Alkaloid fingerprinting resolves *Huperzia selago* genotypes in Iceland

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Abstract

The club moss family Lycopodiaceae produces a diverse array of bioactive lycopodium alkaloids (LAs). In particular, the alkaloid huperzine A (hupA) has grasped attention since it is a potent acetylcholinesterase inhibitor of medical interest in Alzheimer's disease. Although the structural diversity and bioactivities of LAs have been studied to some extent, their chemotaxonomic value is mostly unexplored, especially to a lower taxonomic unit (e.g. subspecies or genotypes). This study focused on previously reported Icelandic *Huperzia selago* genotypes, and aimed to evaluate the chemotaxonomic value of LAs in resolving them. Using liquid chromatography-mass spectrometry (LC-MS), alkaloid fingerprints of *H. selago* taxa were subjected to principal component analysis (PCA). Our results revealed that each genotype tends to have its own alkaloid profile. Genotype 1 and 3 form distinct groups in a PCA plot, where genotype 2 is an intermediate between the other two genotypes. HupA and its derivative, huperzine B, both contribute to the differentiation of genotype 3 from the others. Therefore, our study demonstrated the potential of alkaloid fingerprints in resolving deep taxonomic groups and selecting plant taxa of medicinal importance.

Keywords: Lycopodiaceae, lycopodium alkaloids, huperzine A, phylogeny, alkaloid fingerprinting

1. Introduction

Among vascular plants the club moss family Lycopodiaceae represents an ancient lineage dating back to 420 million years ago (Bateman et al., 1992). According to the recently proposed phylogeny of this family, it contains two subfamilies - Lycopodioideae and Huperzioideae (Field et al., 2016). In total three genera are recognized in Huperzioideae, including *Huperzia* Bernh., *Phlegmariurus* Holub. and *Phylloglossum* Kunze, while Lycopodioideae accommodates more than 10 genera, such as *Spinulum* A. Haines, *Lycopodium* L. and *Diphasiastrum* Holub (Field et al., 2016). Morphological characters may mask species diversity, and molecular phylogenetics is expected to provide us with better taxonomic and systematic insights (Testo et al., 2018).

Compared to seed plants, club mosses are much less studied with respect to their phytochemistry. They produce a wide array of bioactive alkaloids, called lycopodium alkaloids (LAs). According to their structural characteristics, LAs are classified into four groups: lycopodane, lycodane, fawcettimine and miscellaneous groups (Aver and Trifonov, 1994). Huperzine A (hupA), a lycodane-type alkaloid, was first discovered from the Chinese medicinal plant *Huperzia serrata* (Thunb. Ex Murray) Trevis (Liu et al., 1986). HupA is a potent acetylcholinesterase inhibitor, and has been proposed as a potential drug lead for the treatment of Alzheimer's disease (Olafsdottir et al., 2013). Increased interest in LAs has led to extensive bioprospecting efforts in other club moss taxa (Ma et al., 2005). The diversity of LAs has been explored in Icelandic taxa, including *Diphasiastrum alpinum* (L.) Holub (Halldorsdottir et al., 2013), *Spinulum annotinum* (L.) A. Haines (Halldorsdottir et al., 2010) and *Huperzia selago* (L.) Bernh. ex Schrank & Mart (Stærk et al., 2004). Up to now, in total 12 LAs (**Fig. 1**) have been reported in *H. selago* belonging to two LA groups: 1) five lycodane-type LAs including hupA, huperzine B (hupB), 6β-hydroxyhuperzine A, α-obscurine and β-obscurine; 2) seven lycopodane-type LAs including selagoline, serratidine, lycopodine, 6α-hydroxylycopodine, lycodoline, isolycodoline and acrifoline (Achmatowicz and Rodewald, 1956; Ayer et al., 1989, 1990; Rodewald and Grynkiewicz, 1968; Stærk et al., 2004; Valenta et al., 1960; Xu et al., 2018). Screenings on acetylcholine inhibitory activity suggest lycodane-type LAs to be more potent than their lycopodane-type counterparts (Halldorsdottir et al., 2013, 2010; Olafsdottir et al., 2013).

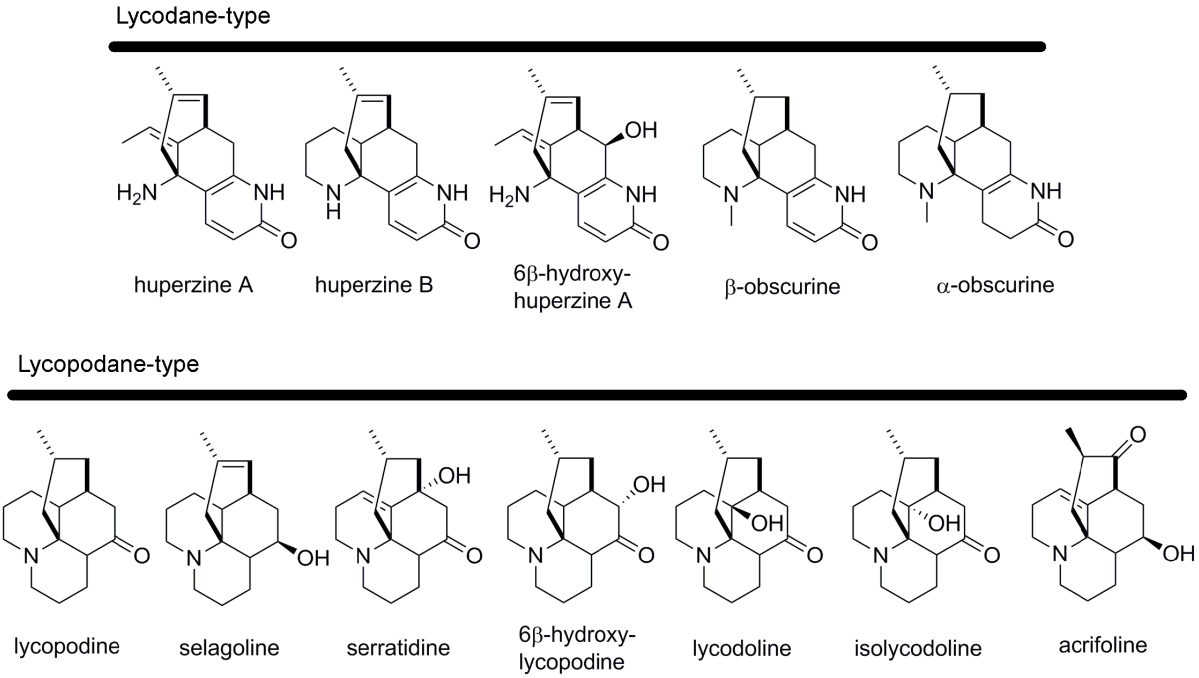

Fig. 1. Lycopodium alkaloids that have been reported in *Huperzia selago*, including lycodane-type and lycopodane-type alkaloids.

Despite the discovery of many new LAs, the chemotaxonomic value of LAs and their distribution patterns between taxa are not well known. Alkaloid profiling of ten major LAs using thin layer chromatography has been carried out in Chinese taxa, and each genus tends to have a genus-specific alkaloid pattern (Ma et al., 1998). A recent review on the LAs present in the genus *Diphasiastrum* shows that this genus is abundant of lycopodane-type alkaloids but lack hupA (Halldorsdottir et al., 2015). However, the utility of LA data in resolving lower taxonomic units (species and subspecies) has not been explored. In a previous study, we have reported the presence of three genotypes of *H. selago* in Iceland (Xu et al., 2018), which vary in hupA contents and morphology. The aim of the present study was to explore the potential chemotaxonomic value of LAs in resolving the Icelandic *H. selago* taxa. The diversity of LAs in Icelandic *H. selago* was also characterized in light of reported LAs in literature.

2. Materials and Methods

2.1 Plant materials, genotype identification and chemicals

Taxon sampling of Icelandic *Huperzia selago* specimens included all described morphotypes, "arctica", "appressa" and "selago". Vouchers of plant specimens were stored in Icelandic Institute of Natural History, Akureyri Division, Iceland. Voucher information is provided in Table 1 with representative photographs in Supplementary file Fig. S1. Morphological identification followed former circumscriptions (Jonsell and Karlsson, 2000; Kristinsson, 2010). Intermediate morphotypes were also found and included in our sampling, altogether representing the morphological and genetic diversity in Iceland. It has been under debate whether different morphotypes should be regarded as subspecies or species (Jonsell and Karlsson, 2000; Rothmaler, 1993; Wagner and Beitel, 1993). Our recent phylogenetic analysis using five chloroplastic loci data suggested that each morphotype should be treated as subspecies under *Huperzia selago* (Xu et al., 2018).

Table 1. Voucher information of sampled Icelandic *Huperzia selago*.

SampleID	Herbarium No.	Date	Location	Latitude	Longitude
HS3	VA21572	19.Aug.2014	Reykjanes, Iceland	64,1917	-22,1875
HS5a	VA21574	19.Aug.2014	Reykjanes, Iceland	63,9967	-22,0978
HS5b	VA21575	19.Aug.2014	Reykjanes, Iceland	63,9967	-22,0978
HS5c	VA21576	19.Aug.2014	Reykjanes, Iceland	63,9967	-22,0978
HS5d	VA21577	19.Aug.2014	Reykjanes, Iceland	63,9967	-22,0978
HS6a	VA21578	26.Aug.2014	Snæfellsnes, Iceland	64,9500	-23,0308
HS6b	VA21579	26.Aug.2014	Snæfellsnes, Iceland	64,9500	-23,0308
HS7	VA21580	17.Jul.2014	Aðalvík, Iceland	66,3358	-23,0486
HS8a	VA21581	26.Aug.2014	Snæfellsnes, Iceland	64,9625	-22,9328
HS8b	VA21582	26.Aug.2014	Snæfellsnes, Iceland	64,9625	-22,9328
HS9a	VA21583	26.Aug.2014	Snæfellsnes, Iceland	64,9683	-22,9317
HS9b	VA21584	26.Aug.2014	Snæfellsnes, Iceland	64,9684	-22,9317
HS10a	VA21585	1.Aug.2012	Snæfellsnes, Iceland	64.9403	-22.8942
HS10b	VA21586	1.Aug.2012	Snæfellsnes, Iceland	64.9403	-22.8942
HS11	VA21587	6.Aug.2009	Aðalvík, Iceland	66.3363	-23.0518
HS12a	VA21588	31.Aug.2011	Snæfellsnes, Iceland	64,8972	-22,8453
HS13	VA21590	12.Jun.2012	Snæfellsnes, Iceland	64,9736	-22,9431
HS14	VA21591	6.Aug.2005	Seyðisfjörður, Iceland	65.2602	-13.9915
HS15	VA21592	18.Jul.2013	Skíðadalur, Iceland	65,7878	-18,6206
HS16	VA21593	9.Jul.2013	Aðalvík, Iceland	66,3381	-23,0542
HS17	VA21594	11.Sep.2015	Flateyjardalur, Iceland	66,1235	-17,9746
HS18	VA21595	11.Sep.2015	Flateyjardalur, Iceland	66,1239	-17,9788
HS19	VA21596	9.Jul.2005	Snæfellsnes, Iceland	64.9010	-22.9015
HS20	VA21597	12.Jun.2012	Snæfellsnes, Iceland	64,9736	-22,9431
HS21	VA21598	12.Jun.2012	Snæfellsnes, Iceland	64,9736	-22,9431
HS23	VA21600	12.Jun.2012	Snæfellsnes, Iceland	64,9736	-22,9431
HS24	VA21601	16.Aug.2016	Austur-Island, Iceland	65.8968	-14.8154

HS25	VA21602	16.Aug.2016	Austur-Island, Iceland	65.9283	-14.5265
HS26	VA21603	16.Aug.2016	Nordur-Island, Iceland	66.2869	-15.0258

To facilitate effective communication and consistency of *Huperzia selago* diversity in Iceland, we used the concept of genotypes. Each genotype was recognized by haplotype networking analysis of a concatenated chloroplast gene sequences using the software POPART v1.7 (Leigh and Bryant, 2015). The combined data matrix of 2372 base pair length included three chloroplast genes (i.e. rbcL, matK and psbA-trnH), and their sequences (Supplementary file Table S1) were retrieved from our previous study. Three genotypes were recovered, and their relationship is shown in **Fig. 2** (Xu et al., 2018).

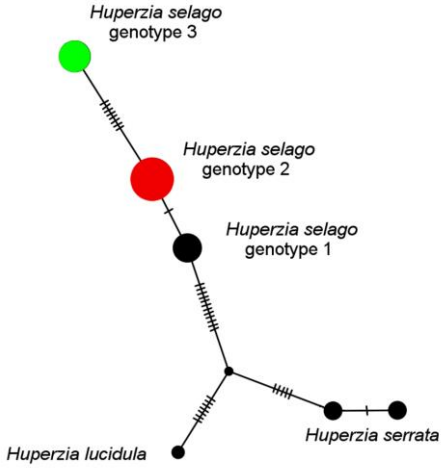

Fig. 2. Haplotype network based on chloroplast genes showing three Icelandic *Huperzia selago* genotypes. Colors of genotypes correspond to the ones used in Fig. 5**.**

All organic solvents of HPLC grade, ammonium acetate and ammonium hydroxide were purchased from Sigma-Aldrich. HupA and hupB (for both, purity > 99% by HPLC-UV) were purchased from PhytoLab GmbH&Co. KG. Water was purified from a Milli-Q water purification system (Millipore GmbH, Darmstadt, Germany).

2.2 Sample preparation

Sample preparation steps follow the procedure we have reported before (Xu et al., 2018). Briefly, whole plant materials were air-dried, powdered in liquid nitrogen and lyophilized. Plant materials (40 mg for each sample; three replicates for each specimen) were weighed and then crude alkaloids were extracted with 2% acetic acid three times. Combined extracts

were washed with dichloromethane. Alkaloids were partitioned to organic phase (dichloromethane) by adding ammonium dropwise until the pH value of the aqueous phase reaches 10. Free alkaloids were extracted with dichloromethane two more times, and combined organic layers were evaporated, dissolved in mobile phase and filtered before UPLC-QToF-MS analysis.

2.3 Alkaloid fingerprinting using LC-MS

Alkaloid fingerprinting was carried out using an Acuity UPLC^{TM} system (Waters corp., Milford, USA) coupled to a QToF SYNAPT G1 mass spectrometer equipped with electrospray ionization (ESI) interface (Waters MS Technologies, Manchester, UK). Separation of LAs was performed on a Luna Omega Polar C-18 column (2.1 mm × 100 mm, 1.6 µm, Phenomenex, UK). Mobile phase contained 10 mM ammonium acetate buffer pH 5.5 (solvent A) and methanol (solvent B). A gradient elution was used as follows: 5% B, 0-0.5 min; linear gradient 5% B-80% B, 0.5-9 min; 80% B, 9-10 min; linear gradient 80% B-5%B, 10-10.1 min; 5% B, 10.1-12 min. Injection volume was 2 µL, and flow rate was 0.4 mL/min. Pooled samples were analyzed as quality control. The SYNAPT G1 mass spectrometer was operated in positive ionization mode with capillary voltage 3.2 kV, cone voltage 42 V, cone gas flow 50 L/h at source temperature 120^{o}C, desolvation temperature 400^{o}C and desolvation gas flow 800 L/h. Collision energy was ramped from 10.0 to 35.0 eV. Ions were scanned at mass to charge ratio (m/z) 100 to 1550. Acquisition and data processing were performed with MassLynx v4.1 (Waters corp., Milford, USA).

2.4 Data processing and multivariate data analysis

MS spectrum alignment and normalization were performed using the software MakerLynx v4.1 (Waters corp., Milford, USA). Peak detection was set from 2-8 min and mass ranging from 100 to 700 Da. Collection parameters were set as follows: marker intensity threshold 250 counts, mass window 0.05 and retention time window 0.2. Processed MS data (Supplementary file Table S2) were subjected to principal component analysis (PCA) using SIMCA v14.1 software (Sartorius Stedim Data Analytics, Umea, Sweden). PCA was used to investigate the potential grouping of specimens or species. Specimens close to each other in the PCA plot indicate similar alkaloid fingerprints. Compounds driving specimen groups differing from each other is visualized using a PCA loading plot. Dots in PCA loading plots

represent detected ions/compounds fulfilling the aforementioned peak detection and collection criteria, which can be annotated by their retention times and base peak m/z values.

3. Results and Discussion

3.1 LC-MS alkaloid fingerprinting

A good alkaloid separation was achieved using Luna Omega Polar C18 column (**Fig. 3**). HupA and hupB were identified in alkaloid extracts by comparing with the commercial standard compounds, which eluted out at 5.61 and 4.69 min, respectively (**Fig. 3A**). Icelandic *H. selago* genotypes 1-3 (**Fig. 3B-3D**) exhibit overall similar alkaloid fingerprints, and they may primarily vary in the contents of certain alkaloids. For example, our previous study shows that the genotype 3 (264 – 679 μg/g) contains significantly ($p < 0.05$) higher amount of hupA than genotype 1 (20 – 180 μg/g), and that intermediate genotype 2 has a broad hupA content (Xu et al., 2018). The current study also revealed a hidden diversity of LAs present in Icelandic *H. selago*. In addition to hupA and hupB, the other three major LAs that have been reported in Icelandic *H. selago*, including lycopodine (m/z 248.2027), selagoline (m/z 248.2001) and serratidine (m/z 262.1803) (Stærk et al., 2004) are detected. The extracted ion chromatogram (EIC) at m/z 248.20 shows three peaks, and two of them may be lycopodine and selagoline, while the third one is unknown (**Fig. 4A**). The alkaloid 6β-hydroxyhuperzine A (t_R = 4.27 min) can be annotated by extracting ion at m/z 259.18 (**Fig. 4B**). An EIC at m/z 262.18 also suggests more serratidine isomers yet to be described (**Fig. 4C**), and one of them might be acrifoline. Another EIC at m/z 264.20 shows five major peaks, which correspond to the molecular mass of protonated 6α-hydroxylycopodine, lycodoline and isolycodoline previously described in *H. selago*, and the remaining two compounds are still unknown. (**Fig. 4D**). Future work should focus on isolation and structural elucidation of the undescribed LAs in *H. selago*.

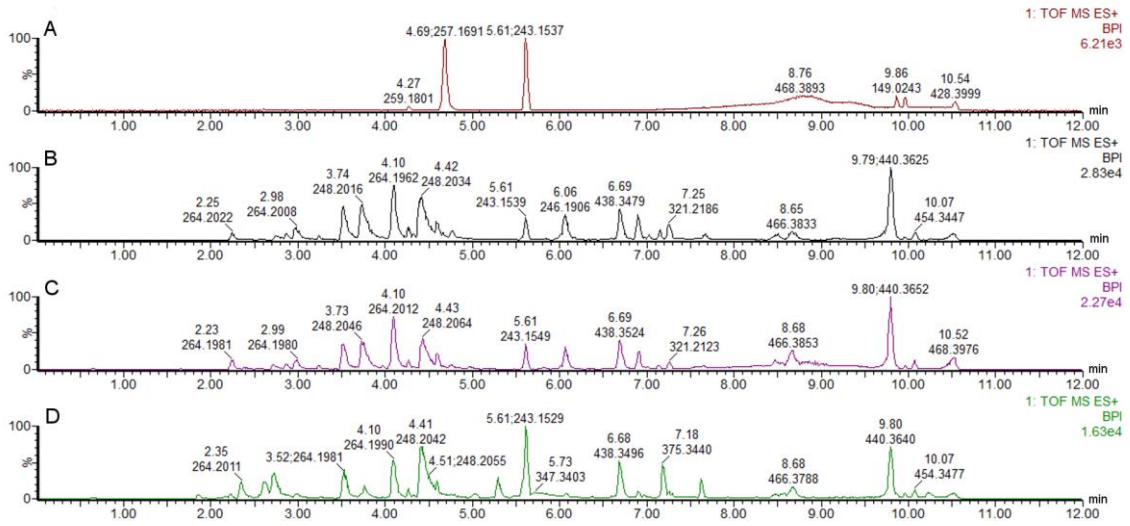

Fig. 3. UPLC-QToF-MS base peak chromatograms of alkaloid samples detected in positive ion mode. A) standard compounds huperzine A (t_R = 5.61 min) and huperzine B (t_R = 4.69 min); (B) Icelandic *Huperzia selago* genotype 1; (C) Icelandic *Huperzia selago* genotype 2; (D) Icelandic *Huperzia selago* genotype 3.

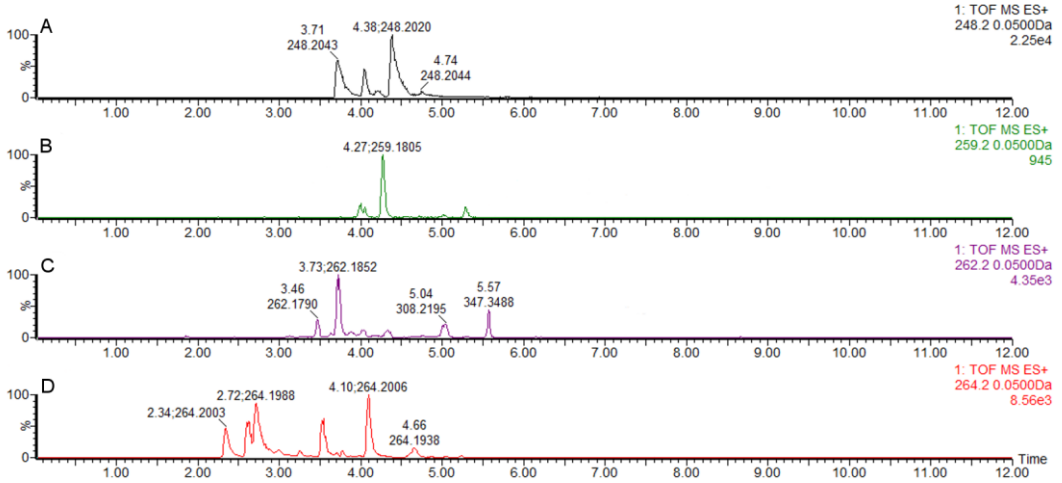

Fig. 4. Extracted ion chromatograms at (A) m/z 248.2 corresponding to lycopodine and selagoline; (B) m/z 259.2 corresponding to 6β-hydroxyhuperzine A; (C) m/z 262.2 corresponding to serratidine; (D) m/z 264.2 corresponding to 6α-hydroxylycopodine, lycodoline and isolycodoline.

The MS fragmentation pattern of hupA (**Fig. S2A**) in our study is in agreement with reference studies (Cuthbertson et al., 2012; Wang et al., 2004; Yang et al., 2017), showing a protonated molecular ion at m/z 243.1424 and a sodium adduct ion at 265.1354. Characteristic fragments result from the loss of NH_3 (m/z 226.1250), followed by further loss of CH_4 (m/z 210.0964) and C_2H_6 (m/z 196.0822). Current collision energy ramp from 10 to 35 eV could generate multiple fragment ions from aromatic LAs (e.g. hupA and hupB), which aids to their structural elucidation. Similarly, hupB (**Fig. S2B**) has characteristic

fragment ions at m/z 240.1394 $[M\text{-}NH_3]^+$ and m/z 198.0966 $[M\text{-}NH_3\text{-}C_3H_6]^+$. The other lycodane-type alkaloid 6β-hydroxyhuperzine A was annotated as a major peak in the extracted ion chromatogram (**Fig. 4B**), and it shows a fragment ion at m/z 242.1576 $[M\text{-}NH_3]^+$ as well as a sodium adduct ion at m/z 281.1936. MS data for identified and annotated lycodane-type alkaloids are summarized in **Table 2**. However, the lycopodine-type alkaloids lack diagnostic fragments at collision energy of 35 eV and lower, and their identification relies on isolated pure standards. It has been reported that lycopodane-type alkaloids with saturated ring structures, such as lycopodine, selagoline and serratidine in *H. selago* (Stærk et al., 2004), are devoid of MS fragments at commonly used collision energy levels around 30-35 eV, and that only a few fragments could be expected to occur at a higher collision energy over 40 eV (Shan et al., 2016). Whether high collision energy levels (> 40 eV) could generate characteristic fragment ions for lycopodane-type LAs remains unexplored.

Table 2. Retention times (t_R), protonated molecular ions ($[M+H]^+$), fragment ions and molecular formula of identified lycodane-type alkaloids in Icelandic *Huperzia selago* at an energy ramp from 10 to 35 eV.

Alkaloids	t_R (min)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	Fragment ions (m/z)	Molecular formula
Huperzine A	5.61	243.1378	265.1323	226.1218, 210.0945,196.0811	$C_{15}H_{18}N_2O$
Huperzine B	4.69	257.1693	279.1502	240.1394, 198.0966	$C_{16}H_{20}N_2O$
6β-hydroxyhuperzine A	4.27	259.1805	281.1936	242.1576	$C_{15}H_{18}N_2O_2$

3.2 Multivariate data analysis

Each genotype tends to form a distinct group in the PCA plot (**Fig. 5A**). The first component could explain 24.98% of the variance found in the PCA analysis, while the second component accounts for 18.19% variance. From the first principal component, it is apparent that genotype 1 and 3 are markedly different from each other. **Fig. 5B** is the PCA loading plot showing detected ions with ion counts over 250. Ions/dots contributing similarly to a certain cluster are grouped together. Dots representing hupA and hupB were located in the loading plot with their respective retention time and m/z values. They contribute to the separation of genotype 3 from genotype 1. In light of their pharmaceutical potential in acetylcholinesterase inhibition (Bai, 2007), genotype 3 should be prioritized for bioprospecting due to its higher contents of hupA and hupB (Xu et al., 2018). This corresponds to our previous results showing that genotype 3 contains significantly higher hupA contents than genotype 1, and

that detectable amount of hupB is only found in genotype 3 (Xu et al., 2018). Genotype 2, the intermediate genotype as shown in haplotype network in **Fig. 2**, also shows an intermediate alkaloid fingerprint between the other genotypes, which in turn supports our previous genetic analysis result (Xu et a., 2018).

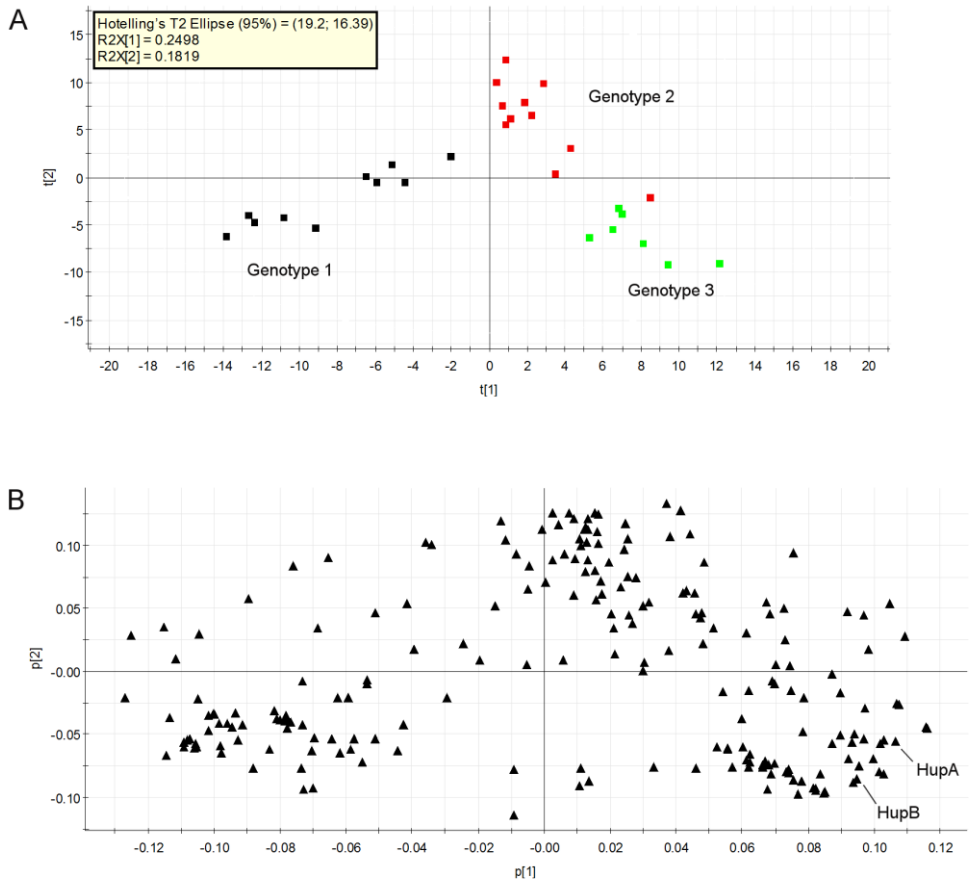

Fig. 5. Multivariate data analysis of alkaloid fingerprints. (A) Principal component analysis (PCA) plot showing genotype-specific groups; (B) PCA loading plot showing that hupA and hupB contribute to the separation of *H. selago* genotype 3 from the other two genotypes.

Although chemotaxonomic values of LAs have been proposed (Ma et al., 1998), our study constitutes the first report using alkaloid fingerprints to resolve taxa at a low taxonomic level – subspecific genotypes. What's more, our chemical results (i.e. PCA plot) well corroborate genetic results (e.g. haplotype network), which provides strong support for the uniqueness of each genotype. The combination of both chemical and genetic approaches suggests that genotype 3 should be prioritized for future bioprospecting, since it contains more of the valuable lycodane-type LAs, such as hupA and hupB.

4. Conclusions

Our results demonstrate the potential chemotaxonomic value of alkaloid fingerprints at subspecific level in Icelandic *H. selago*. They corroborate our previous genetic results showing that Icelandic *H. selago* contains three genotypes that can be considered as subspecies. In addition, the integrated approach of combining alkaloid fingerprinting and genetic analysis can be valuable when selecting plant materials of high medicinal importance.

Acknowledgements

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Conflicts of interest

The authors declare no conflict of interest.

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