Ethnopharmacology of the club moss subfamily Huperzioideae (Lycopodiaceae, Lycopodiophyta): a phylogenetic and chemosystematic perspective

Maonian Xu[a], Starri Heidmarsson[b], Hugo J. de Boer[c], Anneleen Kool[c], Elin Soffia Olafsdottir[a]

Affiliation

[a] Faculty of Pharmaceutical Sciences, University of Iceland, IS-107 Reykjavik, Iceland

[b] Akureyri Division, Icelandic Institute of Natural History, IS-600 Akureyri, Iceland

[c] Natural History Museum, University of Oslo, NO-0318 Oslo, Norway

Correspondence

Elin Soffia Olafsdottir, PhD, Professor

Faculty of Pharmaceutical Sciences

University of Iceland

Hagi, Hofsvallagata 53,

IS-107 Reykjavik, Iceland

Email: elinsol@hi.is

Abstract

Ethnopharmacological relevance: The most speciose subfamily Huperzioideae (Lycopodiaceae, Lycopodiophyta) contains about 276 species, and some (ca. 20 species) have traditionally been used for the treatment of e.g., dementia, rheumatism and traumatic injury. Ethnopharmacological studies have also contributed to the development of huperzine A as a drug lead, a compound first isolated from the club moss *Huperzia serrata* (Thunb. ex Murray) Trevis.

Aim of the review: This review, with a phylogenetic and chemosystematic perspective, intends to highlight plant identification challenges in these taxa with examples from club moss phytochemical and ethnopharmacological studies, as these lead to data inconsistency and confusion. We suggest that future studies should include more details on plant identification including for example plant specimen images and DNA barcoding data. An integrative approach combining DNA barcoding and chemical fingerprinting is also introduced.

Materials and methods: Literature concerning ethnopharmacology and chemosystematics of Huperzioideae club mosses was searched from databases, e.g. PubMed, Web of Science, SciFinder, etc. Plant names were retrieved from original publications, and compared with up-to-date taxonomic and phylogenetic status. Ethnobotanical uses and herbal preparations were summarized. Production of certain pharmaceutically interesting compounds, such as the alkaloid huperzine A, was explored in a phylogenetic context.

Results: Most traditionally used club mosses are associated with psychoactivity, followed by medicinal uses against rheumatism and traumatic injury. Herbs are often prepared as infusions, decoctions or tinctures, and this implies importance of water- or aqueous-alcohol-soluble substances, such as alkaloids. Most ethnopharmacological papers on club mosses need to update or correct plant names according to recent taxonomic nomenclature, and there are still a number of unidentified species with traditional use. Advanced LC-MS chemical profiling techniques, enable distinction of genotypes of the same species as well as annotation of potential chemotaxonomic markers. In combination with DNA barcoding, chemosystematics could also help us select plant taxa with higher pharmaceutical potential. Caution should be taken when interpreting bioassay results, in terms of compounds or extract preparation and bioassay standardization.

Conclusion: Huperzioideae club mosses have interesting pharmaceutical potential supported by ethnopharmacological investigations. Bioprospecting of these plants should be preceded by careful plant identification to produce consistent and reproducible data. We expect that DNA barcoding and LC-MS-based chemical fingerprinting could facilitate and improve ethnopharmaceutical studies in selection of club moss taxa.

Keywords: chemosystematics, club moss, Huperzioideae, Lycopodiaceae, phylogeny

1 Introduction

Club mosses represent an ancient and conserved vascular plant lineage in parallel with ferns and bryophytes. They produce a diverse array of structurally unique alkaloids – lycopodium alkaloids, some of which show pharmaceutical potential (Ma and Gang, 2004; Olafsdottir et al., 2013). The research interest in club moss phytochemistry has been boosted by the discovery of the alkaloid huperzine A (hupA) in Chinese *Huperzia serrata*, which was found to be a potent acetylcholinesterase (AChE) inhibitor (Liu et al., 1986). HupA has been suggested as a drug lead for the treatment of Alzheimer‘s disease, and it has already been made commercially available with two clinical trials passed as reviewed by Olafsdottir et al. (2013). This was followed by a major research effort which resulted in many phytochemical papers focusing on isolation and identification of novel lycopodium alkaloids (Ma and Gang, 2004). The discovery of these alkaloids and their activities have an ethnopharmacological origin as *H. serrata* has been traditionally used for centuries against ailments, e.g. contusions, strains, neurological disorders, schizophrenia and myasthenia gravis (Ma et al., 2007). Closely related taxa, e.g. *Phlegmariurus saururus* (Lam.) B. Øllgaard, have also been used by different ethnic groups for the treatment of dementia, rheumatism and traumatic injury, etc (Armijos et al., 2016; Ortega et al., 2004). In Europe, *Huperzia selago* has been used as a powerful emetic and against lice in livestock (Allen and Hatfield, 2004). This is also reflected in the name of the plant in Norway, lusegras, lice-grass (Høeg, 1974), where the plant is used against lice and for other purposes (diuretic and laxative agents). Faroese uses are somewhat different: an infusion of *H. selago* is used against cough (Svanberg, 1998), possibly reflecting regional phytochemical variations.

The taxonomy of club mosses has been updated and revised extensively in the last two decades, and this was considerably advanced by ultrastructural investigation with microscopic techniques and phylogenetic inferences using DNA sequencing (Field et al., 2016; Øllgaard, 1987; Schuettpelz et al., 2016). However, as the number of both phytochemical and taxonomic papers increases, we have observed a widening taxonomic gap, and phytochemical and ethnopharmacological studies do not keep pace with recent taxonomic updates. Ambiguous plant names in ethnopharmacological and phytochemical papers also bring about confusion in terms of the plant(s) in use. The recommended plant taxonomy database (e.g. The Plant List) (Heinrich and Verpoorte, 2014) has not been updated since 2013.

It should be emphasized, given the growing number of club moss ethnopharmacological studies that uncertainty of sampled club mosses may lead to inconsistent data in downstream phytochemical and bioassay screenings, altogether affecting data reproducibility. In this review, we aim to raise the awareness on thorough plant identification in club moss ethnopharmacological and phytochemical studies. It is suggested that future studies should include detailed plant identification information, including, when possible, DNA barcoding sequence data and specimen photographs. In addition, we introduce an integrative approach combining DNA barcoding and chemical fingerprinting, and discuss how this approach could contribute to future studies, including selection of medicinal plants for bioprospecting. Ethnopharmacology, as a transdisciplinary subject, could benefit considerably from even closer collaboration between taxonomy, phytochemistry and pharmacology.

This review focuses on the subfamily Huperzioideae (Lycopodiaceae, Lycopodiophyta), mainly because: 1) the drug lead alkaloid hupA is phylogenetically confined to this subfamily, and the enclosed taxa are of high ethnopharmacological interest; 2) high taxonomic uncertainty exists in this subfamily, which phylogenetic analyses and DNA barcoding may contribute to resolve.

2 Phylogenetic systematics of Huperzioideae: current status and challenges

The taxonomy of the club moss family Lycopodiaceae has been in a flux (mainly at the generic level), but a fundamental consensus has been achieved that this family is composed of three subfamilies, including Huperzioideae, Lycopodioideae and Lycopodielloideae (Øllgaard, 2012; Schuettpelz et al., 2016). The group is estimated to have originated during the Cenozoic, along with most land plants (Testo et al., 2018a). Subfamilies are phylogenetically supported (**Fig. 1**), morphologically recognizable and lack inter-subfamily and intergeneric hybrids (Øllgaard, 2012). Huperzioideae do not have strobili and creeping or looping stems as opposed to other subfamilies, and more specific ecological (e.g. habitat type), morphological (e.g. presence or absence of gemma) and ultrastructural characters (e.g. spore shape) have enabled their species and generic delimitation (Øllgaard, 2012, 1987).

Phylogenetic analyses based on plastid DNA sequence and morphological data propose a three-genus scenario including *Phylloglossum* Kunze, *Huperzia* Bernh. and *Phlegmariurus* Holub (Field et al., 2016). Recently, efforts have been focusing on the most speciose genus *Phlegmariurus*, inferring its evolutionary patterns in morphological characters and geographic distribution (Bauret et al., 2018; Testo et al., 2018b). In contrast, the phylogeny of the genus *Huperzia* is much less studied. One recent study focusing on Chinese taxa proposed that the genus *Huperzia* has two sections, differing mainly in leave shapes (Ji et al., 2008). Apparently this is an interesting working hypothesis that awaits further investigation incorporating a worldwide sampling effort. Even though the currently proposed species number of the genus *Huperzia* is 25 (Schuettpelz et al., 2016), no formal assessment has been carried out globally. The relationship between the three genera *Huperzia*, *Phlegmariurus* and *Phylloglossum* is still ambiguous according to Field et al. (2016), and it seems that *Phylloglossum* is sister to the other two genera (Bauret et al., 2018).

Recent phylogenetic analyses using plastid loci of club mosses have provided valuable insights into the evolution and genus-level classification. However, phylogenetic relationships between species (and even genera) is still poorly understood. That is partly due to the limitation of plastid markers, which might be less informative than nuclear markers and are maternally inherited. Plastid data can ideally be combined with nuclear markers if there is sufficient congruency. This might, however, be impeded by the lack of established reference nuclear sequence data and available primers in Lycopodiaceae. Meanwhile, it is also noteworthy when testing nuclear markers that amplicons may originate from different alleles, and cloning may be necessary (Aagaard et al., 2009; Klahr et al., 2018). Alternatively, phylogenetic relationships can sometimes be resolved using more advanced sequencing techniques, such as Restriction site Associated DNA sequencing (RAD-Seq) (Eaton and Ree, 2013). Finally, a thorough sampling is needed, especially for the genus *Huperzia*.

3 Ethnobotanical uses of club mosses in Huperzioideae

3.1 Ethnobotanical uses

Most ethnobotanical uses of Huperzioideae club mosses (**Table 1**) are recorded in tropical or subtropical regions, especially high-altitude areas like the Andes (Armijos et al., 2016; Bussmann and Sharon, 2006). Among all ethnobotanical uses, club moss herbal preparations are mainly appreciated for their psychoactivity, claimed to be hallucinogenic, aphrodisiac and memory-improving (Armijos et al., 2016; Birri et al., 2017, 2014; De Feo, 1992; Goleniowski et al., 2006; Luo et al., 2018; Ma et al., 2007; Silalahi et al., 2015; Vallejo et al., 2007). Those effects may be derived from aqueous or alcoholic extracts as mentioned in original publications, such as infusions, decoctions, bath ingredients, tincture or even chewed juices (Ortega et al., 2006). It has also been recorded that *H. serrata* can be used for the treatment of rheumatism and traumatic injury (Luo et al., 2018; Ma et al., 2007). Huperzioideae club mosses are also locally used for the treatment of elephantiasis in Venezuela (Frye, 1934), alcoholism in Ukraine (Stryamets et al., 2015), cancer, bone fractures, colds, inflammation, kidney and liver diseases in Ecuador (Bussmann and Sharon, 2006). Ritual or cultural events also use club mosses, such as prohibition of evil spirits in Nigeria (Nwosu, 2002) and spiritual cleansing in Peru (Bussmann et al., 2010). Even though the ethnobotanical uses are well documented, essential information with regard to herb preparation and/or plant parts used is still missing in some publications.

Adverse effects, such as vomiting and diarrhoea, and intoxication may appear after taking (concentrated) club moss herbal preparations (De Feo, 2003; Ortega et al., 2006). Less concentrated preparations could be used as a purgative or laxative agent (De Feo, 2003; Ortega et al., 2006), demonstrating presumable effects on gut metabolism. Similar toxicity has also been recorded for *Huperzia selago* (L.) Bernh. ex Schrank & Mart in Norway, which is believed to be poisonous (Høeg, 1974). The purgative effects have not been well studied. Toxicity of eight unidentified Huperzioideae club moss extracts (i.e. aqueous and ethnolic) has been evaluated using a brine shrimp lethality assay (Bussmann et al., 2011). The authors found that a much higher toxicity was found in ethanolic extracts than aqueous ones but with large variations between taxa.

3.2 Plant name matters

In light of the intensive taxonomic revisions of nomenclature in the subfamily Huperzioideae, phytochemical and ethnopharmacological studies should keep pace with the most recent updates. Plant names have been updated in **Table 1**, particularly species in the genus *Phlegmariurus*, which were listed as *Huperzia* species in most papers. It is noteworthy that there is still a large uncertainty regarding to the identity of the club mosses with recorded ethnobotanical uses. For example, three *Huperzia* species in Peru (section "unidentified" in Table 1) have not been characterized (Bussmann, 2013; De Feo, 2003), and might turn out to be terrestrial *Phlegmariurus* species. Also, the identity of "*Lycopodium selago*" in early ethnobotanical publications (Knobloch and Correll, 1962; Lloyd, 1964; Nwosu, 2002) is very unambiguous: it may represent either *H. selago* or *Phlegmariurus dentatus* (Heter) M.D.Arana, or even other species belonging to the genus *Lycopodium* L. It has actually been shown that some of the formerly recognized *Lycopodium selago* specimens are in fact terrestrial *Phlegmariurus* species (Arana, 2016). Therefore, locally used plant materials should be subjected to a re-examination for identification.

By taking a closer look at vernacular plant names, several different club moss species may share the same vernacular name and even be used in mixtures. For instance, "Agua Minga" could refer to up to nine different club mosses in Loja, Ecuador, while in China, the old name "Shi Song/石松" (nowadays "Qian Ceng Ta/千层塔" specific for *H. serrata*) also stands for several species, not only from the genus *Huperzia*, but also from the sister genera *Diphasiastrum* Holub and *Lycopodium* (Ma et al., 2007). This implicates the insignificant morphological differences and taxonomic uncertainties of club moss species. Thorough plant identification is essential to guarantee consistency and reproducibility of phytochemical and pharmaceutical studies. Nonetheless, taxonomically important characters e.g. morphological or genetic data, are rarely provided in phytochemical papers reporting bioactive compounds in club mosses.

In order to aid accurate plant identification in future phytochemical studies, we highly recommend the recent phytochemical research guideline (Zidorn, 2017) for providing essential information, including plant identification monographs, plant specimen photograph(s) and geographic locations. We also suggest that at least one DNA barcode sequence should be provided for future club moss phytochemical and ethnopharmacological studies, such as the widely used chloroplast *rbcL*, *psbA*-*trnH* and *matK* loci, which enable comprehensive comparison with publicly available databases (e.g. NCBI GenBank). Even though variation of nucleotide sequences may not be enough for species-level specimen identification, these markers can assign specimens with high certainty in closely related plant groups. Since taxa of Huperzioideae often have minor morphological differences that are not easily discernible, we believe the supplementation of those data will improve the accuracy and consistency of the downstream phytochemical and phytopharmaceutical results.

4 Chemosystematics of Huperzioideae club mosses

4.1 Rationale of plant chemosystematics

The subject chemosystematics holds the hypothesis that closely related taxa usually share a similar biogenic pool of metabolites (Larsson et al., 2008). Dealing with closely related taxa, chemosystematics should incorporate a phylogenetic approach by investigating the production of metabolites in a phylogenetic context (Larsson, 2007; Wink, 2003). It could be used to generate hypotheses on the evolution of biosynthetic pathways, and in return it contributes to phylogeny (Reynolds, 2007). Application of chemosystematics is very well suited for the selection of alternative extant medicinal plant resources when the original plant taxa cannot be sourced in a sustainable manner (Larsson et al., 2008). This application is supported by recent studies indicating that medicinal plants are often phylogenetically clustered, and sister taxa to traditionally used ones could be explored for their pharmaceutical potentials (Saslis-Lagoudakis et al., 2012; Zhu et al., 2011). When the originally used medicinal plants are endangered, the selection of alternative ones should be chemically compared with original species and finally validated by pharmacological tests. However, it should be noted that the production of similar compounds by related plant taxa may not ascertain that they are solely biosynthesized by plants, since they might involve a complex interaction between plants and microbes (e.g. taxol produced by *Taxomyces* species) (Walker and Croteau, 2001).

4.2 Club moss chemosystematics in general

Chemosystematics of Huperzioideae club mosses is mostly carried out in a larger taxonomic context including taxa from the other two subfamilies. In general, previous studies have shown differences mainly between subfamilies and genera, although usually only local taxa were used for chemosystematic investigations. Using thin layer chromatography (TLC) of 10 lycopodium alkaloids, Ma et al., (1998) studied their distribution in 37 Chinese club mosses, which included 29 taxa from Huperzioideae. The authors found that lycodoline, lucidioline and lycopodine are consistently present in genera *Huperzia* and *Phlegmariurus*, with variation or deficiency of hupA and hupB in *Phlegmariurus*. They also reported that serratine and serratinine are potential chemotaxonomic markers differentiating *Huperzia* (present) and *Phlegmariurus* (absent). This may have evolutionary implications, and future studies should include a worldwide sampling of *Huperzia* and *Phlegmariurus* species and characterization of the potential divergence of alkaloid biosynthesis between the two genera. In another study (Choo et al., 2018), high performance liquid chromatography-photodiode array detection was used to acquire metabolite fingerprints of methanol extracts from 25 Malaysian club mosses (including seven *Phlegmariurus* species), and chemical differences assessed using principle component analysis (PCA) of fingerprints. A clear separation of *Phlegmariurus* taxa from *Lycopodium* and *Lycopodiella* taxa was observed from the PCA plot, and no distinct chemical groups were found in the *Phlegmariurus* group. No candidate peaks or metabolites have been identified driving such observed chemical differences. In a quite early study focusing on phenolic acids in club mosses (Pedersen and Øllgaard, 1982), very interesting chemical differences were found between Huperzioideae and the other two subfamilies after examining 137 specimens of 49 species: the former tends to produce esters of dihydrocaffeic acid, while the latter two do not. Differences between Huperzioideae species were also found, but the TLC method did not enable identification of the marker phenolics.

Although a large number of papers have been published describing alkaloids in Huperzioideae club mosses, the ambiguous plant identification in those phytochemical papers makes chemosystematic investigations based on them rather inconsistent and unreliable. This can be illustrated by the spread of obsolete plant names in combination with lack of cited voucher specimens, such as *Lycopodium serratum* var. *longipetiolatum* (Ishiuchi et al., 2016) and *Lycopodium chinense* (Morita et al., 2003). Due to the high morphological similarity between club moss congeners, we again suggest that future phytochemical studies provide at least one DNA barcode sequence from a cited reference voucher to ensure plant identity. Of utmost importance, a thorough chemosystematic study should include a worldwide sampling incorporating all or most carefully identified (or genetically confirmed) extant species.

4.3 Club moss chemosystematics: an example of hupA production

It is noteworthy that hupA and its analogue huperzine B (hupB) are not consistently produced in all *Phlegmariurus* species, and they seem to be absent from some traditionally used *Phlegmariurus* taxa with reported psychoactivity, e.g. *P. saururus* (Ortega et al., 2004; Vallejo et al., 2007). Instead, *P. saururus* produces sauroxine (**Fig. 2**) as the major alkaloid, an analogue of hupA, hupB and *N*-demethyl-sauroxine (Vallejo et al., 2013). All belong to the lycodane-type lycopodium alkaloid group, but differ mainly in the saturation status in the 2-pyridone ring (**Fig. 2**). It has been speculated that some cytochrome P450 enzymes may catalyze a series of oxidative reactions (Yang et al., 2017), taking place in the final biosynthetic steps of hupA and hupB. Future studies may focus on those oxidative enzymes, and investigate their evolutionary implications that may lead to observed chemical differences between hupA-producing and hupA-deficient taxa. The content of hupA may also be associated with the genotype of its resource plant (Xu et al., 2019b). To this end, comparative omics may help elucidate the biosynthesis of hupA. For example, by designing comparative transcriptomic studies focusing on closely related *Huperzia* taxa (or *Phlegmariurus* taxa) with known hupA production differences, the gene expression differences in the hupA biosynthetic pathway could be characterized. In addition, hupA is also absent in two *Phlegmariurus* species collected in Australia, including *P. tetrastichus* (Kunze) A.R.Field & Bostock and *P. subtrifoliata* (Brownlie) A.R.Field & Bostock growing in nursery stock (Goodger et al., 2008), but all investigated congeners growing in natural populations produce high amounts of hupA and hupB. This also raises the question – to which extent do the environment and growing conditions influence the biosynthesis of hupA and hupB? Future experiments using cultivated plants in controlled conditions may answer this question.

Given the number of club moss taxa of Huperzioideae that have been screened, there is still a large number of species that have not been phytochemically investigated. No alkaloid chemistry has for example been reported from the species *Phylloglossum drummondii* Kunze, which is endemic to Oceania. Future research may well focus on those unstudied species with ethnobotanical records, or the ones having close phylogenetic relatedness to traditionally used taxa. Caution should be taken while working on endangered plant species to avoid unsustainable harvesting of material for research or commercialisation.

There is still some uncertainty about the production of hupA in certain Huperzioideae species, which necessitates an urgent re-appraisal of plant identification. For example, *H. lucidula* was generally believed to produce hupA (Ma et al., 2007), but contradictory results are found in two studies (Cuthbertson et al., 2012; Ma et al., 2005). The hupA content in Chinese *H. lucidula* is around 54.94 µg/g, while American *H. lucidula* is devoid of hupA. A later taxonomic study investigating the presence of *H. lucidula* in China finds that this Chinese *H. lucidula* should belong to another species, named *H. asiatica* (Shrestha and Zhang, 2015). Phylogenetic analysis using plastid loci reveals that *H. asiatica* is more closely related to *H. selago* than *H. lucidula* (Shrestha and Zhang, 2015). The other study (Cuthbertson et al., 2012) did not provide identification details for the American specimen, and old literature studying American *H. lucidula* seems to support the absence of hupA (Ayer et al., 1979; Manske and Marion, 1946). This needs to be re-examined using very careful plant identification to reach a final conclusion. Also, it reflects the importance of collaboration between plant taxonomists and phytochemists.

Due to the apparently inconsistent production of hupA and hupB in *Huperzia* species, they should not be used as conclusive chemotaxonomic markers. Instead, we recommend the use of more informative chemical fingerprints for chemotaxonomic purposes.

4.4 Plant preservation: call for new analytics

Phytochemical studies usually include isolation and purification of new bioactive compounds, which may require kilograms of starting plant materials. It might not be an issue for fast-growing or cultivated plants, but it is indeed a problem for slow-growing club mosses. It has been estimated that it takes about 15 years from spore to mature sporophyte (Ma and Gang, 2008), and the whole plants weigh roughly grams to tens of grams. There is a risk of over-harvesting leading to the decline of club mosses, especially the ones with medicinal properties (Ma et al., 2006). Certain club moss taxa have been listed as endangered plants, such as *P. saururus* in Ecuador (Ortega et al., 2004). The International Union for Conservation of Nature (IUCN) red list shows that there are around 12 endangered Huperzioideae club moss species, with *P. nutans* being critically endangered (CR).

Sustainable approaches to bioprospecting of medicinal club mosses are needed. A good approach is plant tissue cultivation at optimized conditions, which additionally shortens plant growth time and enables biological studies requiring a controlled environment (Ma and Gang, 2008). More effective and sensitive chemical methods that only require small amounts of plant materials, should be developed for metabolite fingerprinting and compound dereplication. By combining liquid chromatography-mass spectrometry (LC-MS) chemical profiling and chemometrics, interesting unknown peaks/compounds can be targeted for isolation (Yang et al., 2013). Chemosystematics and phylogenetics may also be useful to select alternative taxa when the original one is endangered.

4.5 Selecting medicinal club moss taxa: an example of Icelandic *Huperzia selago*

Selection of medicinal club mosses could be aided with an integrative approach combining plant barcoding and alkaloid fingerprinting. A recent study focusing on Icelandic *H. selago* found this species to have three genotypes using DNA barcode sequences. Genotype 3 contains significantly higher amount of hupA than genotype 1, and hupB is found in genotype 3 but is not detected in genotype 1 (Xu et al., 2019b). Therefore, *H. selago* of genotype 3 which has dark green color and reflexed leaves, is suggested to be a good source for hupA and analogues. A further investigation on alkaloid fingerprints of these genotypes reveals that each genotype has a distinct alkaloid fingerprint, observed as a clustered group in a PCA plot (Xu et al., 2019a). Alkaloids driving group separation have been annotated, and some of them are only present in one genotype (unpublished). The differences between genotypes may easily be missed during plant collection, and a mixture of genotypes with chemical differences be collected. These studies have shown the power of integrating DNA barcoding and chemical fingerprinting for identifying club moss taxa with higher contents of interesting metabolites. It also highlights the importance of careful plant identification, even on a subspecies level, prior to phytochemical studies. This is especially important for morphologically similar plant taxa like club mosses.

The current challenge in lycopodium alkaloid fingerprinting lies in the lack of a standardized sample preparation method (e.g. extraction solvents, chromatographic methods, etc.), which would affect the obtained fingerprints and chemosystematic conclusions. Therefore, future studies should compare different sample preparation methods and select the optimal one as a standardized alkaloid fingerprinting method.

5 Pharmaceutical screening

Ethnopharmacology seeks a pharmaceutical understanding of traditionally used plants, and the assumption is that herbal preparation (or isolated compounds from the preparation) could have desired effects on the claimed disease(s) (Gertsch, 2009). Current research trends tend to focus on purified alkaloids from herbal preparations, which are most frequently screened for *in vitro* AChE inhibitory activities. However, studies of the bioactivity of one component can not be directly equalized with effects of consuming the whole plant, since other components and their bioactivities have not been investigated. Possible synergistic effects of multiple components in herbal preparations could be explored (Gertsch, 2011), and different omics techniques may be useful in that respect (Wang et al., 2009, 2005). Lycopodium alkaloids may affect multiple neuropharmacological drug targets to exert pharmaceutical efficacy. This is well exemplified by the most intensively studied hupA reviewed by Olafsdottir et al. (2013).

Interestingly, isolated alkaloids or alkaloid fractions of a few traditionally used *Phlegmariurus* species did not show potent *in vitro* AChE inhibitory activity, although the psychoactivity of these species is well documented (Vallejo et al., 2009). A possible reason might be lack of standardization of the AChE inhibition test, where discrepancies are found in terms of enzyme source and the positive control used. Occasionally, alkaloid extracts could not be fully dissolved in test solutions (e.g. DMSO) (Armijos et al., 2016), and this may lead to the underestimation of their inhibitory effects. It is also not uncommon that alkaloids in small amount may be lost during prolonged isolation steps (Ortega et al., 2004). Thus, phytochemical efforts are still needed for the Huperzioideae subfamily. Discrepancy between reported effects, i.e. psychoactivity, and absent activity in bioassays, could also be due to the compounds affecting unknown molecular pathways. Further observations on the in vitro activity of lycopodium alkaloids await the testing of more biological targets and the development of new bioassays (Adams et al., 2007).

Conclusion

Huperzioideae club mosses have interesting pharmaceutical potential in light of ethnopharmacological investigations, and the alkaloid hupA is phylogenetically confined to taxa in this subfamily. Bioprospecting of these plants and their alkaloids should be preceded by careful plant identification to ensure data consistency and reproducibility. Herein we suggest that future studies should provide DNA barcoding data from a referenced voucher specimen to aid club moss identification. For the purpose of plant conservation and effective medicinal plant selection, we emphasize a chemosystematic approach integrating DNA barcoding and LC-MS-based chemical fingerprinting. We anticipate that these recommended approaches could facilitate and improve ethnopharmaceutical studies via a close collaboration between researchers in different subjects, i.e. taxonomy, phytochemistry and pharmacology.

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Figure legends

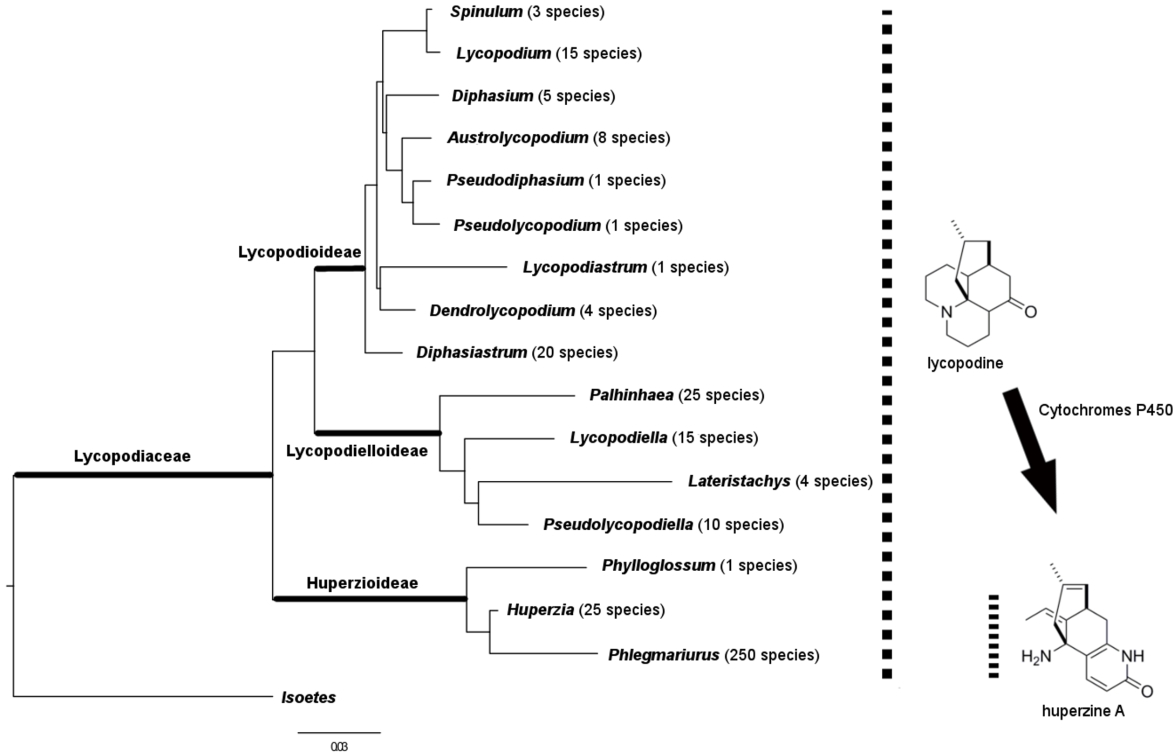

Fig. 1. Phylogenetic tree of the club moss family Lycopodiaceae, consisting of three subfamilies – Huperzioideae, Lycopodioideae and Lycopodielloideae. The alkaloid lycopodine is widely distributed among all the subfamilies, while huperzine A (hupA) is phylogenetically confined to the subfamily Huperzioideae. Lycopodine is hypothesized to undergo cytochrome P450-catalyzed reactions and convert to hupA.

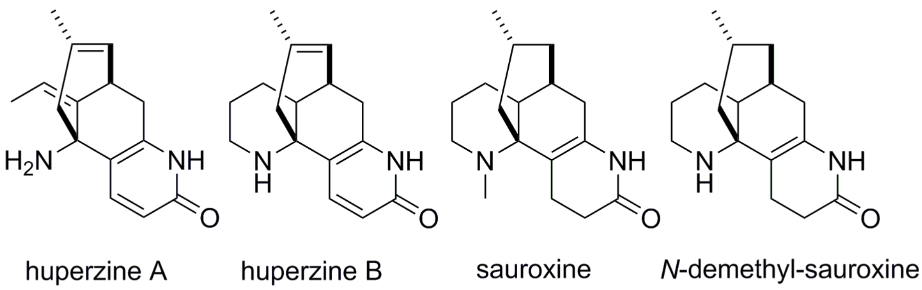

Fig. 2. Molecular structures of representative lycopodium alkaloids in traditionally used Huperzioideae club mosses. HupA and B are found in *Huperzia serrata* and *H. selago*, while sauroxine and *N*-demethyl-sauroxine are found in *Phlegmariurus saururus*.

Table 1. Ethnobotanical uses of species in the club moss subfamily Huperzioideae.

Taxon[1]	Vernacular name (language)	Country (region)	Described use	Reference
Genus *Huperzia* Bernh.				
H. selago (L.) Bernh. ex Schrank &Mart.	Баранець (RU)	Ukraine (Roztochya)	Prepared as tincture for alcoholism treatment	Stryamets et al., 2015
	Lusegras, lomrøk, hårsmåttmåså, luselomme (NO)	Norway	Against lice, to wash hair, as a diuretic, as a laxative, to cause abortions and against udder problems in cows	Høeg, 1974
	-	Faroe Island	Against cough and respiratory problems	Svanberg, 1998
H. serrata (Thunb. ex Murray) Trevis	千层塔, 石松 (ZH)	China (south)	External used to promote blood circulation, stop bleeding, relieve pain, treat senile dementia and traumatic injury; Treatment of contusions, strains, swellings, schizophrenia and myasthenia gravis.	Luo et al., 2018; Ma et al., 2007
Genus *Phlegmariurus* Holub				
P. brevifolius (Hook. & Grev.) B. Øllgaard (*H.brevifolia*)	Agua minga (ES)	Ecuador (Loja)	Bath ingredient for liver, kidney health and prevention of fever, inflammation and colds	Bussman and Sharon, 2006
P. carinatus (Desv.) Ching (*H.carinatum*)	Tamtam jumalo (JW)	Indonesia (Kabanjahe)	Treatment of cancer and bone fractures	Silalahi et al., 2015
P. columnaris (B. Øllg.) B. Øllgaard (*H.columnaris*)	Agua minga (ES)	Ecuador (Loja)	Bath ingredient for liver, kidney health and prevention of fever, inflammation and colds	Bussman and Sharon, 2006
P. compactus (Hook.) B. Øllgaard (*H.compacata*)	Agua minga (ES)	Ecuador (Loja)	Bath ingredient for liver, kidney health and prevention of fever, inflammation and colds	Bussman and Sharon, 2006
P. espinosanus (B. Øllg.) B. Øllgaard (*H.espinosanus*)	Agua minga (ES)	Ecuador (Loja)	Bath ingredient for liver, kidney health and prevention of fever, inflammation and colds	Bussman and Sharon, 2006
P. fargesii (Herter) Ching	-	China (Hunan)	Ingredient in medicinal bath, to treat traumatic injury and rheumatism	Luo et al., 2018
P. hypogaeus (B. Øllg.) B. Øllgaard (*H.hypogaea*)	Auga minga (ES)	Ecuador (Loja)	Bath ingredient for liver, kidney health and prevention of fever, inflammation and colds	Bussman and Sharon, 2006
P. kuesteri (Nessel) B. Øllgaard (*H.kuesteri*)	Agua minga (ES)	Ecuador (Loja)	Bath ingredient for liver, kidney health and prevention of fever, inflammation and colds	Bussman and Sharon, 2006
P. nummulariifolius (Blume) Ching	Tamtam jumalo (JW)	Indonesia (Kabanjahe)	Treatment of cancer and bone fractures	Silalahi et al., 2015
P. phlegmaria (L.) T.Sen & U. Sen	Tara tinggi (JW)	Indonesia (Kabanjahe)	Treatment of cancer and kidney disease	Silalahi et al., 2015
P. proliferus (Blume) A.R.Field & Bostock	Sijergal (JW)	Indonesia (Kabanjahe)	Aphrodisiac agent and treatment of bone fractures	Silalahi et al., 2015
P. ruber (Cham. & Schltdl.) B.Øllgaard (*Lycopodium rubrum*)	-	Venezula	Leaves used to treat elephantitis	Frye, 1934

P. saururus (Lam.) B. Øllgaard ex	Condor blanca (ES)	Peru (Huncabamba)	An ingredient in hallucinogenic beverage	De Feo, 1992
H. saururus (Lam.) Trevis	Cola de quirquincho (ES)	Argentina (Cordoba)	Consumed as infusion and decoction. Aphrodisiac and antialopecic agents; memory-improving agent; treatment of mood and sentimental disorders. Adverse effects when concentrated decoction consumed include vomiting, diarrhea, convulsions and even death	Birri et al., 2017, 2014; Goleniowski et al., 2006; Ortega et al., 2006; Vallejo et al., 2007
P. tetragonus (Hook & Grev) Trevis	Trencilla roja (ES)	Ecuador (Loja)	Laxative	Armijos et al., 2016
Unidentified				
"*Huperzia* sp."	Huaminga (ES)	Peru (Ayabaca)	Concentrated decoction as a strong purgative agent, while diluted one as vermifuge; alcoholic and water extracts may cause diarrhea	De Feo, 2003
"*Huperzia* sp."	-	Peru (north)	Ingredients for cleansing bath and hallucinogenic preparations	Bussmann, 2013
"*Huperzia* sp."	Cabello del bosque (ES)	Peru (Ayabaca)	Juice from pounded fresh plants to promote hair growth	De Feo, 2003
"*Huperzia* sp."	Condor Misha (ES)	Peru (north)	Spiritual cleansing	Bussmann et al., 2010
"*Lycopodium selago*"	Agwo, Ude, Otuen (Igbo)	Nigeria (south)	Leave extracts to treat snake bite; plants hung on doors to prohibit evil spirits; Powders of spore and root mixed with palm kernel oil (*Elaeis guineense*) as external uses to treat eczema and cuts	Nwosu, 2002
"*Lycopodium selago*"	-	Mexico	Spores as vermifuge	Knobloch and Correll, 1962
"*Lycopodium selago*"	-	USA (California)	Stems chewed and juice swallowed leading to intoxication. Three plants for mild intoxication, while eight affecting behavior ("stupified the users")	Lloyd, 1964
Mixture				
P. brevifolia, *P. compactus*, *P. espinosanus*, *P. tetragonus*, etc.	Trencilla, wamingas (ES)	Ecuador (Loja)	Psychoactive preparation using alcohol extracts of plant mixtures including *Phlegmariurus* species and other plants, such as *Echinopsis pachanoi* and *Nicotiana tabacum*.	Armijos et al., 2016

[1] Older synonyms used in original publications are provided in parenthesis.