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Full Paper

Chemical profiles and chemometric analysis of selected *Leucobryum* species (Bryophyta, Leucobryaceae) from Thailand

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Abstract: *Leucobryum* is a moss genus that exhibits various bioactivities. However, the identification of *Leucobryum* species with morphology alone remains difficult. Chemical profile analysis provides additional tools for plant classification. This method uses chemical similarities to identify the differences among some plants, especially the different varieties of plant species. The objective of this study was to obtain the chemical profiles and use chemometrics to identify selected *Leucobryum* species found in Thailand. Lipophilic extracts from 18 samples of five taxa were analysed with thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Principal component analysis (PCA) and hierarchical cluster analysis were used to investigate the similarities of the chemical profiles. Permutational multivariate analysis of variance (PERMANOVA) was performed to determine the difference among taxa. The taxonomic identities were also verified with a molecular marker. Morphological and molecular identification were consistent for five taxa. The TLC profile could separate only the genus of the sample, while HPLC chromatograms showed separation among the *Leucobryum* taxa and consistent patterns within the same species. In addition, the chemical profiles of *Leucobryum* species were separated at the species level in PCA. The PERMANOVA showed significantly different profiles among the species (*P* value = 0.002). Therefore, this work illustrates the potential of chemometrics as supporting evidence for species delimitation in the moss genus *Leucobryum*.

Keywords: *Leucobryum* species, bryophytes, mosses, chemical profiles, cluster analysis, Thailand

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INTRODUCTION

The moss genus *Leucobryum* Hampe is placed in the family Leucobryaceae [1], with 80–100 species distributed in temperate and tropical areas [2]. In Thailand eight species and two varieties have been reported [3]. *Leucobryum* can be found on various substrates and throughout the country [4], and may be at risk because it is used in multiple commercial purposes including floral displays, potting medium, pot soil covering, and terrarium [5-6]. However, species identification remains unclear due to morphological variation and taxonomic confusion of *Leucobryum* in Thailand. Identifying these small plants based on morphology and anatomy can be difficult. There are many misidentifications in bryophytes because the morphological characters are often the result of convergences, parallelisms and reductions [7]. In modern systematics, additional types of comparative data, such as anatomy and physiology, chemistry, embryology, palynology, reproductive biology and molecular genetics, can be helpful for species separation and analysis [8].

Chemometrics is a technique that uses multivariate data sets of chemical profiles to support taxonomic classification [9]. Chemical profiles can be obtained from either thin-layer chromatography (TLC), high-performance thin-layer chromatography, or high-performance liquid chromatography (HPLC) for evaluating the similarity or difference between various plant species including bryophytes, ferns and angiosperms [10-12]. The chemistry of *Leucobryum* was first analysed using TLC, but a significant amount of flavonoids was not detected [13]. However, subsequent studies have identified various chemicals in *Leucobryum*. The first known compound groups from *Leucobryum* are fatty acids and sterols [14-15]. In subsequent studies additional compounds were identified from *Leucobryum*, including fatty aldehydes, hydrocarbons (alkenes, carbocyclic compounds and ester), phenolics, terpenes (monoterpene and sesquiterpene), and alkaloids [16-17]. Many of these substances have the potential for further utilisation. For example, *Leucobryum aduncum* and *L. glaucum* extracts exhibit antioxidant and antibacterial activities [18-19]. Nevertheless, no detailed chemical profiles or chemometric reports have been conducted on *Leucobryum*. The profile of compounds could provide an additional tool for species separation and classification of *Leucobryum* species.

Therefore, the objective of this study is to use the chemical profiles and chemometrics with chromatographic data (TLC and HPLC) to support classification within the genus of *Leucobryum* found in Thailand. Two approaches, principal component analysis (PCA) and hierarchical cluster analysis (HCA), were used to determine differences in the chemical profiles among the studied *Leucobryum* species. In addition, a molecular phylogeny was also constructed using a nuclear marker to verify the taxonomic identities of the studied samples. The results showed the potential of a chemotaxonomic approach in bryophyte classification and future prospecting of bioactive compounds in bryophytes.

MATERIALS AND METHODS

Plant Material and Extract Preparation

Plants of the genus *Leucobryum* were obtained from a local plant market in Bangkok, Thailand, with origins from the northern and north-eastern parts of Thailand. From these materials, eighteen samples from three *Leucobryum* taxa and two outgroup taxa (Table 1) were identified to the species level using available taxonomic keys and other related taxonomic literature. These taxa were verified to ensure that they were previously reported from Thailand. Voucher specimens were kept in the Department of Botany, Faculty of Science, Kasetsart University.

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The plant materials were dried under shade and extracted with methanol for seven days at room temperature in the dark to prevent denaturation or changes in the structure of compounds during extract preparation. Subsequently, the solution was evaporated, and solid crude extracts were separated in purified water and chloroform. The lipophilic extract was concentrated until dry using a rotary evaporator, weighed and kept in a freezer at -34 °C for further analyses (Table 1).

Table 1. Data of studied plant materials and extracts of *Leucobryum* species

Species	Sample code	Air-dried material [g]	Lipophilic extract [mg]	Mass of lipophilic extract/Mass of plant material [mg/10g]	Voucher specimen no.	NCBI accession number
Leucobryum aduncum Dozy & Molk. var. *aduncum*	AD1	14.16	130.2	91.95	2019-PT16	OQ557096
	AD2	7.02	89.50	127.49	2020-PT18	OQ557097
	AD3	6.23	103.5	166.13	2020-PT19	OQ557098
	AD4	15.18	140.0	92.23	2019-PT11	OQ557091
	AD5	7.02	134.1	191.03	2019-PT13	OQ557092
	AD6	3.98	74.10	186.18	2020-PT20	OQ557093
	AD7	5.50	69.70	126.73	2020-PT21	OQ557094
Leucobryum bowringii Mitt.	BO1	9.26	182.7	197.30	2019-PT12	OR842736
	BO2	3.83	78.20	204.18	2019-PT44	OR842737
Leucobryum sanctum (Nees ex Schwägr.) Hampe	SA1	6.28	193.8	308.60	2019-PT10	OR842738
	SA2	6.44	136.0	211.18	2019-PT14	OR842739
	SA3	7.57	130.3	172.13	2019-PT15	OR842740
Ochrobryum subulatum Hampe	OS1	2.16	61.70	285.65	2020-PT41	-
	OS2	1.67	23.60	141.32	2020-PT42	-
	OS3	2.20	55.60	252.73	2020-PT43	-
Campylopus ericoides (Griff.) A. Jaeger	CE1	33.77	335.1	99.23	2020-PT37	-
	CE2	22.59	326.2	144.40	2020-PT38	-
	CE3	16.90	244.4	144.62	2020-PT39	-

Note: NCBI = National Center for Biotechnology Information

TLC Development

The lipophilic extracts of *Leucobryum* and outgroup taxa were analysed using TLC on silica gel 60 F254 (0.25-mm thickness, Merck) coated on the glass plate at 25–30 °C. The concentration of the lipophilic extract was adjusted to 10 mg/mL. The solvent system was developed for the phytochemical screening using chloroform and hexane in the 7:3 (v/v) ratio. Each developed plate was sprayed with anisaldehyde-sulfuric acid and Dragendorff's reagents to detect phenolics, sterols, terpenes and alkaloids in the plant extracts [20].

HPLC Analysis

HPLC was used to analyse the lipophilic extract of studied plant samples. The concentration was adjusted to 10 mg/mL, similar to the TLC analysis. The analysis was conducted using an Agilent 1100 series with reverse-phase BDS hypersil™ C18 column (250 × 4.6 mm) and UV

photodiode array detector eluted with MeOH in aq. buffer (15 mM ortho-H_3PO_4 and 1.5 mM Bu_4NOH, pH3) with run time of 30 min. The flow rate and injection volumes were 1.0 ml/min. and 20 µl respectively. The linear gradient started from 60% MeOH to 90% in 17 min. to 100% in 20 min. and was kept for 8 min. The detection signal was set at 230 nm.

Chemometric Analysis

The HPLC chromatograms at 230 nm wavelength with default thresholds at retention time between 5–20 min. were selected to represent the chemical profiles of the *Leucobryum* taxa and the outgroups. The chromatograms and peak areas at each retention time were exported as csv files from the Agilent ChemStation software attached to the HPLC machine. The areas under the peaks, which had more than one per cent of the total area from the selected part of the chromatogram, were used as multivariate chemical profiles to compare among the studied samples. Statistical analyses including PCA and HCA, were performed using the R program v.3.6.1 [21]. Before PCA and HCA, the pairwise Bray-Curtis distance was calculated to emphasise the shared presence of detected chemicals. The PCA and hierarchical cluster analysis were performed on the distance matrix using the functions 'prcomp' and 'hclust' in the package 'stats.' Permutational multivariate analysis of variance (PERMANOVA) was performed to determine if the chemical profiles are significantly different among taxa, using the function 'adonis' in the package 'vegan' [22-23].

Phylogenetic Identification of Samples

The internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA was amplified from the *Leucobryum* samples to verify the taxonomic identity of these samples. The ITS2 region has been shown to be an effective barcoding marker for various groups of plants and animals and thus was chosen for this study [24-25]. A dried sample was homogenised in liquid nitrogen. Then the genomic DNA was extracted from the samples using the NucleoSpin® Plant II Kit (Macherey-Nagel GmbH & Co. KG, Germany) following the manufacturer's user manual. The ITS2 region was amplified with polymerase chain reactions using the primers and conditions in Bonfim Santos and Stech [26]. The polymerase chain reaction products were cleaned with ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, USA) for bidirectional Sanger sequencing at Macrogen Inc., Seoul, South Korea. The chromatograms and nucleotide sequence data were assembled manually using the Geneious Prime [27].

The resulting DNA sequences were aligned with the publicly available sequences in The National Center for Biotechnology Information database to determine whether the studied samples belonged to the known identified *Leucobryum* species. The sequences included two sequences from *L. aduncum* (AB763350, AB763352), one sequence from *L. sanctum* (KY618964), and one sequence from *L. bowringii* (KY618952). A sequence of *Ochrobryum subulatum* was chosen as an outgroup (KY618933). These sequences used the MUSCLE algorithm [28] available in Geneious Prime [27]. The resulting alignment was also subjected to Gblocks version 0.91b, available on the web server of phylogeny.fr [29], to remove ambiguously aligned positions and reduce the uncertainty of phylogenetic reconstruction [30].

Phylogenetic trees were constructed using the maximum likelihood on the IQTree web server, using the automatic 'Model Finder' option to find the best-fit substitution model [31]. The branch support value of the maximum likelihood tree was estimated using the ultrafast bootstrap algorithm with 1,000 bootstrap replicates [32]. The phylogenetic trees were then visualised using

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Figtree ver. 1.4.4 [33]. A bootstrap support value of 70 or greater from the maximum likelihood analysis was considered strong support for a clade.

RESULTS AND DISCUSSION

TLC Profiles

Developed plates sprayed with anisaldehyde-sulfuric acid and Dragendorff's reagents revealed potential terpenes, sterols, phenolics and alkaloids (Figure 1). No differences within *Leucobryum* taxa were detected based on the band patterns observed with these two reagents. However, the plate developed with an anisaldehyde-sulfuric acid reagent showed the separation of the genus *Leucobryum* from *Campylopus* and *Ochrobryum*. This result indicated a difference in the chemical composition of *Leucobryum* compared to the other genera, suggesting the potential use of TLC profiles for distinguishing *Leucobryum* from closely related genera. Several genera such as *Leucophanes, Ochrobryum* and *Octoblepharum* share the 'Leucobryoid' morphology of short tufted whitish leaves. These genera are easily confused in the field and require detailed examination of leaf anatomy for identification. Having a TLC profile may help separate *Leucobryum* from similar-looking genera.

The results from the developed plates sprayed with Dragendorff's reagent showed that alkaloid compounds were present in all extract samples, turning orange at the starting point (R_f value = 0; Figure 1A). The alkaloids do not move with the mobile phase because these compounds are generally more polar than other compounds such as phenolics and terpenes as shown in Figure 1B. Dragendorff's reagent test showed that this test cannot separate the genus *Leucobryum* from other genera. This assay is consistent with a previous study, which found that *Leucobryum* contains alkaloid compounds based on the comparisons of retention time and mass spectrum data with the existing database [17]. The exact identity of these alkaloids will have to be verified from pure compounds through detailed separations in the future.

The assay with the anisaldehyde-sulfuric acid reagent can separate *Leucobryum* from other genera. This reagent detected phenolic, terpene, sugar and sterol compounds with blue, red, grey and green colours on the developed TLC plates respectively. This assay showed a high content of phenolic and terpene compounds in all *Leucobryum* samples at R_f value = 0.79 and 0.92 respectively. Samples of *L. aduncum* var. *aduncum* and *L. sanctum* showed sterols at R_f value = 0.73 (Figure 1B). These groups of compounds were previously reported from the species of *Leucobryum* such as *L. javense* and *L. glaucum* [14, 34].

The inability to separate species within the genus *Leucobryum* may result from an unsuitable solvent system. The thick band at the end of each track (Figure 1B) showed that the current solvent system could not separate many compounds with low polarity in this study. Highly polar compounds such as alkaloids were not well separated either, as shown in the thick orange colour bands in Figure 1A. In preliminary tests several systems from previous studies were examined for separation of the moss species, especially species of *Leucobryum* from other genera [35-36]. Unfortunately, none of these systems proved suitable for effectively separating the studied species, and none of the reported solvent systems can distinguish *Leucobryum* from other genera. This TLC study shows that the moss genus *Leucobryum* contains mostly terpenes, sterols, phenolics and alkaloids. Consequently, we will have to develop an additional solvent system for TLC if we need to discriminate among the species of *Leucobryum* found in Thailand.

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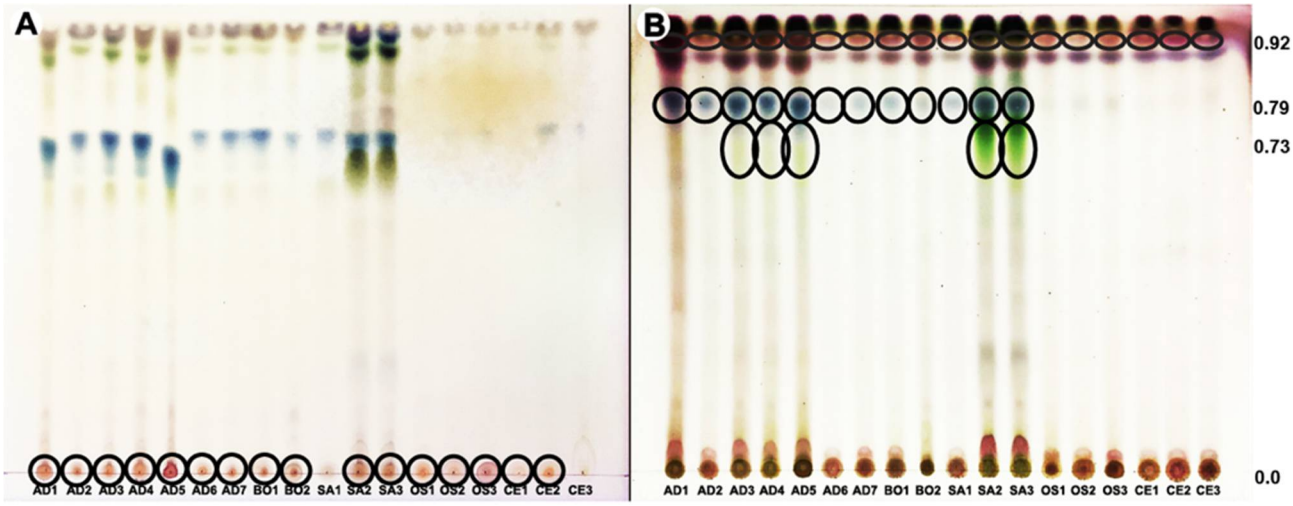

Figure 1. TLC-profiles of *Leucobryum* lipophilic extracts: sprayed with (A) Dragendorff's reagent; (B) anisaldehyde-sulfuric acid reagent. Abbreviations are species names followed by sample number: AD, *Leucobryum aduncum* var. *aduncum*; BO, *L. bowringii*; SA, *L. sanctum*; OS, *Ochrobryum subulatum*; CE, *Campylopus ericoides*. The right side shows R_f values of detected compounds (black circles)

HPLC Profiles and Chemometrics Analysis

The same set of lipophilic extracts from TLC was also analysed using HPLC. While TLC profiles showed no differences among *Leucobryum* taxa, the HPLC profiles exhibited clear distinctions among the studied taxa. The chemical profiles of individuals within the same taxa were similar (Figure 2). We analysed the HPLC profiles using HCA and PCA. The results from HCA showed that most *Leucobryum* samples were clustered together, separated from the outgroup (Figure 3). According to the cluster dendrogram, 18 samples were divided into four clusters. All samples of *L. aduncum* var. *aduncum* were placed in cluster I. Cluster II contained all samples of *L. sanctum* and one sample of *L. bowringii* (B01). Cluster III contained only *Ochrobryum subulatum* samples (outgroup). Finally, Cluster IV contained all samples of *Campylopus ericoides*, the outgroup, and one sample of *L. bowringii* (B02).

These results were consistent with the classification of *Leucobryum* species, with a noted exception of *L. bowringii*. The HPLC profiles also showed different patterns of peaks between two samples of *L. bowringii* around the retention times of 5-7 min. and again at 15 min. onward. This lack of internal consistency within the species is likely to contribute to the clustering of *L. bowringii* samples with the other taxa. The difference in chemical profiles within *L. bowringii* may result from different environmental conditions under which these plant samples were collected. In *Leucobryum*, differences in chemical constituents among the same species from different habitats have been reported for *L. javense* [17]. Increasing the number of samples within each species may help enhance the within-taxa consistency in chemical profiles.

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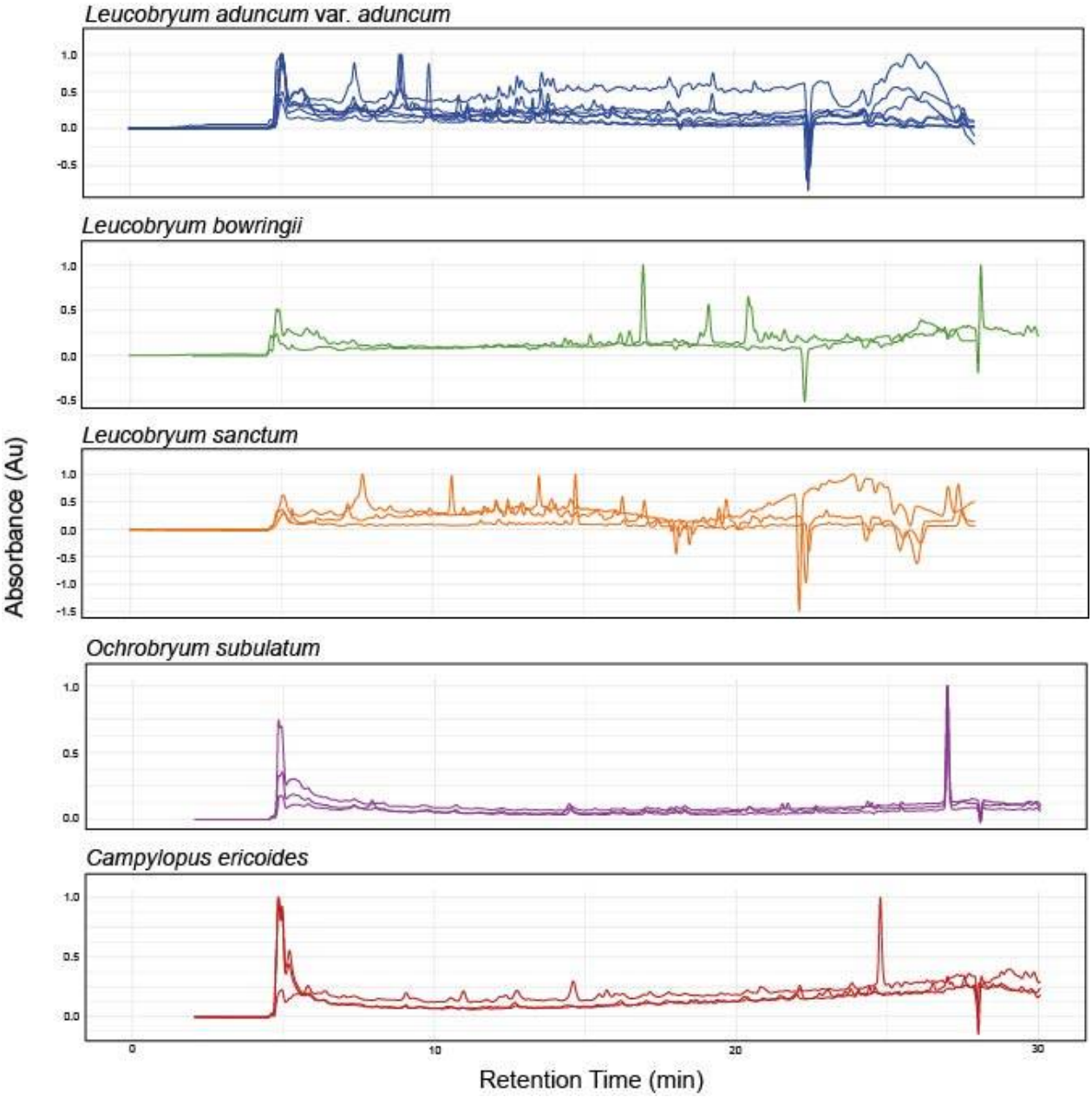

Figure 2. HPLC profiles of *Leucobryum* and outgroup lipophilic extract

Overall, the PCA results confirmed the difference in the chemical profiles among the three *Leucobryum* taxa (Figure 4). Out of the overall variance explained (35%), the first component (PC1) accounted for 22%, and the second component (PC2) accounted for 13%. Three *Leucobryum* taxa (*L. aduncum* var. *aduncum*, *L. bowringii* and *L. sanctum*) showed three distinct, non-overlapping clusters, indicating that the three species were separate. The PERMANOVA showed significant differences among five studied taxa at *P*-values = 0.002 and *F*-values = 2.4973. This multivariate analysis showed that the studied *Leucobryum* species differed markedly from each other and the outgroups in their HPLC chemical profiles.

While the HCA could not group the samples of *L. bowringii*, the PCA readily distinguished this taxon from the others. The observed discrepancy highlights the importance of choosing analytic tools in chemometrics. PCA and HCA are both powerful techniques for chemometric analysis. They were developed for different purposes and have distinct advantages. In the case of comparing

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chemical profiles, PCA uses dimension reduction techniques while HCA relies on clustering the groups based on the distance matrix. PCA is inherently better than HCA in dealing with multicollinearity and highly correlated variables. Therefore, PCA might be able to position the slightly different chemical profiles of *L. bowringii* closer to each other than HCA. In our case PCA was more effective at separating taxa within the genus *Leucobryum*, but the results could differ from those of other chemometric data sets.

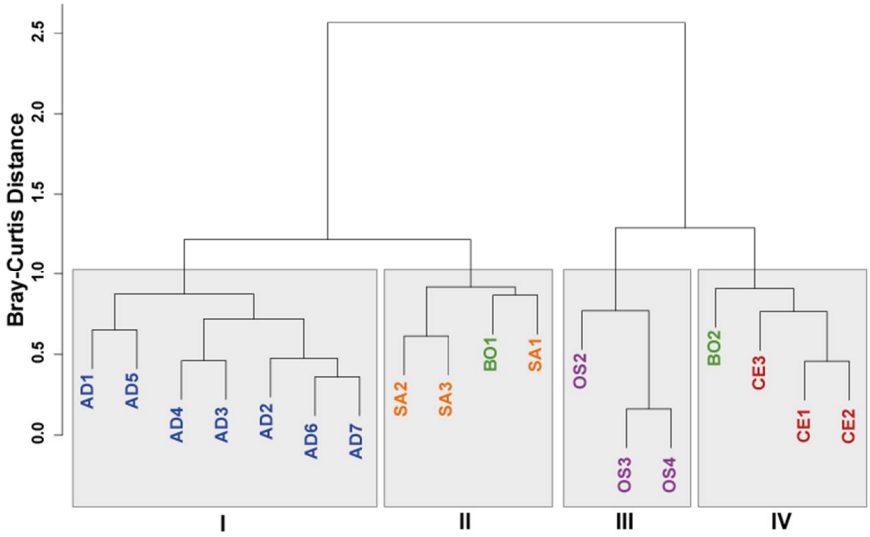

Figure 3. Cluster dendrogram of HPLC chemical profiles of lipophilic extracts of *Leucobryum* and outgroup taxa. Four groups (I–IV) are identified from the analysis. Abbreviations are species name followed by sample number: AD, *Leucobryum aduncum* var. *aduncum*; BO, *L. bowringii*; SA, *L. sanctum*; OS, *Ochrobryum subulatum*; CE, *Campylopus ericoides*

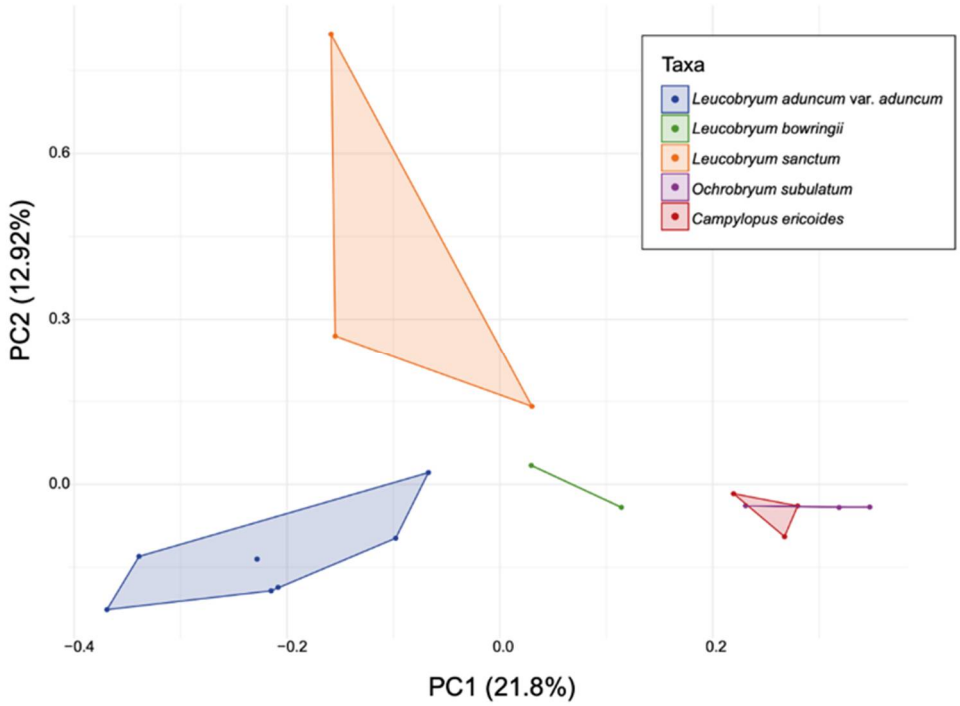

Figure 4. PCA based on HPLC chemical profiles of lipophilic extracts of *Leucobryum* and outgroup taxa (*Ochrobryum subulatum* and *Campylopus ericoides*). The groups are significantly different at *P*-values = 0.002 (PERMANOVA).

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Phylogenetic Identification of Samples

The initial alignment of ITS2 regions of 17 sequences resulted in 656 positions. The Gblocks algorithm yielded the final alignment of 201 unambiguously aligned positions including 161 constant sites and 29 parsimony informative sites. The best-fit substitution model was Tne+I (Tamura-Nei model with equal base frequencies while allowing for a proportion of invariable sites), with a BIC score of 1182.2309. The log-likelihood of the final tree was -527.475555. The final tree showed that all studied specimens formed monophyletic groups with their respective species with strong bootstrap supports (Figure 5). All of the sequences of *Leucobryum aduncum* var. *aduncum* were placed in a monophyletic clade with a bootstrap support of 89. Sequences of *L. sanctum* and *L. bowringii* formed a well-supported clade with a bootstrap value of 87. This clade was divided into two reciprocally monophyletic clades of each species with high bootstrap support values at 92 and 97 for *L. sanctum* and *L. bowringii* respectively.

The results demonstrate that the studied specimens of *Leucobryum* are correctly identified to the species level. While our samples include only a few species, the results show the potential of using ITS2 as a barcoding region for bryophytes. Previous work has generally proposed chloroplast markers such as *rbcL*, *trnL-F* and *rps4* for barcoding purposes in mosses [37]. The utility of the ITS regions has also been shown to do relatively well for separating closely related species of mosses [38]. In the case of *Leucobryum* the utilisation of DNA barcoding remains to be explored, possibly due to unresolved species complexes with unclear boundaries [26]. A thorough taxonomic revision of the genus is required before we can thoroughly investigate the potential of DNA barcoding for species identification of *Leucobryum*.

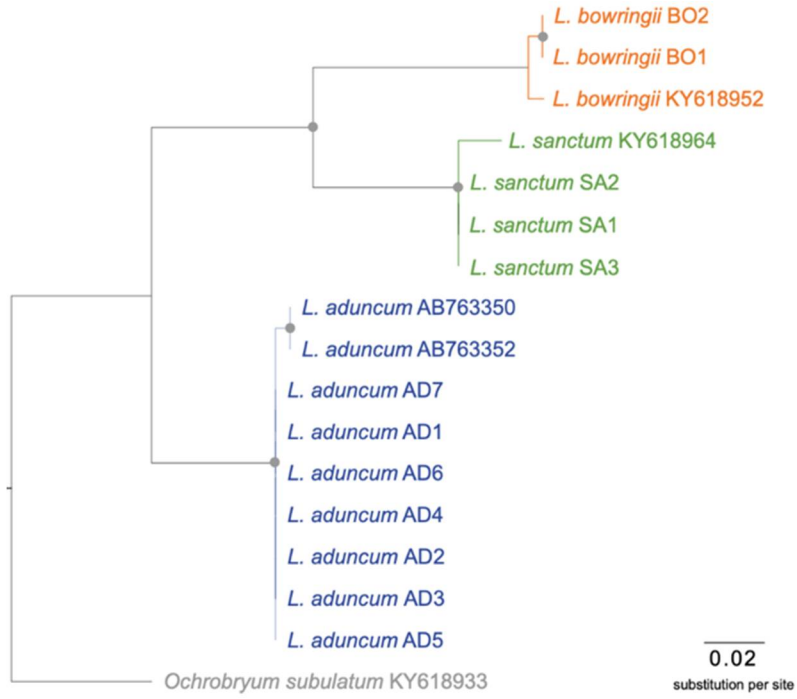

Figure 5. Maximum likelihood phylogram from ITS2 regions from *Leucobryum* specimens and the reference sequences from NCBI nucleotide database. Samples from current study are indicated with sample codes (see Table 1), whereas the sequences from NCBI database are indicated with the accession numbers. Grey circles at the nodes marked the well-supported nodes with bootstrap values greater than 75.

The reconstructed phylogeny offers additional insights into the resulting chemical profiles through chemometric analysis. The small branch lengths among specimens of the same species from Thailand suggest limited intraspecific genetic variation. The reduced genetic diversity may stem from sampling within the same or genetically connected populations. Interestingly, the chemical profiles within each species exhibit a higher diversity level than their genetic profiles. For example, in the case of *L. bowringii*, the slightly different HPLC profiles of the two samples led to their placement into different clusters (Figure 3) despite nearly identical ITS2 sequences. This contrast highlights the impact of environmental factors on secondary metabolite production in bryophytes. More in-depth analyses focusing on population genetics and chemical diversity at the population level are necessary to understand these observed differences better.

CONCLUSIONS

The chemical profile studies of the moss in the genus *Leucobryum* found in Thailand by TLC and HPLC have shown that the TLC profiles can only separate the genus from the outgroups. HPLC chromatograms can separate the moss from the outgroups and classify species within the genus using PCA and HCA. PERMANOVA shows significant separation of the studied *Leucobryum* species by their chemical profiles. This work provides additional data for species delimitation of the moss in the genus *Leucobryum* and serves as an example of the use of chemometrics in the classification of bryophytes. Future studies on *Leucobryum* species and their chemistry should consider the following aspects: First, better identification of the secondary metabolites should be performed using more powerful techniques such as high-resolution liquid chromatography-mass spectrometry. Second, collection of studied samples should be performed from natural habitats so that the environmental conditions and microhabitat conditions can be observed.

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