

**Genetic diversity of *Patellifolia* species  
(GeDiPa)**

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From left to right. The first four pictures show morphological variation within a *P. procumbens* occurrence growing on a site in northern Tenerife. The fifth picture was taken in La Palma and shows *P. patellaris*. © L. Frese, JKI.

**July 2017**

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**Final Activity Report**

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**CONTENTS**

<b>INTRODUCTION .....</b>	<b>1</b>
<b>APPROACH.....</b>	<b>1</b>
Project workshop .....	1
Gap analysis .....	2
Sampling.....	2
Development of SSR markers.....	3
Diversity analysis .....	3
Genetic markers .....	3
Cytological analysis .....	5
<b>RESULTS.....</b>	<b>6</b>
Taxonomic standards.....	6
Gap analysis and collection to close gaps .....	7
Sampling.....	8
Development of SSR markers.....	8
Diversity analysis .....	9
Genetic diversity .....	9
Cytological diversity.....	12
<b>RECOMMENDATIONS .....</b>	<b>12</b>
<b>FINAL CONCLUSIONS.....</b>	<b>14</b>
<b>BIBLIOGRAPHY .....</b>	<b>14</b>
<b>ACKNOWLEDGEMENTS.....</b>	<b>15</b>
<b>ANNEXES .....</b>	<b>16</b>
Annex 1. Gap analysis and recommendation of MAAs .....	17
1a. <i>P. procumbens</i> .....	17
1b. <i>P. webbiana</i> .....	18
1c. <i>P. patellaris</i> .....	19
Annex 2. List of MAA candidates .....	20
Annex 3. Overview on collected leaf and seed samples .....	21

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# Genetic diversity of *Patellifolia* species (GeDiPa)

## Final Activity Report

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### INTRODUCTION

Although the genus *Patellifolia* (syn. *Beta* section *Procumbentes*) consists of only three recognized species (Scott et al. 1977), several taxonomic problems still need to be solved. Wagner et al. (1989) doubted that *Patellifolia webbiana* (Moq.) A. J. Scott, Ford-Lloyd & J. T. Williams and *Patellifolia procumbens* (Smith) A. J. Scott, Ford-Lloyd & J. T. Williams are distinct diploid species. Thulin et al. (2010) proposed to treat all variants within the genus as a single variable species, called *Patellifolia procumbens*. *Patellifolia patellaris* (Moq.) A. J. Scott, Ford-Lloyd & J. T. Williams was considered a tetraploid species and thus readily distinguishable from the diploid species until the detection of triploid forms within a *P. patellaris* locality. A survey of herbarium specimens carried out at a later stage of the GeDiPa project underpinned the need for investigations into the variation patterns of the species and the need for a reliable taxonomic key to the species. A sound classification of collected material is at the core of any genebank quality management system as stipulated by the AEGIS Quality System (AQUAS). Therefore, this action aims at a better understanding of the genetic diversity within the genus *Patellifolia* and at the establishment of taxonomic standard accessions to facilitate the classification of genebank accessions.

As *Patellifolia* species are very difficult to use for the genetic enhancement of cultivated beets, this action also aims to establish a small but representative set of the Most Appropriate Accessions (MAAs). For that purpose, passport data from genebank holdings were compiled and the spatial distribution of the species was reviewed to enable the identification of MAAs for AEGIS and Most Appropriate Wild Populations (MAWPs) for the settlement of genetic reserves, following the MAWP concept described by Maxted et al. (2015).

As announced in the interim report published in January 2016 (covering the period February to December 2015), this final report provides the results of the genetic diversity study undertaken in 2016.

### APPROACH

#### Project workshop

A project workshop hosted by the Universidad Rey Juan Carlos, Madrid (Spain) took place from 16 to 18 February 2015. During the meeting, a detailed guideline for data documentation and sampling of material for genetic and cytological analysis was discussed and agreed. Examples considered typical for *P. procumbens*, *P. webbiana* and *P. patellaris* (Figure 1 a, b and c), were selected to facilitate the categorization of collected material.

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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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**Figure 1a.** *P. procumbens* (Smith) A.J.Scott, Ford-Lloyd & J.T.Williams. © L. Frese, JKI (left), A. Santos Guerra (right).



**Figure 1b.** *P. webbiana* (Moq.) A.J.Scott, Ford-Lloyd & J.T.Williams. © for both photos: L. Frese, JKI.



**Figure 1c.** *P. patellaris* (Moq.) A.J.Scott, Ford-Lloyd & J.T.Williams. © for both photos: L. Frese, JKI.

### Gap analysis

Information on the geographic distribution of *Patellifolia* was extracted from the literature and from the information systems. This gap analysis yielded a summary table (Annex 1, pages 17-19) with the three species (*P. procumbens*, *P. webbiana* and *P. patellaris*) and 30 geographic subunits. The potential distribution area was checked against known findings of occurrences.

### Sampling

The GeDiPa partners marked out the areas where *Patellifolia* can occur and organized four field missions to collect samples. The interest was to locate the places and validate the literature data or the data documented in the information systems. Samples were collected in south-eastern Spain, Tenerife (Canary Islands), mainland Portugal and on the archipelagos of Madeira and Cape Verde. If

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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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mature seeds were available at the time of harvesting leaf samples (for genetic and cytological analysis), these were collected to cover geographic gaps in the European *Patellifolia* holdings. At the same time, leaf samples of approximately 800 individual plants in the natural habitat were harvested and prepared for SSR marker analysis. Also fresh leaves were taken from the same plants and were sent to the Centre for Functional Ecology (Department of Life Science, University of Coimbra) for flow cytometric analysis of the ploidy level of each individual, enabling to evaluate the possibility for the occurrence of hybridization between *P. patellaris* and *P. procumbens* by the detection of triploid individuals at areas where the two species occur in close proximity.

In addition to data on individual plants and probes, passport data on populations (population identifier) and seed collections (collection number) were also recorded, and further sent to the Julius Kühn-Institut (JKI), Quedlinburg (Germany) and compiled. During the collecting mission to southeast Spain all individual plants sampled were geo-referenced.

The sampling procedure was as follows:

- For the molecular analysis, fresh leaf probes of 0.5-1 g from 20 (minimum) to 40 (maximum) individual plants were sent to the Julius Kühn-Institut, Quedlinburg (Germany).
- For the flow cytometric analysis, a branch with at least three leaves per individual was sent to S. Castro, Centre for Functional Ecology, Department of Life Science, University of Coimbra (Portugal).
- For the herbarium specimens, at least two flowering plants were sent to M.C. Duarte, Faculty of Sciences, University of Lisbon (Portugal), for later identification.
- Seed samples were sent to the national genebanks (PRT001, ESP004) and ISOPlexis Genebank (PRT102) at the University of Madeira.

### Development of SSR markers

Microsatellite primers were developed to promote studies on the relationship between the three species of the genus *Patellifolia* and the patterns of genetic diversity within species. 543 MB representing 72,453 single sequences with an average size of 7499 nt of the unpublished genome assembly Papro-1.0 from the *P. procumbens* accession BGRC 35335 [renamed by the genebank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, (Germany), as BETA 951] were screened for SSRs using SciRoKo v3.4 software (Kofler et al. 2007) and default search parameters. We were able to identify 3648 SSRs. A subset of 53 was validated using 24 individuals of *P. procumbens*, *TPH0604151144* from Tenerife, Punta del Hidalgo (Spain); of *P. webbiana*, *grais1* from Gran Canaria, La Isleta (Spain); and of *P. patellaris*, *AZO2403151630* from Murcia, La Azohía, Playa de la Azohía (Spain) (Nachtigall et al. 2016).

### Diversity analysis

#### Genetic markers

Three groups of materials have been analysed in the laboratory (Table 1). The term “study” refers to distinct data sets kept in Access databases. The results of study 3 were already published by Nachtigall et al. (2016) and are not presented here.

- Study 1 included *P. procumbens* from Tenerife and *P. webbiana* from Gran Canaria. This group of materials was designated as study 1 in a database developed to assist documentation and statistical analysis of microsatellite data. This tool was developed by Enders (2010) and improved during the GeDiPa project.

## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

- Study 2 included *P. patellaris* from the Iberian Peninsula.
- Study 4 included *P. patellaris* from the Canary Islands, Madeira, and Cape Verde.

**Table 1.** Population identifier and number of analysed plants per population (see also Annex 3).

Study	Population identifier	Site	No. of plants analysed	Species
Study 1	TPH0604151144	TPH	24	<i>Patellifolia procumbens</i>
	PWGI	graisl1	24	<i>Patellifolia webbiana</i>
	TAL	TAL	23	<i>Patellifolia procumbens</i>
	TBA	TBA	24	<i>Patellifolia procumbens</i>
	TGA	TGA	24	<i>Patellifolia procumbens</i>
	TPC	TPC	30	<i>Patellifolia procumbens</i>
	TPS	TPS	23	<i>Patellifolia procumbens</i>
Study 2	AZO2403151630	AZO	25	<i>Patellifolia patellaris</i>
	BAL2104150900	BAL	40	<i>Patellifolia patellaris</i>
	CGO3103151000	CGO	35	<i>Patellifolia patellaris</i>
	CNE2303151030	CNE	40	<i>Patellifolia patellaris</i>
	COL2005151000	COL	13	<i>Patellifolia patellaris</i>
	FM-1	FM	4	<i>Patellifolia patellaris</i>
	MOR0903151000	MOR	20	<i>Patellifolia patellaris</i>
	PCA3003151000	PCA	28	<i>Patellifolia patellaris</i>
	PS-1	PS	28	<i>Patellifolia patellaris</i>
STE2104151800	STE	39	<i>Patellifolia patellaris</i>	
Study 4	BG	BG	6	<i>Patellifolia patellaris</i>
	CL	CL	16	<i>Patellifolia patellaris</i>
	CT	CT	15	<i>Patellifolia patellaris</i>
	FX	FX	23	<i>Patellifolia patellaris</i>
	H	H	24	<i>Patellifolia patellaris</i>
	HS	HS	24	<i>Patellifolia patellaris</i>
	PPL	PPL	24	<i>Patellifolia patellaris</i>
	PS32	PS32	16	<i>Patellifolia patellaris</i>
	RSJ	RSJ	14	<i>Patellifolia patellaris</i>
	S	S	24	<i>Patellifolia patellaris</i>
	TES	TES	22	<i>Patellifolia patellaris</i>
	TLG	TLG	24	<i>Patellifolia patellaris</i>
	TLS	TLS	24	<i>Patellifolia patellaris</i>
	TPA	TPA	24	<i>Patellifolia patellaris</i>
TPH0604151200	TPH	8	<i>Patellifolia patellaris</i>	
TPM	TPM	22	<i>Patellifolia patellaris</i>	

The microsatellite analysis was conducted using a capillary electrophoresis GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter). Fragment sizes were determined and documented within a database developed by Enders (2010). The analysis of a probe was repeated once in case of absence of the expected fragment. If the fragment remained absent, the allele was recorded as a null allele. The ALLELE procedure of SAS (version 9.3) was used to compile fragment sizes for all used markers. In case of a 1 nt deviation from a marker-specific frequent fragment size, a manual binning procedure was performed to avoid overestimation of genetic diversity (Amos et al. 2007). The binned data was used for the statistical analysis, while the raw data was archived in a separate database. The microsatellite markers used were all derived from diploid *P. procumbens*. Most of the individual plant x

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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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marker combinations showed two fragments. Statistical procedures for diploid species were therefore employed in subsequent analyses (Nachtigall et al. 2016).

The statistical analysis was completed for study 2. The numbers of SSR alleles and polymorphism information content (PIC) were calculated for each locus over all individuals. The deviation from Hardy-Weinberg Proportions (HWP) was assessed for each individual occurrence using the  $\chi^2$ -test. The ALLELE procedure of SAS (version 9.3) was used for all calculations. The factorial analysis was conducted with the DARwin software (Perrier and Jacquemond-Collet 2006) to illustrate the variation patterns.

DifferInt was used for statistical analysis of the genetic data. This computer programme is applicable to a set of co-dominant marker data without null alleles. The measure is free of assumptions such as the existence of HWP in panmictic populations. Null alleles can be included in the analysis, but then results must be interpreted as phenotypic differences (Gillet 2013b). Null alleles for microsatellites are caused by a different genetic phenomenon than genetic polymorphism, in that mutations in the flanking regions of microsatellites prevent the primer from annealing to template DNA during the PCR-amplification of the microsatellite locus. For this reason, all individuals having a homozygous null allele at any of the loci need to be removed from the data set. The smaller data set can then be used to calculate the pairwise genetic distance  $\Delta$  between occurrences and the complementary compositional differentiation  $\Delta_{SD}$ . The genetic distances were further used to construct a UPGMA tree. To this end, the matrix of pairwise genetic distances was loaded into DARwin.

A detailed discussion of the statistical procedures is beyond the scope of this report. The applied methods as well as the results of study 2 have been described in the paper “Genetic diversity of *Patellifolia patellaris* from the Iberian Peninsula, a crop wild relative of cultivated beets” (Frese et al. [2017], accepted for publication in *Euphytica*). The chapter “Results”, subchapter “Genetic diversity” starts with the results on the completed study 2 (*P. patellaris*), continues with study 4 (*P. patellaris*), and ends with study 1 (*P. procumbens*, *P. webbiana*).

### Cytological analysis

Large-scale sampling of over 40 natural populations and 588 individuals of *P. patellaris*, *P. procumbens* and *P. webbiana* was performed across south-eastern Spain, Tenerife (Canary Islands), mainland Portugal and the archipelagos of Madeira and Cape Verde as described in the chapter “Sampling”. Fresh leaves and/or seeds were collected to analyse genome size and DNA-ploidy level using flow cytometry following Galbraith et al. (1983) procedure for nuclear isolation. Nuclear suspensions were obtained by chopping with a razor blade fresh leaf tissue or one seed of *Patellifolia* and fresh leaf tissue of an internal reference standard over a Petri dish containing 1 ml of WPB buffer (Loureiro et al. 2007). The nuclear suspension was then filtered and 50  $\mu\text{g/ml}$  of propidium iodide and 50  $\mu\text{g/ml}$  of RNase were added to sample tubes to stain the DNA and avoid staining of double stranded RNA, respectively. Samples were analysed in a Partec CyFlow Space flow cytometer (Partec GmbH., Görlitz, Germany) equipped with a 532 nm green solid-state laser, operating at 30 mW. Results were acquired using Partec FloMax software v2.4d (Partec GmbH, Münster, Germany) in the form of four graphics: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. To analyse only intact nuclei, the FL histogram was gated using a polygonal region defined in the FL vs. SS cytogram. At least 13000 particles were analysed per peak. Only CV values of *Patellifolia* peaks below 5% were accepted otherwise samples were repeated. The holoploid genome size in mass units (2C in pg; sensu Greilhuber et al. 2005) was assessed using the formula: *Patellifolia* spp. 2C nuclear DNA content (pg) = (*Patellifolia* G0/G1 peak mean / reference standard G0/G1 peak mean) \* genome size of the reference standard. DNA ploidy levels were inferred for all the individual plants using the chromosome counts available in the literature and the comparison among the results obtained.

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# Genetic diversity of *Patellifolia* species (GeDiPa)

## Final Activity Report

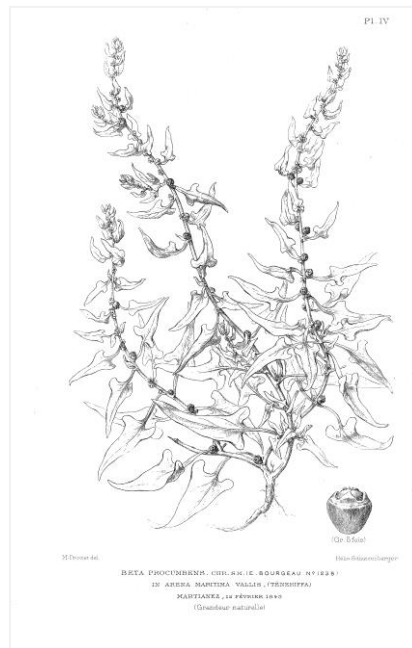
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### RESULTS

#### Taxonomic standards

The key to the species published by Bramwell and Bramwell (1984) and drawings published by Vilmorin (1923) were used to categorize the samples during the field work. Bramwell and Bramwell (1984) separate the species into an annual species with cordate leaf basis and usually single fruits, *P. patellaris*, and two perennial species (*P. procumbens*, *P. webbiana*) having a hastate or saggitate leaf shape basis and 1 to 3-flowered cymes. If the leaves are more ovate or deltoid the species is called *P. procumbens*, and if the leaves are more or less linear, the species is called *P. webbiana*. Bramwell and Bramwell (1984) described *P. procumbens* as a very variable species. The drawing Pl. IV published in Vilmorin (1923) (Figure 2) depicts what the GeDiPa team considered a typical *P. procumbens*.



**Figure 2.** *P. procumbens* (syn. *Beta procumbens*) (Vilmorin 1923).

During sampling of the research material, two distinct morphological types within *P. patellaris*, i.e. slender plants with large thin leaves (Figure 1c) and plants with smaller and succulent leaves (Figure 3) were observed. These types resemble plants depicted by Vilmorin (1923) on drawings labelled Pl. I and III, respectively. The newly discovered population FM-1 (Annex 1c) shows more lanceolate leaves which is quite unusual for a *P. patellaris* and a new variant.



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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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**Figure 3.** A succulent type of *P. patellaris* observed at Punta del Hidalgo, Tenerife. © L. Frese, JKI.

Vilmorin (1923) also reported on distinct fruit shapes at maturity. It seems that traits like keels on the fruit basis (*P. webbiana*) or the type of perianth segment at maturity (oppressed on pericarp cap, *P. patellaris*) or erect ("*Beta campanulata*") can also serve for the development of a key to the species.

Standard accessions for different purposes were defined, including the BETA 534 (BGRC 57667), Almeria, Carboneras, 5 km N of Playa del Algarrobo (Spain) which is the international molecular standard named *P. patellaris*, and BETA 951 (BGRC 35335) named *P. procumbens* of which the collecting site is unknown. BETA 951 was used for the sequencing of the *P. procumbens* genome and thus was considered a reference standard. Accession NC064146 held by ESP003 and collected at the location "Confital/Isleta" according to EURISCO can perhaps be used as standard for *P. webbiana*. Alternatively, an accession collected on Gran Canaria, La Isleta in future would be the best taxonomic standard for *P. webbiana*.

The accessions can be considered cornerstones helping the development of a reliable taxonomic key to the species of the genus *Patellifolia*. To this end the variation of taxonomically significant traits has to be measured under standardized growing conditions. Such work was beyond the capacities of the GeDiPa project.

### Gap analysis and collection to close gaps

The *Patellifolia* species occur in Portugal, Spain, Cape Verde, Morocco, Algeria and Italy. The distribution area was divided in 26 geographic subunits, including eco-geographic zones, province, island or even islets (Annex 1). We assumed that occurrences within these subunits are spatially isolated and thus represent a specific fraction of intraspecific diversity. While *P. patellaris* can potentially be found in the 26 geographic regions, there are good reasons to assume that *P. procumbens* and *P. webbiana* can only be found in 15 and 1 of these geographic units, respectively (Annex 1).

Breeding researchers need a set of well described and readily available accessions. A *Patellifolia* AEGIS collection should therefore be composed by one seed accession from each of the 26, 15 and 1 targeted geographic units of *P. patellifolia*, *P. procumbens* and *P. webbiana*, respectively. Therefore,

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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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for each geographic subunit, accessions with passport data recorded according to the FAO/Bioversity Multi-Crop Passport Descriptors (MCPDs) (Alercia et al. 2012) were identified in genebank information systems and selected as MAA candidates, resulting in 7 MAA candidates for *P. procumbens* and 14 candidates for *P. patellaris*. The set of AEGIS accessions can be completed by accessions held by the United States Department of Agriculture, Agricultural Research Service, National Plant Germplasm System (USDA-ARS-NPGS).

Annex 2 (page 20) lists MAA candidates and describes action to be undertaken before accessions can be nominated by the holding genebanks. Accession B0591 (holder genebank GBR003 [School of Biological Sciences, The University of Birmingham, UK])<sup>1</sup> was regenerated by the JKI in 2015. Accession numbers BETA 882, BETA 862, BETA 622, BETA 928, and BETA 534 (holder genebank DEU146 [Genebank, Leibniz Institute of Plant Genetics and Crop Plant Research]) also belong to the set of 14 MAA candidates of *P. patellaris*. The latter five have been nominated as Multilateral System (MLS) accessions and are safety-duplicated at Spitzbergen (holder NOR051 [Svalbard Global Seed Vault, Norway]). In conclusion, out of 42 accessions needed for building a representative AEGIS *Patellifolia* collection, 22 accessions are stored in genebanks or have been collected recently and would in principle be eligible.

The International Treaty calls for the implementation of complementary conservation actions. Information on recommended genetic reserve sites for *Beta* and *Patellifolia* is available at: [www.agrobiodiversidad.org/aegro](http://www.agrobiodiversidad.org/aegro). *Ex situ* and *in situ* conservation actions can be organized for accession ISOP (holder PRT102 [Banco de Germoplasma ISOPlexis – Universidade da Madeira, Portugal]). The sample was collected within the Parque Natural da Madeira – Ponta de São Lourenço (Portugal), PTMAD0003. The establishment of a genetic reserve site for *Beta patula* Aiton is being planned by the responsible authorities (Pinheiro de Carvalho et al. 2012), which would also cover the site of *P. procumbens*. A genetic reserve site for *P. webbiana* within the protected Area Marítima de La Isleta (Spain), ES7010016, was also proposed. Accession B0614 (*P. procumbens*) and B0597 (*P. patellaris*), holder GBR003, were once collected near Punta del Hildalgo, Tenerife (Spain). This site is located just outside the protected area ES0000109 (Anaga, Bird Directive). The site would be very valuable for the *in situ* conservation of wild beet genetic diversity. *Beta macrocarpa* Guss., *P. patellaris* and *P. procumbens* all occur in the area. The latter two species seem to cross and form a hybrid swarm as indicated by the high morphological diversity (see composite photo of the cover page) and the occurrence of di-, tri- and tetraploid plants.

### Sampling

New populations were found in southeast Spain, Portugal and Cape Verde. Annex 3 (page 21) provides an overview of the material sampled in 2008 and in 2015. Not all of the populations visited where as rich in number of individuals as expected, so not all the populations have the same size of individuals available for analysis.

### Development of SSR markers

Twenty-five SSRs proved to be polymorphic in the three species except for marker *JKIPat16* which is specific for *P. webbiana*. The number of alleles varies between the three *Patellifolia* species. Altogether, the 25 polymorphic SSR loci bear 87, 187, and 227 alleles in *P. patellaris*, *P. procumbens*, and *P. webbiana*, respectively. The number of alleles per locus within a species ranges from 1 to 8 (*P. patellaris*), 2 to 15 (*P. procumbens*) to 4 to 15 (*P. webbiana*). With these new 25 markers, the populations of the genus *Patellifolia* stored in genebanks will be characterized. The 25 primers were resequenced and the data have successfully been submitted to GenBank. More information on this new set of genetic markers has been published in the primer note “Functional SSR markers for the genus *Patellifolia* (Chenopodiaceae)” (Nachtigall et al. 2016).

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<sup>1</sup> Institute codes of holding genebanks according to [FAO WIEWS](http://www.fao.org/wIEWS)

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# Genetic diversity of *Patellifolia* species (GeDiPa)

## Final Activity Report

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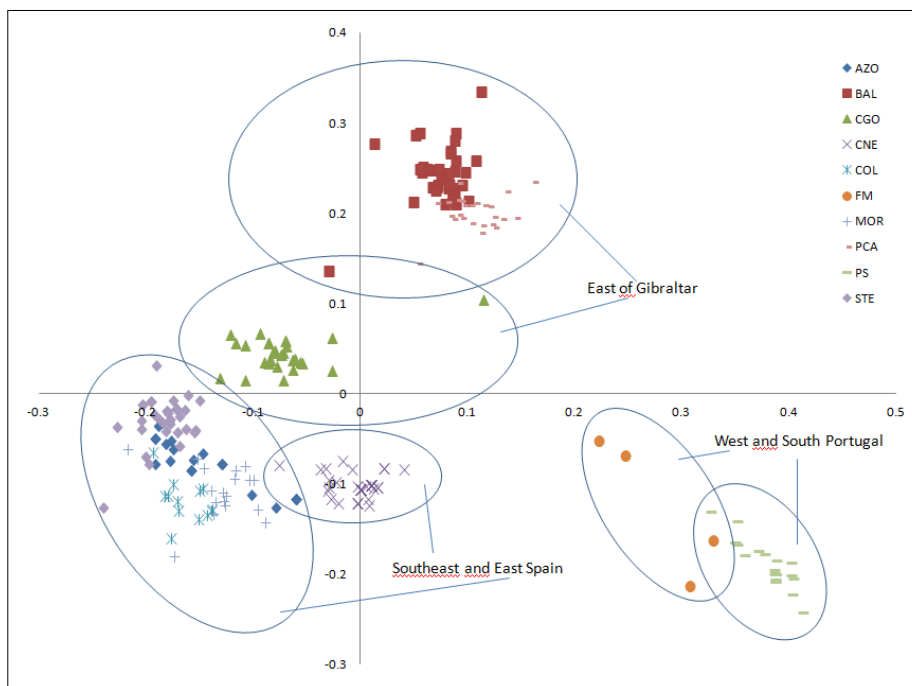
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### Diversity analysis

#### Genetic diversity

- **Study 2 (*P. patellaris* from the Iberian Peninsula)**

A total of 271 individuals was analysed using 24 microsatellite markers. The factorial analysis separated the material into two occurrences from Portugal (FM, PS), two occurrences sampled east of Gibraltar (PCA, BAL) and six occurrences (CGO, STE, CNE, AZO, MOR, COL) from the eastern Spanish coast (see Table 1). The factorial analysis (Figure 4) as well as the genetic distance and differentiation analysis indicate that the two occurrences from Portugal are clearly genetically different from the Spanish group of occurrences (Frese et al. [2017], accepted for publication in *Euphytica*). Both occurrences have low population sizes and are therefore highly endangered. *In situ* conservation actions are urgently required for both occurrences.



**Figure 4.** Factorial analysis for Study 2 (*P. patellaris* from the Iberian Peninsula).

Legend: codes used for population identifiers

AZO	AZO2403151630	FM	FM-1
BAL	BAL2104150900	MOR	MOR0903151000
CGO	CGO3103151000	PCA	PCA3003151000
CNE	CNE2303151030	PS	PS-1
COL	COL2005151000	STE	STE2104151800

# Genetic diversity of *Patellifolia* species (GeDiPa)

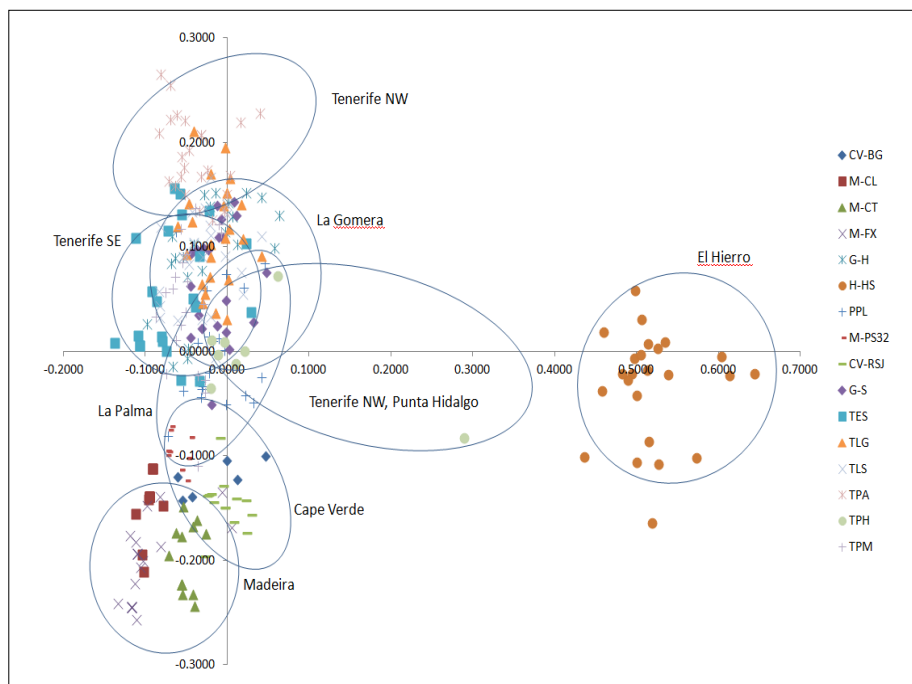
## Final Activity Report

Based on the analysis, additional candidates for the network of MAWPs can be chosen from the PCA-BAL and CGO-STE-CNE-AZO-MOR-COL clusters. If the capacities for the establishment and management of a genetic reserves' network are limited, BAL and MOR, respectively, should be the first choice. The establishment of genetic reserves for few selected MAWPs should not be misunderstood as a license to negligent conservation actions for other occurrences in the distribution area. The total number of individuals existing at the sites of PCA, CGO, AZO, MOR and COL is less than 40. All of these occurrences seem to be threatened and should at least be monitored to prevent accidental loss (Frese et al. 2017, accepted).

Further investigations are needed to organize better informed conservation actions for *P. patellaris*, namely to study genetic relationships between occurrences distributed on the Canary Islands, Madeira and Cape Verde Islands. To this end the data set of study 4 will be used.

### • Study 4 (*P. patellaris* from the Canary Islands, Madeira, and Cape Verde)

The preliminary results of a factorial analysis of the data set (310 individual plants x 24 markers) are presented in Figure 5. The distribution pattern of individuals in the scatter plot coincides well with the geographic origin of the sampled occurrences. The material from Tenerife and La Gomera form a swarm, the samples from El Hierro form a separate cloud and the individuals from Madeira and Cape Verde group together.



**Figure 5.** Factorial analysis for Study 4 (*P. patellaris* from the Canary Islands, Madeira, and Cape Verde).

Legend: codes used for population identifiers

CV-BG	CV.SV.BG-1	CV-RSJ	CV.SV.RSJ-1
M-CL	CLM0707151601	G-S	S
M-CT	CTM0707151415	TES	TES
M-FX	FXM0707151719	TLG	TLG
G-H	H	TLS	TLS
H-HS	HS	TPA	TPA
PPL	PPL	TPH	TPH0604151200
M-PS32	PSM2704151936	TPM	TPM

# Genetic diversity of *Patellifolia* species (GeDiPa)

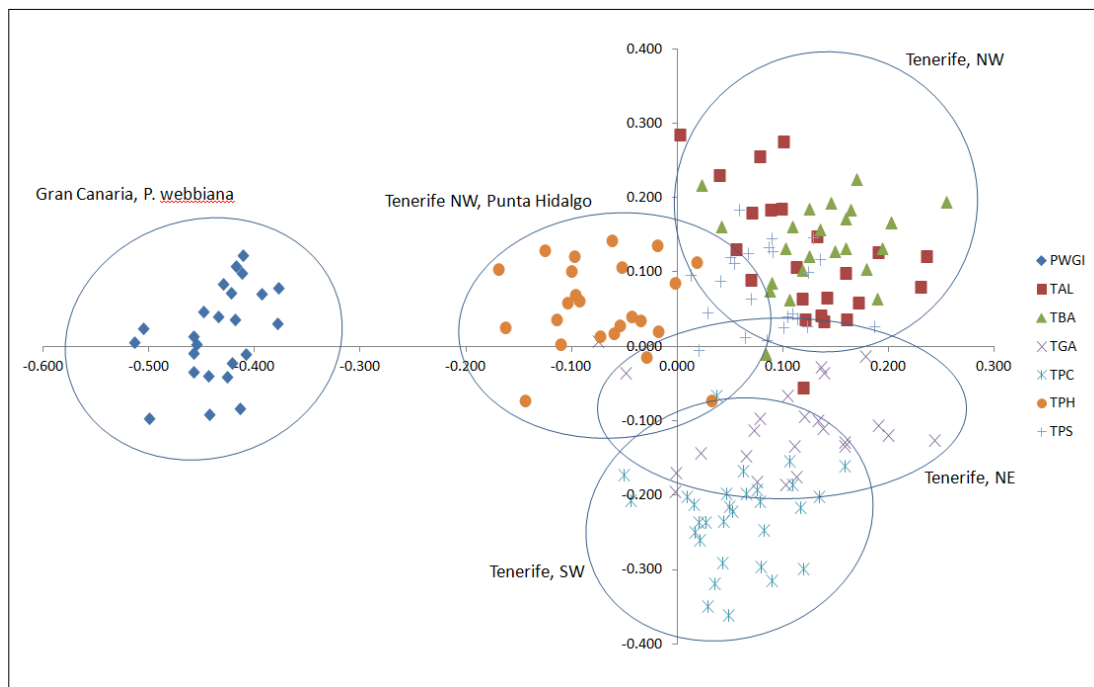
## Final Activity Report

The Cape Verde archipelago was uninhabited until Portuguese explorers discovered and colonized the islands. There must have been shipping traffic between Madeira and the Cape Verde since the 16th century (Vieira 1996) and thus a chance for *P. patellaris* to migrate from Madeira to Cape Verde or vice versa. This is a working hypothesis that deserves further investigations.

- **Study 1 (*P. procumbens* from Tenerife and *P. webbiana* from Gran Canaria)**

Figure 6 displays the preliminary results of the factorial analysis. In total 172 plants were analysed using 22 SSR markers. Again, the patterns coincide well with the geographic origin of the sampled occurrences. The dots located in the left part of the figure represent *P. webbiana* individuals collected on Gran Canaria at La Isleta. The dots in the right part of the scatter plot all represent *P. procumbens*. All plants from three sites of the northern coastline of Tenerife form a swarm (TAL, TBA, TPS), although TPH, also located on the northern coastline, forms a separate group. The north-eastern occurrence is located between those groups and the south-western TPC occurrence.

*P. procumbens* as well as the few *P. patellaris* plants sampled at the site Punta del Hidalgo (TPH) are located in the centre of the scattergrams. At this site, both species grow sympatrically and the morphological variation within "*P. procumbens*-plants" is high. It seems that this site is of particular scientific interest as it can be a site where interspecific hybrids occur and genetic variation is generated. This is a working hypothesis that needs to be verified or discharged in future.



**Figure 6.** Factorial analysis for Study 1 (*P. procumbens* from Tenerife and *P. webbiana* from Gran Canaria).

Legend: codes used for population identifiers

PWGI	grais1	TPC	TPC
TAL	TAL	TPH	TPH0604151144
TBA	TBA	TPS	TPS
TGA	TGA		

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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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#### Cytological diversity

Overall, *P. patellaris* was mainly tetraploid, while *P. procumbens* and *P. webbiana* were diploid. However, the cytogenetic diversity in certain regions and taxa was higher than expected: *P. patellaris* was ploidy variable with diploids being found in southeastern Spain and mainland Portugal; and, in Tenerife, *P. patellaris* and *P. procumbens* co-occur and seem to cross and form a hybrid swarm supported by the occurrence of diploid, triploid and tetraploid plants and by the high morphological diversity. The plant group sampled at Punta del Hidalgo in the north-western part of Tenerife presumably is such a hybrid swarm. It stands out from the others with respect to morphological variation, as illustrated by the photos on the report's cover page. In addition, diploid, triploid and tetraploid plants were detected on this site.

The results indicate that cryptic diversity and interspecific hybridization generates novel genetic variation within the genus, which is positive for the species survival and from the plant breeder's user perspective. The possible presence of cryptic diversity may also explain why the delineation of the three species is a challenge to collectors.

#### RECOMMENDATIONS

1. Information on the extent of morphological variation within the individual species is a condition for the development of a key to the species that works in the field. We recommend the measurement of taxonomically relevant traits within a set of accessions representing the distribution area of the genus and the statistical analysis of the data set.
2. For the time being, BETA 951 (BGRC 35335) can be used as taxonomic standard for *P. procumbens*, NC064146 for *P. webbiana* and BETA 534 (BGRC 57667) for *P. patellaris*. The standards should be used by genebank curators to categorize accessions, and when the taxonomic problems will be solved, to classify the accessions.
3. Genebank curators should focus seed multiplication efforts on those accessions that were identified as MAAs. The genebank at Madrid could rejuvenate accession NC064146 or initiate the recollection at La Isleta. Accessions held by the Spanish and Portuguese genebanks need to be nominated as MLS first.
4. All MAA-suggested accessions should be included in EURISCO (as a pre-requisite to enter AEGIS).
5. All *Patellifolia* accessions shown in Annex 1 that were already included in the MLS should be included in AEGIS.
6. There is a clear need for closing geographic gaps in *ex situ* holdings and a need for the establishment of genetic reserves for *Patellifolia* species.
7. A set of 25 polymorphic genetic markers is available and can be used to study different aspects of genetic diversity in *Patellifolia*. Observations made in the field and during reproduction of genebank accessions conserved since decades indicate that, in particular, *P. procumbens* populations growing in the natural habitat are much more diverse than accessions kept in genebanks. It can be assumed that the seed regeneration procedures of the hard-seeded species caused genetic bottleneck and loss of genetic variation in the accession. A comparison of the amount of genetic diversity kept in genebanks with the amount of genetic diversity available in a number of geographically distinct sites of *P. procumbens* and *P. patellaris* would show how much of the diversity was captured in the past by collectors and is currently being conserved *ex situ*. Such investigation would contribute to the quality of managing genetic diversity of *Patellifolia*.

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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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8. A significant number of *Patellifolia* accessions are being conserved by the USDA-ARS-NPGS and other partners outside Europe. We suggest therefore setting up a joint EU/USA project dealing with aspects outlined in recommendation 7.
9. The thorough analysis of genetic and cytological diversity will take more time and the results will be published later. Nevertheless, the results of the genetic and cytological analysis presented in the report justify the following preliminary recommendations for the selection of MAWPs and MAAs in the distribution area. The designation of a genetic reserve will depend to a large extent on the interest in the species and the capacities of local nature conservation agencies. After all, it will be the task of local conservation agencies to care for a certain degree of disturbance of the site which *Patellifolia* species need to grow and propagate. Therefore, we do not usually suggest a very narrow and specific location, but rather a larger part of the distribution area harbouring, to our current knowledge, a discernible fraction of genetic diversity of the given species. In most cases an area is listed instead of a specific population (see also Annex 1, columns "Proposed Genetic Reserve"). Within the following areas of distribution of the three species, genetic reserves should be designated and managed and a backup sample conserved in genebanks:

#### ***P. procumbens***

##### **9a. Madeira**

The Parque Natural da Madeira - Ponta de São Lourenço - PTMAD0003 is already foreseen as a genetic reserve site for *Beta patula* (Pinheiro de Carvalho et al. 2012). The genetic reserve can be planned as a multi-species site and genetic reserve for *Patellifolia* as well.

##### **9b. Tenerife**

Plant groups sampled in the north-eastern, north-western and south-western areas can be distinguished. In the north-western part, *P. procumbens* also occurs in the protected area named Anaga (Bird Directive) ES000109. The most interesting site and plant group is located just outside a protected area. Particular efforts should be undertaken to establish a genetic reserve at Punta del Hidalgo. This site should be proposed as Site of Community Importance (SCI) to establish a legal basis for a genetic reserve.

#### ***P. webbiana***

##### **9c. Gran Canaria**

We propose the designation of a genetic reserve within the Area Marítima de La Isleta - ES7010016. It is the only confirmed distribution area of the species.

#### ***P. patellaris***

##### **9d. Mainland Spain**

At least two genetic reserves should be established, one east of Gibraltar and one on the eastern coast of Spain. The final choice will depend on the interest and capacity of nature conservation agencies of the provinces Almería, Málaga, Murcia, and Alicante.

##### **9e. Mainland Portugal**

The species is very rare in mainland Portugal and the occurrences are clearly different from those distributed in the Spanish part of the Iberian Peninsula. The establishment of genetic reserves for both occurrences is suggested.

##### **9f. Madeira and adjacent islets and Cape Verde**

The results of the factorial analysis suggest that all analysed individuals belong to the same group. The maintenance of genetic diversity of *P. patellaris* contained in this group within the suggested multi-species genetic reserve within Parque Natural da Madeira - Ponta de São Lourenço - PTMAD0003 would be a pragmatic solution.

##### **9g. Canary Islands**

The genetic diversity of the species can be maintained by designating genetic reserves in the north-western (including Punta del Hidalgo) and south-eastern part of Tenerife, a suitable

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# Genetic diversity of *Patellifolia* species (GeDiPa)

## Final Activity Report

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*P. patellaris* site in La Gomera, a site on La Palma and on El Hierro. Recommendations for *P. patellaris* known to occur on Lanzarote and Fuerteventura cannot be given due to lack of research material.

### FINAL CONCLUSIONS

All products of the GeDiPa activity listed in Annex A of the letter of agreement signed by JKI and ECPGR have been delivered. A set of taxonomic standard accessions for *P. procumbens*, *P. webbiana* and *P. patellaris* has been proposed (product 2). A primer note was published, i.e. a report on the development of a set of polymorphic SSR markers for *Patellifolia* delivered (product 3). This report includes a description of the geographic patterns of genetic diversity of *Patellifolia* species. In 2017 a poster on the results of the cytological studies was presented. Product 4 encompasses this report as well as the above-mentioned paper accepted for publication in *Euphytica* and the poster presentation. Suggestions for further research activities can be found in the report. In addition, a large part of the sampled and desiccated leaf material has been handed over to a USDA/ARS research partner who intends to analyse the material with the restriction site associated DNA (RAD) method. It delivers high resolution population genomic data and has found application in wild species population studies. Moreover, partners of the GeDiPa project participated in the preparation of an Innovative Training Networks (ITN) project proposal (Marie Skłodowska-Curie actions, call: H2020-MSCA-ITN-2017). The proposal includes an early-stage researcher (ESR) project aiming at the investigation, conservation and use of *Patellifolia* genetic resources (product 1).

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## Genetic diversity of *Patellifolia* species (GeDiPa)

### Final Activity Report

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**Genetic diversity of *Patellifolia* species  
(GeDiPa)**

**Final Activity Report**

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**ANNEXES**

Annex 1. Gap analysis and recommendation of MAAs .....	17
1a. <i>P. procumbens</i> .....	17
1b. <i>P. webbiana</i> .....	18
1c. <i>P. patellaris</i> .....	19
Annex 2. List of MAA candidates .....	20
Annex 3. Overview on collected leaf and seed samples .....	21

# Genetic diversity of *Patellifolia* species (GeDiPa)

## Final Activity Report

### Annex 1. Gap analysis and recommendation of MAAs

<p>Aim: One accession per species per known distribution area</p> <p>Rationale: Breeding researchers do not need many accessions, but a well described set of accessions. The AEGIS <i>Patellifolia</i> holding should facilitate access to a set of accessions that represents all known distribution areas (islands / provinces) of the species.</p> <p>Selection criteria for accession numbers: Passport data with description of collecting site</p>
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#### 1a. *P. procumbens*

Country	Location	<i>procumbens</i>	Accession	Holder	Multi-lateral System	Duplication Site	Proposed Genetic Reserve
Portugal (PRT)	Madeira	Madeira, main island	?				
		Ilheu do Desembarcadouro	x	ISOP 1510	PRT102		Parque Natural da Madeira - Ponta de São Lourenço - PTMAD0003
		Ilheu Chao	x				
		Ilheu Farol	x				
		Porto Santo	x	ISOP 1902	PRT102		
	Salvage Islands	x					
	PRT Mainland	Setúbal					
	Faro						
Cape Verde Republic (CPV)	Cape Verde	São Vicente					
Spain (ESP)	Canary Islands	El Hierro	x				
		Fuerteventura	?	B0594	GBR003		
		Gran Canaria	x	B0554	GBR003		
		La Gomera	x	B0628	GBR003		
		Lanzarote	?				
		La Palma	x	B0618	GBR003		
		Tenerife	x	B0614	GBR003		Anaga (Bird Directive) ES0000109 North-eastern area North-western area South-western area
	ESP Mainland	Almería					
		Málaga					
		Murcia					
Alicante							
Morocco (MAR)	Zone E						
	Zone C						
	Zone D (Aglou)						
	Zone D (Agadir)						
Algeria (DZA)	Ahaggar	?					
	Tassili des Ajjer	?					
Italy (ITA)	Linosa <sup>1)</sup>	x					
Lybia (LBY)		?					
Yemen (YEM)	Sokotra	?					
Somalia (SOM)		?					
	<b>Number of geo-graphic subunits with <i>Patellifolia</i></b>	<b>19</b>					

 = species does likely not occur in the subunit

? = the authors are not sure if the species occur in the subunit

<sup>1)</sup> *P. procumbens* has been recently documented in Linosa : [http://www.actaplantarum.org/flora/flora\\_info.php?id=1187](http://www.actaplantarum.org/flora/flora_info.php?id=1187). A series of detailed photos not only illustrates the discovery but also the taxonomic problem addressed in the introductory chapter. The plant resembles a succulent type of *P. patellaris*. The leaf basis of the photographed plants has a unique form never reported before.

**Genetic diversity of *Patellifolia* species  
(GeDiPa)**

**Final Activity Report**

**1b. *P. webbiana***

Country	Location	<i>webbiana</i>	Accession	Holder	Multi-lateral System	Duplication Site	Proposed Genetic Reserve	
Portugal (PRT)	Madeira	Madeira, main island						
		Ilheu do Desembarcadouro						
		Ilheu Chao						
		Ilheu Farol						
		Porto Santo						
	PRT Mainland	Salvage Islands						
		Setúbal						
Cape Verde Republic (CPV)	Cape Verde	Faro						
Spain (ESP)	Canary Islands	El Hierro						
		Fuerteventura						
		Gran Canaria	x	NC064146	ESP003			Area Marítima de La Isleta – ES7010016
		La Gomera						
		Lanzarote						
		La Palma						
	ESP Mainland	Tenerife	(x)					
		Almería						
		Málaga						
		Murcia						
Morocco (MAR)		Alicante						
		Zone E						
		Zone C						
		Zone D (Aglou)						
Algeria (DZA)		Zone D (Agadir)						
		Ahaggar						
Italy (ITA)		Tassili des Ajjer						
Lybia (LBY)		Linosa						
Yemen (YEM)		Sokotra						
Somalia (SOM)								
	<b>Number of geographic subunits with <i>Patellifolia</i></b>		1(2)					

= species does likely not occur in the subunit

# Genetic diversity of *Patellifolia* species (GeDiPa)

## Final Activity Report

### 1c. *P. patellaris*

Country	Location	<i>patellaris</i>	Accession	Holder	Multi-lateral System	Duplication Site	Proposed Genetic Reserve	
Portugal (PRT)	Madeira	Madeira, main island	x	BETA 882	DEU146	y	NOR051	
		Ilheu do Desembarcadouro	?					Parque Natural da Madeira – Ponta de São Lourenço – PTMAD0003
		Ilheu Chao	?					
		Ilheu Farol	?					
		Porto Santo	x	ISOP2834	PRT102			
		Salvage Islands	x					
	PRT Mainland	Setúbal	x	FM-1	PRT001			Fojo dos Morcegos
	Faro	x	PS-1	PRT001			Ponta de Sagres	
Cape Verde Republic (CPV)	Cape Verde	São Vicente	x	CV.SV.RSJ-1 or CV.SV.BG-1 (*)	Genebank, Braga			
Spain (ESP)	Canary Islands	El Hierro	x					El Hierro area
		Fuerteventura	x	B0591	GBR003			
		Gran Canaria	x	BETA 862	DEU146	y	NOR051	
		La Gomera	x	BETA 622	DEU146	y	NOR051	La Gomera area
		Lanzarote	x	BETA 928	DEU146	y	NOR051	
		La Palma	x	B0617	GBR003			La Palma area
		Tenerife	x	B0597	GBR003			Anaga (Bird Directive) ES0000109 North-western area South-eastern area
	ESP Mainland	Almería	x	BETA 534	DEU146	y	NOR051	Almería area
		Málaga	x	ESP/2015/JM I&LdH-2	ESP004			Málaga area
		Murcia	x	ESP/2015/M LRT&PS-1	ESP004			Murcia area
Alicante		x	ESP/2015/PF G&EL-1	ESP004			Alicante area	
Morocco (MAR)	Zone E	x						
	Zone C	x						
	Zone D (Aglou)	x	W6 44512	USA008				
	Zone D (Agadir)	x						
Algeria (DZA)	Ahaggar	x						
	Tassili des Ajjer	x						
Italy (ITA)	Linosa	?						
Lybia		x						
Yemen (YEM)	Sokotra	x						
Somalia (SOM)		x						
	<b>Number of geographic subunits with <i>Patellifolia</i></b>	<b>30</b>						

? = the authors are not sure if species occur in the subunit      y = yes (declared by the holding country as part of the Multilateral system)

(\*) (CV.SV.RSJ-1) and (CV.SV.BG-1): both accessions have been collected on Cape Verde. The legal status of the accessions still needs to be clarified.

**Genetic diversity of *Patellifolia* species  
(GeDiPa)**

**Final Activity Report**

**Annex 2. List of MAA candidates**

Species	Accession number	Population ID	Holder	Action to be taken	Number of candidates
<i>P. procumbens</i>	ISOP 1510		PRT102	Check seed quality and quantity, add to MLS/AEGIS	7
	ISOP 1902		PRT102	Check seed quality and quantity, add to MLS/AEGIS	
	B0594		GBR003	Check seed quality and quantity, add to EURISCO and MLS/AEGIS	
	B0554		GBR003	Check seed quality and quantity, add to EURISCO and MLS/AEGIS	
	B0628		GBR003	Check seed quality and quantity, add to EURISCO and MLS/AEGIS	
	B0618		GBR003	Check seed quality and quantity, add to EURISCO and MLS/AEGIS	
	B0614		GBR003	Check seed quality and quantity, add to EURISCO and to MLS/AEGIS	
<i>P. patellaris</i>	BETA 882		DEU146	Add to MLS/AEGIS	14
	ISOP2834		PRT102	Check seed quality and quantity, add to MLS/AEGIS	
		(FM-1)	PRT001	Seed multiplication, then add to MLS/AEGIS	
		(PS-1)	PRT001	Seed multiplication, then add to MLS/AEGIS	
		CV.SV.RSJ-1 or CV.SV.BG-1 (*)	PRT001	Clarify legal status. If available for exchange, add sample to MLS/AEGIS and increase seed sample.	
	B0591		GBR003	Add to MLS/AEGIS	
	BETA 862		DEU146	Add to MLS/AEGIS	
	BETA 622		DEU146	Add to MLS/AEGIS	
	BETA 928		DEU146	Add to MLS/AEGIS	
	B0617		GBR003	Check seed quality and quantity, add to MLS/AEGIS	
	B0597		GBR003	Check seed quality and quantity, add to MLS/AEGIS	
	BETA 534		DEU146	Add to MLS/AEGIS	
		ESP/2015/JMI&LdH-2	ESP004	Check seed quality and quantity, add to MLS/AEGIS	
		ESP/2015/MLRT&PS-1	ESP004	Check seed quality and quantity, add to MLS/AEGIS	
	ESP/2015/PFG&EL-1	ESP004	Check seed quality and quantity, add to MLS/AEGIS		

(\*) (CV.SV.RSJ-1) and (CV.SV.BG-1): both accessions have been collected on Cape Verde. The legal status of the accessions still needs to be clarified.

**Genetic diversity of *Patellifolia* species  
(GeDiPa)**

**Final Activity Report**

**Annex 3. Overview on collected leaf and seed samples**

Population identifier	Legend in Figures 4 to 6	Code of country of origin	Site location	Species	Number of individual leaf samples	Seed sample
CV.SV.RSJ-1	CV-RSJ	CPV	Cape Verde, São Vicente Island	<i>patellaris</i>	14	Yes
CV.SV.BG-1	CV-BG	CPV	Cape Verde, São Vicente Island	<i>patellaris</i>	6	No
PS-1	PS	PRT	Faro	<i>patellaris</i>	28	Yes
FM-1	FM	PRT	Setúbal	<i>patellaris</i>	4	Yes
PSM2704151447		PRT	Madeira	<i>patellaris</i>	2	Yes
PSM2704151525		PRT	Madeira	<i>procumbens</i>	-	Yes
PSM2704151633		PRT	Madeira	<i>patellaris</i>	-	Yes
PSM2704151128		PRT	Madeira	<i>patellaris</i>	-	Yes
PSM2704151706		PRT	Madeira	<i>patellaris</i>	-	Yes
PSM2704150948		PRT	Madeira	<i>patellaris</i>	-	Yes
PSM2704151044		PRT	Madeira	<i>procumbens</i>	-	Yes
PSM2704151848		PRT	Madeira	<i>patellaris</i>	-	Yes
PSM2704151936	M-PS32	PRT	Madeira	<i>patellaris</i>	17	Yes
FXM0707151719	M-FX	PRT	Madeira	<i>patellaris</i>	23	Yes
CLM0707151601	M-CL	PRT	Madeira	<i>patellaris</i>	17	Yes
CTM0707151415	M-CT	PRT	Madeira	<i>patellaris</i>	15	Yes
MOR0903151000	MOR	ESP	Alicante	<i>patellaris</i>	20	No
CNE2303151030	CNE	ESP	Murcia	<i>patellaris</i>	40	No
AZO2403151630	AZO	ESP	Murcia	<i>patellaris</i>	25	No
PCA3003151000	PCA	ESP	Málaga	<i>patellaris</i>	28	Yes
CGO3103151000	CGO	ESP	Málaga	<i>patellaris</i>	35	No
BAL2104150900	BAL	ESP	Almería	<i>patellaris</i>	40	Yes
STE2104151800	STE	ESP	Almería	<i>patellaris</i>	40	Yes
COL2005151000	COL	ESP	Alicante	<i>patellaris</i>	13	No
HS	H-HS	ESP	El Hierro	<i>patellaris</i>	46	No
S	G-S	ESP	La Gomera	<i>patellaris</i>	42	No
H	G-H	ESP	La Gomera	<i>patellaris</i>	42	No
PPL	PPL	ESP	La Palma	<i>patellaris</i>	26	No
TLG	TLG	ESP	Tenerife	<i>patellaris</i>	25	No
TPM	TPM	ESP	Tenerife	<i>patellaris</i>	22	No
TES	TES	ESP	Tenerife	<i>patellaris</i>	22	No
TPA	TPA	ESP	Tenerife	<i>patellaris</i>	25	No
TLS	TLS	ESP	Tenerife	<i>patellaris</i>	25	No
TAL	TAL	ESP	Tenerife	<i>procumbens</i>	23	No
TBA	TBA	ESP	Tenerife	<i>procumbens</i>	25	No
TPS	TPS	ESP	Tenerife	<i>procumbens</i>	22	No
TPC	TPC	ESP	Tenerife	<i>procumbens</i>	30	No
TGA	TGA	ESP	Tenerife	<i>procumbens</i>	25	No
TPH0604151144	TPH	ESP	Tenerife	<i>procumbens</i>	25	No
TPH0604151200	TPH	ESP	Tenerife	<i>patellaris</i>	8	No
TJ0604151815		ESP	Tenerife	<i>patellaris</i>	6	No
TB0604051905		ESP	Tenerife	<i>procumbens</i>	7	No
grais1	PWGI	ESP	Gran Canaria	<i>webbiana</i>	24	No