

# Succulent protocol for the DBGI

Molecular cartography of the Botanical Garden of the University of Fribourg

Lëndita Schwegler

Bachelor thesis at the Department of Biology, University of Fribourg

Under the direction of Dr. Pierre-Marie Allard

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## Abstract

This paper presents the development of a digitisation protocol to capture the chemical structure of succulents as part of the Digital Botanical Gardens Initiative (DBGI) project. Succulents, with their unique adaptation to dry conditions, present challenges in sample preparation. The protocol outlines the steps involved in collection, preparation, classification using iNaturalist, extraction, and mass spectrometry analysis. The descriptive results include an overview of the samples collected, classifications based on order, family, and genus, and an identification of unclassified samples. In terms of the actual protocol, the paper suggests the use of a red arrow to highlight the sampled plant, explains that there is no need to excessively cut a sample, stresses the need to use three cycles of lyophilisation, and shows that certain classification tasks cannot be outsourced and must be performed by the researcher.

# 1 Introduction

The following paper and the work behind it are a continuation of the main project of the Digital Botanical Gardens Initiative (DBGI Consortium 2022; see here). The DBGI explores innovative digital solutions for managing information about living botanical collections. One focus is on characterising chemodiversity through mass spectrometric approaches to improve our understanding of ecosystems and contribute to biodiversity conservation.

There are at least two reasons why such a project could help preserve biodiversity: Firstly, research into chemical characterisation can help in the development of new drugs. Such discoveries then lead to a new reason to conserve biodiversity (i.e. to protect ecosystems). Secondly, understanding chemistry helps us to understand an ecosystem better: what would be potentially dangerous or particularly important if we wanted to protect something.

Developing such a project in the context of a botanic garden offers a head start: The DBGI can use the living collections of the Swiss Botanic Gardens to develop reliable workflows for digitising biodiversity. They provide all the infrastructure to test different methods, which can then be scaled up. In addition, the gardens have a large collection of different species: They are artificial biodiversity hotspots. The ultimate goal is to be able to extend the developed workflows globally to wildlife ecosystems (cf. The Earth Metabolome Initiative, EMI, see here).

As part of the DBGI, various sampling methods and storage techniques have already been tested and established (Brülhart 2022; see here). The next step was to develop a suitable protocol for a particularly demanding organism: succulents. The development of this protocol is presented and documented in this paper. This includes aspects of processing, digitisation, and the use of Open Science. In addition, the newly developed protocol provided an opportunity to further test the already established workflows.

## 2 Material & method

As briefly mentioned, succulents were the focus of my work. These plants present a particular challenge for the digitisation process used in the DBGI project so far. Succulents are plants adapted to extremely dry conditions (see Griffiths and Males 2017). They are characterised by their fleshy leaves, stems, or roots that serve to store water. Succulents come in a variety of shapes, sizes, and colours and they belong to a large family of plants that includes cacti, agaves, aloes, and echeverias.

Because of their ability to store liquids, succulents use water efficiently and can survive for long periods without watering. However, this characteristic meant that the plant posed a challenge for the DBGI project: It was not clear, whether the usual drying process would work that has been used to prepare samples for the mass spectrometry. Therefore, we wanted to develop a new protocol for succulents which is explained here.

### 2.1 Procedure to collect and prepare samples

The procedure to collect and prepare the succulent samples involved the following six steps: (1) The first step was to locate a point in the botanical garden using an app: QField (see here). The app contains a map of the botanical garden, which helped to determine the location of a specific succulent. The point that was determined in the app was then used over and over again as a frame of reference for further metadata. Prior to the development of this app, it was not possible to locate the geographical point – but this is now possible.

(2) In a second step, an identification number for the plant was given in relation to the geographical point in the app (i.e. “dbgi\_000970”). Then, each sampling point was provided with five photographs: (i) a photo of the panel (plant label in the botanical garden), (ii) a photo of the whole plant and its surroundings with a red arrow pointing to the plant, (iii) a detailed photo e.g. of flowers/fruits/seeds/leaves, (iv) a photo of the cut where the sample was taken, and (v) a photo of the sample plastic tube with the assigned identification

number as well as a QR-code (again, representing the identification number). These images were not only taken to document the sampling process. In addition, they were used for the iNaturalist website (see below).



Figure 1: Five pictures of collection process

The red arrow in the third photo is a new technique we have developed for this project. It allowed us to do the following: to identify the plant from which the sample was taken, but also to look at its surroundings. This can ensure that the same plant can be found again at a later date (particularly when such a process is taken to the wildlife).

- (3) The third step is to separate and prepare the cut-off part of the plant shown in the fourth photo: The sample is wrapped in a coffee filter and placed in a plastic tube. The purpose of the coffee filter is to catch any leaking liquids and to speed up the drying process (see Funk et al. 2017, p. 12). As part of the developing the succulent protocol, we tried out a technique of further cutting the samples into small pieces and checked, whether this could help with the drying process.

**It is apparent that further cutting of a sample into small pieces is not necessary and doesn't help the drying process.**

- (4) The sample is then stored in liquid nitrogen. Through this process, the material is quickly and uniformly frozen prior to the actual drying process (see below).<sup>1</sup> This has two advantages: First, it minimises stress reactions in the plant sample caused by the cut. Otherwise, chemical reactions and metabolic processes in the plant could be triggered, potentially altering the original chemical structures of the sample. Secondly, the cut-off part is transferred to a solid state in preparation for the following steps.

- (5) The fifth step is intermediate storage at -80°C for at least one hour. On the one hand, this is to ensure that the sample is completely frozen and therefore solid. On the other hand, this intermediate storage also allows a possible bridging of time: the time when the central machine, the freeze dryer or lyophiliser, is not available (because another scientist may be using it).

<sup>1</sup>Silica gel is very suitable for processing in difficult areas where liquid nitrogen is not available (see Brülhart 2022). Silica gel, also known as desiccant, is used during the drying process to absorb moisture from the ambient air. The difference to liquid nitrogen (followed by lyophilization) is that silica gel takes about a month to dry the samples.

(6) The samples are then placed in the freeze dryer. The lyophilisation process that takes place removes water from the biological material: It is pressurised, which causes the water to pass directly from the solid to the gas state, and the sample is dried under vacuum. This preserves the structure and properties of the material and makes it durable. The samples collected so far in the project could be freeze-dried in one cycle of 24 hours (Brülhart 2022; see here).

When testing out the process for the succulents, we immediately went with 48 hours (i.e. two cycles). It turned out that half of the samples were not completely dry after this time. In these unsuccessful cases (see picture on the left), the coffee filter was still soft and wet, while the samples were squishy and liquid escaped them when pressure was applied. In the successful cases (see picture on the right), the filter was completely hard and the samples crumbled when touched.



Figure 2: Comparison of samples

**To ensure that all succulent samples are completely dry, three cycles or 72 hours in the lyophiliser are necessary.**

After the preparation of the sample and the successful lyophilisation, it is not always possible to check the samples immediately. To ensure flexibility, the samples can be stored again at -80°C. This protects the material from reactions that would make further work impossible.

## 2.2 Classification of samples with iNaturalist

After sampling in the field, the collected plants are further classified. This is done using the iNaturalist platform. The website and app are used to document observations in wildlife and cultivated settings. It allows users to upload and share observations of plants and animals. Each sample was therefore recorded using iNaturalist: The name of plant, date of sampling, coordinates, and the five images taken were imported.

For this project, iNaturalist was not only used to document the observations. The platform was also important as other users were able to classify the plants. Through this, an effortless extension of a classification to order, family, genus, and species was possible. Also, the platform's user double checked the classification done so far.<sup>2</sup>

<sup>2</sup>There were some wrong classifications on our end. Some plants grew in a way that the panels in the botanical garden were “wrong”, so to speak. Here, the platform helped to double check.

## 2.3 Extraction

In order to obtain the chemical structure of the succulents, an extraction procedure took place first. It consists of the following nine steps (Brülhart 2022; see here):

1. Weighting: For each sample, 50 micrograms of dried plant material are weighted (error of 5%/±2.5 micrograms is possible).
2. Filling: The weighted plant samples are filled into Eppendorf tubes with rounded bottoms (2 ml), together with three 4 mm metal beads.
3. Labelling: Each sample tube is labelled with the previous identification number and QR-code.
4. Milling: The samples are then milled in a Retsch machine at 25 Hz for 2.5 minutes until a powder is formed.
5. Addition of solution: 1,7 ml of a mixture of 80 % methanol, 20 % distilled water and 0,1 % formic acid is added to the samples. This mixture is mixed in the Retsch machine at 25 Hz for 2,5 minutes.
6. Centrifugation: The tubes are then centrifuged in a centrifuge machine for 2 minutes at 13,000 rpm.
7. Supernatant collection: Approximately 1.4 ml of supernatant is removed and transferred to 1.5 ml tubes, which are sealed with unsplit caps.
8. Storage: The final samples are stored again at -80°C.
9. Control: A control process was performed using three different techniques to ensure the success of the procedure (Brülhart 2022; see here).

## 2.4 Mass spectrometry analysis

The extracted material was then analysed via mass spectrometry. This allowed the chemical composition of the plant samples to be determined by measuring the mass and charge of the molecules contained in the sample. As part of this paper, however, no chemical structure is analysed or further discussed.

## 3 Results

This section provides an overview of the 138 samples collected. The classifications from the iNaturalist platform are mainly used before a final comparison with the Lotus-Frozen dataset (Rutz et al. 2022) takes place. According to the community classifications on iNaturalist, 116 different species of succulents were sampled. One species was sampled three times (“Pachysedum”) and five species were sampled twice each (“Aloes and allies”, “Senecio”, “Haworthia cymbiformis”, “Robertson Oxtongue” and “Window Haworthia”).

**In addition to the 116 classified species, 16 samples were not classified by the iNaturalist community and were not featured in downloaded data. Therefore, some samples need to be classified manually/by the researcher herself.**

There seem to be a few reasons why these classifications were missing. The following two were fairly obvious (but there may be others): Firstly, endangered species were missing from the dataset. Secondly, only known cultivated species (i.e. cultivated in a botanical garden) were not classified by the iNaturalist platform.

The missing classifications belong to: (1) Kalanchoe daigremontiana Raym. - Hamet et H. Perrier (dbgi\_000904), (2) Echeveria x titubanus (dbgi\_000923), (3) Echeveria x pulchella A. Berger (dbgi\_000924), (4) X Astroworthia bicarinata (Haw.), G.D. Rowley (dbgi\_000935), (5) Pelargonium triste

(L.) L'Hr. (dbgi\_000937), (6) Haworthia viscosa (L.) Haw. (dbgi\_000950), (7) Pelargonium 'Dimeierii Lila' (dbgi\_000953), (8) Crassula deceptor Schnland et Baker f. (dbgi\_000957), (9) Pelargonium klinghardtense R. Knuth (dbgi\_000980), (10) Pelargonium tongaense Vorster (dbgi\_000982), (11) Argania spinosa (L.) Skeels (dbgi\_000986), (12) Sedum mexicanum Britton (dbgi\_000996), (13) Pleiospilos compactus (Aiton) Schwantes subsp. compactus (dbgi\_000997), (14) Zephyranthes andersoniana Benth. et Hook. f. (dbgi\_001000), (15) Pelargonium 'Lavender Lad' (dbgi\_001002), and (16) Tischleria peersii Schwantes (dbgi\_001119).

### 3.1 Classification overview

The following three barplots give an overview of the sampled specimens based on the classifications “order”, “family” and “genus”.

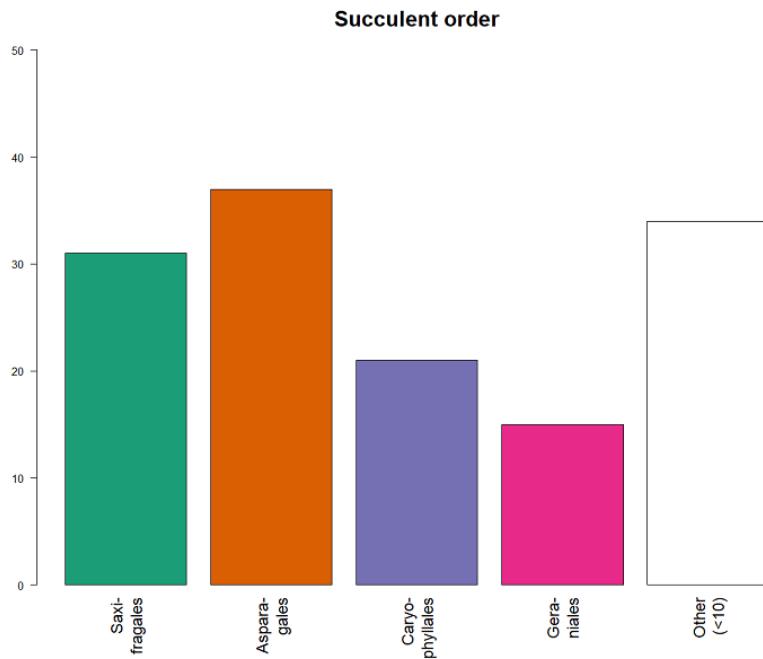


Figure 3: Overview of sampled succulent, order

Regarding the first classification, it is clear that most of the sampled succulents belong to the order “Saxifragales”, closely followed by “Asparagales” with 37 and 31 specimens respectively. “Caryophyllales” and “Geriales” are represented by 21 and 15 plants respectively. Then there are “Other”-order classifications that occur less than 10 times, such as “Rosales”. In total, 16 different orders have been assigned by the iNaturalist community.

The second barplot shows the classification by family. Here “Crassulaceae” and “Asphodelaceae” occur most frequently with 36 and 22 times respectively. Then four different families were sampled 8 to 10 times, such as “Geraniaceae”. The category “Other” now includes those families that were sampled less than 8 times. In total, 24 succulent families were sampled.

In terms of genus, 61 different classifications were found. Important: Here the iNaturalist community did not allow a complete classification and the genus of 5 succulents could not be determined (“NA” on the plot’s far right). “Pelargonium” was the most common with 15 samples. On the right side of the graph “Aeonium”, “Euophoriba”, “Haworthia”, “Pachyphytum”, and “Sedum” were sampled 5 times each. The category “Other” with less than 5 occurrences was sampled a total of 77 times, but for presentation purposes only a range up to 50 is shown.

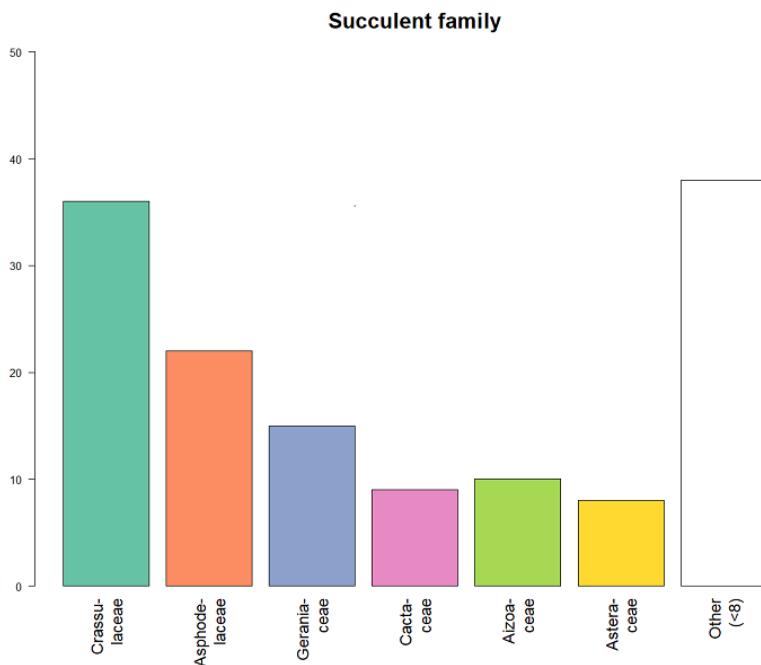


Figure 4: Overview of sampled succulent, family

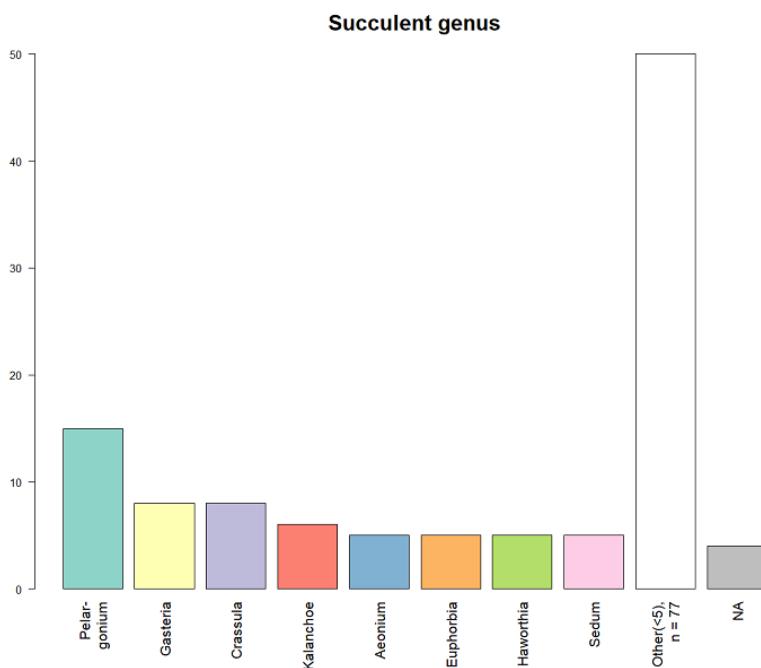


Figure 5: Overview of sampled succulent, genus

### 3.2 Comparison with Lotus-Frozen dataset

The collected samples were further compared with the Lotus-Frozen dataset (Rutz et al. 2022). The aim here was to be able to determine from which of the succulents data on chemical structures had already been collected and for which new information could be provided within the framework of this project.

The literature dataset already contains data on the following 21 samples: (1) *Zephyranthes candida* (Lindl.) Herb. (dbgi\_000912), (2) *Zephyranthes minuta* (Kunth) D. Dietr. (dbgi\_000999), (3) *Carpobrotus edulis* (L.) Bolus (dbgi\_000993), (4) *Haemanthus albiflos* Jacq. (dbgi\_000972), (5) *Canarina canariensis* (L.) Vatke (dbgi\_000956), (6) *Beaucarnea recurvata* Lem. (dbgi\_001019), (7) *Cotyledon orbiculata* L. var. *orbiculata* (dbgi\_000952), (8) *Lavandula canariensis* Mill. (dbgi\_000906), (9) *Senecio crassissimus* Humbert (dbgi\_001007), (10) *Veltheimia capensis* (L.) DC. (dbgi\_000943), (11) *Kalanchoe fedtschenkoi* Raym.-Hamet et H. Perrier (dbgi\_001036), (12) *Kalanchoe tubiflora* Eckl. et Zeyh. (dbgi\_000963), (13) *Euphorbia resinifera* O. Berg (dbgi\_000505), (14) *Pelargonium fulgidum* (L.) L'Hr. (dbgi\_001018), (15) *Xerosicyos danguyi* Humbert (dbgi\_000968), (16) *Simmondsia chinensis* (Link) C.K. Schneid. (dbgi\_000905), (17) *Opuntia microdasys* (Lehm.) Pfeiff. (dbgi\_000948), (18) *Oscularia deltoides* (L.) Schwantes (dbgi\_000958), (19) *Eriocephalus africanus* L. (dbgi\_000994), (20) *Sansevieria cylindrica* Bojer (dbgi\_000512), and (21) *Kalanchoe daigremontiana* Raym. - Hamet et H. Perrier (dbgi\_000904).

## 4 Discussion & acknowledgement

The additions this project made in terms of developing a new protocol for succulents have been highlighted in the “2. Materials & methods” section and in the introduction to the “3. Results” section. These were the following: (i) a red arrow can be used to highlight the sample plant in the wild; (ii) further cutting of a succulent sample is not necessary; (iii) three cycles (72h) in the lyophiliser are necessary to freeze dry the succulents; and (iv) some classification work cannot be outsourced to iNaturalist but has to be done by the researcher. Now that only descriptive results have been presented, another possibility can be discussed with the actual data – the chemical structures.

As mentioned above, a method of further cutting the samples was tested. Although it's not necessary for the actual protocol to do this, a comparison is now possible. There are two times five samples of certain succulents, one sliced, one whole.<sup>3</sup> For these five succulents it can be tested whether further cutting of a sample changes the chemical structure of a plant as a metabolic stress response.

Last but not least: I would like to thank my supervisor, Dr. Pierre-Marie Allard, for giving me the opportunity to participate in his project, and I would also like to thank my colleague Edouard Brülhart for his guidance and support throughout the project. I would also like to thank all the members of this project for the informative team meetings and the friendly atmosphere!

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<sup>3</sup>These five samples were (1) dbgi\_000921: sliced and dbgi\_000919: not sliced, (2) dbgi\_000992: sliced and dbgi\_001013: not sliced, (3) dbgi\_000991: sliced and dbgi\_000913: not sliced, (4) dbgi\_000915: sliced and dbgi\_001017: not sliced, (5) dbgi\_000922: sliced and dbgi\_000998: not sliced.

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