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Metabolomics and evolution of chemical traits in the subtribe Espeletiinae (Asteraceae)

Metabolômica e evolução de caracteres químicos na subtribo Espeletiinae (Asteraceae)

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RESUMO

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A subtribo Espeletiinae (Asteraceae) representa um exemplo clássico de adaptação em ecossistemas tropicais de altitudes elevadas. No entanto, estudos que combinem diferentes campos de pesquisa ainda são necessários para entender este caso proeminente de radiações adaptativas rápidas nos trópicos. Esta tese fornece uma abordagem multidisciplinar combinando informação metabolômica, biogeográfica, taxonômica, evolutiva, química, molecular e ecológica, para um estudo aprofundado da subtribo Espeletiinae e do seu gênero irmão *Smallanthus*. Através de análises metabolômicas baseadas em cromatografia líquida de ultra-alta eficiência acoplada a espectrometria de massas, nós fornecemos, pela primeira vez, evidências metabolômicas de segregação alopátrica em Espeletiinae e evidência metabolômica apoiando a possível segregação do gênero *Espeletia* em dois gêneros diferentes com distintas impressões digitais metabólicas. Em combinação com a filogenia molecular da subtribo e amplificações por PCR, demonstramos que a evolução dos caracteres químicos em Espeletiine seguiu cenários complexos de mudança química com alguns caracteres representando sinapomorfias químicas e outros representando múltiplos ganhos e perdas, implicando em evolução convergente. Por fim, analisando os padrões de expressão dos principais genes envolvidos na biossíntese de ácidos clorogênicos, flavonoides e lactonas sesquiterpênicas, em combinação com análises metabolômicas e informações ambientais, relatamos a regulação ambiental e de desenvolvimento do metabolismo secundário de *Smallanthus sonchifolius*, fornecendo informações relevantes para o entendimento dos fatores regulatórios e possíveis papéis adaptativos dos metabólitos secundários em táxons andinos. Em conclusão, esta tese fornece uma compreensão holística de uma linhagem que representa um exemplo clássico de radiações adaptativas rápidas nos Andes tropicais, abrindo uma nova perspectiva intrigante de pesquisa em outros grupos.

Palavras-chave: Espeletiinae, evolução química, metabolômica, *Smallanthus*.

ABSTRACT

PADILLA GONZÁLEZ, G. F. **Metabolomics and evolution of chemical traits in the subtribe Espeletiinae (Asteraceae).** 2018. 181s. Thesis (Doctorate). Faculty of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2018.

The subtribe Espeletiinae (Asteraceae) represents a classic example of adaptation in tropical high-elevation ecosystems. However, studies bringing different research fields are still necessary to understand this prominent case of rapid adaptive radiations in the tropics. This dissertation provides a multidisciplinary approach combining metabolomic, biogeographic, taxonomical, evolutionary, chemical, molecular and ecological information, for an in-depth study of the subtribe Espeletiinae and its sister genus *Smallanthus*. Through metabolomic analyses based on ultrahigh-performance liquid chromatography-mass spectrometry, we provide, for the first time, metabolomic evidence of allopatric segregation in Espeletiinae and metabolomic evidence supporting a putative segregation of the genus *Espeletia* in two different genera with distinctive metabolic fingerprints. In combination with the molecular phylogeny of the subtribe and PCR amplifications, we also demonstrate that the evolution of chemical traits in Espeletiinae followed complex scenarios of chemical change with some traits representing chemical synapomorphies and other traits being gained and lost multiple times implying convergent evolution. Lastly, by analyzing the expression patterns of key genes involved in the biosynthesis of chlorogenic acids, flavonoids and sesquiterpene lactones, in combination with metabolomic analyses and environmental information, we report the developmental and environmental regulation of the secondary metabolism of *Smallanthus sonchifolius*, providing relevant information towards the understanding of the regulatory factors and possible adaptive roles of secondary metabolites in Andean taxa. In conclusion, this dissertation provides a holistic understanding of a lineage representing a classic example of rapid adaptive radiations in the tropical Andes, opening an intriguing new perspective of research in other groups.

Keywords: Espeletiinae, metabolomics, chemical evolution, *Smallanthus*.

CHAPTER 1: GENERAL INTRODUCTION

METABOLOMICS

As one of the most recent “omics” sciences, metabolomics constitutes a relatively new research field aimed at studying the whole set of metabolites synthesized by a given biological system and their response to genetic or environmental changes (FIEHN, 2002; OLIVER, 1998). The metabolome, similarly to the definition of the proteome (the set of proteins) or the transcriptome (the set of RNAs), can be defined on all levels of complexity, from single cells or cell compartments to tissues and organisms (FIEHN, 2002). Considering that metabolites are the end products of regulatory processes occurring at the cellular level, the types of metabolites and their concentration levels are often the result of complex response mechanisms of biological systems to genetic or environmental disturbance. Thus, the metabolome of a given organism, tissue or cell is often chemically complex in terms of its qualitative and quantitative composition, which represents an important challenge to the current analytical platforms. As a consequence, different metabolomic approaches have been developed to precisely detect, identify and quantify metabolites at different levels, from single compounds from a given chemical class to broad methods aiming to detect as many metabolites as possible in a given organism.

Metabolomic approaches

Given their high structural complexity and the fact that “metabolites have a greater variability of atoms and groups compared to the linear 4-letter codes for genes or the linear 20-letter codes for proteins” (FIEHN, 2002), determining the elemental composition, the linkage order among atoms and the stereochemical configuration of all metabolites in a given biological system remains an elusive task. Furthermore, due to their highly diversified physicochemical properties, varying concentrations and structural complexity, no single analytical platform or extraction protocol is currently able to simultaneously extract and detect all metabolites from a given system. Instead, different metabolomic approaches have been designed in order to answer specific types of questions. In general terms, metabolomic analyses can be categorized in two broad types: targeted and untargeted analyses. Targeted

metabolomics refers to the detection, identification and quantification of a small set of known metabolites in a certain system. On the other hand, untargeted metabolomics is focused on the detection of as many metabolites as possible through comprehensive analyses of complex biological matrices, without previous knowledge of the compounds identities or classes (FIEHN, 2002). More recently, different terms have been adopted by the scientific community and three main metabolomic approaches have been proposed: (1) metabolite targeted analysis, which refers to the “detection and precise quantification of a single or small set of target compounds”, (2) metabolic profiling, which focuses on the “identification and approximate quantification of a group of metabolites associated to specific biosynthetic pathways” and (3) metabolic fingerprinting, which is used for “complete metabolome comparison without knowledge of compounds identities”, usually by spectral fingerprinting of crude extracts, biological fluids or by direct analysis in real time (KRASTANOV, 2010).

Analytical techniques

By definition, metabolomics, and especially metabolic fingerprinting, must aim at detecting all metabolites present in a given biological system by using comprehensive extraction protocols and analytical techniques. However, this is a very ambitious goal still far from reality when considering the high chemical complexity of biological matrices and the limitations of comprehensive extraction methods and the current analytical platforms in terms of sensitivity and dynamic range of detection (SUMNER; MENDES; DIXON, 2003). Accordingly, no single analytical technique is currently able to detect all metabolites from a complex biological matrix and, similarly, no single extraction protocol is currently able to extract all metabolites from a given system. As a truly comprehensive analysis of the metabolome is currently not feasible, in practice a comprehensive metabolomic analysis should at least include multiple metabolic pathways and cover as many metabolites as possible (SUMNER; MENDES; DIXON, 2003). Therefore, different analytical techniques have been routinely used in metabolomic analyses, including ultraviolet (UV) and infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) and mass spectrometry (MS), among others (SUMNER; MENDES; DIXON, 2003). Since “the dynamic range of many techniques can be severely limited by the sample matrix or the presence of interfering and competing compounds” (SUMNER;

MENDES; DIXON, 2003), in most cases, analytical detectors are coupled to chromatographic separation techniques like gas chromatography (GC) or high-performance liquid chromatography (HPLC) to avoid overlapping or suppression of signals in complex matrices.

Considering that different analytical techniques have different sensitivities and dynamic ranges of detection, the selection of the most suitable technology is generally a compromise between speed, selectivity and sensitivity (SUMNER; MENDES; DIXON, 2003). NMR and MS constitute the two most commonly used methods in metabolomics. However, they still have some limitations. For example, NMR constitutes a widely used technique because of its high dynamic range of detection and speed, but its very low sensitivity (approx. 10^{-6} mol) compared to mass spectrometry (approx. 10^{-15} mol) represents a serious concern as it fails to detect low concentrated metabolites (SUMNER; MENDES; DIXON, 2003). On the other hand, mass spectrometry presents a very good sensitivity able to detect compounds in minor concentrations but its selectivity can be compromised. In order to be detected, metabolites should be first ionized and, for example, in electrospray ionization (ESI), the most commonly used MS-ionization method in metabolomics, differences in the ionization behavior of different chemical classes compromise their detection as compounds in the extremes of the pH scale are more prone to gain or lose protons (or other atoms to form specific adducts) than neutral species favoring their ionization and subsequent detection. Other ionization methods are also possible, but each of them has its own merits and drawbacks are reported by Ernst et al. (2014). In spite of this inherent limitation, MS represents the technique of choice for the analysis of cellular proteins and metabolites (proteome and metabolome respectively) due to the recent development of high-resolution analyzers (such as time-of-flight - TOF, Orbitraps and ion cyclotron resonance - ICR), the different ionization methods available (such as electron impact - EI, ESI, matrix-assisted laser desorption/ionization- MALDI, etc.) and the possibility of performing tandem MS analyses as well as coupling different detectors in a single equipment allowing the detection of a wide range of metabolites on a scale previously unimaginable.

Applications and importance

Metabolomics was first considered as a functional genomics tool aiming to link genotypes with phenotypes by helping in the identification of gene function (FIEHN,

2002). However, in addition to this specific application, which requires its integration with other “omics” sciences (e.g. genomics and transcriptomics), metabolomics is applied in many biological systems, including human, plants and microorganisms as a transdisciplinary science comprising of ‘organic chemistry’, ‘analytical chemistry’, ‘chemometrics’ and ‘informatics’, among others (KRASTANOV, 2010). Through the use of statistical multivariate pattern recognition methods, metabolomics enables the classification and discrimination of diverse biological status like sample origin and quality, the characterization of metabolic and phenotypic alterations between genetically manipulated and wild-type lineages or between healthy and diseased tissues, the investigation of food composition and quality, the discovery of biomarkers involved in certain biological activity, the monitoring of metabolic responses to developmental or environmental alterations, the understanding of metabolic networks established in symbiotic associations and the fingerprinting of species for taxonomic purposes, among others (KRASTANOV, 2010; SUMNER; MENDES; DIXON, 2003; VILLAS-BÔAS; RASMUSSEN; LANE, 2005). Thus, given its transdisciplinary nature, metabolomics has been applied in several research fields. Yet, plant science is one of the fields where metabolomics has found many of its current applications, especially in the chemical characterization and quality control of botanicals, phytomedicines and crop plants, as well as in the assessment of plant responses to genetic and environmental alterations.

THE SUBTRIBE ESPELETIINAE

The subtribe Espeletiinae Cuatrec. (tribe Millerieae, Asteraceae) represents a morphologically diverse group of 144 species distributed in eight genera: *Carramboa* Cuatrec., *Coespeletia* Cuatrec., *Espeletia* Mutis ex Humb. & Bonpl., *Espeletiopsis* Cuatrec., *Libanothamnus* Ernst, *Paramiflos* Cuatrec., *Ruilopezia* Cuatrec., and *Tamania* Cuatrec., popularly known as “frailejones”, endemic to the high altitudes of the tropical Andes of South America (CUATRECASAS, 2013; DIAZGRANADOS, 2012; DIAZGRANADOS; BARBER, 2017). Since the first collection and description of an Espeletiinae species in 1792 by José Celestino Mutis during the “Expedición Botánica del Nuevo Reino de Granada”, frailejones have intrigued the scientific community for their unusual life forms, impressive morphological diversity (Figure 1.1) and numerous adaptations to the harsh environmental conditions prevailing at high Andean altitudes (CUATRECASAS, 2013; MONASTERIO; SARMIENTO, 1991).

Figure 1.1 Morphological diversity within the subtribe Espeletiinae Cuatrec. Capitula of: A, *Espeletiopsis pannosa* (Standl.) Cuatrec.; B, *E. purpurascens* (Cuatrec.) Cuatrec.; C, *Tamania chardonii* (A.C.Sm.) Cuatrec.; D, *Espeletia batata* Cuatrec. Leaves of: E, *E. grandiflora* Humb. & Bonpl.; F, *E. chocontana* Cuatrec.; G, *E. barclayana* Cuatrec.; H, *Ruilopezia jabonensis* (Cuatrec.) Cuatrec. Young leaves of:

I, *E. murilloi* Cuatrec.; J, *E. lopezii* Cuatrec.; K, *Coespeletia palustris* Diazgr. & Morillo; L, *E. boyacensis* Cuatrec. Trichomes of: M, *Paramiflos glandulosus* (Cuatrec.) Cuatrec.; N, *R. floccosa* (Standl.) Cuatrec.; O, *E. aristeguietana* Cuatrec.; P, *R. marcescens* (S.F.Blake) Cuatrec. Rosettes of: Q, *R. jahnii* (Standl.) Cuatrec.; R, *E. discoidea* Cuatrec.; S, *Espeletiopsis caldasii* (Cuatrec.) Cuatrec.; T, *T. chardonii*. Habits of: U, *Carramboa badilloi* (Cuatrec.) Cuatrec.; V, *Coespeletia timotensis* (Cuatrec.) Cuatrec.; W, *E. jimenez-quesadae* (Cuatrec.) Cuatrec.; X, *Libanothamnus arboreus* (Aristeg.) Cuatrec. (Photographs from M. Diazgranados.)

Morphologically, frailejones exhibit an exceptional diversity of growth-forms including large trees, dichotomous trees, shrubs and the iconic giant rosettes (Figure 1.1), an unusual life form found only in a few other high elevation ecosystems. Their leaves, reproductive organs and indument display a comparable level of variation (Figure 1.1). The enormous morphological diversity of Espeletiinae is also represented by their remarkable ecological specialization and associations with numerous species of insects and microorganisms (CUATRECASAS, 2013; FAGUA; GONZALEZ, 2007; MONASTERIO; SARMIENTO, 1991). Furthermore, frailejones represent key species of critical ecological importance because they contribute to regulating the hydrologic cycle and prevent soil erosion in the high altitude Andean grasslands (GARCÍA, N., CALDERÓN, E., GALEANO, 2005).

Distribution and biogeography

Espeletiinae comprises a monophyletic group entirely restricted to the high Andean forests and páramos of Colombia, Venezuela and Ecuador, usually located between 2,000 to 4,700 m above the sea level (CUATRECASAS, 2013). Páramos, represent high elevation ecosystems located “along the crests of the highest mountain ranges or on isolated mountaintops, like islands in a sea of forest” (LUTEYN, 1999). The broken topography and altitudinal restriction of Andean páramos, makes them a particular ecosystem that biogeographically function as islands acting as barriers promoting allopatric speciation (LUEBERT; WEIGEND, 2014), a mechanism that has shaped the evolution and biogeographic history of several plant groups including the subtribe Espeletiinae (DIAZGRANADOS; BARBER, 2017; MADRIÑÁN; CORTÉS; RICHARDSON, 2013). Considering that even highly variable molecular markers commonly used to reconstruct phylogenies

and assess biogeography do not have the resolving power needed for a detailed geographic discrimination in Espeletiinae, in chapter 2 we present a metabolomics approach based on ultrahigh-performance liquid chromatography-mass spectrometry to provide metabolomic evidence of allopatric segregation in this Andean-endemic subtribe. Our approach allows distinct degrees of discrimination of Espeletiinae taxa at different geographic scales with characteristic metabolic fingerprints related to the species' country of origin in a global scale, to their páramo massifs in a regional scale and to their páramo complexes in a local scale, revealing inter- and intraspecific metabolic variations, and demonstrating that metabolomic data can provide relevant information for understanding allopatric segregation in recently diversified plant groups where the genetic divergence is still at an early stage.

Evolutionary history and classification

Since the official publication of the genus *Espeletia* by Humboldt and Bonpland in Plantae Aequinoctiales (1809[1808]) and the early works of José Cuatrecasas (1976, 1986), the taxonomy of the frailejones has been the focus of great debate and much disagreement persists over the taxonomic subdivisions of the subtribe, as well as its phylogeny (DIAZGRANADOS; BARBER, 2017; POUCHON et al., 2018). Recent phylogenetic studies based on nuclear (ITS and ETS), chloroplast (rpl16) and AFLPs molecular markers provided the more complete phylogeny of Espeletiinae to date (although with a low resolution), suggesting a very strong influence of geography on phylogenetic relationships and concluding that the current generic classification of the subtribe needs to be deeply revised, in view that most of the genera were recovered as paraphyletic (DIAZGRANADOS; BARBER, 2017). Recently, the first fully resolved phylogeny of all morphological groups of Espeletiinae, using whole plastomes and about one million nuclear nucleotides, was published and it confirmed the strong influence of geography on the evolutionary history of the group and the role of adaptive morphological evolution in the exceptionally rapid radiation of the frailejones (POUCHON et al., 2018). By using a metabolomics, phylogenetic and biosynthetic approach, in chapter 3 we explored chemotaxonomic relationships in Espeletiinae, determined ancestral states of chemical traits and explored the presence of a key gene (Germacrene A oxidase, GAO) involved in the biosynthesis of secondary metabolites in selected members of the subtribe. Our results not only contribute to clarify taxonomic relationships in

Espeletiinae but suggest that specialized metabolites might have contributed to the adaptive success of this subtribe to the Andean páramos.

Secondary metabolites and their regulatory factors

Because secondary metabolites possess a broad range of ecological roles, serving as chemical interface between plants and their environment, it is likely that some of them played a crucial role in evolutionary success of Espeletiinae by helping the plants to survive in their highly specialized ecological niche, the Andean páramos. However, the regulatory factors of the secondary metabolism in Andean taxa from a metabolomics perspective remain poorly understood, and the adaptive roles that secondary metabolites played in Andean groups have yet to be described. By analyzing gene expression patterns, metabolic fingerprints and environmental variables in an integrated approach, in chapter 4 we report the developmental and environmental regulation of the secondary metabolism of *Smallanthus sonchifolius* (yacón), an important Andean crop with medicinal use, considered a sister taxa of the subtribe Espeletiinae. Our results demonstrate that a complex interplay between environmental factors and plant development regulate the secondary metabolism of yacón, providing relevant information towards the understanding of the regulatory factors and possible adaptive roles of secondary metabolites in Andean taxa.

Thus, through a multidisciplinary approach combining metabolomic, biogeographic, taxonomical, evolutionary, chemical, molecular and ecological information, this dissertation provides an in-depth study of the subtribe Espeletiinae and its closely related species, *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson. The results obtained herein contribute not only towards a holistic understanding of the biogeographic and taxonomic relationships of a lineage representing a classic example of rapid adaptive radiations in the tropical Andes, but to the understanding of chemical evolution as a whole, including the processes and regulatory factors influencing the metabolic fingerprints of several Asteraceae species, opening an intriguing new perspective of research in other plant groups with adaptive radiations.

CONCLUSIONS

The research detailed in this dissertation integrates metabolomic, biogeographic, taxonomical, evolutionary, chemical, molecular and ecological information for the study of the subtribe Espeletiinae and its sister genus *Smallanthus*. Metabolomic analyses by UHPLC-UV-HRMS of 211 samples and correlation with biogeographic data provided metabolomic evidence for allopatric segregation in Espeletiinae by identifying quantitative metabolic differences at higher taxonomic levels and lower geographic scales compared to the previous reports. Species displayed characteristic metabolic fingerprints related to their country of origin in a global scale, to their páramo massifs in a regional scale and to their páramo complexes in a local scale, revealing inter- and intraspecific metabolic variations. Additionally, this study allowed establishing chemotaxonomic relationships in Espeletiinae based on whole-metabolome comparisons, providing metabolomic evidence to support a putative segregation of the genus *Espeletia* into two different genera based on their characteristic metabolic fingerprints: one comprising Colombian taxa and the other Venezuelan representatives. We also provided the first ancestral states reconstructions of chemical traits in Andean taxa, demonstrating complex scenarios of chemical evolution with some traits representing direct descent from ancestral taxa, other traits resulting from evolutionary innovation, and several traits being gained and lost multiple times implying convergent evolution. Amplification of GAO in species-producing and species-lacking STLs suggested that the capacity of biosynthesizing STLs is putatively present and evolutionary conserved in the whole subtribe Espeletiinae. Lastly, metabolomic analyses of *S. sonchifolius* in combination with gene expression data provided relevant information towards the understanding of the regulatory factors shaping the metabolic fingerprints of Andean taxa, demonstrating that the secondary metabolism of yacón is regulated by a complex interplay between both plant development and abiotic environmental conditions and providing the first information about the genes involved in the biosynthesis of flavonoids, chlorogenic acids and STLs in the genus *Smallanthus*, which might effectively promote the discovery of new genes involved in the secondary metabolism of other related taxa in the future.

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