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Population and Species Level Molecular Analyses of Bolete-infecting *Hypomyces* Species

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Author
Uribe Valdez, Gilberto

Publication Date
2013

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UNIVERSITY OF CALIFORNIA
RIVERSIDE

Population and Species Level Molecular Analysis of Bolete-infecting *Hypomyces* Species

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Plant Pathology

by

Gilberto Uribe Valdez

June 2013

Thesis Committee:
- Dr. Greg W. Douhan, Chairperson
- Dr. James G. Borneman
- Dr. Jason E. Stajich

The Thesis of Gilberto Uribe Valdez is approved:

Committee Chairperson

University of California, Riverside

Acknowledgments

There are many people without whom completion of this work would have been much more difficult, if not impossible, and I would like to take a moment to express my gratitude as best I can.

I would like to begin by thanking my major professor, and mentor, Dr. Greg Douhan. I came to UC Riverside specifically to work with Dr. Douhan and I am extremely grateful for the many things I have learned from him, which are not limited to my research topic. His generosity, willingness to help others, honesty, capacity to continuously publish, obtain funding, and still manage to be a devoted and involved husband and father are a true inspiration to what a scientist should be. I would also like to thank him for allowing me the freedom to learn and experience many things on my own while always being there to guide me back on course when I started veering too far from it, and for making this not only a great learning experience, but in many ways an enjoyable and fun experience. Most importantly, I would like to express my gratitude for making me feel like the things I did during my time here were for no one else's benefit but my own– to make me a better researcher. Also, I am grateful for the financial support he provided to me and to this project. This can only begin to express the gratitude I feel toward Dr. Douhan.

I would like to thank my committee members, Dr. Jason Stajich and Dr. James Borneman for serving on my committee despite some of the last-minute arrangements. I am also grateful for their support and encouragement while I was trying to decide what to do with my degree and for making me feel that it was my best interest that they had in mind. For this I am truly grateful.

I wish to extend my gratitude to all our lab members, past and present, who have made working at the Douhan lab an enjoyable experience. To Dr. Deborah Pagliaccia for her help and guidance, Javier Perez for all his help during the initial stages of the project, and all past members who helped collect and maintain specimens and cultures used in this work.

To my mother and father, Norma and Gilberto Uribe, for their unconditional support in my educational pursuits. I can never repay all that you have done for me, nor fully express what you mean to me. [Para mi madre y padre, Norma y Gilberto Uribe, por su apoyo incondicional en torno a mis objetivos educativos. Nunca les podre pagar todo lo que han hecho por mi, ni podre por completo expresar lo que ustedes significan para mi]. To my little sisters Norma and Victoria for all their love and kindness which did nothing but inspire and motivate me. I would like to thank my aunt and uncle María de Jesus and Manuel for supporting our family and me in our times of need, as well as my little cousins Karyme and Denia who are virtually my sisters. And to my best friend and brother Moisés Uribe whose help and silent company are invaluable to me.

To all my close friends who in some way helped me keep my sanity, either through kind, motivating words, or by joining me in activities that reminded me that there was a life beyond work. I would also like to thank my undergraduate professors who have maintained contact with me and provided me with motivation and support.

Finally, to Christine Hayes, my fiancée, for her help and support, despite concurrently working on her own thesis, I have my sincerest gratitude and love to express.

Chapter I of this thesis, in part or in full, is a reprint of the material as is appears in *Mycologia* January/February 2012. The co-author Greg W. Douhan listed in that publication directed and supervised the research which forms the basis for this thesis.

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CHAPTER I. Geographic structure of a bolete infecting cryptic species within the *Hypomyces microspermus* species complex in Western California

ABSTRACT

Fungi in the genus *Hypomyces* are mycoparasites of various fungal groups. Previous studies within this genus have dealt primarily with taxonomic descriptions and no studies have been published on the population biology of these fungi in natural ecosystems. In this study, genetic structure within and among six California (CA) bolete-infecting *H. microspermus* populations were analyzed using amplified fragment length polymorphism (AFLP). Four of the populations were collected in relative close proximity to each other, south of the Transverse Mountains in Southern CA, whereas the other populations were from the central coast of CA (Pismo Beach) and from Northern CA (Humboldt County). Thirty-nine genotypes out of 68 isolates were found based on 30 AFLP markers. This was a higher degree of genotypic diversity than expected because no sexual fruiting bodies were ever observed while collecting, suggesting that sexual reproduction occurs infrequently. These observations were also supported by multilocus disequilibrium analyses. Overall, the populations were significantly differentiated ($\theta=0.321$, $P < 0.001$) and most population pair-wise F_{st} values were also significantly different from one another. The populations sampled also fit an isolation by distance model based on a Mantel test ($P < 0.001$). The overall results suggest that this pathogen has a primarily clonal population genetic structure and that spore dispersal was restricted across the sampled populations.

INTRODUCTION

Species of *Hypomyces* are common mycoparasites of diverse fungal hosts including agarics, boletes, and polypores (Rogerson and Samuels 1989, 1993, 1994). Host specificity has been noted for many species of *Hypomyces* with the level of specificity varying; some appear to be generically host specific, such as *H. copletus* and *H. transformans* on *Suillus* spp. or *H. melanocarpus* on *Tylopilus* spp., whereas others appear to have a much broader host range (Rogerson and Samuels 1989). Therefore, resource and habitat specialization appears to be a driving force shaping the evolution of *Hypomyces* and their mushroom hosts, resulting in varying levels of specificity between certain host and parasite species.

Bolete-parasitizing *Hypomyces* species are extremely common mycoparasites and are generally found in all natural ecosystems where boletes fruit. *Hypomyces* species parasitic on boletes and bolete relatives have anamorphs classified in the genus *Sepedonium* (Rogerson and Samuels 1989, Sahr *et al*. 1999). There are seven species recognized within *Sepedonium* associated with the teleomorph genus *Hypomyces* (Rogerson and Samuels 1989) and an additional five taxa that have not had the teleomorph found or have not been formally described (Sahr *et al*. 1999). *Hypomyces* species with *Sepedonium anamorphs* reproduce asexually by forming persistent thick-walled aleurioconidia that likely aid in the survival and long distance dissemination of these fungi. However, they also produce thin walled phialoconidia that are likely important for localized secondary infections. Sexual fruiting structures are rarely ever encountered in nature, so the importance of sexual reproduction within

Sepedonium/*Hypomyces* is not known, and no work that we are aware of has examined any aspect of population biology of these common bolete parasites. However, we will refer to all bolete-infecting parasites as members of the genus *Hypomyces* throughout the text since phylogenetic analyses clearly support a monophyletic generic classification (Sahr *et al*. 1999).

One of the most commonly found species of *Hypomyces* associated with boletes is *H. chrysospermus*. This species is a cosmopolitan generalist parasite that infects many genera within the Boletales. In contrast, *H. microspermus* forms a sister taxon to *H. chrysospermus* but host specificity to members of the *Xerocomus chrysenteron* group has been observed in Europe (Sahr *et al*. 1999) and more recently within California (Douhan and Rizzo 2003). However, two cryptic species, each within *H. chrysospermus* and *H. microspermus* s.l., have recently been observed based on amplified fragment length polymorphism (AFLP) analysis and phylogenetic analysis of the ITS regions (Douhan and Rizzo 2003). Moreover, this has also been further supported by sequence analysis of actin and elongation factor regions (Douhan, unpublished). It should also be noted that members of the *X. chrysenteron* group are often included in the genus *Boletus* in the North American literature due to Smith and Thiers (1971) placing all *Xerocomus* species in the genus *Boletus*. However, *Xerocomus* is widely recognized as a distinct genus and will be recognized as such in this paper.

These recent findings of cryptic species found within *Hypomyces* demonstrates that adequate identification first needs to be made before studies aimed at understanding the population genetic structure can be undertaken. Moreover, these findings also put into question some of the previous observations of particular host associations that have been described thus far. With this in mind, the objective of this study was aimed at quantifying the level of genetic variation found within and among six California bolete-infecting *Hypomyces* populations of one particular cryptic species in the *H. microspermus* clade (Douhan and Rizzo 2003). Since sexual fruiting bodies of *Hypomyces* were not observed on any infected bolete sampled, we hypothesized that we would find significant clonal genotypic diversity within isolated populations and we also hypothesized that the populations would be significantly structured and would fit a model of isolation by distance based on our sampling strategy.

MATERIALS AND METHODS

Hypomyces Collection, Culturing, and DNA Extraction

Hypomyces infected boletes were sampled from five locations in California dominated by coast live oak (*Quercus agrifolia*) and one location in California dominated by various conifer species between November and February 2004-2009 (Fig 1.1). For the purposes of this study, each location was considered to represent a separate population and therefore isolates collected from the same location were also pooled by year to increase the sample size from populations that were sampled more than one year. Each population was separated by approximately 5 to 1,075 km and consisted of 16, 20, 11, 4, 7, and 10 isolates in locations FP, FS, HC, LE, OH, and PB, respectively (Fig 1.1). Population FP was collected from Felicita County Park, Escondido, San Diego Co.; population FS was collected from Finn Springs County Park, El Cajon, San Diego Co.; population HC was collected from East Fork campground in the Six Rivers National

Forest, Humboldt Co.; population LE was collected right outside of Lake Elsinore off of Highway 74 in the Cleveland National Forest, Riverside Co.; population OH was collected near the town of El Cariso off of Forest Service route 6S05 road on Highway 74 in the Cleveland National Forest, Riverside Co.; and population PB was collected near the town of Pismo Beach next to US Highway 101, San Luis Obispo Co.

To isolate the parasites, a flame-sterilized loop was used to streak out spores on modified Melin-Norkrans's media (Marx 1969) amended with streptomycin (50 mg/l) or several small pieces of the subiculum were incubated on potato dextrose agar (Difco, Detroit, MI, USA). Each plate was incubated for several days at room temperature and the leading edge of a colony was transferred to potato dextrose agar (Difco, Detroit, MI, USA) and again incubated at room temperature for 1 to 2 weeks. Mycelium was then scraped off with a flame-sterilized blade and placed into 1.5 ml centrifuge tubes and frozen at –20° C until DNA extraction. DNA was extracted using the method of Gardes and Bruns (1993). All isolates used in this study belonged to one of the two known cryptic species found within *H. microspermus* from California. These isolates belong to the AFLP group I clade as determined by Douhan and Rizzo (2003) and were also identified based on ITS sequence analysis (Douhan, unpublished).

Amplified fragment length polymorphism (AFLP)

The AFLP protocol used was modified from Vos *et al*. (1995) as described by Douhan and Rizzo (2003). Briefly, extracted Hypomyces genomic DNA was subjected to a restriction reaction using EcoRI and MseI, followed by a ligation reaction using specific EcoRI and MseI adaptors. Six sets of primers with the addition of two nucleotides at the three prime ends were screened using a subset of isolates in order to find two primer pairs that yielded high quality fingerprints under our experimental conditions. Two sets of primers where chosen for the final analysis (E+TC/ M+AT and E+CA/M+AG). Restriction-ligation mixture was diluted 10-fold with TE (10mM Tris, 0.1mM EDTA pH 8.0) and 10 µl were used as template in a single amplification reaction.

Each 20 µl amplification reaction contained 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 2.5 mM $MgCl_2$, 2.5 mM each dNTP (Invitrogen), 0.375 µM of each primer, 0.5 U of Taq polymerase (Invitrogen), and 10µl of template from the restriction ligation dilution. Six isolates were used as controls by conducting independent DNA extractions, restriction-ligation reactions, and PCR amplifications. Thermocycling conditions consisted of an initial hold at 72° C and 94° C for 1 and 4 min, respectively, followed by 44 cycles with an annealing temperature of 65° C for 30 sec and an extension temperature of 72° C for 1 min with the annealing temperature reduced by 1° C for the first 9 cycles. All amplifications were performed in a MyCycler (Bio-Rad Laboratories Inc).

Following amplification, 5µl of loading dye (98% formamide, 10mM EDTA pH8.0, 0.25% bromphenol blue, 0.25% xylene cyanole) were added to each reaction, and the reaction mix was denatured for 4 min at 94° C and cooled on ice. Three to four µl of each sample were loaded and separated on 4% acrylamide gels buffered in TBE (1.35 M Tris pH 8.0, 0.45 M boric acid, 25 mM EDTA) for approximately 2 hr at 1800-1900 volts using a Bio Rad Sequi-Gen GT Sequencing Cell (Bio-Rad Laboratories Inc., Hercules, CA). The gels were then silver-stained by the method of Bassam *et al*. (1991) and dried overnight before visually scoring bands.

AFLP allele scoring and analysis

Putative alleles at each polymorphic AFLP locus were scored using a binary code (1, 0) corresponding to positive and null alleles, respectively. For the selection of AFLP markers, only polymorphic loci that were reproducible and easily identifiable were chosen (i.e., well-separated from adjacent bands and intensively stained).

Cluster analysis on genetic distances using the mean character difference option and unweighted pair-group method with arithmetic averaging (UPMGA) were conducted using PAUP* (version 4.0 beta 1) (Swofford 2002). Confidence in tree topology was examined using bootstrap with 1000 pseudoreplicates.

Population genetic analyses

To determine if we scored enough markers to adequately analyze the data, the program MULTILOCUS (Agapow and Burt 2001) was used to calculate genotypic diversity using 1 to 30 loci based on 1,000 random permutations from the combined data for all six populations. This data was then graphically viewed by placing the number of markers on the X axis and genotypic diversity on the Y axis to determine approximately how many markers were needed to explain at least 95% of the variation.

Weir and Cockerham's (1984) population differentiation statistic θ was calculated using FSTAT (Goudet 1995) to test for overall population differentiation. The significance level was based on 1,000 randomizations of the data by permuting genotypes among samples not assuming Hardy Weinberg equilibrium and the P-value was calculated based on the log-likelihood statistic G (Goudet *et al.* 1996). For the pair-wise comparisons, F_{st} values were calculated using FSTAT and the significance level was based on 1,000 randomizations of the data using log-likelihood statistic G (Goudet *et al.* 1996). The resulting pair-wise P-values were also then subjected to Bonferonni corrections as implemented in FSTAT.

Isolation by distance was tested by computing the regression of F_{ST} /1-F_{st} on geographic distances and the level of significance determined by performing a Mantel test using ISOLDE in GenePop (Raymond and Rousset 1995) based on 1,000 randomizations of the data.

Multilocus gametic disequilibrium was tested using the Index of Association (I_A) statistic as implemented in the program MULTILOCUS (Agapow and Burt 2001) using both full data sets and clone corrected data sets for all 30 loci scored. The analyses were also repeated on a subset of the data for loci (n =10) with allele frequencies between 0.20 and 0.80 since rare, absent, or fixed alleles can influence these analyses. I_A has an expected value of zero if there is no association of alleles at unlinked loci as expected in a randomly mating population (Brown *et al.* 1980, Maynard Smith 1993). The significance of I_A was tested by randomization (1,000 times) procedures by comparing the observed value of I_A to that expected under the null hypothesis of random mating (Agapow and Burt 2001, Burt *et al.* 1996).

RESULTS

Genotypic diversity and cluster analysis

A total of 30 polymorphic AFLP markers were scored based on the two primer sets used which yielded 39 genotypes out of 68 isolates (Fig 1.2). Based on the analysis of the number of markers scored to evaluate genotypic diversity, it was found that approximately only 20 markers out of the 30 scored were needed to account for ~95% of the variation observed (Fig 1.3). Twelve clonal genotypes were found consisting of 2 to 10 isolates with most clonal genotypes collected from the same population. Only 4 clonal genotype clades were found that consisted of isolates from two separate populations each, which were also from the geographically close populations in Southern California (OH, LE, FP, FS). Only four clades were found that had bootstrap support of 50% or higher. One major clade was also found that consisted of most of the more Northern California collections from PB and HC, but this clade did not have any bootstrap support (Fig 2).

Population genetic structure

Allele frequencies for each population are presented in Table 1.1. A total of 5 private alleles were found with 3 of them occurring in population LE. Likewise, in comparing allele frequencies between the populations, many of the alleles were absent in one or more of the population comparisons (Table 1.1). Allele frequencies for most loci varied considerably across the populations and the overall population differentiation statistic was significant ($\theta=0.321$, $P < 0.001$). For pair-wise comparisons, 13 out of the 15 F_{st} values were found to be significantly different from one another without Bonferroni corrections and 5 out of 15 with Bonferroni corrections. F_{st} values ranged from -0.080 to 0.524 and in general, populations that were closer to one another geographically also yielded smaller F_{st} values (Table 1.2). This trend was also supported by an analysis of isolation by distance based on the Mantel test (Fig 1.4) in which the obtained P-value was highly significant ($P < 0.001$). The hypothesis of random mating was rejected ($1.096 < I_A < 11.350$; $0.002 < P$- value < 0.001) for all comparisons except for the clone corrected data set for population FS in which random mating could not be rejected (I_A = 0.283; P-value = 0.157) (Table 1.3). Based on the reduced data with only 10 loci, the same results were also found but the p-value for this analyses was just outside of the significance level (I_A = 0.388; P-value = 0.063) (Table 1.3).

DISCUSSION

To our knowledge this is the first study to investigate the population biology of any *Hypomyces* species collected from natural populations. A higher degree of genotypic diversity was found among our isolates than expected given that sexual fruiting bodies have never been observed for this mycoparasite from any sampling location throughout California. However, we found that the populations were geographically structured and fit a model of isolation by distance, which we did hypothesize given the spatial scale that was sampled. One potential criticism that could be made is that the population sample sizes were relatively small, especially after clone correcting, but many loci were scored to try and overcome this limitation. Unfortunately, collections of the parasites for this study were conducted over 5 years of sampling and this was the fraction of the collections that corresponded to one of the known cryptic species within *H. microspermus* s.l. (Douhan and Rizzo 2003). Obtaining large sample sizes for these parasites is difficult because this is a multi-species system, mushroom hosts are ephemeral, and fruiting is highly dependent upon rainfall, which is very sporadic, especially in Southern CA.

The population differentiation analysis found that most pairs of populations were significantly different from one another, suggesting little to no gene or genotype flow between populations. This was also supported by the fact that some private alleles were found only in certain populations, and many of the loci were either absent or fixed between population comparisons or they were in very low or high frequency. The populations also fit an isolation by distance model as determined by the Mantel test ($P < 0.001$) (Fig 1.2). However, two (HC & PB) of the six populations were sampled at considerably further distances from the populations in Southern California which influenced this analysis because when the HC and PB populations were removed from the analysis, the Mantel test P-value increased to $P = 0.06$ (data not shown). This indicates that there is likely a greater barrier to gene flow between Northern and Southern populations of *Hypomyces* which may be due to the Transverse Mountain Ranges in Southern California which has been identified as a significant barrier to gene flow for many other organisms (Carlsbeek *et al*. 2003, Jockusch 2001).

Populations FS and FP were two interesting populations with respect to contradicting our hypothesis of finding significant clonal diversity. In the 2008 collections from FS, 4 genotypes out of 15 isolates were found with one dominant genotype accounting for 10 isolates. We predicted that the same clones would be found in 2009 since the sampling location was identical between years and only encompassed an approximate sampling area of 15 m^2 from this location. However, none of the same clones were found. Instead, three additional clonal genotypes were found out of 5 isolates collected in 2009. Similarly for the FP collections, 2, 7, and 4 genotypes were found in 2007, 2008, and 2009, respectively, but none of the same clonal genotypes were collected between years.

It is possible that the variation observed is a result of improper digestion of the DNA samples during their preparation (Mueller and Wolfenbarger 1999, Vos *et al*. 1995) or poor selection of scoreable loci. However, we obtained identical fingerprints on the 6 isolates used as controls. The most likely explanation may be that such variation is due to competition between related asexual genotypes between any given year or it may be possible that asexually derived spores were randomly blown in from different locations, adding to the genotypic variation in a given area. Slight differences in the time of year that the collections were made may have also affected our findings, possibly resulting from competition between clonal genotypes to become established and dominant in a given location.

Multilocus disequilibrium analyses based on the I_A indicated that we could reject random mating in 5 out of the 6 populations sampled but the P-value for the reduced locus data set for population FS was just outside of the 95% significance level. Therefore, it appears that sexual reproduction is likely not occurring regularly in these fungi, which is consistent with our observations of never finding perithecia on infected boletes in California based on over 400 collections (Douhan, unpublished). Moreover, when looking at the allele frequencies within and between the populations (Table 1.1), it is obvious that most of the loci are either absent or fixed between populations or they are in very low or high frequencies, indicating that few polymorphisms separate the genotypes. Therefore, most pairs of loci within a given population were in gametic disequilibrium, which is most consistent with a clonal population structure, and the variation observed between the isolates may simply be the result of mutations.

It is typical of other host-parasite systems to demonstrate temporal changes in allele frequencies as both the parasites and hosts struggle to maintain a high enough level of virulence and resistance, respectively (Lively 1996, Sasaki 2000). For this reason, perhaps it would seem unlikely that a given parasitic clone would be able to persist for long periods of time before the more resistant host genotypes begin to form a larger part of the population, forcing the parasite to change as well. However, it remains unknown how much of a role *Hypomyces* has on the genetic fluctuations of its host and vice versa.

We were not able to identify all of the hosts from this study due to significant colonization by the parasites from most collections. However, in the FP population, all hosts were *Xerocomus dryophilus* and it is likely that each fruiting body collected over both years were the same clonal genet, since most studies based on bolete fruiting bodies find that they often produce large clonal genets. For example, Fiore-Donne and Martin (2001) found that two *Xerocomus* species each produced a single persistent genet, each covering an area of at least 40 m^2 and 100 m^2 in one of their 100 m^2 plots during three consecutive years. Bonello *et al*. (1998) also found a single *Suillus pungens* genet, covering an area of at least 300 m^2, in a 1200 m^2 plot. Therefore, if we assume that host genotypes are persistent over many years due to their mycorrhizal associations with their plant hosts, it is likely that competition among parasite genotypes would be important for successful colonization and dissemination.

We did find four clonal genotype clades in which putative clones were shared between populations, but the shared genotypes were found only from the mycoparasite populations within relatively close proximity in the southern California area. No shared putative clonal genotypes were found between the most distantly sampled populations of PB and HC or even PB and the other southern populations. This suggests the potential movement of aleuroconidia at the scale of a couple 100 km, but not across larger expanses of the Transverse Mountain Range. These survival structures are thick walled, highly pigmented, and seem 'designed' for long-term survival and potentially long distant movement by either wind and or animals. Aleuroconidia are physically very similar to 'rust' spores, which have been shown to travel 1,000's of kilometers, i.e, the 'Puccinia Pathway,' and have been found in the atmosphere at an elevation of 5,000 m (Stakman 1934). Therefore, long distance movement of *Hypomyces* asexual spores may be occurring to some degree.

This is the first attempt to study the population biology of any *Hypomyces* mycoparasite from a natural ecosystem. Understanding the genetic structure of the various species of bolete-associated *Hypomyces* will aid in understanding how these pathogens reproduce and spread in the environment and proper identification will also lead to a better understanding of host parasite relationships (e.g., host specificity and potential coevolution among hosts and pathogen) within this system. The overall results suggest that this specific cryptic species within *H. microspermus* has a primarily clonal population genetic structure and that spore dispersal was restricted across the sampled populations. However, the population genetic structure of the other species in this group remains to be determined which will be important for a better understanding the overall biology of this host parasite system.

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Table 1.1. FSTAT calculated allele frequencies for six populations of *Hypomyces*. Fixed and null alleles are in bold and private alleles are italicized.

	Population allele frequencies					
	FP	FS	HC	LE	OH	PB
Locus	N = 13	N = 8	N = 6	N = 4	N = 5	N = 7
A500	0.923	0.500	0.833	**1.000**	0.800	0.714
A440	**0.000**	**0.000**	**0.000**	*0.250*	**0.000**	**0.000**
A425	**0.000**	**0.000**	**0.000**	*0.250*	**0.000**	**0.000**
A410	**0.000**	**0.000**	*0.167*	**0.000**	**0.000**	**0.000**
A400	0.923	0.750	**0.000**	**1.000**	0.800	**0.000**
A375	**0.000**	**0.000**	0.500	0.250	**0.000**	0.571
A340	**1.000**	0.875	0.833	**1.000**	0.800	**1.000**
A300	**0.000**	**0.000**	**0.000**	0.250	**0.000**	**0.000**
A270	**1.000**	**1.000**	0.167	**1.000**	0.800	0.857
A260	0.385	0.375	**0.000**	**0.000**	0.200	**0.000**
A250	0.308	**0.000**	**1.000**	0.500	0.800	0.857
A238	0.077	**0.000**	**0.000**	0.000	0.200	**0.000**
A230	0.154	**1.000**	**0.000**	0.250	0.400	**0.000**
A215	0.077	**0.000**	0.167	0.250	**0.000**	**0.000**
A203	**1.000**	**1.000**	0.833	**1.000**	0.800	**1.000**
A195	0.077	0.125	0.833	0.250	0.200	0.857
A185	0.769	0.375	0.167	0.500	0.800	**0.000**
A150	0.308	0.125	**0.000**	0.250	**0.000**	**0.000**
A115	**0.000**	**0.000**	0.833	0.250	0.200	**0.000**
A110	**0.000**	**0.000**	0.167	0.250	**0.000**	**0.000**
A102	0.231	0.125	0.167	0.250	**0.000**	0.429
B270	0.769	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
B240	0.923	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
B233	**0.000**	0.125	**0.000**	0.000	**0.000**	**0.000**
B218	0.923	**1.000**	**1.000**	0.250	0.600	**1.000**
B215	0.692	0.625	**0.000**	0.750	0.800	0.143
B167	0.923	**1.000**	**1.000**	0.250	0.600	**1.000**
B150	0.308	0.500	**1.000**	0.250	0.200	0.857
B125	0.077	**0.000**	0.167	**0.000**	**0.000**	**0.000**
B118	0.923	**1.000**	0.833	0.250	0.400	**1.000**

Table 1.2. Population differentiation analyses based on pair-wise F_{st} comparisons.

	Pairwise F_{st} Values				
	FS	HC	LE	OH	PB
FP	0.150*	0.472**	0.158*	0.073	0.415**
FS		0.524**	0.294*	0.202*	0.480**
HC			0.401*	0.364*	0.184*
LE				-0.080	0.431*
OH					0.404**

* Significance at the 95% confidence level based on 10,000 randomizations of the data using FSTAT

** Significance at the 95% confidence level based on 10,000 randomizations of the data using FSTAT after Bonferroni corrections

Table 1.3. Tests for random mating based on the Index of association statistic (IA) for six populations of *Hypomyces microspermus* using all isolates and clone corrected data sets as well as full locus data sets (N =30) and a reduced locus data set (n = 10) with allele frequencies between 0.20 and 0.80.

Population[a]	No. of isolates	No. of loci	I_A Value	P-Value[b]
FPCC	13	30	1.096	0.002
FPNC	16	30	1.327	<0.001
FSCC	8	30	0.283	0.157
FSNC	20	30	2.056	<0.001
HCCC	6	30	7.375	<0.001
HCNC	11	30	7.243	<0.001
LECC	4	30	11.350	<0.001
LENC	4	30	11.350	<0.001
OHCC	5	30	1.951	0.005
OHNC	7	30	2.660	<0.001
PBCC	7	30	1.515	0.007
PBNC	10	30	1.828	<0.002
FPCCFC	13	10	0.796	0.002
FPNCFC	16	10	0.980	<0.001
FSCCFC	8	10	0.388	0.063
FSNCFC	20	10	2.020	<0.001
HCCCFC	6	10	3.000	0.008
HCNCFC	11	10	3.000	0.002
LECCFC	4	10	3.213	0.001
LENCFC	4	10	3.213	0.001
OHCCFC	5	10	1.852	0.009
OHNCFC	7	10	2.284	<0.001
PBCCFC	7	10	1.007	0.027
PBNCFC	10	10	1.140	0.011

[a] The first two letters denote the populations as described in the text. CC = clone corrected, NC = not clone corrected, FC = reduced locus data sets in which rare, fixed or absent loci were not included in the analyses.
[b] Significance was determined by 1,000 permutations of the data using MULTILOCUS with the null hypothesis of random mating.

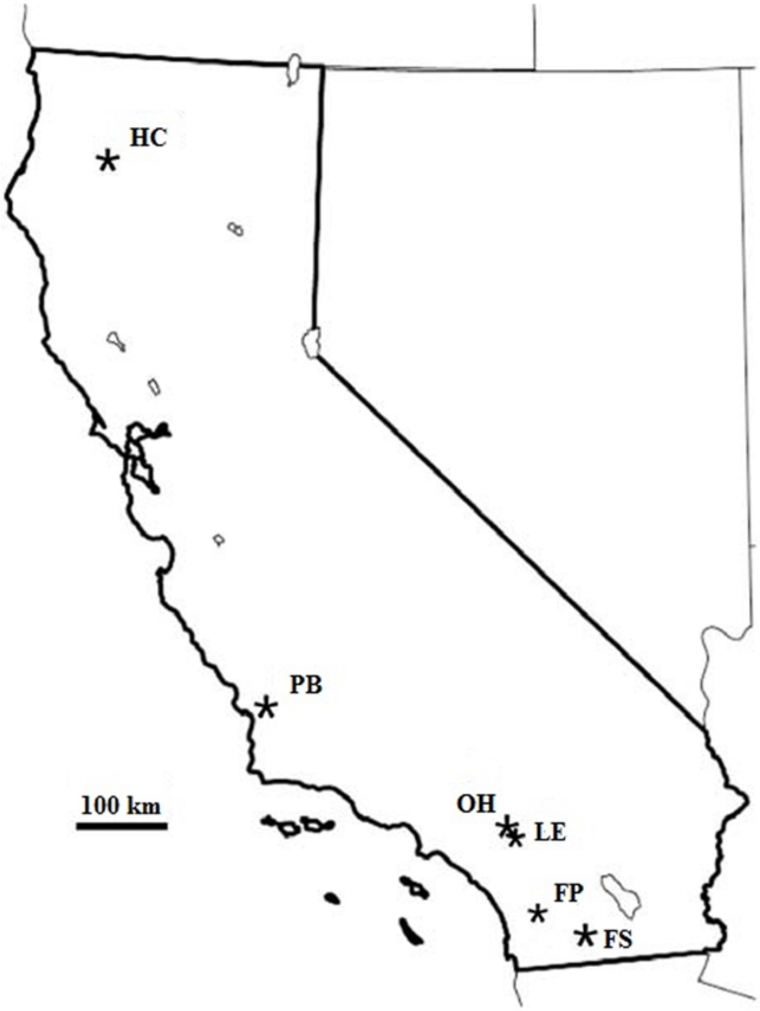

Fig 1.1 Collection sites for six populations of *Hypomyces microspermus* collected for this study. Collections for each of the locations took place in the following years: HC (East Fork campground in the Trinity National Forest, Humboldt Co.): 2005; PB (Pismo Beach area right off of US Highway 101, San Luis Obispo Co.): 2004; OH (El Cariso area off of Forest Service route 6S05 road in the Cleveland National Forest, Riverside Co.): 2008; LE (Lake Elsinore area off of Highway 74 in the Cleveland National Forest, Riverside Co.): 2008; FP (Felicita County Park, Escondido, San Diego Co.); 2007, 2008, 2009; FS (Finn Springs County Park, El Cajon, San Diego Co.): 2008, 2009.

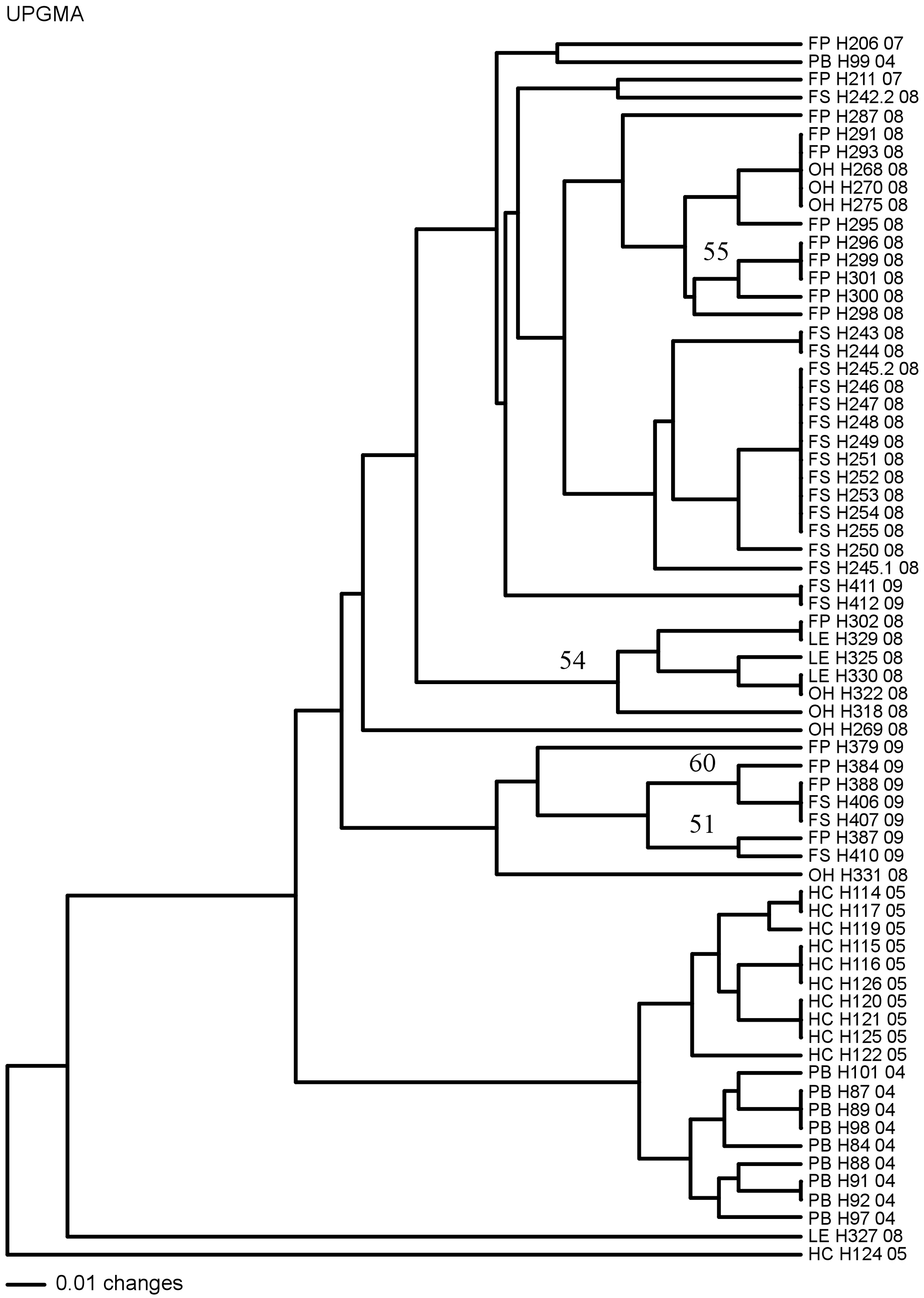

Fig 1.2 UPGMA cluster analysis constructed in PAUP based on 30 AFLP markers. Bootstrap values of 50% or greater are shown based on 1,000 replicates using PAUP.

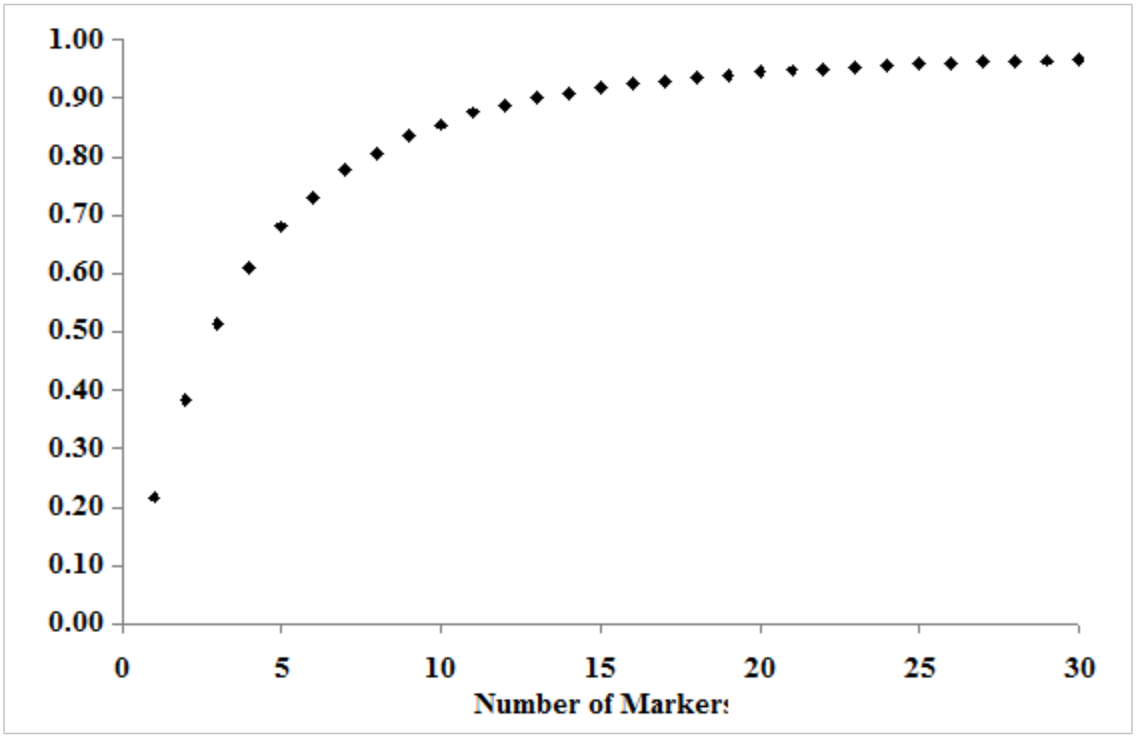

Fig 1.3 Graphical representation of genotypic diversity (Y-axis) calculated from 1 to 30 randomly chosen markers (X-axis) based on 1,000 randomizations of the combined data for all six populations using the program MULTILOCUS.

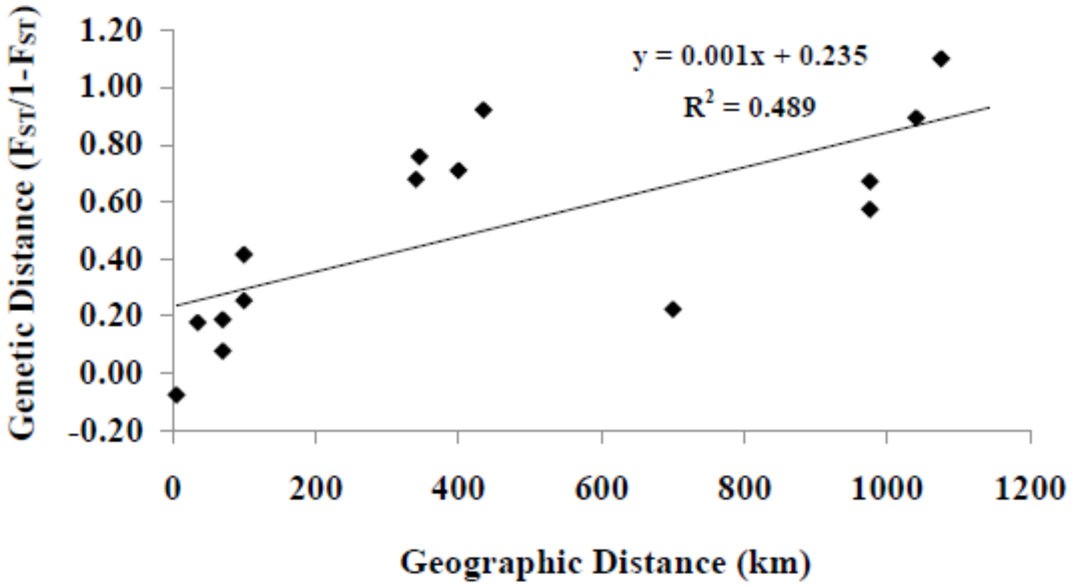

Fig 1.4 Isolation by distance analysis based on a Mantel test (1,000 replicates of the data (P <0.001)) using the program ISOLDE from GENEPOP.

CHAPTER II. Multigene phylogenetic analysis of bolete-infecting *Hypomyces* species

ABSTRACT

Hypomyces species are common mycoparasites of diverse macro fungal hosts and are readily found in all natural ecosystems where their hosts produce fruiting bodies. For this study, we are interested in *Hypomyces* species associated with bolete hosts. Only a single molecular systematics study has been conducted on this group of parasites and it was only based on a single locus and the phylogenetic relationships among all studied taxa were not completely resolved. Moreover, data from a more recent study suggested that additional cryptic species might also be found within these fungi. Therefore, our objective was to perform a multigene analysis of this group using maximum parsimony and neighbor joining analyses based on eight loci: ITS rDNA (ITS), 18s rDNA (18S), 28s rDNA (28S), Actin, β-tubulin, RNA polymerase II subunit RPB1 (RPB1), RNA polymerase II subunit RPB2 (RPB2), and translational elongation factor 1-α (EF). Maximum parsimony (MP) and Neighbor-joining (NJ) analyses of the concatenated multigene data were able to resolve the relationships among the species with strong bootstrap support delineating all species studied, which included *H. ampullosporum*, *H. chlorinus*, *H. chlorinigenus*, *H. chalcipori*, *H. chrysospermus*, *H. boletiphagus*, *H. laevigatum*, *H. microspermus*, and *H. tulasneanum*. It was also confirmed that at least two cryptic species exist within both *H. chrysospermus* and *H. microspermus* based on significant bootstrap support (>97%) at the nodes delimiting each of these clades. Analyses of individual loci were generally in agreement, with the exception of 18S that was too conserved to resolve most species relationships. For this locus, only the more

divergent *H. boletiphagus* and *H. chlorinigenus* were well resolved. In conclusion, this study adds to the growing body of evidence within the field: that multilocus data sets are essential compared to single gene analyses because some loci are simply too conserved while others can be to divergent to be useful for molecular systematic investigations.

INTRODUCTION

With the advent of molecular techniques and the inclusion of molecular data in systematics, it quickly became apparent that relationships for organisms hypothesized solely on morphological characteristics would, in many instances, require taxonomic revisions. Not only have revisions among relationships of taxa been made at generic or higher levels, there are many instances of organisms that were once thought to be a single species based on morphological characters that have subsequently been split into multiple species based on molecular analyses (e.g. Brown *et al.* 2007; Elmer *et al.* 2007). Species that are indistinguishable from one another morphologically but are distinct enough genetically are often referred to as cryptic species. There are many examples of cryptic species or species complexes within most, if not all of the major taxonomic groups (e.g. Bass *et al.* 2007; Cooke *et al*. 2012; Pfenninger & Schwenk, 2007; Knowlton, 1993). This has proven to be case even for taxa with suites of complex morphological and phenotypic characters such as the giraffe (Brown *et al.* 2007), frogs (Elmer *et al.* 2007), and fish (Cooke *et al.* 2012). However, the use of molecular data to distinguish species within microorganisms has been especially significant because microbial organisms have less complex morphologies and therefore fewer characters to distinguish among species (Driver *et al.* 2000; Taylor *et al.* 2006).

Most modern studies investigating phylogenetic relationships within the fungi now take a multigene approach when attempting to delimit species boundaries because of the lack of robust morphological differences found within many fungal groups (e.g. Bischoff *et al.* 2009; Cruse *et al.* 2002; Kauserud *et al.* 2007; Miller and Huhndorf, 2004; Pringle *et al.* 2005; Schoch *et al.* 2006; Spatafora *et al.* 2006). Prior to this, many earlier studies used molecular data based on a single locus, which often produced unresolved phylogenetic relationships setting a precedent for the use of multigene studies in fungal systematics (Leigh *et al.* 2008). Taylor *et al.* (2000) synthesized this concept in an important review article and argued that within the fungi, the use of phylogenetic species concept using multiple loci is more appropriate than biological and morphological species concepts because genetic changes can happen before morphological and behavioral changes (i.e. reproductive behaviors). They also suggested that this approach was the most appropriate technique because of the high degree of genetic divergence found among morphologically identical isolates, coupled with the difficulty or impossibility of carrying out mating tests with putatively asexual fungi as a method to confirm biological species concepts.

Our interests lie within a group of mycoparasitic fungi in the anamorphic genus *Sepedonium*, which are linked to *Hypomyces spp.* teleomorphs. *Hypomyces* species are common mycoparasites of diverse fungal hosts including agarics, boletes and polypores (Rogerson and Samuels 1989, 1993, 1994). Bolete-parasitizing *Hypomyces* species have a worldwide distribution and are found generally in all natural ecosystems where boletes fruit. There are seven species recognized within *Sepedonium* associated with the teleomorph genus *Hypomyces* (Rogerson and Samuels 1989). There are an additional five taxa that have not been connected to a teleomorph or been formally described (Sahr *et al.* 1999).

For the purpose of this study, we will use the teleomorphic names associated with *Hypomyces* associated with boletes since, the study by Sahr *et al.* (1999) determined that all taxa appeared to be monophyletic based on an analysis of the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA genes using neighbor-joining (NJ) and parsimony analyses (see Sahr *et al.* 1999). Although tree topologies resulting from NJ and parsimony analyses were mostly in agreement, there was one branch from the Sahr *et al.* (1999) study that changed positions depending the analysis performed, indicating that more robust data was required to more adequately resolve phylogenetic relationships within these parasites. For example, parsimony analysis resulted in the placement of *H. ampullosporum* between *H. chrysospermus* and *H. microspermus*, while their NJ results placed *H. chrysospermus* and *H. microspermus* as a sister group to *H. ampullosporum*. They also noted that the strains of *H. chrysospermus* and *H. microspermus* clustered as subgroups, which suggested that more 'species diversity' may be present within the known morphological species that have been described for bolete-infecting *Hypomyces* taxa.

A more recent study by Douhan & Rizzo (2003) found more evidence for the presence of two cryptic species existing within *H. microspermus* and *H. chrysospermus* based on the analysis of amplified fragment length polymoprhisms (AFLP) of isolates collected from California. This finding was also substantiated by the analysis of ITS sequences from a subset of the California isolates and sequences of the isolates used in Sahr *et al.* (1999) obtained from GenBank. Therefore, our objective for this study was to produce a well resolved phylogenetic hypothesis to establish the relationships among bolete-infecting *Hypomyces* and tease out the presence of cryptic species using a multigene phylogenetic approach that included ITS rDNA (ITS), 18s rDNA (18S), 28s rDNA (28S), Actin, β-tubulin, RNA polymerase II subunit RPB1 (RPB1), RNA polymerase II subunit RPB2 (RPB2), and translational elongation factor 1-α (EF).

MATERIALS AND METHODS

Isolate Collections and Growth

Isolates spanning wide geographic and temporal locations were chosen for this study to better understand the relationships among these widely distributed species. The goal was also to include as many of the bolete-infecting *Hypomyces* species as possible. Therefore, isolates from North America, Europe, and Japan were included in this study, as indicated in Table 2.1. Six isolates, which represented the known species of *Hypomyces* associated with boletes, *H. ampullosporum* (S15), *H. tulasneanum* (S29), *H. chalcipori* (S32), *H. chrysospermus* (S47 & S68), and *H. laevigatum* (S50), were obtained from Dr. Helmut Besl, Universität Regensburg, Institut für Botanik, D-93040 Regensburg. These are the same isolates that were used in the Sahr *et al*. (1999) study, which was the first study to investigate phylogenetic relationships among these parasites. Isolates representing *H*. *boletiphagus* (UAMH6602, UAMH6604, UAMH6612) were originally collected from the eastern United States and were obtained from the University of Alberta Microfungus Collection and Herbarium. A representative isolate of *H*.

chlorinus (ATCC28784) was collected in France and was obtained from the American Type Culture Collection (ATCC). Representative isolates of *H. chlorinigenus* (NBRC105334 & NBRC105335) were both collected in Japan and were obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC) in Japan. Isolates 03-47, 05-68, 05-79, 05-80, 05-94, 05-95, 05-105, 05-109, and 05-112 are all European isolates and were originally obtained from Dr. Kadri Põldmaa, Institute of Ecology and Earth Science, and Natural History Museum, University of Tartu, Estonia, and were only identified as *Hypomyces*/*Sepedonium spp*. isolated from various bolete hosts. All other isolates with the identifying designation ending in 'H' were from our 'in-house' culture collection and were originally collected from various parts of California and Oregon (USA) and Quebec, Canada. Putative identifications, (Table 2.1) were based on the analysis of ITS sequences from comparisons with isolates from Douhan & Rizzo (2003), named isolates obtained from culture collections, and the isolates that were used by Sahr *et al*. (1999). A strong effort was made to include as many of the same isolates used by Sahr *et al.* (1999). However, we had to obtain some samples from culture collections to represent species we did not collect or obtain from collaborators. *Hypomyces brunneum* was the only species that was used by Sahr *et al.* (1999) that we were unable to obtain.

To isolate the parasites, a flame-sterilized loop was used to streak spores on potato dextrose agar (Difco, Detroit, Michigan) and or by placing small pieces of clean subiculum directly onto the agar dishes. Each plate was incubated several days at room temperature, and the leading edge of a colony was transferred once again to potato dextrose agar (Difco, Detroit, Michigan) and incubated at room temperature for approximately one week to ensure no contaminants were present. Mycelium was scraped off with a flame-sterilized microspatula and placed into 1.5mL centrifuge tubes with C-tab buffer (Gardes & Bruns 1993) and frozen at -20°C until DNA extraction using protocols outlined in Douhan & Rizzo (2003). Quality and quantity of genomic DNA was visually inspected on 0.8% agarose gels after electrophoresis. Genomic DNA was then diluted 1:25 in sterilized H_2O before being used for PCR.

DNA Amplification & Sequencing

A total of eight different regions were amplified for each of the isolates 56 isolates used based on published primers and/or primers that we developed (Table 2.2). For each PCR amplification, a 20µl PCR reaction was set up using 2µL of a diluted template DNA, 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 2.5µL $MgCl_2$, 0.2µL each dNTP (Invitrogen), 3.75µL of each primer, and 0.5µL of Taq polymerase (Invitrogen).

Subsets of representative isolates from different known species were used in initial screenings to ensure consistent amplification success using primers and thermocycling conditions from the literature (see Table 2.2). Most amplifications were successful except when attempting to amplify RPB1 and RPB2, for which success was sporadic. In those instances, sequences for isolates that amplified successfully were aligned and new primers were designed using Primer3web V4 (Rozen & Skaletsky, 2000) using other conserved regions found within the successfully amplified fragments. Several new primers were then designed, tested in the same manner described above, and the combinations that had the highest amplification success were used (Table 2.2). To amplify RPB1, the custom designed HRPBIguFb and HRPBIguRb primers were used for most isolates, as were HRPBIIguFa and HRPBIIguRc for RPB2. Thermocycling conditions for all loci followed the same pattern, with the exception of changes to the corresponding annealing temperatures which were adjusted to the lowest melting temperature (Tm) for either of the two primers being used (Table 2.2). Thermocycling settings consisted of an initial hold at 95°C for 5 min, followed by 35 cycles of 94°C (30 s), variable Tm (see TABLE) (30 s), 72°C (1 min), and a final hold of 72°C for 10 min.

All amplifications were done using a MyCycler (Bio-Rad Laboratories Inc., Hercules, CA) and the PCR products were cleaned using ExoSapit (USB Amersham, Uppsala, Sweden) following the manufacturer’s instructions. Each locus was sequenced in both directions using Big Dye® Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA) at the Core Instrumentation Facility (CIF) of the University of California’s Institute of Integrative Genome Biology at UC Riverside.

Phylogenetic analysis

The sequences were edited using Sequencher (version 4.9, Gene Codes Corporation, Ann Arbor, MI, USA) with reads from both directions for all primers used. The sequences were aligned using the ‘E-INS-i’ strategy on the online application MAFFT version 7 (Katoh & Standley, 2013) and manually edited in MacClade version 4 (Maddison & Maddison, 2001). Data sets for each individual locus were created as well as a concatenated data set for isolates with at successful amplification of at least four loci using Sequence Matrix (Vaidya *et al.* 2011) and analyzed in the same manner as individual loci following the ‘total evidence’ principle of Kluge (1989). Maximum Parsimony (MP) analysis, using the heuristic search procedure with 1,000 random, stepwise-additive replicates, were conducted using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was analyzed using bootstrap with 10,000 replicates under the fast-heuristic search. Neighbor joining (NJ) analyses were performed using the online application MAFFT version 7 (Katoh & Standley,2013) based on the conserved sites method with 1,000 resamplings to calculate bootstrap values. After the alignments were generated using the same program, the alignments were visually inspected using the Jalview (Waterhouse *et al.* 2009) option within MAFFT. *Hypomyces boletiphagus* was used as the outgroup to root trees based on our analysis of the 18S locus (see results) and was also one of the most distantly related species based on the results of Sahr *et al*. (1999). If *H*. *boletiphagus* data was unavailable for rooting, *H*. *chlorinigenus*, *H. chlorinus*, or *H. chalcipori* were used to root the trees, in decreasing order of choice based on their positioning in Sahr *et al.* (1999).

RESULTS

A total of eight loci successfully amplified: 18S, 28S, ITS, RPB1, RPB2, β-tubulin, EFα-1, and Actin. Seven loci were used for individual MP and NJ analyses, including 18S, 28S, ITS, RPB1, RPB2, β-tubulin, EF1-α, and Actin. EF1-α was too variable to align with confidence and was therefore not included in the analyses. Overall, generalized patterns in tree topology were consistent among most trees generated from the different gene fragments, though bootstrap support above 50% for all clades was not always obtained. Results from both MP and NJ analyses were largely the same, so only results for MP will be presented. Some isolates also failed to amplify or produce useable sequences despite many attempts to reamplify and sequence them. Therefore, the total number of isolates used for each analysis will be clearly noted (Table 2.3).

The analysis of the 18S small subunit locus resulted in 1563 equally parsimonious trees. Forty-nine isolates were included in the analysis based on a total of 1021 characters with ten parsimony-informative sites and seven parsimony-uninformative sites. The analysis of the 28S large subunit locus resulted in 150 equally parsimonious trees. Forty-two isolates were included in the analysis based on a total of 1149 characters with 36 parsimony-informative sites and 18 parsimony-uninformative sites. The analysis of the Actin locus resulted in six equally parsimonious trees. Forty-eight isolates were included in the analysis based on a total of 312 characters with 43 parsimony-informative sites and 25 parsimony-uninformative sites. The analysis of the β-tubulin locus resulted in 16 equally parsimonious trees. Forty-eight isolates were included in the analysis based on a total of 687 characters with 122 were parsimony-informative sites and 27 parsimony-uninformative sites. The analysis of the ITS locus resulted in 41 equally parsimonious trees. Forty-eight isolates were included in the analysis based on a total of 522 characters with 26 were parsimony-informative sites and 31 parsimony-uninformative sites. The analysis of the RPB1 locus resulted in three equally parsimonious trees. Forty-five isolates were included in the analysis based on a total of 572 characters with 89 were parsimony-informative sites and 56 parsimony-uninformative sites. The analysis of the RPB2 locus resulted in one tree. Fifty-three isolates were included in the analysis based on a total of 633 characters with 133 were parsimony-informative sites and 16 parsimony-uninformative sites. The analysis of the multigene concatenated dataset resulted in 16 equally parsimonious trees. Fifty-two taxa were included in the analysis based on a total of 4913 characters with 447 parsimony-informative sites and173 parsimony-uninformative sites.

The resulting patterns in tree topology for MP analyses were generally consistent for all loci examined. The phylogram for the multigene concatenated data had strong bootstrap support for most clades and their relationship to each other (Fig 2.8), which was consistent with their positioning and placement in Sahr *et al*. (1999). High bootstrap support was found at nodes where putative species delimitations took place for most species and was most notable for the putative cryptic species within *H. chrysospermus* and *H. microspermus* groups with bootstrap values ≥97%, except for outlier isolates 05-68 and 171H, which occasional clustered in unexpected clades (Figs 2.2-4) but with no bootstratp support. Isolates for *H. tulasneanum* and *H. laevigatum* were placed as sister taxa with bootstrap support of 56%. That clade, in turn, was placed as a sister group to the H. *chrysospermus* and *H. microspermus* groups with a bootstrap support of 65. Also, isolates for both *H. laevigatum* and *H. ampullosporum* had consistent geographic patterns in their clustering, with isolates from Germany and Canada clustering closer together than isolates of the same species collected in California from the Western United States. We had no isolates from other locations to compare with our isolates from Japan, but their placement relative to the other clades was in agreement with its position in Sahr *et al.* (1999).

The resulting tree for 18S locus deviated from all the other loci in patterns of tree topology, as most of the isolates and taxa were unresolved; only *H. boletiphagus* and *H. chlorinigenus* resolved into branches with high bootstrap support (Fig 2.1). Also, results for RPB1 placed the *H. laevigatum* and *H. tulasneanum* clade as a sister clade to the *H. microspermus* group (Fig 2.6). The bootstrap support at the nodes delimiting these groups is relatively high, 72% for *H. microspermus* and 73% for *H. laevigatum* and *H. tulasneanum*, but bootstrap support for their positions relative to each other, as well as all the other species, had bootstrap values under 50%.

DISCUSSION

Our multigene analysis confirms the previous results of Douhan & Rizzo (2003) that cryptic species exist within *H. chrysospermus* and *H. microspermus* sensu lata and we also produced a well resolved phylogeny among these parasites using a multigene approach compared to the single gene analysis of Sahr *et al.* (1999). Overall, our phylogenetic analyses of the bolete-infecting *Hypomyces* fungi has resulted in a relatively well supported and consistent establishment of relationships among the species comprising this group based on the concatenated data as well as for most of the single loci (Figs 2.2-8). The largest discrepancy was found for the 18S locus, which was highly conserved. However, this analysis demonstrated that most species associated with the boletes fall into one monophyletic group and that *H. boletiphagus* and *H. chlorinigenus* were the more distantly related species.

We also found that isolates from both North America and Europe routinely clustered closely together, demonstrating that the same species can have very wide geographic distributions. This is in concordance with hypotheses that some microbes have the capacity to establish cosmopolitan distributions due to their high fecundity and ease of dispersal (Fenchel & Finlay, 2004; Finlay, 2002; Finlay & Fenchel 2004). However, Taylor *et al.* (2006) argues that generally, especially for fungi, the putative geographic range will depend on the method utilized to delimit species. As such, methods unable to detect cryptic taxa will erroneously infer a very wide distribution for some species (i.e. morphospecies). However, some of the clustering within species show topological patterns consistent with geographic location. For example, the *H. laevigatum* isolates from Canada and Germany consistently clustered more closely together than *H. laevigatum* isolates from California (Figs 2.4-7). This was also found for isolates of *H. ampullosporum* because isolates from Germany and Canada, also clustered closer together than to *H. ampullosporum* isolates from California in the Western United States. However, considering the fact that these are parasitic species, their consistent grouping patterns may reflect host distribution and may be limited by such, especially considering patterns described by both Sahr *et al.* (1999) and Douhan & Rizzo (2003) in which evidence of varying degrees of host specificity among the groups comprising *H. chrysospermus* and *H. microspermus* was presented.

Despite overall patterns and the results of the multigene dataset which resulted with relatively high bootstrap support, especially near the nodes where one would expect the species boundaries to be (Fig 2.8), there was some incongruence in the tree topology for the cryptic species within *H. chrysospermus* and *H. microspermus* (Fig 2.2), while tree topology remained consistent for all other species. This suggests that there may be some level of incomplete lineage sorting within these species complexes. However, as suggested by Maddison & Knowles (2006), increasing the number of loci will increase the chances that phylogenetic relations will be determined in spite of recent, or in-progress speciation events. For these reasons, multigene approaches can be more powerful than studies based on single loci. This is made especially evident within our study because the more conserved 18S and 28S loci were not as informative as the more variable loci used. Data from 18S was not able to resolve the relationships beyond that of *H. boletiphagus* and *H. chlorinigenus*, while data from 28S was unable to adequately resolve the relationships among the cryptic species of the *H. chrysospermus* and *H. microspermus* species complexes.

In terms of the widespread distribution of some of the species, either recent geographic expansion events, or periodic genetic exchanges serve as possible explanations for such a high degree of genetic similarity among such widely distributed organisms. The genetic diversity found within species complexes, despite morphological similarities, are also an indicator of a relatively recent divergence in evolutionary history between the two groups. Allopatry within cryptic organisms would provide a straightforward explanation as to the cause in genetic divergence. Sympatry would be more difficult to explain, although in the case of parasitic organisms, host specialization is a very likely driver for speciation. Sahr *et al.* (1999) and Douhan & Rizzo (2003) presented some evidence of differential host preference between the different cryptic species, and a larger scale study looking at host specificity with a large sample size from wide geographic ranges and molecular techniques to identify hosts would be an appropriate next step.

In addition, finer scale population level studies may reveal the geographic origin for the group, as well as when, and how their geographic dispersal took place and originated. Also, despite the lack of diversity seen in our phylogenetic analyses within some of the cryptic species, a study by Uribe Valdez & Douhan (2012) presented evidence that isolates from one of the putative cryptic species of *S. microspermum* from California fit isolation by distance models based on AFLP data. This may indicate that given longer periods of evolutionary time, and greater geographic separation, the cryptic species will differentiate themselves from their sister taxa, and the populations within them may follow suit.

Although molecular data has made it possible to more accurately tease out and approximate the true species makeup of a species complex and their relationships to one another, not all molecular data is equally useful. This is because not all molecular data will yield the same results (Planet, 2006). For example, Schoch *et al.* (2012) studied six DNA gene fragments to determine if any of them would be suitable to use as a barcode for fungal identification. They concluded that many were not suitable on their own to provide sufficient information to distinguish between species or were too variable or too conserved to serve as a reliable source for comparison (Schoch *et al.* 2012). They also found that even though protein coding regions such as RPB1 and RPB2 provided more resolving power in identifying species, ITS was chosen as the barcode because it had higher PCR amplification success across divergent fungal taxa. For these reasons most studies within the fungi use the ITS1 and ITS2 regions for identification purposes as well as to produce phylogenies, usually at the genus level. However, this region is often to variable to produce reliable alignments. In our study, ITS was a good marker for species identification within this group of fungi and was also a reasonable locus for phylogeny construction, but additional loci were needed to better resolve relationships. As with other studies, we also found that some loci were too variable or too conserved to be used as reliable markers alone for identification and or phylogeny construction. For example, the EF1-α locus was too variable to produce a useful alignment that could be used with confidence. On the other hand, the loci for 18S and 28S were too conserved, either producing unresolved trees (Fig 2.1), or trees with topologies that are incongruent to the trees produced by other loci and multigene data (Fig 2.2).

To conclude, the multigene approach using ITS rDNA, 18s rDNA, 28s rDNA, actin, EF1-α, β-tubulin, RPB1, and RPB2 resulted in a well supported phylogenetic study of the bolete-infecting *Hypomyces* species, with enough resolution to tease out cryptic species within *H. microspermus* and *H. chrysospermus*. Members of these cryptic species were found distributed across both North America and Europe. Further analysis from other parts of the world may demonstrate that these cryptic species also have an even broader global distribution. Also, this study adds to the growing body of evidence within the field: that multilocus data sets are essential and more robust when compared to single gene analyses because some loci are simply too conserved while others can be to divergent to be useful for molecular systematic investigations.

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Table 2.1. Isolates with a first character designation of 'S' correspond to isolates used in the Sahr *et al.* (1998) study. Isolates designated 'UAMH' were obtained from the University of Alberta Microfungus Collection and Herbarium. The isolate with the 'ATCC' designation was obtained from the American Type Culture Collection. The isolates with 'NBRC' designations were obtained from NITE Biological Resource Center in Japan. All other isolates were obtained either by members of the Douhan Lab or personal collaborators. Roman numeral designations after the putative identity denote the AFLP clade from Douhan & Rizzo (2003) to which the isolate belongs.

Isolate	Location	Date Collected†	Putative Identity
S15	Germany	08/30/94	*H. ampullosporum*
S29	Germany	06/04/86	*H. tulasneanum*
S32	Germany	08/17/87	*H. chalcipori*
S47	Germany	10/17/95	*H. chrysospermus*
S50	Germany	08/11/95	*H. laevigatum*
S68	Germany	10/04/95	*H. chrysospermus*
UAMH6602	NC	N/A	*H. boletiphagus*
UAMH6612	NC	N/A	*H. boletiphagus*
UAMH6604	VA	N/A	*H. boletiphagus*
ATCC28784	France	N/A	*H. chlorinus*
NBRC 105334	Japan	N/A	*H. chlorinigenus*
NBRC 105335	Japan	N/A	*H. chlorinigenus*
28H	CA	01/01/04	*H. laevigatum*
40H	Canada	08/13/03	*H. laevigatum*
44H	Canada	08/13/03	*H. laevigatum*
45H	Canada	08/13/03	*H. ampullosporum*
48H	CA	N/A	*H. ampullosporum*
81H	CA	N/A	*H. ampullosporum*
82H	CA	N/A	*H. ampullosporum*
83H	CA	N/A	*H. ampullosporum*
66H	CA	12/04/04	*H. ampullosporum*
03-47	Estonia	N/A	*H. microspermus* I
05-79	Italy	N/A	*H. microspermus* I
05-94	Italy	N/A	*H. microspermus* I
52H	CA	03/04	*H. microspermus* I
80H	CA	N/A	*H. microspermus* I
91H	CA	12/23/04	*H. microspermus* I
112H	OR	11/12/05	*H. microspermus* I
126H	OR	11/12/05	*H. microspermus* I
141H	OR	10/22/05	*H. microspermus* I
142H	OR	10/19/05	*H. microspermus* I
161H	CA	02/06	*H. microspermus* I
169H	CA	02/06	*H. microspermus* I
170H	CA	12/05	*H. microspermus* I
172H	CA	12/05	*H. microspermus* I
104H	CA	11/12/05	*H. microspermus* II
168H	CA	02/06	*H. microspermus* II
174H	CA	12/05	*H. microspermus* II
05-68	Estonia	N/A	*H. chrysospermus* III
05-80	Latvia	N/A	*H. chrysospermus* III
05-105	Estonia	N/A	*H. chrysospermus* III
05-112	Finland	N/A	*H. chrysospermus* III
4H	CA	01/29/03	*H. chrysospermus* III
55XH	CA	11/18/04	*H. chrysospermus* III
96H	CA	12/23/04	*H. chrysospermus* III
135H	CA	11/12/05	*H. chrysospermus* III
171H	CA	12/05	*H. chrysospermus* III
173H	CA	12/05	*H. chrysospermus* III
05-95	Estonia	N/A	*H. chrysospermus* IV
05-109	Estonia	N/A	*H. chrysospermus* IV
14H	CA	01/31/03	*H. chrysospermus* IV
20H	CA	01/31/03	*H. chrysospermus* IV
60H	CA	11/26/04	*H. chrysospermus* IV
102H	CA	12/23/04	*H. chrysospermus* IV
106H	CA	11/12/05	*H. chrysospermus* IV
132H	CA	11/12/05	*H. chrysospermus* IV

Table 2.2. Primers and melting temperatures for each primer that was used or developed during the course of this study.

Gene/Locus	Primer	Sequence (5'-3')	Direction	Tm°C	Source
ITS	ITS1-f	CTTGGTCATTTAGAGGAAGTAA	F	49.7	Gardes & Bruns, 1993
	ITS-4	TCCTCCGCTTATTGATATGC	R	58.0	White et al., 1990
EF-1α	EF-728F	CATCGAGAAGTTCGAGAAGG	F	58.0	Carbone & Kohn, 1999
	EF1986R	TACTTGAAGGAACCCTTACC	R	58.0	Carbone & Kohn, 1999
Actin	Act512F	ATGTGCAAGGCCGGTTTCGC	F	64.5	Carbone & Kohn, 1999
	Act783R	TACGAGTCCTTCTGGCCCAT	R	62.4	Carbone & Kohn, 1999
18S	SR1R	TACCTGGTTGATQCTGCCAGT	F	57.1	Vilgalys*
	NS4	CTTCCGTCAATTCCTTTAAG	R	56.0	White et al., 1990
28S	LR0R	GTACCCGCTGAACTTAAGC	F	52.4	Vilgalys and Sun, 1994
	LR7	TACTACCACCAAGATCT	R	45.6	Vilgalys and Sun, 1994
β-tubulin	T12	TAACAACTGCTGGGCCAAGGGTCAC	F	63.3	O'Donnell and Cigelnik, 1997
	T22	TCTGGATGTTGTTGGGAATCC	R	54.9	O'Donnell and Cigelnik, 1997
RPB1	CRPB1A	CAYCCWGGYTTYATCAAGAA	F	51.3	Castlebury et al., 2004
	RPB1Cr	CCNGCDATNTCRTTRTCCATRTA	R	54.2	Castlebury et al., 2004
	HRPBIguFb	TGGAGATTGTCTGYCACAAC	F	53.8	This Study
	HRPBIguRb	CATGTATGTCGCCACATGGT	R	55.3	This Study
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	F	51.9	Liu et al., 1999
	fRPB2-7cR	CCCATRGCTTGTYYRCCCAT	R	59.1	Liu et al., 1999
	HRPBIIguFa	ATTTGCCTCGACCTTGTCAC	F	55.5	This Study
	HRPBIIguRc	GCGCARATRCCMAGAATCAT	R	54.8	This Study

*Unpublished [http://www.biology.duke.edu/fungi/mycolab/primers.htm]

Table 2.3. Loci used in this study. Successfully sequenced loci are designated with an 'X' for the corresponding locus and isolate. Isolates with an 'X' for 'Concat.' were included in the concatenated multigene analysis.

Isolate	18S	28S	Actin	B-tubulin	ITS	RPB1	RPB2	Concat.
S15	X	X	X	X	X	X	X	x
S29	X	X	X	X	X	X	X	x
S32	X	X	X		X	X	X	x
S47	X	X	X	X	X	X	X	x
S50		X		X		X	X	x
S68	X	X	X	X	X	X	X	x
UAMH6602	X	X		X			X	x
UAMH6612	X	X		X			X	x
UAMH6604	X	X		X			X	x
ATCC28784				X			X	
NBRC10533	X	X				X	X	x
NBRC10533	X							
03-47	X	X	X	X	X	X	X	x
05-68	X	X	X	X	X	X	X	x
05-79	X		X	X	X	X	X	x
05-80	X		X	X	X	X	X	x
05-94	X	X	X	X	X	X	X	x
05-95	X		X	X	X	X	X	x
05-105	X	X	X	X	X	X	X	x
05-112	X	X	X	X	X	X	X	x
05-109	X	X	X	X	X	X	X	x
4H	X	X	X	X	X	X	X	x
14H	X	X	X		X	X	X	x
20H	X	X	X		X		X	x
28H	X	X	X	X	X	X	X	x
40H	X	X	X		X	X	X	x
44H	X		X	X	X	X	X	x
45H	X	X	X	X	X	X	X	x
48H	X	X	X	X	X	X	X	x
52H			X	X	X	X	X	x
55xH	X	X	X	X	X	X	X	x
60H	X	X	X	X	X	X	X	x
66H		X	X	X	X	X	X	x
80H	X	X	X	X	X	X	X	x
81H			X		X			
82H	X		X	X	X	X	X	x
83H	X		X	X	X	X	X	x
91H		X	X	X	X	X	X	x
96H	X	X	X	X	X	X	X	x
102H	X	X	X	X	X	X	X	x
104H	X	X	X	X	X	X	X	x
106H	X	X	X	X	X	X	X	x
112H	X	X	X	X	X	X	X	x
126H	X	X	X	X	X		X	x
132H	X	X	X	X	X	X	X	x
135H	X		X	X	X	X	X	x
141H	X	X	X	X	X	X	X	x
142H	X	X	X	X	X	X	X	x
167H	X	X	X	X	X	X	X	x
168H	X	X	X	X	X	X	X	x
169H	X	X	X	X	X	X	X	x
170H	X	X	X	X	X	X	X	x
171H	X		X	X	X		X	x
172H	X							

Fig 2.1. Maximum Parsimony tree showing one of 1536 most parsimonious trees based on the analysis of the 18s rDNA locus. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces boletiphagus* was used as the outgroup.

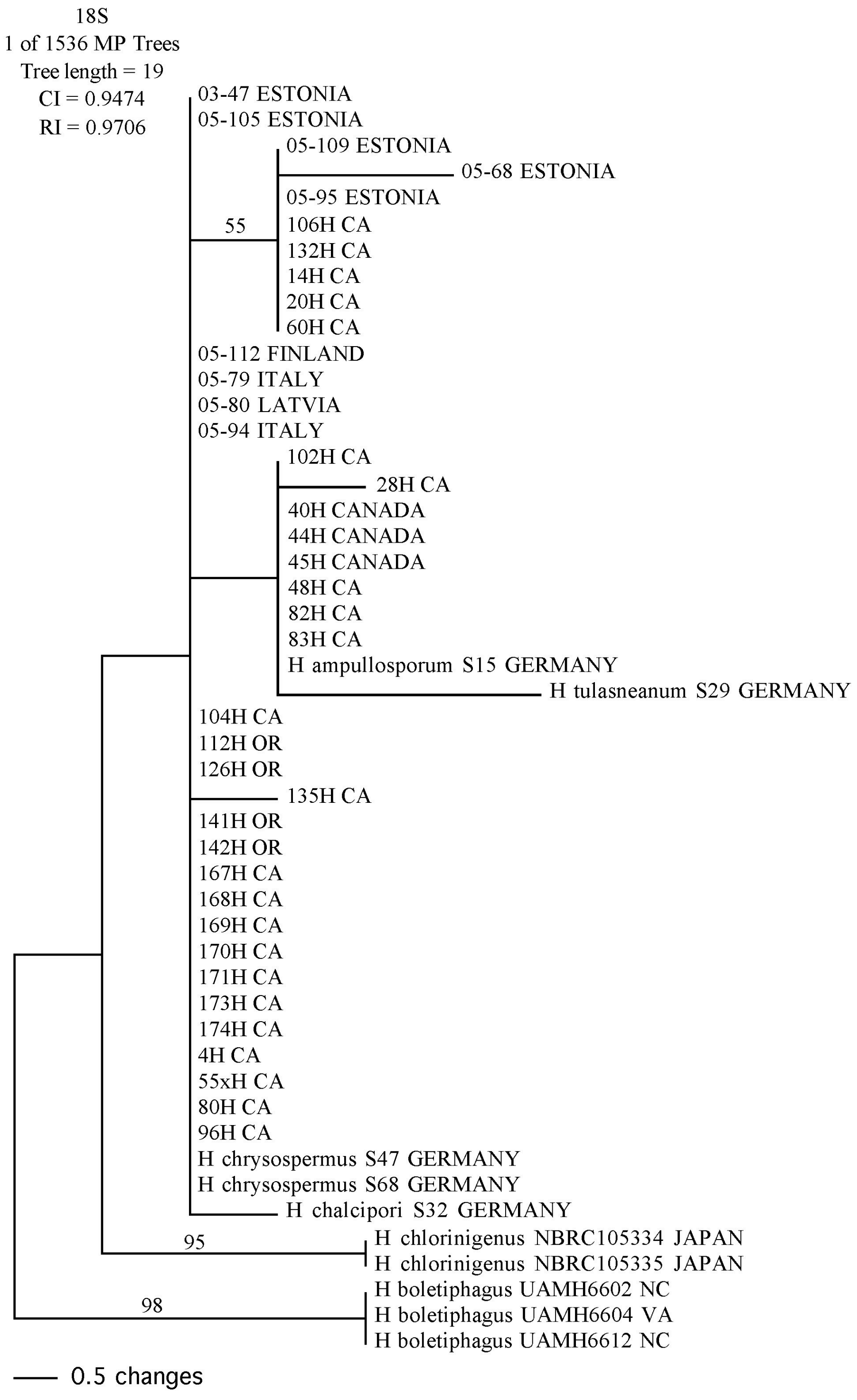

18S
1 of 1536 MP Trees
Tree length = 19
CI = 0.9474
RI = 0.9706
03-47 ESTONIA
05-105 ESTONIA
05-109 ESTONIA
05-68 ESTONIA
05-95 ESTONIA
55
106H CA
132H CA
14H CA
20H CA
60H CA
05-112 FINLAND
05-79 ITALY
05-80 LATVIA
05-94 ITALY
102H CA
28H CA
40H CANADA
44H CANADA
45H CANADA
48H CA
82H CA
83H CA
H ampullosporum S15 GERMANY
H tulasneanum S29 GERMANY
104H CA
112H OR
126H OR
135H CA
141H OR
142H OR
167H CA
168H CA
169H CA
170H CA
171H CA
173H CA
174H CA
4H CA
55xH CA
80H CA
96H CA
H chrysospermus S47 GERMANY
H chrysospermus S68 GERMANY
H chalcipori S32 GERMANY
95
H chlorinigenus NBRC105334 JAPAN
H chlorinigenus NBRC105335 JAPAN
98
H boletiphagus UAMH6602 NC
H boletiphagus UAMH6604 VA
H boletiphagus UAMH6612 NC
0.5 changes

Fig 2.2. Maximum Parsimony tree showing one of 150 most parsimonious trees based on the analysis of the 28s rDNA locus. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces boletiphagus* was used as the outgroup.

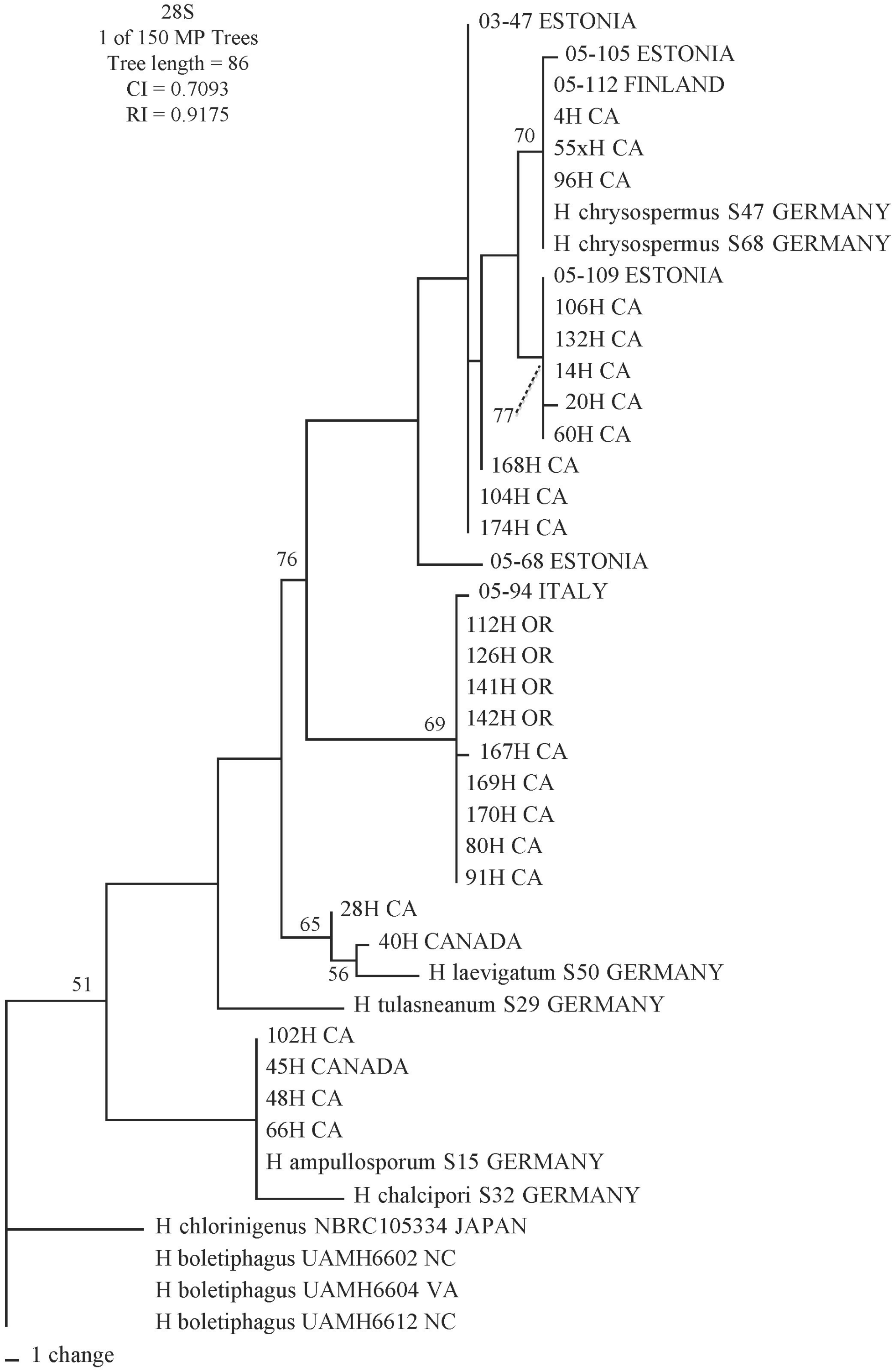

28S
1 of 150 MP Trees
Tree length = 86
CI = 0.7093
RI = 0.9175
03-47 ESTONIA
05-105 ESTONIA
05-112 FINLAND
4H CA
70
55xH CA
96H CA
H chrysospermus S47 GERMANY
H chrysospermus S68 GERMANY
05-109 ESTONIA
106H CA
132H CA
14H CA
20H CA
77
60H CA
168H CA
104H CA
174H CA
76
05-68 ESTONIA
05-94 ITALY
112H OR
126H OR
141H OR
142H OR
69
167H CA
169H CA
170H CA
80H CA
91H CA
28H CA
65
40H CANADA
56
H laevigatum S50 GERMANY
51
H tulasneanum S29 GERMANY
102H CA
45H CANADA
48H CA
66H CA
H ampullosporum S15 GERMANY
H chalcipori S32 GERMANY
H chlorinigenus NBRC105334 JAPAN
H boletiphagus UAMH6602 NC
H boletiphagus UAMH6604 VA
H boletiphagus UAMH6612 NC
1 change

Fig 2.3. Maximum Parsimony tree showing one of 6 most parsimonious trees based on the analysis of the Actin locus. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces chalcipori* was used as the outgroup.

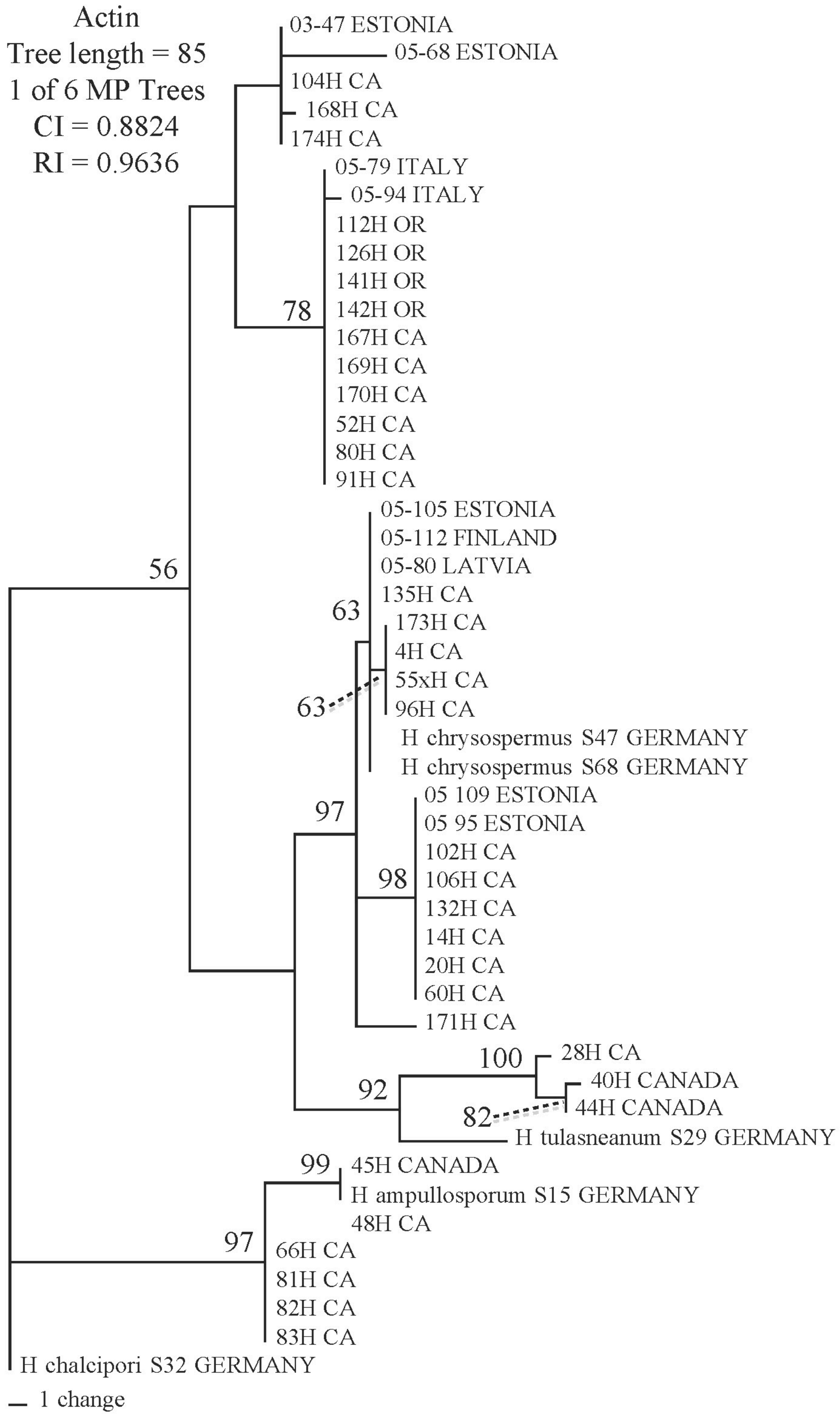

Actin
Tree length = 85
1 of 6 MP Trees
CI = 0.8824
RI = 0.9636
03-47 ESTONIA
05-68 ESTONIA
104H CA
168H CA
174H CA
05-79 ITALY
05-94 ITALY
112H OR
126H OR
141H OR
142H OR
167H CA
169H CA
170H CA
52H CA
80H CA
91H CA
78
56
05-105 ESTONIA
05-112 FINLAND
05-80 LATVIA
135H CA
63
173H CA
4H CA
55xH CA
63
96H CA
H chrysospermus S47 GERMANY
H chrysospermus S68 GERMANY
97
05 109 ESTONIA
05 95 ESTONIA
102H CA
106H CA
98
132H CA
14H CA
20H CA
60H CA
171H CA
100
28H CA
40H CANADA
92
44H CANADA
82
H tulasneanum S29 GERMANY
99
45H CANADA
H ampullosporum S15 GERMANY
48H CA
97
66H CA
81H CA
82H CA
83H CA
H chalcipori S32 GERMANY
1 change

Fig 2.4. Maximum Parsimony tree showing one of 16 most parsimonious trees based on the analysis of the β-tubulin locus. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces boletiphagus* was used as the outgroup.

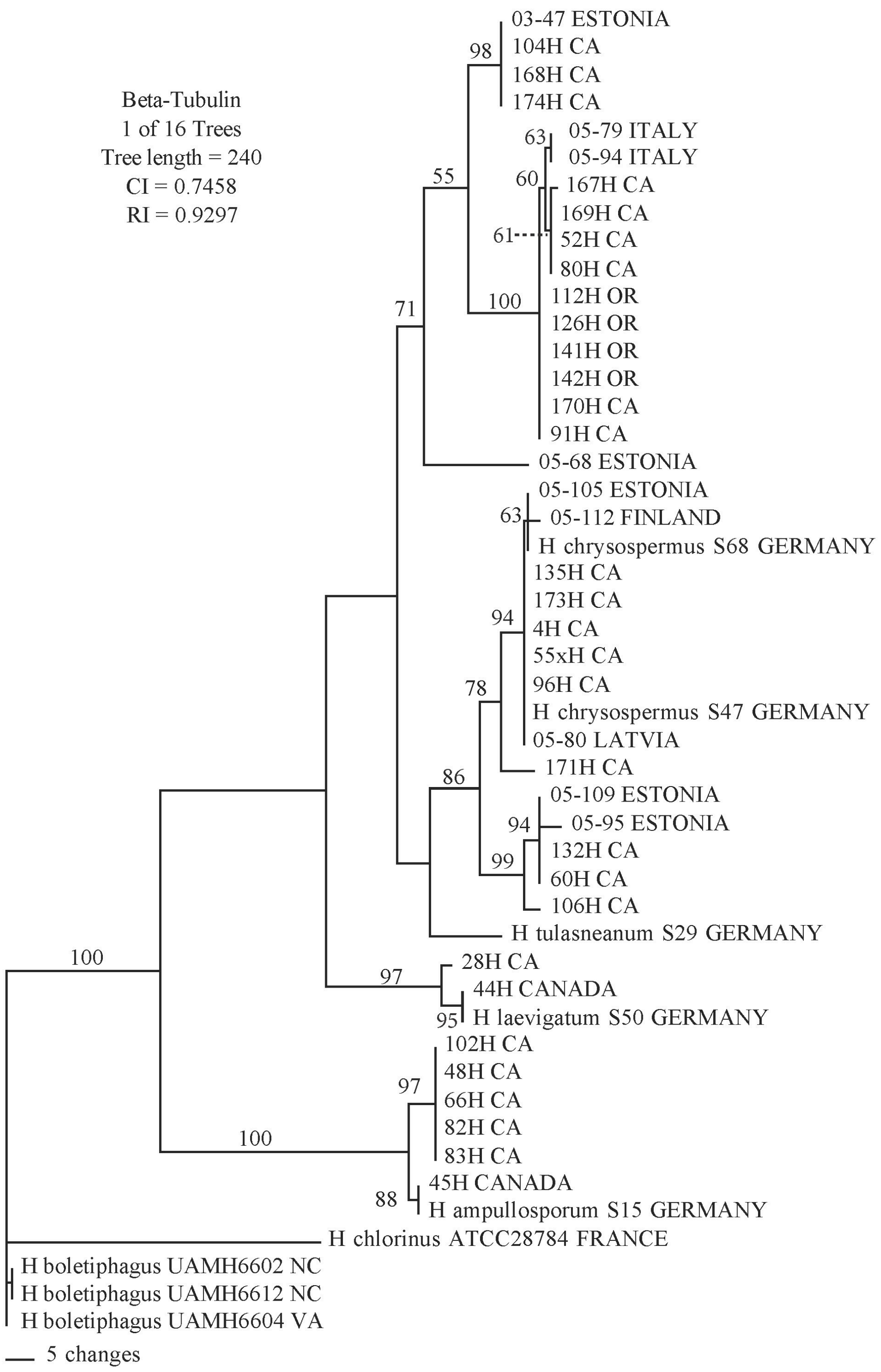

Beta-Tubulin
1 of 16 Trees
Tree length = 240
CI = 0.7458
RI = 0.9297
98
03-47 ESTONIA
104H CA
168H CA
174H CA
63
05-79 ITALY
05-94 ITALY
60
167H CA
169H CA
61
52H CA
80H CA
55
100
112H OR
126H OR
141H OR
142H OR
170H CA
91H CA
71
05-68 ESTONIA
05-105 ESTONIA
63
05-112 FINLAND
H chrysospermus S68 GERMANY
135H CA
173H CA
94
4H CA
55xH CA
96H CA
78
H chrysospermus S47 GERMANY
05-80 LATVIA
171H CA
86
05-109 ESTONIA
94
05-95 ESTONIA
132H CA
99
60H CA
106H CA
H tulasneanum S29 GERMANY
100
28H CA
97
44H CANADA
95
H laevigatum S50 GERMANY
102H CA
48H CA
97
66H CA
82H CA
100
83H CA
45H CANADA
88
H ampullosporum S15 GERMANY
H chlorinus ATCC28784 FRANCE
H boletiphagus UAMH6602 NC
H boletiphagus UAMH6612 NC
H boletiphagus UAMH6604 VA
5 changes

Fig 2.5. Maximum Parsimony tree showing one of 41 most parsimonious trees based on the analysis of the ITS rDNA locus. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces chalcipori* was used as the outgroup.

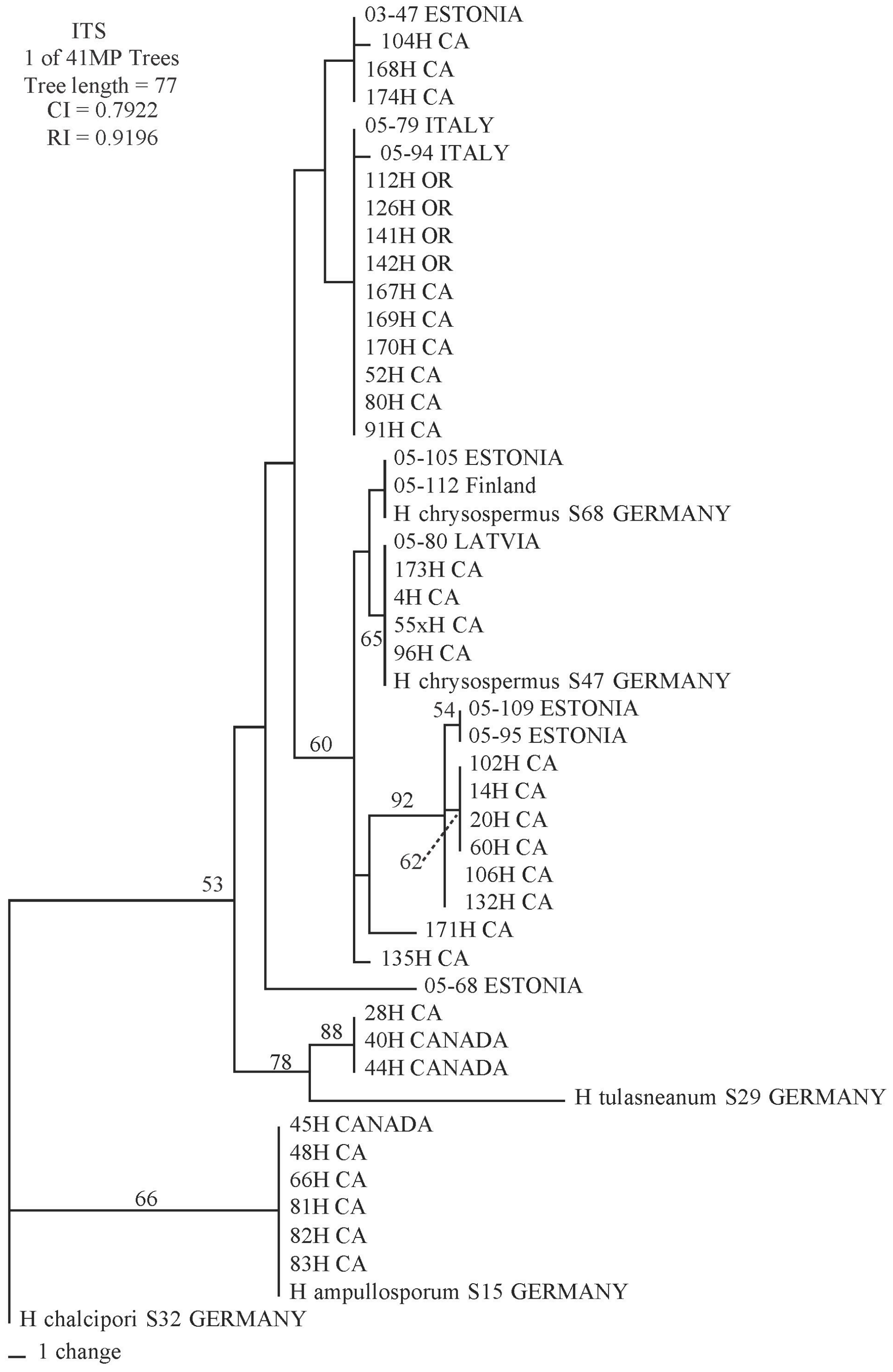

ITS
1 of 41MP Trees
Tree length = 77
CI = 0.7922
RI = 0.9196
03-47 ESTONIA
104H CA
168H CA
174H CA
05-79 ITALY
05-94 ITALY
112H OR
126H OR
141H OR
142H OR
167H CA
169H CA
170H CA
52H CA
80H CA
91H CA
05-105 ESTONIA
05-112 Finland
H chrysospermus S68 GERMANY
05-80 LATVIA
173H CA
4H CA
55xH CA
96H CA
65
H chrysospermus S47 GERMANY
54
05-109 ESTONIA
05-95 ESTONIA
60
102H CA
14H CA
92
20H CA
60H CA
62
106H CA
132H CA
53
171H CA
135H CA
05-68 ESTONIA
28H CA
88
40H CANADA
78
44H CANADA
H tulasneanum S29 GERMANY
45H CANADA
48H CA
66H CA
66
81H CA
82H CA
83H CA
H ampullosporum S15 GERMANY
H chalcipori S32 GERMANY
1 change

Fig 2.6. Maximum Parsimony tree showing one of 3 most parsimonious trees based on the analysis of the RNA polymerase II subunit RPB1 locus. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces chalcipori* was used as the outgroup.

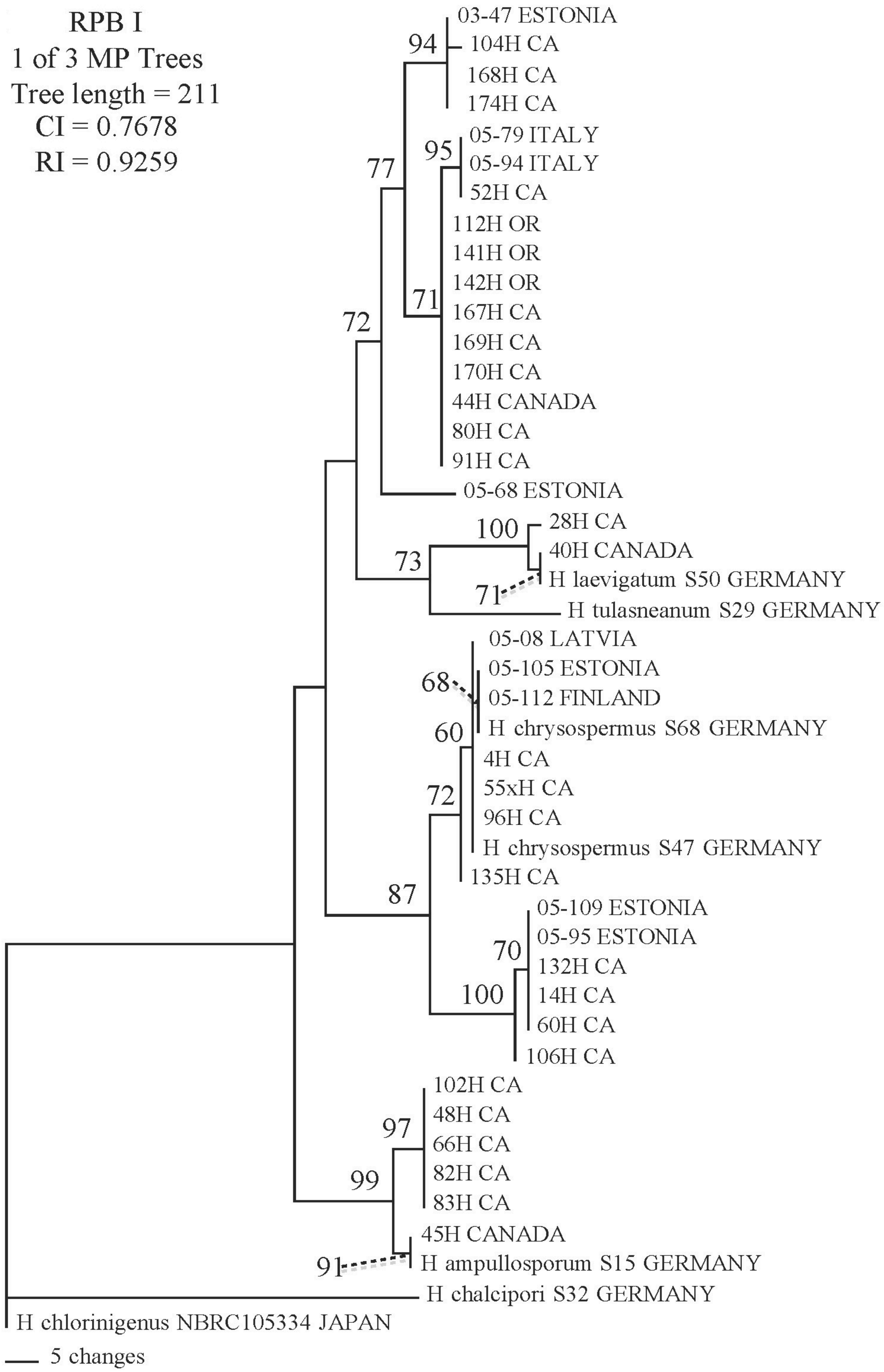

RPB I
1 of 3 MP Trees
Tree length = 211
CI = 0.7678
RI = 0.9259
03-47 ESTONIA
104H CA
168H CA
174H CA
05-79 ITALY
05-94 ITALY
52H CA
112H OR
141H OR
142H OR
167H CA
169H CA
170H CA
44H CANADA
80H CA
91H CA
05-68 ESTONIA
28H CA
40H CANADA
H laevigatum S50 GERMANY
H tulasneanum S29 GERMANY
05-08 LATVIA
05-105 ESTONIA
05-112 FINLAND
H chrysospermus S68 GERMANY
4H CA
55xH CA
96H CA
H chrysospermus S47 GERMANY
135H CA
05-109 ESTONIA
05-95 ESTONIA
132H CA
14H CA
60H CA
106H CA
102H CA
48H CA
66H CA
82H CA
83H CA
45H CANADA
H ampullosporum S15 GERMANY
H chalcipori S32 GERMANY
H chlorinigenus NBRC105334 JAPAN
5 changes
94
95
77
71
72
100
73
71
68
60
72
87
70
100
97
99
91

Fig 2.7. Maximum Parsimony tree showing the only resulting tree based on the analysis of the RNA polymerase II subunit RPB2 locus. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces boletiphagus* was used as the outgroup.

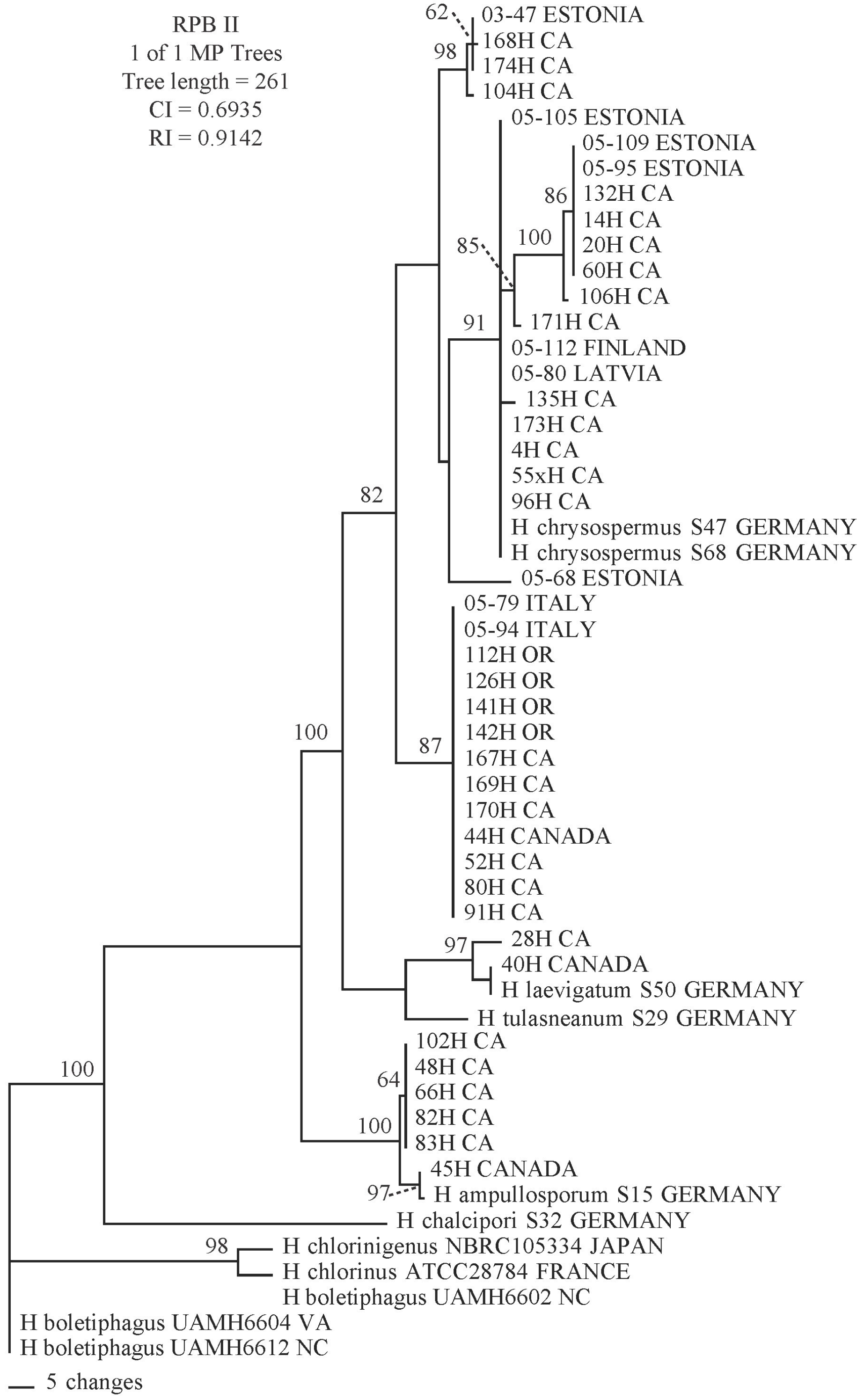
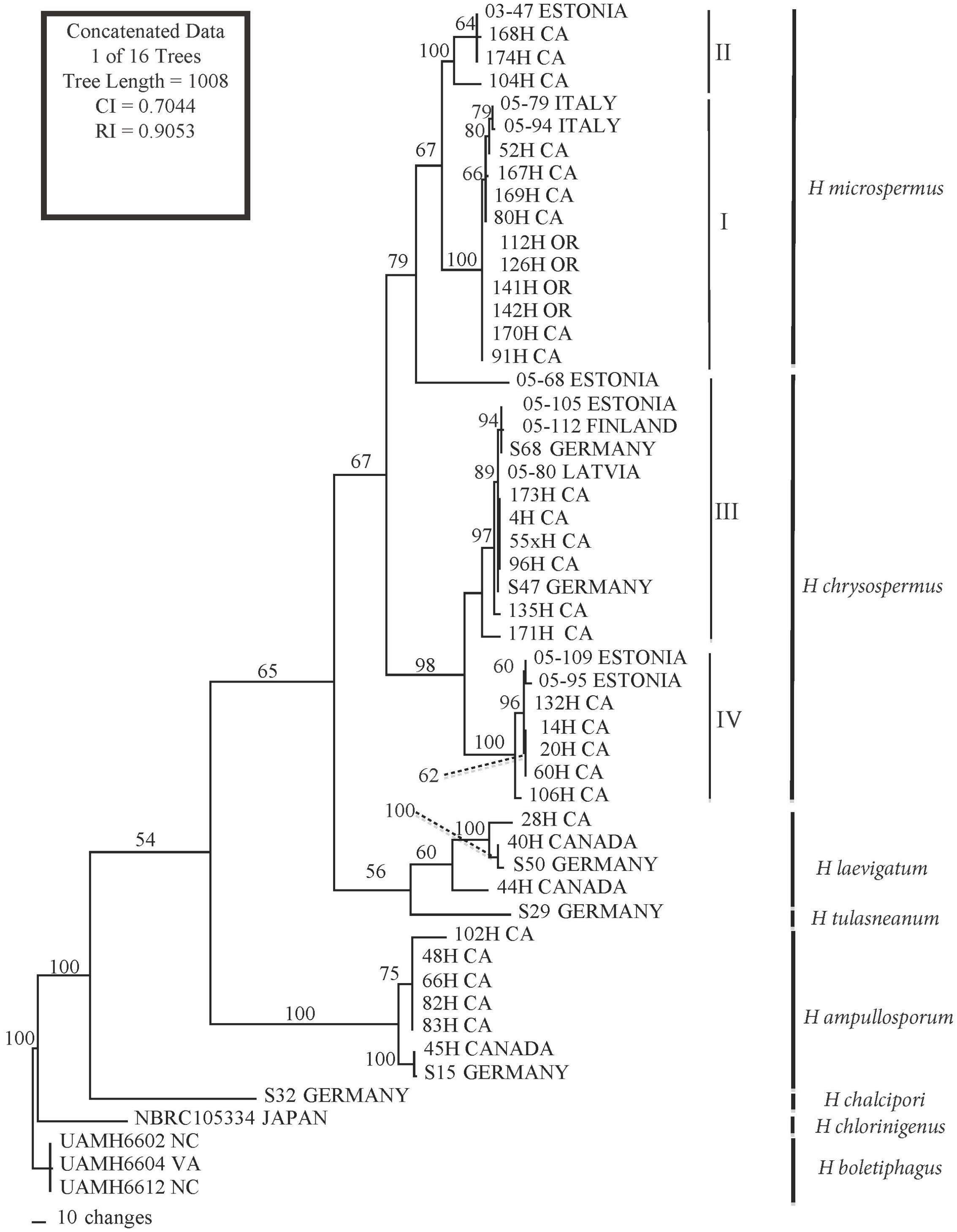

RPB II
1 of 1 MP Trees
Tree length = 261
CI = 0.6935
RI = 0.9142
62
98
03-47 ESTONIA
168H CA
174H CA
104H CA
05-105 ESTONIA
05-109 ESTONIA
05-95 ESTONIA
86
132H CA
14H CA
100
20H CA
85
60H CA
106H CA
171H CA
91
05-112 FINLAND
05-80 LATVIA
135H CA
173H CA
4H CA
55xH CA
96H CA
82
H chrysospermus S47 GERMANY
H chrysospermus S68 GERMANY
05-68 ESTONIA
05-79 ITALY
05-94 ITALY
112H OR
126H OR
141H OR
142H OR
100
87
167H CA
169H CA
170H CA
44H CANADA
52H CA
80H CA
91H CA
97
28H CA
40H CANADA
H laevigatum S50 GERMANY
H tulasneanum S29 GERMANY
102H CA
100
48H CA
64
66H CA
82H CA
100
83H CA
45H CANADA
97
H ampullosporum S15 GERMANY
H chalcipori S32 GERMANY
98
H chlorinigenus NBRC105334 JAPAN
H chlorinus ATCC28784 FRANCE
H boletiphagus UAMH6602 NC
H boletiphagus UAMH6604 VA
H boletiphagus UAMH6612 NC
5 changes

Fig 2.8. Maximum Parsimony tree showing one of 16 most parsimonious trees based on the analysis of the concatenated multigene dataset. The analysis was conducted using the heuristic search procedure with 1,000 random and stepwise additive replicates using PAUP* version 4.0 beta 10 for Macintosh (Swofford 2002). Confidence in tree topology was examined using bootstrap with 10,000 replicates under the fast-heuristic search option. Only bootstrap support values above 50% are shown and *Hypomyces chlorinigenus* was used as the outgroup.


Concatenated Data
1 of 16 Trees
Tree Length = 1008
CI = 0.7044
RI = 0.9053
64
03-47 ESTONIA
168H CA
100
174H CA
104H CA
II
79
05-79 ITALY
80
05-94 ITALY
67
52H CA
66
167H CA
169H CA
80H CA
I
H microspermus
112H OR
79
100
126H OR
141H OR
142H OR
170H CA
91H CA
05-68 ESTONIA
05-105 ESTONIA
94
05-112 FINLAND
S68 GERMANY
67
89
05-80 LATVIA
173H CA
4H CA
III
97
55xH CA
96H CA
S47 GERMANY
H chrysospermus
135H CA
171H CA
98
05-109 ESTONIA
65
60
05-95 ESTONIA
96
132H CA
14H CA
IV
100
20H CA
62
60H CA
106H CA
100
28H CA
100
40H CANADA
54
60
S50 GERMANY
H laevigatum
56
44H CANADA
S29 GERMANY
H tulasneanum
102H CA
100
48H CA
75
66H CA
82H CA
100
83H CA
H ampullosporum
100
45H CANADA
100
S15 GERMANY
S32 GERMANY
H chalcipori
NBRC105334 JAPAN
H chlorinigenus
UAMH6602 NC
UAMH6604 VA
H boletiphagus
UAMH6612 NC
10 changes