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The systematic and evolutionary significance of phytochemical variation in the Eurasian sedoideae and sempervivoideae (crassulaceae)

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

1995

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Stevens, J. F. (1995). *The systematic and evolutionary significance of phytochemical variation in the Eurasian sedoideae and sempervivoideae (crassulaceae)*. Ponsen & Looijen BV, Wageningen.

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SUMMARY

Approximately 120 species of the stonecrop family (Crassulaceae) from the Utrecht Botanic Gardens were examined for the presence of the three main classes of secondary metabolites (alkaloids, flavonoids, and terpenoids) with the aim of determining their systematic and evolutionary value. In our studies, emphasis lay on the phytochemistry of the Eurasian Sedoideae and Sempervivoideae, two subfamilies of the Crassulaceae which are thought to be closely related. For many species included in our phytochemical surveys, data from morphological, cytological, hybridization, and molecular studies were available for comparison with our chemical data to assess their systematic and evolutionary value. After all, hypothetical evolutionary scenarios gain probability by correlation of characters with others.

Before we could make a start with the phytochemical surveys, fast and reliable analysis methods had to be developed which are suitable for the examination of small samples of cultivated plant material. Thin-layer chromatography was used for the detection of the classes of secondary metabolites with selective spray reagents. For the identification of individual compounds, gas chromatography and coupled gas chromatography-mass spectrometry was performed on a routine basis. Plant constituents that could not be analysed with gas chromatography were examined with high-performance liquid chromatography in combination with diode array detection. As far as possible, the presence of putative compounds was confirmed by chromatographic comparison with authentic markers, which were purchased from commercial sources, isolated from a readily available natural source, or prepared chemically.

Fifty-five species of *Aeonium*, *Bryophyllum*, *Crassula*, *Echeveria*, *Greenovia*, *Kalanchoe*, *Pachyphytum*, *Sedum*, and *Sempervivum* were investigated for the presence of alkaloids. Only *Echeveria venezuelensis* and species of *Sedum* section *Sedum* were found to contain alkaloids which were identified as pyrrolidines and piperidines. Pyrrolidines had not been reported from species of Crassulaceae, while a number of new piperidine alkaloids bearing C₄ and C₅ side chains were detected in Macaronesian Sedoideae. The distribution of the alkaloids is in good agreement with a phylogeny of the family based on molecular characters: alkaloids are restricted to species belonging to the so-called *Acre* lineage, which also includes the central American genus *Echeveria*. The structural diversity of the alkaloids proved to be of systematic value at the series level within *Sedum*. The studies are described in chapters 3, 4, and 5.

The ecological function of alkaloids as feeding deterrents is well-documented. Species of Crassulaceae lacking alkaloids seem to rely on condensed tannins (proanthocyanidins) and galloyl esters in their defence against herbivore pests and pathogens. As evolution proceeds by the gradual transition from general quantitative (e.g. tannins) to specific qualitative (e.g. alkaloids) defence agents, the presence of alkaloids in the Crassulaceae most probably represents the derived rather than the primitive condition, which is in agreement with a molecular phylogeny of the family but not with the apparently primitive floral structures of the *Acre* clade species, however. Another aspect of the alkaloid/tannin dichotomy concerns the

occurrence of proanthocyanidins which is frequently associated with the woody habit of higher plants. In various angiosperm lineages the transition from woody to herbaceous growth-forms is often accompanied by loss of proanthocyanidins. The correlation between the presence of proanthocyanidins and woodiness, however, is completely absent in the Crassulaceae. This observation lends support to the hypothesis that the woody taxa of the Crassulaceae are derived from herbaceous ancestors.

Leaf tissue flavonoids in acid hydrolysates of 30 species of *Sedum* and 35 species of *Sempervivum* (including *Jovibarba heuffelii*) were qualitatively and quantitatively determined by gas chromatography after trimethylsilylation. Ten flavonol aglycones were detected and identified as kaempferol, quercetin, isorhamnetin, and myricetin, as well as their 8-hydroxy and/or their 8-methoxy derivatives. The flavonoid variation proved to be of little evolutionary value due to a high degree of parallel evolution. By contrast, multivariate analysis of quantitative flavonoid data furnished useful systematic information. *Sempervivum* could be distinguished from *Sedum* by the dominant presence of kaempferol in the former. The uniform flavonoid patterns of species of *Sempervivum* reflect the similarity in ecological preference of the species as well as the morphological uniformity of the genus. The flavonoid chemistry of *Sedum* is much more diverse. Hydroxylation and methoxylation of the 8-position of common flavonols is widespread in Eurasian *Sedum*, which seems to mirror the enormous morphological and cytological variation present in this taxon. This flavonoid survey is described in chapter 6.

The exudate (externally accumulated) flavonoids of the predominantly Macaronesian genus *Aeonium* are the subject of chapter 7. The excretion of free flavonoids, usually in highly methylated form, together with other lipophilic constituents is frequently encountered in species from (semi)arid habitats. In leaf exudates of 32 species of *Aeonium*, a total of 32 flavonoids were identified in about half of the species. The exudate flavonoid patterns are by and large in agreement with infrageneric classifications based on morphological and molecular characters. Methoxylation of the 6-position of flavonoids is typical of some species of *Aeonium*. A molecular phylogeny of the genus points to a unique gain of this feature early in the evolution of the genus and subsequent loss of it in various lineages. A similar explanation is proposed for the distribution of the methylethers of myricetin which have only been retained in the sections *Goochia* and *Petrothamnium* of *Aeonium*. It is generally understood that the variation in growth-forms in *Aeonium* evolved through adaptive radiation. The Macaronesian species are primarily single island endemics and occupy a wide range of different ecological niches. The presence of exudate flavonoids shows no obvious correlation with habitat, however.

The epicuticular waxes from 30 species of *Sedum* were also analysed with the aim of determining the systematic and evolutionary value of wax constituents (chapter 8). The waxes of *Sedum* consist of alkanes, alcohols, fatty acids, aldehydes, alkyl esters, and pentacyclic triterpenes. Of the aliphatic compounds, only the alkanes had some systematic value. Multivariate analysis of the alkane profiles

resulted in three groups which could be related to the infrageneric classification of *Sedum* based on biosystematic data. Triterpenes are abundantly present in waxes from glaucous and pruinose leaves. Comparison of wax composition and wax ultrastructure of glaucous and glossy plants led to the conclusion that triterpenoids are primarily responsible for glaucousness in *Sedum*. To some extent, the triterpene variation agrees with the infrageneric classification of *Sedum*. A number of series, including *Sedum* series *Alpestris* and *S. series Rupestris*, could be distinguished by a unique combination of triterpenes. Triterpenyl formates (not previously reported from a natural source) are characteristic of the latter series. In a more extensive study of the waxes of the seven species of *S. series Rupestris* (chapter 9), triterpene variation supported a molecular phylogeny of this taxon.

Chapter 10 consists of a synopsis of the chapters 3-9 and a general discussion of the main results from a taxonomic point of view. In conclusion, the phytochemical approach in helping solve taxonomic problems made a significant contribution to the present ideas of the evolutionary relationships within and between the Eurasian Sedoideae and Sempervivoideae which will see implementation in a revised intrafamilial classification of the Crassulaceae.

Uitkomst van een evolutionaire hypothese van het licht van de verwantschapen met elkaar in overeenstemming zijn.

Alvorens een begin kan worden gemaakt met het beschrijven van de plantestoffen in soorten van de volkscin, werden enkele en beschrijvende methoden ontwikkeld voor de analyse van kleine hoeveelheden gekweekt vegetatief plantemateriaal. Damschlagchromatografie werd gebruikt voor de detectie van stofgroepen tertiairalkalen, flavonoiden, terpenoiden met aldehyd en spulmateriaal. Gaschromatografie is dan niet in combinatie met massaspectrometrie werd op grote schaal gebruikt voor de scheiding en identificatie van individuele componenten. Voor stoffen die niet makkelijk gechromatografie worden worden gebruikt voor een beroep gedaan op hogedruk-vloeistofchromatografie in combinatie met diode-array detectie. De aanwezigheid van verwante verbindingen werd zoveel mogelijk bevestigd door chromatografische vergelijking van plantextracten met referentiestoffen, welke werden bereikt van gespecialiseerde firma's of, indien niet verkrijgbaar, werden verkregen door isolatie uit plantemateriaal of chemische synthese (alkaloiden). Middels de ontwikkelde analytische methoden werden meer dan 50 plantestoffen in verschillende soorten aangetoond.

Vertegenwoordigers van de geslachten *Arrostia*, *Bryophyllum*, *Crossula*, *Echeveria*, *Grevillea*, *Kalanchoe*, *Pachyphyllum*, *Sedum* en *Sempervivum* (in totaal 55 soorten) werden onderzocht op de aanwezigheid van alkaloiden. Alleen *Echeveria renardii* en soorten van *Sedum* welke heten in staat alkaloiden te accumuleren, ze konden worden geïdentificeerd als pyrrolidine en piperidine alkaloiden. Pyrrolidines waren nog niet eerder aangetoond in de verpluimfamilie, terwijl een aantal nieuwe tertiaire piperidines met zijketen bestaande uit 4- en 5- koolstofatomen werden aangetoond in *Sedum* soorten van de Canarische eilanden en Madeira. De verspreiding van de alkaloiden stemt uitstekend overeen met een stamboom van de verpluimfamilie welke is gebaseerd op