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A multilocus phylogeny of Hericium fungi in Canada and their production of Erinacine A

Julien Koga,

Supervisor: Thorn, R. Greg, *The University of Western Ontario*
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Abstract

The genus *Hericium* contains species demonstrated to produce bioactive secondary metabolites with potential medical applications. Previous phylogenies of the genus *Hericium* based on the internal transcribed spacer (ITS) region struggle to support monophyly at the species level, hindering biochemical research efforts. Using concatenated sequence data from ITS, the large ribosomal subunit (LSU), translation elongation factor 1-alpha (*tef1*) and RNA polymerase subunit 2 (*rpb2*) genes, *Hericium* species from North America and key taxa from Europe were resolved as monophyletic groups. Several species of *Hericium* from North America also demonstrated production of erinacine A, a secondary metabolite with nerve-growth factor stimulatory activity, and liquid culture conditions were modified for enhanced erinacine A biosynthesis in each species. Together with clear distinction of the species in *Hericium*, the findings presented herein will contribute to natural products research and medical applications of these edible and medicinally valuable mushrooms.

Keywords: *Hericium*, phylogenetics, systematics, biogeography, erinacine A, liquid culture

Summary for Lay Audience

Hericium is a genus of fungi that produce edible fruit bodies (mushrooms) with a history of use in East Asian medical traditions. Evidence of their beneficial health effects has increased the demand for the cultivated species, *H. erinaceus,* known in Europe and North America as the lion's mane mushroom. However, prior works have shown that there are uncertainties in the correct names used for species in the genus and how these species are related to each other.

To better understand the evolutionary relationships in *Hericium*, I analyzed data from several genes of various *Hericium* species. The results of this analysis were significantly more informative of species boundaries than previous studies that used only a single gene. I also found that a species previously known as *H. erinaceus* is actually four distinct species.

Hericium mushrooms are considered medicinal mushrooms as these fungi are sources of bioactive compounds. One of the compounds produced by *Hericium*, erinacine A, is being considered as a potential therapeutic tool in neurological diseases. However, the production of erinacine A is only known from a few *Hericium* species. In this study, I found evidence of erinacine A production in several species of which erinacine A production was previously unknown. I also aimed to maximize erinacine A production in each species. This study provides evidence that several *Hericium* species may be used as sources for erinacine A in medical research and describes methods to optimize its production in those species.

Co-Authorship Statements

This project was conceptualized and developed with input from Dr. R. Greg Thorn, Dr. Mark Bernards, and industrial sponsors Tyler Watson and Murray Good of WhiteCrest Innovations Inc. Chapter 1 was written by me and edited by R.G. Thorn and M. Bernards. A version of Chapter 2 has been submitted for publication to the Canadian Journal of Microbiology titled "A multilocus phylogeny of *Hericium* in Canada" (CJM-2023-0231) with coauthors R.G. Thorn and Dr. Ewald Langer of the University of Kassel, Kassel, Germany. The manuscript was conceptualized by myself and R.G. Thorn, investigation conducted by myself and E. Langer. R.G. Thorn conducted the data curation, formal analysis, visualization, and provided funding for the research. Writing, editing, and reviewing was conducted by myself, R.G. Thorn, and E. Langer. Chapters 3 and 4 were written by me and edited by R.G. Thorn and M. Bernards.

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List of Abbreviations

ANOVA	One way analysis of variance
bss	Bootstrap support
DAOM	Canadian National Mycological Herbarium
DAOMC	Canadian Collection of Fungal Cultures
DNA	Deoxyribonucleic acid
EIC	Extracted ion chromatogram
GYE	Glucose yeast extract
HPLC	High performance liquid chromatography
ITS	Internal transcribed spacer
LSU	Large subunit
ME	Malt extract
MS	Mass spectrometry
NGF	Nerve growth factor
RNA	Ribonucleic acid
rpm	Revolutions per minute
SLCF	Submerged liquid culture fermentation
m/z	Mass-to-charge ratio
rDNA	Ribosomal deoxyribonucleic acid
rpb2	RNA polymerase subunit II
tef1	Translation elongation factor 1-alpha

List of Terminology

Applanate	Flattened
Basionym	Original name
Context	The flesh of the fruit body
Dimidiate	Fan-shaped
Epitype	The specimen or illustration to serve as the type when the holotype, lectotype, or neotype is ambiguous or cannot be critically evaluated
Hispid	Covered by stiff hairs, bristles, or short spines
Imbricate	Shelving, tiled
Holotype	The type specimen designated at the time of publication
Lectotype	The specimen or illustration to serve as the type when no holotype was designated at the time of publication
Neotype	The specimen or illustration to serve as the type when all material on which a name is based is missing
sensu auct.	*Sensu auctorum*; In the sense of the authors
sp. nov.	*Species nova*; A new species
Terete	Approximately cylindrical with tapering ends
Triquetrous	Having three angles

Chapter 1

1.1 An Overview of *Hericium* Diversity

1.1.1 Characteristics of *Hericium* Mushrooms

Members of the genus *Hericium* Pers. are wood-decomposing fungi (phylum Basidiomycota) that produce large, white fruit bodies (mushrooms) with hanging toothy spines covered by a fertile hymenial surface. Species of *Hericium* are distributed throughout the world on every continent excluding Antarctica and, although morphology is variable across the genus, are readily recognized in nature by their charismatic fruit bodies. Fruit bodies are produced annually on dead wood (in some species, on dead heartwood of living trees) and vary from fleshy to brittle or pliant, shelving to hemispherical or intricately branched, with spines mostly hanging down (positively geotropic) (Banker 1906; Das et al. 2011). Tissues of the fruit bodies, including the hymenium, turn blue in reaction to iodine (are amyloid) and contain swollen, oil-filled conducting cells called gloeocystidia (Harrison 1973; Ginns 1985). Basidiospores are smooth or covered in warts, white, amyloid, subglobose to oblong, and vary in shape and size depending on the species (Banker 1906; Das et al. 2011). *Hericium* fungi are generally considered to be saprotrophs and they are commonly found on hardwoods and, to a lesser extent, softwoods, decomposing lignocellulosic substrates to cause a white rot (Wolters et al. 2015). In certain instances where fruit bodies emerge from old dying trees, *Hericium* may be considered weakly parasitic (Banker 1906; Larsson and Larsson 2003). Mycelial cultures of *Hericium* grow in concentrically zoned mats of tissue that are

cottony, felty, or downy in texture, with clamp connections and variably ornamented gloeocystidia (Ginns 1985; Das et al. 2011; Jumbam et al. 2019).

1.1.2 Conservation Status

In temperate climates, *Hericium* fruits in the summer and fall seasons, and their occurrence depends on the availability of substrates as old trees and fallen logs (Ouali et al. 2020; Kujawska et al. 2021). The presence of *Hericium* may therefore be used as an indicator of natural old-growth forests where an abundance of woody material associated with these forests support the growth of *Hericium* mushrooms (Christensen et al. 2005; Kujawska et al. 2021). Two species of *Hericium* have been officially evaluated by the IUCN Red List: *H. erinaceus* and *H. rajchenbergii*, both of which are decreasing globally due to habitat loss and degradation and are listed as least concern and critically endangered, respectively (Kałucka and Ibarguren 2019; Robledo et al. 2020). Beyond these two species, regional assessments of fungal community composition and population integrity have raised concerns over the conservation status of other *Hericium* species in some countries. Smith et al. (2016) used available population data for regional *Hericium* spp. in the U.K. to generate an unofficial red list which includes *H. cirrhatum* (vulnerable), *H. coralloides* (endangered), and *H. erinaceus* (vulnerable), under the IUCN Criterion D: very small or restricted population (IUCN Red List Categories and Criteria, 2014). Elsewhere, *Hericium erinaceus* is protected by law in Croatia, Hungary, Poland, Serbia, Slovenia, the U.K., and Sweden, and is red listed at a regional scale in thirteen European countries (Thongbai et al. 2015; Kałucka and Ibarguren 2019). Beyond the official IUCN Red List that includes *H. erinaceus* and *H. rajchenbergii*, no such regional

red lists exist that detail the conservation status of *Hericium* in Canada, where five species are known to occur (Harrison 1973; Harrison 1984; Ginns 1985).

1.1.3 Diversity

The disproportionate concern over the conservation of *H. erinaceus* relative to other species of *Hericium* is largely due to its status as a culinary and medicinal mushroom in various traditions, which has made *H. erinaceus* the most widely known species of *Hericium* to the general public. Approximately seventeen species of *Hericium* are reported globally. In North America, five species are known: *H. abietis* (Weir ex Hubert) K.A. Harrison*, H. americanum* Ginns, *H. cirrhatum* (Pers.) Nikol., *H. coralloides* (Scop.) Pers., and *H. erinaceus* (www.indexfungorum.com; Harrison 1973; Ginns 1985). A summary of the key features of these North American species is presented in Table 1-1.

Table 1-1. Summary of characteristics of North American *Hericium* species.

	Distribution	Substrate	Morphological features
H. erinaceus	Widespread in North America; Southern U.S.A (Florida) to Ontario.	Dead hardwood; rarely living hardwood.	Fruit body unbranched, covered in long fleshy spines (up to 4 cm); spores 5.5 – 6.8 x 4.5 – 5.6 µm.
H. coralloides	Widespread in North America; Alaska to Nova Scotia down to Michigan and California.	Dead hardwood.	Fruit body openly branched; spines < 1 cm; spores 3.5-5 x 2.8-5 µm.
H. cirrhatum	Boreal and montane regions of western North America.	Dead hardwood, particularly *Populus.*	Fruit body shelving, triquetrous to applanate; lower surface covered with hanging spines; spores 4.0–4.5 (–4.8) × 3.2–3.5 (–4.0) µm.
H. americanum	Northeast North America; east of the Great Plains up to Nova Scotia in the north and Tennessee/North Carolina in the south.	Dead hardwood.	Fruit body tightly branched, fleshy branches arising from common base covered in long hanging spines (>1 cm); spores 4.5 – 6.5 x 4.5 – 6.0 µm.
H. abietis	Western North America; Alaska to California.	Dead conifers.	Fruit body tightly branched, fleshy branches arising from common base; covered in hanging spines (>1 cm); spores 4.5-5 x 4-4.5 µm.

Harrison (1973, 1984); Hallenberg et al. (2013); Kuo (2022); Baroni (2017)

Hericium fruit bodies are difficult to identify to species due to subtle differences in key morphological characteristics (spine length, orientation, spore size and shape) in different stages of development, leading to errors in identification of specimens which has contributed to blurred species delineation based on morphology alone. Identification of young fruit bodies to species based on morphology is particularly difficult for non-

experts due to misinterpretations of underdeveloped spines and the branching pattern in their mature state (Hallenberg et al. 2013). Additional difficulties in identification arise from species-level differences in substrate-specificity, which, together with non-rigorous evaluation of the developmental stage of a sample, often results in incorrect labelling of observations and collections. Because of the familiarity of *H. erinaceus* as a culinary and medicinal mushroom among the general public, lesser known and rarer *Hericium* species are often mislabelled as *H. erinaceus* resulting in misleading overrepresentation of *H. erinaceus* observations made to databases such as iNaturalist.com and as sequence data in GenBank (Hallenberg et al. 2013; Cesaroni et al. 2019). However, even among experts, nomenclatural issues persist due to ambiguous boundaries of species delineation, lack of type specimens, and consequent confusion about application of species names.

1.2 Nomenclatural Concerns

1.2.1 Classification History

The genus *Hericium* was originally described by Persoon (1794) with *Hydnum coralloides* (Scopoli 1772) as the type species for the genus, however no type specimen is available for this species name. As a result, the nomenclatural status of several *Hericium* species, and of *H. coralloides* in particular, has been a subject of considerable debate. In Europe, the name *H. coralloides* has been applied by some authors to a species occurring on hardwoods, particularly beech (*Fagus*) (Harrison 1973; Laessoe and Petersen 2019), and by others to a separate species that occurs on fir (*Abies*) (Maas Geesteranus 1971). The species on *Abies* is referred to as *H. alpestre* (Hallenberg 1983; Hallenberg et al. 2013; Cesaroni et al. 2019) or as *H. flagellum* (Stalpers 1996; Laessoe and Petersen 2019;

Kujawska et al. 2021), the latter another name that has also been applied, perhaps more correctly, to the species on hardwoods (Banker 1906). The species in North America that was previously known as *H. coralloides* (Harrison 1973) is mating-incompatible with *H. coralloides*, and has been redescribed as a separate species, *H. americanum* (Ginns 1984, 1985). Resolving these nomenclatural concerns requires critical evaluation of taxonomic history, which includes the establishment of lectotypes and epitypes for critical collections, in conjunction with a thorough analysis of DNA sequence data.

1.3 Modern Phylogenetics

1.3.1 Limits of the ITS Locus

Analyses of sequence data from the internal transcribed spacer (ITS) region of *Hericium* samples have shown that this locus is extremely similar across *Hericium* species and does not provide the resolving power necessary for satisfying species delineation (Hallenberg et al. 2013; Cesaroni et al. 2019; Jumbam et al. 2019). Phylogenies that have been produced using this locus struggle to support monophyly at the species level and provide evidence of cryptic species in several subclades (Hallenberg et al. 2013; Cesaroni et al. 2019). Sequences of *H. erinaceus* from geographically distinct regions appear in separate clades, suggesting *H. erinaceus* may be a species complex with distinct North American, European, and Asian species (Hallenberg et al. 2013; Cesaroni et al. 2019).

The ITS region has become the standard locus in assignment of taxonomy of fungal rDNA sequences since its designation as the primary fungal barcode by the International Fungal Barcoding Consortium in 2012. The frequency with which ITS is

sequenced and deposited in genomic sequence databases (GenBank) relative to other loci has amplified the power of ITS in assignment of taxonomy of unknown specimens, and it is phylogenetically informative for groups in which ITS is highly variable (Schoch et al. 2012; Xu 2016). For groups in which ITS is highly similar between species, such as *Hericium*, additional molecular markers (sequences from protein coding genes) are necessary for phylogenetic resolution (Matheny et al. 2007). There is a scarcity of such sequences available in GenBank, and most ITS sequences that are available are listed as *H. erinaceus*, limiting the breadth of taxa available for thorough phylogenetic analysis. There is also an abundance of sequences from commercial strains of *H. erinaceus* used in the mushroom cultivation industry, due to the ease of access of mycelial cultures, for which voucher specimens are unknown or unavailable. Sequences of commercial strains of *H. erinaceus* may confound the results of phylogenetic studies as these strains are often the result of selective breeding for the expression of specific phenotypes of fruit bodies, and may be representative of a potential, but not necessarily realized, mating event where strains from different geographic origin are mated (Wang et al. 2018). As a precaution, phylogenetic studies must take into consideration the origin of sequence data and omit any sequences that are generated from strains from industrial sources, which is especially important for resolving the *H. erinaceus* species complex.

The ITS locus is also limited in its resolution of the *H. coralloides* complex. According to ITS, the *H. coralloides* species complex consists of distinct European and North American clades (Hallenberg et al. 2013; Cesaroni et al. 2019; Jumbam et al. 2019). On its own, ITS is too limited in its polymorphisms for robust species delineation and thorough phylogenetic analysis of *Hericium*, particularly with respect to *H. erinaceus*

and *H. coralloides*. To approach a resolved phylogeny wherein the *H. erinaceus* and *H. coralloides* species complexes can be clarified, analysis of sequences from other gene regions would be necessary.

1.3.2 Multilocus Phylogenetic Analysis

In groups where ITS does not sufficiently resolve interspecific relationships, additional molecular markers such as protein-coding genes have been useful in providing greater phylogenetic resolution (Matheny et al. 2007). With the exception of a single study (Jumbam et al. 2019) known to this author in which ITS was combined with the adjacent large subunit (LSU) rDNA gene, which the authors determined to be a phylogenetically uninformative locus, a thorough multi-gene phylogenetic study of *Hericium* has not been conducted. Protein coding genes *rpb2*, which codes for the second largest subunit of RNA polymerase II, and the translation elongation factor 1-alpha coding gene, *tef1*, have been demonstrated to be useful loci for phylogenetic analyses in mushroom fungi (Matheny et al. 2007). While *rpb2* is more variable than *tef1,* the combination of both genes with ITS and LSU contributes to higher resolution at lower taxonomic levels, provided by *rpb2* and ITS, along with recovery of higher order relationships by the more conserved *tef1* and LSU (Matheny et al. 2007). The combination of these loci has displayed sufficient phylogenetic signals to delimit closely related species in other genera such as *Trametes* (Carlson et al. 2014) and *Tolypocladium* (Dong et al. 2022), for which ITS alone provides little resolution due to limited intrageneric variability. A major objective of the present work is to provide a better resolution to the interspecific relationships of *Hericium* species known from North America using multi-gene phylogenetic analysis, and to position these species in relation

to global *Hericium* diversity. Resolving the phylogenetics of *Hericium* is particularly important as *Hericium* mushrooms are sources of medically important compounds, some of which are under clinical investigation. While taxonomic idiosyncrasies are often not of primary importance for researchers in the applied fields of pharmacology, biomedical sciences and in industrial mushroom production generally, precise identification of study organisms is necessary for instances where the production of medically or industrially important biologically active compounds is species-specific (Cesaroni et al. 2019). The uncertain nomenclatural status of *H. coralloides* in Europe and in North America and the delineation of cryptic species within the *H. erinaceus* species complex, both of which have been shown to produce secondary metabolites of research interest, remain as important questions that require an integrated approach using taxonomic history and modern phylogenetics. An improved phylogeny of *Hericium* would also inform the validity of names originally designated for European species in North America. Evidence for the misapplication of names in *Hericium,* should it arise, should be rectified with the rules of the International Code of Nomenclature for algae, fungi, and plants (the Code).

1.3.3 The Importance of Phylogenetics in Applied Contexts

The correct application of species names is paramount to progressing our understanding of fungal biodiversity, evolution, and to define and achieve conservation objectives. In the case of economically important organisms, such as *Hericium* mushrooms, properly defining species delimitations also has economic and medicinal consequences. *Hericium erinaceus* is a mushroom that is commonly used in East Asian medical traditions, such as in traditional Chinese medicine (TCM), and significant efforts have been made over the past thirty years to evaluate the therapeutic properties of these

mushrooms (Kawagishi et al. 1994, 1996a – b; Mori et al. 2008; Li et al. 2020). Vague or poorly defined species concepts can lead to misidentification of specimens used in biomedical and pharmacological studies, where researchers may characterize the biological effects of a specimen known to them by one name, but which is known to others as another. For example, Suruga et al. (2015) compared the biological effects of extracts of *H. erinaceus* to those from a specimen collected in Japan identified as *H. ramosum* and found that the desired bioactivity was greater in the latter than that of the medically esteemed *H. erinaceus*. However, *H. ramosum* is a synonym for *H. coralloides* under the current rules of the Code*,* and both *H. coralloides* and *H. erinaceus* are European names that may apply to different species than are found in Japan. Consistent application of correct names is important in the context of biomedical research and natural products discovery.

1.4 *Hericium* Mushrooms as Medicinal Mushrooms

1.4.1 Traditional Uses

In East Asia, the interest in *Hericium* mushrooms as medicinal and edible mushrooms is a millennium-old phenomenon, where *H. erinaceus,* known as the monkey head mushroom (Houtou) in China and as Yamabushitake in Japan, has been used as both a nutritional and medicinal resource for over one thousand years (Bhandari et al. 2014; Li et al. 2014). The Ming dynasty's Compendium of Materia Medica describe the benefits of *H. erinaceus* to the five internal organs and in aiding with digestion, and several products containing *H. erinaceus* intended to treat gastric conditions have been granted patents by the Chinese Food and Drug Administration (He et al. 2017; Qi et al. 2024). Claims of the

efficacy of *H. erinaceus* in treating gastric conditions in this tradition have received modest support by recent scientific studies where polysaccharides isolated from *H. erinaceus* exhibited protective activity in both *in vitro* and *in vivo* models primarily through antioxidant activity at the epithelial lining of the intestine (Gitter et al. 2001; Jena et al. 2012; Wang et al. 2015, 2019; Chen et al. 2020; Hou et al. 2022). While the traditional medicinal value of *H. erinaceus* has focussed on treating digestive ailments by consumption of fruit bodies, investigation of the bioactivity of both fruit body and mycelial extracts have extended the scope of its medicinal potential to a range of biological conditions beyond the digestive system. Over 80 bioactive secondary metabolites have been identified from both the fruit body and the mycelium; these are classified as polysaccharides, erinapyrones, sterols (erinol, hericerins, hericenes), cyathane diterpenoids (erinacines), and benzaldehyde derivatives (hericenones) (Chen et al. 2017; Corana et al. 2019). The bioactivity of some of these compounds has been documented, with a wide range of effects including anti-inflammatory (Chiu et al. 2018; Wang et al. 2019), antimicrobial (Kim et al. 2000; Wong et al. 2009), anti-cancer (Li et al. 2014), and neuroprotective (Suruga et al. 2015; Chen et al. 2016) properties; the clinical value of several of these metabolites continues to be evaluated. The clinical potential of two classes of compounds, hericenones and erinacines, are of particular interest to pharmacologists and natural products researchers as they have demonstrated potent neuroprotective properties in animal models, human cell lines, and in human subjects themselves (Kawagishi et al. 1991, 1994, 1996a – b; Shimbo et al. 2005; Mori et al. 2008; Rascher et al. 2020; Li et al. 2020).

1.4.2 *Hericium* Metabolites for Neurodegenerative Diseases

The neuroprotective properties of erinacines and hericenones have become the subject of extensive study to medical researchers with interests in natural products discovery as 1) the prevalence of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) is increasing significantly faster than is drug development for such conditions, and 2) few natural products have demonstrated bioactivity in the central nervous system (CNS) of the character and to the degree of erinacines and hericenones (Guan et al. 2023; Aramsirirujiwet et al. 2023). Both AD and PD are associated with decreased levels of nerve growth factor (NGF), a protein that is variously involved in neuronal survival, promotion of neurite outgrowth and differentiation, and maintenance of synaptic connectivity (Chao 2003). Though other physiological processes are involved in the pathogenesis of AD and PD, increasing NGF levels in patients at early stages of neurodegeneration has been of interest to researchers and clinicians due to the neuroprotective function of NGF (Price et al. 2007). However, NGF is a large protein complex composed of α-, β-, and γ-subunits, making it unable to pass from the bloodstream through the blood-brain barrier (BBB) to the CNS to exert the desired effect if administered exogenously. The prohibitively high cost of screening and manufacturing NGF precursor compounds has limited further development of these drugs for clinical trials, and no drug for the treatment of AD has been approved since 2003 (Hung and Fu 2017). This has created a unique situation that has encouraged the search for small natural compounds that act as precursors for NGF synthesis, particularly among medicinal fungi. Both erinacines and hericenones, produced in the mycelium and the fruit body of *Hericium* fungi, respectively, have demonstrated stimulatory activity to NGF

synthesis and are being considered as candidates for the treatment of neurodegenerative diseases (Kawagishi et al. 1991, 1994; Li et al. 2020).

1.5 Erinacines

1.5.1 In Vitro and In Vivo Studies

In the early works by Kawagishi, mouse astroglial cells were incubated in the presence of various hericenones, labelled in the order of discovery as hericenones C, D, E (Kawagishi et al. 1991), or erinacines A, B, C, and later D (Kawagishi et al. 1994; 1996a), E, F, G (Kawagishi et al. 1996b) (Fig. 1-1). The concentration of secreted NGF from these neuronal cells after exposure to these compounds were measured. Erinacine C demonstrated the strongest effect on NGF secretion (299.1 ± 59.6 pg/mL), followed closely by erinacine A (250.1 ± 36.2 pg/mL) and to a lesser extent erinacine F (175 ± 5.4 pg/mL) (Kawagishi et al. 1994, 1996b). By comparison, the NGF secreting properties of the hericenones were significantly lower than those of the erinacines, ranging from 10.8 ± 0.8 pg/mL by hericenone D to 23.5 ± 1.0 pg/mL by hericenone C (Kawagishi et al. 1991). Mori et al. (2008) investigated the NGF-stimulating effects of *H. erinaceus* extracts and metabolites on human astrocytoma cells and found that while *H. erinaceus* fruit body extracts exert this effect on human astrocytoma 1321N1 cells *in vitro*, hericenones were found in significantly lower concentrations in extracts than the effective concentrations used in the study by Kawagishi et al. (1991). Moreover, isolated hericenones did not exhibit the same effects on human astrocytoma cells as they had in previously reported studies on mouse astroglial cells (Kawagishi et al. 1991; Mori et al. 2008). The authors concluded that hericenones are not responsible for the NGF-stimulating effects of *H.*

erinaceus in human cells (Mori et al. 2008). In contrast, Rupcic et al. (2018) found that erinacines isolated from the mycelium of *H. erinaceus* induced NGF release in 1321N1 cells, which was supported by the findings of Rascher et al. (2020) wherein 1321N1 cells incubated with erinacine C significantly upregulated expression of *ngf* in astrocytes. In their study, Rascher et al. (2020) report the differentiation of PC12 cells into neuronal-like cells after incubation in a culture medium conditioned with erinacine C-treated human astrocytoma cells as a result of upregulating the expression of several neurotrophic factors, including NGF. Evidence for the *in vivo* bioactivity of erinacines reported by Shimbo et al. (2005) strengthens the research interest in prioritizing erinacines as target compounds among *H. erinaceus* metabolites where oral treatment with an erinacine A solution (8 mg/kg body weight erinacine A) led to significantly higher levels of NGF in the locus coeruleus and the hippocampus of rats, both of which are areas associated with learning and memory, compared to the control group (Fortin et al. 2002; Shimbo et al. 2005; James et al. 2021). In a double-blind placebo-controlled study with human subjects, Li et al. (2020) found that daily administration of *H. erinaceus* mycelium (350 mg) enriched with erinacine A (5 mg/g) for 49 weeks to 68 patients with mild AD resulted in improvements in markers for activity of daily living and cognitive ability.

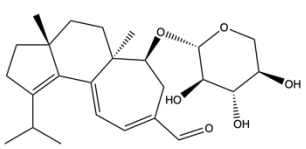
Coinciding with the increase in research attention generated by the neuroprotective activity of *H. erinaceus* and erinacines demonstrated in these studies, *H. erinaceus* has become a popular health supplement in the nootropic and nutraceutical markets for their reported neuroprotective and cognitive enhancing effects. However, the *H. erinaceus* supplement market is dominated by products containing *H. erinaceus* fruit

bodies exclusively due to the established techniques and processes of fruit body cultivation, yet the neuroprotective properties of this fungus are largely, though not exclusively, a function of erinacines isolated from the mycelium (Mori et al. 2008). Polysaccharides isolated from both the fruit body and mycelium of *H. erinaceus* have also demonstrated neuroprotective properties under *in vitro* conditions in PC12 cells and in *in vivo* studies with mice models (Cheng et al. 2016; Zhang et al. 2016). The neuroprotective properties of *H. erinaceus* are likely to be the result of several secondary metabolites; nevertheless, products intended to provide maximum neuronal health benefits should be comprised of mycelium enriched with erinacine A.

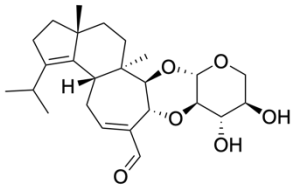
1.5.2 Erinacine A as a Target Erinacine

Despite erinacine C showing higher NGF-secreting effects than erinacine A in the inaugural work of Kawagishi et al. (1994), erinacine A has attracted significantly more attention by the research community as it is biosynthesized in the highest proportions of all erinacines (A-K, P-T, Z1, Z2) (Gerbec et al. 2015). The chemical structures of known erinacines are provided in Fig. 1-1. Chiu et al. (2018) discovered that mycelial extracts of *H. erinaceus* contained 5.010 mg/g (mycelium dry weight) erinacine A compared to 0.019 mg/g erinacine C, 0.094 mg/g erinacine Q, and 0.374 mg/g erinacine S. Moreover, the bioactive properties and biosynthesis of erinacine A are understood to a significantly greater degree than other erinacines, as most pharmacological studies of erinacines have focussed almost exclusively on erinacine A (Chen et al. 2017). The total and partial synthesis of erinacine A has been described by Snider et al. (1996, 1998), however these are long, complex, multi-step, and low-yielding syntheses that make synthetic erinacine A prohibitively expensive for most applications. Currently the most effective and

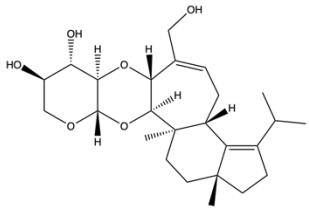
economically viable method of erinacine A production is from *H. erinaceus* mycelium cultivation and its subsequent isolation. Krzyczkowski et al. (2010) described the enrichment of mycelial cultures with erinacine A by manipulation of culture conditions, which may be used as a baseline to obtain erinacine A for research and commercial purposes.



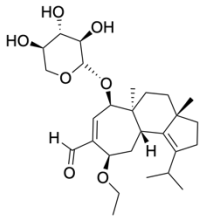
Erinacine A



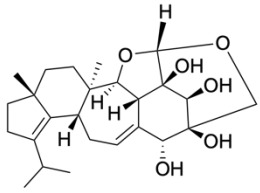
Erinacine B



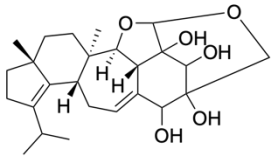
Erinacine C



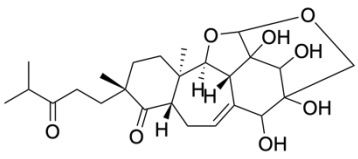
Erinacine D



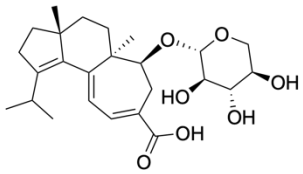
Erinacine E



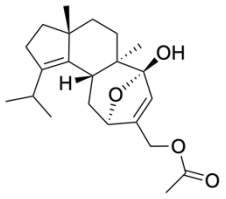
Erinacine F



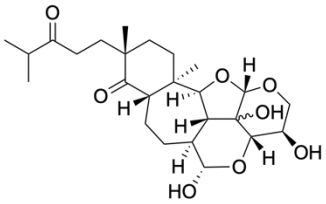
Erinacine G



Erinacine H



Erinacine I



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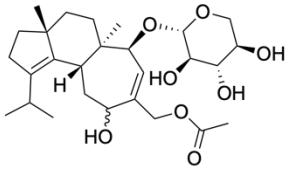
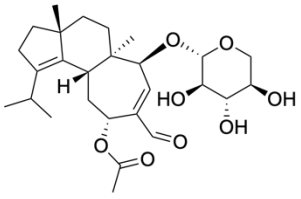
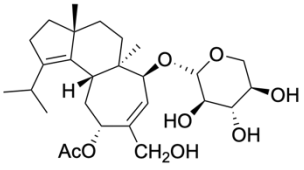


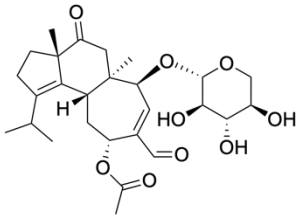
Erinacine K



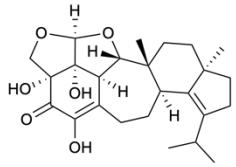
Erinacine P



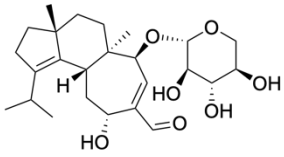
Erinacine Q



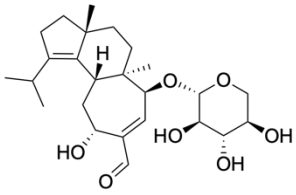
Erinacine R



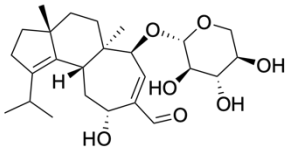
Erinacine S



Erinacine T



Erinacine Z1



Erinacine Z2

Figure 1-1. Chemical structures of known erinacines. Adapted from (Kawagishi et al. 1994, 1996a – b, 2006; Kenmoku et al. 2000, 2002; Chen et al. 2016; Chiu et al. 2018; Li et al. 2018; Rupcic et al. 2018; Wei et al. 2023). Structures produced in ChemDraw version 22.2.0.3348 (PerkinElmer, USA).

1.6 Production of Erinacine A by Submerged Liquid Culture Fermentation (SLCF)

1.6.1 Submerged Liquid Culture Fermentation (SLCF)

Submerged liquid culture fermentation (SLCF) is a common technique employed to grow mycelium of fungi in a liquid medium for the rapid production of metabolites. Examples of compounds produced by SLCF of filamentous fungi include the production of enzymes (cellulase from *Aspergillus* spp. and *Trichoderma* spp.), antibiotics (cephalosporin from *Acremonium* spp. and *Fusarium* spp.; penicillin from *Penicillium* spp., *Aspergillus* spp., *Acremonium* spp.), and organic acids (Papagianni 2004). The industrial scale production of mycelium of mushroom-forming fungi is also technically viable as demonstrated by the cultivation of *Cordyceps militaris* mycelium for the production of cordycepin, a potent anti-tumour (Lee et al. 2012) and anti-inflammatory (Kim et al. 2006) compound, and of *Ganoderma lucidum* mycelium for the production of polysaccharides and triterpenes including ganoderic acid (Fang and Zhong 2002), which utilizes bioreactors and other equipment common in industrial production of fermented products (Núñez-Ramírez et al. 2012). With the recent interest in mycelium-specific compounds demonstrated to confer health benefits produced from macrofungi, the cultivation of mycelium has expanded from mold fungi to basidiomycetes (Tang et al.

2007). Understanding the conditions necessary for the cultivation of *H. erinaceus* mycelium enriched with erinacine A by SLCF is necessary prior to any large-scale production of such products.

The SLCF of fungi in industrial settings requires consistent production of both the mycelium and of target metabolites, which necessitates the development of a standardized liquid medium in which it is grown. Carbon and nitrogen sources, pH, temperature, agitation regime, light exposure, and mineral composition of the liquid medium are the most important factors to consider when designing SLCF processes and therefore receive the most attention from researchers and industrial producers aiming to target the improved biosynthesis of certain compounds, increase tissue yields, reduce fermentation time, or a combination thereof (Malinowska et al. 2009; Krzyczkowski et al. 2010). Several studies have investigated the optimization of SLCF conditions for mycelium yields, polysaccharides, and erinacine biosynthesis of *H. erinaceus* (Malinowska et al. 2009; Cui et al. 2010; Krzyczkowski et al. 2010; Wolters et al. 2015; Chang et al. 2016; Chen et al. 2016; Ofosu et al. 2016). In a series of optimization experiments, Krzyczkowski et al. (2010) developed an improved medium for the production of erinacine A by *H. erinaceus* which was implemented by Chang et al. (2016) to achieve a concentration of 225 mg/L erinacine A by a different strain of *H. erinaceus*, the highest reported production of erinacine A known to this author. An important consideration when designing an optimized medium is that culture modification is strain-specific, as growth and metabolite production under a given set of SLCF conditions varies from one organism to the next. While the conditions described in Krzyczkowski et al. (2010) can serve as a general guide to producing erinacine A-enriched *H. erinaceus*

mycelium, the cultivation of different strains may require individual parameter optimization (Elisashvili et al. 2009; Atila et al. 2021). This becomes increasingly relevant when extending the scope of erinacine production to other *Hericium* species known or suspected to produce erinacines.

1.6.2 Production of Erinacine A by Other *Hericium* Species

Though the majority of studies concerned with the production of erinacine A isolate the compound from mycelium of *H. erinaceus,* erinacine A has also been detected in *H. alpestre* (as *H. flagellum*) (Rupcic et al. 2018) and *H. rajendrae* (Wei et al. 2023). As mentioned, erinacine A is produced in the highest concentrations relative to other erinacines and is therefore most commonly used in pharmacological studies evaluating erinacine bioactivity. However, Wolters et al. (2015) described the optimized conditions for the biosynthesis of erinacine C in *H. erinaceus* by SLCF. Other erinacines identified in the *Hericium* genus include erinacine E isolated from *H. coralloides* (as *H. ramosum*) (Saito et al. 1998), erinacines B, G, E, F, T, and Z1 from *H. rajendrae* (Wei et al. 2023), erinacines B, C, E, F, and Z2 from *H. alpestre* (as *H. flagellum*) (Rupcic et al. 2018), and erinacine E from an unknown North American *Hericium* sp. (Song et al. 2020) (Fig. 1-1). Screening of wild isolates is an important aspect of culture library maintenance and improvement to mushroom growers for the production of cultivated edible species, such as *Pleurotus* and *Lentinula*, where wild collected strains are developed into high yielding strains of considerable commercial value (Stamets 1993; Atila et al. 2021). Bioprospecting *Hericium* in North America for the production of erinacines, and in particular erinacine A, is therefore necessary to evaluate the biosynthesis and industrial-

scale production of this important metabolite. The evidence of its production in *H. alpestre* (Rupcic et al. 2018) and *H. rajendrae* (Wei et al. 2023) would suggest that the production of erinacine A may be a common trait that is shared across *Hericium* species, and there is a knowledge gap with respect to the relative amount of erinacine A produced in different species. While *H. erinaceus* has thus far been the main source of erinacine A, other species that have yet to be investigated might represent alternative sources for this compound.

Following the first objective of this project, which is the resolution of *Hericium* phylogeny (Chapter 2), the second major objective is to evaluate the production of erinacine A by North American species of *Hericium* from which erinacine A biosynthesis is unknown. For species that show evidence of erinacine A biosynthesis, SLCF conditions will be modified for improved erinacine A production in a species-specific manner (Chapter 3). An improved phylogenetic understanding of the genus will support research on *Hericium* metabolites by establishing consistent nomenclature for species of medical interest and information exchange thereof.

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Chapter 2

2 A Multilocus Phylogeny of *Hericium* in Canada

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2.1 Introduction

Species of *Hericium* Pers. are wood-decomposing basidiomycetes that produce large, white fruit bodies with a toothed hymenophore or fertile surface. In nature, *Hericium* are found on wood of deciduous trees (hardwoods), and to a lesser extent that of conifers (softwoods), causing a white rot (Ginns 1985; Das et al. 2011). Considered both a highly prized edible mushroom as well as a valuable medicinal resource, *H. erinaceus* (Bull.) Pers. has a long history of use in the treatment of gastric ailments in East Asia, where *H. erinaceus* is known as the monkey head mushroom (Houtou) in China and as Yamabushitake in Japan (Bhandari et al. 2014). Analyses over the past three decades of the compounds present in *H. erinaceus* fruit body and mycelium, which include erinacines, hericenones, pyrones, sterols, and polysaccharides, suggest that these mushrooms, and their secondary metabolites in particular, do indeed possess medicinal qualities by modern standards (Kawagishi et al. 1994, 1996a–b; Chen et al. 2017; Corana et al. 2019). Evidence of their bioactivity by *in vitro* and *in vivo* studies have demonstrated that isolated compounds as well as whole extracts possess various bioactivity profiles including anti-inflammatory (Chiu et al. 2018; Wang et al. 2019), antimicrobial (Kim et al. 2000; Wong et al. 2009), anti-cancer (Li et al. 2014), and neuroprotective (Suruga et al. 2015; Chen et al. 2016) properties. Because of these

potential health-promoting properties, together with its culinary value as an edible mushroom, the demand for *H. erinaceus* has extended beyond East Asia into Europe and North America in culinary and nutraceutical markets (Thongbai et al. 2015).

Since the description of *Hericium* by Persoon (1794), with *H. coralloides* (Scop.) Pers. as the type and only species, nomenclature of *Hericium*, and of *H. coralloides* in particular, has been controversial, as no original specimen is available for a holotype (Jumbam et al. 2019). Approximately seventeen species of *Hericium* are currently recognized globally, with five occurring in North America (Harrison 1984; Ginns 1985; Van Der Merwe et al. 2023). Harrison (1973b) treated the four species then known to occur in North America as *H. erinaceus*, *H. abietis* (Weir ex Hubert) K.A. Harrison, *H. ramosum* (Bull.) Letell., and *H. coralloides*, of which all but *H. abietis* were originally described from Europe. Neotypification of *H. coralloides* by Hallenberg (1983) placed *H. ramosum* in synonymy with *H. coralloides*, confirming the synonymy indicated by Bulliard (1791) and Fries (1821). Subsequently, Ginns (1985) used mating intercompatibility tests to show that cultures of the species in North America that Harrison (1973b) and others referred to as *H. ramosum* were compatible with European *H. coralloides*, whereas cultures of the species known by Harrison (1973b) and others in North America as *H. coralloides* were not. Based on preliminary results of these studies, Ginns (1984) proposed the name *H. americanum* Ginns for this North American species, and the published mating tests (Ginns 1985) supported the redescription of *H. coralloides* sensu auct. N. Amer. as *H. americanum*. The fifth *Hericium* species in North America is *Hericium cirrhatum* (Pers.) Nikol., known for many years as *Creolophus cirrhatus* (Pers.) P. Karst. (Harrison 1984). Analyses of sequences of the internal transcribed spacer (ITS)

region by Hallenberg et al. (2013) support the concept of *H. coralloides* sensu lato as a species complex, within which collections of *H. coralloides* sensu stricto from Europe were placed on a separate branch from those of North American origin. The use of the name *H. coralloides* in North America was therefore considered uncertain by these authors. Additionally, several authors have shown that ITS sequences of *H. erinaceus* of Asian origin cluster separately from those of North American and European origin (Hallenberg et al. 2013; Cesaroni et al. 2019; Jumbam et al. 2019). The interpretation of *Hericium* species is complicated by the high frequency of misidentified specimens and their subsequent deposit as mislabelled sequences in public databases, as well as the high number of sequences from commercial strains of *H. erinaceus*, for which voucher specimens are unknown or unavailable.

Despite the progress made in our understanding of *Hericium* taxonomy by ITS sequence analysis, polymorphisms at this locus are minimal in *Hericium,* and phylogenies produced using ITS alone have provided unsatisfactory species delimitations with little resolution to interspecific relationships (Hallenberg et al. 2013; Das et al. 2013; Cesaroni et al. 2019; Ouali et al. 2020). Combination of ITS and the large subunit (LSU) of nuclear rDNA regions with protein-coding genes *rpb2* and *tef1* have provided stronger phylogenetic signals to delimit species in other groups, such as *Trametes* (Carlson et al. 2014) and *Tolypocladium* (Dong et al. 2022), for which ITS alone provides little resolution. Here, I present the first multilocus phylogenetic study of the genus *Hericium,* with a particular focus on species occurring in North America and Europe. The first major objective of the present work is to provide a higher resolution to the interspecific relationships of *Hericium* species known from North America using ITS, LSU, *rpb2*, and

tef1, and to position these species in relation to global *Hericium* diversity. Secondly, to help stabilize the nomenclature of *Hericium*, I designate lectotypes, and epitypes where possible, for critical European species, *H. coralloides, H. erinaceus*, *H. flagellum* (Scop.) Pers., and *H. clathroides* (Pall.) Pers.

2.2 Materials and Methods

2.2.1 Sampling and Collecting

From September 2022 to August 2023, specimens of *H. americanum* and *H. coralloides* fruit bodies were collected from forests in Southern Ontario, and mycelial cultures were isolated by transferring clean internal tissue of fruit bodies to malt extract (ME) agar containing 50 mg/L chloramphenicol (Nobles 1948) as sources of genomic DNA for sequence analysis. Fruit bodies of collected specimens were dried at 45ºC for 48 hours to serve as voucher specimens and were deposited in the Dr. Laurie L. Consaul Herbarium, London, Canada (UWO). Cultures of other *Hericium* species were obtained from the Canadian Collection of Fungal Cultures (DAOMC) (Table 2-1) to extend the breadth and depth of the taxon sample for DNA analysis. With the exception of DAOMC 195739 (as *H. erinaceus*, Japan), DAOMC 172297 (*H. alpestre*, France), and DAOMC 251013 (as *H. clathroides*, Czech Republic), which were included for assessment of geographic

Table 2-1. Sequences included in the multilocus phylogeny, including taxon names as identified in this study, geographic origin, voucher or culture number, and GenBank accession numbers.

			Accession Number[4]			
Species	Origin	Voucher	ITS	LSU	*tef1*	*rpb2*
Amylostereum areolatum	China	HG-01[1]	SAXG01000196	SAXG01000196	SAXG01000196	SAXG01000196
Auriscalpium vulgare[2]	WA, USA	AFTOL-ID 1897	DQ911613	---	---	---
	IN, USA	FP105234-sp[1]	---	JAFLMV010000061	JAFLMV010000061	JAFLMV010000061
Auriscalpium vulgare	Netherlands	CBS 236.39[1]	JAHBBC010000018	JAHBBC010000018	JAHBBC010000018	JAHBBC010000018
Dentipellis fragilis	Germany	DSM 105465[1]	SEOQ01000808.1	SEOQ01000808.1	SEOQ01000031.1	SEOQ01000005.1
Dentipellis sp.	South Korea	KUC8613[1]	OL898488	OL898488	NSJX01000003.1	NSJX01000003.1
Hericium abietis (HB1)[3]	BC, Canada	DAOMC17054	*OR793932*	*OR793932*	*OR829831*	*OR829871*
H. abietis (HB2)	BC, Canada	DAOMC251004	*OR793933*	*OR793933*	*OR829832*	*OR829872*
H. abietis (HB3)	BC, Canada	DAOMC196447	*OR793934*	*OR793934*	*OR829833*	*OR829873*
H. abietis (HB4)	BC, Canada	DAOMC22748	*OR793935*	*OR793935*	*OR829834*	*OR829874*
H. abietis (HB5)	BC, Canada	DAOMC16601	*OR793936*	*OR793936*	*OR829835*	*OR829875*
H. abietis (HB6)	BC, Canada	DAOMC251005	*OR793937*	*OR793937*	*OR829836*	*OR829876*
H. abietis (HB7)	BC, Canada	DAOMC251006	*OR793938*	*OR793938*	*OR829837*	*OR829877*
Hericium alpestre	Germany	DSM 108284[1]	SFCI01000210	SFCI01000210	SFCI01000210	SFCI01000210
H. alpestre (HL1)	France	DAOMC172297	*OR793952*	*OR793952*	*OR829851*	*OR829891*
Hericium americanum	MA, USA	AFTOL-ID 469	DQ206987	DQ411538	DQ028585	DQ408127
H. americanum (HA1)	ON, Canada	UWO-F1485	*OR793913*	*OR793913*	*OR829812*	*OR829852*
H. americanum (HA2)	ON, Canada	---	*OR793914*	*OR793914*	*OR829813*	*OR829853*
H. americanum (HA3)	ON, Canada	UWO-F1487	*OR793915*	*OR793915*	*OR829814*	*OR829854*
H. americanum (HA5)	ON, Canada	UWO-F1488	*OR793916*	*OR793916*	*OR829815*	*OR829855*
H. americanum (HA6)	ON, Canada	UWO-F1489	*OR793917*	*OR793917*	*OR829816*	*OR829856*
H. americanum (HA8)	ON, Canada	UWO-F1490	*OR793918*	*OR793918*	*OR829817*	*OR829857*
H. americanum (HA9)	ON, Canada	---	*OR793919*	*OR793919*	*OR829818*	*OR829858*

H. americanum (HA10)	ON, Canada	UWO-F1492	*OR793920*	*OR793920*	*OR829819*	*OR829859*
H. americanum (HA11)	ON, Canada	UWO-F1493	*OR793921*	*OR793921*	*OR829820*	*OR829860*
H. americanum (HAX1)	ON, Canada	DAOMC21467	*OR793928*	*OR793928*	*OR829827*	*OR829867*
H. americanum (HAX2)	PA, USA	DAOMC251011	*OR793929*	*OR793929*	*OR829828*	*OR829868*
H. americanum (HC3)	ON, Canada	UWO-F1482	*OR793925*	*OR793925*	*OR829824*	*OR829864*
H. americanum (HC4)	ON, Canada	UWO-F1483	*OR793926*	*OR793926*	*OR829825*	*OR829865*
H. americanum (HC5)	ON, Canada	UWO-F1484	*OR793927*	*OR793927*	*OR829826*	*OR829866*
H. americanum (WCHA)	ON, Canada	---	*OR793922*	*OR793922*	*OR829821*	*OR829861*
H. americanum (EON1)	ON, Canada	DAOM 241459	*PP786302*	*PP786302*	*PP793770*	*PP793775*
Hericium asiaticum (as *Hericium erinaceus*)	China	CS-4[1]	SZZO02000036	SZZO02000036	SZZO02000036	SZZO02000036
H. asiaticum[2] (as *H. erinaceus*)	China	Dai 14392	MH085955	MH085955	---	---
	Taiwan	0605[1]	---	---	JABWEG010000004	JABWEG010000004
H. asiaticum (HE9) (as *H. erinaceus*)	Japan	DAOMC195739	*OR793950*	*OR793950*	*OR829849*	*OR829889*
Hericium carolinense (HE2) (as *H. erinaceus*)	MD, USA	DAOMC251034	*OR793940*	*OR793940*	*OR829839*	*OR829879*
H. carolinense (HE3) (as *H. erinaceus*)	MD, USA	DAOMC251033	*OR793941*	*OR793941*	*OR829840*	*OR829880*
H. carolinense (HE4) (as *H. erinaceus*)	GA, USA	DAOMC251031	*OR793942*	*OR793942*	*OR829841*	*OR829881*
H. carolinense (HE6) (as *H. erinaceus*)	PA, USA	DAOMC251030	*OR793943*	*OR793943*	*OR829842*	*OR829882*
Hericium cirrhatum	Germany	KAS F794	AF506385	AF506385	---	---
H. cirrhatum (HCi1)	AB, Canada	UWO-F1892	*PP786306*	*PP786306*	*PP793774*	*PP793777*

Hericium coralloides	China	tvtc0002[1]	QUOP01000211	QUOP01000211	QUOP01000211	QUOP01000211
H. coralloides (HC2)	ON, Canada	UWO-F1481	*OR793924*	*OR793924*	*OR829823*	*OR829863*
H. coralloides (HC6)	ON, Canada	RGT 230902/02	*OR793947*	*OR793947*	*OR829846*	*OR829886*
H. coralloides (HCNB)	NB, Canada	RGT 220816/29	*OR793944*	*OR793944*	*OR829843*	*OR829883*
H. coralloides (HCX1)	MI, USA	DAOMC251025	*OR793930*	*OR793930*	*OR829829*	*OR829869*
H. coralloides (HCX3)	VA, USA	DAOMC251017	*OR793931*	*OR793931*	*OR829830*	*OR829870*
H. coralloides (HCX4)	England	DAOMC22531	*OR793946*	*OR793946*	*OR829845*	*OR829885*
H. coralloides (HCX5)	ON, Canada	DAOMC251022	*OR793945*	*OR793945*	*OR829844*	*OR829884*
H. coralloides (HD1)	Czech Republic	DAOMC251013	*OR793951*	*OR793951*	*OR829850*	*OR829890*
Hericium erinaceus (HE7)	Netherlands	CBS 202.31	*OR793948*	*OR793948*	*OR829847*	*OR829887*
H. erinaceus (HE8)	England	DAOMC251032	*OR793949*	*OR793949*	*OR829848*	*OR829888*
H. erinaceus (WCHE)	N/A	Commercial strain	*OR793923*	*OR793923*	*OR829822*	*OR829862*
Hericium oregonense (EBC1) (as *Hericium erinaceus*)	BC, Canada	DAOM 193728	*PP786303*	*PP786303*	*PP793771*	*PP793776*
H. oregonense (EBC2) (as *H. erinaceus*)	BC, Canada	DAOM 193896	*PP786304*	*PP786304*	*PP793772*	---
H. oregonense (EBC3) (as *H. erinaceus*)	BC, Canada	DAOM 185101	*PP786305*	*PP786305*	*PP793773*	---

[1] Whole genome sequence (WGS) data

[2] Missing ITS_LSU region filled in with sequence data from geographically close specimens

[3] Koga & Thorn culture code

[4] Accession numbers in *italics* for sequences generated in the present study

diversity in relation to North American *Hericium* species, all DAOMC cultures were those used by Ginns (1985) in his treatment of the genus in North America and are therefore formally representative of *H. erinaceus, H. americanum, H. coralloides,* and *H. abietis* as they are currently understood. Cultures were maintained on ME agar at room temperature in the dark and transferred as needed. Additional voucher specimens identified as *H. erinaceus* from British Columbia (DAOM 193896, DAOM 193728, DAOM 185101) and Ontario (DAOM 241459) were obtained from the National Mycological Herbarium of Canada (DAOM), along with a specimen of *H. cirrhatum* obtained from Stacey Scorda, a naturalist in northern Alberta (UWO- F1892).

2.2.2 Phylogenetic Analysis

For each strain, mycelium was grown in liquid culture by transferring 5 × 5 mm sections of colonized ME agar at the leading edge of the mycelium into autoclaved liquid V8 medium (20% V8 vegetable juice, 80% deionized H_2O) and grown at room temperature on a rotary shaker to obtain biomass for DNA extractions. Small portions of dried fruit bodies of voucher specimens for which no cultures were available were ground in liquid nitrogen, and genomic DNA from these and of mycelial cultures was extracted using a CTAB and chloroform extraction and ethanol precipitation (Lee et al. 1988; Bainbridge et al. 1990; Möller et al. 1992). The ITS and LSU regions of nuclear rDNA were amplified as a single ~1200 bp amplicon using primers ITS1F/LR3 (Gardes and Bruns 1993; Hopple and Vilgalys 1994). Amplification of protein coding genes *tef1* (~600 bp) and *rpb2* (~800 bp) were amplified using primers RR.EF1-983F (5’-GCY CCY GGH CAY CGY GAY TTY AT-3’)/RR.EF1-1567R (5’-ACH GTR CCR ATA CCR CCR ATC TT-3’; both modified from Rehner and Buckley 2005) and bRPB2-6F/bRPB2-7.1R

(Liu et al. 1999; Matheny et al. 2007), respectively. Successful PCR products were cleaned using the Bio Basic EZ-10 Spin Column PCR Products Purification Kit and sequenced by Sanger sequencing at the London Regional Genomics Centre.

Sequence files were cleaned and assembled in SeqEd v1.03 (ABI Software) and BLAST searches of ITS–LSU, *rpb2*, and *tef1* were used to select reference sequences for inclusion in the analyses. Forty-three strains of *Hericium* from the present study were sequenced for ITS–LSU (OR793913–OR793952; PP786302- PP786306), *tef1* (OR829812–OR829851; PP793770- PP793774), and *rpb2* (OR829852–OR829891; PP793774-PP793777), and 10 strains from GenBank for which all three loci were available and one of *Hericium cirrhatum* for which only ITS–LSU data were available were added to the analysis (Table 2-1). Most sequences of *Hericium* that were available on GenBank could not be included in my analyses because they included only ITS or LSU regions, which are largely uninformative for *Hericium* taxonomy (Hallenberg et al. 2013; Jumbam et al. 2019), and no *tef1* or *rpb2* sequences were available for the same samples. In addition, several sequences are apparently obtained from commercial strains for which the natural origins are unknown. Datasets for each locus were aligned individually with MAFFT (Multiple Alignment using Fast Fourier Transform) software version 7 (Katoh and Standley 2013) with the options for iterative G-INSi algorithm and aligning gappy regions invoked. Alignments were manually improved and forward and reverse primers trimmed in MEGA 11 (Tamura et al. 2021). For sequences with degenerate bases (two clear peaks in both forward and reverse reads), the canonical base for the taxon was selected for analysis. Where ITS–LSU regions were not detectable in whole genome sequences of *Auriscalpium vulgare* FP105234-Sp (accession

JAFLMV010000061) and *Hericium erinaceus* 0605 (accession JABWEG010000004), ITS–LSU sequences from geographically close specimens were used to fill in the gaps. *Auriscalpium vulgare* AFTOL-ID 1897 (accession DQ911613) was used to fill in the ITS for *A. vulgare* FP105234-Sp, and the ITS–LSU of *H. erinaceus* Dai 14392 (accession MH085955) was used to complement the missing sequence data of *H. erinaceus* 0605. After determining the most appropriate model of evolution for each gene region in MEGA 11 and confirming congruence in phylogenies based on each gene region (data not shown), the trimmed and aligned sequences of ITS–LSU, *rpb2*, and *tef1* were concatenated and used to generate phylogenetic trees using Maximum Likelihood with 100 bootstrap replicates, using the T93+G model selected using BIC in MEGA 11 (Tamura et al. 2021). To further assess tree topology and branch support, a maximum parsimony analysis of the same dataset was run in MEGA with 1000 bootstraps (retaining up to 10,000 trees at each step), followed by a Bayesian analysis in MrBayes v3.2.7 (Ronquist et al. 2012) with 25 million generations and a burnin of 25%, at which point the average standard deviation of split frequencies had stabilized below 0.001.

Phylogenetic trees from MEGA 11 were generated as pdf files and brought into Adobe Acrobat Pro 2020 to add italics and labels, then brought into Adobe CS3 to set image size and resolution. Historic illustrations (all in the public domain except Fig. 2-2C; specific sources cited below) were acquired into Photos v8 to adjust brightness and contrast, then these and photographs of specimens in the field were brought into Adobe Photoshop CS3 to set the image size and resolution and apply figure dividing lines and labels.

2.3 Results

The maximum likelihood tree of aligned and concatenated ITS, LSU, *tef1*, and *rpb2* sequences yielded a well-supported Hericiaceae and *Hericium,* each with 100% bootstrap support (bss) (Fig. 2-1). The topology and species-level clades obtained in maximum parsimony and Bayesian analyses were identical, so the maximum likelihood tree is shown with branch support from all three analyses. Seventeen representatives of *H. americanum* (including one identified as *H. erinaceus*)*,* all of which originated from specimens growing on hardwood (*Fagus*, *Acer*, *Carya*) in eastern North America, formed a well-supported (100% bss) clade that is sister to a branch (95% bss) that includes the European conifer-dwelling species *H. alpestre* (81% bss) and the conifer-dwelling species from western North America, *H. abietis* (100% bss). Sister to all of these is a clade representing the *H. erinaceus* complex (99% bss), subdivided into strains from Europe (100% bss), western North America (99% bss), Asia (74% bss), and eastern North America (100% bss). A collection of *H. cirrhatum* from Alberta clustered with the single ITS–LSU sequence of this species from Germany (96% bss) and sister to *H. coralloides* from Europe, Asia, and North America (96% bss), and both as sister to the remainder of the genus. Eight representatives of *H. coralloides* from Europe, Asia, and North America formed a clade with some internal geographic structure, with a clade of four isolates from Ontario and the eastern United States (100% bss) subtended by a grade of isolates from Sweden, Czech Republic, China, and Great Britain. Analysis of a set of ITS–LSU sequences of *H. coralloides* separated a New Brunswick sequence as part of a subclade with collections from Norway and Russia on *Populus* and from Sweden on conifer wood

(100% bss), with the remainder showing little geographic resolution and with the sequence of the epitype specimen (designated below) nested in this subclade (Fig. A-1).

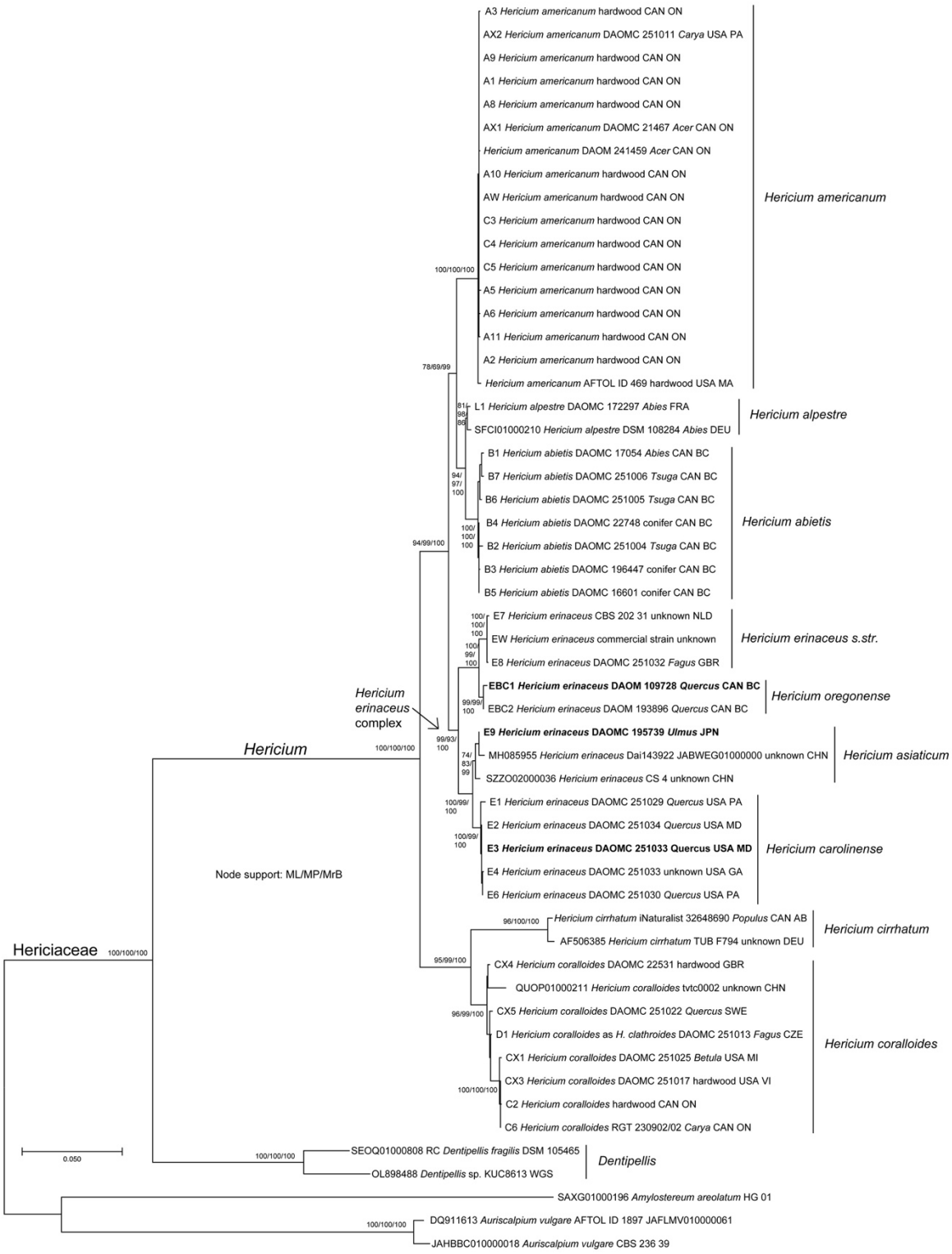
A3 Hericium americanum hardwood CAN ON
AX2 Hericium americanum DAOMC 251011 Carya USA PA
A9 Hericium americanum hardwood CAN ON
A1 Hericium americanum hardwood CAN ON
A8 Hericium americanum hardwood CAN ON
AX1 Hericium americanum DAOMC 21467 Acer CAN ON
Hericium americanum DAOM 241459 Acer CAN ON
A10 Hericium americanum hardwood CAN ON
AW Hericium americanum hardwood CAN ON
C3 Hericium americanum hardwood CAN ON
C4 Hericium americanum hardwood CAN ON
C5 Hericium americanum hardwood CAN ON
A5 Hericium americanum hardwood CAN ON
A6 Hericium americanum hardwood CAN ON
A11 Hericium americanum hardwood CAN ON
A2 Hericium americanum hardwood CAN ON
Hericium americanum AFTOL ID 469 hardwood USA MA
Hericium americanum
100/100/100
78/69/99
L1 Hericium alpestre DAOMC 172297 Abies FRA
SFCI01000210 Hericium alpestre DSM 108284 Abies DEU
Hericium alpestre
81/98/86
B1 Hericium abietis DAOMC 17054 Abies CAN BC
B7 Hericium abietis DAOMC 251006 Tsuga CAN BC
B6 Hericium abietis DAOMC 251005 Tsuga CAN BC
B4 Hericium abietis DAOMC 22748 conifer CAN BC
B2 Hericium abietis DAOMC 251004 Tsuga CAN BC
B3 Hericium abietis DAOMC 196447 conifer CAN BC
B5 Hericium abietis DAOMC 16601 conifer CAN BC
Hericium abietis
94/97/100
100/100/100
94/99/100
E7 Hericium erinaceus CBS 202 31 unknown NLD
EW Hericium erinaceus commercial strain unknown
E8 Hericium erinaceus DAOMC 251032 Fagus GBR
Hericium erinaceus s.str.
100/100/100
100/99/100
EBC1 Hericium erinaceus DAOM 109728 Quercus CAN BC
EBC2 Hericium erinaceus DAOM 193896 Quercus CAN BC
Hericium oregonense
99/99/100
Hericium erinaceus complex
E9 Hericium erinaceus DAOMC 195739 Ulmus JPN
MH085955 Hericium erinaceus Dai143922 JABWEG01000000 unknown CHN
SZZO02000036 Hericium erinaceus CS 4 unknown CHN
Hericium asiaticum
99/93/100
74/83/99
Hericium
100/100/100
E1 Hericium erinaceus DAOMC 251029 Quercus USA PA
E2 Hericium erinaceus DAOMC 251034 Quercus USA MD
E3 Hericium erinaceus DAOMC 251033 Quercus USA MD
E4 Hericium erinaceus DAOMC 251033 unknown USA GA
E6 Hericium erinaceus DAOMC 251030 Quercus USA PA
Hericium carolinense
100/99/100
100/99/100
Node support: ML/MP/MrB
Hericium cirrhatum iNaturalist 32648690 Populus CAN AB
AF506385 Hericium cirrhatum TUB F794 unknown DEU
Hericium cirrhatum
96/100/100
Hericiaceae 100/100/100
CX4 Hericium coralloides DAOMC 22531 hardwood GBR
QUOP01000211 Hericium coralloides tvtc0002 unknown CHN
CX5 Hericium coralloides DAOMC 251022 Quercus SWE
D1 Hericium coralloides as H. clathroides DAOMC 251013 Fagus CZE
CX1 Hericium coralloides DAOMC 251025 Betula USA MI
CX3 Hericium coralloides DAOMC 251017 hardwood USA VI
C2 Hericium coralloides hardwood CAN ON
C6 Hericium coralloides RGT 230902/02 Carya CAN ON
Hericium coralloides
95/99/100
96/99/100
100/100/100
0.050
SEOQ01000808 RC Dentipellis fragilis DSM 105465
OL898488 Dentipellis sp. KUC8613 WGS
Dentipellis
100/100/100
SAXG01000196 Amylostereum areolatum HG 01
DQ911613 Auriscalpium vulgare AFTOL ID 1897 JAFLMV010000061
JAHBBC010000018 Auriscalpium vulgare CBS 236 39
100/100/100

Figure 2-1. Maximum likelihood tree based on concatenated sequences of the nuclear ribosomal internal transcribed spacer region (ITS), adjacent 3'-end of the large ribosomal subunit (LSU), and portions of the translation elongation factor 1-alpha (*tef1*), and second largest subunit of RNA polymerase II (*rpb2*). Numbers at nodes represent bootstrap support from 100 replicates run in MEGA 11 (Tamura et al. 2021), bootstrap support from a maximum parsimony analysis with 1000 replicates in MEGA 11, and Bayesian posterior probabilities from an analysis with 25 million generations run in MrBayes 3.2.7 (Ronquist et al. 2012). *Auriscalpium vulgare* Gray and *Amylostereum areolatum* (Chaillet ex Fr.) Boidin were used as the outgroup.

2.4 Discussion

The use of multiple loci in phylogenetic studies has become a valuable method for obtaining a stronger phylogenetic signal amongst closely related taxa for which the use of the commonly used ITS region alone does not provide enough resolution to accurately delimit them to a satisfactory degree (Matheny 2005; Matheny et al. 2007; Carlson et al. 2014; Dong et al. 2022). Such constraints have limited the understanding of global *Hericium* diversity as demonstrated by previous phylogenetic studies (Hallenberg et al. 2013; Jumbam et al. 2019; Cesaroni et al. 2019). The limited discrimination of species traditionally recognized through characters of morphology and mating compatibility (e.g., Hallenberg 1983; Ginns 1984, 1985) has raised concerns over accurate nomenclature and species delineation. The present multilocus analysis using sequence data from ITS, LSU, *tef1* and *rpb2* provides a significant improvement in the resolution of interspecific variation of *Hericium* from North America, Europe, and Asia, and allows us to resolve some nomenclatural issues.

The maximum likelihood tree generated in this analysis resolves *Hericium* species into distinct, well-supported monophyletic clades and subclades. Sequences obtained from *H. americanum* are grouped together in a subclade with 100% bss, consistent with Ginns' (1984, 1985) recognition of *H. americanum* based on interfertility tests using single-spore isolates. *Hericium abietis*, consisting of sequences obtained from specimens isolated from conifer wood (*Abies, Tsuga*) in western North America, is also well resolved with respect to the closely related *H. americanum* and *H. alpestre*. The name *Hericium alpestre* is used by some authors (Hallenberg 1983; Ginns 1985; Hallenberg et al. 2013), but others (Stalpers 1996; Laessoe and Petersen 2019; Kujawska et al. 2021; indexfungorum.org) place that name in synonymy with *H. flagellum*, an earlier name of very uncertain application, which I argue below applies to *H. coralloides*, not *H. alpestre*. Several of these species are of conservation concern in Europe: in Great Britain, *H. coralloides* is listed as near threatened (Evans et al. 2006) or endangered (Smith et al. 2016), and in Czechia, *H. alpestre* (as *H. flagellum*) is listed as near threatened (Holec and Beran 2006; Kujawska et al. 2021). Although the *H. coralloides* clade contains a relatively high degree of intraspecific variability with respect to other clades in the analysis, *H. coralloides* nevertheless forms a monophyletic grouping of sequences originating from specimens from North America, Europe, and Asia, all but one of which were isolated from hardwoods, consistent with the findings of Ginns (1985) of intercompatibility among isolates of *H. coralloides* from Europe and North America. A collection of *Hericium cirrhatum* from Alberta clustered with the single available ITS–LSU sequence of *Hericium cirrhatum* from Germany, and represents the first confirmed Canadian record of the species, previously known from Colorado and New Mexico in the

United States (Harrison 1984). The Alberta collection also provides the first sequence data for North American material of this species, which is reported from boreal and montane regions of North America from New Brunswick to Alberta and south to New Mexico, and is associated with *Populus* (Harrison 1984; iNaturalist.com).

The multilocus analysis clearly distinguishes four taxa within the *H. erinaceus* species complex delineated by geographic origin of the specimens. Prior works using single-gene sequence data (Hallenberg et al. 2013; Cesaroni et al. 2019) were suggestive of geographically isolated cryptic species within *H. erinaceus*. However, these studies did not display sufficient resolution of cryptic species from one another to formally establish novel species within the *H. erinaceus* complex. Although there are no apparent morphological differences between these four taxa that I am aware of, the mutually monophyletic clades obtained from multigene sequence data suggest a lack of genetic recombination among these populations (Taylor et al. 2000). This, together with the geographic separation, supports the recognition of four distinct species within the *H. erinaceus* species complex, two of which were recently proposed as new species, *H. carolinense* Koga & Thorn and *H. asiaticum* Koga & Thorn (Koga and Thorn 2023), and a third described below as *H. oregonense* sp. nov.

2.5 Taxonomy

Hericium abietis (Weir ex Hubert) K.A. Harrison, Can. J. Bot. 32: 1208 (1964)

≡ *Hydnum abietis* Weir ex Hubert, Outline of Forest Pathology: 305 (1931)

Holotype none; lectotype BPI 9964, on *Abies grandis*, Priest River, Idaho, USA, 19 September 1916, R.J. Weir (designated by Maas Geesteranus 1960). Maas Geesteranus (1959, 1960) was uncertain whether this species was a synonym of *H. americanum* (as *H.*

coralloides) or *H. coralloides* (as *H. ramosum*) but settled on the former. However, sequence data support *H. abietis* as a good species, closely related to *H. alpestre* of Europe and *H. americanum* of North America (Fig. 2-1). Detailed descriptions were provided by Harrison (1964, 1973b), and the species has been illustrated by Smith (1963, as *Hericium weirii*, nom. inval.), Bandoni and Szczawinski (1976, as *H. coralloides*), Tylutki (1979), Arora (1986), Trudell and Ammirati (2009), Desjardin et al. (2015), and MacKinnon and Luther (2021). This species occurs on coniferous wood, both of standing trees and fallen logs, in the Pacific Northwest, causing a heart rot of living trees (Hubert 1931). Observations from central and eastern Canada posted to iNaturalist.ca appear to be misidentifications of *H. americanum* or *H. coralloides*.

Hericium alpestre Pers., Mycol. Eur. (Erlanga) 2: 151 (1825)

Holotype none; neotype (designated by Hallenberg et al. 2013) Romania, Suceava region, Codrul Secular Slatioara, on a dead standing tree of *Abies*, 1985-10-16, coll. Nils Hallenberg, NH 9161 (GB), GenBank accession JQ716936 (ITS). Hallenberg (1983; Hallenberg et al. 2013) mentions the presence of what is possibly original material of the species in the Persoon herbarium in Leiden (L 910256–1300), but the packet has no other label data beyond the name. In the absence of original material or an illustration cited in the original description, the neotype designated by Hallenberg is compliant with the Code (Turland et al. 2018). Of the names that have been applied to this species, which is restricted to growing on fir (*Abies alba*) in southern and central Europe north to the Netherlands (Laessoe and Petersen 2019), *H. alpestre* is the only one that can reliably be associated with this conifer-dwelling species, as previously indicated by Hallenberg

(1983; Hallenberg et al. 2013). The basionyms of both *H. clathroides* and *H. flagellum* are more likely to apply to the hardwood inhabiting species correctly known as *H. coralloides*, as suggested by their original descriptions and illustrations. *Hericium alpestre* is well-supported in the multilocus phylogeny (Fig. 2-1) as a sister taxon to *H. abietis*, the conifer-dwelling species of the Pacific Northwest (above), and these two as sister to the North American hardwood-dwelling species *H. americanum*. Basidiospores of all three species are larger than those of *H. coralloides*, and the fruit bodies are more densely branched, with shorter branches with longer spines at their tips. Fruit bodies of *H. alpestre* develop pinkish staining in age, as do *H. americanum* and *H. abietis*. The ITS sequences of *H. yumthangense*, described from *Abies* in Sikkim state in northeastern India (Das et al. 2013), suggest its synonymy with *H. alpestre* (data not shown).

Hericium americanum Ginns, Mycotaxon 20(1): 43 (1984)

= *Hericium coralloides* auct. N. Amer. (e.g., Harrison 1973b; Smith and Smith 1973)

Holotype on *Platanus*, Pennsylvania, USA, 5 November 1931, J.W. Sinden, L.O. Overholts 14844 (PAC); ex-type culture preserved as DAOMC 2167. The species was briefly described by Ginns (1984) when differentiated from *H. coralloides* by mating tests, and described in full by Harrison (1973b, as *H. coralloides*). It occurs on wood of diverse hardwoods, and rarely on wood of conifers (*Tsuga*), mostly in eastern North America, east to Nova Scotia (Harrison 1973b; Pomerleau 1980, as *H. coralloides* and *H. abietis*; Ginns 1984); records on iNaturalist from the West appear to be misidentifications of *H. coralloides*. It is certainly the most common species in Ontario. *Hericium*

botryoides S. Ito & Otani, growing on living trunks of *Quercus myrsinifolia* in Nara, Japan, was described as cauliflower-like, with 5–10 mm spines covering the surface of multiple globular outgrowths of a solid core, salmon pink or light orange-yellow in colour, with non-amyloid basidiospores 4.5–6.5 × 4.5–6.0 µm (Otani 1957). The type collection and recent collections should be examined and sequenced to determine the correct disposition of this species, which seems most like *H. americanum* but might be quite unrelated. Harrison (1973a) has similarly raised the possibility that two species of the "*H. coralloides*" group occur on hardwood substrates in Europe as they do in North America, citing a specimen from *Fagus* in Hungary with spores 5.0–6.2(–6.8) × 4.8–5.6(–6.4) µm, matching the spore size of what Harrison knew as *H. coralloides* (now *H. americanum*). It is hard to imagine how a vicariant of *H. americanum* could have been overlooked in both Europe and Asia, except perhaps because the mature, undisturbed deciduous forests supporting their growth and fruiting are now rare (Harrison 1973b).

Hericium asiaticum Koga & Thorn, Index Fungorum 569: 1 (2023)

Registration: MB# 850907

Holotype: on fallen hardwood log, Horoiwayama, Saroma-cho, Tokoro-gun, Hokkaido, Japan, 27 September 1984, I. Ohira, N. Maekawa and E. Nagasawa, TMI-8380. A culture derived from this collection (TMIC-30293) was sent to Ottawa for study by J.H. Ginns and is maintained there as DAOMC 195739.

Description: Fruit body ovate to globose, 5–10 cm broad, white at first, yellowing with age and becoming brown when dried, the upper surface roughened-hispid, the lower surface composed of spines 1–5 cm long, 1–2 mm thick at their base and tapering to

needle tips, with solid, spongy white flesh. Gloeocystidia present, up to 7.5 µm broad. Basidiospores subglobose, smooth, amyloid, 6.5–7.5 × 5.0–5.5 µm. On beech, oak, and chestnut, causing a white rot (Teng 1963; Imazeki et al. 1988; Imazeki and Hongo 1989). Comments: I am not aware of any characters of macro- or micromorphology that differentiate the members of the *H. erinaceus* species complex, which includes *H. erinaceus* s.str., *H. asiaticum*, *H. carolinense*, and now *H. oregonense* sp. nov., and mating tests by Ginns (1985) indicated that strains which he identified as *H. erinaceus* from Europe, eastern North America and Asia were all interfertile. Unfortunately, neither I nor Ginns (1985) had living material available of the population of *Hericium erinaceus* occurring on the West Coast of North America, as reported by Siegel and Schwarz (2016) and MacKinnon and Luther (2021), which is described as *H. oregonense* sp. nov. Nonetheless, since sequences from the four geographic regions form mutually monophyletic clades, I feel that it is worthwhile distinguishing all these entities at the species level to allow precise designation of the subjects of study by mushroom cultivators, biochemists, and medical researchers. Only ITS sequences are available for *Hericium rajendrae* U. Singh & K. Das, described from a living tree of *Quercus* in Uttarakhand state in northern India (Singh and Das 2019), and these place it in or near the *H. erinaceus* complex (data not shown). Multilocus sequence data from type or authentic material of *H. rajendrae* are desirable to better place this species.

Hericium carolinense Koga & Thorn, Index Fungorum 569: 1 (2023)

Registration: MB# 850975

Holotype: on *Quercus* in oak stand on Laurel-Bowie Road, Laurel, Maryland, USA, 21 November 1965, John Lindsay, O.K. Miller, Jr 3766, VPI-F-0001744. A culture derived from this collection (OKM 3766-S) was sent to Ottawa for study by J.H. Ginns and is maintained there as DAOMC 251033.

Description: Fruit body ovate to globose, sometimes lobed, up to 25 cm broad, white at first, then yellowing in age and browning when dry, the upper surface a coarsely matted tangle of mycelial strands, the lower portion composed of spines up to 4 cm long, tapering to needle tips, with soft, solid to porous white flesh. Gloeocystidia arising in subhymenium, up to 7 µm broad. Basidiospores subglobose, amyloid, smooth to finely roughened (finely ridged under SEM, Pegler and Young 1972, referring to *H. erinaceus* s.str.), 5.5–6.8 × 4.5–5.6 µm. Usually growing from cracks or knot holes in living trees, recorded on *Quercus, Fagus, Platanus,* and *Acer* (Harrison 1973b).

Comments: Neither Harrison (1973b) nor Ginns (1985) knew of any confirmed records of this species from Canada. Some recent observations from southern Ontario posted to iNaturalist.ca appear plausible (e.g., Fig. 2-3E), but none are substantiated with a specimen or by DNA sequence data. One specimen in DAOM from Ontario annotated by Ginns as *H. erinaceus* was sequenced in this study and proved to be *H. americanum*. Below, I describe another vicariant of the *H. erinaceus* complex, on *Quercus garryana* from southern British Columbia, as a new species *H. oregonense* sp. nov.

Hericium cirrhatum (Pers.) Nikol., Acta Inst. Bot. Acad. Sci. USSR Plant. Crypt., Ser. II 6: 343 (1950)

≡ *Hydnum* cirrhatum Pers. [as '*cirratum*'], Neues Mag. Bot. 1: 109 (1794), nom. sanct., Fr., Syst. Mycol. 1: 411 (1821)

≡ *Creolophus cirrhatus* (Pers.) P. Karst., Meddn. Soc. Fauna Flora Fenn. 5: 42 (1879)

Holotype none; no material or plates were cited that could be available for lectotypification. A well-documented recent specimen from the collecting areas of Fries or Persoon should be designated as neotype.

Description (microscopic details from Harrison 1984): Pilei uniformly white to cream when fresh, drying grey to brown, compound, imbricate, segments broadly triquetrous to applanate, 3–10 cm broad by 1–4 cm thick at the base, surface dry and rough, usually with distinct fine spines at least toward the acute margin; lower surface covered with fine to coarse spines, terete to flattened and sometimes branched; context soft and white, sometimes drawn out into a pseudostipe to 5 × 3 cm. Basidiospores white, amyloid, broadly ellipsoid to oval, smooth, 4.0–4.5 (–4.8) × 3.2–3.5 (–4.0) µm; context and hymenium inamyloid, hymenium mostly of gloeocystidia that originate deep in the context, 6–8 µm broad.

Comments: This species, which in Europe is known mostly from *Fagus*, occurs rarely in boreal and montane North America on *Populus* (Harrison 1984; records from iNaturalist.ca), but until now there were no sequences from any North American material to assess if it is the same as the European species. I was fortunate to receive a voucher of an observation published on iNaturalist.ca from northern Alberta (iNaturalist 32648690), from which I was able to get high-quality ITS–LSU, *tef1* and *rpb2* sequence data, making this the first sequence-confirmed record of the species from North America and the first

published record of the species in Canada. Good quality photographs of this highly distinctive species support additional observations from New Brunswick (iNaturalist 89219181, 142821513), Saskatchewan (iNaturalist 95907531, 124501128), and Alberta (iNaturalist 175651143, 179090349, 183457588, 184213002).

Hericium coralloides (Scop.) Pers., Neues Mag. Bot. 1: 109 (1794)

≡ *Hydnum coralloides* Scop., *Fl. Carniol.*, Edn. 2 (Wien) 2: 472 (1772b), nom sanct., Fr., *Syst. Mycol.* 1: 408 (1821)

Holotype none; lectotype (MBT 10016573) Schaeffer, Fung. Bavar. Palat. Nasc. (Ratisbonae) 2: Tab. 142 (1763), specifically, the copy in the Bayerische Staatsbibliothek München, posted online as https://commons.wikimedia.org/wiki/File:Hericium_coralloides_(Schaeffer,_1763).jpg (Fig. 2-2A). A neotype designated by Hallenberg (1983) is not Code compliant because of the existence of original icons. I hereby designate as epitype (MBT 10016816) the collection NW-FVA 2023-90-2, on rotten log of *Fagus sylvatica*, in mixed old growth forest with *Fagus sylvatica, Quercus petraea,* and *Carpinus betulus*, Ederseerandstrasse, 34513 Waldeck, Hesse, Germany, 51.20110° N, 9.06083° E, 28 August 2023, Gitta Langer & Ewald Langer (KAS).

= *Hericium clathroides* (Pall.) Pers., Comm. Fung. Clav. (Lipsiae): 23 (1797)

≡ *Hydnum clathroides* Pall., Reise Prov. Russ. Reichs 2(2): 744 (1773), nom. sanct., Fr., Syst. Mycol. 1: 409 (1821)

Holotype none; lectotype (MBT 10016572), Pallas, Reise Prov. Russ. Reichs 2(2): Tab. K, Fig. 3 (1773) (Fig. 2-2E). This species has been listed in the synonymy of (Stalpers

1996), or as the correct name for (Maas Geesteranus 1959, 1971), *H. coralloides*. Pallas' specimen was found on wood in coniferous forest ("*in Pineto*") near the Ob River in central Siberia (Pallas 1773). However, there are multiple observations on iNaturalist of typical *H. coralloides*, often on *Betula*, from this region and the only observation on iNaturalist from Russia labelled *H. alpestre* is on *Abies* in the Ussuri region of far eastern Siberia. The illustration by Pallas (1773) is a better match for *H. coralloides* than *H. alpestre*. A collection from the Tomsk or southern Krasnoyarsk regions of Russia should be selected as epitype. Although the basionyms of both *H. coralloides* and *H. clathroides* were sanctioned by Fries (1821), *Hydnum coralloides* Scop. (1772b) is one year older than *Hydnum clathroides* Pall. (1773), so it has priority if the two are synonyms. However, a phylogeny based on ITS data (Fig. A-1) indicates considerable variation within *H. coralloides* as I am recognizing it. There is a well-supported, basal subclade including collections on *Populus* from western Norway and Bryansk Oblast, Russia, a collection from conifer wood in Sweden, and one from hardwoods in New Brunswick, Canada (Fig. A-1); should this prove to be specifically distinct through further study, the name *Hericium clathroides* may be suitable for it.

= *Hericium flagellum* (Scop.) Pers., Comm. Fung. Clav. (Lipsiae): 25 (1797)

≡ *Manina flagellum* Scop., Diss. Sci. Nat., Edn. 1: 97 (1772a) (not sanctioned by Fries)

Holotype none; lectotype (MBT 10016818) Scop., Diss. Sci. Nat., Edn 1: Tab. XI (1772a) (Fig. 2-2B); epitype (MBT 10016819) on rotten log of *Fagus sylvatica*, in mixed old growth forest with *Fagus sylvatica, Quercus petraea,* and *Carpinus betulus*, Ederseerandstrasse, 34513 Waldeck, Hesse, Germany, 51.20110° N, 9.06083° E, 28

August 2023, Gitta Langer & Ewald Langer NW-FVA 2023-90-2 (KAS). The original description and illustration emphasize the highly branched nature of the fruit body, with spines lining the undersides of the branches, characteristics of *H. coralloides* (Fig. 2-2C), not *H. alpestre.*

= *Hericium ramosum* (Bull.) Letell., Hist. Descr. Champ. (Paris): 43 (1826)

≡ *Hydnum ramosum* Bull., Herb. Fr. 9: pl. 390 (1789).

Holotype none; lectotype Bulliard, Herb. Fr. 9: pl. 390, 1789 (designated by Harrison 1973b) (Fig. 2-2F). Bulliard (1791) expressly stated that *H. ramosum* was a synonym of *H. coralloides*, citing the same plate by Schaeffer as Scopoli (1772b) had, and Fries (1821) agreed, thus designation of an epitype to settle the application of the name is unnecessary. The highly branched fruit bodies with fine short spines lining the lower sides of the branches, and the small basidiospores are diagnostic for this species. Micheli (1729) illustrated this species (as Tab. 64, Fig. 2; Fig. 2-2D), and was perhaps the first to introduce the confusion among the branched species of *Hericium* occurring in Europe on hardwoods (this species) and fir (*H. alpestre*), since he listed the hosts of the species illustrated in Tab. 64 Fig. 2 as *Ilex, Morus, Fagus,* and *Abies*. This confusion was not solved for over 250 years (Hallenberg 1983). This species is most common on hardwoods, but collections from coniferous wood in Europe are known. The distribution extends across Eurasia and in North America extends from the island of Newfoundland west to the Yukon, British Columbia and California (Harrison 1973b; Tylutki 1979; Pomerleau 1980; Ginns 1984, 1985; Arora 1986; Schalkwijk-Barendsen 1991; Hallenberg et al. 2013; Desjardin et al. 2015; this study).

Hericium erinaceus (Bull.) Pers., Comm. Fung. Clav. (Lipsiae): 27 (1797)

≡ *Hydnum erinaceus* Bull., Herb. Fr. (Paris) 1: pl. 34 (1780), nom. sanct., Fr., Syst. Mycol. 1: 407 (1821)

Holotype none; lectotype (MBT 10016574) Bulliard, Herb. Fr. (Paris) 1: pl. 34 (1780) (Fig. 2-2G). A neotype designated by Hallenberg (1983) is not Code compliant because of the existence of an original icon, but the same or another specimen could be designated as epitype if desired. This species appears to be restricted to Western Europe, where it occurs on *Fagus* and *Quercus* (Hallenberg et al. 2013; this study). I recognize vicariants in eastern North America (*H. carolinense*), western North America (*H. oregonense* sp. nov.) and Asia (*H. asiaticum*) as distinct species, albeit morphologically indistinguishable and mating compatible (Ginns 1985).

Hericium oregonense Koga & Thorn, **sp. nov.**

MB # 854007

Holotype: on *Quercus garryana*, Oak Bay, Vancouver Island, British Columbia, Canada, (approximately 48.45 N, 123.31 W), 8 Nov. 1984, R.S. Hunt and A. Hunt, DAOM 193728 (isotype, DAVP 23114), ITS–LSU sequence PP786303.

Etymology (Latin): of the oregonian ecoregion where the host, *Quercus garryana* (and other *Quercus* species further south to California) lives.

Diagnosis: Fruit body ungulate to globose, 5–20 cm broad, white at first, becoming creamy yellow, the upper surface roughly hairy and becoming brown in age, the lower surface composed of spines 1–6 cm long, 1–3 mm thick at their base and tapering to needle tips, white when young but discolouring yellow or orange-brown, with solid,

fibrous-spongy white flesh, especially tough towards base. Gloeocystidia present, up to 7 µm broad. Basidiospores broadly elliptical to subglobose, smooth to finely roughened, amyloid, 5.0–6.5 × 4.0–5.5 µm.

Ecology and distribution: On *Quercus garryana*, presumably causing a white rot, coastal southern Vancouver Island, the Gulf Islands and dryer portions of the Lower Mainland of British Columbia, Canada, and apparently occurring on this and other species of *Quercus* as well as introduced *Eucalyptus* further south through coastal Washington, Oregon and California in the United States based published and iNaturalist records as *H. erinaceus* (Harrison 1973b; Arora 1986; Desjardin et al. 2015; Siegel and Schwarz 2016; MacKinnon and Luther 2021).

Comments: I am not aware of any characters of macro- or micromorphology that reliably differentiate the members of the *H. erinaceus* species complex, which includes *H. erinaceus* s.str.*, H. asiaticum, H. carolinense*, and *H. oregonense* sp. nov. The spines of *H. oregonense* sp. nov. may be slightly longer than those of *H. carolinense*, and its spores slightly smaller than those of *H. asiaticum,* but these details should be confirmed on a larger sample. To my knowledge, there are no publicly available cultures of this species, nor studies of mating intercompatibility that have included strains of the *H. erinaceus* complex from western North America with strains from Europe or eastern North America. Nonetheless, since sequences (ITS, LSU, *tef1*, and *rpb2*) from the four geographic regions form mutually monophyletic clades, I feel that it is worthwhile distinguishing these entities at the species level to allow precise designation of the subjects of study by mushroom cultivators, biochemists, and medical researchers. According to observations on iNaturalist, members of the *H. erinaceus* complex are

relatively common across the southern United States, from Florida through Louisiana, Texas, Arizona, and California. A comprehensive analysis should be carried out of the genetic variation across the range of the complex in North America. Based on my small sample, the western *H. oregonense* sp. nov. is more closely related to European *H. erinaceus* s.str. than to *H. carolinense* of eastern North America.

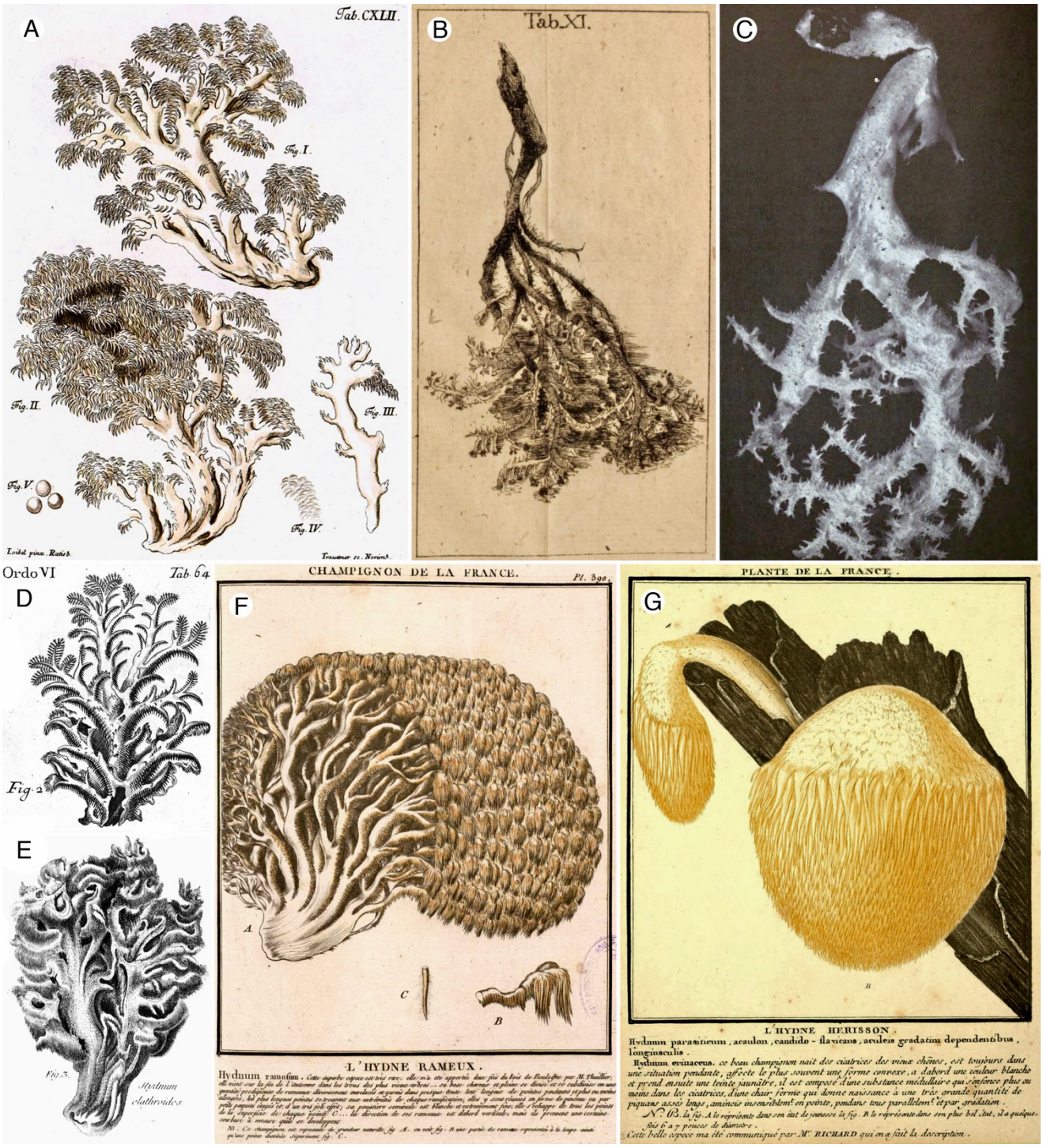
A
Tab. CXLII.
Fig. I.
Fig. II.
Fig. III.
Fig. V.
Fig. IV.
B
Tab. XI.
C
Ordo VI
Tab. 64
D
Fig. 2
E
Fig. 3.
Hydnum clathroides
CHAMPIGNON DE LA FRANCE.
Pl. 390.
F
A
C
B
L'HYDNE RAMEUX.
Hydnum ramosum.
PLANTE DE LA FRANCE.
G
B
L'HYDNE HERISSON.
Hydnum paradoxum, acaulon, candido-flavicans, aculeis gradatim dependentibus, longiusculis.
Hydnum erinaceus.

Figure 2-2. Lectotypes and other historic illustrations helping to establish the identities of key species in *Hericium*. A) The lectotype of *Hydnum coralloides* Scop., Tab. 142 in Schaeffer (1763); B) the lectotype of *Manina flagellum* Scop., Tab. 11 in Scopoli (1772a); C) *Hericium coralloides* (photo by A.H. Smith in The Michigan Botanist 12: 187; Harrison 1973b, Fig. 5, as *H. ramosum*) – note the similarity in form to B; D) Tab. 64, Fig. 2 from Micheli (1729), cited by Scopoli (1772b) and Fries (1821) as *Hydnum coralloides*; E) the lectotype of *Hydnum clathroides* Pall., Plate K, Fig. 3 (Pallas 1773); F) the lectotype of *Hydnum ramosum* Bull., Plate 390 in Herbier de la France, vol. 9 (Bulliard 1789); G) the lectotype of *Hydnum erinaceus* Bull., Plate 34 in Herbier de la France, vol. 1 (Bulliard 1780).

Key to species of *Hericium* in North America, with comments on extralimital species

1a. Fruit bodies unbranched, either globular or dimidiate 2

1b. Fruit bodies branched 3

2a (1a). Fruit bodies ovate or globular, with coarsely hairy upper surface and lower surface with spines 1-5 cm long; basidiospores 5.5–6.8 × 4.5–5.6 µm ..... *Hericium carolinense* (Fig. 2-3E)

[Morphologically identical species in western Europe, Asia and western North America are *H. erinaceus* s.str., *H. asiaticum*, and *H. oregonense* sp. nov., respectively.]

2b. Fruit bodies dimidiate, often imbricate, thin-fleshed, with hispid to spinose upper surface and short spines on the lower surface; basidiospores 4.0–4.5(–4.8) × 3.2–3.5 (–4.0) µm (Harrison 1984); on dead aspens (*Populus*) in boreal-montane regions .... *Hericium cirrhatum* (Fig. 2-4)

[In Europe, this species is found primarily on *Fagus* and reported to have basidiospores 3.5–4.5 × 2.5–3.5 µm (Stalpers 1996).]

3a (1b). Fruit bodies massive, on wood of conifers (*Abies, Tsuga*) in the humid Pacific Northwest; basidiospores 4.5–5.5 × 4.0–4.5 µm (Harrison 1973b) ..... *Hericium abietis* (Fig. 2-3C–D)

3b. Fruit bodies more highly branched, typically on hardwoods, widespread in North America 4

4a (3b). Fruit bodies with open, lacy branching, with short teeth (to 5 mm) along (beneath) the branches as well as at the tips; basidiospores 3.1–5.0 × 3.0–4.0 µm (Harrison 1973b) ..... *Hericium coralloides* (Fig. 2-3B)

[This species also occurs across Eurasia, where it usually occurs on hardwoods, but rarely also on conifers.]

4b. Fruit bodies stockier, with short branches and longer teeth (to 1 cm); basidiospores 5.5–7.0 × 4.5–6.0 µm (Harrison 1973b) ..... *Hericium americanum* (Fig. 2-3A)

[A similar and closely related species, *H. alpestre* (sometimes referred to as *H. clathroides* or *H. flagellum*) occurs on wood of conifers (*Abies*) in Europe; basidiospores are similarly (5.0–)5.5–6.5(–7.0) × 4.5–6.0(–6.5) µm (Stalpers 1996). For other similar species, see discussion above under *H. americanum*.]

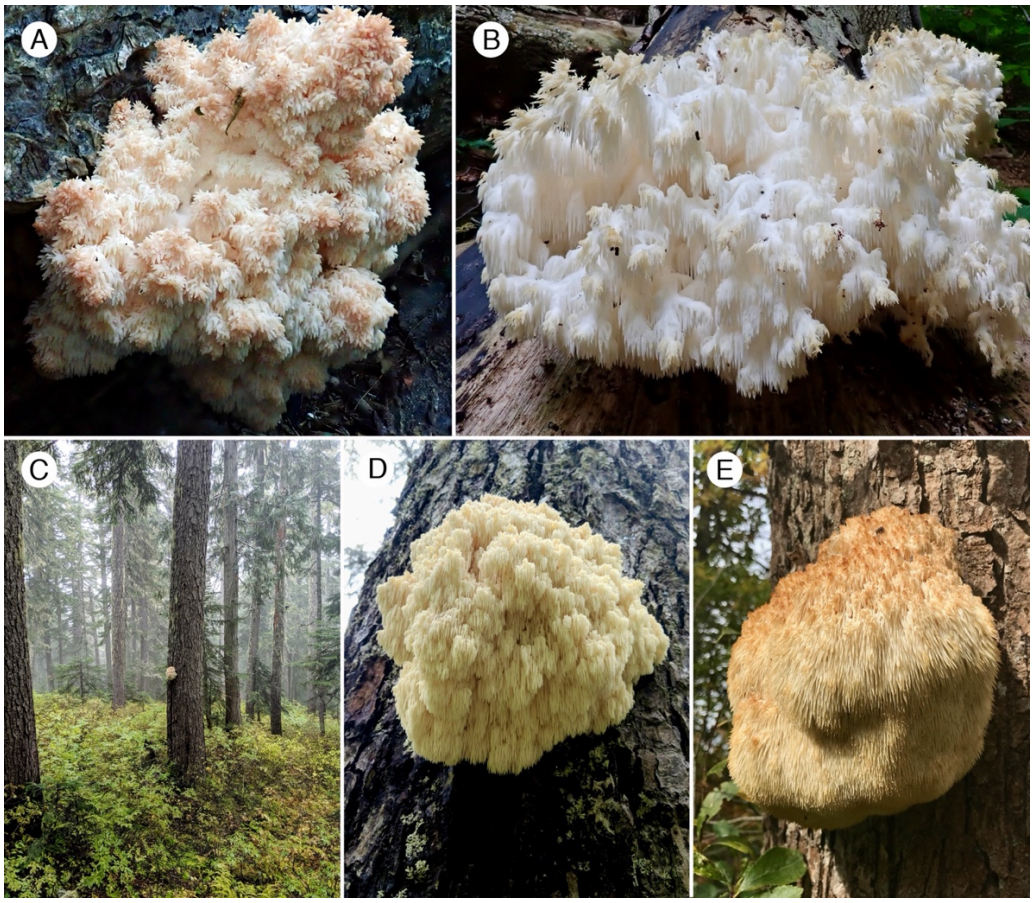

Figure 2-3. North American species of *Hericium*. A) *Hericium americanum*, on hardwood log, Point Pelee National Park, Ontario, RGT 230830/s.n. (UWO); B) *Hericium coralloides*, on fallen bitternut hickory (*Carya cordiformis*), London, Ontario, RGT 230902/02 (UWO); C) *Hericium abietis*, habitat, near Needle Peak, British Columbia, iNaturalist 176674507 (photo, Drew Brayshaw); D) *Hericium abietis*, on standing conifer, near Needle Peak, British Columbia, iNaturalist 176674507 (photo, Drew Brayshaw); E) possibly *Hericium carolinense*, on standing soft maple (*Acer* sect. *Rubra*), near Caledonia, Ontario, iNaturalist 138570613, (photo Justin Brodeur). Another Ontario specimen on the same host that looked very similar and was tentatively identified by J.H. Ginns as *H. erinaceus* (DAOM 241459) proved to be *H. americanum* by sequence data (Fig. 2-1).

Figure 2-4. *Hericium cirrhatum*, on dead standing *Populus tremuloides* L., near Last Lake, Alberta, iNaturalist 32648690 (photo Stacey Scorda). Note the fine spines on the upper surface of the dimidiate-imbricate fruit bodies, as well as the longer spore-bearing spines on the underside. Sequences of this specimen are included in Fig. 2-1.

2.6 Conclusion

Improved understanding of species delimitations and overall phylogeny in *Hericium* has potential economic consequences especially with respect to the use of *H. erinaceus* s.l. in medicinal applications and in nutraceutical markets. In certain East Asian traditions, *H. asiaticum* (as *H. erinaceus*) is highly regarded as a medicinal mushroom and research evaluating specific bioactivity profiles of its secondary metabolites over the past thirty years has demonstrated a wide range of potential health promoting properties of these mushrooms (Kawagishi et al. 1994, 1996a – b; Suruga et al. 2015; Chen et al. 2017; Corana et al. 2019). These studies have increased research efforts in natural

products discovery and have driven a growing demand for *H. erinaceus* s.l. supplement products globally (Thongbai et al. 2015). Additionally, several authors have discovered certain bioactive secondary metabolites originally isolated from *H. erinaceus* s.l. in other members of *Hericium* including *H. coralloides* (Saito et al. 1998; Wolters et al. 2015), *H. novae-zealandiae* (Chen et al. 2022), *H. alpestre* (Rupcic et al. 2018), and *Hericium* sp. WBSP8 (Song et al. 2020), which I determined as *H. americanum* according to ITS sequence data (data not shown). However, poorly defined species delineation in *Hericium* has led to misidentification of specimens used in biomedical and pharmacological studies, where researchers may characterize the biological effects of a specimen known to them by one name, but which is known to others as another. For example, Rupcic et al. (2018) evaluated the production of erinacines by *H. erinaceus* and *H. flagellum* but the latter name refers to *H. alpestre* or *H. coralloides,* depending on the species concept of the authors. Similarly, Suruga et al. (2015) evaluated the bioactivity of extracts of *H. erinaceus* and what they refer to as *H. ramosum*; again, the identity of the latter is uncertain although most likely *H. coralloides*. Multiple *Hericium* strains available from international culture collections are misidentified, listed under the identity provided by the supplier, just as is the case with sequences deposited to GenBank (Bidartondo 2008). For instance, CCBAS 654 and 837 are listed as *H. erinaceus*, but were collected on *Abies alba* and thus likely represent *H. alpestre*; whereas CCBAS 663 and 664 are listed as *H. coralloides* but are cultures of *H. abietis* (DAOMC 16601 and 22748), and CCBAS 662 is listed as *H. coralloides* but represents *H. americanum* (DAOMC 21467) (https://www.biomed.cas.cz/ccbas/fungi.htm). The problem is not new; in his studies of mating compatibility among *Hericium* strains, Ginns (1985) used two cultures sent to him

by Nils Hallenberg as *H. alpestre* that later were identified as *Dentipellis fragilis* (Pers.) Donk (Hallenberg et al. 2013). The origins of some *Hericium* strains for which sequences are available are not indicated in GenBank, with some appearing to be commercial strains from countries other than where the sequences were deposited from. I was fortunate that the majority of the strains used in the phylogenetic analyses were wild-collected and assuredly native to the regions where they grew. However, in future, the phylogeography of *Hericium* species may be obscured if non-indigenous, cultivated strains escape into the wild, as has been documented recently for another cultivated mushroom, *Pleurotus citrinopileatus* Singer (Bruce 2018). Given that there may not be barriers to interbreeding among closely related species from different continents, such escapes could also erode the genetic identities of indigenous species of *Hericium*. Evidently, misapplication of names of mushroom specimens used in pharmacological research may have consequences in future research and the present work seeks to firmly establish the nomenclatural status of *Hericium* species of high research interest. Additionally, discrimination of *H. erinaceus* s.str., *H. carolinense*, *H. oregonense* sp. nov., and *H. asiaticum* may have potential consequences in supporting breeding programs for improved strain development, where mushroom growers may more accurately focus breeding strategies on certain lineages to achieve desirable phenotypes.

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Chapter 3

3 Production of Erinacine A by *Hericium abietis*, *H. americanum*, *H. carolinense*, *H. coralloides*, and *H. erinaceus* and its Optimization in Liquid Culture

3.1 Introduction

Hericium erinaceus has long been considered a medicinal mushroom in East Asian medical traditions, and the first records of its medicinal properties were recorded in the Sui dynasty (581 – 618 CE), with written accounts of its beneficial effects to the five internal organs (Tan et al. 2024). The Compendium of Materia Medica of the Ming dynasty (1368 – 1644) also describe the benefits of *H. erinaceus* in supporting digestion (Tan et al. 2024). Under these traditional frameworks, *H. erinaceus* is still largely regarded as a medicinal mushroom that promotes gastrointestinal health generally, and it is recommended for digestive ailments (Mizuno 1999). Recent investigations have demonstrated modest support for these claims by modern scientific standards, wherein polysaccharides produced by *H. erinaceus* have shown antioxidant and anti-inflammatory activity in epithelial cell cultures of the intestine and in rodent models (Gitter et al. 2001; Jena et al. 2012; Wang et al. 2017, 2019; Hou et al. 2022; Chen et al. 2020). However, the bioactivity potential of compounds produced by *Hericium* extends beyond its effects in the gastrointestinal system as these fungi are unusually rich sources of diverse secondary metabolites. Over 80 bioactive compounds isolated from the fruit body and mycelium of these fungi have been reported with various biological activities including anti-inflammatory (Chiu et al. 2018; Wang et al. 2019), antimicrobial (Kim et al. 2000; Wong

et al. 2009), anti-cancer (Li et al. 2014), and neuroprotective (Suruga et al. 2015; Cheng et al. 2016) properties. Bioactive compounds isolated from *Hericium* have been categorized variously as erinacines (cyathane diterpenoids), hericenones (benzaldehyde derivatives), sterols, pyrones, alkaloids, and polysaccharides (Wang et al. 2015a, 2015b; Chen et al. 2017; Corana et al. 2019). Two of these classes of compounds, erinacines and hericenones, produced in the mycelium and fruit body, respectively, have generated significant research interest since their discovery in *H. erinaceus* by Kawagishi et al. (1991, 1994) in which they were shown to stimulate nerve-growth factor (NGF) secretion by mouse astroglial cells. Further study of the NGF-stimulating effects of erinacines and hericenones in animal models and human cell lines have demonstrated that while both erinacines and hericenones exhibit this effect on mouse astroglial cells, only erinacines have demonstrated this effect *in vivo* and in human neuronal cells (Shimbo et al. 2005; Mori et al. 2008; Tsai-Teng et al. 2016; Rupcic et al. 2018; Li et al. 2020; Tsai et al. 2021). Erinacine A, the main representative of erinacines, was shown to cross the blood-brain-barrier (BBB) to exert the NGF-stimulating effect in the central nervous system (CNS) of rats (Tsai et al. 2021). Penetration of the BBB by NGF-stimulating compounds is a critical requirement for a candidate therapeutic in the prevention and treatment of conditions associated with decreased NGF levels such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Adessi and Soto 2002). Additionally, erinacine A has been demonstrated to attenuate ischemia-induced reperfusion injury to neuronal cells (Lee et al. 2014), promote neurogenesis and ameliorate amyloid beta plaque formation (Tsai-Teng et al. 2016), inhibit colorectal cancer cell growth (Lu et al. 2016), and reduce metastatic activity of gastric cancer cells (Kuo et al. 2017). Erinacine A

is therefore being considered as a potential therapeutic tool in the treatment of neurodegenerative diseases and in certain cancer treatments.

Erinacines are classified as cyathane diterpenoids, a class that includes thirty-seven known compounds from the fungi *Hericium*, *Cyathus*, *Phellodon,* and *Sarcodon* (Kawagishi et al. 1994; Fang et al. 2010; Bhandari et al. 2014; Bai et al. 2015). Twenty erinacines have been reported thus far, labelled in the order of discovery as erinacines A-L, P-T, Z1, and Z2 (Kawagishi et al. 1994, 1996a–b, 2006; Kenmoku et al. 2000, 2002; Chen et al. 2016; Chiu et al. 2018; Li et al. 2018; Rupcic et al. 2018; Wei et al. 2023). Studies on the bioactivity profiles of erinacines and relative production of the several erinacines produced by *H. erinaceus* have promoted erinacine A as the erinacine of highest research interest, as the biological effects of erinacine A on neuronal cells has been studied most extensively relative to other erinacines. While the early works by Kawagishi et al. (1994) demonstrated a slightly higher NGF-secreting effect by erinacine C than erinacine A, erinacine A is produced in the highest concentrations of all erinacines by *H. erinaceus* and is therefore considered as the compound of highest clinical potential (Chiu et al. 2018).

The total synthesis of erinacine A has been described by Snider et al. (1996, 1998), however its synthesis is long, multi-step, and low yielding. The majority of studies evaluating the pharmacology of erinacine A therefore isolate it from the mycelium of *H. erinaceus*, a commercially cultivated species for which many industrial strains have been developed and are accessible through culture libraries. Several other erinacines have been discovered from wild-collected *Hericium* species including erinacine E isolated from *H. coralloides* (as *H. ramosum*) (Saito et al. 1998), erinacines A, B, E, F, G, T, and Z1 from

H. rajendrae (Wei et al. 2023), erinacines A, B, C, E, F, and Z2 from *H. alpestre* (as *H. flagellum*) (Rupcic et al. 2018), erinacine E from an unknown North American *Hericium* sp. (Song et al. 2020; identified as *H. americanum* in Chapter 2), and erinacines A-C from *Dentipellis fragilis*, a member of Hericiaceae (Ha et al. 2021). While some of these more cryptic erinacines have demonstrated similar bioactivity profiles as erinacine A, erinacine A nevertheless remains as the erinacine of highest interest due to its high concentration in mycelial extracts. However, the production of erinacine A by other species of *Hericium* is understudied and a systematic evaluation of erinacine A biosynthesis across *Hericium* has yet to be conducted.

Evidence of erinacine production by other species of *Hericium* suggests the production of erinacines may be a shared trait across the *Hericium* genus and represents an opportunity to evaluate wild collections and local species beyond the commercially available cultures for the production of this and other compounds. Bioprospecting wild-collected species is a common method for the discovery of new and known compounds in fungi, and there is no reason to believe that the biosynthesis of a given secondary metabolite is highest in a commercially cultivated species. Atila (2019) evaluated total phenolic content and antioxidant production of *Hericium* species and discovered higher values in *H. coralloides* than *H. erinaceus*. Should erinacine A continue to be of medical and economic interest, other species within the genus should be assessed for their relative production of this compound.

The first aim of this study is the evaluation of erinacine A production in wild-collected species of *Hericium*, with a focus on the species that are known from North America. The second objective is the improvement of culture conditions for the enhanced

production of erinacine A in species that demonstrate its production. This is the first report on the detection of erinacine A in the critically identified *H. abietis*, *H. americanum*, *H. carolinense*, and of *H. coralloides*, as well species-specific modifications to cultivation parameters for the improved production of erinacine A in these species and for *H. erinaceus* s.str.

3.2 Materials and Methods

3.2.1 Erinacine A Standard

To generate a reference standard of erinacine A and calibration standards for the quantification of erinacine A in crude extracts, mycelium of *H. erinaceus* s.str. (laboratory code WCHE) was grown in liquid culture. Ten 5 x 5 mm sections of colonized malt extract (ME) agar were transferred into 500 mL of autoclaved V8 liquid medium [20% V8 vegetable juice, 80% deionized (di) H_2O]. After twenty-one days of growth, the culture was homogenized with a sterilized hand blender, and 50 mL of this starter culture was used to inoculate 1L Erlenmeyer flasks containing 500 mL of autoclaved V8+ media: 20% (v/v) V8 vegetable juice, 80% (v/v) diH_2O, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 2.0 g/L yeast extract. After three weeks of growth, mycelium was harvested by filtering and dried at 50^{o}C for 48 hours for a final dry weight of 59.12 g. Erinacine A was extracted following the procedures described by Krzyczkowski et al. (2009). Mycelium was powdered and extracted by Soxhlet extraction with 80% ethanol at 80^{o}C. After 24 hours, the solvent was removed by rotary evaporation and the concentrated extract was partitioned between water and ethyl acetate (1:1). The organic layer was collected, washed with an equal volume of diH_2O saturated with NaCl and dried over anhydrous

$MgSO_4$ to remove residual H_2O, and solvent was removed by rotary evaporation. The oily extract was reconstituted in hexane and separated into fractions by silica column chromatography with hexane – ethyl acetate as the mobile phase in a gradient elution. Beginning with 100% hexane (100 mL), the polarity of the mobile phase was gradually increased by increasing the proportion of ethyl acetate in 10% increments up to 100% ethyl acetate; eleven fractions were collected, dried, and reconstituted in 50% methanol. Fractions were then diluted by 50x and analyzed by HPLC-UV-ESI/MS. Analyses were performed on an Agilent Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA), Agilent 1260 Infinity binary pump, Agilent 1260 Infinity high performance degasser, Agilent 1260 Infinity high performance autosampler, and Agilent 1290 Infinity thermostatted column compartment (Agilent Technologies, Santa Clara, CA, USA). Detection of the eluted analytes was performed at 340 nm with an Agilent 1260 Infinity variable wavelength detector (Agilent Technologies, Santa Clara, CA, USA). The HPLC was coupled to an Agilent 6230 TOF LC/MS with an electrospray ionization interface under positive ion conditions. The analytical column [Zorbax Eclipse Plus C18 column 3.0 x 100 mm, 1.8 µm particle size (Agilent Technologies, Santa Clara, CA, USA)] was maintained at 40°C; the injection volume was 5 µL with a flow rate of 0.25 mL/min. The mobile phase consisted of HPLC grade water + 0.1% formic acid (solvent A) and 90% acetonitrile + 0.1% formic acid (solvent B) eluted linearly in the following gradient: 0 – 2 min (50% B), 2 – 12 min (50% - 75% B), 12 – 13 min (75% - 100% B), 13 – 15 min isocratic at 100% B, 15 – 16 min (100% - 50% B).

Fractions ten and eleven demonstrated peaks in the total ion chromatogram at 10.12 minutes and 10.10 minutes associated with diagnostic erinacine A ion fragment at *m/z*:

301.215, and corresponding peaks in the UV spectrum at 9.99 minutes and 9.98 minutes, respectively. Erinacine A is known to absorb UV at 340 nm, and the *m/z*: 301.215 generated by loss of sugar from the parent compound has been shown to be a reliable indicator of erinacine A (Tsai et al. 2021). To remove co-eluents from the extracts, fractions ten and eleven were pooled and cleaned over a silica column with chloroform:methanol (95:5); ten fractions of 25 mL each were collected and analyzed by HPLC-UV-ESI/MS according to the parameters described above. Fractions three to seven demonstrated evidence for the presence of erinacine A by a *m/z*: 301.215 peak in the extracted ion chromatogram (EIC) at 10.07 minutes and associated UV absorbance at 9.98 minutes. These fractions were pooled and purified with a solid phase extraction using a 75 mL C18 Extra-Clean SPE cartridge (Grace Discovery Science, Deerfield, IL, USA). The column was washed with 100% acetonitrile, conditioned and loaded with 50% acetonitrile, and the sample was eluted by increasing the proportion of acetonitrile in 10% increments to a final mobile phase of 100% acetonitrile. Six fractions were collected and analyzed by HPLC-UV-ESI/MS. Clean peaks in the chromatograms consistent with previous erinacine A spectral data were detected in the fraction eluted with 50% acetonitrile, with retention times shifted to 10.74 minutes in the UV spectrum and 10.90 minutes in the EIC due to column replacement. This fraction was then dried and reconstituted in 50% methanol to make a 1.00 mg/mL stock erinacine A solution that was used to prepare calibration standards from 0.01 mg/mL to 1.0 mg/mL (N=3). To generate a calibration curve, peak area from the peak in the EIC was plotted against concentration (mg/mL) and linear regression analysis was used to establish a line of best fit.

From the peak area of erinacine A in subsequent analyses of crude extracts, the equation of the line and the dilution factors from the preparation of analytical samples were used to calculate total erinacine A (µg/mL). In some samples, the peak area detected was outside the linear range of the calibration curve, and are considered approximate estimates of the concentration of erinacine A.

3.2.2 Bioprospecting *Hericium* spp. for Erinacine A production

Of the *Hericium* species that exist globally, *H. erinaceus, H. rajendrae*, and *H. alpestre* (referred to as *H. flagellum*) are the only species in which erinacine A has been confirmed (Kawagishi et al. 1994; Rupcic et al. 2018; Wei et al. 2023). To evaluate the production of erinacine A in North American species, thirty-three *Hericium* strains representing *H. americanum* (15), *H. abietis* (7), *H. carolinense* (6), *H. coralloides* (4), as well as a single strain from the European species *H. erinaceus* s.str. were examined; a list of strains used in this study is presented in Table 3-1. In replicates of 3 cultures for each strain, a 5 x 5 mm disk of colonized ME agar was transferred to 125 mL Erlenmeyer flasks containing 50 mL of autoclaved V8+ media. The cultures were incubated at room temperature at 100 rpm for 14 days, after which the mycelium was collected by filtering through 50 mm Whatman cellulose filter paper under vacuum, washed with distilled water, ground in liquid nitrogen, and lyophilized for 24 hours. Lyophilized mycelium was extracted with 70% ethanol in a water bath at 50^{o}C for 24 hours (Gerbec et al. 2015), centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected in fresh 1.5 mL centrifuge tubes. Extracts were dried under N_2 using a Rapidvap Vertex Dry Evaporator (Labconco, MO, USA), reconstituted in 1 mL 50% methanol, centrifuged at 10,000 rpm for 10 minutes, and 200 µL of the supernatant was transferred into LC-MS

vials. The samples were analyzed by HPLC-UV-ESI/MS operating according to the conditions described earlier, and spectral data was analyzed for the presence of erinacine A. The peak area of the ion mass fragment (*m/z*: 301.215) was used to calculate total erinacine A produced by each strain using the calibration curve.

Table 3-1. Mycelial cultures used in the bioprospecting experiment, including species names, strain voucher or culture numbers, and geographic origin.

Species	Strain	Source	Origin	Voucher Number
H. abietis	HB1[1]	DAOMC	British Columbia	DAOMC 17054[2]
	HB2	DAOMC	British Columbia	DAOMC 251004
	HB3	DAOMC	British Columbia	DAOMC 196447
	HB4	DAOMC	British Columbia	DAOMC 22748
	HB5	DAOMC	British Columbia	DAOMC 16601
	HB6	DAOMC	British Columbia	DAOMC 251005
	HB7	DAOMC	British Columbia	DAOMC 251006
H. americanum	HA1	Wild Collected	Ontario	UWO-F1485[3]
	HA2	Wild Collected	Ontario	UWO-F1486
	HA3	Wild Collected	Ontario	UWO-F1487
	HA5	Wild Collected	Ontario	UWO-F1488
	HA6	Wild Collected	Ontario	UWO-F1489
	HA8	Wild Collected	Ontario	UWO-F1490
	HA9	Wild Collected	Ontario	UWO-F1491
	HA10	Wild Collected	Ontario	UWO-F1492
	HA11	Wild Collected	Ontario	UWO-F1493
	HC3	Wild Collected	Ontario	UWO-F1482
	HC4	Wild Collected	Ontario	UWO-F1483
	HC5	Wild Collected	Ontario	UWO-F1484
	WCHA	Wild Collected	Ontario	NA
	HAX1	DAOMC	Ontario	DAOMC 21467
	HAX2	DAOMC	Pennsylvania	DAOMC 251011
H. carolinense	HE1	DAOMC	Michigan	DAOMC 251029
	HE2	DAOMC	Maryland	DAOMC 251034
	HE3	DAOMC	Maryland	DAOMC 251033
	HE4	DAOMC	Georgia	DAOMC 251031
	HE5	DAOMC	Virginia	DAOMC 196448
	HE6	DAOMC	Pennsylvania	DAOMC 251030
H. coralloides	HC2	Wild Collected	Ontario	UWO-F1481
	HCX1	DAOMC	Michigan	DAOMC 251025
	HCX3	DAOMC	Virginia	DAOMC 251017
	HCNB	Wild Collected	New-Brunswick	RGT 220816/29
H. erinaceus s.str.	WCHE	Commercial Strain	Unknown	NA

[1]Laboratory culture code

[2]Culture code from the Collection of Fungal Cultures (DAOMC)

[3]Voucher number in the Dr. Laurie L. Consaul Herbarium, London, Canada (UWO)

3.2.3 Experiment 1 – Effect of Agitation Regime on Erinacine A

Based on the results from the previous experiment where each of the strains of *H. abietis* (7), *H. americanum* (15), *H. carolinense* (6), *H. coralloides* (4), and *H. erinaceus* s.str. (1) were evaluated by HPLC-UV-ESI/MS for erinacine A production, the strain producing the highest total erinacine A content was selected from each species for liquid culture medium improvement. The redescription of the *H. erinaceus* species complex (Chapter 2) was not completed until after the bioprospecting experiment; *H. erinaceus* was therefore treated in the broad sense without distinguishing between *H. erinaceus* s.str. and *H. carolinense*. The first variable assessed for its effect on erinacine A production was the effect of the agitation regime for each of the chosen strains, of which seven treatments were evaluated: 0 rpm for 5 days, 0 rpm for 10 days, 75 rpm for 5 days, 75 rpm for 10 days, 150 rpm for 5 days, 150 rpm for 10 days, and a two-stage regime where cultures were incubated at 150 rpm for 5 days followed by a static incubation (0 rpm) for 5 days. For each strain, a starter culture was prepared by transferring three 5 x 5 mm sections of colonized ME agar at the leading edge of the mycelium into 100 mL of an autoclaved glucose-yeast extract (GYE) medium with the following composition: 30 g/L glucose, 2 g/L yeast extract, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, pH 5.0. Starter cultures were incubated in 250 mL beakers at room temperature for ten days at 100 rpm. Each starter culture was homogenized with a sterilized hand blender and 5 mL was transferred to 125 mL Erlenmeyer flasks containing 50 mL of autoclaved GYE medium. Cultures from each species were subjected to each agitation treatment in replicates of three. Once the incubation period was completed, the mycelium in each flask was harvested by filtering through 50 mm Whatman cellulose filter paper, rinsed with diH_2O, ground in liquid

nitrogen, and lyophilized for 24 hours. At this stage, mycelium biomass yield as dry mycelium weight was recorded. Crude extracts were prepared for each sample using a 24-hour ethanol extraction protocol according to Gerbec et al. (2015); lyophilized tissue was extracted with 70% ethanol for 24-hours in a water bath at 50ºC. After extraction, the tubes containing mycelium and ethanolic extracts were centrifuged at 10,000 rpm for 10 minutes and the supernatant was transferred to fresh 1.5 mL Eppendorf tubes. Ethanolic extracts were dried under N_2 and reconstituted in 100 µL 50% methanol, centrifuged at 10,000 rpm for 10 minutes, and 40 µL of the supernatant was transferred into LC-MS vials with micro-inserts. The samples were placed onto the HPLC-UV-ESI/MS operating according to the conditions described earlier, and spectral data was analyzed for the presence and concentration of erinacine A in each extract. The agitation regime resulting in highest erinacine A production for each species was considered the optimal agitation regime and was implemented in following experiments.

3.2.4 Experiment 2 – Effect of Carbohydrate on Erinacine A

Based on the results from experiment 1, each species' optimal agitation regime was implemented for the evaluation of the effect of carbohydrate source on erinacine A production. Three types of carbohydrates were evaluated: glucose, sucrose, and malt extract (ME). The effect of carbohydrate source on erinacine A production was determined by substituting glucose with each carbohydrate in the GYE medium in the concentration of 30 g/L. A starter culture of GYE medium was prepared for each species as described in section 3.2.3. After ten days of incubation at 100 rpm, 5 mL of the homogenized starter culture was distributed into 125 mL Erlenmeyer flasks containing 50 mL of autoclaved GYE medium modified with the specific carbohydrate source and

incubated under the agitation regime determined to be optimal for each species in experiment 1. All cultures were incubated at room temperature. After ten days, the mycelium in each flask was harvested and crude extracts were prepared for analysis by HPLC-UV-ESI/MS as described earlier, and the concentration of erinacine A produced by each treatment was calculated. The carbohydrate resulting in highest erinacine A production for each species was considered the optimal carbohydrate source and was implemented in following experiments.

3.2.5 Experiment 3 – Plant Oil Supplementation

The final culture condition to be evaluated was the supplementation of the liquid medium with a plant oil. Three plant oils were evaluated: olive oil, canola oil, and corn oil. As described previously, 5 mL of homogenized starter culture of GYE medium from each species was used to inoculate 125 mL Erlenmeyer flasks containing 50 mL of medium consisting of the carbohydrate production the highest erinacine A for each species, determined in experiment 2, supplemented with 1% (v/v) of either olive oil, canola oil, or corn oil (N = 3), and incubated under the agitation regime producing the highest erinacine A determined in experiment 1. After the incubation period, mycelium was harvested and mycelial extracts were prepared for analysis by HPLC-UV-ESI/MS.

3.2.6 Experiment 4 – Final Comparison

To evaluate the effect of modifications made to the liquid culture media on erinacine A production for each species, the medium optimized for agitation regime, carbohydrates, and plant oil supplementation (experiments 1 – 3) was compared to the unmodified GYE medium (30 g/L glucose, 2.0 g/L yeast extract, 1.0 g/L KH_2PO_4, 0.5 g/L

$MgSO_4$, pH 5.0) directly. Cultures grown in the unmodified GYE medium were incubated for 10 days at 100 rpm. Based on observations of rapid mycelial growth of several species in a V8 liquid medium, which is commonly used in our lab, a third treatment of V8 (20% V8 juice, 80% diH_2O) liquid medium was also included in the final analysis. In each treatment, the liquid medium (50 mL) was inoculated with 5 mL of homogenized starter GYE culture. For each species, cultures for each treatment were prepared in triplicate, and mycelial extracts were prepared and analyzed according to the methods described above.

3.2.7 Statistics

In the bioprospecting experiment, the production of erinacine A (µg/mL) by different strains of the same species was evaluated. For experiments 1 – 4, the effect of the different treatments on erinacine A production (µg/mL) was evaluated within each species. Each dataset was subjected to a Shapiro-Wilk test of normality and to Levene's test for equal variances to assess whether the data met the assumptions of normal distribution and homogeneity of variances required for a one-way analysis of variance (ANOVA). Datasets that met both assumptions ($p > 0.05$), in either their raw or logarithmically transformed forms, were subjected to one-way ANOVAs and Tukey HSD post-hoc tests ($p = 0.05$). Non-parametric datasets that did not meet ANOVA assumptions were evaluated by Kruskal-Wallis test and Dunn's test as non-parametric equivalents to ANOVA and post-hoc tests. The experimental results are expressed as means $\pm$ SE of triplicates. Statistical analyses were performed in Rstudio version 2023.09.1+494.

3.3 Results

3.3.1 Erinacine A Isolation

A total of 7.0 mg of erinacine A was isolated from the mycelium (59.12 g dried weight) of *H. erinaceus* s.str. (WCHE). The identity of erinacine A was confirmed by HPLC-UV-ESI/MS, where the extract, in a 1.0 mg/mL concentration, produced a highly resolved peak at 10.74 minutes in the UV spectrum at 340 nm, with a corresponding peak of *m/z*: 301.215 in the EIC at 10.90 minutes, characteristic of erinacine A (Krzyczkowski et al. 2010; Chiu et al. 2018; Tsai et al. 2021) (Fig. 3-1, 3-2). In these experiments, the ion mass fragment of *m/z*: 301.215 in the EIC was a more reliable signal for erinacine A than the parent ion (*m/z*: 433.5) and was therefore used as the primary signal for erinacine A identification. The peak areas from the calibration standards were used to produce a calibration curve in the range of 0.05 to 1.0 mg/mL ($R^2 = 0.9963$) (Fig. 3-3). In subsequent experiments, erinacine A (μg/mL culture medium) was calculated from the peak area using the equation of the line and dilution factors used in the preparation of analytical samples ($y = 530911x – 13701$) (Fig. 3-3).

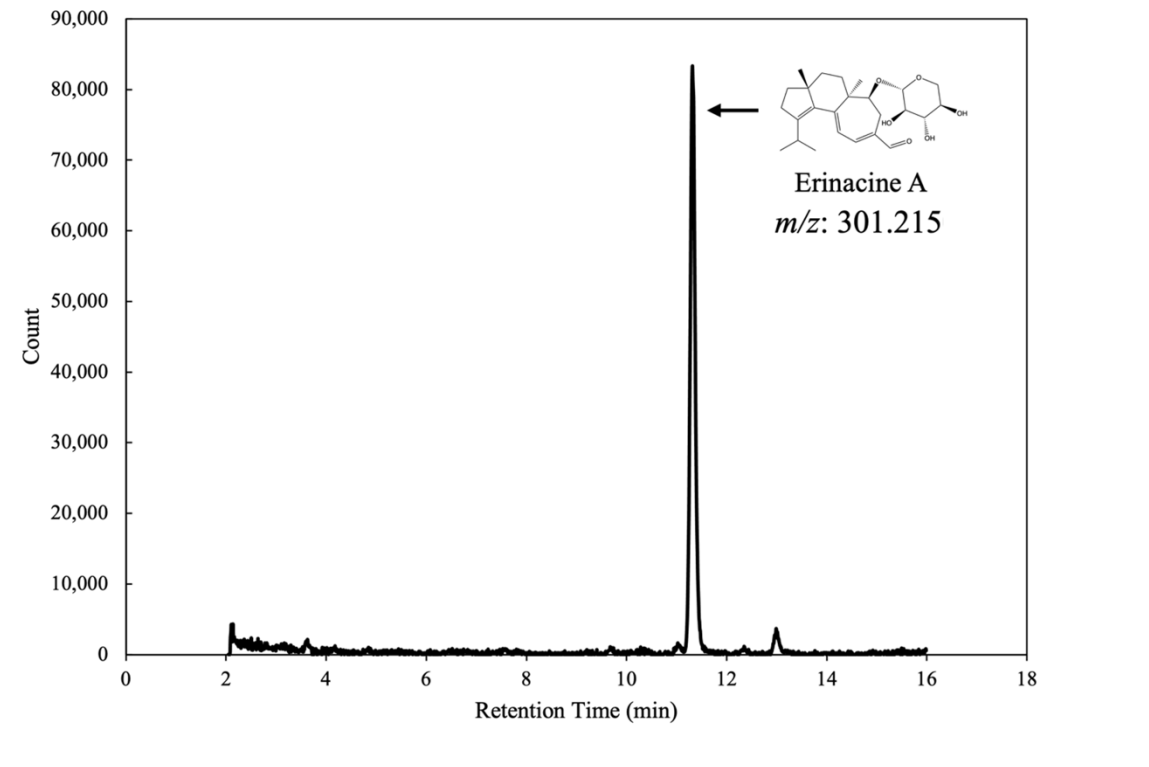

Figure 3-1. Extracted ion chromatogram of erinacine A standard (1.0 mg/mL) isolated from *H. erinaceus* in positive ion mode by HPLC-UV-ESI/MS. The major peak is generated by the ion fragment of erinacine A after the loss of the D-xylose moiety at a *m/z*: 301.215. The intact structure of erinacine A ($C_{25}H_{36}O_6$, 432.5 g/mol) generates a small peak at a *m/z*: 433.5 (not shown).

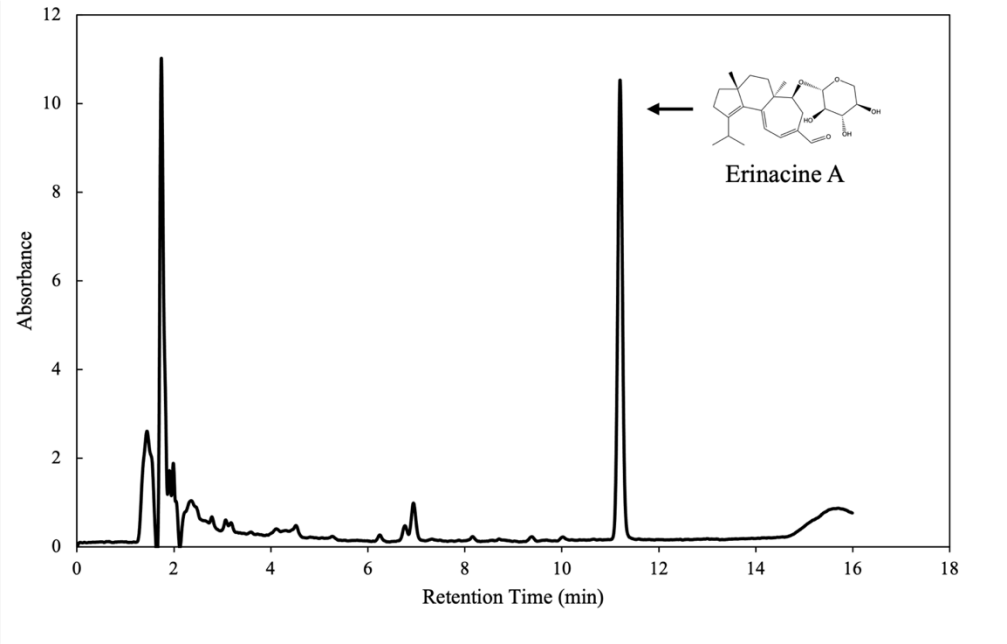

Figure 3-2. Total UV chromatogram of erinacine A standard (1.0 mg/mL) by HPLC-UV-ESI/MS at 340 nm.

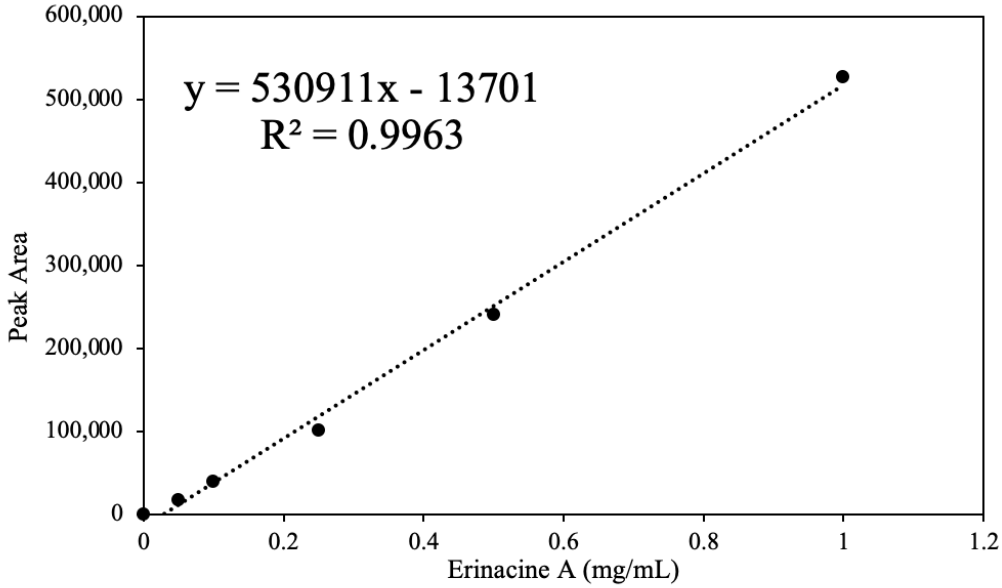

Figure 3-3. Calibration curve of erinacine A from 0.0 to 1.0 mg/mL, produced from peak areas of the ion fragment (*m/z*: 301.215) detected at ~10.9 min. Points represent mean peak area of each calibration standard (N =3).

3.3.2 Bioprospecting *H. abietis, H. americanum, H. coralloides*, and *H. erinaceus* for Erinacine A

The mycelium of strains of *H. abietis, H. americanum*, *H. carolinense, H. coralloides*, and *H. erinaceus* s.str. were grown in V8+ liquid culture for 14 days. The complete analysis included 7 strains of *H. abietis*, 15 strains of *H. americanum*, 6 strains of *H. carolinense,* 4 strains of *H. coralloides*, and 1 strain of *H. erinaceus* s.str. The bioprospecting experiment was conducted prior to the redescription of the *H. erinaceus* species complex (Chapter 2). Strains of *H. carolinense* were then considered to be conspecific with *H. erinaceus* s.str. and were treated as such in the experimental design, and discrimination of *H. carolinense* and *H. erinaceus* s.str. was only possible in hindsight. The results of the bioprospecting experiment are provided in Fig. 3-4. Erinacine A was detected in each of the 7 strains of *H. abietis*, 13 of the 15 strains of *H. americanum*, 2 of the 4 strains of *H. coralloides*, in 5 of the 6 strains of *H. carolinense*, and in the single strain of *H. erinaceus* s.str.

In *H. abietis*, strain HB5 produced the highest mean concentration of erinacine A of 2.82 $\pm$ 2.07 µg/mL of culture medium or 2.45 $\pm$ 1.19 µg/mg dried biomass, however no significant differences were found between HB5 and any other strains of *H. abietis* ($p = 0.13$, $F = 2.03$) (Table B-1; Fig. 3-4). For *H. americanum*, the highest concentration of erinacine A was detected in HAX1 at 0.44 $\pm$ 0.12 µg/mL culture medium or 0.41 $\pm$ 0.18 µg/mg dried biomass; HAX1 produced significantly more erinacine A than HC4, HC5, HA1, and HA9 ($p = 0.0089$, $\eta^2 = 0.82$) (Table B-1; Fig. 3-4). For *H. coralloides*, HCX3 produced the highest concentrations of erinacine A at 0.45 $\pm$ 0.30 µg/mL culture medium

or 0.30 ± 0.18 µg/mg dried biomass; HCX3 produced significantly more erinacine A than HC2 and HCNB ($p = 0.033$, $\eta^2 = 0.95$) (Table B-1; Fig. 3-4). Finally, WCHE produced the highest concentration of erinacine A at 6.12 ± 1.29 µg/mL culture medium or 5.31 ± 0.88 µg/mg dried biomass, which is significantly more than HE1, HE2, HE4, and HE5 ($p = 0.0055$, $\eta^2 = 0.88$) (Table B-1; Fig. 3-4). For each species, the strain that produced the highest concentration of erinacine A (µg/mL) was selected for the optimization of culture medium in the following experiments.

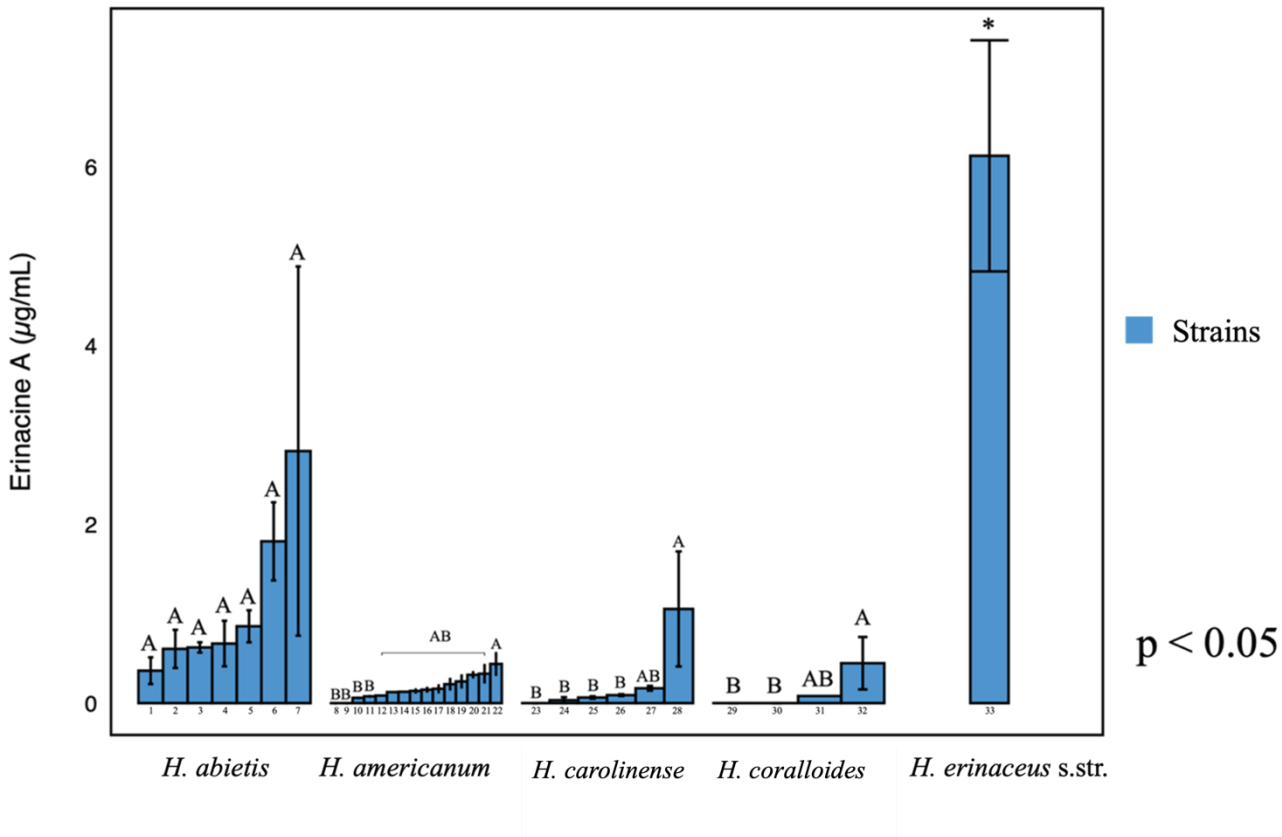

Figure 3-4. Bioprospecting mycelial extracts of strains of *Hericium abietis*, *H. americanum*, *H. carolinense, H. coralloides*, and *H. erinaceus* s.str. for the production of erinacine A by HPLC-UV-ESI/MS. Presence of erinacine A was determined from the peak area in the EIC of *m/z*: 301.215 at ~10.9 minutes, and concentration calculated using the line of best fit of the calibration curve. For each species, individual bars represent different strains from *H. abietis* (1 = HB3, 2 = HB4, 3 = HB1, 4 = HB7, 5 = HB6, 6 = HB2, 7 = HB5), *H. americanum* (8 = HA1, 9 = HC4, 10 = HA9, 11 = HC5, 12 = HA3, 13 = HA10, 14 = HA8, 15 = HAX2, 16 = HA11, 17 = WCHA, 18 = HC3, 19 = HA2, 20 = HA5, 21 = HA6, 22 = HAX1), *H. carolinense* (23 = HE1, 24 = HE4, 25 = HE5, 26 = HE2, 27 = HE3, 28 = HE6), *H. coralloides* (29 = HC2, 30 = HCNB, 31 = HCX1, 32 = HCX3), and *H. erinaceus* s.str. (33 = WCHE); mycelial cultures were prepared in triplicate. Data presented as mean ± SE. * Peak area outside linear range of calibration

curve. Letters indicate significant differences ($p < 0.05$) relative to the highest producing strain for each species.

3.3.3 Experiment 1 – Optimization of the Agitation Regime

The strain that produced the highest concentration of erinacine A (HB5, HAX1, HCX3, and WCHE) as identified in the previous experiment, was chosen as a representative strain for each species for the optimization of culture medium conditions. As representatives for each species, each strain will henceforth be referred to as *H. abietis* (HB5), *H. americanum* (HAX1), *H. coralloides* (HCX3), and *H. erinaceus* s.str. (WCHE). The first condition evaluated for the optimization of culture medium for erinacine A production was the effect of the agitation regime. The agitation regime is a critical component in the SLCF of fungi for both biomass and metabolite production as the culture must be sufficiently agitated to ensure homogenous nutrient distribution, mass, and heat transfer, but not so high that the mycelium will experience decreased production from excessive shear stress (Kwon et al. 2009). The results of experiment 1 are presented in Fig. 3-5.

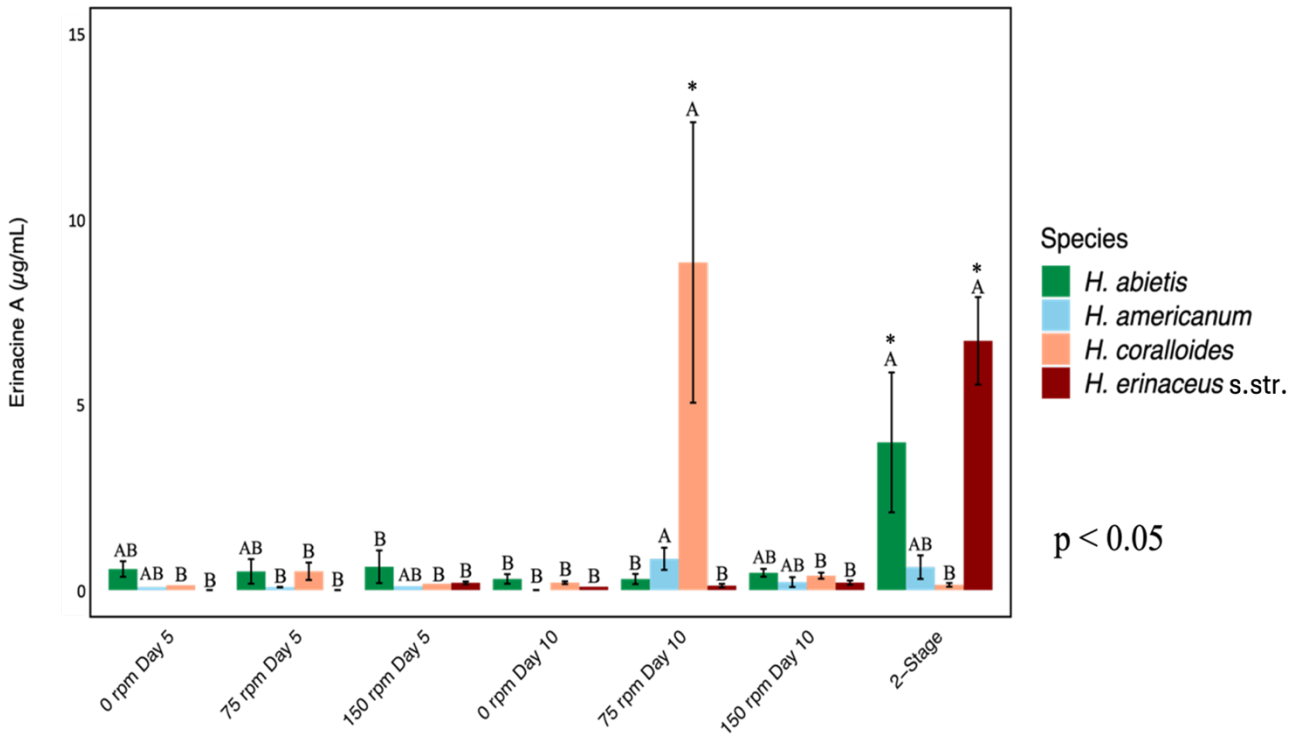

Figure 3-5. Effect of agitation regime on the production of erinacine A by mycelial cultures of *Hericium abietis*, *H. americanum*, *H. coralloides*, and *H. erinaceus* s.str. Presence of erinacine A determined from the peak area in the extracted ion chromatogram of *m/z*: 301.215 at ~10.9 minutes, and concentration calculated using the line of best fit of the calibration curve. Mycelial cultures were prepared in triplicate, data presented as mean ± SE. Significance codes convey within-species comparisons only. * Peak area outside linear range of calibration curve.

Both *H. abietis* and *H. erinaceus* s.str. produced the most erinacine A under a two-stage agitation regime, at concentrations of 3.99 ± 1.89 µg/mL and 6.73 ± 1.18 µg/mL, respectively. For *H. abietis*, two-stage agitation produced significantly more erinacine A than 0 rpm for 10 days, 150 rpm for 5 days, and 75 rpm for 10 days ($p = 0.031$, $F = 3.39$)

(Fig. 3-5). For *H. erinaceus* s.str.*,* the two-stage agitation regime produced significantly more erinacine A than all other agitation regimes ($p = 1.12e-08$, $F= 116.2$) (Fig. 3-5). An agitation regime of 75 rpm for 10 days resulted in the highest erinacine A concentration for *H. americanum* at 0.84 ± 0.30 µg/mL, which was significantly more than 0 rpm for 10 days and 75 rpm for 5 days ($p = 0.013$, $\eta^2 = 0.85$) (Fig. 3-5). For *H. coralloides,* the agitation regime of 75 rpm for 10 days also resulted in the highest erinacine A concentration at 8.85 ± 3.79 µg/mL, which was significantly higher than all other agitation regimes ($p = 6.21e-05$, $F = 16.64$) (Fig. 3-5). For each species, the agitation regime that resulted in the highest erinacine A concentration was implemented in the subsequent experiments.

3.3.4 Experiment 2 – Carbohydrate Optimization

The second culture medium condition to be evaluated for its effect on erinacine A production was the carbohydrate source used. In studies of SLCF medium modification of fungi, the type and complexity of carbohydrate used is critical as it is the primary source of nutrition for the fungus. While some have shown that simple carbohydrates result in the rapid growth of mycelial tissues (Wu et al. 2003; Krzyczkowski et al. 2010), others consider complex carbohydrates as optimal sources of carbon to support the biosynthesis of secondary metabolites (Wolters et al. 2015). Three carbohydrates of ranging complexities were evaluated: glucose, sucrose, and ME at a concentration of 30 g/L added to a YE medium (30 g/L carbohydrate, 2 g/L yeast extract, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, pH 5.0) under the optimal agitation regime identified for each species in experiment 1. The results of this experiment are presented in Fig. 3-6.

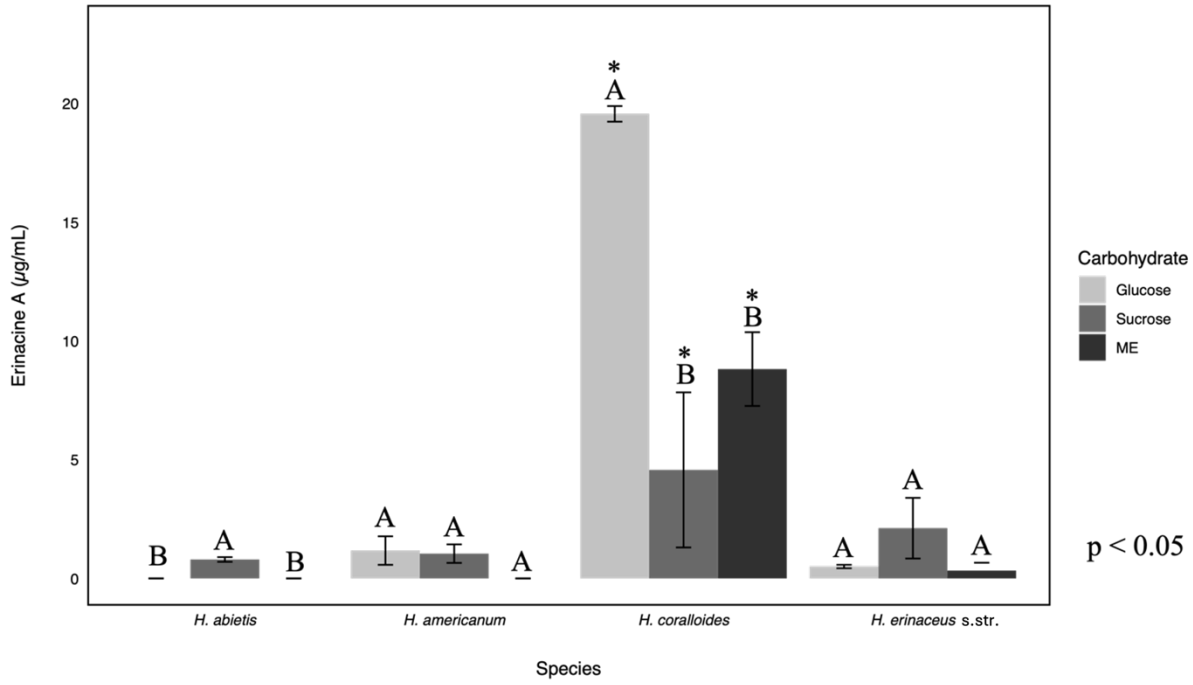

Figure 3-6. Effect of glucose, sucrose, and malt extract (ME) on the production of erinacine A by mycelial cultures of *Hericium abietis*, *H. americanum*, *H. coralloides*, and *H. erinaceus* s.str. Presence of erinacine A determined from the peak area in the extracted ion chromatogram of *m/z*: 301.215 at ~10.9 minutes, and concentration calculated using the line of best fit of the calibration curve. Mycelial cultures were prepared in triplicate, data presented as mean ± SE. Significance codes convey within-species comparisons only. * Peak area outside linear range of calibration curve.

The liquid medium containing glucose resulted in the highest erinacine A production in *H. coralloides,* at a concentration of 19.57 ± 0.33 µg/mL ($p = 0.0059$, $F = 13.6$) (Fig. 3-6). The medium containing sucrose resulted in the highest erinacine A concentration for *H. abietis* at 0.80 ± 0.10 µg/mL ($p = 0.022$, $\eta^2 = 0.94$) (Fig. 3-6). The effect of carbohydrate source on erinacine A production was not significant for *H. americanum* ($p = 0.17$, $F = 2.42$) or *H. erinaceus* ($p = 0.27$, $F = 1.67$) (Fig. 3-6). For each

species, both the agitation regime and the carbohydrate resulting in the highest erinacine A concentration were implemented in the following experiments.

3.3.5 Experiment 3 – Supplementation of Liquid Culture with a Plant Oil

The supplementation of liquid culture media with plant oils is used in the SLCF of fungal mycelium as they have demonstrated stimulatory effects on the biosynthesis of metabolites (Hsieh et al. 2008; Zhang et al. 2019). However, the majority of articles describing the beneficial effects of plant oils and fatty acids on biosynthesis have targeted the production of polysaccharides, and there is limited knowledge on the effects of plant oils on non-polysaccharide secondary metabolite production in fungi. In this experiment, the effect of the addition of three plant oils (olive oil, canola oil, corn oil) at a concentration of 1% (v/v) on erinacine A production by *Hericium* spp. were evaluated. The results of experiment 3 are presented in Fig. 3-7.

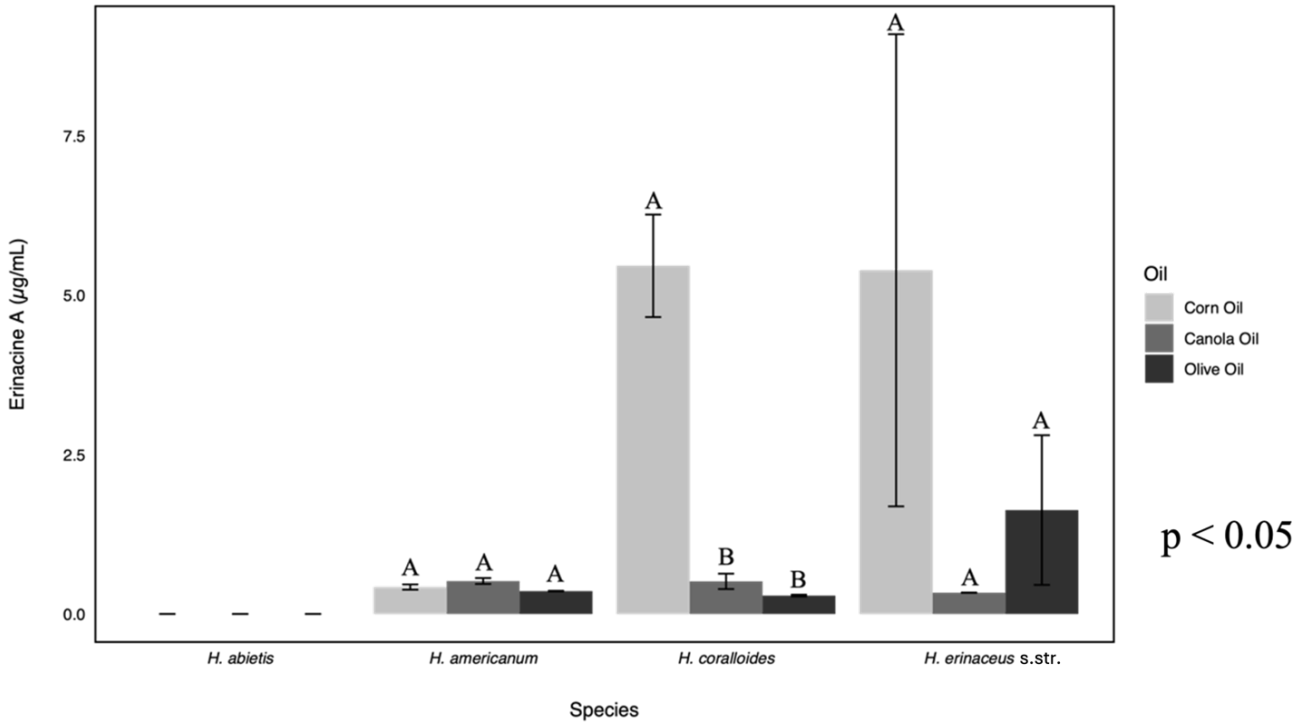

Figure 3-7. Effect of corn oil, canola oil, and olive oil on the production of erinacine A by mycelial cultures of *Hericium abietis*, *H. americanum*, *H. coralloides*, and *H. erinaceus* s.str. Presence of erinacine A determined from the peak area in the extracted ion chromatogram of *m/z*: 301.215 at ~10.9 minutes, and concentration calculated using the line of best fit of the calibration curve. Mycelial cultures were prepared in triplicate, data presented as mean ± SE. Significance codes convey within-species comparisons only.

Hericium coralloides produced the most erinacine A in the medium supplemented with corn oil, at 5.46 ± 0.80 µg/mL ($p = 0.00037$, $F = 38.79$) (Fig. 3-7). The choice of added plant oil had no significant effect on erinacine A production in *H. americanum* ($p = 0.053$, $F = 4.98$) or in *H. erinaceus* ($p = 0.32$, $F = 1.37$) (Fig. 3-7). Finally, all three plant oils inhibited the production of erinacine A in *H. abietis*; no peaks corresponding with erinacine A were detected in either the UV spectrum or EIC. The optimal plant oil

treatment for each species as identified in this experiment, together with the optimal agitation regime and carbohydrate source were combined in a final experiment to evaluate the production of erinacine A in the optimized medium compared to the unmodified GYE medium and V8 liquid medium.

3.3.6 Experiment 4 – Optimized Medium vs. GYE vs. V8

To evaluate the effect of GYE medium optimization on erinacine A production, each species was grown in a basic GYE medium incubated for 10 days at 100 rpm and in the optimized medium conditions. The optimized medium conditions were as follows: 75 rpm for 10 days, sucrose, and canola oil (*H. americanum*); two-stage agitation, sucrose, and no plant oil (*H. abietis*); 75 rpm for 10 days, glucose, and corn oil (*H. coralloides*); two-stage agitation, sucrose, and corn oil (*H. erinaceus* s.str.). A third treatment of V8 liquid medium (20% V8 juice, 80% diH_2O) was prepared for each species as an additional treatment. The results of this final comparison are presented in Fig. 3-8.

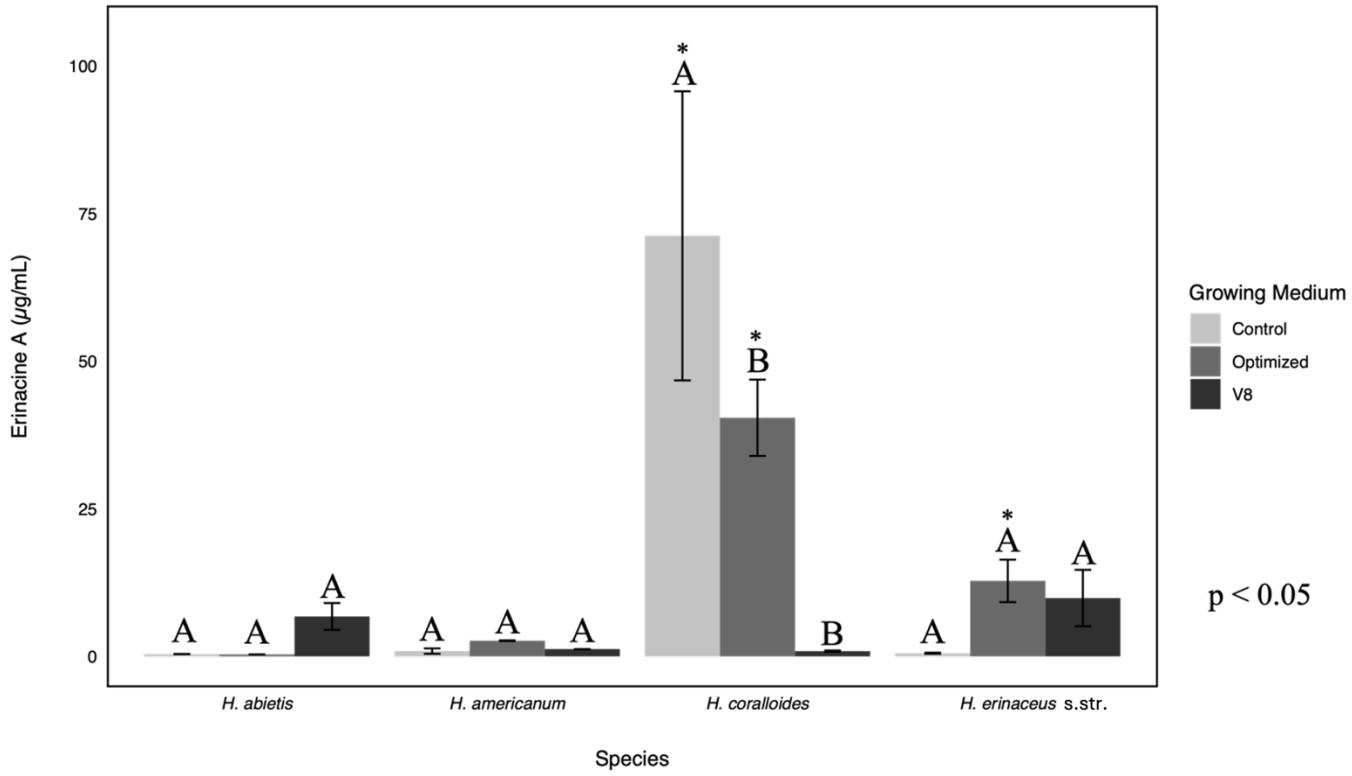

Figure 3-8**.** Erinacine A production by mycelial cultures of *Hericium abietis*, *H. americanum*, *H. coralloides*, and *H. erinaceus* s.str. in an unmodified glucose-yeast extract (GYE) medium, the optimized medium as determined by Experiments 1 – 3, and a V8 liquid medium. Detection of erinacine A determined from the peak area in the extracted ion chromatogram of *m/z*: 301.215 at ~10.9 minutes, and concentration calculated using the line of best fit of the calibration curve. Mycelial cultures were prepared in triplicate, data presented as mean ± SE. Significance codes convey within-species comparisons only. * Peak area outside linear range of calibration curve.

The highest concentration of erinacine A was detected in the unmodified GYE medium for *H. coralloides* (71.22 ± 24.48 µg/mL), which was significant compared to the V8 medium ($p = 0.039$, $F = 5.82$) but not in comparison the optimized medium (Fig. 3-8). The choice of medium had no significant effect on erinacine A production by either

H. abietis ($p = 0.39$, $F = 1.58$), *H. americanum* ($p = 0.061$; $\eta^2 = 0.60$), or *H. erinaceus* s.str. ($p = 0.10$, $F = 3.44$) (Fig. 3-8).

3.4 Discussion

3.4.1 Erinacine A Production by *Hericium*

Hericium erinaceus has commonly been used as the source of erinacine A in studies of its biological effects wherein methods for its isolation allow researchers to reliably obtain the compound from *H. erinaceus* mycelium, for which culture materials are widely available. Slight modification of the methods described by Krzyczkowski et al. (2009) for its isolation produced a relatively pure sample of erinacine A from *H. erinaceus* that served as a reference and calibration standard for qualitative and quantitative evaluation of its production in other species of *Hericium*. Similarly to Tsai et al. (2021), these analyses of the reference standard by HPLC-UV-ESI/MS showed that the ion fragment with a *m/z*: 301.215 was a more reliable signal for erinacine A than the parent ion ($C_{25}H_{36}O_6$ $[M+H]^+$, *m/z:* 433.5). Unfortunately, there was an insufficient quantity of the reference standard for compound verification by nuclear magnetic resonance (NMR). However, the signal generated by the ion fragment was associated with a peak in the UV spectrum (340 nm), which is also characteristic of erinacine A (Krzyczkowski et al. 2009, 2010) and was therefore satisfactory for the purposes of compound identification in the absence of structural verification. A total of 7.0 mg of erinacine A was recovered from 59.12 g of dry mycelium.

This is the first report of the production of erinacine A by *H. abietis*, *H. americanum*, *H. carolinense*, and *H. coralloides*. In addition to several other cryptic erinacines of

which less is known of their specific bioactivity, the production of erinacine A has been demonstrated in *H. rajendrae* (Wei et al. 2023), *Dentipellis fragilis* (Ha et al. 2021), and a specimen referred to as *H. flagellum* by Rupcic et al. (2018) but which is more likely to be *H. alpestre*. In their study, Rupcic et al. (2018) describe the specimen as originally isolated from *Abies* in Romania (CBS 103681) known to them as *H. flagellum* with the synonym *H. alpestre*. However, under the most recent phylogenetic treatment of *Hericium* (Chapter 2), the species occurring in Europe on conifers is *H. alpestre*. The original discovery of erinacine A by Kawagishi et al. (1994) was made using a specimen known to them as *H. erinaceus,* but which, under the recently clarified *H. erinaceus* species complex is now known as *H. asiaticum*. Therefore, in addition to the reported production of erinacine A by *H. alpestre*, *H. asiaticum*, *H. erinaceus*, *H. rajendrae*, and *D. fragilis*, the findings of the current study are the first to demonstrate its production by *H. abietis*, *H. americanum*, *H. carolinense*, and *H. coralloides*. In the bioprospecting experiment, in which cultures were grown in a V8+ medium, the highest concentration of erinacine A was detected in the only strain of *H. erinaceus* (WCHE) included in the study (6.12 ± 2.24 µg/mL). However, in subsequent medium optimization experiments, the highest concentrations of erinacine A were produced by *H. coralloides.*

3.4.2 Medium Improvement in Species-Specific Manner

In SLCF, the agitation regime is an important consideration for mycelium growth and metabolite production as agitation can have a positive influence on substrate, heat, and dissolved oxygen transfer (Kwon et al. 2009). However, rapid agitation creates shear stress to the mycelium, which has been shown to cause morphological changes to mycelium with opposing effects on cell growth and metabolite production such that high

agitation can lead to high cell growth but low metabolite yield (Papagianni 2004). In this study, the optimal agitation for erinacine A production was species-specific. A moderate agitation regime of 75 rpm for 10 days was optimal in the production of erinacine A for both *H. americanum* and *H. coralloides*, whereas a two-stage agitation regime was optimal for *H. erinaceus* s.str. and *H. abietis*. The two-stage agitation regime is thought to strike a balance between high oxygen diffusion and faster carbohydrate consumption in the first stage for rapid cell growth, followed by high metabolite synthesis from higher amounts of biomass in the static phase (Cui and Zhang 2011; Feng et al. 2015). For all species, the total incubation time of 10 days resulted in higher erinacine A production than the incubation period of 5 days at 0 rpm, 75 rpm, and 150 rpm. In their study on the optimization of erinacine A in cultures of *H. erinaceus*, Krzyczkowski et al. (2010) found a rapid increase in erinacine A after day 5 of incubation owing to glucose depletion in the culture medium, with the maximum erinacine A produced on day 8 of incubation and decreasing concentrations afterwards. The authors conclude that erinacine A is a growth-associated metabolite that is synthesized at higher rates as nutrients become depleted, and itself becomes degraded over time through conversion to another type of cyathane diterpenoid (Krzyczkowski et al. 2010). The results from the current study where the 10-day incubation period led to the highest erinacine A concentrations are consistent with these findings.

Studies investigating the optimal carbohydrate source for secondary metabolite production in *Hericium* have shown that mycelial growth and compound biosynthesis respond differently to different types of carbohydrates. A single carbohydrate type is rarely optimal for both growth and biosynthesis, and the complexity of the carbohydrate

is one of the most important considerations in SLCF. In their attempts to optimize the production of erinacine C from *H. erinaceus* mycelia, Wolters et al. (2015) found that a medium comprised of oatmeal resulted in the highest erinacine C concentrations while the highest biomass was recorded in a glucose-based medium. The beneficial effect of oatmeal on erinacine biosynthesis is also reported by Shen et al. (2015). The authors speculate the favourable response to oatmeal is due to its high complexity, which may more closely mimic carbohydrate sources that the fungus would encounter in natural settings and therefore stimulate secondary metabolite biosynthesis (Wolters et al. 2015). This is consistent with the carbon catabolite repression mechanism whereby the fungus prioritizes cellular growth in the presence of a simple carbon source, and the expression of genes and utilization of metabolic pathways required for the utilization of more complex sources that may have cross-talk with biosynthetic pathways, are inhibited (Tang et al. 2018; Xu et al. 2008). In contrast, Krzyczkowski et al. (2010) found that higher complexity carbohydrates resulted in higher biomass of *H. erinaceus* while a medium containing glucose as the sole carbon source resulted in the highest erinacine A concentrations. Using the glucose-based medium described as optimal for erinacine A production by Krzyczkowski et al. (2010), Chang et al. (2016) reported an erinacine A concentration of 225 mg/mL, the highest concentration of erinacine A reported from the literature. The results from the current study support simple carbohydrates as the optimal carbon sources for erinacine A production, in which *H. americanum* and *H. coralloides* produced more erinacine A in a glucose-based medium, and sucrose was optimal for *H. abietis* and *H. erinaceus* s.str. None of the species responded most favourably to ME. The increased production of secondary metabolites in the presence of simple carbohydrates by

other species of fungi has been hypothesized to be due to a mycelial autolysis mechanism whereby the easily accessible glucose supports rapid mycelial growth in the early stages of incubation. Upon glucose depletion, an increase in metabolite production may indicate that the main source of energy for continued biosynthesis is mycelial autolysis (Ma et al. 2023). These findings suggest that the effect of carbohydrate sources on erinacine A biosynthesis is species-specific, although simple carbohydrates resulted in higher erinacine A concentrations than the complex source.

The supplementation of liquid media with plant oils is considered by some authors to be a method to promote mycelial growth and polysaccharide yield in the SLCF of fungi (Hsieh et al. 2008). Fatty acids in the plant oils can be incorporated into the cell membranes to increase nutrient uptake from the culture medium to support mycelial growth (Melhuish et al. 1975). Triglycerides also support biosynthesis of metabolites by providing an alternative complex carbon source to avoid the carbon catabolite repression mechanism that occurs under rapid glucose depletion (Tang et al. 2018). The stimulatory effect of plant oils on mycelial growth is largely dependent on the fatty acid profile of a given plant oil as short chain fatty acids with carbon chains of 10 or less (capric acid, caproic acid, propionic acid, acetic acid) have been demonstrated to be inhibitory to fungal growth, whereas longer chained fatty acids such as palmitic acid, oleic acid, and linoleic acid stimulate mycelial growth (Stanisopoulos and Saviour 1990; Yang et al. 2000; Tang et al. 2018; Meng et al. 2021). Polysaccharide production has also been shown to be influenced by the fatty acid composition of plant oils (Yang et al. 2000; Hsieh et al. 2008). However, there are few reports on the effects of plant oils on the biosynthesis of non-polysaccharide metabolites. This study evaluated the effects of three

plant oils with varying fatty acid profiles on the production of erinacine A. Supplementation of the culture medium with 1% corn oil, which is comprised of upwards of 50% linoleic acid (Kim et al. 2020) resulted in the highest production of erinacine A by *H. coralloides* and *H. erinaceus* s.str., which was significantly more than olive and canola oil for *H. coralloides* but not for *H. erinaceus* s.str. For *H. americanum*, the medium containing canola oil, which is composed of 15% linoleic acid and 58% oleic acid (Sairin et al. 2022), resulted in slightly more erinacine A than corn and olive oil, but the effect was not significant. Finally, all three plant oils inhibited erinacine A production in *H. abietis* in which no erinacine A was detected in the mycelial extracts of any treatment. Consistent with the results in experiments 1 and 2, the results from this experiment emphasize the importance of considering media optimization in a species- and strain-specific manner. While the results from this and other studies may serve as general guidelines for industrial producers or researchers aiming to maximize erinacine A yield by modification of SLCF conditions, it is recommended that culture media be tailored to the specific strain being used as the source of erinacine A.

The final experiment compared the optimized medium for each species with the unmodified GYE medium and a V8 liquid medium. Due to the nature of the experiments, chemical analyses of mycelial extracts by HPLC-UV-ESI/MS were performed at different times and internal standards were not included in each analysis to normalize spectral data for comparison of erinacine A peak areas across experiments. Therefore, a final analysis that directly compared the erinacine A production in the unmodified GYE medium and the optimized medium was required. The use of V8 vegetable juice as a culture medium for fungi was first reported by Wickerham (1951) and has become a popular liquid

medium in our laboratory for the rapid generation of mycelium from fungi of various taxa. Observations of its beneficial effect on mycelium growth of *H. erinaceus* s.str. in earlier experiments prompted an investigation into its effect on erinacine A production by other *Hericium* species. Compared to the control and V8 media, cultures of *H. erinaceus* and *H. americanum* produced the highest concentrations of erinacine A in the optimized medium, and the V8 medium resulted in the highest erinacine A concentration for cultures of *H. abietis*, although these differences were not significant (Fig. 3-8). Interestingly, the highest concentration of erinacine A for cultures of *H. coralloides* was detected in the unmodified GYE medium, which was significantly higher than the concentrations produced in the optimized and V8 media (Fig. 3-8). This finding can be explained by a possible inhibitory effect of corn oil on erinacine A biosynthesis by *H. coralloides*, as with *H. abietis*, that was not accounted for in earlier experiments. While cultures of *H. coralloides* grown in the unmodified GYE medium were incubated at 100 rpm, which was not included as a treatment in experiment 1 which identified 75 rpm as the optimal agitation regime for *H. coralloides*, the large difference in erinacine A production is not likely due to the minor difference in the agitation speed. Experiment 2 compared the effects of the different carbohydrates in the optimized (sucrose) and unmodified (glucose) media directly (Fig. 3-6), and therefore cannot explain the difference found the final analysis. In the evaluation of the effect of plant oils on erinacine A production (experiment 3), a culture medium without a plant oil was not included. While the medium that included corn oil resulted in the highest erinacine A production for *H. coralloides* compared to those supplemented with olive oil and canola oil, corn oil may have a partial inhibitory effect on erinacine A biosynthesis by *H.*

coralloides. The higher production of erinacine A in the unmodified GYE medium (71.22 ± 42.41 µg/mL) compared to the optimized medium (40.41 ± 11.20 µg/mL) may be explained by the absence of any plant oils in the former. Further investigation on the inhibitory effects of plant oils on erinacine A biosynthesis in *H. coralloides* would be necessary to evaluate this hypothesis. As mentioned, the lack of internal standards included in each analysis prevents the direct comparison of erinacine A concentrations between experiments as data were not normalized across analyses. Therefore, the high concentration of erinacine A produced by *H. coralloides* in the GYE medium in experiment 4 (71.22 ± 24.48 µg/mL, Fig. 3-8) compared to the highest detected concentration for the same species in the same medium in experiment 2 (19.57 ± 0.33 µg/mL, Fig. 3-5), for example, cannot be compared directly. Finally, the culture medium itself was not analyzed for erinacine A. The increase in membrane permeability provided by plant oils has been shown to enhance exo-polysaccharide production in other species of fungi leading to higher concentrations of metabolites in the culture broth (Tang et al. 2018). No studies on the extra-cellular accumulation of erinacine A in SLCF have yet been conducted. For maximum biosynthesis and recovery of erinacine A, the exocytosis effect of plant oils should be investigated.

3.5 Conclusion

This is the first study to report the production of erinacine A by mycelial cultures of *H. abietis*, *H. americanum*, and *H. coralloides*, in addition to its detection in cultures of *H. carolinense*, which was until recently known as *H. erinaceus*. The effects of agitation regime, carbohydrate source, and supplementation of media with plant oils on erinacine A biosynthesis were evaluated. This study highlights the importance of

optimizing culture media in a species- and strain-specific manner as each species responded differently to the experimental treatments evaluated. Following a modified culture medium composition described by Krzyczkowski et al. (2010) for the optimized production of erinacine A in a strain of *H. erinaceus*, the differential response to this medium by *H. abietis*, *H. americanum*, *H. coralloides*, and a different strain of *H. erinaceus* s.str. reinforces the necessity in formulating the culture medium for individual strains. The highest concentration of erinacine A detected in these experiments (71.22 $\pm$ 24.48 µg/mL by *H. coralloides*) was significantly lower than concentrations reported by Krzyczkowski et al. (2010) of 192 mg/mL and by Chang et al. (2016) of 225 mg/mL. While the majority of studies on the biological effects of erinacine A isolate it from the mycelium of *H. erinaceus*, the results from this study are strongly suggestive that *H. coralloides*, in particular HCX3 (DAOMC 251017), is a more productive source of erinacine A than *H. erinaceus* s.str. (WCHE) under the conditions applied in this study. Should erinacine A continue to be a compound of interest for medical research, this study supports the use of *H. coralloides* as a source for its production. Naturally occurring *Hericium* species not yet analyzed for the production of erinacine A should also be screened for its production as alternative sources of erinacine A in the absence of high producing strains available through culture libraries.

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Chapter 4

4 General Discussion

4.1 Improved Phylogeny of *Hericium* by Multilocus Analysis

The improved phylogeny of *Hericium* in Canada by multilocus analysis and the detection of erinacine A in *H. abietis*, *H. americanum*, *H. carolinense*, and *H. coralloides* contribute to a more complete understanding of the genus from its fundamental taxonomy to its medical potential and industrial applications. Previous phylogenetic work using sequence data from the internal transcribed spacer (ITS) region demonstrated the need to consider other molecular markers to approach phylogenetic resolution in *Hericium*, as ITS sequences are highly similar across the genus and do not provide the interspecific variation required for robust phylogenetic inference (Hallenberg et al. 2013; Jumbam et al. 2019; Cesaroni et al. 2019). The multilocus analysis that included sequence data from ITS–LSU and protein coding genes *tef1* and *rpb2* placed species into strongly supported monophyletic clades consistent with the biological species concept that was used by Ginns (1985) in his treatment of the genus in North America. The monophyly of each of *H. americanum, H. alpestre*, and *H. erinaceus*, which was not shown by ITS alone (Hallenberg et al. 2013), represents a major improvement to the phylogeny of *Hericium*.

The existence of cryptic species within the *H. erinaceus* species complex was suggested as a possibility by Hallenberg et al. (2013) and Cesaroni et al. (2019) as ITS sequences from specimens of *H. erinaceus* collected in Asia appeared to be distinct from those of North America and Europe, but clades were not clear and support was not robust. Concatenation of sequence data from ITS–LSU, *tef1*, and *rpb2* supports the clarification

of the *H. erinaceus* species complex into *H. asiaticum* (Asia), *H. carolinense* (eastern North America), *H. oregonense* sp. nov. (western North America), and *H. erinaceus* s.str. (Europe). Ginns (1985) based his treatment of the genus on mating intercompatibility tests, which were not conducted in this study; however, species within *H. erinaceus* s.l. were separated into mutually monophyletic groups according to geographical distribution. Such genomic divergence of geographically isolated taxa is evidence of reproductive isolation of what was formerly recognized as a single, highly variable species (*H. erinaceus*) into *H. asiaticum*, *H. carolinense*, *H. erinaceus* s.str., and *H. oregonense* sp. nov. No mating tests that have included cultures of the *H. erinaceus* species complex have yet been conducted, but such studies would be informative to evaluate interfertility according to the biological species concept; Ginns' (1985) study only included cultures of *H. carolinense* (as *H. erinaceus*, from eastern North America) and *H. erinaceus* s.str. (from Europe).

Multilocus analysis was also able to recover clades within the *H. coralloides* species complex, which formed a monophyletic grouping with sequences from eastern North America on a separate branch from one that includes sequences of *H. coralloides* from Europe and China. While I do not consider this evidence sufficient to support the designation of distinct species within the *H. coralloides* complex, advanced molecular methods such as whole genome sequencing would be informative to evaluate this possibility, and to validate the restructured *H. erinaceus* complex as determined here. Apart from its importance in constructing a robust taxonomic understanding of a given genus, identifying cryptic fungal species has consequences in conservation of at-risk

species, biological control and treatment of pathogens, as well as for the bioprospecting of target metabolites in closely related species (Bickford et al. 2006).

4.2 Resolved Systematics Contribute to Effective Bioprospecting

Bioprospecting North American species of *Hericium* for erinacine A began before the phylogenetic work presented in Chapter 2 was finalized. The *H. erinaceus* species complex was then thought to be a single species and was therefore treated in the broad sense; discrimination of cultures of *H. carolinense* and *H. erinaceus* s.str. was only possible in hindsight. Cultures of *H. asiaticum* and *H. oregonense* sp. nov. were not evaluated for their production of erinacine A (the latter because none were available). In addition to the detection of erinacine A in mycelial extracts of *H. erinaceus,* the species from which erinacine A and other erinacines was first isolated by Kawagishi et al. (1994, 1996a – b), this was the first report of its production by cultures of *H. abietis*, *H. americanum*, *H. carolinense*, and *H. coralloides*. The research conducted by Kawagishi et al. (1994, 1996a – b) is not explicit in the origin of fungal material used in the isolation of erinacines and screening of their bioactivity. As the research was conducted in Japan, the species referred to as *H. erinaceus* in their studies is likely *H. asiaticum* under the current phylogenetic understanding (Kawagishi et al. 1994, 1996a – b).

Erinacine A, in addition to several cryptic erinacines, has been reported from mycelial extracts of *H. alpestre* (as *H. flagellum*) (Rupcic et al. 2018), *H. rajendrae* (Wei et al. 2023), and *Dentipellis fragilis,* a related species in Hericiaceae (Ha et al. 2021). Erinacine E has been reported from the mycelial extracts of *H. coralloides* (as *H. ramosum*) (Saito et al. 1998) and in a North American sample of *Hericium* sp. (WBSP8) collected in Minnesota that is most similar to *H. americanum* in morphology, but analysis of its ITS

sequence data placed it closer to *H. abietis* and *H. alpestre* according to Song et al. (2020). Using the ITS sequence data available for this specimen, I determined *Hericium* sp. WBSP8 to be *H. americanum.* Again, misidentification of *Hericium* sp. WBSP8 by Song et al. (2020) emphasizes the phylogenetic limitations of ITS in this group.

The detection of erinacines in mycelial extracts of these *Hericium* species and in *D. fragilis* suggests that erinacine biosynthesis may be a genus- and possibly family-wide trait. The biosynthesis of erinacines, as cyathane diterpenoids, was evaluated by Chen et al. (2017) by genomic and transcriptomic analyses of *H. erinaceus* where they determined that erinacines are synthesized by the common isoprene precursor geranyl-geranyl diphosphate (GGPP), identifying 12 genes involved in the terpenoid backbone synthesis. Heterologous gene expression by Yang et al. (2017) identified the gene cluster *Eri* in the genome of *H. erinaceus*, within which the specific Ubi-A-type diterpene cyclase *EriG* was detected. In addition, heterologous gene expression in *Aspergillus oryzae* by Liu et al. (2019) found that the *Eri* gene cluster includes genes encoding the GGPP synthase *EriE*, an additional Ubi-A prenyl transferase *EriF*, a UDP-glycosyl-transferase *EriJ*, and three cytochrome p450 proteins *EriA*, *EriC*, and *EriI*, all of which are involved in the synthesis of erinacines. The proposed biosynthetic pathway of erinacines as reported by Liu et al. (2019) is presented in Fig. 4-1.

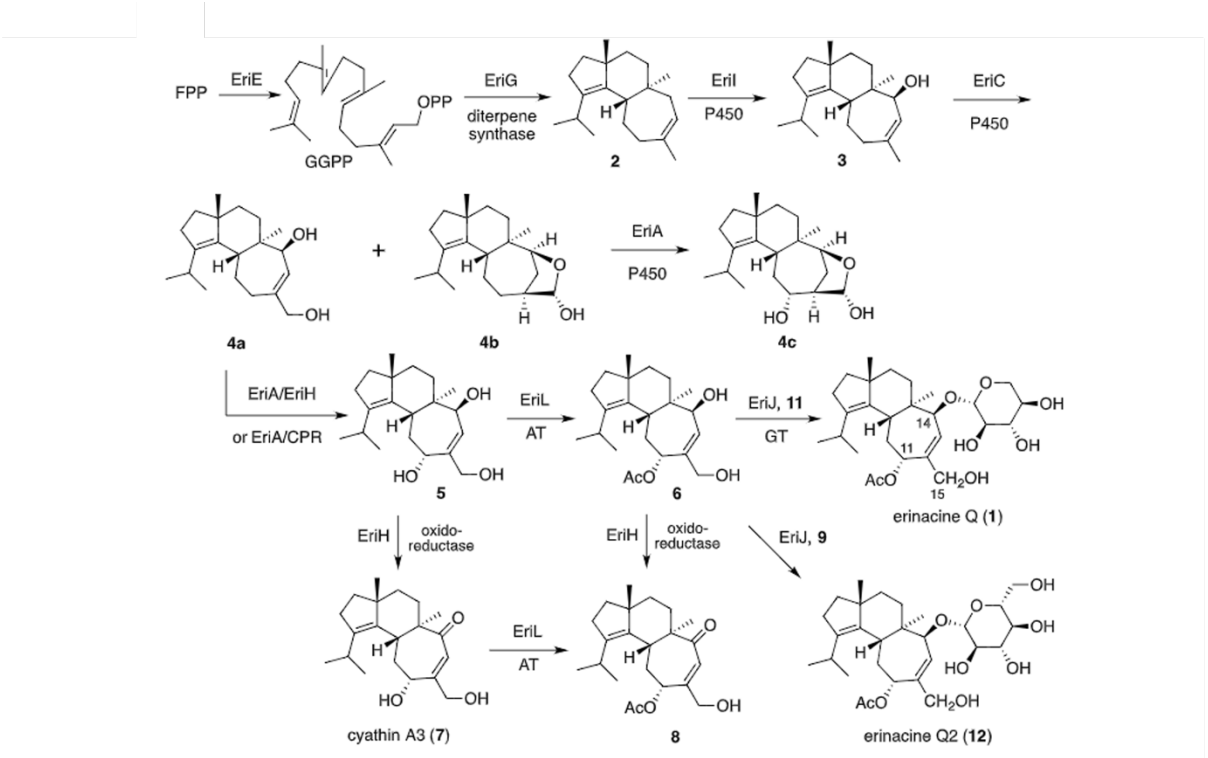

Figure 4-1. The biosynthetic pathway of erinacines proposed by Liu et al. (2019). Intermediates and enzymes were identified by GC-MS, UPLC-MS, ^{1}H or ^{13}C NMR via heterologous gene expression in *Aspergillus oryzae* as erinacine Q (1), cyatha-3,12-diene (2), erinacol (3), cyathadiol (4a), a hemiacetal derivative (4b), cyathatriol (5), 11-*O*-acetyl-cyathatriol (6), cyathin A3 (7), 11-*O*-acetylcyathin A3 (8), a UDP-$_{D}$-glucose dehydrogenase (9), a UDP-xylose (11), and erinacine Q2. Figure reproduced from Liu et al. (2019).

A similar gene cluster (*Cya*) was found in the genome of *Cyathus striatus* (Agaricales, Nidulariaceae) (Yang et al. 2017). The discovery of the *Cya* gene cluster in *Cyathus* is not surprising as these fungi are known producers of cyathane diterpenoids, compounds that have also been detected in *Sarcodon* and *Phellodon* (Thelephorales,

Bankeraceae) (Fang et al. 2010; Bhandari et al. 2014) and in *Laxitextum* (Russulales, Hericiaceae) (Mudalungu et al. 2016). Production of cyathane diterpenoids from these distantly related fungi is likely a result of convergent evolution. Nevertheless, erinacine A biosynthesis by *Hericium* and *Dentipellis*, and cyathane diterpenoid production by *Laxitextum,* is evidence of shared biosynthetic machinery in Hericiaceae and suggests that erinacine A may yet be detected in other members of Hericiaceae. All *Hericium* species considered in these experiments demonstrated erinacine A production.

4.3 Erinacine A Biosynthesis as a Species-Specific Phenomenon

In the bioprospecting experiment, the highest concentration of erinacine A was detected in the only strain of *H. erinaceus* s.str. (WCHE) included in the study (6.12 $\pm$ 1.29 µg/mL). However, in subsequent medium optimization experiments, the highest concentration of erinacine A was produced by *H. coralloides* for a maximum concentration of 71.22 $\pm$ 24.48 µg/mL, compared to the maximum concentration of 12.78 $\pm$ 6.22 µg/mL by *H. erinaceus* s.str. As erinacine A continues to be evaluated for its bioactivity and medicinal potential, these findings emphasize the value in bioprospecting wild-collected specimens of locally available *Hericium* species and mycelial cultures for their relative production of erinacine A and other erinacines. The only *H. erinaceus* s.str. strain considered in this study, WCHE, originates from a strain used in commercial fruit body cultivation of unknown geographic origin but this analysis placed it in the European clade of *H. erinaceus*. While culture materials for commercially important strains are most readily available, this study suggests that other species may be superior sources of erinacine A.

An improved understanding of interspecific relationships provided by the multilocus analysis can support these efforts with accurate and consistent specimen identification. Blurred species delineation and inconsistent interpretation of taxonomy confounds information exchange of specimens used as sources of erinacines as evidenced by the misapplication of names in Saito et al. (1998), Rupcic et al. (2018), and Song et al. (2020). Clear delineation of species within the *H. erinaceus* complex will benefit the continued evaluation of their medicinal properties by pharmacologists and biochemists where medically important lineages can be more precisely identified. Improved resolution at the species level will also support targeted breeding of economically important strains in the industrial cultivation of *Hericium* mushrooms, a global industry that was valued at $978 million USD in 2021 (Niego et al. 2023).

In the submerged liquid culture fermentation (SLCF) of fungi, modification of liquid culture conditions is often necessary to achieve the maximum yield of a target compound, the optimization of which must be species- and compound-specific (Elisashvili et al. 2009). The results from the optimization experiments (chapter 2) are consistent with this concept as the production of erinacine A by *H. abietis*, *H. americanum, H. coralloides*, and *H. erinaceus* s.str. varied in response to culture medium modification in a species-specific manner. Moreover, the conditions resulting in improved production of erinacine A for each species do not appear to be related to the phylogenetic distance between species. Of the species considered in these studies, *H. americanum* and *H. abietis* are the most closely related, which together form a clade that is sister to the *H. erinaceus* species complex. However, the conditions resulting in the highest erinacine A production by *H. americanum* (75 rpm for 10 days, glucose, canola oil) were all different from those for *H.*

abietis (two-stage agitation, sucrose, no oil). The response of *H. americanum* to culture modification was most similar to the conditions optimal for *H. coralloides* (75 rpm for 10 days, glucose, corn oil), yet *H. coralloides* forms its own clade that is sister to the major clade which includes *H. americanum*, *H. abietis*, and *H. erinaceus* s.l. Based on the proximity of interspecific relationships, *H. abietis* is most similar to *H. americanum* but responded most similarly to culture medium modification by *H. erinaceus* s.str. (two-stage agitation, sucrose, corn oil). From these experiments it can be concluded that the biosynthesis of erinacine A by species of *Hericium,* in response to the agitation regime, carbohydrate source, and plant oil, is independent of the phylogenetic distance between species.

4.4 Limitations and Future Considerations

The scarcity of LSU, *tef1*, and *rpb2* sequences of *Hericium* species in genomic databases limited the breadth of species included in this analysis for use as reference sequences. While a few whole genome sequences are available for *H. asiaticum* (CS-4, 0605), *H. alpestre* (DSM 108284), and *H. coralloides* (tvtc0002), the overwhelming majority of available *Hericium* sequences are of the phylogenetically uninformative ITS locus. Additional sequence data of the phylogenetically informative *tef1* and *rpb2* loci from other *Hericium* species would improve the statistical power of phylogenetic analyses and be especially useful in evaluating the taxonomic position of *H. botryoides* S. Ito and Otani, a species described from Japan that appears to be most similar in morphology to *H. americanum* (Otani 1957). Sequence data of *tef1* and *rpb2* from the *Abies*-associated *H. yumthagense* K. Das, Stalpers and Stielow, which is described from northeastern India, would also help clarify its taxonomic positioning as ITS suggests its

synonymy with *H. alpestre* (data not shown), which is known from *Abies* in Europe (Das et al. 2013; Hallenberg et al. 2013). Future studies of the genus would benefit from whole genome sequencing (WGS) to evaluate whether the high intraspecific variability of the *H. coralloides* complex detected in this study reflects species boundaries between geographically distinct taxa. Together with mating intercompatibility tests, which were not performed in this study, WGS may provide higher resolution to the genomic distances and reproductive isolation for species delineation in the *H. coralloides* and *H. erinaceus* complexes.

The isolation of erinacine A followed methods described by Krzyczkowski et al. (2009), with slight modification, for a final yield of 7.0 mg erinacine A from 59.12 g of dry *H. erinaceus* mycelium. The small quantity of erinacine A limited the number of internal calibration standards available for quantification of erinacine A in each analytical run. The inclusion of an internal standard of known concentration would allow for normalization of peak areas from different experiments to account for variations in equipment sensitivity. In the absence of normalized spectral data, the comparison of erinacine A concentrations in each sample is limited to within-experiment comparisons. Future studies, if working with limited quantities of erinacine A, would benefit from diluting analytical samples and standards so that less erinacine A standard is used in calibration curves and make material available for internal standards to normalize spectral data and allow direct comparison between experiments. The small quantity of erinacine A also prevented compound structural verification by nuclear magnetic resonance (NMR) spectroscopy. While the absorbance of 340 nm by the ion fragment (m/z = 301.215) has been reported to be a reliable indicator of erinacine A (Chiu et al. 2018; Tsai et al. 2021),

structural verification would contribute to a more robust experimental outcome. Future studies should aim to increase yields of erinacine A for use as reference and calibration standards following the optimal culture media conditions described in Chapter 3. The analyses also suffered from a high degree of variability and large standard errors within treatment groups due to the small number of replicates for each sample (N=3). If possible, future studies should increase the number of replicates to strengthen statistical analyses. Finally, the culture broth itself was not analyzed for erinacine A and all analyses were of erinacine A extracted from lyophilized mycelium. Erinacine A has been detected in the culture broth of *Dentipellis fragilis* (Ha et al. 2021), *H. erinaceus,* and *H. alpeste* (Rupcic et al. 2018), and it is likely that it would have been detected in the culture filtrate in these experiments. Analyzing the culture broth would provide a more complete understanding of the effects of culture medium modification on total erinacine A biosynthesis as fungi are known to release metabolites into the culture broth to prevent intracellular accumulation of compounds to a toxic level (Stasinopoulus and Seviour 1990). This would be especially important in culture media supplemented with plant oils, where fatty acids incorporated into the cell membranes can increase bidirectional membrane permeability (Tang et al. 2018).

4.5 Conclusion

The sequencing of multiple molecular markers has become a powerful tool in resolving phylogenies of closely related species where the traditionally used ITS region is limited (Matheny 2005; Matheny et al. 2007; Carlson et al. 2014; Dong et al. 2022). The first objective of this study was to resolve interspecific relationships of *Hericium* species using sequence data from ITS, LSU, *tef1*, and *rpb2.* In this study, concatenation of ITS–

LSU, *tef1*, and *rpb2* recovered mutually monophyletic clades of *Hericium* at the species level which represents a major improvement to the taxonomic understanding of the genus. The multilocus analysis recovered cryptic species in the *H. erinaceus* species complex and led to the description of three novel species: *H. asiaticum* Koga and Thorn, *H. carolinense* Koga and Thorn, and *H. oregonense* Koga and Thorn sp. nov., and holotypes for each of these novel species were designated. I report the first sequence-confirmed record of the rare *H. cirrhatum* in North America and the first published record of the species in Canada. Additionally, nomenclatural uncertainty of several European species and the variable interpretation of synonymy was clarified by designation of an epitype for *H. coralloides,* the type species for the genus for which type materials from the original collection are absent. The second major objective of this study was to evaluate the production of erinacine A in North American species of *Hericium.* This was the first report of erinacine A production by mycelial cultures of *H. abietis*, *H. americanum*, *H. carolinense,* and *H. coralloides*. Biosynthesis of erinacine A by each of these species suggests that the isolation of erinacine A for medical research and industrial purposes may be accomplished by a variety of species beyond commercially available strains of *H. erinaceus*. Finally, the response to culture medium optimization for improved erinacine A production emphasizes the need to consider medium modification in a species-specific manner. Under the conditions evaluated in this study, modification of the agitation regime, carbohydrate source, and supplementation with a plant oil in a stepwise fashion resulted in the highest production of erinacine A by a strain of *H. coralloides*.

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Appendices

Appendix A: *Hericium coralloides* species complex.

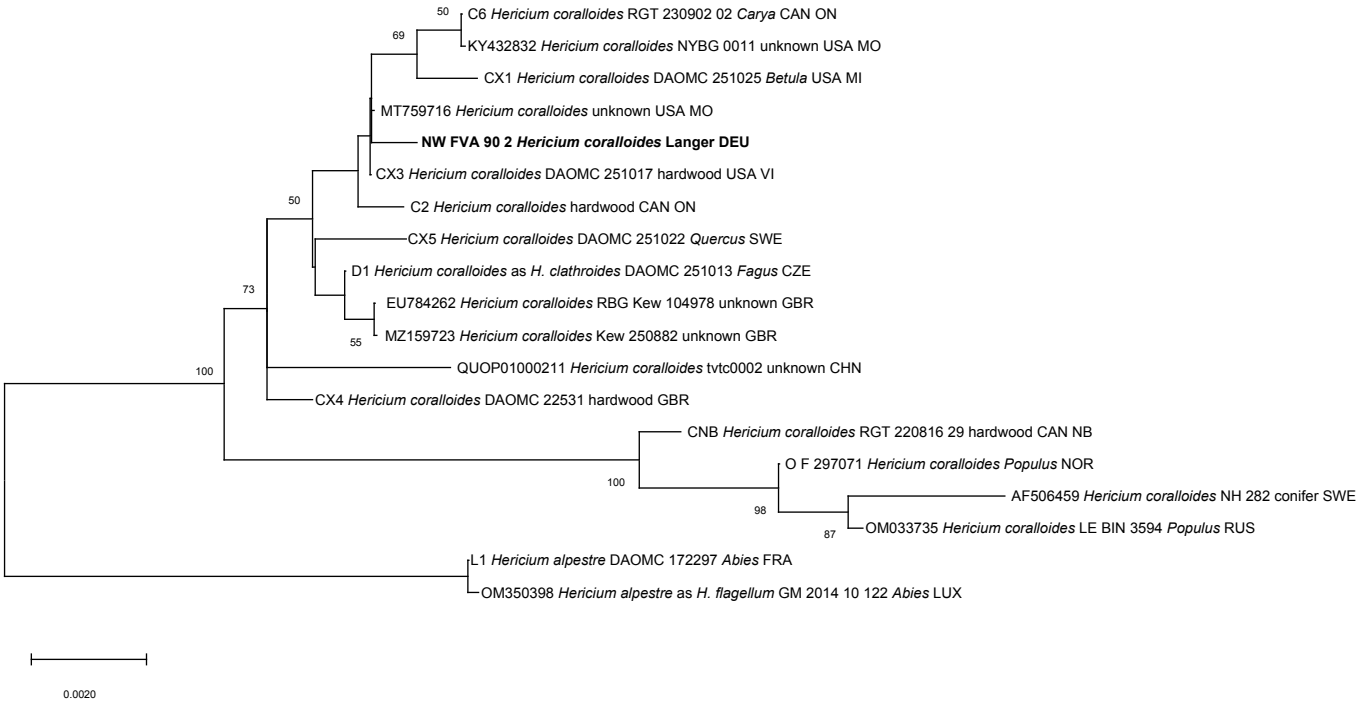

Figure A-1. Maximum likelihood tree based on nuclear ribosomal internal transcribed spacer region (ITS) of the *H. coralloides* species complex. The sequence from the epitype (NW FVA 90 2 *Hericium coralloides* Langer DEU) is in bold. *Hericium alpestre* used as outgroup.

Appendix B: Statistical results from Chapter 3.

Table B-1. Data transformations and results from statistical analyses.

Experiment	Data Transformation	Test	F-value/Effect Size[1]	p-Value	Top Producing
Detection					
H. americanum	None	Kruskal-Wallis	$\eta^2 = 0.82$	0.0089	HAX1
H. coralloides	None	Kruskal-Wallis	$\eta^2 = 0.95$	0.033	HCX3
H. abietis	Log(x+0.1)	ANOVA	F = 2.03	0.13	HB5
H. erinaceus	None	Kruskal-Wallis	$\eta^2 = 0.88$	0.0055	WCHE
Experiment 1					
H. americanum	None	Kruskal-Wallis	$\eta^2 = 0.85$	0.013	75 rpm Day 10
H. coralloides	Log(x+0.1)	ANOVA	F = 16.64	6.21e-05	75 rpm Day 10
H. abietis	Log(x+0.1)	ANOVA	F = 3.39	0.031	Two-Stage
H. erinaceus	Log(x+0.1)	ANOVA	F = 116.2	1.12e-08	Two-Stage
Experiment 2					
H. americanum	None	ANOVA	F = 2.42	0.17	Glucose
H. coralloides	None	ANOVA	F = 13.6	0.0060	Glucose
H. abietis	None	Kruskal-Wallis	$\eta^2 = 0.94$	0.022	Sucrose
H. erinaceus	None	ANOVA	F = 1.67	0.27	Sucrose
Experiment 3					
H. americanum	None	ANOVA	F = 4.98	0.053	Canola Oil
H. coralloides	None	ANOVA	F = 38.79	0.00037	Corn Oil
H. abietis	NA	NA	NA	NA	NA
H. erinaceus	None	ANOVA	F = 1.37	0.32	Corn Oil
Experiment 4					
H. americanum	None	Kruskal-Wallis	$\eta^2 = 0.60$	0.061	Optimized
H. coralloides	None	ANOVA	F = 5.82	0.039	GYE
H. abietis	None	ANOVA	F = 1.58	0.39	V8
H. erinaceus	None	ANOVA	F = 3.44	0.10	Optimized

[1]F-value from ANOVA, effect size (η^2) from Kruskal-Wallis

Curriculum Vitae

Name: Julien Koga

Post-secondary Education and Degrees

University of Manitoba
Winnipeg, Manitoba, Canada
Bachelor of Science with distinction, Biology
2015 – 2020

University of Western Ontario
London, Ontario, Canada
Master of Science, Biology
2022 – 2024

Honours and Awards

Ruth Arnold Fellowship (2023)
Japanese Canadian Legacies Society Scholarship (2023)
Best flash presentation, CanFunNet virtual conference (2023)
UMSU Scholarship (2019, 2020, 2021)
Undergraduate Research Award (2019)

Related Work Experience

Teaching Assistant
The University of Western Ontario
2022 – 2024

Mycology Intern
Whitecrest Innovations Inc.
2023

Research Assistant
University of Manitoba
2019, 2021

Teaching Assistant
University of Manitoba
2020

Publications:

Koga, J., Thorn, R.G., and Langer, E. 2024. A multilocus phylogeny of *Hericium* in Canada. Can. J. Microbiol. [Manuscript under revision].

Presentations:

Koga, J., Thorn, R.G., and Bernards, M. June 9, 2024. An improved multilocus phylogeny of *Hericium* in North America, new species, and their production of erinacine A. Annual meeting of the Mycological Society of America. Markham, Ontario, Canada. [Oral]

Koga, J., Thorn, R.G., and Bernards, M. June 12, 2023. Resolving the systematics of *Hericium* in North America with multigene phylogenetic analysis and its implications for mushroom cultivation programs. CanFunNet annual conference. [Virtual; Oral]

Koga, J., Thorn, R.G., and Bernards, M. June 2, 2023. Resolving the systematics of *Hericium* in North America with multigene phylogenetic analysis and its implications for mushroom cultivation programs. Joint annual meeting of the Canadian Society for Ecology and Evolution & Canadian Botanical Association. Winnipeg, Manitoba, Canada. [Oral]