

Infraspecific Variation of Huperzine A and B in Icelandic *Huperzia selago* Complex

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ABSTRACT

The alkaloids huperzine A and huperzine B were originally isolated from the Chinese club moss *Huperzia serrata*. They are known inhibitors of acetylcholinesterase, and especially huperzine A shows pharmaceutical potential for the treatment of Alzheimer's disease. Its supply heavily relies on natural plant sources belonging to the genus *Huperzia*, which shows considerable interspecific huperzine A variations. Furthermore, taxonomic controversy remains in this genus, particularly in the *Huperzia selago* group. With focus on Icelandic *H. selago* taxa, we aimed to explore the relatedness of *Huperzia* species using multi-locus phylogenetic analysis, and to investigate correlations between huperzine A contents, morphotypes, and genotypes. Phylogenetic analysis was performed with five chloroplastic loci (the intergenic spacer between the photosystem II protein D1 gene and the tRNA-His gene, maturase K, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, tRNA-Leu, and the intergenic spacer region between tRNA-Leu and tRNA-Phe). Huperzine A and huperzine B contents were determined using an HPLC-UV method. The phylogenetic analysis suggests that previously proposed *Huperzia appressa* and *Huperzia arctica* should not be considered species, but rather subspecies of *H. selago*. Three genotypes of Icelandic *H. selago* were identified and presented in a haplotype networking diagram. A significantly ($p < 0.05$) higher amount of huperzine A was found in *H. selago* genotype 3 (264–679 $\mu\text{g/g}$) than genotype 1 (20–180 $\mu\text{g/g}$), where the former shows a typical green and reflexed “selago” morphotype. The huperzine A content in genotype 3 is comparable to Chinese *H. serrata* and a good alternative huperzine A source. Genotype 2 contains multiple morphotypes with a broad huperzine A content (113–599 $\mu\text{g/g}$). The content of huperzine B in Icelandic taxa (6–13 $\mu\text{g/g}$) is much lower than that in Chinese *H. serrata* (79–207 $\mu\text{g/g}$).

Introduction

Lycopodium alkaloids represent a diverse array of alkaloids specific to the club moss family Lycopodiaceae [1]. The club moss *Huperzia serrata* is used as a medicinal herb in China to treat several ailments, e.g., contusions and dementia [2]. The lycopodium alkaloids hupA and hupB shown in ► **Fig. 1** were first isolated from

H. serrata in 1980s [2]. They are potent AChE inhibitors, with hupA being considered a potential drug lead for the treatment of Alzheimer's disease [1–4]. However, further pharmaceutical development is hampered by the lack of a sustainable resupply of the compound [3]. Although a series of techniques have been proposed, such as *in vitro* cultivation of *Huperzia* shoots [5], total synthesis [6], and fermentation of hupA-producing microbes [7], the

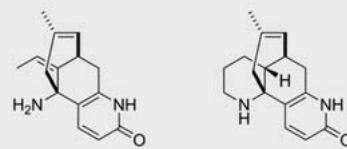
ABBREVIATIONS

AchE	acetylcholinesterase
BS	bootstrap support
d. w.	dry weight
hupA	huperzine A
hupB	huperzine B
LOD	limit of detection
LOQ	limit of quantification
matK	maturase K
ML	maximum likelihood
PP	posterior probability
psbA-trnH	the intergenic spacer between the photosystem II protein D1 gene and the tRNA-His gene
rbcl	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
trnL	tRNA-Leu
trnL-F	the intergenic spacer region between tRNA-Leu and tRNA-Phe

major source of hupA still heavily relies on herbal materials, which could lead to overharvesting [4, 8].

Since considerable variation of hupA content has been found in *Huperzia* species, it is important to identify the high hupA producers. In total, four club moss species grow in Iceland and one of them, *Huperzia selago* (L.) Bernh. Ex Schrank & Mart., produces hupA [9]. The others are *Diphasiastrum alpinum* (L.) Holub, *Spinulum annotinum* (L.) A. Haines (Basionym: *Lycopodium annotinum*) [10], and *Lycopodium clavatum* (L.) [11]. The lycopodium alkaloids isolated and their *in vitro* inhibitory activity against AChE have been studied in Icelandic *D. alpinum* [12], *S. annotinum* [13], and *H. selago* [9]. HupA is by far the most potent AChE inhibitor of all tested lycopodium alkaloids with an IC_{50} value of 72.4 nM, while hupB has an IC_{50} value of 19.3 μ M [14].

The taxonomy of *H. selago* is still controversial, and phylogenetic analysis can be expected to resolve some of the issues. The genus *Huperzia* in the northern areas (boreal and arctic regions) is very polymorphic, and numerous taxonomical treatments have been published so far [15–18]. Some fairly recent European taxonomical treatments of *Huperzia* based on a hypothesis of gradual divergence of races within the genus [15–17, 19] have accepted one species of *H. selago* that includes two or three races, i.e., the temperate to boreal morphotype “selago” (spreading or patent, dentate, green to dark green leaves, and few, if any, bulbils), the more northerly distributed (northern alpine-boreal to southern arctic) morphotype “appressa” (appressed or subappressed, usually dentate leaves), and the most northerly distributed (arctic) morphotype “arctica” (brown leaves that were pressed outwards and bulbils present). Some European authors do not accept the morphotype “appressa” [16, 17] as a subspecies and include it in the subspecies *arctica*. These treatments include almost all taxonomically possible options ranging from recognition of one species (undifferentiated *H. selago*) and one species with local races (treated as subspecies) to the recognition of several independent species. Morphological differences between *H. selago*-related taxa



► Fig. 1 Chemical structure of huperzine A (left) and B (right).

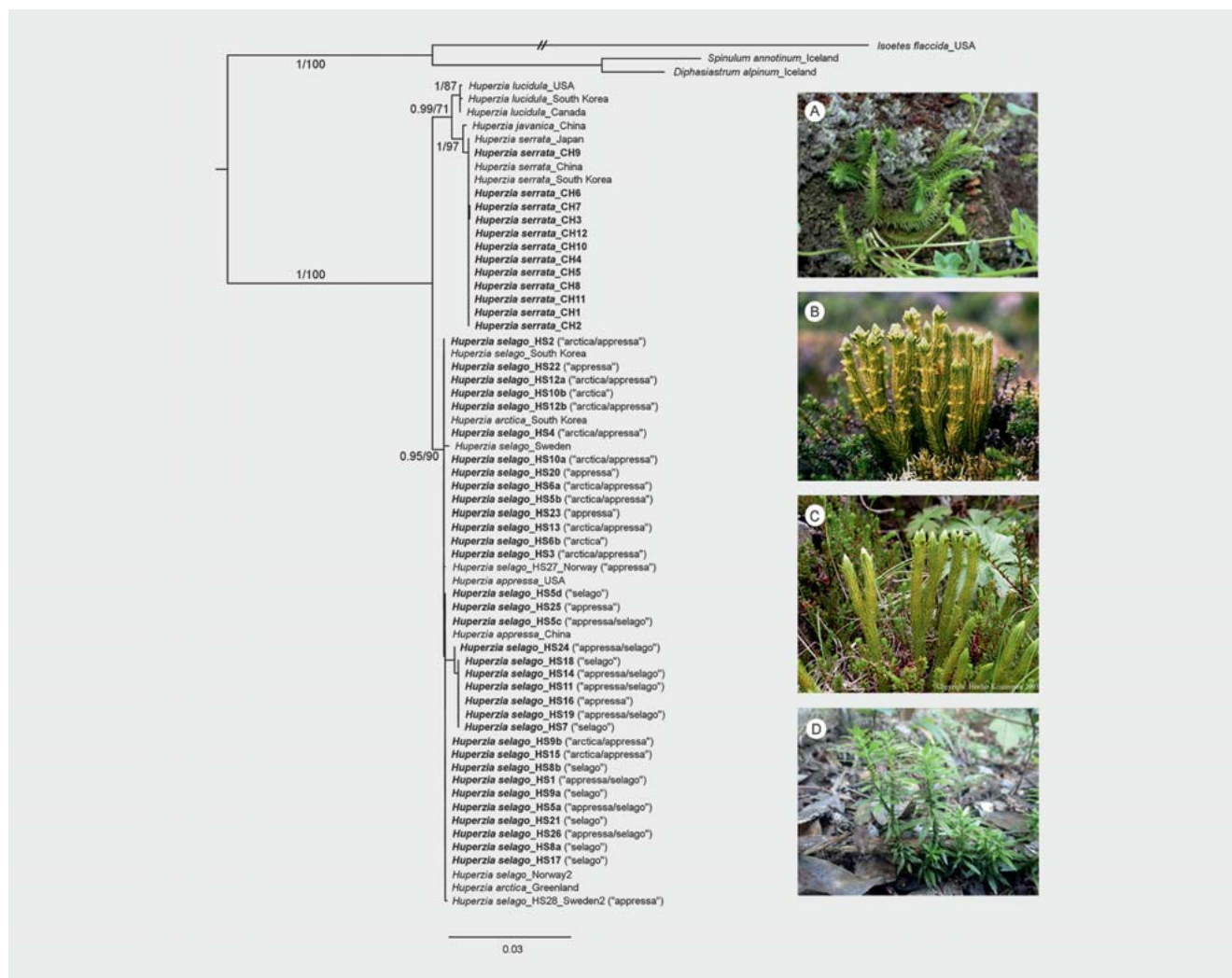
might be trivial, with intermediate morphology being common, and thus they have been called the “*H. selago* group” [20]. This indicates that significant morphological and genetic variations are present within the genus, and that current taxonomical subdivisions of *Huperzia* are more or less provisional. Recent phylogenetic analyses of club mosses used chloroplast DNA markers and revealed a phylogenetic relationship between genera and even species [20–23]. Species delimitation in those studies mainly focused on the sister genus *Phlegmarium* [21, 23], while the species level identification in the genus *Huperzia* was largely neglected.

In Iceland, “arctica” and “selago” morphotypes are regarded as *H. selago* subspecies [8], but the intermediate morphotype resembling the “appressa” morphotype can also be found. Subspecies *arctica* is commonly found in open areas with a yellow to yellowish-green color and outward pressed leaves, and subspecies *selago* usually grows in relatively humid and shady habitats with green to dark green shoots and reflexed leaves. Their taxonomic ranks comply with descriptions in the monograph Flora Nordica [17]. The subspecies *selago* is commonly found in western Iceland, while the subspecies *arctica* has a wider distribution [17]. It is timely to apply genetic tools to clarify this taxonomic uncertainty regarding the “*H. selago* group”, and to investigate the correlations between alkaloid contents, morphology, and genotypes.

Phytochemical studies of club mosses as well as other plants used for medical purposes should always be preceded by accurate identification of the plant material. It is also important to identify the plant species, cultivars, or even populations or genotypes correctly as they might vary considerably in alkaloid content. This study aimed to (1) explore species relatedness in the genus *Huperzia* using multi-locus phylogenetic analysis with a focus on the Icelandic *H. selago* complex, and determine whether this complex group should be recognized as one or more species or subspecies, (2) determine the contents of hupA and hupB in Icelandic *Huperzia* taxa, and explore correlations between hupA contents, morphotypes, genotypes, and phylogeny, and (3) compare the hupA and hupB contents between Icelandic *Huperzia* taxa and the widely consumed Chinese *H. serrata*.

Results

Overall, 203 new sequences of five loci (i.e., psbA-trnH, rbcl, matK, trnL, and trnL-F) were generated in this study. With the newly designed primers, matK regions (ca. 800 bp) of sampled taxa were successfully amplified. The concatenated sequence matrix contained 3385 nucleotide characters with 595 variable sites. Our phylogenetic analysis recovered the genus *Huperzia* as



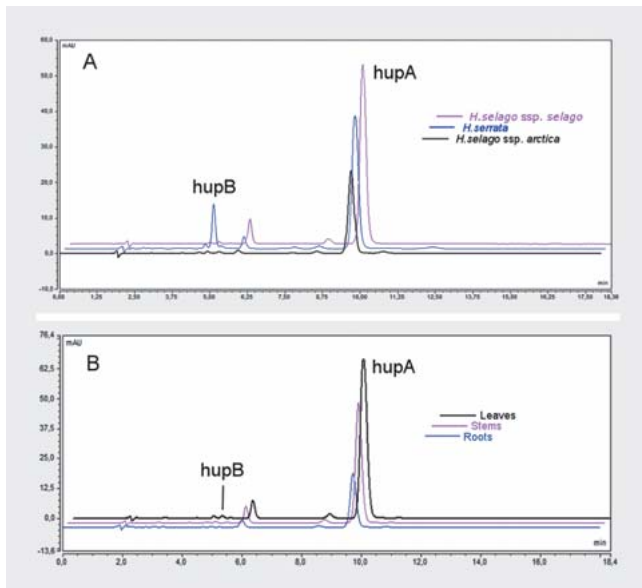
► **Fig. 2** Maximum likelihood tree made from concatenated sequence matrix containing *rbcl*, *matK*, *psbA-trnH*, *trnL*, and *trnL-F*. Sampled Icelandic (HS1–26) and Chinese (CH1–12) club moss specimens are in boldface. *H. selago* morphotypes are indicated in parentheses. Posterior probabilities (PP) over 0.95 and bootstrap (BS) values over 70% are labelled near branches as PP/BS. Representative photographs of sampled taxa including (A) *H. selago* ssp. *selago* (i.e., “selago” morphotype), (B) *H. selago* ssp. *arctica* (i.e., “arctica” morphotype), (C) *H. selago* ssp. *pressa* (i.e., “pressa” morphotype), and (D) *H. serrata*. Photos were taken by Natalia Kowal (A), Hördur Kristinnsson (B, C) and Cheng Zhang (D).

a monophyletic clade with a BS value of 100% and a PP of 1 (► **Fig. 2**). A deeper subgeneric relationship of *Huperzia* species was uncovered showing that the temperate taxa *H. serrata*, *Huperzia javanica*, and *Huperzia lucidula* are more closely related to each other and form a sister clade (BS: 71; PP: 0.99) to the circumpolar species *H. selago*. Previously proposed morphological species *Huperzia arctica* and *Huperzia pressa* are not distinct and form a monophyletic clade (BS: 90; PP: 0.95) with *H. selago*.

The alkaloids hupA and hupB (► **Fig. 1**) were identified by comparing their retention times ($t_R = 9.0$ min and 4.5 min, respectively) (► **Fig. 3A**) with those of commercial standards. The HPLC method showed good performance in separating hupA and hupB (► **Fig. 3A**). Good linearity was obtained ($R^2 = 0.9918$ – 0.9986) in the working linear range of 0.2–25 $\mu\text{g}/\text{mL}$ for both compounds. LOD and LOQ were determined at 0.04 and 0.1 $\mu\text{g}/\text{mL}$ for hupB, and 0.06 and 0.15 $\mu\text{g}/\text{mL}$ for hupA, respectively. Intraday and in-

ter-day variations (measured as relative standard deviation) were 0.5–3.8% and 2.1–6.4%, respectively. A good recovery of 92–105% was obtained for both compounds.

The contents of hupA in the sampled *Huperzia* specimens showed a considerable variation ranging from 20.63–679.82 $\mu\text{g}/\text{g}$ d.w. (► **Table 1**). Low hupA contents (ca. 20–110 $\mu\text{g}/\text{g}$ d.w.) are mostly associated with the “arctica” morphotype, while high hupA contents (ca. 260–680 $\mu\text{g}/\text{g}$) are associated with the “selago” morphotype. The correlation between genotypes and hupA contents in Icelandic specimens was studied and compared using a haplotype network diagram (► **Fig. 4**). It was found that genotype 3 (264.39–679.82 $\mu\text{g}/\text{g}$ d.w.) contained a significantly ($p < 0.05$) higher amount of hupA than genotype 1 (20.63–193.84 $\mu\text{g}/\text{g}$ d.w.). Genotype 3 is a “selago” morphotype with one exception of an “pressa” morphotype, while genotype 1 is uniformly an “arctica” morphotype. The intermediate genotype 2



► **Fig. 3** HPLC-UV chromatograms showing (A) hupA and hupB variations between two *H. selago* subspecies and *H. serrata*, and (B) tissue-dependent hupA contents in *H. selago* ssp. *selago*.

has a much wider hupA range, spanning from 113.13–599.63 $\mu\text{g/g}$ d. w., and seems to contain all three morphotypes. A detectable amount of hupB in Icelandic specimens was only found in genotypes 2 and 3 of the “selago” morphotype with a content variation from 5.97 to 12.92 $\mu\text{g/g}$ d. w., which is much lower than that in *H. serrata* (78.74–206.67 $\mu\text{g/g}$ d. w.).

A tissue-dependent distribution of hupA was observed in *H. selago* of genotype 3 and the “selago” morphotype (► **Fig. 3B**). The specimen (HS18) with the highest overall hupA content (679.82 $\mu\text{g/g}$ d. w.) was investigated, and the results showed that the leaves exhibit the highest hupA content of 922.74 $\mu\text{g/g}$ d. w., followed by stems with 697.51 $\mu\text{g/g}$ d. w., and roots with 343.91 $\mu\text{g/g}$ d. w.

Discussion

The reconstructed phylogeny, achieved by combining five chloroplastic loci, gives an improved subgeneric resolution compared to the most recent phylogeny [22]. Previous phylogenetic analysis mainly used rbcL sequences to reflect a genus- or family-level relationship, and it appears that a subgeneric- and species-level resolution should include more variable sequence data [24, 25]. Phylogenetic analysis focusing on Asian specimens proposed that the genus *Huperzia* contains two subgeneric sections: one section includes *Huperzia asiatica*, *Huperzia miyoshiana* and *H. selago*, and the other section *H. lucidula*, and *H. serrata*, and *Huperzia nanchuanensis* [26, 27]. Our phylogenetic analysis supports the presence of the two-section proposal with more specimens added. However, our study does not brace the species-level classification of “arctica”, “appressa”, and “selago” morphotypes, but it supports that they should be treated as subspecies.

Although the morphological distinction of some *H. selago* specimens into one of the three morphotypes can be challenging, as reflected in **Table S1**, Supporting Information, and ► **Table 1**, it seems that the “arctica” and “selago” morphotypes tend to have their own genetic identity as do genotypes 1 and 3 in the haplotype network diagram. This also correlates with hupA contents: low in the former and high in the latter. The presence of intermediate genotype 2 with mixed and intermediate morphotypes may indicate hybridization, which has been shown to be common among *Huperzia* species [28].

Variation of hupA contents in Icelandic *H. selago* (20.63–679.82 $\mu\text{g/g}$ d. w.) is within the range of reported amounts in the literature, such as hupA in Australian *Huperzia* species (0–1.03 mg/g d. w.) [5] and Chinese taxa (46.85–254.58 $\mu\text{g/g}$) [29]. The highest concentration of hupA reported in wild *Huperzia* species is 1.27 mg/g d. w. in a Polish *H. selago* specimen [5]. Our results of hupA contents in sampled Chinese *H. serrata* (318.71–593.50 $\mu\text{g/g}$ d. w.) are, however, much higher than the reported range (80.16–182.55 $\mu\text{g/g}$) [29]. Such variation might be due to the insufficient drying and grinding of plant materials in the reference study, where plant materials are only air-dried and ground without the assistance of liquid nitrogen, which altogether leads to a lower concentration of hupA. Furthermore, different sample preprocessing methods (e.g., storage and grinding, etc.), solvents, and extraction time might contribute to such variations. Another reason might be the inaccurate identification of plant materials in the reference study, where no morphological or genetic data were shown.

A study on Chinese *Huperzia* species and related taxa suggests that the genus *Phlegmariurus* (241.84–560.46 $\mu\text{g/g}$) should be a better source of hupA than its sister genus *Huperzia* (46.85–254.58 $\mu\text{g/g}$) [29]. Other studies have shown the contrary, i.e., no detectable amount of hupA were found in Australian *Phlegmariurus phlegmaria* and *Phlegmariurus tetrastrichus* [5]. *Phlegmariurus* species in the latter study are from Nursery stock, while the taxa in the former study are wild. Future studies should investigate the possible influence of environmental conditions and seasonal fluctuation on the production of hupA. In addition, the current study also supported the tissue-dependent distribution of hupA, which is concentrated in the aerial part. A high content of hupA (415 $\mu\text{g/g}$) in spore-bearing leaves (i.e., sporophyte) has been reported [30]. Samples in the current study were mostly collected in late summer and early September when spores have been released, so an early sampling strategy in spring may be considered to compare seasonal variation with and without spores.

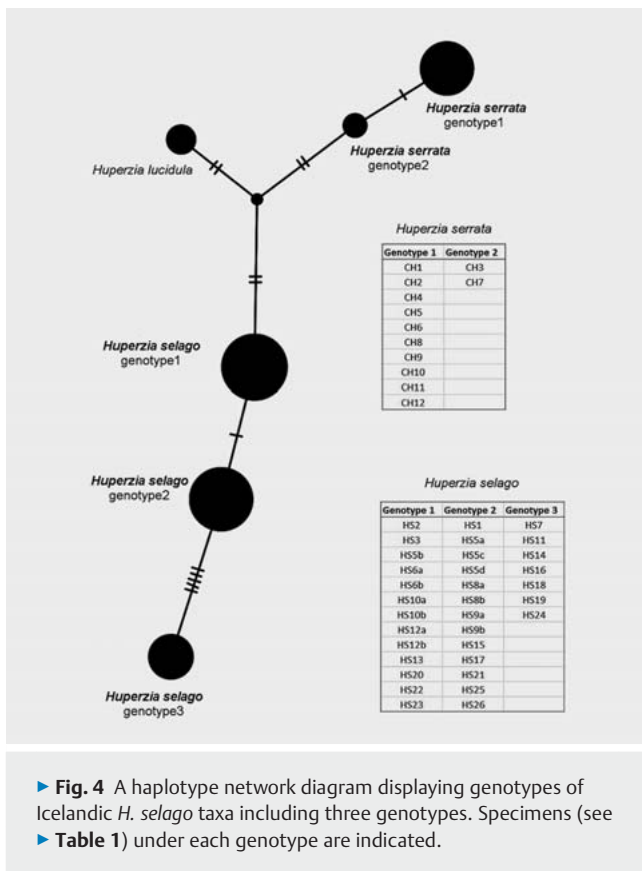
Considerable hupB variation has been reported in *Huperzia* and *Plagmarium* species ranging from 0 to 302 $\mu\text{g/g}$ [31, 32], and our results of 5.97–12.92 $\mu\text{g/g}$ for *H. selago* and 78.74–206.67 $\mu\text{g/g}$ for *H. serrata* fall within this range. Although hupB is a relatively strong AChE inhibitor (IC_{50} : 19.3 μM), its content in the plants is usually much lower than the more potent hupA (IC_{50} : 72.4 nM) [14, 29], and therefore hupB has gained much less attention. The large hupB differences between Icelandic *H. selago* and Chinese *H. serrata* can result from both environmental and genetic factors.

To conclude, our phylogenetic analysis suggests that previously proposed *H. appressa* (i.e., “appressa” morphotype) and *H. arctica* (i.e., “arctica” morphotype) should be regarded as sub-

► **Table 1** HupA and hupB contents in sampled *Huperzia* species^a.

SampleID ^b	Herbarium No.	Morphotypes ^c	HupA (µg/g) ^d	HupB (µg/g) ^e
HS1_G2	VA21570	appressa/selago	179.41 ± 1.71	n. d.
HS2_G1	VA21571	arctica/appressa	–	–
HS3_G1	VA21572	arctica/appressa	180.37 ± 16.42	n. d.
HS4_G1	VA21573	arctica/appressa	83.33 ± 10.56	n. d.
HS5a_G2	VA21574	appressa/selago	214.82 ± 23.35	n. d.
HS5b_G1	VA21575	arctica/appressa	150.76 ± 12.37	n. d.
HS5c_G2	VA21576	appressa/selago	428.40 ± 11.33	n. d.
HS5d_G2	VA21577	selago	277.11 ± 16.23	n. d.
HS6a_G1	VA21578	arctica/appressa	58.36 ± 6.80	n. d.
HS6b_G1	VA21579	arctica	31.98 ± 1.85	n. d.
HS7_G3	VA21580	selago	306.22 ± 21.85	n. d.
HS8a_G2	VA21581	selago	333.06 ± 31.82	n. d.
HS8b_G2	VA21582	selago	271.41 ± 4.00	9.07 ± 0.09
HS9a_G2	VA21583	selago	242.86 ± 28.77	n. d.
HS9b_G2	VA21584	arctica/appressa	113.14 ± 7.23	n. d.
HS10a_G1	VA21585	arctica/appressa	20.63 ± 2.80	n. d.
HS10b_G1	VA21586	arctica	28.63 ± 2.61	n. d.
HS11_G3	VA21587	appressa/selago	477.01 ± 6.65	6.72 ± 0.44
HS12a_G1	VA21588	arctica/appressa	64.88 ± 10.09	n. d.
HS12b_G1	VA21589	arctica/appressa	–	–
HS13_G1	VA21590	arctica/appressa	–	–
HS14_G3	VA21591	appressa/selago	264.39 ± 18.86	n. d.
HS15_G2	VA21592	arctica/appressa	285.24 ± 17.29	n. d.
HS16_G3	VA21593	appressa	413.02 ± 41.66	n. d.
HS17_G2	VA21594	selago	517.77 ± 33.65	5.97 ± 0.53
HS18_G3	VA21595	selago	679.82 ± 16.25	8.91 ± 0.95
HS19_G3	VA21596	appressa/selago	336.27 ± 34.06	n. d.
HS20_G1	VA21597	appressa	–	–
HS21_G2	VA21598	selago	599.77 ± 40.55	n. d.
HS22_G1	VA21599	appressa	–	–
HS23_G1	VA21600	appressa	159.09 ± 8.40	n. d.
HS24_G3	VA21601	appressa/selago	631.99 ± 27.92	12.92 ± 1.77
HS25_G2	VA21602	arctica/selago	–	–
HS26_G2	VA21603	appressa/selago	599.63 ± 35.29	n. d.
HS27_G1	VA21604	appressa	–	–
HS28_G2	VA21605	arctica/appressa	–	–
CH2	VA21607	–	543.10 ± 16.51	195.14 ± 17.71
CH3	VA21608	–	593.50 ± 35.03	143.94 ± 8.87
CH5	VA21610	–	568.29 ± 13.88	206.67 ± 5.00
CH9	VA21614	–	318.71 ± 10.90	78.74 ± 9.47

^a Specimens of small quantity (i.e., less than 0.3 g) were only used for phylogenetic investigation but not hupA and hupB determination; ^b Genotypes G1, G2, and G3 refer to ► **Fig. 4**; ^c Morphotype assignment refers to the morphological description in the “Introduction” section; ^{d, e} HupA and hupB contents in specimens were shown as dry weight matters



species of *H. selago*. HupA content differs substantially in the three genotypes found within the Icelandic *H. selago* complex. Genotype 3 corresponding to the “selago” morphotype consistently contains a high amount of hupA, and genotype 1 corresponding to the “arctica” morphotype contains a significantly ($p < 0.05$) lower amount of hupA. Genotype 3 of *H. selago* can be considered a good alternative hupA source to the widely consumed Chinese *H. serrata*, but hupB content turned out to be much lower in the *H. selago* specimens than that in *H. serrata*. The results emphasize the importance of carefully identifying plant materials down to the subspecies and genotype level, especially for medicinal plants aimed for human consumption or extraction of valuable compounds such as hupA and hupB.

Materials and Methods

Chemicals and biochemicals

Acetic acid, ammonium acetate, dichloromethane, and methanol were purchased from Sigma-Aldrich. Organic solvents are of HPLC grade. Commercial standard compounds huperzine A and B (purity > 99% by HPLC) were from PhytoLab GmbH&Co. KG. A Plant DNeasy Mini Kit was purchased from Qiagen. Taq DNA polymerase was from New England Biolabs. Gel stain SYBR safety was from Invitrogen. Exonuclease I (EXO) and Shrimp alkaline phosphatase (SAP) were from Fermentas.

Plant materials and sampling

In total, 58 specimens were collected for the current study. Among them, 34 specimens of Icelandic *H. selago* were collected around Iceland, including morphotypes “selago”, “appressa”, and “arctica” (see below for description). Additional two Scandinavian *H. selago* specimens with an “appressa” morphotype were kindly provided by Dr. Hugo de Boer (Natural History Museum, University of Oslo). The species *D. alpinum* (four specimens) and *S. annotinum* (six specimens) were also collected, as they were used for phylogenetic analysis as an outgroup [22]. In addition, Chinese *H. serrata* (Thunb.) Trevis (12 specimens) was included in the current study in order to compare the hupA and hupB contents between the widely used *H. serrata* and Icelandic *H. selago*. Vouchers of all collected specimens are deposited in the Icelandic Institute of Natural History, Akureyri Division (AMNH) under the herbarium numbers VA20570-21629. *H. selago* specimens were distinguished to three morphotypes referring to their description in the “Introduction” section. Representative photos of the morphotypes are shown in ► **Fig. 2A–C**. Intermediate morphotypes were also commonly found, as reported by other taxonomists [17], and were assigned as “arctica/appressa” or “appressa/selago” (**Table 1S**, Supporting Information).

Assignment of morphotypes is shown in **Table 1S**, Supporting Information, and ► **Table 1**, together with voucher information and GenBank accession numbers. Plant materials for downstream DNA extraction and chemical analysis were dried in silica gel and air-dried for 10 days, respectively. Dried plant materials were stored in darkness at room temperature. Specimens of small quantity (i.e., less than 0.2 g) were only used for the phylogenetic investigation and deposited in herbarium.

Sample preparation for alkaloid analysis

Air-dried plant materials (ca. 0.3–0.5 g) were ground in liquid nitrogen into fine powders. Then, pulverized materials were lyophilized overnight before alkaloid extraction. Lyophilized materials were accurately weighed (40 mg) in glass tubes, and the alkaloids were extracted in a sonicator for 30 min with 0.9 mL 2% acetic acid in Milli-Q water. Organic solvents were not used due to the lower extraction efficiency of hupA [29]. Extraction was repeated twice and all extracts were combined and washed twice with dichloromethane. The supernatant was transferred to another glass tube, and 0.9 mL dichloromethane was added. The pH of the aqueous layer was adjusted to 9–10 with ammonium hydroxide. The organic layer was separated, and the aqueous layer was then extracted two times with dichloromethane. All three organic layers were combined and evaporated under reduced pressure. Dried residues were dissolved in methanol and filtered (0.45 µm; Millipore) before the HPLC analysis. Alkaloid extraction of each specimen was carried out in triplicate.

Quantitation of huperzine A and B

An HPLC method was developed following a reported procedure [20] with minor modifications to determine hupA and hupB contents in club moss samples. Chromatographic analysis was carried out using the Dionex UltiMate 3.0 HPLC system, controlled by Dionex Chromeleon software v7.2. The HPLC system consisted of a column oven compartment, an autosampler with temperature

► **Table 2** Primers and their annealing temperatures.

Primer	Sequence (5'-3')	T _a	Reference
rbcl-1F	ATGTCACCACAAACGGA	55	[24]
rbcl-1409R	TCAAATTCAAACCTTGATTCTTTCCA		[24]
psbA-F	GTTATGCATGAACGTAATGCTC	50	[38]
trnH-2R	CGCGCATGGTGGATTACAATC		[39]
Hup-matK-F	TGGAGGACCATTTTTTCACAT	51	This study
Hup-matK-R	TTAAATTGGTTAGGAATGTCA		This study
B49317	CGAAATCGGTAGACGCTACG	51	[40]
A50272	ATTTGAACTGGTGACACGAG		[40]

control, an UltiMate 3000 pump, and an UltiMate 3000 photodiode array detector. Separation of alkaloids was performed on an Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm; Agilent). The column oven was kept at 30 °C, and the autosampler at 10 °C. The mobile phase contained MeOH:ammonium acetate buffer (15 mM, pH 5.5) (25:75, v/v). Each sample of 20 μL injection volume was run using isocratic elution for 18 min. The flow rate was 0.8 mL/min. A standard mixture containing 50 μg/mL hupA and 50 μg/mL hupB in methanol was prepared, followed by the preparation of a series of standard dilutions (0.5, 1, 2.5, 5, 10, and 25 μg/mL) of hupA.

Validation of the HPLC method included the LOD (signal-to-noise ratio = 3:1), LOQ (signal-to-noise ratio = 9:1), linearity of the calibration curve and linear range, and a precision and recovery test. Precision was evaluated as intraday and inter-day precision by analyzing the standard dilutions at concentrations from 1 to 50 μg/mL in five replicates. To measure the loss of hupA during sample preparation, a recovery test of hupA was performed. Lyophilized plant materials (40 mg) were spiked with a known amount of hupA and hupB (10 μg for each) before extraction. The peak areas of spiked and unspiked samples were used to calculate the percentage recovery (R%). Recovery tests were carried out in five replicates.

$$R\% = \frac{\text{Peak area (spiked before extraction)} - \text{peak area (unspiked)}}{\text{Peak area (spiked after extraction)} - \text{peak area (unspiked)}} \times 100\%$$

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from silica-dried plant materials (ca. 15–20 mg) using a Qiagen DNeasy Plant Mini Kit following the manufacturer's instructions. Three chloroplast loci were amplified using PCR, including psbA-trnH, rbcl, matK, trnL, and trnL-trnF. Primers of the loci psbA-trnH, rbcl, trnL, and trnL-F were chosen from published articles, while two new matK primers were designed (Oligo, v7; National Biosciences Inc.) to amplify the partial matK-encoding region. The design of the new matK primers refers to published whole chloroplast genomes of *H. serrata* (NC033874) and *H. lucidula* (NC006861) as well as complete or partial matK regions of other club moss species (DQ465956-DQ465964, KT821301-KT821304, and EU749488-EU749492). Primer sequences and their annealing temperatures are listed in

► **Table 2**.

► **Table 3** Statistics of sequence alignment.

Partitions	Length (base pair)	Variable characters	Substitution model
rbcl	1278	191	TRN+G
psbA-trnH	317	51	HKY+I
matK	777	279	TVM+I
trnL	423	55	K81UF
trnL-F	590	19	HKY+I

The PCR master mix (25 μL) contained 1 × standard Taq DNA polymerase, 2.5 μL standard PCR reaction buffer, 1–3 μL DNA template, 200 μM dNTPs, 0.2 μM forward/reverse primer, and PCR-grade water. PCR conditions for rbcl and psbA-trnH were as follows: an initial denaturation at 94 °C for 3 min, then 32 cycles of 94 °C for 50 s, 55 °C (rbcl) or 53 °C (psbA-trnH) for 50 s, and 68 °C for 1 min, followed by a final extension of 5 min. The amplification of matK and trnL & trnL-F regions used a touchdown PCR program: 94 °C for 3 min, six cycles of 94 °C for 50 s, 55–50 °C (decreasing 1 °C per cycle) for 50 s, and 68 °C for 1 min, then 30 cycles of 94 °C for 50 s, 55–50 °C (decreasing 1 °C per cycle) for 50 s, and 68 °C for 1 min, followed by a final extension at 68 °C for 7 min. Gel electrophoresis of PCR products used 1.3% agarose stained by SYBR Safe DNA stain. Successfully amplified PCR products were subjected to EXO-SAP purification before Sanger sequencing by Macrogen Inc. The same sets of forward and reverse PCR primers were used for sequencing.

Phylogenetic and haplotype network analysis

Genetic sequences of 65 specimens were subjected to phylogenetic analysis, which included newly generated sequences from 50 collected Icelandic and Chinese specimens and reference sequences of 15 specimens from GenBank (**Table S1**, Supporting Information). Sequence assembly and primary sequence alignment were performed using PhyDe-Phylogenetic Data Editor version 0.9971. Multiple sequence alignments of three loci were then conducted using MAFFT v7 [33], followed by manual refinement. Alignment data are summarized in ► **Table 3**.

A concatenated sequence matrix was used to infer the phylogeny of the club mosses. Before that, a gene tree of each locus was estimated using an ML method to see if there were incongruences of tree topology. No well-supported incongruences were observed, and the sequences of five loci were concatenated. ML analysis was performed using the software RaxML GUI v1.3 [34]. In total, 1000 bootstrap pseudoreplicates were used to estimate nodal support values, and the GTRGAMMA model was implemented. Two partition schemes were compared: (1) the whole psbA-trnH and the 1st, 2nd, and 3rd codon position of rbcL and matK, and (2) each locus as a single partition. Similar nodal support values were generated using both partition schemes, and the 2nd partition scheme was used for downstream phylogenetic analysis. Nucleotide substitution models of each locus was estimated using jModeltest. Bayesian trees were constructed using the software MrBayes v3.2.6 [35], and the analysis was conducted for 10 million generations with the first 25% of the tree discarded. The software FigTree v1.4.2 was used for the visualization of the phylogenetic trees. Branches with BS values over 70% and PP values over 95% were considered well supported.

A TCS haplotype network diagram [36] was created using the software POPART v1.7 [37] to visualize the subspecies-level relationships of Icelandic *H. selago* taxa. Individual genotypes were identified and specimens under each genotype were also represented.

Supporting information

Voucher information and GenBank accession numbers of sequenced loci are provided as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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