 failed in spite of dilutions and other attempts to germinate them. Based on the results, age did not seem to be crucial for the success of PCR amplification or sequencing reaction. It appears that the condition of the specimens both in field and during storage is the most important factor.

Directed sequencing from PCR products was used to yield a consensus sequence and reduce problems associated with errors made by *Taq* polymerase for the heavily damaged DNA samples of herbarium specimens. In addition, it saves much labor and time compared with sequencing from cloning (Hillis, *et al.*, 1996). Also, cycle sequencing (PCR sequencing) was used instead of normal sequencing, and it largely reduced the amount, and to some extent, the quality of template necessary for the sequencing reaction. Moreover, it is quick and efficient and leaves less room for experimental error (Hillis, *et al.*, 1996).

Phylogenetic relationships

Neither geographic isolation was found in the phylogenetic trees based on ITS sequence data, nor correlation between spore size and phylogenetic relatedness was observed. Additional morphological characters that were examined were not corresponded with the genetic data (Fig. 12 and 13).

The bootstrap values are relatively low and some of the branches were not strongly supported in phylogenetic construction, which indicated that similarity is very high with few divergences, and the grouping are not statistically significant. The high similarity among geographically isolated populations of *Montagnea* might be mostly caused by recent evolution of this fungus as it has become adapted to similar desert habitats all over the world.

Several reasons may exist for lack of phylogenetic resolution. First, the data may not have enough characters to resolve all relationships. This is certainly a problem here, since the resolution of 24 internal branches was attempted with only 23 parsimony-informative characters. Second, characters may conflict with one another due to parallelisms or reversals (jointly termed homoplasy). Third, some branches connecting genotypes may be very short, with few or no substitutions supporting them. The overall levels of genetic divergence prove to be low among the 24 collections of *Montagnea*, and sequences even more variable (i.e. the NTS region of rDNA) are needed for comparison. In previous studies, ITS region was often found highly variable among species (Gardes and Bruns, 1991; Lee and Taylor, 1992; Goosen and Debets, 1996). But it has also been reported that in some cases, ITS variation was insufficient to separate morphologically distinct, reproductively isolated fungal species, probably because such species could evolve more quickly than mutations could accumulate in the relatively short ITS regions (Anderson and Stasovski, 1992). The reason that the NTS sequence analysis was not employed here is that it might be too long (1kb to 2kb) to be amplified from herbarium specimens.

Study of these kinds of close relationships may lie more within the realm of population genetics, where large samples of strains and assay of many genetic loci are required. The sample size in this study was not adequate to provide a definitive conclusion on classification. The distribution of ITS variation needs to be compared within, between, and among species to evaluate whether their morphological differences represent intra- or inter-specific variation. From the result of phylogenetic study of Agaricales based on sequence data of large subunit rDNA (Vilgalys *et al.*, unpublished), *Montagnea* is most closely related to *Coprinus comatus*, and the latter was chosen at first as an outgroup when constructing *Montagnea* phylogenetic trees. But even *C. comatus* has an ITS sequence which is too different to align with the ones of *Montagnea* and largely reduced resolution.

Summary

Reid and Eicker (1991) described *M. haussknechtii* Rab. with small spores, *M. arenaria* (DC.) Zeller with larger spores, and *M. arenaria* var. *macrospora* Reid & Eicker, producing 2-3-spored basidia and even larger spores. But the spore size was found not to be significant in either genetic or phylogenetic study in this thesis. In addition, no 2- or 3-spored basidia were observed. There is no evidence, from the results of this research, that speciation at any level has taken place in *Montagnea arenaria*. Therefore, hypothesis 1 on page 11 should be rejected.

The mating system of *Montagnea* Fr. is very complicated and was not clearly determined in this study. Therefore, the hypothesis 2 that *Montagnea arenaria* is

tetrapolar could not be accepted or rejected due to the unusual nuclear behavior. Evidence of partial compatibility corresponds with the phylogenetic relationships among collections of *Montagnea*. Populations from different locations are partial compatible and hypothesis 3 (page 11) could be rejected. The combination of mating compatibility studies with morphological studies and molecular phylogenetic analyses did not show a uniform or integrated picture of *Montagnea*. Neither phylogenetic relationships nor micromorphological measurements cluster collections geographically. These results indicate that genetically controlled characters, from molecular to morphological, evolve at independent, nonlinked rates.

Additional sequence evidence showed a close relationship among collections from widely distributed populations, which are all closely related to the genus *Coprinus* in Agaricales. Thus, hypothesis 4 (page 11) could be accepted.

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APPENDIX: ITS rDNA SEQUENCES OF MONTAGNEA

	50	100
M.arenaria VT2931 Idaho	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria VT2932 California	?????????GAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria VT2505 California	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria VT2466 California	ATCATTATTGAATAAACATGGTGGGTTGTAACTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.radiosus Rea1031 California	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.sp KJ91042706 Namibia	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.sp KJ91050801 Namibia	?TCATTATTGGATAAACATGGTGGGTTGTAGCTGGTCTTTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.radiosus var tenuis Rea200 Ca	??CGGA?CATGTGCACGC?TG?CGTCTTTATCCATCCAACCTGTGCACACATTGTAGTCTTTGG	
M.candollei AFW360824 Arizona	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACA?TGTAGTCTTTGG	
M.arenaria ET4918 Idaho	????TTATTGAATAAACATGGTGGGTTTAAAGCTG?TCCCTCGGAGCATGTGCACGCCTGCCGTCT?TATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria HAT720415 Washington	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria HTW680300 Mexico	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria VT3750 Montana	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria SF925 Wyoming	?????????????AAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria F247 Utah	??CATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria VT2933 Oregon	AGGATTC?AGAA?AGGAAGAGAGAGGTTGTAACCT?GCACC?CGGA?CATGTGCACGCCTGT?GTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.candollei GMB620700 Oregon	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria ET3833 Oregon	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria OKM20923 California	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria DEB350703 California	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria EL73003 Arizona	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria MV690700 NewMexico	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.arenaria JT871100 Mexico	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
M.candollei EAS351021 Texas	ATCATTATTGAATAAACATGGTGGGTTGTAGCTGGCTCCTCGGAGCATGTGCACGCCTGCCGTCTTTATCCATCCACCTGTGCACACATTGTAGTCTTTGG	
	150	200
M.arenaria VT2931 Idaho	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTG	
M.arenaria VT2932 California	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCRCGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTG	
M.arenaria VT2505 California	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTG	
M.arenaria VT2466 California	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTG	
M.radiosus Rea1031 California	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTAG	
M.sp KJ91042706 Namibia	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTAG	
M.sp KJ91050801 Namibia	AGGCG?C?GCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTAG	
M.radiosus var tenuis Rea200 Ca	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTG	
M.candollei AFW360824 Arizona	AGGCGACATCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGKTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTCAG	
M.arenaria ET4918 Idaho	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCATCGTCCTCTGAAGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria HAT720415 Washington	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTCAG	
M.arenaria HTW680300 Mexico	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria VT3750 Montana	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria SF925 Wyoming	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCATCGTCCTCTGARGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria F247 Utah	AGGCGACATCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTCAG	
M.arenaria VT2933 Oregon	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCATCGTCCTCTGAAGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.candollei GMB620700 Oregon	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCATCGTC?CTGAAGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria ET3833 Oregon	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTTG	
M.arenaria OKM20923 California	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria DEB350703 California	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCATCGTCCTCTGAAGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria EL73003 Arizona	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria MV690700 NewMexico	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.arenaria JT871100 Mexico	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATCATAAACCACGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	
M.candollei EAS351021 Texas	AGGCGACAGCTGTCGCGGGGCCCTAGGCCCGTCGTCCTCTGAGGTCTATGTTTTATTATACACCATGTAGTCAGTCGCAGAATGAAGTATTTGGGCCTTAG	

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M.arenaria VT2931 Idaho	GACTACCCGCTGAA
M.arenaria VT2932 California	GACTACCCGCTGAA
M.arenaria VT2505 California	GACTACCCGCTGAA
M.arenaria VT2466 California	GACTACCCGCTGAA
M.radiosus Rea1031 California	GACTACCCGCTGAA
M.sp KJ91042706 Namibia	GACTACC?GCTGAA
M.sp KJ91050801 Namibia	??????????????
M.radiosus var tenuis Rea200 Ca	??????????????
M.candollei AFW360824 Arizona	GACTACCCGCTGAA
M.arenaria ET4918 Idaho	GAGTA?C?G?TGAA
M.arenaria HAT720415 Washington	GACTACCCGCTGAA
M.arenaria HTW680300 Mexico	GACTACCCGCTGAA
M.arenaria VT3750 Montana	GACTACCCGCTGAA
M.arenaria SF925 Wyoming	GACTACCCGCTGA?
M.arenaria F247 Utah	GACTACCCGCTGAA
M.arenaria VT2933 Oregon	G??????????????
M.candollei GMB620700 Oregon	GACTACCCGCTGAA
M.arenaria ET3833 Oregon	GACTACCCGCTGAA
M.arenaria OKM20923 California	GACTACCCGCTGAA
M.arenaria DEB350703 California	GACTACCCGCTGAA
M.arenaria EL73003 Arizona	GACTACCCGCTGA?
M.arenaria MV690700 NewMexico	GACTACCCGCT???
M.arenaria JT871100 Mexico	GACTACCCGCTGAA
M.candollei EAS351021 Texas	GACTACCCGCTGAA

VITA

Chang Chen was born in a Chinese family in Beijing, China in September 1969. She was not very strong and had some heart disease when she was a baby. All her parents wanted was for her to grow up healthy. As Chang got older, she became stronger and stronger and got rid of her disease little by little. Her parents were so happy seeing her going to schools like any other child, especially when she enrolled in college.

Chang chose microbiology as her undergraduate major and received her Bachelor of Science degree at Beijing Agricultural University in July 1992. After graduation, she stayed and worked at her school as an assistant faculty member. She also worked at some part time jobs during school breaks.

Chang came to Department of Biology of Virginia Tech in August 1996 and continued her study as a Master of Science student in Mycology under Dr. Orson K. Miller, Jr., her thesis advisor. Here she finished her degree and also got to know her husband Ming Qian who was a student in the Department of Electronic Engineering. Chang will join Ming after graduation and move to Raleigh, North Carolina where Ming is working.

Chang likes to travel around and she hopes that she could visit many of the places that Dr. Miller has been to. Chang also enjoys collecting stamps in her spare time, but did not and won't spend too much money on it.