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Molecular Investigation of the Bioluminescent Fungus Mycena chlorophos: Comparison between a Vouchered Museum Specimen and Field Samples from Taiwan

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by

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ABSTRACT

There are 71 species of bioluminescent fungi belonging to at least three distinct evolutionary lineages. *Mycena chlorophos* is a bioluminescent species that is distributed in tropical climates, especially in Southeastern Asia, and the Pacific. This research examined *Mycena chlorophos* from Taiwan using molecular techniques to compare the identity of a named museum specimens and field samples. For this research, field samples were collected in Taiwan and compared with a specimen provided by the National Museum of Natural Science, Taiwan (NMNS). Specific locations for collection were chosen based on previous GIS data provided by NMNS. FTA DNA extraction cards were used to collect fresh DNA from fruiting bodies. The samples were brought back to the lab at SUNY-ESF located in Syracuse, N. Y. where DNA extraction, PCR amplification and restriction fragment length polymorphism (RFLP) analysis of the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS region) were performed. The ITS region, or fungal barcode was sequenced from these PCR products. The RFLP patterns and ITS sequences showed that the field samples and museum specimen were probably the same species, supported by BLAST search in Genbank. This study has raised questions about the mechanisms of long distance fungal spore dispersal, and suggests possible future research in taxonomic and interdisciplinary study of *Mycena chlorophos*.

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ACKNOWLEDGMENTS

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INTRODUCTION

Bioluminescent fungi are often labeled as “mystery species,” “ghost fungi,” and “fox fire” (Coder, 1999). The phenomenon of fungi that luminesce is fascinating and attracts many scientists and the general public who encounter these interesting fungi glowing in the forest. For a long period of time no one knew for sure how or why they are luminescent. It has been suggested that bioluminescent basidiocarps attract invertebrates to aid in fungal spore dispersal (Desjardin et al., 2008). Some other hypotheses stated that the luminescence of fungi might serve as a warning signal to repel nocturnal fungivores, or might attract predators or parasitoids of fungivores (Desjardin et al., 2008). The ecological and evolutionary functions of the bioluminescent fungi are still unknown. One of the bioluminescent fungi species that this research focused on was *Mycena chlorophos* ((Berk. & M.A.Curtis Sacc.) Figure 1, 2 and 3). From previous research, the data about bioluminescent fungi from Malaysia, Japan and the South Pacific were invalidly published. “In addition, the protologues provided only limited morphological data making identification of subsequent collections difficult, and consequently, vouchered reports of most of the Asian species have not been published since” (Desjardin et al., 2008). One of the reasons that this research has been conducted is because there are no valid reports published from Taiwan. This research focused on comparing field specimens of *Mycena chlorophos* to those deposited and held in the Taiwan National Museum of Natural Science and to sequence data in Genbank from named vouchered specimens.

Recently, the development of molecular techniques has allowed scientists to identify the basic chemistry of the luminescent substance in the fungi. In general, most

species emit light of maximum wavelength about 530 nm continuously day and night from the fruiting body, the mycelium or both (Mori et al., 2011). Scientists have reported that the bioluminescence system of luminescent fungi exhibited a luciferin-luciferase type reaction in the presence of NADH and two enzymes (Mori et al., 2011). However, the isolation of luciferin from the fungi has not yet been accomplished. Bioluminescent fungi are widely distributed in the world, and the majority of species belong to the class Basidiomycetes (Mori et al., 2011). Collectively, there are 71 known species of bioluminescent fungi belonging to at least three distinct evolutionary lineages: Omphalotus, Armillaria and mycenoid (Desjardin et al., 2008). According to Desjardin et al. (2010) *Mycena chlorophos* (Berk. & M.A. Curtis Sacc. 1887), typified by material collected in the 1850s from the Bonin Islands, Japan, has been used for bioluminescent agarics occurring in Australia, Brazil, Southeastern Asia, and the Pacific. Unfortunately, the only published descriptions of *Mycena chlorophos* focus on a subset of specimens collected from Sri Lanka, Malaysia, Borneo and Brazil. Several mycologists in Taiwan noticed that *Mycena chlorophos* can be found in various locations across the country. As part of the museum species database research, samples of *M. chlorophos* were collected and stored at the Museum of Natural Science. The information about the existence of *M. chlorophos* was later mentioned in the mushroom field guild "Mushrooms of Taiwan" (2004) and in some advertisements for community ecotourism. *M. chlorophos* is a unique species that is well adapted to Taiwan's subtropical weather. According to Mori et al. (2011), the fruiting bodies of *M. chlorophos* develop only on the day after rain falls during the rainy season (June/July and September/October). The limited distribution and

limited season for the formation of the fruiting bodies has made the field collection difficult.

In this research, we used restriction fragment length polymorphism (RFLP) of the nuclear ribosomal DNA (rDNA) internal transcribed spacer region (ITS) to help identify the luminescent mushrooms I found in Taiwan. RFLP is a way to detect differences in homologous DNA sequences. These differences can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. Isolation of sufficient DNA for RFLP analysis is time consuming and labor intensive. However, PCR can be used to amplify very small amounts of DNA, usually in two to three hours, to the levels required for RFLP analysis. Therefore, many samples can be analyzed in a short amount of time. According to Horton and Bruns (2001), this method is easy to use and also relatively cheap compared to many other techniques.

The ITS of the nuclear ribosomal DNA (rDNA) is the most widely sequenced DNA region for fungi (Schoch et al. 2012). Initially, it has typically been most useful for molecular systematics at the species level, but it has been found to be too variable and so now has been primarily used for diagnostics. According to Schoch et al. (2012), the ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. Because of its higher degree of variation than other genetic regions of rDNA (small subunit 'SSU' and large subunit 'LSU'), we can use the ITS region to identify inter-individual variation in sequence. In this research, ITS1F and ITS4B primers were used, a *schematic* diagram of ITS primers and their location can be found in Figure 4.

METHODS

COLLECTION OF SPECIMEN DNA

For this research, field samples were collected in Taiwan and compared with the specimen provide by National Museum of Natural Science. Specific locations for collection were chosen based on previous GIS data, and FTA cards (Whatman, Inc.) were used to collect fresh DNA of mushroom fruiting bodies. From the DNA analysis data, the collected samples were compared for their genetic similarities and differences. After the PCR, variation in the DNA sequence was analyzed using RFLP analysis and direct sequence analysis (which included BLAST search in Genbank).

In this project, specimens were collected in Taiwan over the summer (July, 2012). Using the GPS coordinates given from Taiwan National Museum of Natural Science (exact GPS coordinates cannot be released due to museum concern); locations were selected. Collected sites included (Figure 5): Kenting National Park-SheDing (墾丁國家公園–社頂), National Pingtung University of Science and Technology campus at Neipu Township, Pingtung County (屏科大後山), Takeng Mountain region, Taichung City (台中大坑風景區), and Alishan Township, Chia-Yi County (嘉義阿里山).

Overall observations and descriptions of each collection site were recorded (details on temperature, weather, surrounding vegetation, forest type, relative humidity, GPS coordinates, and the morphology of mushroom's fruiting body; Table 1.) For each collection site, at least three random patches of *M. chlorophos* that exhibited luminescent fruiting bodies were chosen for analysis. From each chosen patch, a single sporocarp was picked and smashed onto an FTA card for DNA collection.

There were five *M. chlorophos* specimens collected and deposited in the herbarium of the National Museum of Natural Science in Taiwan from the years 2001, 2006, 2008 and 2009. Five specimens were obtained from the herbarium of National Museum of Natural Science in Taiwan. Even though the FTA card approach to obtaining DNA from dry specimen is not as effective as from fresh specimen, because this was the only method available, dry specimens were directly transferred to the FTA cards and then put the FTA cards in a small plastic bag to bring back to the United States for further analysis.

EXTRACTION, PCR AND RFLP ANALYSIS OF SPECIMEN DNA

DNA extraction was followed by the manufacturer's instruction manual for the FTA cards. The CTAB extraction method was also used for samples that did not get DNA from the FTA card method. PCR and RFLP analyses were performed in the laboratory in the United States. The ITS region of the rDNA was amplified by using PCR and the primer pair was ITS-1f (fungus specific primer) and ITS-4b (basidiomycete-specific primer). The sequence for primer ITS-1f is 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and the sequence for primer ITS-4b is 5′-CAGGAGACTTGTACACGGTCCAG-3′ (Gardes and Bruns, 1993).

Amplification occurred in the thermo cycler with the following program: 1) initial denaturation temperature of 94°C for 3 minutes, 2) melt temperature of 94°C for 35 seconds, 3) annealing temperature of 53°C for 55 seconds, 4) extending temperature of 72°C for 45 seconds + 2 seconds per cycle, 5) repeat steps 2-4 with 34 more cycles, final extension temperature of 72°C for 10 minutes.

One of the key features of *Mycena chlorophos* is that it has cheilocystidia that looks like a champagne flute/prestige cuvee (Figure 6). Cheilocystidea are sterile cells that are located on the edges of the gills.

RFLP patterns were generated with restriction digest enzymes *Hae* III and *Hinf* I. The *Hae* III enzyme cuts DNA at the specific recognition sequence of *GGCC* whereas *Hinf* I is a 5 base pair cutter that will recognize the sequence of GANTC (N can be any letter). According to Horton and Bruns (2001), usually two or three restriction enzyme digests are enough to distinguish most species and the reason for that is the sequence differences between taxa are generally the result of insertions or deletions of nucleotides that cause differences in length (i.e., restriction length polymorphisms). The ITS bands were identified by gel electrophoresis on a 3 percent agarose with 1kb reference ladder. After the RFLP gel, we picked the best products with the clearest bands and sent subsamples to the Eurofins MWG Operon Company for sequencing. ITS sequence data were analyzed by the SEAVIEW (Version 4.4.0) software. The forward and reverse sequences for each sample were aligned with Genbank number: AB512312.1 and a consensus sequence was generated also with the same number. The identity of the consensus sequence was confirmed using a BLAST search to known sequences in NCBI Genbank.

RESULTS

Collection site description:

This field research for this project was conducted in Taiwan at four collecting sites (Kenting National Park-SheDing; National Pingtung University of Science and Technology campus at Neipu Township, Pingtung County; Takeng Mountain region,

Taichung City; Alishan Township, Chia-Yi County). *M. chlorophos* were only found at two sites: Kenting National Park-SheDing and Alishan Township, Chia-Yi County. The week before the samples were collected at the Alishan Township, the weather was dry, hot with burning sun. The day the samples were collected, there was a thunder storm that caused trees to fall down and soil erosion from the mountain to the roads. Due to the unusual weather the previous summer, a tour to all the routes where *M. chlorophos* might be found was not possible. The ecosystem is composed of bamboo forests, the temperature was 20°C and the relative humidity was 90% after the rain. Unfortunately, only one sporocarp was discovered on a dead bamboo twig along the trail; this sporocarp was collected and smashed onto an FTA card.

At SheDing village, in Kenting National Park, the ecosystem is a mix forest that is composed of bamboo, *Diospyros discolor*, *Murraya paniculata, Koelreuteria elegans*, *Aglaia formosana*, and *Champereia manillana*. From my observation, some of the fruiting bodies of *M. chlorophos* grow on rotting wood, rotting bamboo, on twigs, and on woody fences. SheDing's land formation is special in that the topography of the entire area is comprised of raised coral reef within which grassland, cracked reef gorges and limestone caverns are found everywhere. Therefore, this special topography allowed the scrub plant community to be created on a high level coral atoll. The relative humidity was 85-90% and the temperature was 25°C on the collection date. There was also a stream running underneath the "glowing bridge" (the name the local people used) where lots of *M. chlorophos* were found. A total of eight sporocarps were collected from this area and were smashed onto the FTA cards.

Molecular Results:

Six samples were loaded to the electrophoresis gel (samples number: SC32, SC35, SC36, SC38, SC39 and M5). Compared between the two enzymes *Hae* III and *Hinf* I (see Figure 7), samples SC32, SC35, SC36, and SC38 appear to be the same species. Sample number SC39 did not show any bands on the gel image. M5 was the museum sample, the bright bands indicated the DNA material was over amplified and thus the bands were hard to see the on the gel image, we should have loaded another gel with half or less of the DNA to account for this problem. M5 looks similar to the other four samples, but the location of double bands shifted; this might be due to gene insertion or deletion. The blurry lines behind the marker out front are called primer dimers, and they are caused by primers binding to themselves or each other. The blurry lines can also be due to nonspecific amplification products.

The experiment yielded three relatively good sequences, SC 35, SC38 and M5, where SC35 and SC38 were from one site but different patches, and M5 was the specimen from the museum that was collected in 2008(Table 1). Interestingly, all of the three specimens were from Kenting Sheding Park. Specifically, the sequences alignment (Figure 8) showed the variation between the museum specimen (M5) and the two field specimens (SC35 and SC38) was lower than 3% which suggests that they are the same species.

Schoch et al. (2012) suggested that the average distance for barcode gap for intraspecific variation can range from 0 to 58% and can range from 0 to 72% for interspecific variation. Despite the wide range for intraspecific variation, most of the intraspecific variation is usually less than 5% on the percentage of pairwise comparisons

graph. According to Horton (2002), generally if the ITS variation is less than 3% then it would be considered to be the same species but if the ITS variation is greater than 5% it would be considered a new species. However, there was no absolute percentage as the cut off line because the percentage depends on the lineage. In the ITS region, the 5.8S is a conserved region that is approximately about 150 base pairs (bp). From the results, the sequences of the field sample SC35 is 576 bp, SC38 is 567 bp and the museum M5 specimen is 420 bp. The sequences include a portion of the ITS1 region, the complete 5.8s gene, and a portion of the ITS2 region. This is sufficient for identification purposes (Horton, pers. comm.). The 5.8S region among those sequences mentioned above (Figure 8) was identified by using ITS-2 and ITS-3 primer sequences from White et al. (1990), which are known to anchor in the 5.8S region. Compared with the two fresh field samples, the museum specimen was older in terms of collection date. It was possible that poorer results for this specimen were related to its age.

DISCUSSION

From the gel image (Figure 7), there is a possibility of intraspecific genetic variation between the museum specimen and field samples. Horton and Bruns (2001) review the molecular techniques applied to studies of ectomycorrhizae communities. Their findings suggested that the RFLP typing using ITS sequences is an effective tool for identification purposes, but does have its limitations. For instance, many types remain unknown because no matching RFLP type is observed in available sporocarp databases. We sequenced from both directions—using the ITS-1f primer and the ITS-4b primer—but because there were homopolymers at both ends, there are still gaps in the middle of the sequence making it difficult to sequence.

From the BLAST search function from Genbank, a sequence was produced with significant alignments to a *M. chlorophos* collection from Japan (*Mycena chlorophos* genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence, strain: 305759; Genbank: AB512312.1). Compared to the Japanese strain, the query coverage for SC35 is 73%, maximum identity is 99%, max score is 1048, total score is 1048, and E-value is 0.0; the query coverage for SC38 is 72%, maximum identity is 95%, max score is 939, total score is 939, and E-value is 0.0; the query coverage for M5 is 36%, maximum identity is 99%, max score is 353, total score is 493, and E-value is 1e-101. This might be due to some possible mutations between Japanese specimen and my Taiwanese specimens. Max identity was calculated from the homology and length, the higher the number the more homologous the sequences are. Query coverage indicates how long the piece of the DNA sequence is covered by the one found. By comparing them, it strongly suggests that the samples that I collected from the different fields are *Mycena chlorophos*.

From these results, one of the questions that came up was how come there is very little polymorphism between the species at Taiwan and the species at Japan? The geographic distance of Taiwan from Japan is 1,945 kilometers; it is possible that spores had traveled by airflow during recent geological times. My hypothesis would be that this species has not evolved much in recent times, therefore there are little changes in its gene pool with respect to the highly conserved ITS region. Human cultivation of these species might have occurred in Taiwan a long time ago, but there is no historical data to prove that hypothesis. *Mycena chlorophos* seems only to occur in certain places in Southeastern Asia and the Pacific islands, which made the origin untraceable. From my observations, they usually grow in high humidity areas, only fruiting above ground after

tremendous rain. There are several possible directions for further study on this subject such as the study of evolutionary lineage of *Mycena chlorophos*, species genome comparison among different countries, and global species distribution or biogeographic patterns.

CONCLUSIONS

This research was a novel project to compare the species from specimens in a museum herbarium with the newly collected samples by using molecular techniques. It was interesting to see the comparison result from the DNA sequence. The sequence from the field samples seems to match up with the museum specimen collected from 2008. We can conclude that they were probably the same species. The results give us an idea of the degree of genetic polymorphism between individuals within a species; in this experiment we did not find much polymorphism within a species. Since the ITS region is very close to evolutionarily neutral, single nucleotide polymorphisms it is probably rarely acted on by selection. The results of the sequences will be submitted to Genbank for other scientists to use.

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APPENDICES

Figure 1 (Left) & Figure 2 (Right). The fruiting bodies of *Mycena chlorophos* that are luminescent. Photograph used with kind permission of Ryder Lin. Copyright 2012.

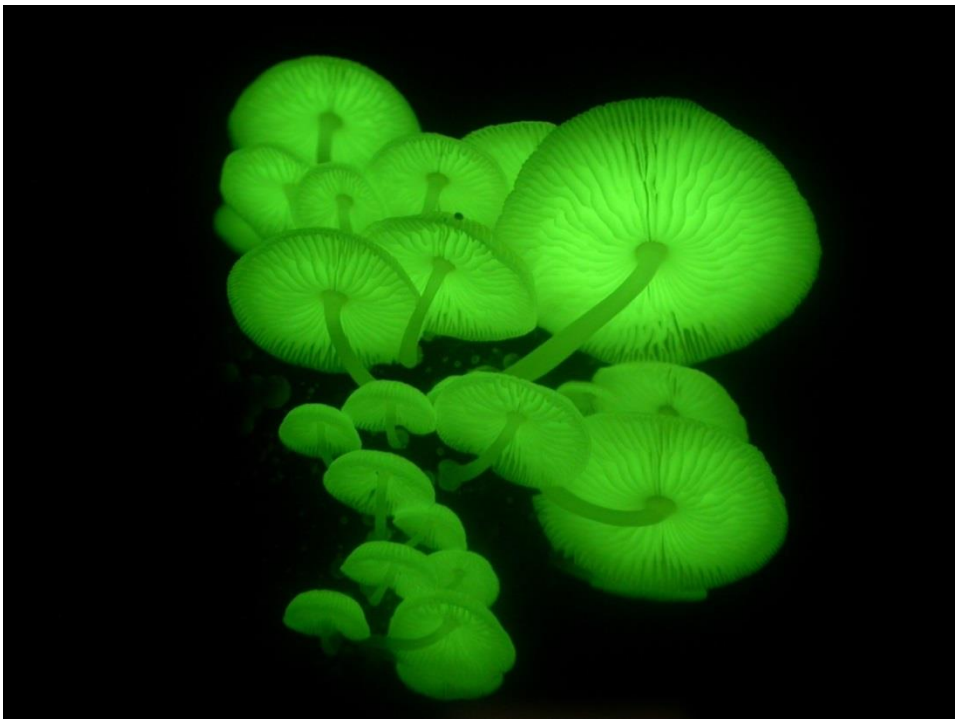

Figure 3. The ventral view of *Mycena chlorophos* showing luminescent lamellae. Photograph used with kind permission of Chia-Liang Yeh. Copyright 2012.

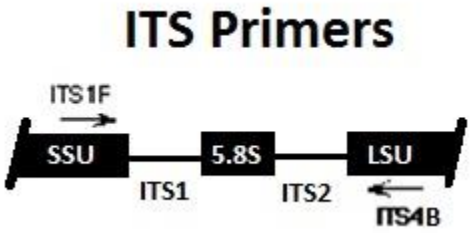

Figure 4. ITS primers and their locations, adapted from Vilgalys lab, Duke University

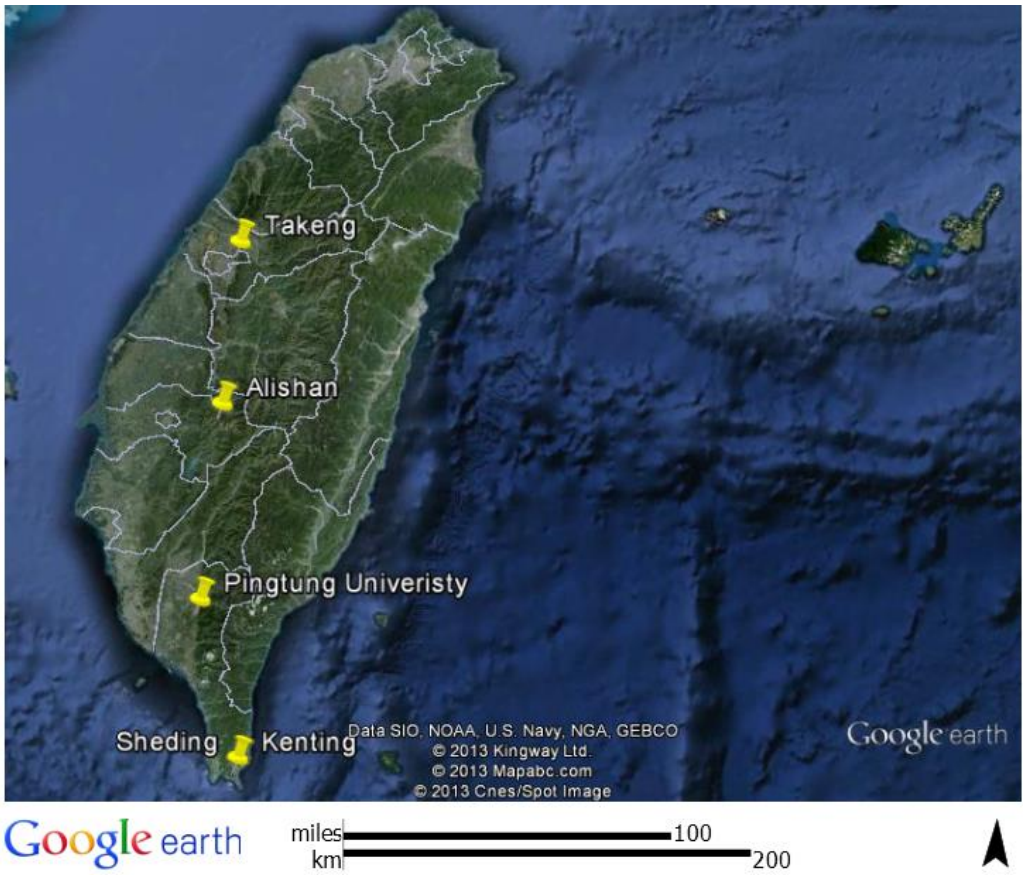

Figure 5.

Four collecting sites were (from top to bottom): Takeng Mountain, Taichung City; Alishan Township, Chia-Yi County; National Pingtung University of Science and Technology campus at Neipu Township, Pingtung County; and Kenting National Park-SheDing.

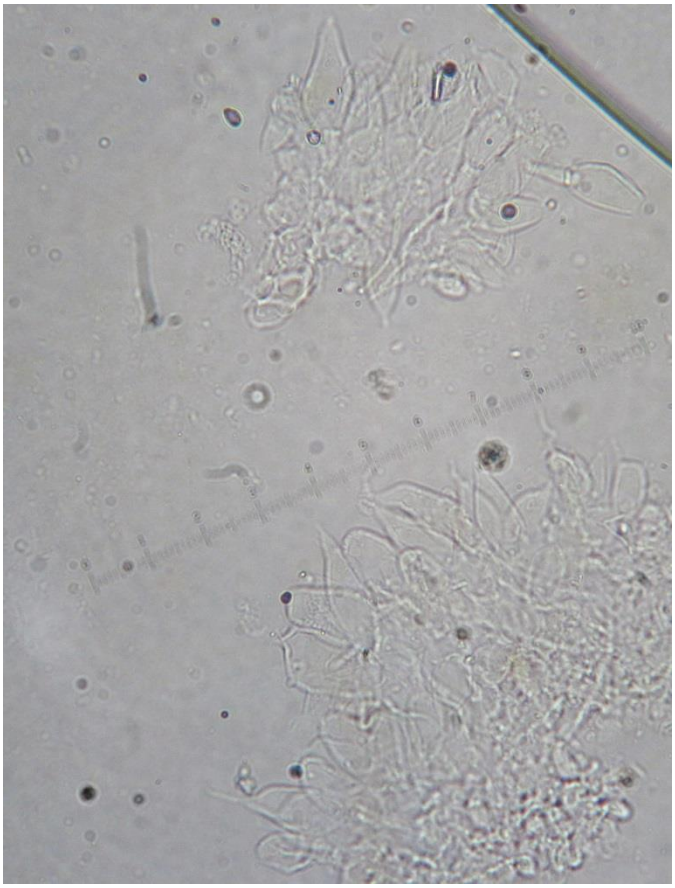

Figure 6. Cheilocystidia: 400X magnification on *Nikon* Microscope *ECLIPSE E200*, camera: Nikon Coolpix 995

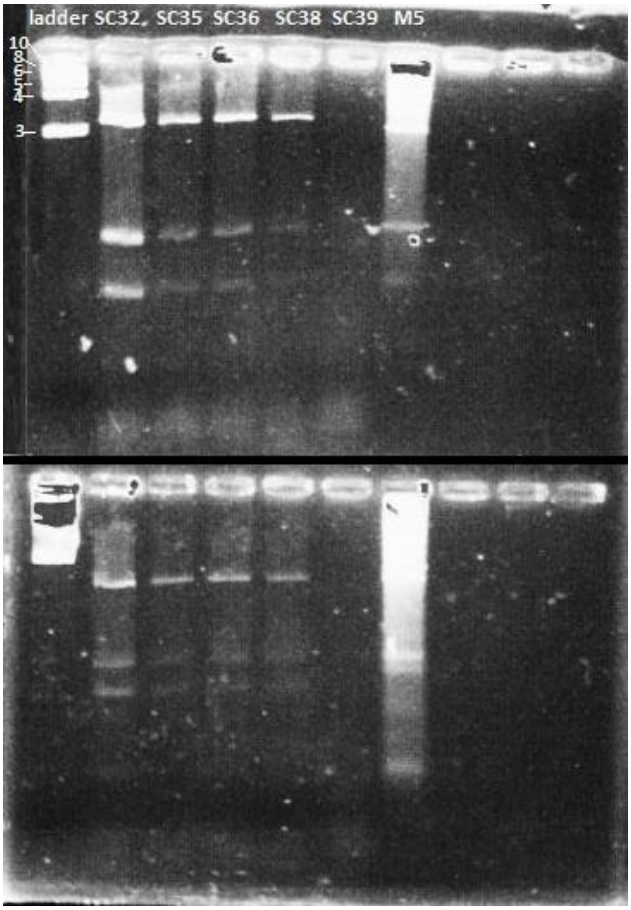

Figure 7. ITS PCR-RFLP patterns digested with HaeIII (top half) and Hinf I (bottom half). The digestions were resolved using 3% agarose gel electrophoresis at 90 V for 50 minutes. As a size marker, 1kb DNA Ladder was used.

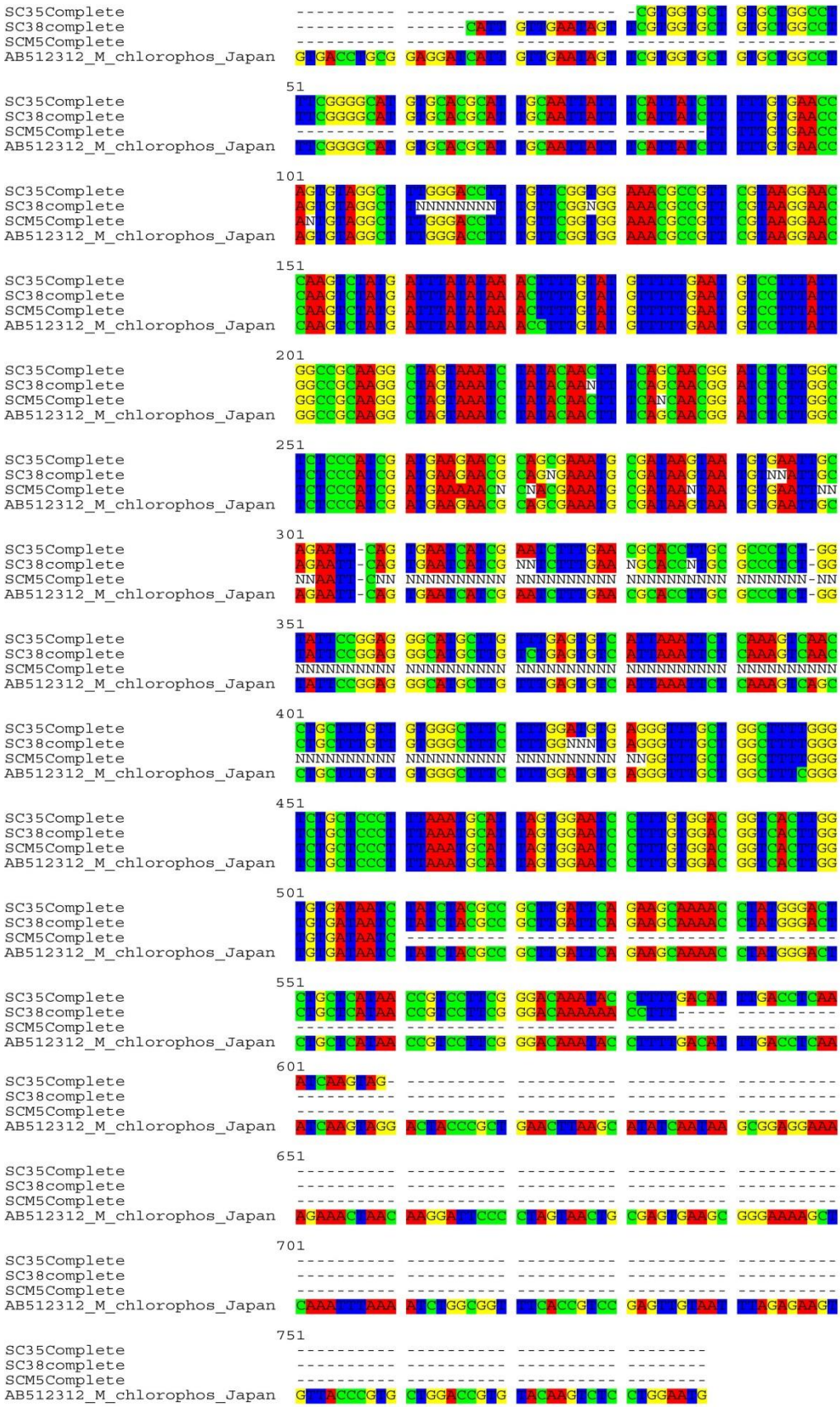
SC35Complete
SC38complete
SCM5Complete
AB512312_M_chlorophos_Japan
51
101
151
201
251
301
351
401
451
501
551
601
651
701
751

Figure 8. ITS sequences for 4 sporocarps of *Mycena chlorophos*. SC35 and SC38 are field samples, M5 is from the museum and AB512312.1 is from Genbank. Gaps introduced for alignment are indicated (–). Unknown nucleotides are indicated (N).

Table 1. Summary of environmental conditions pertaining to collected samples.

Collection number	Collection Date	Location	Temperature (°C)	Weather	surrounding vegetation	forest type	Relative humidity	Description
SC32	7/12/2012	23.57,120.68	20	Raining	Bamboo, mix deciduous trees	Bamboo	85%	Fruiting body grow in solitary on bamboo, cap white with sticky and smooth surface, spore print color is white
SC35	7/27/2012	21.95,120.81	25	1 days after 1 weeks of heavy rain	Similar to SC38	Mix forest	85%	Gregarious and solitary. Fruiting body white with conical shape, grow on twigs, rotting bamboo
SC38	7/27/2012	Taiwan, Pingtung, Kenting Sheding Park (near glowing bridge)	25	1 days after 1 weeks of heavy rain	*Diospyros discolor*, *Murraya paniculata*, *Koelreuteria elegans*, *Aglaia formosana*, and *Champerei a manillana*	Mixt forest	90%	Gregarious , fruiting bodies grow on rotting wood, rotting bamboo, on twigs, and on woody fence
M5(herbarium specimen)	8/06/2008	Taiwan, Pingtung, Kenting Sheding Park	Not available	Not available	Not available	Not available	Not available	Was found on bamboo