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Digestive glands extraction and precise pigment analysis support the exclusion of the carnivorous plant *Dionaea muscipula* Ellis from the Caryophyllales order

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ABSTRACT

In the order Caryophyllales, plants synthesize betalains instead of anthocyanins, with only two exceptions, the Caryophyllaceae and Molluginaceae. *Dionaea muscipula* Ellis was included in the Caryophyllales order but recent research based on genetic studies proposed the consideration of the Droseraceae family into the Nepenthales order. In this work we face the dilemma of the phylogenetic classification of *Dionaea* from a phytochemical point of view. *Dionaea*'s pigments were analyzed by using techniques of structural analysis. Extracts from the leaves, mature stem and flowers of different specimens of *Dionaea* were analyzed, to find possible differences in the types of pigments or in their proportion in different parts of the plant. These extracts were analyzed by spectrophotometry, HPLC co-elution and ESI-MS/MS. In addition, digestive glands were extracted from the snap trap with minor sample manipulation and by reducing the non-pigmented plant tissue. Considering only the digestive glands instead of whole snap traps, the analyses allowed to quantitate and elucidate the structure of the compounds responsible for the red coloration: delphinidin-3-*O*-glucoside (myrtillin), cyanidin-3-*O*-glucoside (kuromanin) and a third compound, the aglycone cyanidin, detected in the species for the first time. The unambiguous results of the present work support the exclusion of *Dionaea* from the Caryophyllales.

1. Introduction

Dionaea muscipula Ellis, commonly known as Venus flytrap, is a carnivorous and autotrophic plant. It commonly occupies relatively closed ecosystems where the soil is poor in nutrient substances, acid and wet, as in its natural habitat in North and South Carolina in the USA. These plants take in and absorb nutrients directly from small animal resources by means of carnivorous leaves [1,2]. The Venus flytrap was already described by Charles Darwin as "one of the most wonderful plants in the world" [3] and evolved from an ancestor that was already carnivorous. Although carnivory may seem an unusual feature for a plant, it has evolved independently at least six times in plants [4,5]. The world of carnivorous plants is diverse, with these plants found in different taxonomic groups [5].

Dionaea muscipula Ellis is a small plant, consisting of a rosette of leaves [1]. It has snap traps consisting of two lobes and with spikes at the end of them. Springing from the upper surface of the two lobes are six slender, mechanosensitive hairs, three on each side in a triangular position. The rest of the surface is covered quite densely with digestive glands [6,7]. The attraction of insects is due to the combination of the shape and color of these traps, in addition to the emission of volatile organic compounds. These compounds might serve as a first signal to attract prey insects from distant locations and entice them towards the plant [8]. Currently, there are very few references about the pigment that this plant has in its lobes, although the red coloration they present is common in *Drosera* and *Dionaea* species [2].

Dionaea muscipula Ellis belongs to the Droseraceae family which only recently is considered part of the order Nepenthales [10]. Droseraceae was moved into the Nepenthales from the order Caryophyllales. The order Nepenthales, comprising a carnivorous and non-carnivorous clade, is characterized by the frequent presence of acetogenic napthoquinones and by the lack of betalain pigments that characterize their sister group Caryophyllales [9,10]. In the Caryophyllales order, betalains substitute the otherwise ubiquitous anthocyanins [11,12], which are the dominant form of pigmentation across land plants [13,14]. Betalains and anthocyanins are two types of water

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soluble pigments with analogous coloration and similar functions [15]. Anthocyanins and betalains are mutually exclusive and have never been found together in the same plant [16,17]. The origin of this exclusion is unclear and only recently are investigations beginning to clarify how betalains could have arisen [14]. In the Caryophyllales, only the coloration of the Caryophylaceae and Molluginaceae is due to anthocyanins [18,19], and these are the only two exceptions identified to date [20]. Droseraceae is a family of carnivorous herbs in the Nepenthales considered as "non-core Caryophyllales" (APG IV 2016) [9,21]. *Dionaea* is a monotypic genus, comprising the sole extant especies *Dionaea muscipula*. Complex evolution has recently been described for carnivorous plants. Contemporary researchers have used molecular systematics to demonstrate that carnivory evolved independently among flowering plants at least 10 times. However, carnivory likely arose once in the Nepenthales [10,22]. Unambiguous determination of pigments present in *Dionaea* may help in the taxonomic and evolution analysis of the plant.

Betalains are water-soluble, nitrogen containing pigments. These are divided into two groups: the violet betacyanins and the yellow betaxanthins. Glycosylation and acylglycosilation of one or two hydroxyl groups are possible in betacyanins, and complex pigment structures can be obtained [23–25]. In contrast, betaxanthins are yellow and no glycosylation has ever been reported. Both groups share betalamic acid as the structural and chromophoric unit. It is condensed with amines and amino acids in betaxanthins and with *cyclo*-DOPA in betacyanins [26]. Anthocyanins are also water-soluble plant pigments, but have no biosynthetic or structural relationship with betalains. They are responsible for the red, purple and blue tones of flowers and fruits of most of the plants. These pigments are flavonoids that are derived from the shikimic acid pathway [27]. Anthocyanins, like betacyanins, are constituted by an aglycone, which is anthocyanidin, to which a sugar is bound by a beta-glycosidic bond. The chromophoric aglycones (anthocyanidins) are red polyhydroxylated salts, which are seldom found in their free form in plant tissues [28]. Despite the structural and biosynthetic differences between betalains and anthocyanins [14], their distribution within the plant and their functions, both vegetative and reproductive, are essentially identical [16]. The two pigments are found in fruits, flowers, leaves, stems and roots [19,29]. In addition, the two pigments have a high antiradical capacity and are potent antioxidants in plants [19,30].

In spite of the abundant scientific literature on the pigments of betalains and anthocyanins, pigments of *Dionaea muscipula* Ellis have been poorly characterized, with limited references. A first article reports the analysis of its pigment (only one compound was found) through paper chromatography and absorption spectra, identifying a single colored compound with similar properties to cyanidin-3-glucoside [28]. The second, and last, study was carried out using plants grown *in vitro* in culture medium. It exhibited a second pigment, tentatively identified as delphinidin-3-*O*-glucoside [2]. This last article does not use plants under natural conditions and does not show structural evidence of the nature of the pigments. Furthermore, in both articles the red pigment was obtained from whole leaves, while it is known that the coloration is restricted to the digestive glands [31].

This paper aims to evaluate the existence of pigments, anthocyanins and betalains in the species *Dionaea muscipula* Ellis, through the use of modern and sensitive techniques like mass spectrometry and high resolution liquid chromatography (HPLC). Glands were extracted in order to give higher accuracy to the previous partial results. The exclusion from its former phylogenetic order is considered in terms of precise pigments analysis and discussed in relation to anthocyanins pigmentation in the Nepenthales order.

2. Materials and methods

2.1. Chemicals

Chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). Solvents were from Merck Chemicals Ltd. (Dorset, England). HPLC-grade acetonitrile and methanol were purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Bedford, MA, USA).

2.2. Plant material

Dionaea muscipula Ellis plants were obtained from "Viveros Murcia" (Murcia, SE Spain), selecting those that had a deep red color inside the surface of the snap traps.

2.3. Glands extraction

A new extraction system was honed to obtain the molecules responsible for the red color, confined in the glands. A scalpel was used and the inner surface of the leaf was scraped to obtain the glands containing the plant pigment. A Leica-Z6-APO macroscope was used to verity the success of the separation of the glands from the snap trap. Between 4 and 6 leaves of the plant were used for the collection of the glands by assay. After separation, glands were placed into eppendorf tubes and weighed, obtaining weights around 1.6 mg per assay. In addition to digestive gland extracts, samples of mature stem and flowers were obtained. Extracts of the flowers were made from petals and in the case of the mature stem, the reddish epidermis found at the base of the stem was extracted. Mature stems were considered to be fully developed when holding open flowers.

2.4. Preparation of extracts

For the disaggregation of the samples (digestive glands, epidermis of the base of the mature stems and flower petals) and for the extraction of the pigments to be analyzed, a solution of methanol and hydrochloric acid in a 1000: 1 ratio was used, for 4 h [2,31]. A glass stirring rod was used to help the disaggregation and grinding of the glands. In this way pigments were extracted and kept stable. Preliminary analyses were also performed with acetate buffer pH 5.0, 20 mM containing 10 mM ascorbic acid. The samples were then centrifuged for 5 min at 14,000 rpm. The supernatant was used for further analysis by reversed phase chromatography (HPLC) and spectrophotometry. Extracts were repeated with 100% methanol [32] and equivalent results were found.

2.5. Standard anthocyanins and betalains

Cyanidin chloride, delphinidin chloride, kuromanin and myrtillin from Sigma-Aldrich (St. Louis, EEUU) were used as standard anthocyanins. Known pigments extracted from characterized plant sources [33], were used as standard betacyanins. Betanin was obtained from roots of *Beta vulgaris* and betanidin was obtained from violet flowers of *Lampranthus productus* [34]. All compounds were characterized spectrophotometrically, chromatographically, and by electrospray ionization mass spectrometry (ESI-MS/MS).

2.6. UV–vis spectroscopy

A V-630 spectrometer (Jasco Corporation, Tokyo, Japan) attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain) was used for UV–vis spectroscopy. For quantitation of anthocyanins, pigment concentration was evaluated using molar extinction coefficients of $\varepsilon = 34{,}000\ M^{-1}\,cm^{-1}$ at 530 nm for cyanidin chloride, $\varepsilon = 30{,}200\ M^{-1}\,cm^{-1}$ at 530 nm for kuromanin, and $\varepsilon = 29{,}000\ M^{-1}\,cm^{-1}$ at 543 nm for myrtillin. The baseline was made with methanol and