TURUN YLIOPISTO
UNIVERSITY OF TURKU

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Suvi Vanhakylä

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SARJA – SER. AI OSA – TOM. 740 | ASTRONOMICA – CHEMICA – PHYSICA – MATHEMATICA | TURKU 2025

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ABSTRACT

In this multidisciplinary thesis, thousands of plant species were screened to study their major foliar polyphenol groups and their distribution across the plant kingdom. While plants produce thousands of polyphenol compounds primarily for defensive purposes, current research has predominantly focused on health benefits of certain species and compounds, often employing variable methodologies. In contrast, older large-scale chemotaxonomy studies were limited by the methods available at the time. This study addresses these gaps by employing a comprehensive plant screening approach with updated and consistent methodologies throughout the data.

The first part of this thesis establishes a basis for extensive plant screening. A novel graphical method was developed to examine species-specific quantitative and qualitative variations in polyphenol profiles and their seasonal changes. It was observed that while the species expressed certain levels of quantitative variation, the polyphenol profile remained recognizable. The seasonal changes were relatively subtle and most of the changes were detected in the species producing ellagitannins and proanthocyanidins. Additionally, linking the polyphenol results to significant bioactivities revealed specific compound types that influence these activities. The findings from various plant species suggest that plant collection methods used in large-scale plant screening, which do not strictly define timing and use pooled samples from multiple individuals, effectively represent the species.

The second part of this thesis integrates chemical data from hundreds of plant families with contemporary plant phylogenies, providing a biological context for the chemical findings. It was observed that the distribution of eight polyphenol groups likely reflects key evolutionary events in plants at deeper phylogenetic levels, while ongoing ecological processes influence polyphenol profiles at lower levels closer to the species level. Flavonol derivatives, quinic acid derivatives and proanthocyanidins were the most widely distributed polyphenol classes, while hydrolysable tannins were even more restricted to specific clades than previously assumed. Within these main classes, the biosynthetically simpler subgroups were more frequently observed compared to biosynthetically later counterparts.

KEYWORDS: bioactivities, evolution, phylogeny, plant kingdom, polyphenols

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TIIVISTELMÄ

Tässä poikkitieteellisessä väitöskirjassa kartoitettiin tuhansien kasvilajien lehtien pääpolyfenoliyhdisteryhmiä ja niiden jakautumista kasvikunnassa. Vaikka kasvit tuottavat polyfenoleja pääasiassa puolustustarkoituksiin, nykyinen tutkimus on keskittynyt lähinnä yksittäisten kasvilajien ja yhdisteiden terveysvaikutuksiin käyttäen vaihtelevia menetelmiä. Toisaalta vanhempia laaja-alaisia kemotaksonomiatutkimuksia rajoitti saatavilla olevat menetelmät. Tämä tutkimus pyrkii täydentämään näitä puutteita kattavalla kasvikunnan kartoittamisella hyödyntäen uusia ja yhteneviä menetelmiä koko aineistolle.

Väitöskirjan ensimmäisessä osassa luotiin pohja laajalle kasvikunnan kartoitukselle. Uusi visuaalinen työkalu kehitettiin kartoittamaan lajityypillistä polyfenoliprofiilien kvantitatiivista ja kvalitatiivista vaihtelua ja niiden kausittaista muutosta. Havaittiin, että lajien polyfenoliprofiilien kvantitatiivisesta vaihtelusta huolimatta lajiprofiilit pysyivät tunnistettavina. Havaitut kausittaiset vaihtelut olivat melko vähäisiä, ja suurimmat muutokset näkyisät lajeilla, jotka tuottivat ellagitanniineja ja proantosyanidiineja. Lisäksi polyfenolitulosten yhdistäminen merkittäviin bioaktiivisuuksiin paljasti poikkeavia yhdistetyyppejä aktiivisuuksien taustalla. Useiden kasvilajien tulokset vahvistavat, että laajassa kasvien kartoituksessa käytetyt keräysmenetelmät, jotka eivät ole tiukasti keräysaikaan sidottuja ja joissa näyte koostuu useammasta kasviyksilöstä, kuvaavat hyvin tutkittavaa kasvilajia.

Väitöskirjan toinen osa yhdisti satojen kasviheimojen kemiallisen aineiston nykyaikaisiin kasvien fylogeneettisiin sukupuihin, mikä tarjosi biologisen kontekstin kemiallisille tuloksille. Havaittiin, että kahdeksan polyfenoliryhmän jakautuminen todennäköisesti heijastaa suuria evolutiivisia tapahtumia kasvikunnan vanhemmissa haaroissa, mutta nykyiset ekologiset prosessit vaikuttavat polyfenoliprofiileihin lähempänä lajitasoa. Flavonolijohdannaiset, kviinihappojohdannaiset ja proantosyanidiinit olivat laajimmin levittäytyneitä polyfenoliluokkia, kun taas hydrolysoituvien tanniinien esiintyminen oli jopa oletettua rajoittuneempaa vain tiettyihin kasviryhmiin. Näiden pääluokkien sisällä havaittiin, että biosynteettisesti yksinkertaisemmat alaryhmät olivat yleisempiä verrattuna vastaaviin, biosynteettisesti seuraaviin ryhmiin.

ASIASANAT: bioaktiivisuudet, evoluutio, fylogenia, kasvikunta, polyfenolit

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Abbreviations

APG	Angiosperm Phylogeny Group
CQA	Caffeoylquinic acid
DAD	Diode array detector
F–C	Folin–Ciocalteu
FL	Flavonol
G	Galloyl
HHDP	Hexahydroxydiphenoyl
HT	Hydrolysable tannin
KA	Kaempferol
MS	Mass spectrometer
MY	Myricetin
OX	Oxidative activity
PA	Proanthocyanidin
PC	Procyanidin
PCoA	Principal coordinate analysis
PD	Prodelphinidin
PGLS	Phylogenetic generalized least squares
PPC	Protein precipitation capacity
QA	Quinic acid
QqQ	Triple quadrupole
QU	Quercetin
RDA	Radial diffusion assay
SM	Specialized metabolite
UHPLC	Ultrahigh-performance liquid chromatography
WFO	World Flora Online

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Vanhakylä, S., Salminen, J.-P. Mass spectrometric fingerprint mapping of polyphenols reveals species-specific patterns in 31 biologically diverse plant species. *Molecules*. 2023; 28: 6388.

II Vanhakylä, S., Salminen, J.-P. Seasonal variation in plant polyphenols and related bioactivities across three years in ten tree species as visualized by mass spectrometric fingerprint mapping. *Molecules*. 2023; 28: 6093.

III Vanhakylä, S., Segar, S. T., Volf, M., Drozd, P., Endara, M.-J., Foley, W. J., Forrister, D. L., Furlan, C. M., Kim, J., Koivuniemi, A., Lamarre, G. P. A., Marsh, K. J., Moles, A. T., Murakami, M., Novotny, V., Ossipov, V., Pearse, I. S., Rothman J. M., Sedio, B. E., Wright, S. J., Salminen, J.-P. Kingdom of polyphenols: Evolution and distribution patterns in plants. To be submitted to *Ecological Monographs*, 2025.

Articles **I** and **II**, copyright © 2023 MDPI, published under an open access Creative Commons Attribution (CC BY 4.0) license.

1 Introduction

Plant evolution is estimated to have begun over 1.5 billion years ago from aquatic unicellular green algae.[1,2] Over time, these algae evolved into multicellular forms, eventually giving rise to terrestrial spore-bearing plants such as bryophytes, lycophytes and ferns, followed by the emergence of seed-bearing plants, including gymnosperms and angiosperms. Over millions of years of evolution, plants have diversified into approximately 380,000 recognized species today, with over 350,000 of these being flowering plants.[3,4]

During millions of years, plants have encountered a wide range of abiotic and biotic threats and changes, such as geographical and climate changes, pathogens, pollinators, herbivores and competing plant species. These have driven the development of a diverse array of features enhancing plants' survival. In addition to morphological innovations and adaptations, phytochemical characteristics have been crucial in the evolutionary processes of plants.[5–7]

Besides the vital primary metabolites, such as lipids, proteins, nucleic acids and carbohydrates, plants produce thousands of specialized metabolites (SM) that provide indirect benefits to plants. The major classes of plant SMs include terpenes, alkaloids, phenolics and glucosinolates. Among these, terpenes and phenolics are the most widely distributed SMs in the plant kingdom, while the others are more specialized to certain plant groups.[8–12] Phenolics, ranging from simple phenolic acids to larger polyphenols, play an important role in attracting pollinators, signalling purposes and protection against pathogens, herbivores and harmful UVB radiation. The diverse biological activities of polyphenols, deriving from their various structural features, have evidently been key factors in plant evolution.

Several significant evolutionary milestones in plant evolution have likely influenced the development of modifications in polyphenol structures (**Figure 1**). For instance, the transition of the ancestors of bryophytes (mosses, liverworts and hornworts) and all other land plants from aquatic environments to land habitats with increased UV radiation exposure is associated with the emergence of flavonoid biosynthesis, as flavonoids function as photoprotective compounds.[13–15] The development of vascular tissue with phenolic-based lignin provided structural support and enabled long-distance transportation of water and nutrients within

plants, revolutionizing their growth patterns and providing a significant competitive edge in the competition for light and space. The further modifications in flavonoid biosynthesis, leading to the formation of proanthocyanidin oligomers and polymers in the ancestors of ferns and seed plants, expanded the potential for structural diversification and new bioactivities.[16,17] Concurrently, the trihydroxy-substitution of the B-ring in the flavonoid structure introduced novel bioactive functions.[13]

The emergence of different herbivores tentatively may have influenced the evolution of polyphenols, as these compounds are strongly linked to plants' defense mechanisms. Two major pulses of arthropod herbivore emergence and one major emergence of tetrapod herbivores have been identified in the history of plants (**Figure 1**).[18,19] Given the substantial differences in the digestive systems of invertebrates and vertebrates, as well as the diversity of digestive mechanisms within these groups, plants have developed a wide array of polyphenol-based defensive strategies, especially within seed plants.

Over time, especially angiosperms, the so called flowering plants, have amassed an extraordinary diversity of species and became the dominant plant group in relatively short evolutionary period, a phenomenon that Charles Darwin referred as 'an abominable mystery'.[20] Flowering plants display a remarkable range of morphological, functional and ecological versatility.[21] Besides these factors, the diverse chemical defenses in angiosperms are believed to be an intrinsic factor that has allowed them to diversify.[22]

Angiosperms can be further categorized into several hierarchical lower taxa, depending on the classification system. This thesis employs the most recent, mainly molecular-based system of taxonomy by Angiosperm Phylogeny Group IV[23] (APG IV) as a general framework. In the APG IV classification, taxonomically lower ranks follow the botanical classification from highest to lowest: orders, families, genera and species, each potentially including super- and subcategories. The higher taxonomic ranks are not strictly classified according to botanical nomenclature but are commonly used in botany (**Figure 2**).

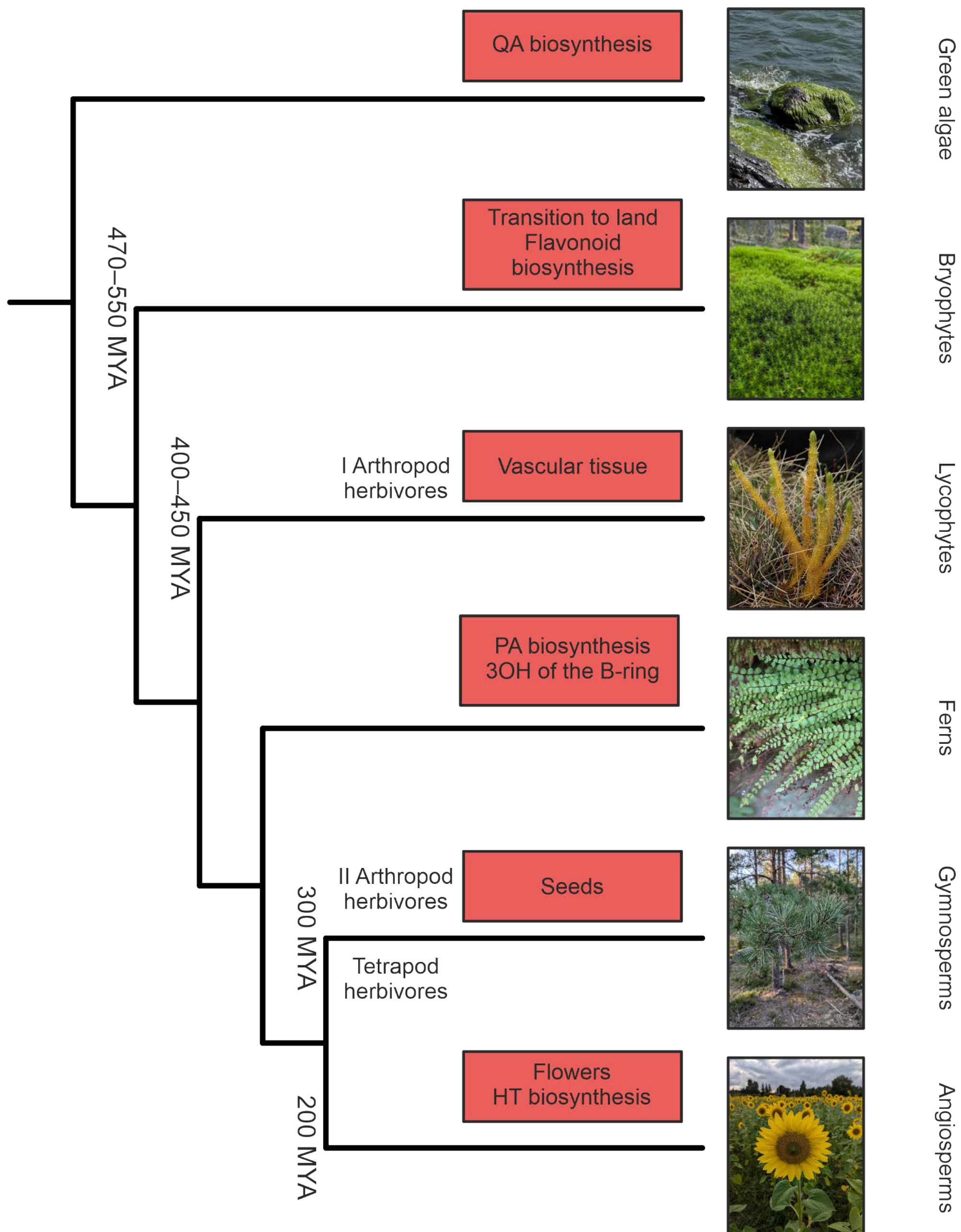


Figure 1. The most important milestones in plant evolution, specifically those with potential effect on polyphenol development. Abbreviations: QA: quinic acid derivatives, PA: proanthocyanidin, 3OH: trihydroxy-substitution, HT: hydrolysable tannin, MYA: million years ago.

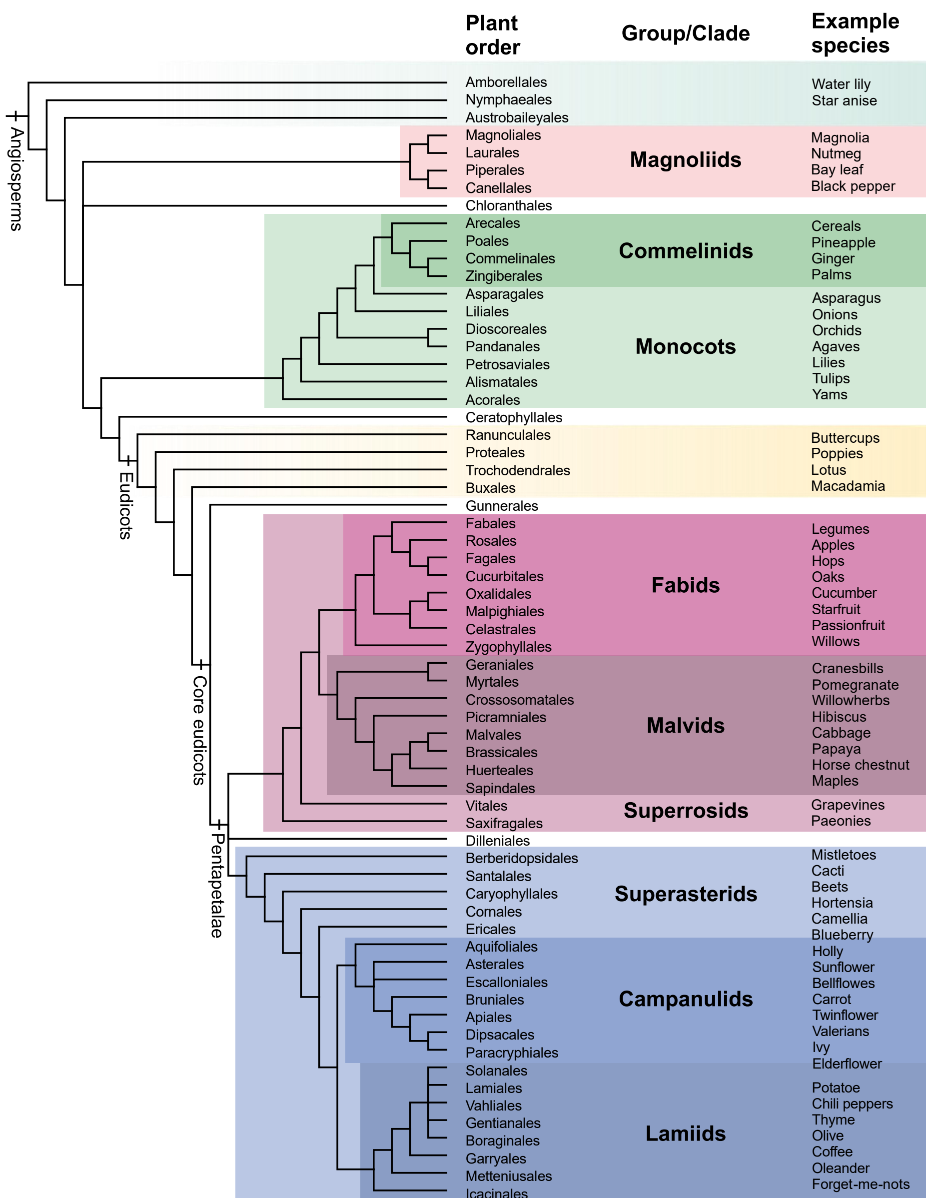
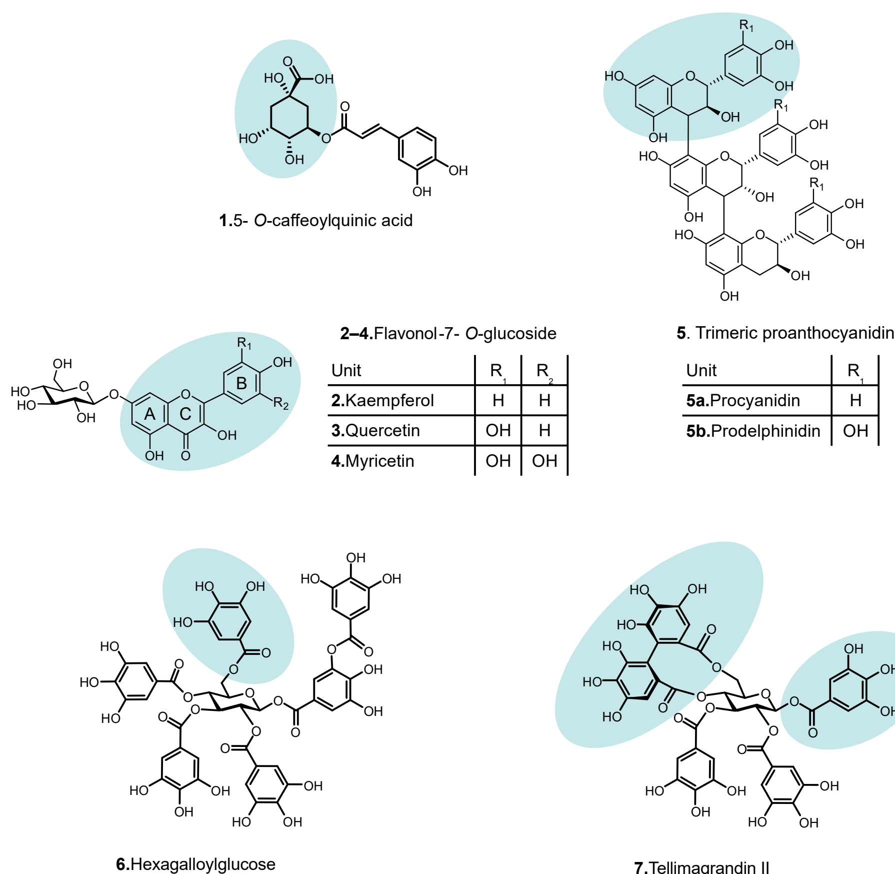


Figure 2. Phylogenetic relationships of angiosperm orders with example species from each plant group or clade. Consensus tree modified from: The Angiosperm Phylogeny Group et al. (2016)[23]. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV, *Botanical Journal of the Linnean Society*, 181(1), 1–20 by permission of Oxford University Press.

Currently, more than 10,000 polyphenols have been identified.[24,25] These compounds can be classified into several classes based on their functional units. Since a compound can contain units from multiple classes, the classification can partially overlap. Examples of compounds from different classes are presented in **Figure 3**.



Unit	R_1	R_2
2.Kaempferol	H	H
3.Quercetin	OH	H
4.Myricetin	OH	OH

Unit	R_1
5a. Procyanidin	H
5b. Prodelphinidin	OH

Figure 3. Model compounds and their subgroups, highlighted in blue, detected and quantified using group-specific methods.[26,27] Compound **1** is a quinic acid derivative, compounds **2–4** are kaempferol, quercetin and myricetin glucosides, which belong to flavonol derivatives, while **5a** is procyanidin and **5b** prodelphinidin subunit of proanthocyanidins. Compounds **6** and **7** belong to hydrolysable tannins: **6** is a gallotannin with only galloyl groups and **7** is an ellagitannin with both hexahydroxydiphenoyl and galloyl groups.

Although quinic acid (QA) is not a phenolic compound itself, it commonly forms esters with phenolic acids. Therefore, QA can be used in detection to reveal phenolic QA derivatives. Caffeoylquinic acids (CQA, e.g. 5-*O*-caffeoylquinic acid; **1**), which

are esters of caffeic acid and QA, are among the most abundant polyphenols.[28] Also, other esters of QA with other cinnamic acid derivatives, such as *p*-coumaric and ferulic acid, are common.[29] CQAs are present in almost all plants and have been detected even in some bryophyte species.[30,31] It is suggested that CQA could potentially be produced even by green algae.[32] In addition, galloylquinic acids, another type of QA derivatives with one or more galloyl (G) units, have been characterized in several vascular plant clades from distinct branches of the plant phylogenetic tree.[33–36]

Flavonoids are a diverse group of polyphenols characterized by a basic $C_6C_3C_6$ skeleton structure. They are primarily classified into subclasses based on variations in the C-ring structure, with additional structural differences arising from hydroxylation, methoxylation, and glycosylation at different sites. Flavonol (FL) derivatives, mainly FL glycosides, are one of the most common flavonoid classes exhibiting a nearly ubiquitous distribution in plants. FLs can be divided into subgroups by the degree of hydroxylation in the B-ring. Among these, kaempferol (KA; **2**) and quercetin (QU; **3**) glycosides with one and two hydroxyl groups in the B-ring, respectively, are predominantly observed across the plant kingdom while trihydroxy-substituted myricetin (MY; **4**) glycosides are comparatively rare. Even some mosses are reported to produce KA and QU derivatives, but not MY derivatives, which emerged later in ferns.[13,37,38] The diversity of flavonol glycosides is influenced not only by the degree of hydroxylation but also by the variety of sugar moieties and the specific sites where these sugars attach to the flavonol unit.

Proanthocyanidins (PA, syn. condensed tannins), are prevalent throughout vascular plants. PAs consist of flavan-3-ol subunits, primarily of (epi)catechins and (epi)gallocatechins forming the most common PA classes in plants: procyanidins (PC; **5a**) with two OH-groups in the B-ring and prodelphinidins (PD; **5b**) with three OH-groups. Propelargonidins with only one OH-group have more limited prevalence. The subunits form complex oligo- and polymers with several structural variations including the degree of polymerization, stereochemistry of the subunits and the quantity and arrangement of the bonds between them. PAs can display additional structural substitutions, such as G units. All these structural details can lead to very complex PA-structures and their mixtures in a single plant organ.

The occurrence of another tannin group, hydrolysable tannins, (HT) is predominantly restricted to specific parts of dicotyledonous plant orders, with only one known exception, the Nymphaeales order.[39,40] HTs are based on a polyol core, typically glucose, to which variable amounts of gallic acid moieties can esterify. HTs can be classified into simple gallic acid derivatives, for instance galloylglucoses, containing 1–5 G moieties, gallotannins (**6**), with digalloyl or trigalloyl groups and ellagitannins with hexahydroxydiphenoyl (HHDP; **7**) group(s) where two G units are linked by a C–C bond. The complexity of ellagitannins is amplified by the cyclic or

acyclic nature of the glucose core and by the variable positioning of the HHDP group and G units. Furthermore, the HHDP group can undergo additional modifications. Ellagitannins can exist in various forms from monomers to oligomers and even polymers[41], each with diverse types of linkages.

The biosynthesis of the major polyphenols occurs through two main, partially overlapping biosynthetic pathways.[42–44] In short, FLs and PAs are synthesized utilizing both the acetate/malonate and shikimate pathways. In contrast, HTs and the quinic acid moiety of QA derivatives are derived solely from the intermediate compound of the shikimate pathway. The simultaneous presence of compounds from the same biosynthetic pathway is frequently observed.[45,46] For example, within flavonoids this occurs because intermediate compounds are diverted into alternative routes within flavonoid pathway, contributing to the synthesis of other flavonoid compounds as well.[47,48] However, several plant defense theories suggest that resource limitations and the allocation of resources to specific defenses can lead to negative trade-offs among defenses.[49,50] Negative correlations between polyphenols and other defense mechanisms, as well as among specific polyphenol subgroups within plant species, have been observed.[51–56] Since intermediates from the shikimate pathway are shared, especially the efficient production of HTs can negatively impact the synthesis of PAs and FLs.[57] However, the extent to which these compounds from different pathways occur simultaneously and the underlying reasons for this phenomenon are not well understood. For instance, it has been suggested that the ecological benefits of producing a wide variety of defenses against different threats, such as various herbivores, may compensate for the negative trade-offs.[54,58,59] Although these correlations between polyphenol subgroups are not yet fully understood, they could shed light on the evolutionary pressures that shape the diversity of polyphenol compounds in different plant species.

Recently, the majority of the polyphenol research has been highly human-centered and plant polyphenols have gained attention mainly for their nutritional and medical benefits.[25,60–62] The main interest towards them lies especially on their ability to prevent oxidative damage by their antioxidant activity.[25,62–64] In general, these health benefits derive from the same bioactivities that help plants protect themselves.[25] Specifically, in plants, the primary role of polyphenols is to defend against herbivores.

Currently, two primary modes of action have been identified. The traditional studied biological activity of tannins is their capability to precipitate proteins and other substrates in water solution.[63,64] Precipitation can reduce digestibility of plant proteins, resulting in a lower nutritional value of the plant material from the herbivore’s perspective.[65] This is particularly relevant for mammal herbivores with a low to neutral pH in their oral cavity and part of their digestive system, as an acidic environment can promote protein precipitation, acting as a feeding deterrent and

making the proteins less accessible for digestion and absorption.[66–70] In contrast, the high gut pH of several caterpillars may significantly reduce hydrogen bonding and, consequently, protein precipitation.[66]

The oxidative activity (OX) is another crucial defense mechanism against herbivores. While several polyphenols are known for their antioxidant properties, they can also act as oxidants under certain physiochemical conditions.[66] In the presence of oxygen, oxidation of polyphenols can produce reactive oxygen species and quinones, which can directly damage the herbivore's gut or further enhance oxidative stress reactions, such as the Fenton reaction.[71,72] These reactions can be triggered by the alkaline conditions in the gut fluids of many insect herbivores, such as butterflies and moths (Lepidoptera), beetles (Coleoptera) and true flies (Diptera).[73–75] Alternatively, they can be initiated by the release of polyphenol oxidizing enzymes already present in the plant, for example by herbivore chewing the plant material.[76] In mammalian herbivores, only enzymatic oxidative activity, which takes place already in the mouth and does not require alkaline conditions, plays a significant role.[57,66,69,77]

It is essential to understand that, even within a single species, plants can exhibit significant intraspecific variation in the expression of these chemical traits. The polyphenol content of different plant organs often varies greatly.[78–81] For instance, reproductive tissues are reported to exhibit higher polyphenol quantities compared to vegetative tissues.[7,81] The polyphenol content in different organs reflects their adaptation to various functions. Flowers, which are specialized to attract pollinators, can have significantly different polyphenol content compared to roots, which are involved in below-ground interactions with nutrients, microbes, and plant-plant allelopathy.[82–85] In contrast, one of the principal roles of polyphenols in plant leaves is defense against herbivores. However, polyphenols exhibit diverse and complementary functions, contributing to the complexity of their variation across plant organs and species. Additionally, the production of defensive chemicals can be either constant or induced by abiotic stress or herbivore attack, making it necessary to understand these effects during plant sampling.[86,87]

A significant portion of the variation is due to the natural phenotypic and genotypic plasticity in SM production, allowing plants to acclimate and adapt to various environmental changes. Especially in natural environments with varying seasons, plants can exhibit significant changes not only in their easily detectable visible morphological features (**Figure 4**), but also in their polyphenol content.[56,88–90] This is particularly seen at the beginning of the growing season.[91–93] The content can vary across different ontogenetic stages; for instance, young plants can differ significantly from fully-grown individuals.[94,95] Therefore, systematic plant sampling and understanding natural plasticity are pivotal in research, which aims to conclude general species-specific patterns.

In this thesis, the focus was on foliar chemistry, as these tissues can be sampled from the majority of species. Vegetative tissues evolved before more specialized structures such as roots, flowers and seeds, providing insights into the early evolutionary stages of the plant kingdom. Given that leaves are particularly susceptible to herbivory, this thesis specifically investigates the impact of herbivory on foliar chemistry and related bioactivities. However, it must be noted that this approach captures only a part of the evolutionary journey of plants, as the effects of pollination and seed dispersal, for instance, represent other fascinating aspects that are beyond the scope of this thesis.

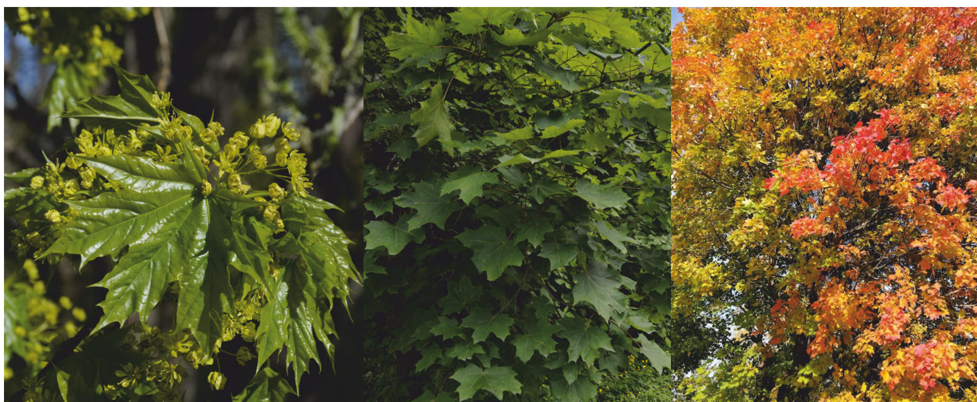


Figure 4. Besides the visible morphological changes, plants also exhibit seasonal variations in their polyphenol production.

The overall distribution of polyphenols in the plant kingdom is influenced by several factors. The evolutionary history of plants plays a significant role in shaping their metabolic capabilities, as ancestral environmental conditions have partially constrained the range of compounds they can produce. Ongoing evolutionary pressures and ecological interactions further shape the diversity and distribution of these compounds. However, evolution is not a straightforward process; it involves the gain or modification of new SMs, the loss of previously developed ones and the possible reoccurrence of these traits, sometimes due to convergent evolution.[96] The chemical content is partially influenced by the phylogenetic relationships of plants, as genetic factors and similar evolutionary circumstances contribute to the observed patterns, making similar SM patterns more likely within closely related plants.[96] The co-occurrence of different polyphenol groups, possible trade-offs of specific compounds, evolutionary history and gene silencing events all contribute to the complex landscape of polyphenol distribution in plants. Although fossil records can shed light on the evolutionary history of plant chemistry to some extent, limitations such as the availability of fossil data, the longevity of chemical constituents, and

potential contaminations can hinder research.[97,98] Therefore, large-scale screening of extant plant species allows for the study of evolutionary events with a broader range of data and less laborious methods.

Altogether, the overall picture of the evolution and distribution of phenolics and their bioactivities throughout the plant kingdom remains incomplete. The most extensive chemotaxonomic reviews and screening studies were conducted decades ago using simpler methodologies available at that time.[13,47,99–105] Even today, numerous studies present results as imprecise total amounts. Recent research employing modern methodologies has primarily focused on specific polyphenol compounds within a narrow range of plant species often with a strong emphasis on human health. Conversely, research aiming to detect all compounds produced by plant species often yields results with significant uncertainties. The wide-ranging methodologies and perspectives reduce the comparability of the results. However, the task of identifying every phenolic compound from thousands of plant species may not be a practical or even necessary to gain a comprehensive understanding of the plant kingdom and its significant evolutionary milestones. At the moment, characterizing larger, representative entities is more efficient and sufficient to signify the pivotal evolutionary steps.

The primary aims of this PhD project encompass three distinct aspects, which create a continuum that spans from the detailed study of single plant species to extensive large-scale plant screening. The main aims were:

1. Compare polyphenol profiles within plant populations across a diverse selection of plant species to assess the level of natural variation (Article **I**). This information will then be applied to large-scale plant collections (Article **III**).
2. Investigate seasonal variations in polyphenol profiles of ten woody species during their seemingly steady growth phases and analyze interannual differences to identify large-scale variability patterns (Article **II**).
3. Study the distribution of foliar polyphenols across the plant kingdom to reveal key factors affecting the polyphenol content of plants, utilizing consistent methodologies on data from thousands of plant species across the phylogenetic tree (Article **III**).

These aims were accomplished by collecting a comprehensive plant library from five continents during ten years with a special focus on the good coverage of the plant kingdom at the family level (Article **III**). The polyphenol profiles were measured utilizing group-specific methods on an ultrahigh-performance liquid

chromatograph coupled with a diode array detector and triple quadrupole mass spectrometer (UHPLC-DAD-QqQ-MS) (Articles **I–III**). The bioactivities were determined with two bioactivity tests: radial diffusion assay for protein precipitation capacity and modified Folin–Ciocalteu assay for oxidative activity (Articles **I** and **II**).

A new graphical mass spectrometric fingerprint mapping tool was created to visualize quantitative and qualitative polyphenol and bioactivity profiles as species-specific 2D maps (Articles **I** and **II**) and plant family-specific maps. The variations observed in Articles **I** and **II** were used to evaluate the sufficiency of plant sampling protocol used in the large-scale global sampling. The chemical results were linked to up-to-date phylogenetic trees and examined in the light of plant evolution (Article **III**).

2 Materials and Methods

2.1 Plant sampling

Plant samples for Articles **I** and **II** were collected from Turku region in South-West Finland. A selected set of 31 chemically and biologically variable plant species for the Article **I** were collected from May till July in 2016 (**Table 1**). Ten mature plant individuals were sampled from each species. Depending on the plant's size and growth type, up to ten undamaged leaves, needles or flowers were collected from various parts of the plant to ensure a representative pooled sample.

Since *Juniperus communis* and *Picea abies* were also part of the seasonal monitoring in Article **II**, their current year needles, which correspond to the leaves of deciduous trees, were sampled. In contrast, older needles of *Pinus sylvestris* were collected to align with the general sampling of mature tissues. Additionally, three species (*Trifolium hybridum*, *Chamaenerion ancgustifolium* and *Primula veris*) were sampled for their floral chemistry due to prior knowledge of their high content of specific polyphenols in these organs.

Ten tree species from article I were selected for the seasonal monitoring in Article **II** (**Table 1**). Leaves and needles that had reached their mature size were collected three times during a growing season after the most intensive growing phase from mature tree individuals. This approach aimed to detect potential changes during the seemingly stable stage of leaf growth. In the seasonal study, samples were gathered from five plant individuals of each species by cutting three branches around the tree and selecting six to 10 healthy leaves or annual shoots per branch. The seasonal monitoring was repeated annually over a three-year period from 2016 to 2018. In these studies, the samples were wrapped in aluminum foil and stored in a cooler during collections. They were transferred to a freezer (-21 °C) within three hours and left to freeze overnight at minimum before a thorough freeze-drying.

Plant collections for the larger data set commenced in 2011 and continued until 2020. Altogether 7090 leaf samples were analyzed on the scope of this research. The data set consisted of repetitions and parallel samples of 3772 plant species, subspecies and varieties in total (**Figure 5**). Samples were collected from 24 countries from five continents. A list of the plant samples and collection sites is presented in the supplementary of Article **III**. Most of the samples were collected

from natural environments and natural parks, with additional collections from arboretums and botanical gardens. Collections were targeted to cover all parts of land plant phylogeny as comprehensively as possible with the main focus on seed plants. The indicative goal was to collect ten species/plant order and five species/plant family, when possible, to create a balanced plant library.

Table 1. Plant species and collected plant organs studied in the Articles **I** and **II**.

Species	**Family**	**Organ**	**Article**
Gymnocarpium dryopteris	Cystopteridaceae	leaflets	I
Juniperus communis	Cupressaceae	annual shoots	I, II
Pinus sylvestris	Pinaceae	needles	I
Picea abies	Pinaceae	annual shoots	I, II
Caltha palustris	Ranunculaceae	leaves	I
Chelidonium majus	Papaveraceae	leaves	I
Ribes alpinum	Grossulariaceae	leaves	I
Paeonia lactiflora	Paeoniaceae	leaves	I
Trifolium hybridum	Fabaceae	flowers	I
Sorbus aucuparia	Rosaceae	leaves	I, II
Prunus padus	Rosaceae	leaves	I, II
Argentina anserina	Rosaceae	leaves	I
Comarum palustre	Rosaceae	leaves	I
Rubus saxatilis	Rosaceae	leaves	I
Geum rivale	Rosaceae	leaves	I
Filipendula ulmaria	Rosaceae	leaves	I
Betula pubescens	Betulaceae	leaves	I, II
Alnus glutinosa	Betulaceae	leaves	I, II
Alnus incana	Betulaceae	leaves	I, II
Quercus robur	Fagaceae	leaves	I, II
Salix phylicifolia	Salicaceae	leaves	I, II
Geranium sylvaticum	Geraniaceae	leaves	I
Geranium pratense	Geraniaceae	leaves	I
Acer platanoides	Sapindaceae	leaves	I, II
Lythrum salicaria	Lythraceae	leaves	I
Chamaenerion angustifolium	Onagraceae	flowers	I
Plantago major	Plantaginaceae	leaves	I
Primula veris	Primulaceae	flowers	I
Lysimachia vulgaris	Primulaceae	leaves	I
Vaccinium myrtillus	Ericaceae	leaves	I
Vaccinium vitis-idaea	Ericaceae	leaves	I

A pooled sample, comprising at least five mature plant individuals and at least five leaves or annual shoots from each, was collected to ensure a representative sample of the species. In the normal protocol in optimal circumstances the samples were stored in dry ice or ice and frozen shortly after collection, as in the Articles **I** and **II**. After a freeze-drying for at least 48 hours, the dry samples were ground into fine powder with a ball mill.

Due to the lack of required drying equipment or demanding field circumstances, some species sets were processed with alternative methods. Samples from Papua New Guinea were collected as small cut leaf discs into 50 mL Falcon tubes filled with acetone. In Uganda the samples were dried at 50 °C to constant weight using a food dehydrator. *Inga* species were collected from understory saplings in Panama, French Guiana, Peru, Ecuador and Brazil. Fresh leaf samples were air-dried in silica gel at room temperature. Samples from the World Herbivory Project by Moles et al. were oven-dried at 55–65°C and treated with gamma irradiation.[106] These exceptions did not significantly impact the results. Additionally, duplicates from other collection sites ensured robust representation.

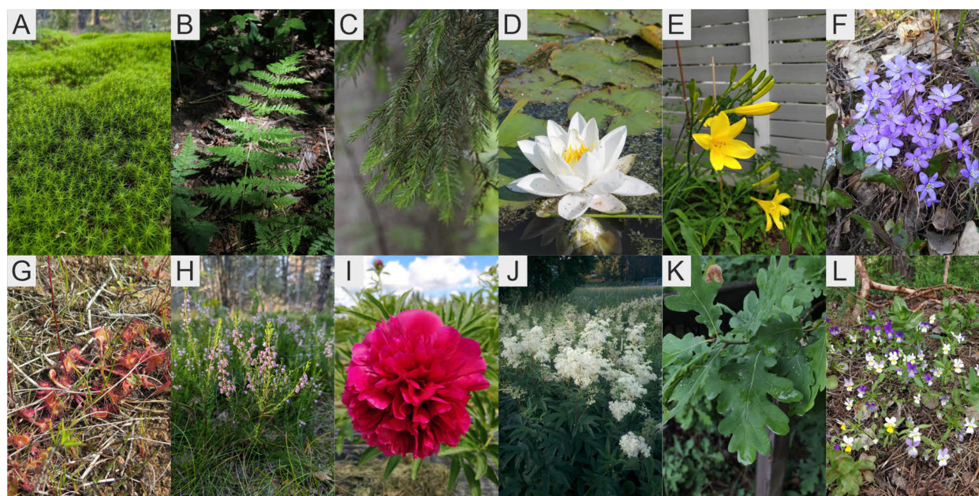


Figure 5. Examples of the variety of collected plant species: (**A**) *Polytrichum sp.*, (**B**) *Dryopteris carthusiana*, (**C**) *Picea abies*, (**D**) *Nymphaea alba*, (**E**) *Hemerocallis lilioasphodelus*, (**F**) *Hepatica nobilis*, (**G**) *Drosera rotundifolia*, (**H**) *Calluna vulgaris*, (**I**) *Paeonia lactiflora*, (**J**) *Filipendula ulmaria*, (**K**) *Quercus robur* and (**L**) *Viola tricolor*. Targeted plant screening covered a wide range of biologically and chemically different plant species from different parts of the plant kingdom.

2.2 Chemical analyses

2.2.1 Extraction

After the normal collection protocol and freezing and drying procedure, 20 mg (± 0.50 mg) of finely powdered sample was mixed with 1400 µL of acetone/water (8/2, v/v) solvent and left to macerate overnight at 4 °C. Extraction was conducted in 2 mL Eppendorf tubes on a planar shaker for 3 hours, samples were centrifuged (14,000 rmp, 10 min) and supernatant was collected. The extraction step was conducted twice and supernatants were combined. Acetone was evaporated with an Eppendorf concentrator at room temperature and the remaining aqueous phase was frozen and freeze-dried. The dried extract was dissolved in 1 mL of ultra-pure water. Before the further steps, the extracts were filtered with 0.2 µm PTFE syringe filters.

The samples from Papua New Guinea required a special extraction protocol since they were collected and stored in acetone in 50 mL Falcon tubes. The storage acetone was poured into a new Falcon tube and 5 mL of ultra-pure water was added to it. Acetone was then evaporated under nitrogen flow in water bath and the remaining water phase was stored at 4 °C overnight. Simultaneously the leaf material was homogenized in grinding capsules (IKA Ultra Turrax, 6 × 5 min, 4,000 rpm) with 30 mL of acetone/water (8/2, v/v). The ground mixture was poured in the original Falcon tubes and filled with acetone/water (8/2, v/v) to the 35 mL line before centrifuging (9,000 rpm for 10 min). The centrifuged solution was combined with the water phase. Acetone was evaporated with water bath and nitrogen flow. The extracts were then frozen and lyophilized. The material was dissolved in 5 mL of ultra-pure water and filtered with 0.2 µm PTFE syringe filters.

2.2.2 UHPLC-DAD-QqQ-MS analyses

Prior to following analyses, all the plant extracts were diluted to one-fifth concentration with ultra-pure water. Every sample in the Articles **I–III** was analysed with an ultrahigh-performance liquid chromatographic system coupled with a diode array detector and triple quadrupole mass spectrometer (UHPLC-DAD-QqQ-MS). The UHPLC system included a sample manager, a binary solvent manager, an Acquity UPLC® BEH Phenyl column (100 mm × 2.1 mm i.d., 1.7 µm; Waters Corporation, Wexford, Ireland), a photodiode array detector and Xevo TQ triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA).

The analysis conditions and methods were as previously published by Engström et al.[26,27] The flow rate of eluent was 0.5 mL/min. Gradient elution used two eluents; acetonitrile (A) and 0.1% aqueous formic acid (B). Each elution was divided in the following sections: 0–0.5 min, 0.1% A in B (isocratic); 0.5–5.0 min, 0.1–30% A in

B (linear gradient); 5.0–6.0 min, 30–35% A in B (linear gradient); and 6.0–9.5 min, column wash and stabilization. Negative ion mode was used on the mass spectrometer. Electrospray ionization was used with the following conditions. The capillary voltage was 2.4 kV. Desolvation and source temperatures were 650 °C and 150 °C, respectively. N_2 was used as desolvation and cone gas with flow rates 1000 L/h and 100 L/h, respectively. Argon was used as collision gas.

UV and MS data were recorded from 0 to 6 min during the years 2013–2015 and from 0 to 7 min in 2015–2022. The DAD operated within an overall detection range of 190 to 500 nm. Catechin solution (5 µg/mL) was injected before and after every ten samples to monitor and correct any changes in MS performance and mixtures of flavonoids and ellagitannins and PA standards were analyzed prior to every data set to monitor the quality and variations in both chromatography and UV and MS responses.

Multiple reaction monitoring and single reaction monitoring methods were utilized for each plant sample to form group-specific polyphenol fingerprints for the eight studied polyphenol groups: QA derivatives, KA, QU and MY-based FL derivatives, PC and PD units of PAs, G derivatives and HHDP derivatives.[95,96] In addition, the ion abundances predicting the linkage site of the sugar moiety, as described in previous studies[26,107–109], were examined in the three FL derivatives by measuring the ratio of formed FL aglycone radical ions to aglycone ions.

First, the UHPLC-separated polyphenols were fragmented with individually optimized cone voltages for each polyphenol group in the ion source. Next, the specific precursor ions, which represent the selected functional units of the polyphenols, are selected in the first quadrupole for fragmentation in the collision cell. In most cases two product ions from the specific precursor ions were selected to exclude possible false positive detections from other compound groups. The product ions are then accumulated with optimal collision energies and subsequently selected by the final quadrupole for detection and creation of group-specific fingerprints. The total concentrations are then calculated from the smoothed and integrated fingerprints (TargetLynx software, Waters Corporation) using external standards and calibration curves specific to each compound group.[110]

2.2.3 Measurement of total phenolic content and oxidative activity

Total phenolics (Articles **I**–**III**) and oxidized phenolics (Articles **I** and **II**) were measured using Multiscan Ascent microplate reader (Labsystems and Thermo Electron Corporation). A modified Folin–Ciocalteu (F–C) assay was utilized on 96-well plates to determine total phenolics before and after alkaline oxidation to resolve the content of easily oxidized phenolics in plant extracts.[57] All the measurements were conducted in triplicates and results were reported in gallic acid equivalents (mg/g).

The oxidation of the 20 µL of the previously prepared plant extracts was initiated by adding 180 µL of sodium carbonate buffer (pH 10) to wells. Samples were incubated exactly 60 min at 25 °C and shaken every min for 10 s. The oxidation was stopped by the addition of 100 µL of 0.6% formic acid lowering the pH to 6. The control samples for total phenolic measurement were prepared by adding 280 µL of the buffer mixture containing both 180 µL of sodium carbonate buffer and 100 µL of 0.6% formic acid to the 20 µL of the plant extracts.

The F–C assay was used for both oxidized and non-oxidized extracts. A portion of 50 µL of the extracts were pipetted on the wells, 50 µL of F–C reagent was added and the plate was shaken for 1 min. After an addition of 100 µL of 20% sodium carbonate (m/v) to activate F–C reagent, the plate was shaken again every minute for 10 s. Absorbance was measured at 742 nm after 30 min and was used to calculate the total phenolics against a gallic acid standard curve (prepared in water: 0 µg/mL, 10 µg/mL, 25 µg/mL, 100 µg/mL).

2.2.4 Radial diffusion assay

Radial diffusion assay[111] (RDA) was used to determine protein precipitation capacity (PPC) of the plant samples. The RDA gel was prepared by dissolving 1 g of the model protein BSA and 10 g of agarose into 1000 mL of RDA buffer consisting of 10.6 mg of ascorbic acid, 2.85 mL of glacier acetic acid and 1 L of ultra-pure water. The mixture was buffered to pH 5 with 2 M NaOH. A 10 mL volume of the prepared gel was pipetted into Petri dishes and let to settle. Nine 4 mm wells were punched into each dish.

The previously prepared plant extracts were concentrated by lyophilizing 200 µL of extract and dissolving them again into 100 µL of ultra-pure water. A portion of 24 µL of the extract was applied into the wells in triplicate. A mixture of pentagalloylglucose/oenothein B (1/1, v/v) was used as a standard (1, 2, 3, 4 and 5 mg/mL in 30% aqueous EtOH) and pipetted into the Petri dishes similarly. The extracts were let to diffuse into the gel and the dishes were sealed with parafilm and incubated upside down in 30 °C for three days. The formed precipitation areas were measured from photographs with ImageJ software.[112] The results were transformed into mg/g (dry weight) concentration using the pentagalloylglucose/oenothein B standard curve.

2.3 Mass spectrometric fingerprint mapping

Mass spectrometric fingerprint mapping tool was created in Excel (Microsoft Corporation) and tested in Article **I** and **II**. Quantitative data of each major polyphenol group (QA, FL, PA and HT) and bioactivities (OX and RDA) were

normalized between 0 and 1 to plot all the groups on the same scale *x*-axis in the mass spectrometric fingerprint maps. The maximal values of each group were set to cover 95% of the samples from the data set in Article **III**, because the highest 5% tended to be scattered on a very large concentration scale. Quantification limits were 0.1 mg/g for QAs, FLs and HTs, producing more easily defined sharp peaks in chromatograms, and 1.0 mg/g for PAs forming oligomer and polymer humps. In the Article **III** and in the family-level fingerprint maps, only the quantitative results exceeding 0.05 threshold at the normalized scale were displayed to exclude insignificant quantities and possible false positive records from other groups. The *y*-axis displays the proportions of the subgroups or subunits belonging to the major polyphenol groups (KA/QU/MY, PC/PD and G/HHDP) and the proportions of QA, OX and PPC against the total phenolic content of the sample.

2.4 Data handling and analysis

2.4.1 Standardization of scientific names

All statistical analyses, tree generating and name standardization were conducted with R (version 4.3.0) using RStudio (version 2023.06.2). Scientific names were standardized with an R package 'U.Taxonstand' (version 1.1.3)[113] with the World Flora Online (WFO)[4] chosen as the taxonomic database for name verification. Since 'U.Taxonstand' package handles binomial names best, all infraspecific taxa, such as subspecies, cultivars, variations and other possible details were excluded from the automated name standardization and manually verified using the WFO database.

2.4.2 Generating phylogenetic trees

After the name standardization, a family-level phylogenetic tree was built. An R package U.PhyloMaker (version 0.1.0)[114] was used to generate the phylogenetic trees. A megatree 'GBOTB.extended.WP.tre' (retrieved 5.12.2024) reported by Jin & Qian[114] including both megatree for seed plants[115] and pteridophyte tree[116] was used as a backbone for the phylogenetic trees. In the family-level phylogenetic tree in Article **III** algae and byophytes were omitted to maintain the main focus on vascular plants. The Interactive Tree Of Life (iTOL) tool[117] was used to annotate and visualize the results in generated trees. The distribution of the studied polyphenol groups was displayed as averages of the families.

2.4.3 Statistical methods

Principal component analysis in Canoco 5[118] was used to explore the variation in the concentration of polyphenol subgroups across the dataset. Plant species were used as samples and log-transformed concentrations as response variables.

To test how much variation in polyphenol profiles was explained by plant phylogeny vs. family identity, series of redundancy analyses were conducted with Canoco software (version 5.0).[118] Plant species were used as samples and log-transformed concentration of individual polyphenol subgroups as response variables. Plant family was used as an explanatory variable in the first redundancy analysis to test its effect on variation in the polyphenol subgroups with Monte-Carlo test with 999 permutations. To test the effect of phylogeny, the phylogenetic data was first transformed into phylogenetic axes with an R package ‘ape’.[119] The phylogenetic principal coordinate (PCoA) axes that significantly influenced the variation in polyphenol profiles were identified using forward selection in redundancy analysis. Log-transformed concentrations of each polyphenol group for the species were used as response variables, with phylogenetic PCoA axes serving as explanatory variables. In the subsequent phase, plant families and the previously selected phylogenetic axes were used as explanatory variables. A Monte Carlo test with 999 permutations was conducted to partition the variation explained by the two sets of variables, following the methodology of ter Braak and Šmilauer.[118]

To study the trait correlations across species while accounting for phylogenetic relationships, we applied phylogenetic generalized least squares (PGLS) with Lambda model using the R packages ‘nlme’ and ‘phytools’.[120,121] Polyphenol concentrations were log-transformed and centered with the R package ‘emmeans’ and p-values were adjusted for multiple comparisons using the false discovery rate method.[122,123].A pairwise correlation matrix based on the PGLS analyses was generated using the R package ‘corrplot’.[124]

3 Results and discussion

3.1 Mass spectrometric fingerprint mapping tool

A new graphical mass spectrometric fingerprint mapping tool was created to visualize quantitative and qualitative data of the eight polyphenol groups and two bioactivities. The normalized quantitative results of the major polyphenol groups from group-specific MS/MS fingerprints (QA, FL=KA+QU+MY, PA=PC+PD, HT=G+HHDP) could be effectively represented within the same 2D chart on the *x*-axis with the two bioactivities (OX and PPC). The ratios of the subgroups (KA/QU/MY, PC/PD and G/HHDP) were plotted on the *y*-axis. For variables lacking subunits, their concentrations were calculated relative to the total phenolics (QA/TP, OX/TP and PPC/TP). Besides these variables, the tool was utilized in more detailed way to monitor ionization efficiencies and fragmentation patterns among the three flavonol groups. The tool was modified to contain the normalized values of KA, QU and MY and the ratios of the flavonol aglycone deprotonated ions and aglycone radical ions indicating the potential position of the sugar moiety.

Since the normalization was based on the comprehensive plant library encompassing nearly 3,800 species, each individual result could be compared against the whole data set. The quantitative results were categorized into five concentration levels at the normalized scale: low (<0.2), lower than intermediate (0.2–0.4), intermediate (0.4–0.6), higher than intermediate (0.6–0.8) and high (>0.8).

In the Article **I** the mass spectrometric fingerprint maps were used to test the capability to discriminate 31 plant species, to estimate the variation within species, search linkage between polyphenol groups and bioactivities and to reveal the ionization patterns of FL derivatives. In Article **II**, the seasonal variation of polyphenols in ten woody species was monitored using fingerprint maps. In this thesis, the fingerprint maps were utilized to display patterns within 286 plant families (**Appendix Figure A1**). In **Figure 6** examples of the both group-specific fingerprint maps (**Figure 6A**) and flavonol ion-pattern maps (**Figure 6B**) are presented at a species-level. The examples of family-level maps used in the Article **III** are presented in the **Figure 6C**. This tool also exhibits potential for applications beyond its initial scope, including the monitoring of biological treatments and environmental

changes. Additionally, the methodology can be adapted for analysis of other compound groups and their respective subclasses.

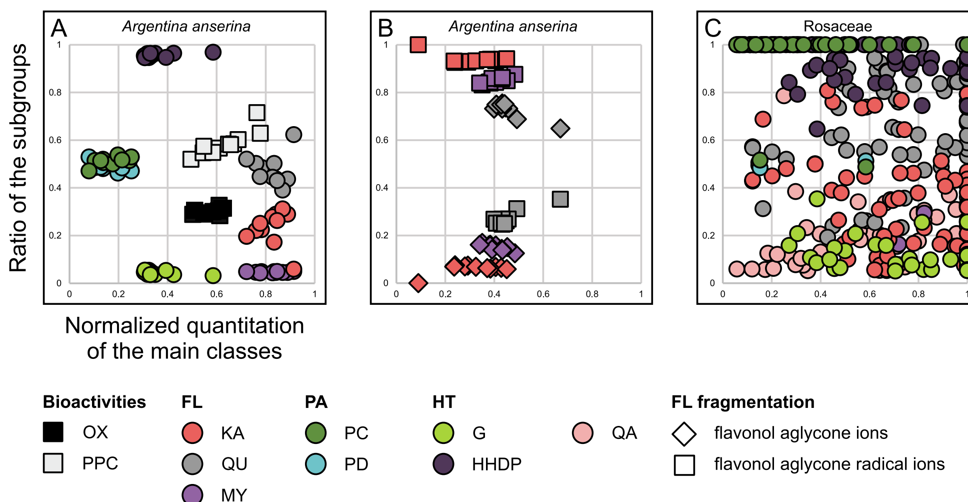


Figure 6. Examples of different types of mass spectrometric fingerprint maps. (**A**) Fingerprint map of eight polyphenol groups and two bioactivities of *Argentina anserina* population (10 individuals). The map shows intermediate hydrolysable tannin (HT) production with hexahydroxydiphenoyl (HHDP) dominance over galloyl (G) derivatives, low proanthocyanidin (PA) content with a balanced procyanidin/prodelphinidin (PC/PD) ratio, high flavonol (FL) derivative production with quercetin (QU) dominance and no quinic acid (QA) derivatives. Both oxidative activity (OX) and protein precipitation capacity (PPC) are at intermediate to higher than intermediate levels, indicating relatively high activity levels of the compounds. (**B**) Flavonol fingerprint map reveals that kaempferol (KA) and myricetin (MY) glycosides primarily produce aglycone radical ions, indicating probable 3-*O*-glycosylation, while QU glycosides producing deprotonated aglycone ions derive from other linkage types. Besides these, other substituents can affect the fragmentation. (**C**) In the family-level fingerprint maps of *A. anserina*'s family Rosaceae, each circle represents the average values of polyphenol groups of a single species. Figure adapted from Article **I**.

3.1.1 Species-specific variation

The mass spectrometric fingerprint mapping tool demonstrated the capability to successfully discriminate species when tested on a selected set of 31 species, suggesting its potential utility as a chemotaxonomic marker. The eight polyphenol groups generally provided sufficient contrast between species. However, for closely similar fingerprint maps, examining the ionization patterns of flavonol derivatives provided more discriminating variables. All the fingerprint maps are presented in Article **I**.

The variation observed in the fingerprint maps was primarily quantitative rather than qualitative within the species. Each species exhibited normal plasticity in its

polyphenol concentrations influenced by biotic and abiotic factors. Different growth types and tissues did not appear to have a clear impact on the variation. However, in some species the replicates were relatively tightly clustered on the fingerprint map, for example in *Chamaenerion angustifolium* (**Figure 4** in Article **I**) and *Argentina anserina* (**Figure 6**), which typically form dense clonal populations. In contrast, more sporadic variation was detected in specific polyphenol groups of two species: HT quantities and G/HHDP ratios within the *Alnus glutinosa* population varied significantly and PAs were particularly scattered in the *Salix phylicifolia* population (**Figure 4** in Article **I**).

In general, several species displayed a broad range of quantitative values across three categories (e.g. from intermediate to high), while the subunit ratios (e.g. PC/PD) remained relatively stable. Notably, at very low quantitative levels, the ratios of subunits showed a wider distribution along the *y*-axis. In the broader context, these very low levels may not be significantly impactful, for instance, for bioactivities. Therefore, results below the 0.05 normalized threshold were excluded from the family-level fingerprint maps.

In the flavonol fingerprint maps, aglycone radical ions were found to be predominated throughout the data, suggesting the presence of 3-*O*-glycosidic forms of FLs.[26,109,125–127] Notably, significant intensities of dominant aglycone ions were observed only for QU derivatives of certain species. Similar to the original fingerprint maps, the ion ratios remained mostly at the same level when the quantitative levels were above 0.1.

Although the closely related species *Comarum palustre* and *Rubus saxatilis* exhibited qualitatively similar fingerprint maps within the Rosaceae family, they could be easily distinguished by their flavonol fingerprint maps, which showed significantly different KA and QU profiles (**Figure 7A**). However, some fingerprint maps of species (*Trifolium hybridum* and *Prunus padus*) from distinct plant families (Fabaceae and Rosaceae) appeared quite similar on both fingerprint map types differing only by the presence of QA derivatives, which enhanced the significance of each variable (**Figure 7B**).

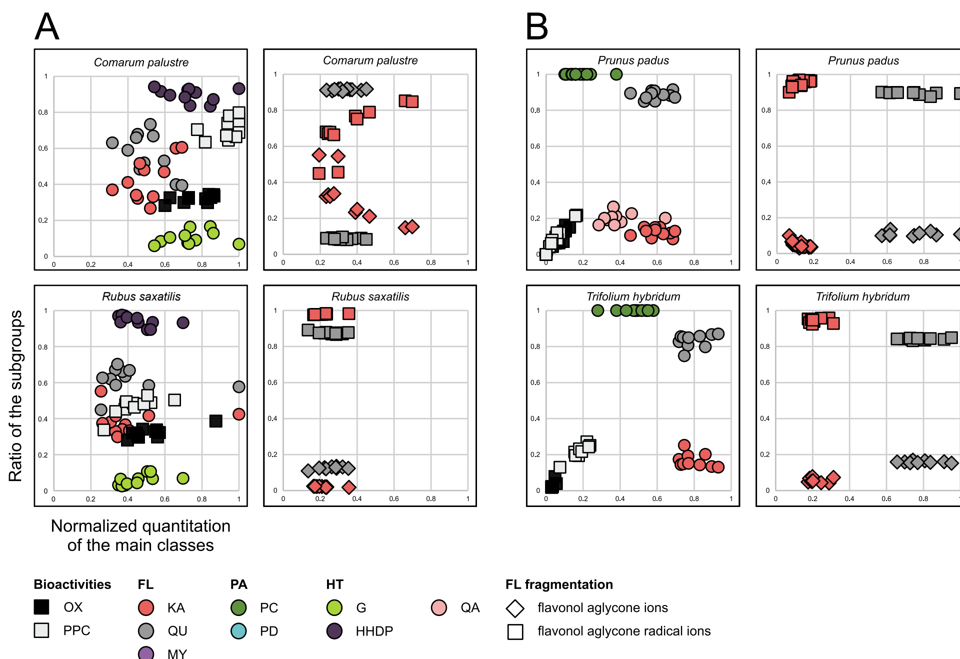


Figure 7. The mass spectrometric fingerprint maps of *Comarum palustre* and *Rubus saxatilis* (**A**) show a close qualitative similarity on the left side. However, their flavonol fingerprint maps on the right show significant differences. In contrast, the fingerprint maps of qualitatively similar *Prunus padus* and *Trifolium hybridum* (**B**) are distinguished mainly by the presence of quinic acid derivatives in *P. padus***.** Abbreviations: OX: oxidative activity, PPC: protein precipitation capacity, FL: flavonol derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PA: proanthocyanidin, PC: procyanidin, PD: prodelphinidin, HT: hydrolysable tannin, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives, QA: quinic acid derivatives. Figure adapted from Article **I**.

Besides the quantitative and qualitative polyphenol profiles, their linkage with bioactivities was able to reveal details about the compounds. The majority of OX levels above intermediate were associated with the presence of HHDP derivatives. For instance, both *Geranium* species demonstrated high activity due to their significantly high HT concentrations (**Figure 8A**), as also reported in previous studies.[90,78,79] In contrast, *Lythrum salicaria* exhibited notably high OX levels despite having lower-than-intermediate HT levels (**Figure 8B**). This indicates the presence of exceptionally active HT types, in this case ellagitannins with acyclic glucose core.[128–130] The other polyphenol groups that increased OX were PD-rich PAs, often found alongside MY derivatives as seen in the fingerprint map of *Ribes alpinum* (**Figure 8C**). Similar to HTs, both of these groups contain a pyrogallol structure, which is characteristic feature of the most easily oxidized phenolics.[40,131] Other phenolic substructure enhancing oxidative activity was the catechol group present for instance in CQAs.[40]

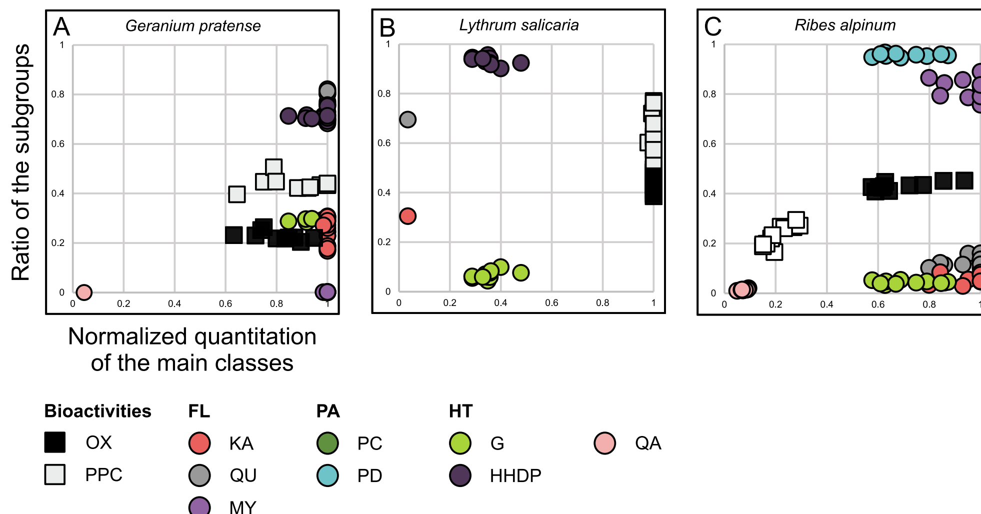


Figure 8. Examples of mass spectrometric fingerprint maps showing high oxidative activity (OX) caused by different compound types. (**A**) *Geranium pratense* exhibits high OX due to a large quantity of moderately active hydrolysable tannins (HT). (**B**) *Lythrum salicaria* shows increased OX with a lower amount of highly active HT compounds. (**C**) In *Ribes alpinum*, both prodelphinidins (PD) and myricetin (MY) derivatives contribute to elevated OX levels. OX: oxidative activity, PPC: protein precipitation capacity, FL: flavonol derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PA: proanthocyanidin, PC: procyanidin, PD: prodelphinidin, HT: hydrolysable tannin, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives, QA: quinic acid derivatives. Figure adapted from Article **I**.

The other bioactivity type, PPC, generally increases with the molecular size and flexibility of the tannin.[132–139] Consequently, the high PPC observed in *Acer platanoides* and *Paeonia lactiflora*, which produce relatively large gallotannins with flexible galloyl and digalloyl groups, was anticipated. These characteristics were detected in pure G derivatives in the fingerprint maps (**Figure 9A & B**). Instead, the highest measured PPC values detected in *L. salicaria* (**Figure 8B** above) derived from dimeric ellagitannins with a flexible linkage type between the ellagitannin monomers and in *C. angustifolium* (**Figure 9C**) from its larger oligomeric ellagitannins.[138,139]

Not only the polyphenols detected with group-specific methods, but also their absence in the presence of either OX or PPC activity was able to enlighten the chemical composition of those plant extracts. For instance, the intermediate oxidative activity detected in *Alnus incana* (**Figure 4** in Article **I**), without the production of HHDP, PD, or MY derivatives, suggests the presence of other functional structures important to OX in compounds that were not visible with the group-specific methods used. These structures could be tentatively characterized

from the full scan MS data, revealing catechol-containing phenylethanoids, with rubranoside A being the main compound.[140]

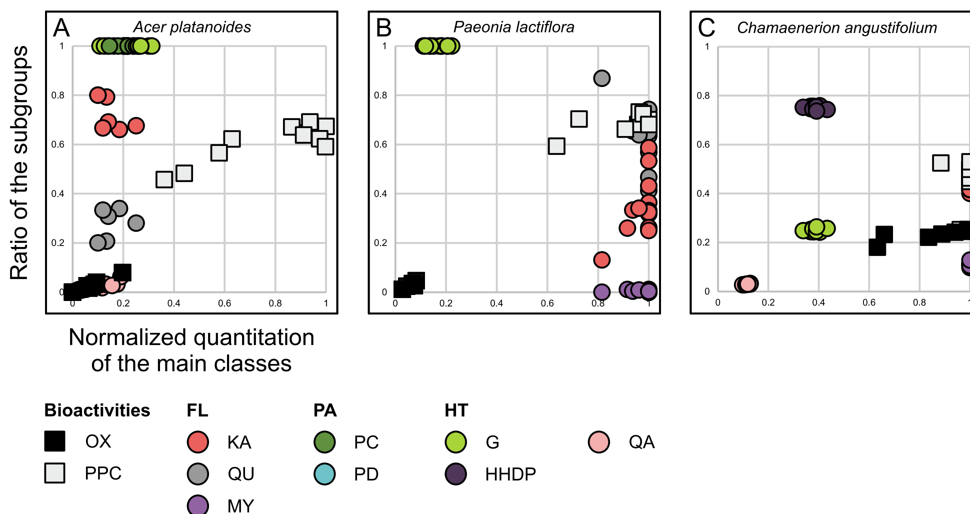


Figure 9. Examples of mass spectrometric fingerprint maps showing high protein precipitation capacity (PPC) caused by different compound types. (**A**) *Acer platanoides* and (**B**) *Paeonia lactiflora* exhibit high PPC due to flexible structure of gallotannins observed as pure galloyl (G) derivatives. (**C**) In *Chamaenerion angustifolium*, oligomeric ellagitannins elevate the PPC. OX: oxidative activity, PPC: protein precipitation capacity, FL: flavonol derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PA: proanthocyanidin, PC: procyanidin, PD: prodelphinidin, HT: hydrolysable tannin, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives, QA: quinic acid derivatives. Figure adapted from Article **I**.

With a limited sampling of flowers, comprehensive conclusions about their chemical variation cannot be drawn. Since flowers may exhibit highly different chemistry and higher quantities of polyphenols, further research is needed to estimate their general variation level.

Overall, the mass spectrometric fingerprint maps demonstrated strong discriminating capability at the species level. Despite some quantitative variations in the species-specific maps, they remained recognizable. This suggests that a pooled sample comprising several individuals from a population effectively represents the species. Consequently, this approach could be applied to larger plant screenings in Article **III**.

3.1.2 Seasonal variation

Given that the plant samples were collected during a relatively stable period in the growing season, the seasonal variations in polyphenol composition were relatively

subtle. However, certain patterns were observed in the data. Species differed in the level of seasonal and yearly variation and the changes were group-specific. The seasonal fingerprint maps are presented in Article **II**.

The most pronounced variation was observed in species producing HHDP derivatives, which exhibited a declining trend in HTs throughout the growing season. In contrast, species producing merely the other HT type, G derivatives, specifically gallotannins, did not display a similar pattern. Instead, the concentration of PAs generally increased towards the end of the growing season. The decreasing trend in PA concentration was rather exceptional, as it was observed in only two species (*P. abies* and *Sorbus aucuparia*) within a single year. FL derivatives expressed mainly decreasing or stable levels across the growing season. An example of decreasing HT and FL levels and increasing PA levels are visible in seasonal fingerprint maps of *Quercus robur* in **Figure 10**.

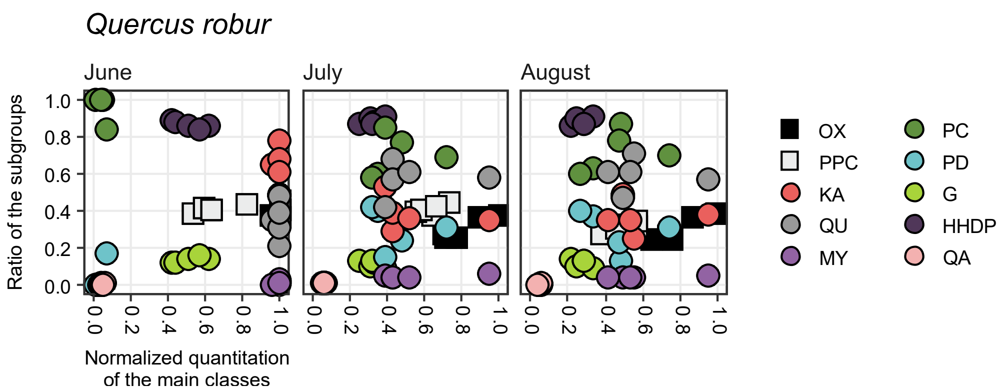


Figure 10. Example of seasonal trends in polyphenol content in *Quercus robur* indicate that HT and FL quantities decrease during the growing season, while PA levels show an upward trend. Simultaneously both bioactivities decreased. OX: oxidative activity, PPC: protein precipitation capacity, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PC: procyanidin, PD: prodelphinidin, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives, QA: quinic acid derivatives. Figure adapted from Article **II**.

The KA/QU/MY ratios remained mainly stable, but in two species (*Q. robur*, *A. platanoides*) the KA/QU ratio shifted from being dominated by KA to being dominated by QU derivatives. Interestingly, FL-fingerprint maps showed the clearest seasonal variation only in the ratios of QU aglycone and aglycone radical ions, whereas the variations in KAs and MYs were less distinct. The most significant changes in QU ratios were observed in *B. pubescens*, *Q. robur*, *A. platanoides*, *A. glutinosa* and *A. incana* (**Figure 2** in Article **II**). Changes in the concentrations of QA derivatives were variable. The trend was decreasing in six species (*P. abies, J. communis, S. aucuparia, P. padus, Betula pubescens* and *A. platanoides*) and slightly

increasing in one (*S. phylicifolia*), while the trends of three species varied between years (*A. incana, A. glutinosa* and *Q. robur*).

These subtle seasonal changes in polyphenol groups also affected their bioactivities. In general, the decreasing HT and increasing PA levels were linked to a clear decrease in OX (**Figure 10**). Conversely, the PD-rich PAs and MY derivatives had an increasing effect on OX. In contrast, PC-rich PAs and pure G derivatives were associated with higher PPC. Furthermore, larger PA polymers also contributed to increased PPC. Seasonal trends in polyphenol groups did not fully explain bioactivity changes in some species, suggesting the presence of undetected bioactive compounds. These observations helped to reveal situations, where a more detailed examination of full scan chromatograms was needed to elucidate the deviations.

Despite the observed seasonal trends, the composition of the polyphenol groups remained consistent, although their quantities and ratios varied slightly. Altogether, the species-specific fingerprint maps remained recognizable regardless of the sampling time or collection year. These results suggest that the timing of plant sampling, whether slightly earlier or later at the stable growing phase, does not significantly affect the outcomes as long as the collection is systematic. The variation among individual plants was often more pronounced than seasonal variation and fell within the species' normal range of plasticity, as noted also in Article **I**.

3.1.3 Variation within plant families

Mass spectrometric fingerprint maps were generated for 311 plant families. Of these, 286 vascular plant families are represented in **Appendix Figure A1**, as no detections were observed in algae and bryophytes. In the family-level maps, each dot represented the average values of a plant species, whereas in the species-specific maps, they represented individual plants. The family-level fingerprint maps exhibited a much larger quantitative variation compared to the species-specific maps and qualitative differences in polyphenol composition were observed within some families. However, since it was not feasible to examine each species individually in detail, these visual maps helped in identifying notable deviations from other families within the same order or deeper clades indicating possible evolutionary adaptation. These intriguing cases could then be subjected to more detailed examination.

Phylogenetic conservatism suggests that closely related plants tend to resemble each other more than randomly selected species, especially at deeper taxonomic levels.[5,141] This was evident as qualitatively similar polyphenol profiles were observed within several clades, for example magnoliids (**Figure 11A**), campanulids (**Figure 11B**) and Myrtales (**Figure 11C**).

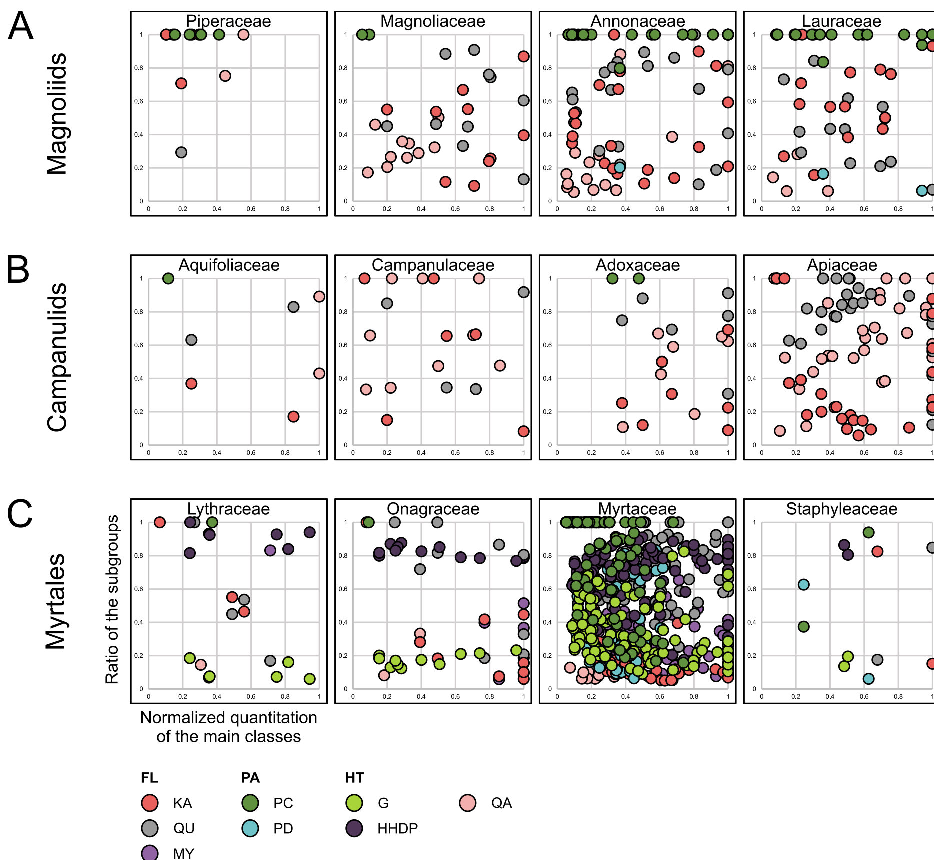


Figure 11. Example clades (**A**–**C**) exhibit highly similar qualitative polyphenol profiles despite significant quantitative variation. Abbreviations: FL: flavonol derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PA: proanthocyanidin, PC: procyanidin, PD: prodelphinidin, HT: hydrolysable tannin, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives, QA: quinic acid derivatives.

In contrast to the examples above, for instance the order Caryophyllales exhibited considerable diversity in the qualitative polyphenol composition of its families. Most families had a relatively simple polyphenol profile, containing only FL derivatives or small amounts of PAs (**Figure 12**). HTs were detected in only a few families. All these families with apparent HTs formed a monophyletic clade within Caryophyllales indicating a unique evolutionary path compared to other branches of the order. These families contained a diverse array of G and HHDP derivatives, including galloylated PCs and PDs, galloylquinic acids and monomeric and oligomeric ellagitannins. Intriguingly, different families within the monophyletic clade exhibited distinct compounds, suggesting either the independent evolution of the necessary biosynthetic pathways within these families or an ancestral capability to produce a wide variety of polyphenols.

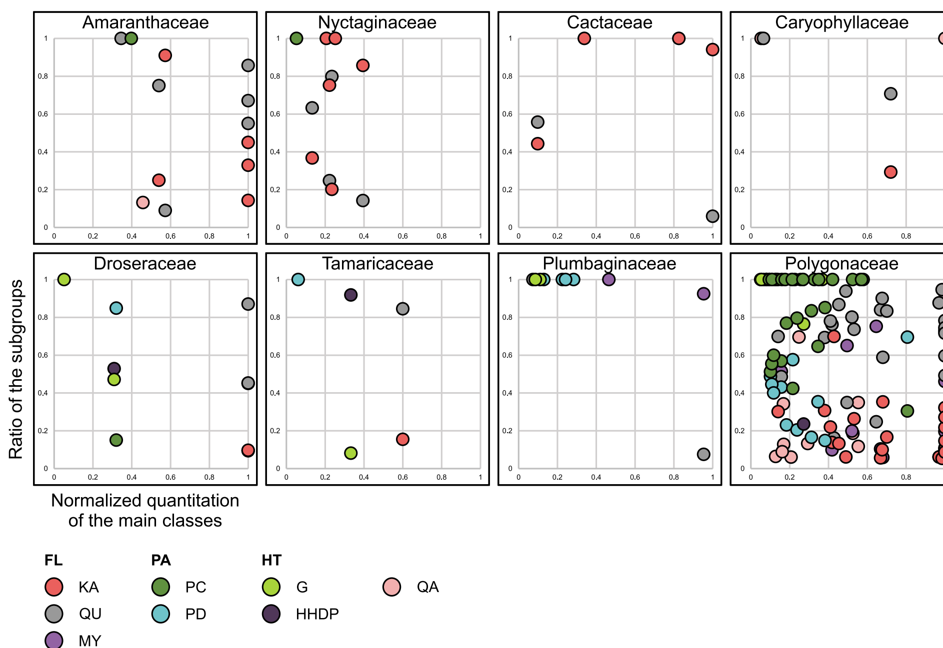


Figure 12. Example families with variable qualitative polyphenol profiles within the order Caryophyllales. The majority of the studied families exhibited relatively similar qualitative polyphenol content (upper row). However, HT derivatives were detected in only a few families (lower row). Abbreviations: FL: flavonol derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PA: proanthocyanidin, PC: procyanidin, PD: prodelphinidin, HT: hydrolysable tannin, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives, QA: quinic acid derivatives.

Particularly within those clades capable of producing HTs, the family-level fingerprint maps often appeared complex. However, a detailed examination could reveal certain internal trends within plant families. These trends were validated by analyzing genus-level fingerprint maps within the families, which could not be included within the scope of this thesis. For example, in Rosaceae family (**Figure 13**) high concentrations of both large-sized tannin groups PAs and HTs are not likely produced simultaneously. When fingerprint maps were looked at genus level it was possible to identify different lineages within the family. The diverse fingerprint map with relatively scattered PA detections of Betulaceae family indicated similar pattern (**Figure 13**). On a larger scale, the fingerprint maps effectively highlighted the evolutionary steps where specific polyphenol subgroups emerged (**Appendix Figure A1**).

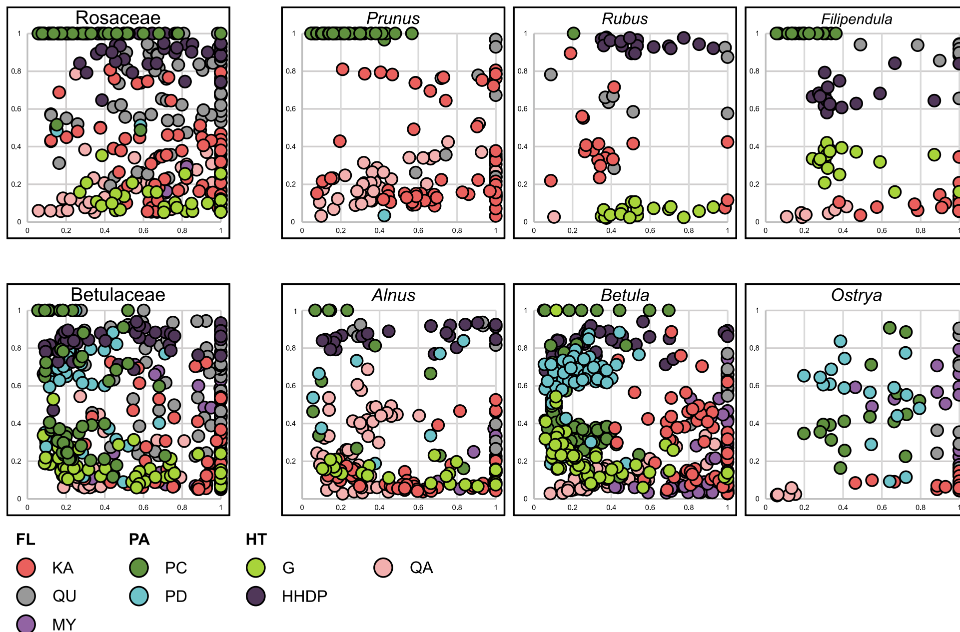


Figure 13. Example families with diverse fingerprint maps, indicating distinct lineages within the family. This was confirmed by examining genus-level fingerprints within the family. Abbreviations: FL: flavonol derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PA: proanthocyanidin, PC: procyanidin, PD: prodelphinidin, HT: hydrolysable tannin, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives, QA: quinic acid derivatives.

3.2 Distribution of foliar plant polyphenols in the plant kingdom

A comprehensive plant library of 3772 plant species from 311 plant families was gathered during the research with an emphasis on vascular plants. This included 246 out of 416 angiosperm families, 11/12 gymnosperm families and 30/51 pteridophyte families.[23,142,143] These families were distributed relatively evenly across the vascular plant phylogeny providing a comprehensive representation of the plant kingdom (**Figure 14**). Overall, the data set covered approximately 1% of the estimated plant diversity.

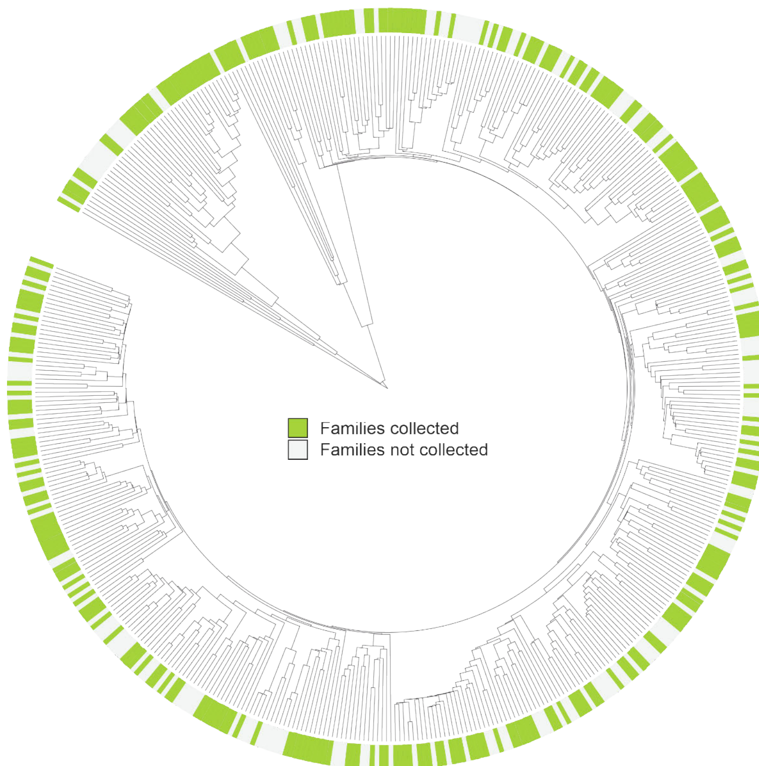


Figure 14. The placement of collected 286 plant families within the vascular plant phylogeny encompassing 477 families demonstrates a relatively even distribution and good coverage across different parts of the phylogeny.

In general, total phenolic levels were relatively low in bryophytes, lycophytes and ferns and were notably low in the monocot clade. In contrast, the total phenolic content was highest in certain parts of superrosids and superasterids, although there was high variability depending on the family (**Figure 3** in Article **III**). Intriguingly, the highest total phenolic quantities and the most diverse polyphenol composition

was detected in the superrosids clade, which is one of the most species-rich group in the plant kingdom.[144] This suggests that a diverse chemical profile may have either significantly influenced plant evolution or resulted from accelerated plant evolutionary processes.

Among the major polyphenol groups analyzed, FL derivatives were most frequently produced by the vascular plants. This was followed by QA derivatives and PAs. HTs were the least common group. Within the main polyphenol groups, the occurrence of subgroups followed biosynthetic pathways. Earlier and simpler counterparts were more commonly detected compared to biosynthetically later compounds. For example, within FLs, the most common subgroup was KA, followed by QU, with MY being the rarest. Similarly, PCs were more common subunits of PAs compared to PDs and Gs were more prevalent than HHDPs. A more detailed distribution and prevalence of each polyphenol subgroup among the plant families can be found in the **Appendix Table A1**.

3.2.1 Quinic acid derivatives

Although QA derivatives are considered nearly ubiquitous in the plant kingdom[28,32,145], significant quantities were detected in only half of the plant families, with their distribution being rather scattered across the phylogeny (**Figure 15**). No significant quantities of QA derivatives were detected in algae, bryophytes and lycophytes, despite previous reports suggest their abundance in these groups.[30–31,146] In contrast, significant quantities of QA derivatives were found in several fern plant families and major clades of angiosperms (**Appendix Figure A1**). Intriguingly, the gymnosperm clade was largely devoid of QA derivatives, yet free QA was detected in the majority of its species.

We detected CQAs, the most common QA derivatives, frequently across various monocot species, which contradicts previous reports suggesting that CQAs are sparse among monocots.[103,147] Another interesting observation was that some clades of the phylogenetic tree, such as campanulids, were almost devoid of PAs and HTs but notably rich in QA derivatives. For instance, the family Asteraceae within campanulids, showed notably high concentrations of QA derivatives, produced by nearly all species in the family (**Appendix Figure A1**). The high quantities of QA derivatives within the campanulids are likely due to the accumulation of complex QA structures, as also noted by Clifford.[146]

The biological activity of QA derivatives highly depends on the linked moieties. For instance, catechol groups in CQAs can undergo enzymatic oxidation and tetra-CQA has been reported to be notably active in inhibiting viral proteases via hydrogen bonding.[131,148] These bioactivities may have been significant evolutionary factors contributing to the high CQA concentrations observed in campanulids. CQAs also

play roles in lignin biosynthesis, defense against pests and microbes and protection against abiotic stress, such as UV radiation, highlighting their importance from the early stage of the plant evolution.[32,149,150] Furthermore, the alternative biosynthetic pathways for CQA production in different plant lineages may influence their distribution across taxa.[28] Our results indicate a widespread capability to produce QA and its derivatives throughout the plant kingdom, while the biosynthesis could have been affected by several possible factors in different plant lineages.

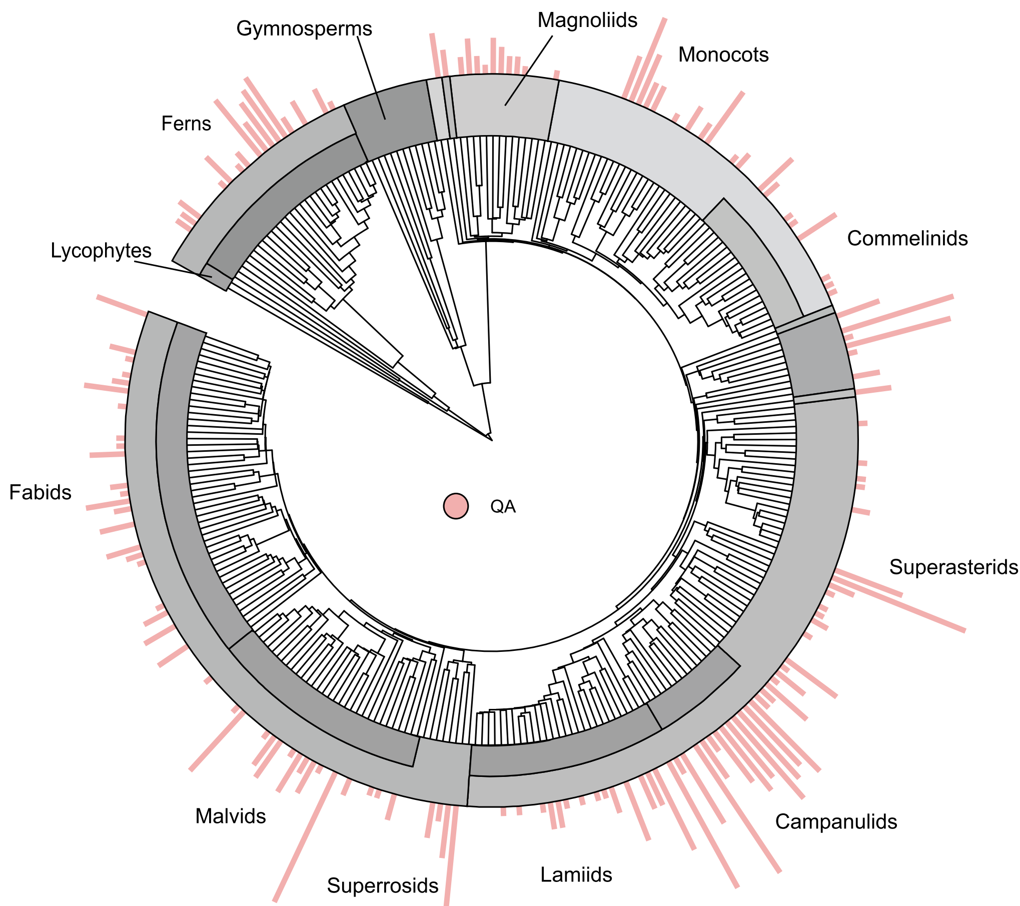


Figure 15. Phylogenetic tree of vascular plant families illustrating the distribution of quinic acid (QA) derivatives. The distribution is wide but uneven with some blank clades and notably QA-rich clades. Figure adapted from Article **III**.

3.2.2 Flavonol derivatives

FLs were the most common and evenly spread group of polyphenols found in land plants (**Figure 16**). This verifies that the development of these compounds originated

in relatively early phase of the plant evolution and they have provided evolutionary advantages throughout history. Previous research indicates that the flavonoid biosynthetic pathway is exclusive to terrestrial plants.[37,151] This pathway is believed to have evolved in the ancestors of bryophytes, serving a crucial role in protecting against UV radiation.[37,151] In accordance to this, no FL derivatives were detected in the algae within our dataset. Interestingly, FL derivatives were also absent in bryophytes, even though they possess the capability for flavonoid biosynthesis, as some species are producing KA and QU glycosides.[31,152]

Both KA and QU derivatives were prevalent in pteridophytes including lycophytes and ferns. However, while the least hydroxylated form of flavonols, KA derivatives, dominate in these clades, QU derivatives were dominant forms in the majority of other clades (**Appendix Figure A1**). The predominance of KA derivatives in pteridophytes may reflect the typical habitat of extant ferns in shaded forest understories with low UV exposure. This environment can reduce the selective pressure for more effective photoprotectants, such as QU derivatives.[153] The emergence of MY derivatives is known to have occurred in the ancestors of ferns and seed plants.[13,37] However, MY derivatives were not detected in ferns, but this absence may be due to gene loss rather than a lack of biosynthetic capability.[13,37,154] This is supported by the presence of trihydroxy-substituted PDs in certain families, indicating that they possess the enzymatic capacity for more complex flavonoid biosynthesis.

Gymnosperms exhibited a dominance of QU derivatives over KAs, likely reflecting an evolutionary response to higher sunlight exposure. They were also found to contain MY derivatives, with their evolutionary emergence coinciding with the second major pulse of arthropod herbivory during the Late Jurassic to Early Cretaceous period. The more oxidatively active MY derivatives[37,75,131] may have played a role in plant defense during this time, contributing to the diversification and ecological success of gymnosperms. The relatively high concentration of MY derivatives in Nymphaeaceae, as reported in previous studies[155], may reflect the high UV stress experienced by species inhabiting open waters, similar to gymnosperms in open land habitats. Interestingly, MY derivatives were entirely absent in magnoliids and relatively rare in certain clades, such as monocots, campanulids, and lamiids. In contrast, MY prevalence was significantly higher in superrosids, suggesting a complex evolutionary trajectory influenced by adaptive pressures across different plant lineages.

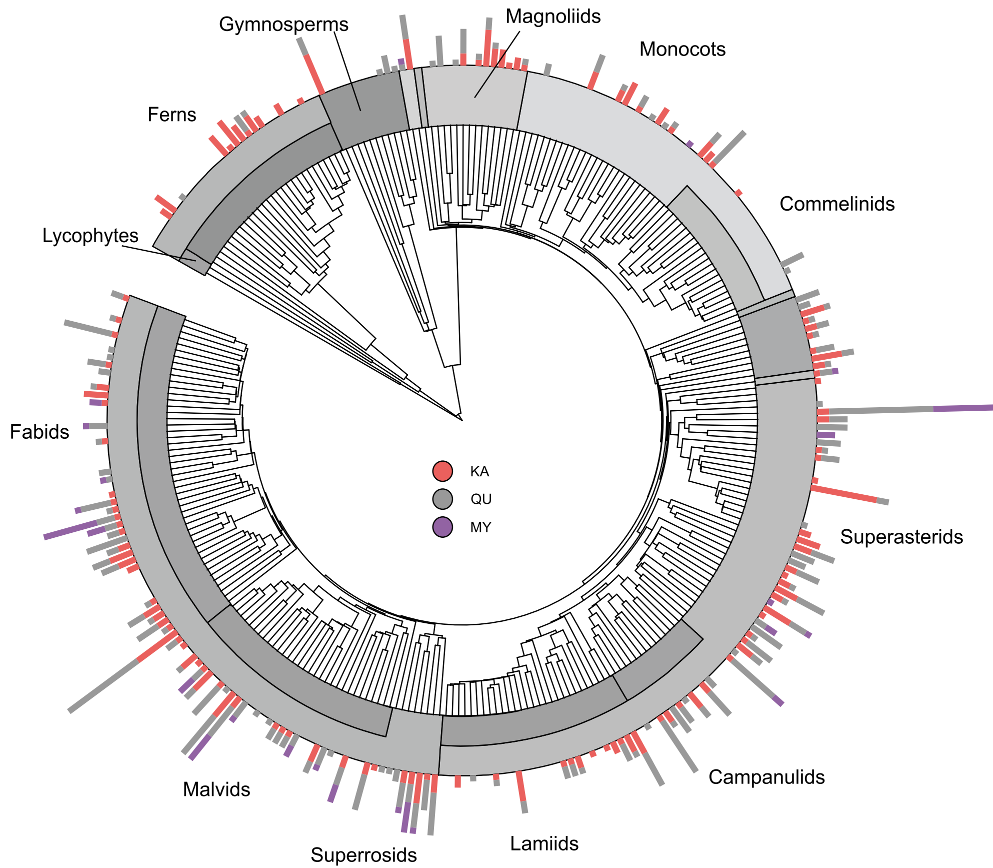


Figure 16. The distribution of flavonol derivatives indicates that kaempferol (KA) and quercetin (QU) derivatives are prevalent throughout the family-level vascular plant tree, while myricetin (MY) derivatives show a more limited and scattered distribution. Figure adapted from Article **III**.

3.2.3 Proanthocyanidins

The capability to produce both PC and PD type PAs emerged simultaneously following the divergence of the lycophytes clade.[13,16,17,37,151] PAs were observed throughout vascular plants, with fewer occurrences in monocots, Caryophyllales, campanulids and lamiids within superasterids and Brassicales within malvids (**Figure 17**, **Appendix Figure A1**). The major clade richest in PAs was the superrosids, particularly the fabids clade. Species producing only PCs were the most prevalent across the phylogenetic tree, followed by those producing PC/PD mixtures. Pure PDs were the rarest throughout the plant kingdom.

Among ferns, PC units were generally more prevalent than PDs. PA content was significantly higher in Polypodiales compared to other orders. Additionally, PDs were exclusively detected within the Polypodiales order, as reported also in previous studies.[156–158] This may indicate a unique evolutionary adaptation specific to this order.

As the first seed plants, gymnosperms likely provided high-quality food for early tetrapod herbivores and omnivores.[159–161] The emergence of tetrapods significantly impacted plant consumption, leading to competition among plants and subsequent rapid diversification.[162] This made PA-based defenses particularly valuable, with both PA subunits detected in the gymnosperm clade. The increased herbivory likely influenced the diversity of PA-based defenses in seed plants.

Certain families within the Caryophyllales order within superasterids completely lacked PAs. This absence aligns with families that also lack anthocyanins in the flavonoid pathway[63,163,164], likely due to shared biosynthetic enzymes and intermediates[165–167]. Among the PA-rich superrosids, the Brassicales order notably lacked PAs, likely because they rely on glucosinolates as alternative defense compounds.[168]

PA oligomers and polymers, with their diverse structural variations, offer a broad spectrum of bioactivities. Key structural features, such as high degree of polymerization, A-type bonding, galloylation and high PD content, enhance their protein-precipitation capacity.[132,135,169,170] This is particularly effective against mammalian herbivores, whereas the alkaline gut pH of many caterpillars may reduce this effect. Interestingly, PA biosynthesis likely evolved before tetrapod animals, indicating their initial roles in other functions, such as defense against pathogens and insect herbivores, mitigating abiotic stress and interacting with soil and microbial communities.[171–181] Especially, a high PD/PC ratio enhances oxidative activity in alkaline conditions indicating the significance against certain insect herbivores. Additionally, the data revealed an intriguing positive correlation between high PD/PC ratios and galloylation, suggesting a potential functional significance for bioactivity.

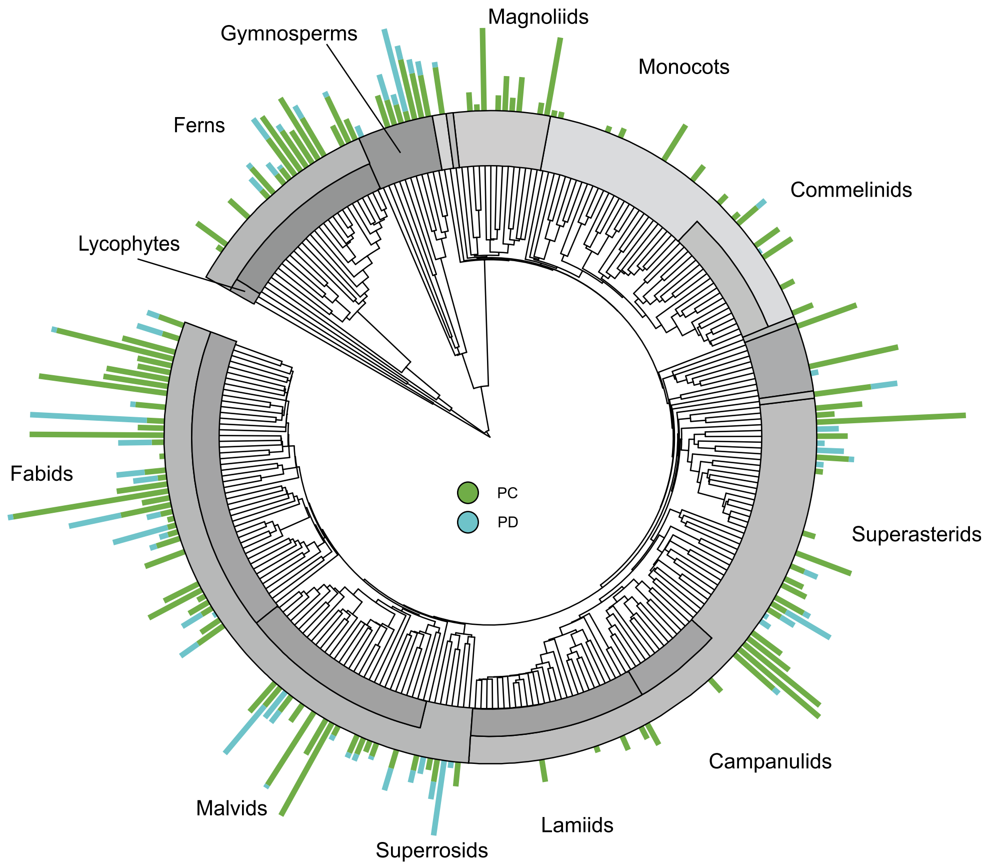


Figure 17. The distribution of proanthocyanidins shows that procyanidins (PC) are more prevalent than prodelphinidins (PD) and their mixtures across 286 vascular plant families. Figure adapted from Article **III**.

3.2.4 Hydrolysable tannins

In line with previous research, hydrolysable tannins were the most constrained polyphenol group in our study (**Figure 18**).[39,40,182] The family Nymphaeaceae (Nymphaeales) stood out due to the notable quantities of hydrolysable tannins detected, aligning with historical research.[101,104] The ability to produce HTs is likely a result of convergent evolution, as no species outside the superrosid and superasterid clades appear capable of producing them. Besides the unique chemistry of Nymphaeales, its other features also underscore its distinct evolutionary trajectory.[183–185] HT production is likely an adaptation to aquatic ecosystems, where high HT contents may play an important role in defense against herbivory and microbes.[186]

It was anticipated that the clade Pentapetalae (**Figure 18**), which includes Dilleniales, superasterids and superrosids, would exhibit an ancestral capacity for HT biosynthesis, as suggested by the presence of gallic and ellagic acid derivatives in these clades.[39,40,182,187–190] However, our results indicate that HTs were even more restricted to specific superasterid and superrosid families. In the Dilleniales order, the detected gallic acid derivatives were primarily (epi)catechin gallates and (epi)gallocatechin gallates, rather than HTs *per se*. The biosynthesis from Gs does not seem to progress to larger galloylglucoses, gallotannins, or ellagitannins. This raises the question of whether HT production was lost in this lineage or never fully evolved. This is particularly intriguing given the controversial classification of Dilleniales within superasterids and superrosids.[23,191,192]

Overall, HT production was rather patchy within superrosids and superasterids, being most consistent in the superrosids clade. All lineages within these two clades seem to retain a tentative ancestral ability to synthesize both G and HHDP derivatives. However, certain lineages within these groups differed significantly. Within superasterids, campanulids and lamiids were nearly devoid of HTs, whereas malvids and fabids within superrosids exhibited high HT concentrations, with HHDP groups being dominant over G groups.

While the group-specific methods can not disclose every structural detail, the ratio of G to HHDP provided some insight into the family-specific HT content. Pure G detections, derived from specific gallotannins, were primarily produced within certain plant orders and families within the superrosids. Species from the families Paeoniaceae, Crassulaceae and Hamamelidaceae within the order Saxifragales, as well as species from the families Anacardiaceae, Sapindaceae, Simaroubaceae and Burseraceae within the order Sapindales, produced gallotannin-type HTs. These can be observed as pure G detections in the fingerprint maps, as well as the galloylated PAs (**Appendix Figure A1**).

No families consisted solely of HHDP derivatives, given that ellagitannins are synthetised from galloylglucoses and often contain G moieties besides HHDP

group(s). The lowest G/HHDP ratios were observed in the families Loranthaceae, Francoaceae, Lythraceae, Tamaricaceae, Elaeagnaceae, Corynocarpaceae, Casuarinaceae and Coriariaceae. Many species of these families exhibit a unique ellagitannin content often containing compounds with C-glycosidic structures that generally have fewer free G moieties.[128,130,193–196] This is due to the presence of the NHTP (nonahydroxytriphenoyl) group, which consists of three G groups bound together via two C–C linkages. In general, the change in the focus of biosynthesis from free G to HHDP or NHTP derivatives implies a change in defence mechanisms from protein-precipitation to oxidative activity, enhancing defence against herbivores with high gut pH. The diversity in HT composition among plant lineages suggests adaptations that balance oxidative and protein-precipitating properties to counter specific herbivore pressures. However, it must be noted that a compound may express both types of bioactivities and the detailed structure and chemical environment determine the final activity.[77,138,197,198]

The evolutionary diversification of HTs likely began within the Pentapetalae clade around 109–115 million years ago.[199,200] After an evolutionary short period (100–50 million years ago), angiosperms expanded alongside key herbivorous and pollinator insect groups.[22,200] Several species especially within Coleoptera and Lepidoptera evolved highly alkaline gut conditions, which enhanced the significance of oxidatively active HTs as defensive compounds.[201] HTs and their oxidative activity seem to have been one important driver of plant-herbivore interactions, as seen in extant communities.[202] However, further research is needed to validate the impact of HTs on these interactions and their role in diversification.

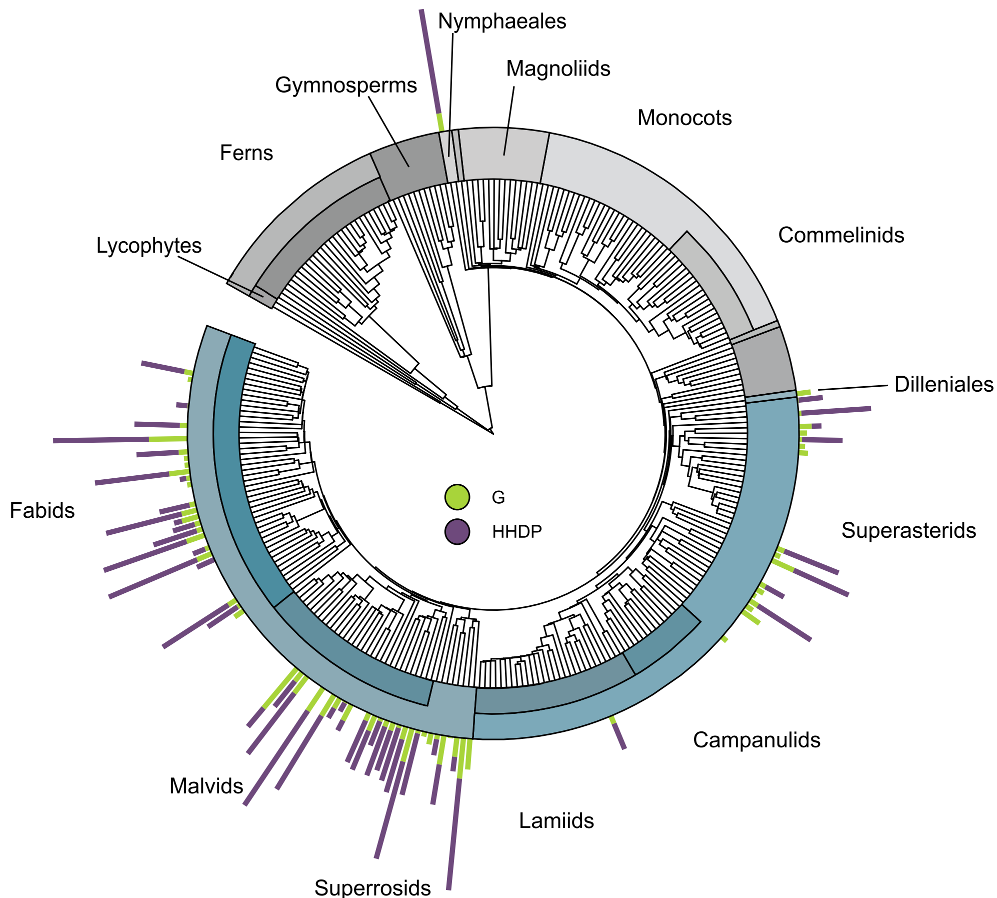


Figure 18. The occurrence of hydrolysable tannins (HTs) is restricted to the Pentapetalae clade (highlighted with blue), excluding the Dilleniales order with galloylated proanthocyanidins. The high quantities of HTs detected in Nymphaeales are likely a result of convergent evolution. Abbreviations: G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives. Figure adapted from Article **III**.

3.2.5 Co-occurrence of chemical defences

It was observed that some clades possessing other defense chemical groups, such as glucosinolates in Brassicales[203,204] were notably deficient in polyphenol defenses. Similar negative trade-offs between SM classes have been reported earlier indicating a possible resource allocation towards specific types of defenses, but contrasting results have also been reported.[52–54] This aspect, however, was not examined in detail in this study but provides an interesting possibility for future research of trade-offs of different defense types.

Chemical traits often play complementary roles in plant ecology.[53,58] Polyphenols, in particular, contribute to protection against abiotic stress and natural enemies, as well as to signalling purposes and attracting pollinators.[205] The co-occurrence of different subgroups can though be influenced by various factors. Previous studies have reported negative correlations within polyphenol groups[54,55], suggesting either competition for substrates or divergent investment in chemical defenses by closely related species.[52,55]

However, when phylogenetic relationships were considered in our data, PGLS correlations among polyphenol subgroups predominantly showed significant positive correlations **(Figure 19)**. These subgroups include those derived from the same compounds, such as HHDPs and Gs from ellagitannins and PD and G from galloylated PDs. Additionally, positive correlations were detected between subgroups produced through similar hydroxylation processes, such as PC and QU derivatives and PD and MY derivatives, as well as steps in metabolic pathways, for instance, from KA to QU derivatives. These patterns primarily indicate positive connections between biosynthetic pathways, with no evidence of internal competition. Our comprehensive view of the plant kingdom suggests that the distribution of polyphenols has been shaped by the gradual diversification of biosynthetic pathways and the emergence of positive correlations between these pathways over evolutionary time.

According to our results the polyphenol profiles of plants were closely linked to their evolutionary history. Specifically, nearly half of the variation in these profiles can be attributed to a small portion of the phylogenetic axes or the identity of the plant family. This indicates that the distribution of polyphenols is mainly influenced by ancient evolutionary branches rather than more recent evolutionary relationships. The distribution at a deeper level supports the view that plant diversification has been influenced by major evolutionary events, as the emergence of novel polyphenol subgroups roughly coincides with transitions to new environments and presumably with the diversification of key herbivores.[14,206]

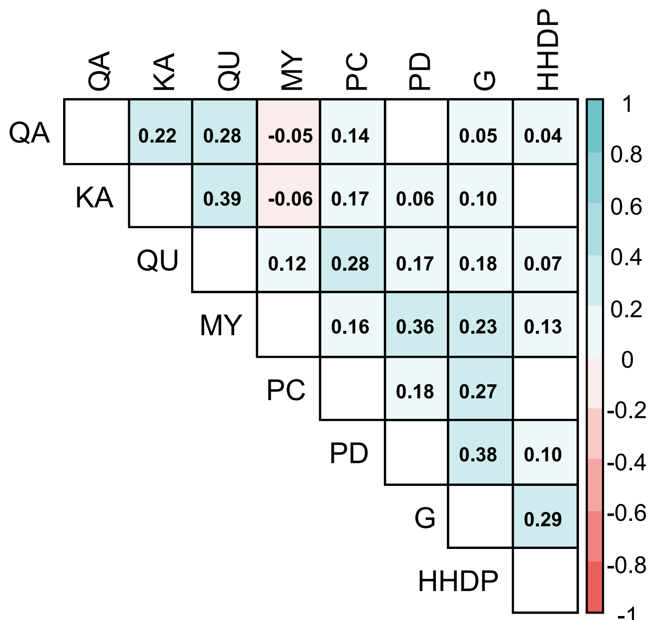


Figure 19. Correlation plot based on pair-wise PGLS correlations between the eight polyphenol subgroups. Only significant ($p<0.05$) correlation coefficients reported. Abbreviations: QA: quinic acid derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PC: procyanidins, PD: prodelphinidins, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives. Figure adapted from Article **III**.

However, the more recently developed phylogenetic levels near the species level may significantly contrast with the polyphenol correlations and distribution observed at deeper levels. This suggests that ecological factors likely influence metabolite correlations at the species level.[52,54] Our findings also indicate that polyphenols frequently undergo losses or convergent evolution across different lineages as seen in previous studies.[96,207]

Summary

This thesis provides a comprehensive overview of the distribution of major foliar polyphenol groups in the plant kingdom (Article **III**) and more detailed view of the species-specific variation (Article **I**) and seasonal changes (Article **II**) of different polyphenol types.

A newly developed mass spectrometric fingerprint mapping tool was used to study species-specific differences of eight common polyphenol groups and related bioactivities (Article **I**) and their seasonal changes (Article **II**). The visual tool demonstrated strong capability to discriminate plant species based on the quantitative and qualitative patterns of the polyphenol groups and bioactivities (Article **I**). Detailed flavonol fingerprint maps provided more discriminating factors and insights into compound structure. Since the quantitative data was normalized using data from thousands of plant species, the results could be compared with global patterns. The linkage between polyphenol groups and bioactivities aligned with existing knowledge about the enhancing effects of certain polyphenol structures. Interestingly, the observed outliers in these linkages revealed exceptional compound types responsible for these activities.

Depending on the plant species, slight changes in the polyphenol content were observed even on the seemingly steady growing phase (Article **II**). The changes were strongest with HHDP-producing species and they were quantitative rather than qualitative. In general, the FL and HT content expressed decreasing trend, while PA content increased during the growing season. However, despite the seasonal fluctuations and interannual variations, the species-specific fingerprint maps remained identifiable, with the observed changes reflecting natural plasticity.

Beyond their initial purpose, the fingerprint maps have potential for a variety of other applications. They can be applied at various taxonomic levels, as demonstrated in Articles **I** and **II** at the species level and in this thesis at the family level. They are also applicable to different compound groups and their subunits. The fingerprint maps can be utilized to monitor for example the effects of changing conditions, such as those resulting from climate change or biological treatments. Overall, the findings from Articles **I** and **II** indicate that plant sampling can be performed with considerable flexibility during the relatively stable growing stage. Additionally, a

pooled sample of a plant population generally provides a good representation of the species. Based on this information, the collection method described in Article **III** can be considered sufficient to accurately describe the plant species.

Thousands of plant species were screened for their foliar polyphenol composition and linked with the information of the evolutionary history. Such a comprehensive study has not been conducted before using modern and consistent biological and chemical methods. Numerous factors have influenced the chemical profile of plants throughout evolution and some of these were tentatively identified in extant plants.

The distribution of polyphenol subgroups appears to be phylogenetically conserved at deep evolutionary levels likely reflecting the important steps of plant evolution, such as the adaptation to terrestrial environments and higher UV radiation. Additionally, there are indications in polyphenol profiles that suggest the coevolution of plants and various herbivores, particularly in the simultaneous emergence of oxidatively active hydrolysable tannins and the proliferation of several insect herbivore groups.

Generally, early-developed polyphenol groups were broadly distributed, with biosynthetically simpler compounds detected widely across the phylogeny. In contrast, biosynthetically more complex counterparts were more restricted. Hydrolysable tannins, in particular, displayed a more limited distribution than previously assumed based on existing knowledge. Potential negative trade-offs were detected between polyphenols and other SM groups, but no significant negative correlations were found among polyphenol subgroups. The observed positive correlations between certain subgroups were attributed to their biosynthesis, shared enzymes, or derivation from the same compounds indicating no evidence for competition between pathways and produced metabolites.

The findings of this thesis significantly supplement the existing knowledge regarding the distribution of major polyphenol groups across the entire plant kingdom. Additionally, it introduces a novel visual tool that is applicable for both extensive screening experiments and precise monitoring tests. These results establish a basis for research across various fields concerning polyphenols and their applications.

However, despite the notable extent of this research, it merely scratches the surface of the broader picture of the chemical defenses. This study rather lays the groundwork for further exploration into various aspects such as the evolutionary processes, plant-herbivore interactions, chemical evolution of other plant organs, impacts of climate change, defense mechanisms against emerging threats, correlation with other defense mechanisms, economic significance of specific species, potential medicinal uses, discovery of new bioactive compounds and the chemical and taxonomical dimensions of biodiversity loss. I sincerely hope that the multidisciplinary nature of this research will advance future studies in both biological and chemical fields.

Appendix

Table A1. Detected polyphenol groups and their average quantities in studied vascular plant families. The quantities of each polyphenol group are normalized according to group-specific values. Categories are consistent with those presented in the mass spectrometric fingerprint maps in Articles I and II: <0.05: insignificant, 0.05–0.2: *, 0.2–0.4: **, 0.4–0.6: ***, 0.6–0.8: **** and >0.8: *****. Abbreviations used: QA: quinic acid derivatives, KA: kaempferol derivatives, QU: quercetin derivatives, MY: myricetin derivatives, PC: procyanidins, PD: prodelphinidins, G: galloyl derivatives, HHDP: hexahydroxydiphenoyl derivatives.

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Selaginellaceae		*						
Lycopodiaceae								
Marattiaceae								
Psilotaceae								
Equisetaceae	*	**	*		*			
Osmundaceae	*	*****	*		**			
Salviniaceae	**							
Marsileaceae			*					
Dicksoniaceae		*			**			
Cyatheaceae	*	*						
Metaxyaceae								
Cibotiaceae								
Lindsaeaceae	**							
Saccolomataceae								
Pteridaceae	*	*	*		*	**		
Dennstaedtiaceae	*	****	*		**	*		
Aspleniaceae		*	*					
Cystopteridaceae	****	*****	*		*	*		

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Thelypteridaceae	**	***			*	*		
Woodsiaceae	***	**	**		**	**		
Athyriaceae	***	***	*		***			
Onocleaceae	****	***	*		**	*		
Blechnaceae	*	*			**			
Hypodematiaceae					***			
Dryopteridaceae	**	**			**	*		
Lomariopsidaceae								
Nephrolepidaceae								
Polypodiaceae	**	**	*		*			
Davalliaceae	*				**	*		
Tectariaceae		*			*			
Ginkgoaceae		*****	***	*		*		
Cycadaceae								
Zamiaceae					**			
Gnetaceae								
Ephedraceae								
Pinaceae		*	*	*	**	**		
Araucariaceae			*		*	*		
Podocarpaceae					*	*****		
Sciadopityaceae			*		***	*		
Taxaceae		*	***		**	*		
Cupressaceae		*	**	*	**	*		
Nymphaeaceae			*	**			***	*****
Schisandraceae	**	*****	***		**	*		
Chloranthaceae	**							
Canellaceae								
Aristolochiaceae	*	*	**					
Saururaceae	**	*	****		*			
Piperaceae	*				*			

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Myristicaceae	*	*	*		****			
Magnoliaceae	**	***	***					
Eupomatiaceae	**	*	*		*			
Annonaceae	*	**	**		**			
Calycanthaceae	*	*****	**		*			
Siparunaceae	*	***	**		**			
Atherospermataceae		***						
Hernandiaceae		**	*					
Monimiaceae		***			*			
Lauraceae	*	**	*		****			
Acoraceae			*		*			
Araceae		*			*			
Tofieldiaceae		*	**					
Juncaginaceae								
Potamogetonaceae		*						
Alismataceae								
Butomaceae								
Hydrocharitaceae								
Dioscoreaceae	**	***	**		*			
Velloziaceae	****		*					
Cyclanthaceae	***				*			
Pandanaceae	*							
Liliaceae	*	**	**					
Philesiaceae								
Smilacaceae	**	*	*		**			
Melanthiaceae	**	***	*					
Alstroemeriaceae		****						
Colchicaceae		*						
Orchidaceae		****						
Asteliaceae	*	*	**					

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Hypoxidaceae	***	**	*					
Tecophilaeaceae								
Doryanthaceae					*			
Iridaceae		*	*	**				
Asphodelaceae	*	*	*					
Amaryllidaceae	*	****	**					
Asparagaceae		**	*					
Typhaceae		*	*****		*			
Bromeliaceae								
Juncaceae	***	*	*					
Cyperaceae	*		*		*			
Restionaceae					**	*		
Flagellariaceae		**						
Joinvilleaceae		*						
Poaceae	*							
Arecaceae	*				**			
Commelinaceae						*		
Philydraceae	**		*		**			
Pontederiaceae								
Haemodoraceae								
Musaceae								
Heliconiaceae		*	*					
Strelitziaceae		*	*		*			
Costaceae	*	*	*					
Zingiberaceae					*			
Cannaceae	*	*	***					
Marantaceae	*		*					
Ceratophyllaceae								
Eupteleaceae	**	*	***		***			
Papaveraceae		*	**					

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Lardizabalaceae	*****	****	**					
Menispermaceae	*	**	*					
Berberidaceae	*****	***	**					
Ranunculaceae	*	**	**					
Sabiaceae		*	*		****			
Proteaceae		**	**	*		*		
Trochodendraceae	**	*****	**					
Buxaceae		***	*					
Dilleniaceae	**	**	**	**	***	**	**	
Olacaceae		**					*	**
Balanophoraceae					**			
Loranthaceae			*		*		*	***
Berberidopsidaceae	*	*	**		*****			
Droseraceae		***	*****	*****		**	**	*
Nepenthaceae		**	**		**		**	
Tamaricaceae		*	***			*	*	**
Plumbaginaceae				***		**	*	
Polygonaceae	*	*	***	*	**	*	**	
Simmondsiaceae		**	*			*		
Amaranthaceae	*	**	**		*			
Caryophyllaceae	*							
Aizoaceae		*						
Nyctaginaceae		**	*					
Phytolaccaceae	*	*****	**					
Petiveriaceae								
Montiaceae								
Basellaceae								
Didiereaceae								
Portulacaceae			*		*			
Anacampserotaceae		***						

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Cactaceae		****						
Hydrangeaceae	****	***	***		***			
Nyssaceae	*****	*	***				**	***
Grubbiaceae	**		**		*	*	*	
Cornaceae	*	***	***				****	***
Marcgraviaceae		*			*			
Balsaminaceae	*	**	*		**			
Diapensiaceae			****				***	
Styracaceae	***	**	*		**			
Clethraceae	*		*		*****			
Ericaceae	***	*	*****	**	***	*	*	
Sarraceniaceae	*	**	****		***			
Actinidiaceae	**	****	**		****			
Polemoniaceae	*	*****	****					
Theaceae	*	***	**		**	*	*	*
Sapotaceae		*	*	**	*	****	**	
Primulaceae	*	*****	**	**	*	**	*	
Lecythidaceae		**	*			*	**	***
Ebenaceae		*	**	***		*	**	
Helwingiaceae	*****							
Aquifoliaceae	****	*	**					
Cardiopteridaceae	*							
Stemonuraceae	*							
Escalloniaceae	*	*	****					
Campanulaceae	***	**	**					
Menyanthaceae	*****	**	*****					
Goodeniaceae								
Asteraceae	****	*	*					
Adoxaceae	****	***	****		*			
Caprifoliaceae	*****	*	*					

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Pittosporaceae	****	****	**					
Araliaceae	****	*	**					
Apiaceae	***	**	**					
Garryaceae	*	*	*					
Eucommiaceae	*****	****	****		*			
Metteniusaceae		***	*		*			
Convolvulaceae	****	**	***					
Solanaceae	**	***	*					
Rubiaceae	**	**	*		*			
Gentianaceae								
Apocynaceae	**	**	*		*			
Gelsemiaceae							**	**
Loganiaceae	***	*	*	*				
Boraginaceae	*	**	**					
Oleaceae	*	**	**					
Calceolariaceae								
Gesneriaceae	*							
Plantaginaceae		*						
Scrophulariaceae	*	*	*					
Linderniaceae	**	*						
Stilbaceae	**	*****	**		*			
Lamiaceae	*	*	*					
Phrymaceae								
Orobanchaceae		*	*					
Verbenaceae	*	*						
Pedaliaceae		*						
Bignoniaceae	*	*	*					
Martyniaceae		*						
Lentibulariaceae		***						
Thomandersiaceae								

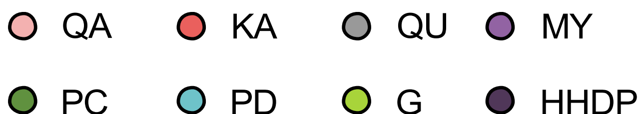
FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Acanthaceae								
Paeoniaceae		***	*****				****	
Cercidiphyllaceae	*****	**	****		*		*****	*****
Hamamelidaceae	***	*****	***	**		*	***	*
Haloragaceae		*	**		*		*	*
Crassulaceae		*	*	*		**	**	
Grossulariaceae	*	**	***	*****		*****	*	
Saxifragaceae	**	**	***	*	*	**	****	**
Vitaceae		*	*	*	*	*	*	
Francoaceae		**	*				*	***
Geraniaceae	**	***	****				*****	*****
Combretaceae			*	*	*	**	**	***
Lythraceae	*	*	*	*			*	***
Onagraceae	*	**	***	***			**	**
Melastomataceae		*	*		*	*	*	***
Vochysiaceae		*	**		*		*	**
Myrtaceae	*	*	***	**	*	*	***	**
Staphyleaceae		****	**		*	*	**	**
Picramniaceae	*****							
Nitrariaceae								
Anacardiaceae	*	*	**	**	*	*	***	*
Burseraceae	**	**	**		*****		*	*
Sapindaceae	*	**	**		**		**	*
Kirkiaceae			*	*			****	*****
Rutaceae	**	*	*		*			
Simaroubaceae	**	**	**				****	****
Meliaceae	*	*	*		****	*		
Thymelaeaceae	**	*						
Muntingiaceae		*****	*****	*	*	****	*****	**
Malvaceae		*	*		**			

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Bixaceae	*	*	**	**	*	*	***	****
Cistaceae		**	****	*****		***	**	**
Tropaeolaceae	****	**	****					
Moringaceae	*		*					
Caricaceae		****	**					
Limnanthaceae		**	**	****				
Salvadoraceae								
Resedaceae		**						
Brassicaceae		***	**					
Capparaceae	*		*					
Cleomaceae		**	*					
Zygophyllaceae		**	**					
Polygalaceae		*****	*****					
Fabaceae		**	*	*	**	**	**	
Rosaceae	**	**	***		*		*	**
Elaeagnaceae		***	*			*	**	****
Rhamnaceae		***	***		*	**		
Ulmaceae	**	*	*		*			
Cannabaceae		*			*			
Urticaceae	**		*		***			
Moraceae	*	*	*		**			
Cucurbitaceae		*						
Datiscaceae		****	***				*	*
Begoniaceae		**	**		**			
Corynocarpaceae		**						*
Coriariaceae		***	**				***	****
Nothofagaceae	*	*	*****	*			***	****
Fagaceae	*	**	**	*	*	*	*	**
Juglandaceae	**	**	**	****	*	*	*	*
Myricaceae		**	**	*****	*	****	***	*

FAMILY	QA	KA	QU	MY	PC	PD	G	HHDP
Casuarinaceae		**	*		*		***	****
Betulaceae	**	**	****	**	*	*	*	**
Celastraceae		**	*		**	****		
Cunoniaceae	*	*	**		**		*	*
Elaeocarpaceae	*		*	*	*	**	***	****
Oxalidaceae	*				**			
Connaraceae	**	*	**	**	*****	*	*	
Humiriaceae					*	**	*	
Peraceae							*	
Euphorbiaceae		**	*		*		**	**
Linaceae	**							
Caryocaraceae	*		***	**	*	***	*****	****
Ochnaceae	*				*****			
Putranjivaceae			*		**		**	**
Chrysobalanaceae		**	*	**	*	*****		
Picrodendraceae		*****	*					
Phyllanthaceae	*	**	*		**	*	*	*
Pandaceae								
Erythroxylaceae	***		**		*****			
Rhizophoraceae	*	**	**	*	***			
Malpighiaceae	*	*	*		***		*	
Calophyllaceae			*		**		**	**
Clusiaceae	*	*			**			
Hypericaceae	**	**	*****		*****	*		
Achariaceae		*	*		***			
Violaceae		**	*					
Passifloraceae					*	**		
Lacistemataceae		*						
Salicaceae	***	**	**	*	**	*		

Figure A1. Mass spectrometric fingerprint maps of 286 vascular plant families arranged in phylogenetic order according to phylogenetic trees in Article III. The family-level fingerprint maps consist of the results of each studied individual plant species belonging to the family. Eight polyphenol groups were measured: gallic acid derivatives (G), hexahydroxydiphenoyl derivatives (HHDP), procyanidin units (PC), prodelphinidin units (PD), kaempferol derivatives (KA), quercetin derivatives (QU), myricetin derivatives (MY) and quinic acid derivatives (QA).

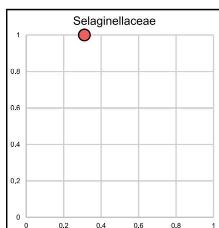
The *x*-axis displays the normalized concentrations of QAs, flavonol derivatives (the sum of KA, QU and MY derivatives), proanthocyanidins (the sum of PCs and PDs) and hydrolysable tannins (the sum of G and HHDP derivatives). The *y*-axis shows the proportions of the subgroups belonging to these main polyphenol groups (KA/QU/MY, PC/PD, G/HHDP) or the proportion of QA of the total phenolic (TP) level (QA/TP).



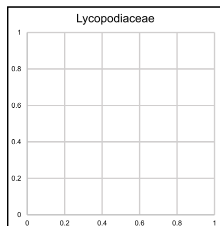
PTERIDOPHYTES

LYCOPHYTES

Selaginellales

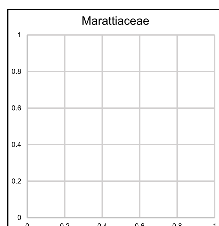


Lycopodiales

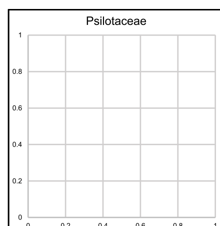


FERNS

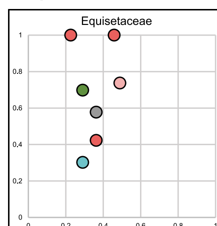
Marattiales



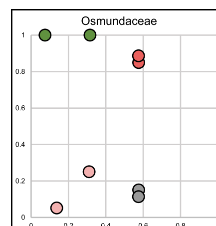
Psilotales



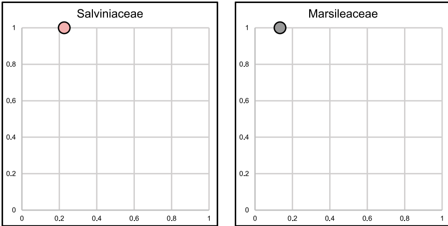
Equisetales



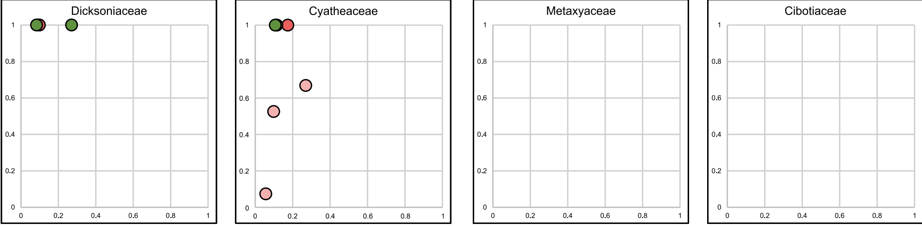
Osmundales



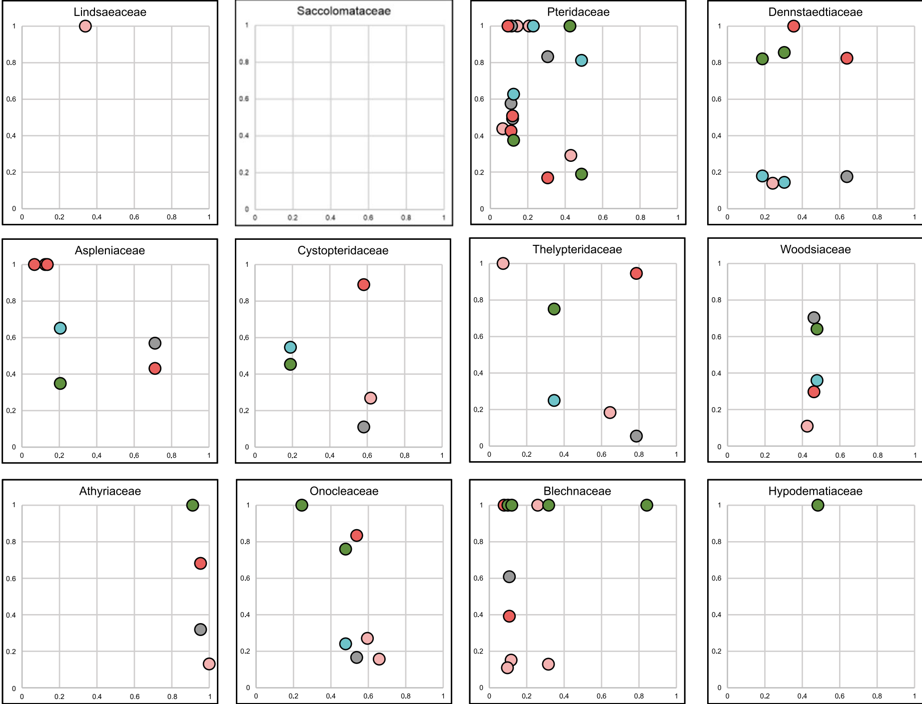
Salviniales

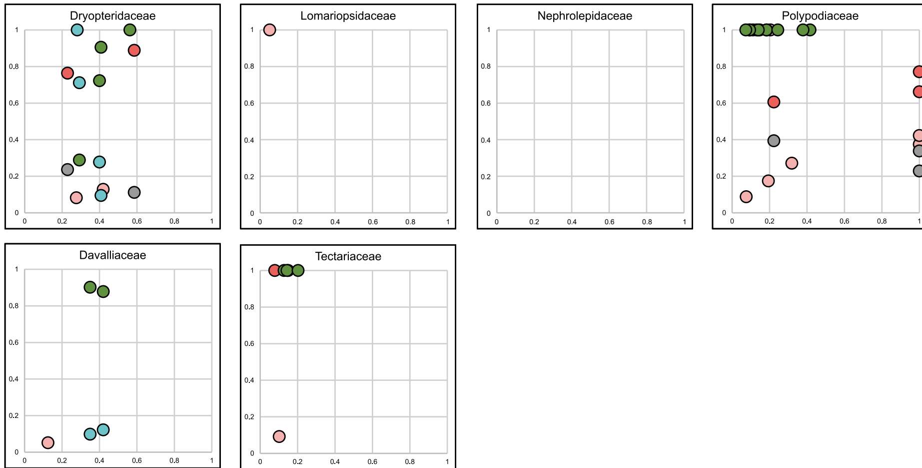


Cyatheales



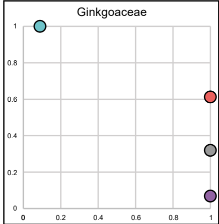
Polypodiales



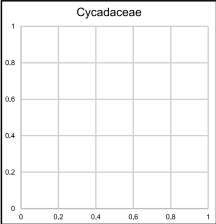

Dryopteridaceae
Lomariopsidaceae
Nephrolepidaceae
Polypodiaceae
Davalliaceae
Tectariaceae
0
0.2
0.4
0.6
0.8
1

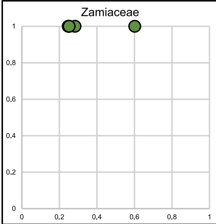
GYMNOSPERMS

Ginkgoales

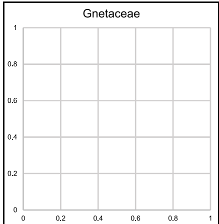


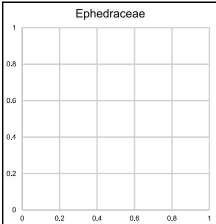
Cycadales



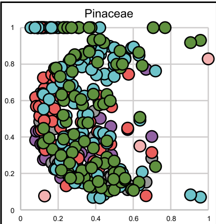


Gnetales

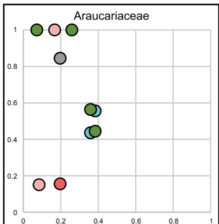


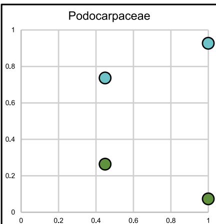


Pinales

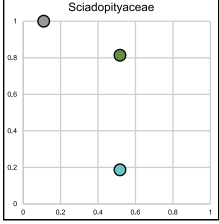


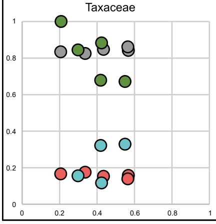
Araucariales

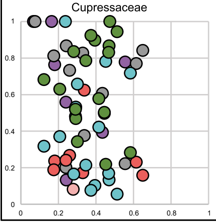




Cupressales



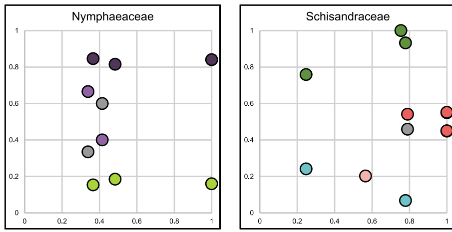




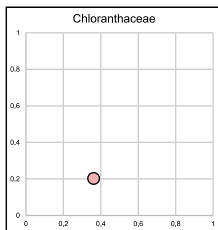
ANGIOSPERMS

Nymphaeales

Austrobaileyales



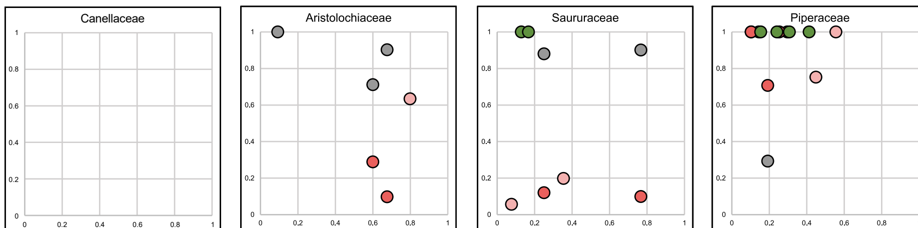
Chloranthales



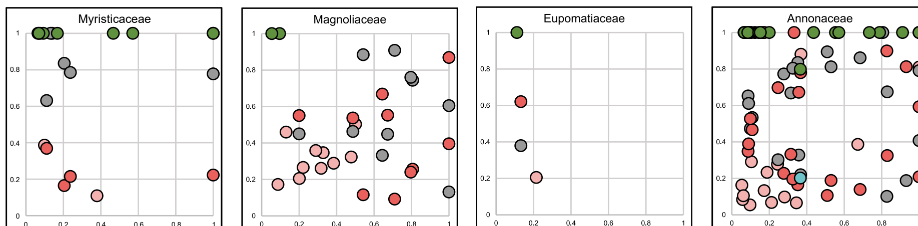
MAGNOLIIDS

Canellales

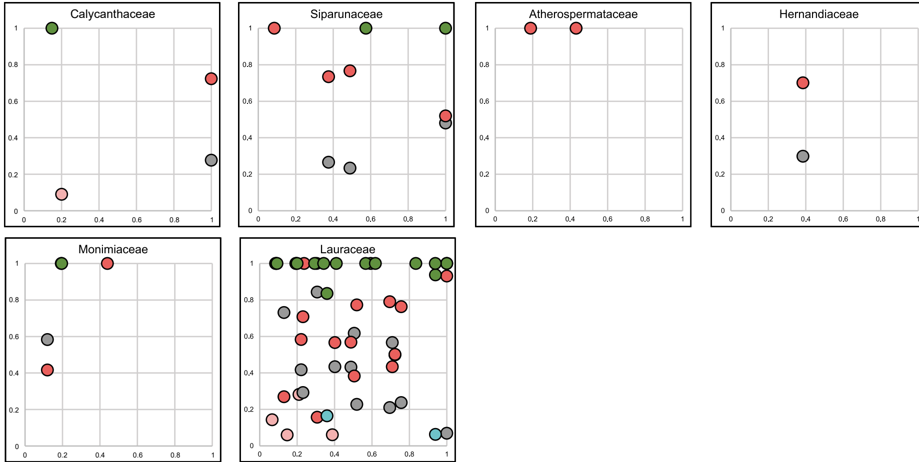
Piperales



Magnoliales

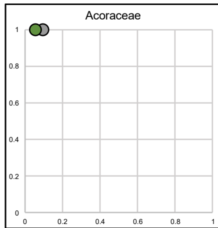


Laurales

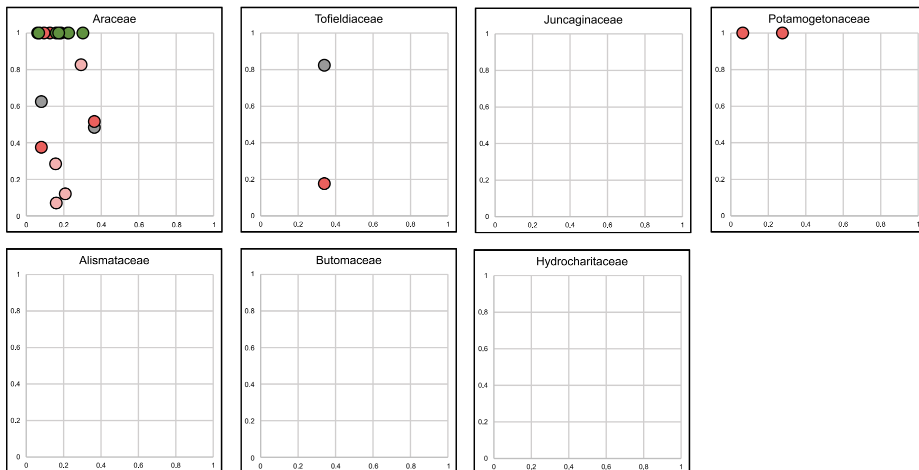


MONOCOTS

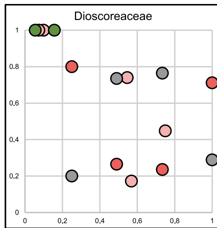
Acorales



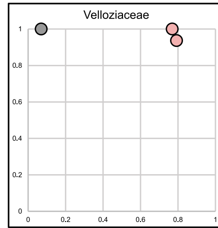
Alismatales

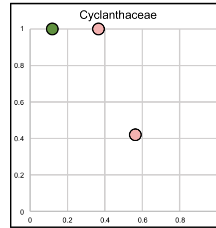


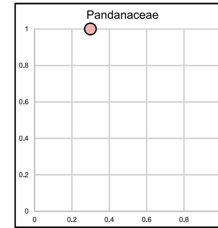
Dioscoreales



Pandanales







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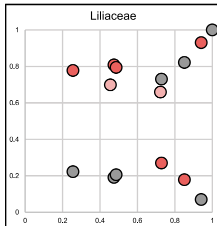
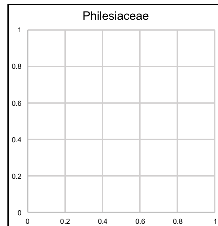
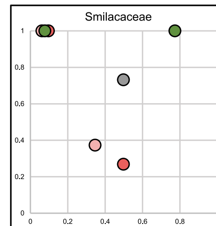
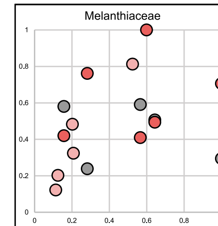
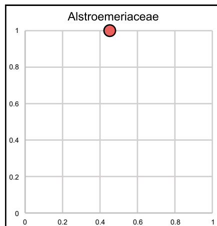
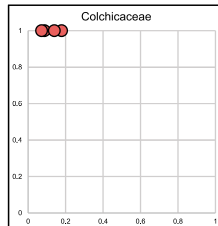




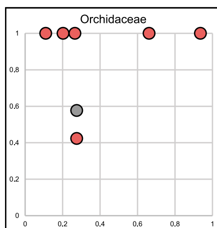


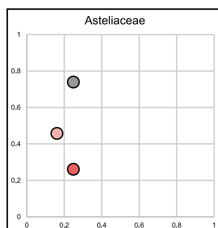


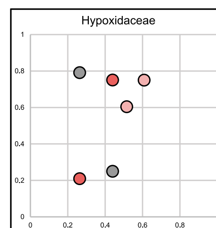


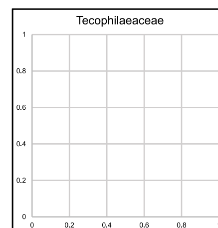


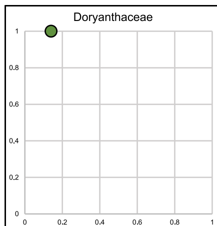
Asparagales

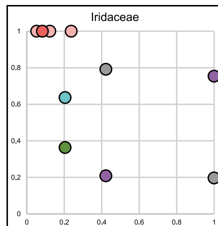


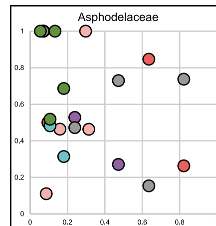


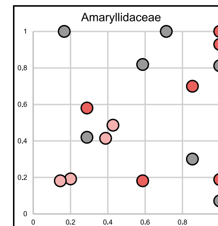


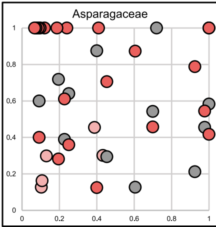






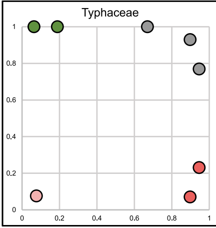


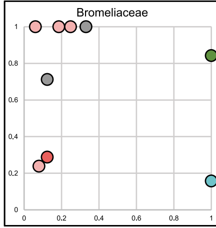


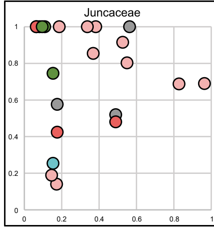


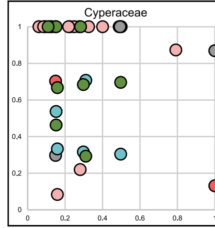
MONOCOTS
Commelinids

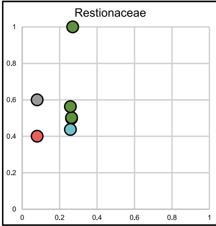
Poales

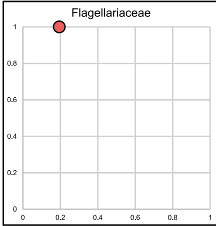


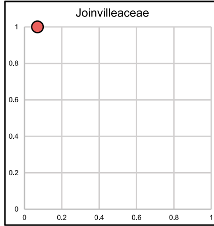


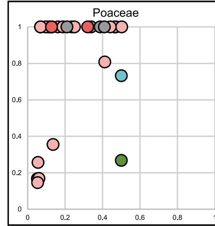




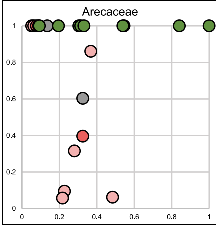




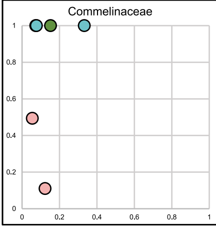


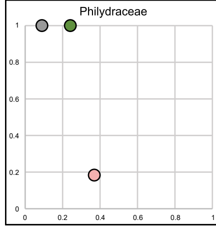


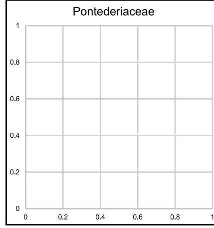
Arecales

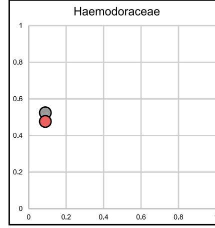


Commelinales

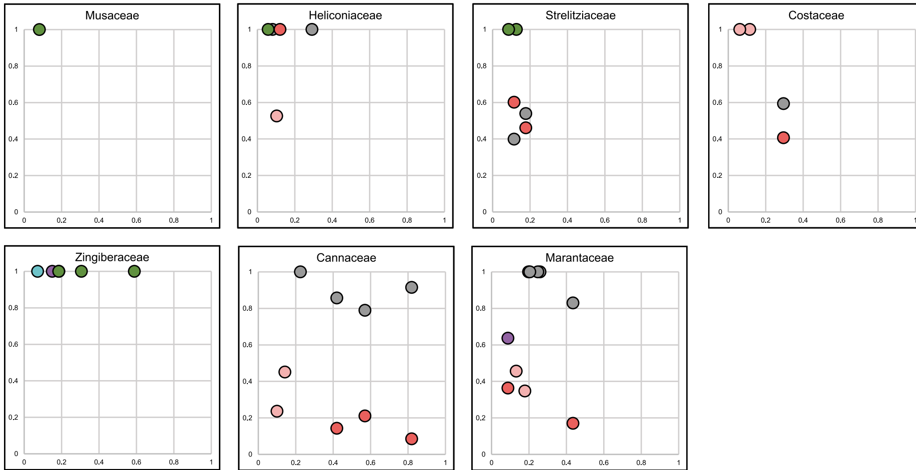




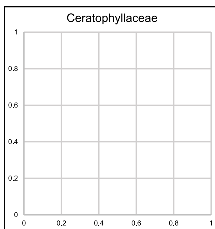




Zingiberales

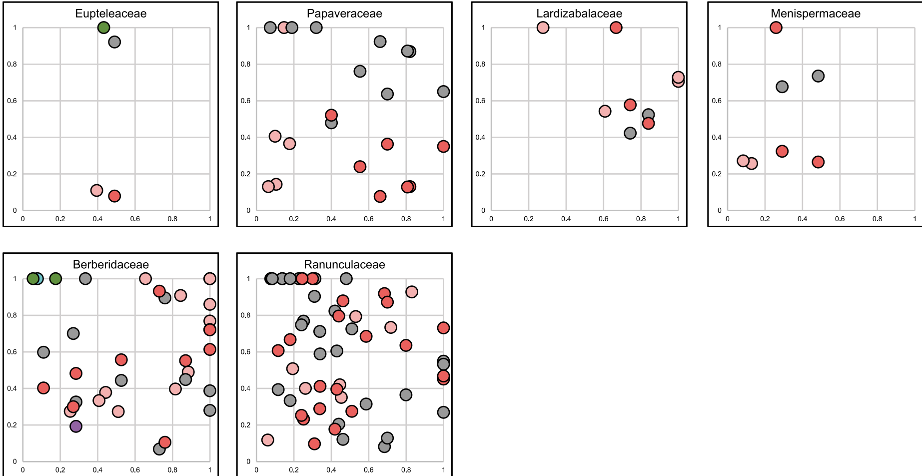


Ceratophyllales



EUDICOTS

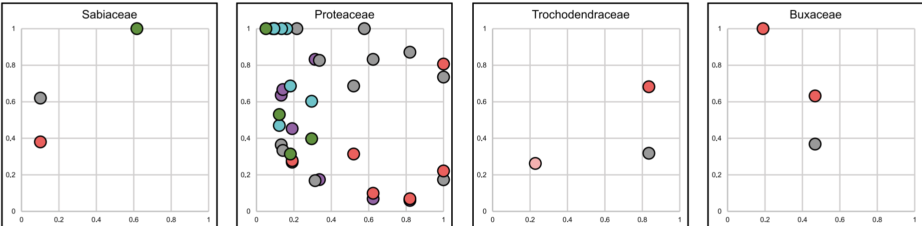
Ranunculales



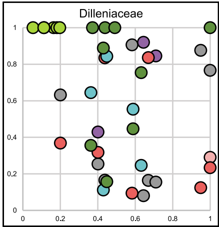
Proteales

Trochodendrales

Buxales

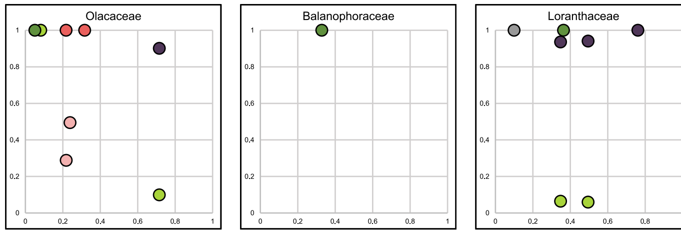


Dilleniales

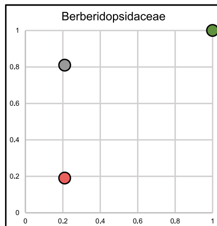


SUPERASTERIDS

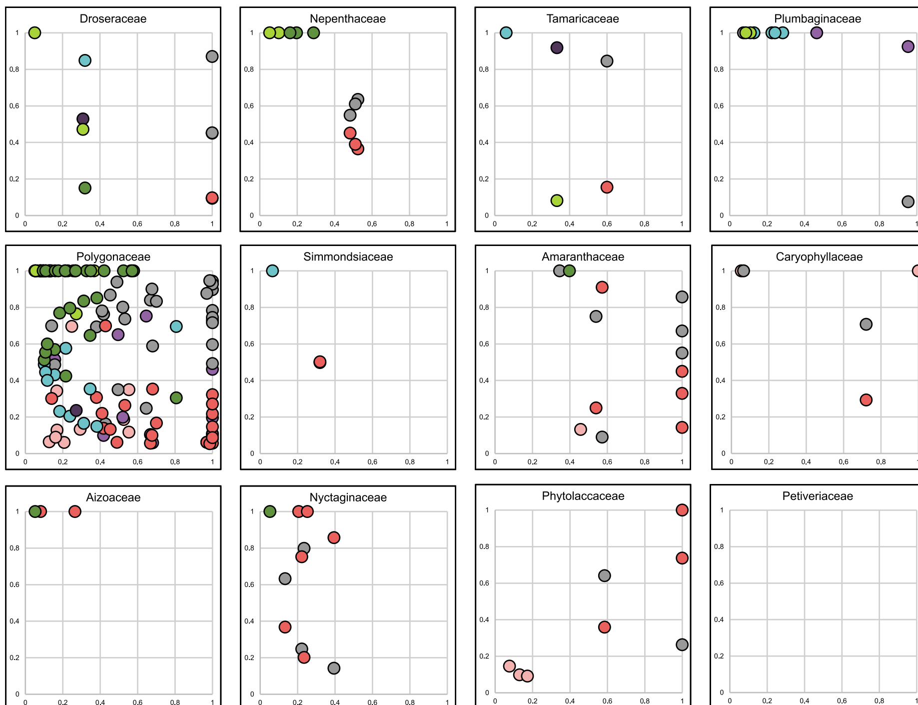
Santalales

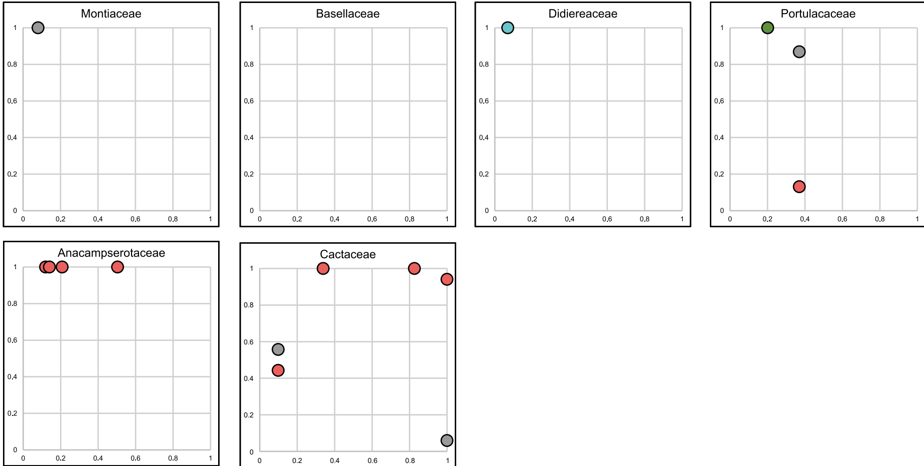


Berberidopsidales

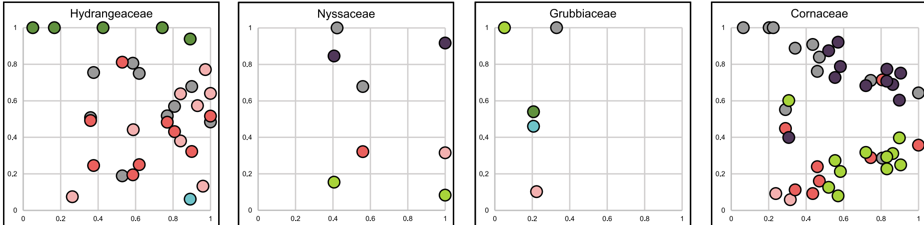


Caryophyllales

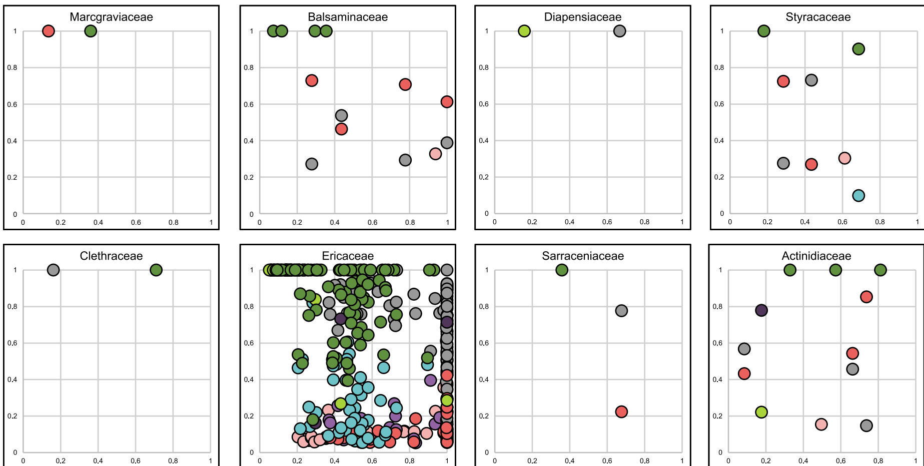


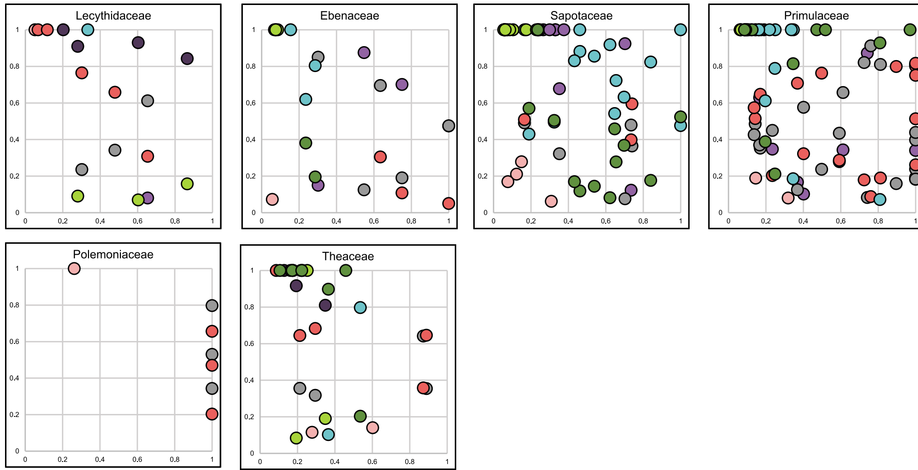


Cornales



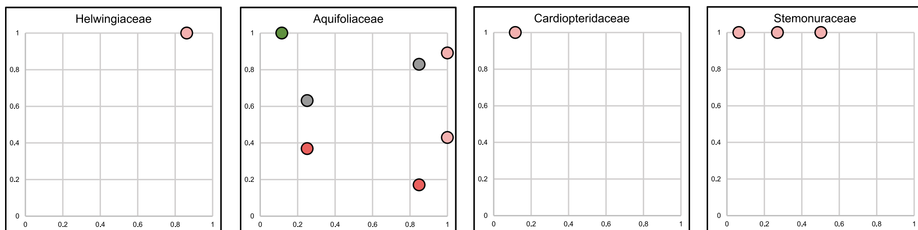
Ericales



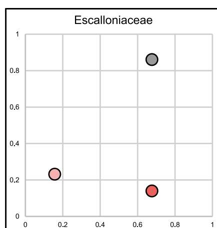


SUPERASTERIDS
Campanulids

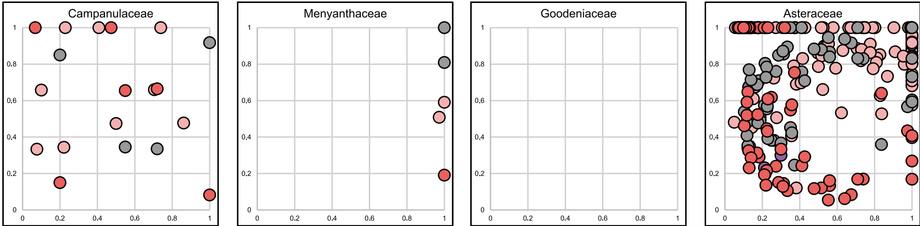
Aquifoliales



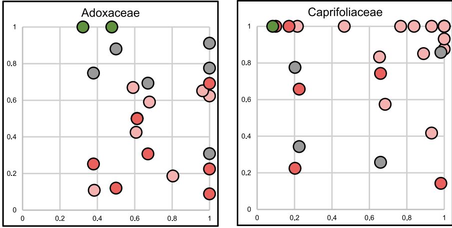
Escalloniales



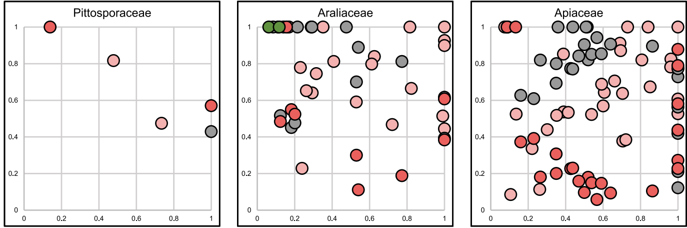
Asterales



Dipsacales

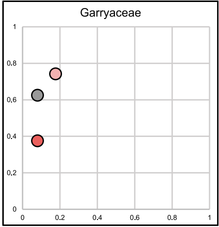


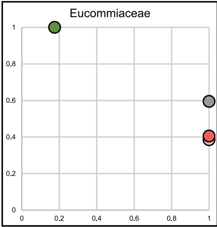
Apiales



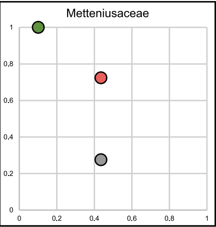
SUPERASTERIDS
Lamiids

Garryales

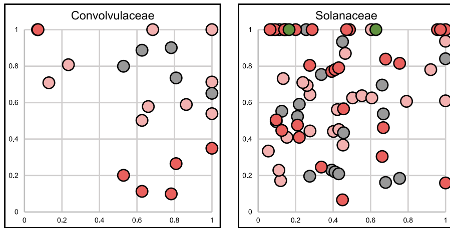




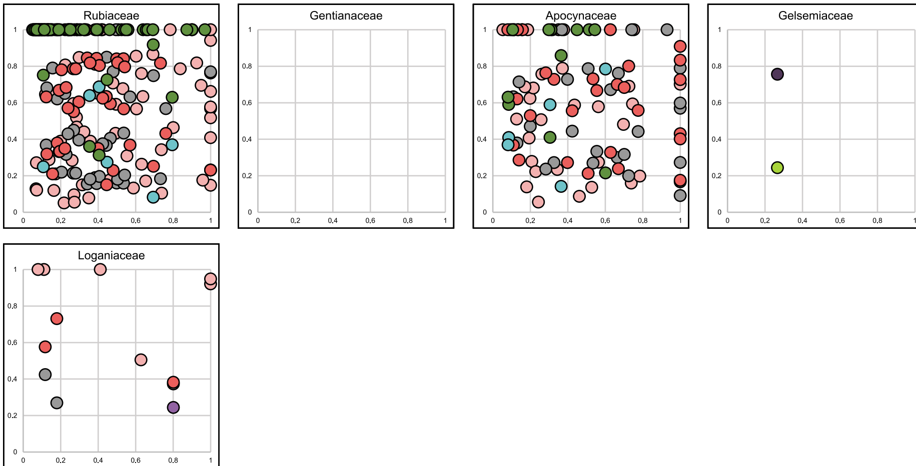
Metteniusales

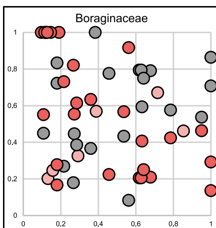


Solanales



Gentianales

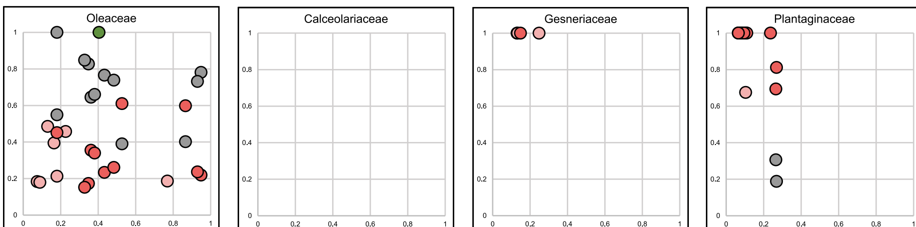




Boraginales

Boraginaceae

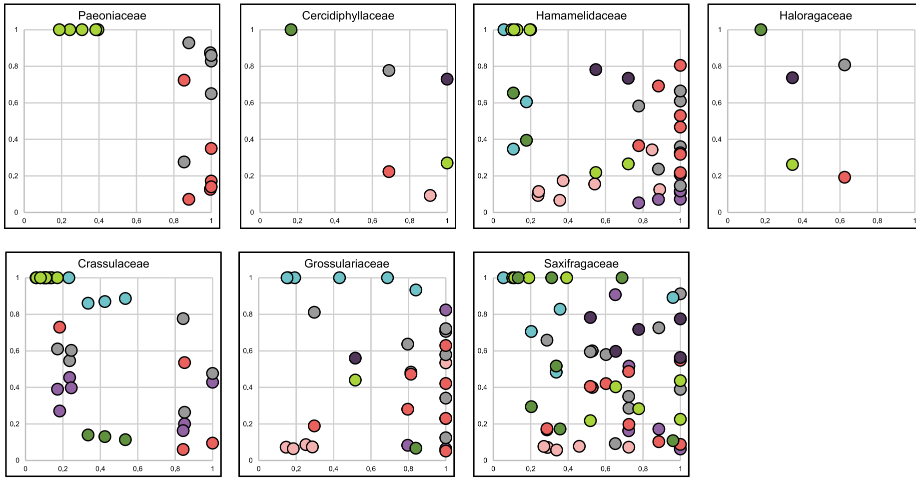
Lamiales



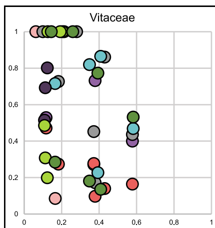
Scrophulariaceae
Linderniaceae
Stilbaceae
Lamiaceae
Phrymaceae
Orobanchaceae
Verbenaceae
Pedaliaceae
Bignoniaceae
Martyniaceae
Lentibulariaceae
Thomandersiaceae
Acanthaceae

SUPERROSIDS

Saxifragales

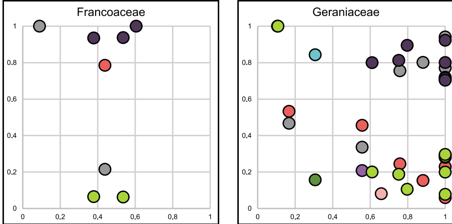


Vitales

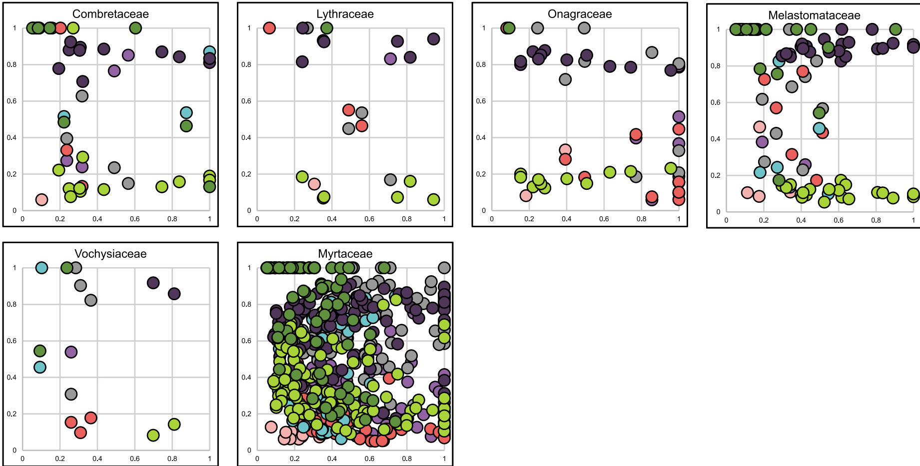


SUPERROSIDS
Malvids

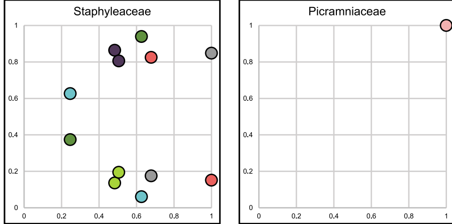
Geraniales



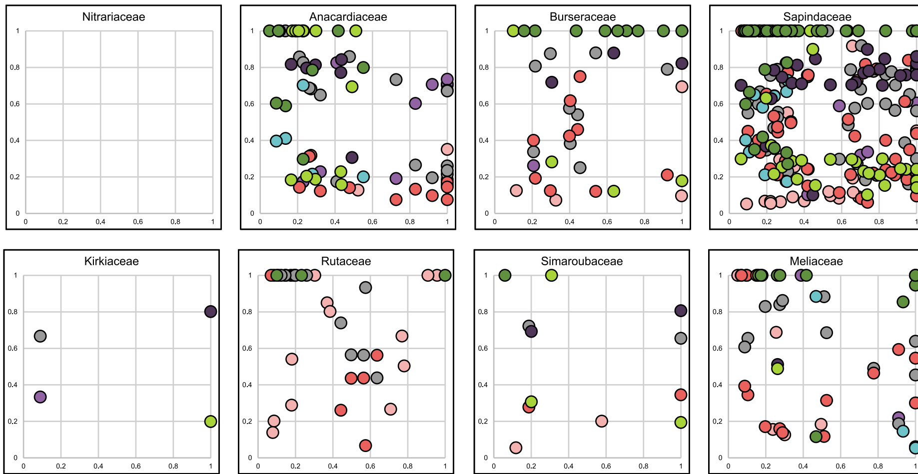
Myrtales



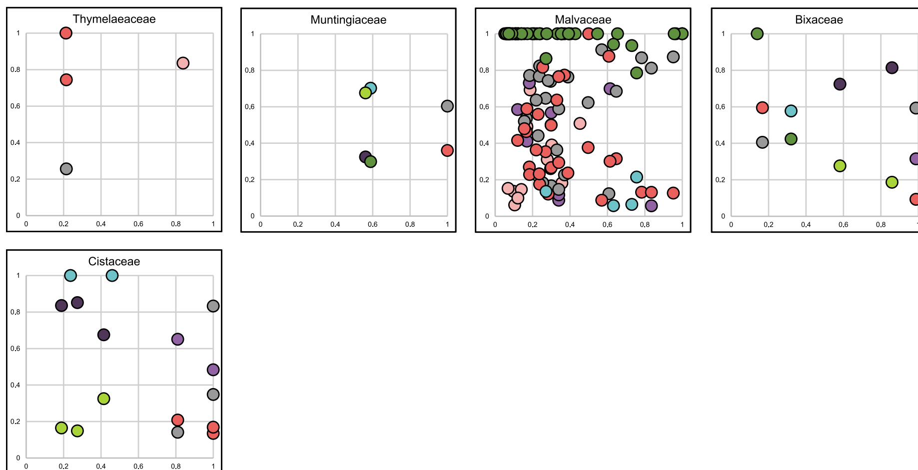
Crossosomatales Picramniales



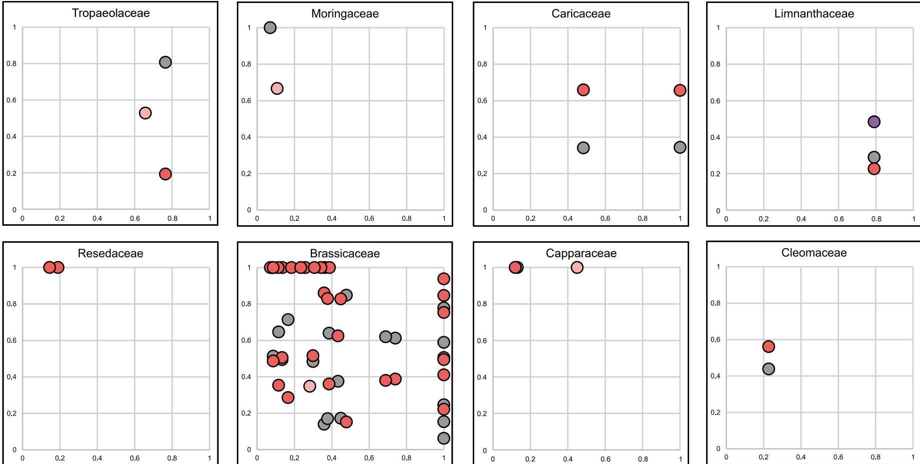
Sapindales



Malvales

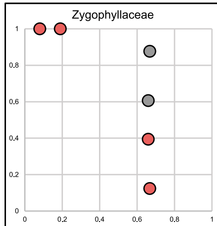


Brassicales

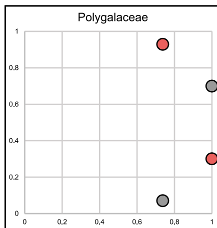


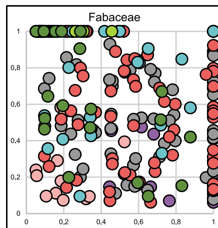
SUPERROSIDS
Fabids

Zygophyllales

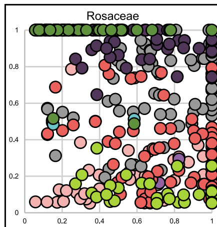


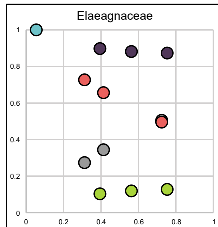
Fabales

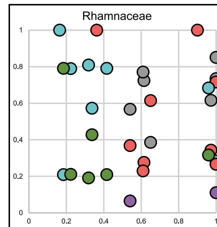


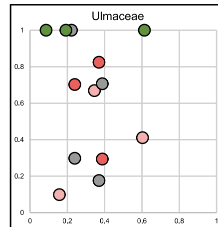


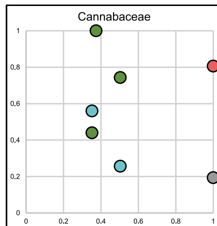
Rosales

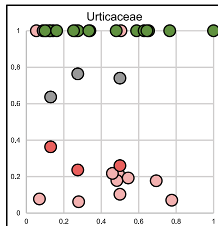


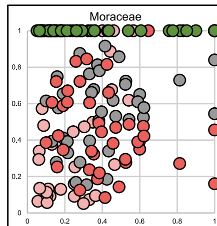




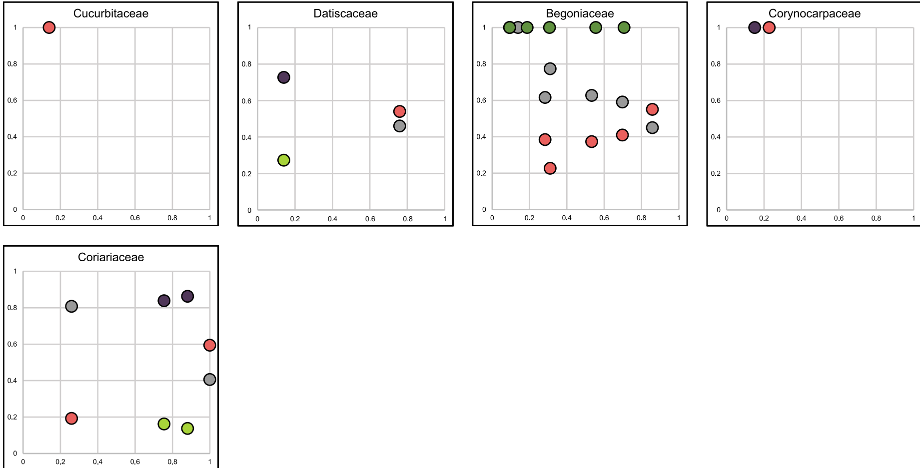




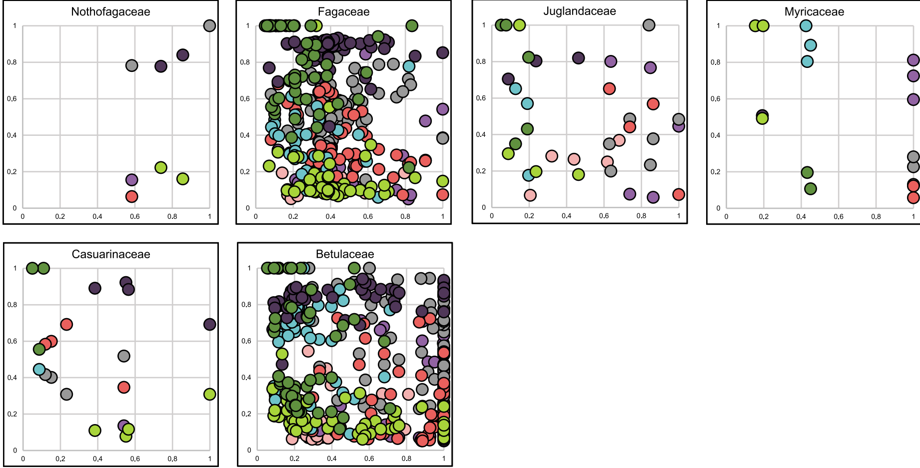




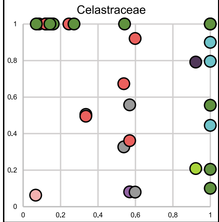
Cucurbitales



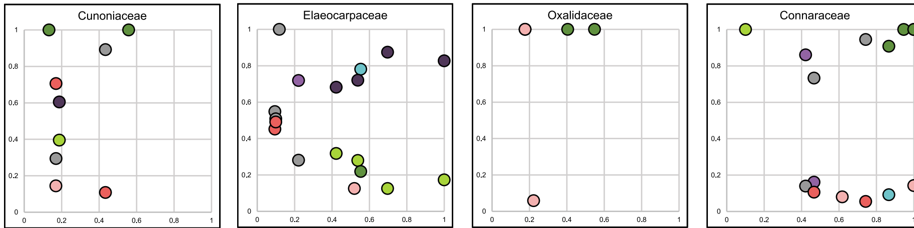
Fagales



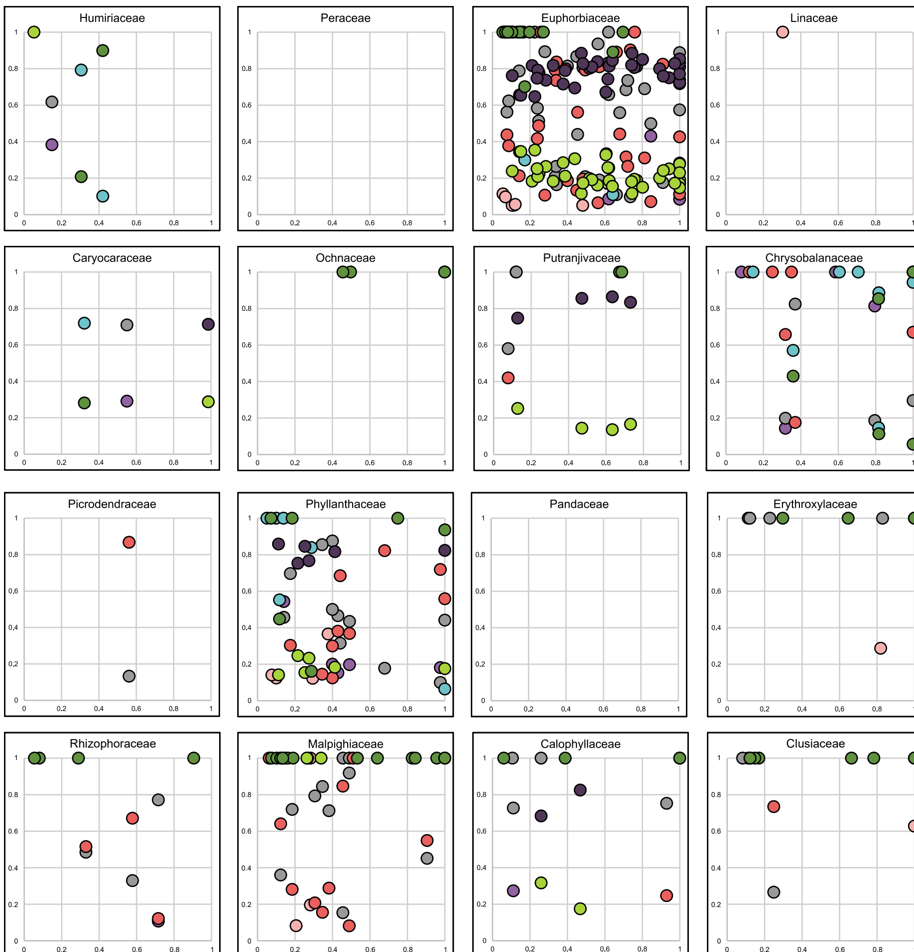
Celastrales

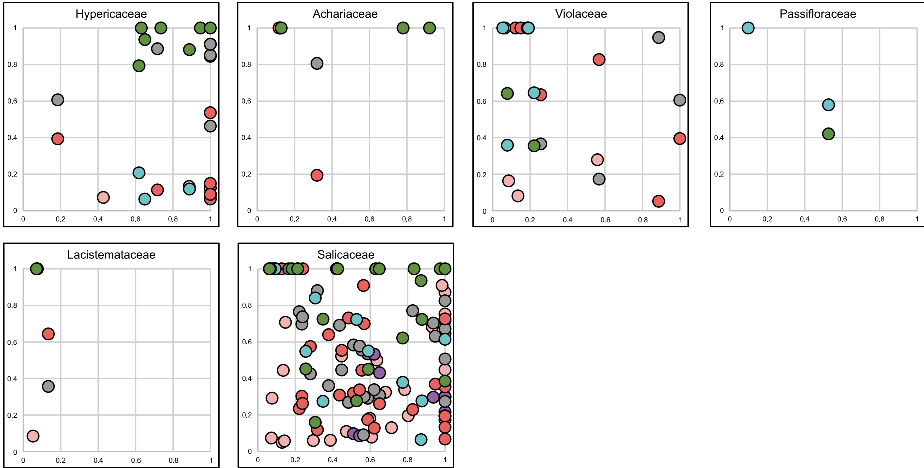


Oxalidales



Malpighiales




Hypericaceae
Achariaceae
Violaceae
Passifloraceae
Lacistemataceae
Salicaceae

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"Nothing in biology makes sense except in the light of evolution."
Theodosius Dobzhansky

"It is simply this: do not tire, never lose interest, never grow indifferent—lose your invaluable curiosity and you let yourself die. It's as simple as that."
Tove Jansson, Fair play

"And I knew exactly what to do. But in a much more real sense, I had no idea what to do."
Michael Scott, The Office

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Suvi Vanhakylä

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