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RESEARCH ARTICLE

Genetic diversity and differentiation in *Patellifolia* (Amaranthaceae) in the Macaronesian archipelagos and the Iberian Peninsula and implications for genetic conservation programmes

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Abstract This is the first comprehensive investigation of the patterns of genetic diversity of *Patellifolia* species. The main objective of our research work is to determine Most Appropriate crop Wild relative Populations (MAWP) suited to conserve in situ wild relatives of the sugar beet. Individual plant samples of *P. patellaris* were collected at 26 and of *P. procumbens/P. webbiana* at seven sites and analysed with 24 and 22 microsatellite markers, respectively. On average 15 alleles per locus were found within the set of 581 *P. patellaris* and an average of 12 alleles per locus in the set of 172 *P. procumbens/P. webbiana* individuals. The factorial analysis showed diversity patterns which agree well with the geographic origin of the samples. The genetic data suggest that *P. patellaris* reproduces mainly by self-fertilisation while *P. procumbens/P. webbiana* have the signature of outbreeders. The measure Δ was used to calculate the genetic distance of each occurrence to the pooled remaining occurrences, the complement. Occurrences with either the lowest or the highest genetic distance to the complement are particularly suited to conserve the genetic diversity of the species. Eight occurrences of *P. patellaris*, two of P. *procumbens* and one for *P. webbiana* were determined according to this scheme, proposed as MAWP and recommended for the establishment of genetic reserves.

Keywords Genetic diversity · Differentiation · In situ conservation · Genetic resources · Microsatellite marker · *Patellifolia*

Introduction

The former *Beta* section *Procumbentes* Ulbr. was ranked as a separate genus *Patellifolia* A. J. Scott, Ford-Lloyd and J. T. Williams (Amaranthaceae) by Scott et al. (1977). *Patellifolia* species are important crop wild relatives (CWR) of cultivated beets (de

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Vilmorin 1923; Jung et al. 1993). One of the four groups of cultivated beets (Hammer 2001), the sugar beet (*Beta vulgaris* L. subsp. *vulgaris* culton group Sugar Beet; Lange et al. 1999), was grown on 3,091,844 ha in Europe in the year 2014. Where the crop is grown in short crop rotations for economic reasons the pest and disease pressure increases and induces a major problem to sugar beet growers (Winner 1981).

Sugar beet breeding has been quite successful in the development of resistant varieties through introgression of novel traits from wild species into the breeding pool (Panella and Lewellen 2007). The species of the genus *Patellifolia* are known to possess resistance to several viruses (curly top, beet mosaic, beet necrotic yellow vein), bacteria (*Erwinia* spp.), fungi (*Erysiphe betae* (Vaňha) Weltzien*, Cercospora beticola* Sacc.*, Polymyxa betae* Keskin), nematodes (*Heterodera* spp., *Meloidogyne* spp.), aphids (*Myzus persicae* Sulzer) or leafminer (*Pegomya betae* Curtis) affecting the sugar beet crop (compiled by Frese 2002a). The introgression of these traits into the cultivated beets is difficult but not impossible as exemplified by the successful transfer of the resistance against the beet cyst nematode (*Heterodera schachtii* Schm.) from *Patellifolia* species into the sugar beet by conventional breeding techniques (Löptien 1984).

According to the gene pool concept of Harlan and de Wet (1971) the genus *Patellifolia* forms the tertiary gene pool of cultivated beets. The genera *Beta* and *Patellifolia* started to diverge at roughly 25.3 million years ago (16.1–34.8 Mya), and currently form two clearly separated monophyletic groups (Romeiras et al. 2016) which explain the strong crossing barriers between *Patellifolia* species and *Beta vulgaris*. Following Scott et al. (1977), the genus *Patellifolia* consists of three species, namely *Patellifolia patellaris* (Moq.) A. J. Scott, Ford-Lloyd and J. T. Williams (syn. *B. patellaris* Moq.), *Patellifolia procumbens* (C. Sm.) A. J. Scott, Ford-Lloyd and J. T. Williams (syn. *B. procumbens* C. Sm.), and *Patellifolia webbiana* (Moq.) A. J. Scott, Ford-Lloyd and J. T. Williams (syn. *B. webbiana* Moq.). However, the difficulties in distinguishing the species lead some authors to refer an uncertain number of species—two or three (e.g. Hohmann et al. 2006; Kadereit et al. 2006) or even only one (e.g. Thulin et al. 2010, see below).

The center of diversity of the genus *Patellifolia* encompasses the Macaronesian archipelagos (Madeira, Salvages, Canary Islands, Cape Verde) where all three species occur. *P. patellaris* is also distributed along the coastline of the Iberian Peninsula and Northwest Africa (Morocco, Algeria, and Mauritania) as well as in a few more isolated localities such as the island Linosa (Italy) and interior localities of Northwest Africa (Anonymous 2017; El Bahloul and Gaboun 2013; Romeiras et al. 2016; Thulin et al. 2010). *Patellifolia* species are adapted to ruderal sites and occur mainly in remnant habitat fragments along the coastline as well as road sites or in abandoned farm fields close to the sea.

Raab-Straube et al. (2016) recently listed the occurrence, in Tunisia, of *Patellifolia procumbens* (syn. *Beta patellaris* Moq. and syn. *Tetragonia pentandra* Balf. f.). The collectors, R. El Mokni and D. Iamonico, used the taxonomic system suggested by Thulin et al. (2010) who recognised *P. procumbens* as the only species within the genus *Patellifolia*. Thulin and co-workers further suggest using this system until it can be disproved by new data. The proposal of Thulin et al. (2010) is based on the analysis of ITS regions of five specimens, namely *P. procumbens* and *P. patellaris* from Gran Canaria, *P. webbiana* and *P. patellaris* from Tenerife as well as one specimen from *Tetragonia pentrandra* from Socotra (Yemen). While the taxonomic debate continues, we propose using the taxonomic system of Scott et al. (1977) which is also the basis used in threat assessment studies. *P. patellaris* and *P. procumbens* are categorized according to the IUCN Red List as "LC" (least concern) while *P. webbiana* is classified as "CR" (critically endangered; Bilz et al. 2011).

Most of the CWR species are not actively managed in or outside protected areas and may therefore get lost accidentally (Maxted et al. 2008). There is therefore the need to organize systematic CWR conservation programmes combining the best elements of the in situ and ex situ conservation strategy. Recently, Maxted and collaborators developed a European concept for in situ conservation where they propose the establishment of genetic reserves networks for CWR (Maxted et al. 2015). The genetic reserve conservation technique is defined as *"The location, management and monitoring of genetic diversity in natural populations within defined areas designated for long-term active conservation"* (Maxted et al. 1997). Following this approach and using the step by step methodology published on the CWR In situ Strategy Helpdesk (http://aegro.jki.bund.de), Kell et al. (2012) already identified seven sites in Europe (five in the Canaries and two in Madeira) which could qualify for the establishment of genetic reserves for *Patellifolia*. According to the proposed quality standards for genetic reserves, Most Appropriate crop Wild relative Populations (MAWP), a term for in situ conservation units first coined by S. Kell (Maxted et al. 2015), have to be identified through a rigorous scientific process (Iriondo et al. 2012) which includes the genetic characterization of populations of the target taxon. The genetic analysis is required to identify a number of individual MAWPs representing altogether the genetic diversity of a taxon (Maxted et al. 2015). Until recently, only few polymorphic microsatellite markers (El Bahloul and Gaboun 2013) were suited to investigate genetic diversity of *Patellifolia*. Nachtigall et al. (2016) developed additional microsatellite markers which were used to study genetic diversity of *P. patellaris* from the Iberian Peninsula (Frese et al. 2017a). The results of a comprehensive analysis of all occurrences of *P. patellaris* and *P. procumbens*/*P. webbiana* sampled in major parts of the distribution area are presented here. The main objective of our research work is to determine MAWPs of *Patellifolia* species.

Materials and methods

According to the definition of Kleinschmit et al. (2004) a collection of individual plants can only be denoted population if the individuals form a reproductively coherent group within spatial limits. The establishment of a reproductively coherent plant group is a task that cannot be performed in exploratory projects such as the one presented here. It requires the establishment of time series data (Kleinschmit et al. 2004). Therefore, the term “occurrence” is used in this paper in place of the term “population.”Occurrence is defined as a collection of individuals growing at a specific locality.

Sampling of leaf material

The shortest distance from Madeira to the Canary Islands, the east coast of Iberia Peninsula and the Cape Verde Islands is approximately 500 km, 1700 km and 2100 km, respectively. The task of collecting plant material was therefore shared by project partners and supported by travel funds provided by the European Cooperative Programme for Plant Genetic Resources (ECPGR, Rome) in the year 2015. Distribution data of *Patellifolia* taxa were downloaded from the Global Biodiversity Information Facility (GBIF 2015), the International Database for Beta (IDBB 2015), the Genetic Resources Information Network (GRIN 2015) and the EURISCO Web Catalogue (EURISCO 2015) and compiled. This data set and in particular the knowledge of local experts was used to identify potential growing sites of *Patellifolia*. The localities were visited by project partners to take plant samples. Further details on the collecting mission are reported by Frese et al. (2017b). Information on the geographic origin of the sampled plant species and the material codes used in tables and figures is given in Table 1. A leaf sample of 0.5–1 g fresh weight was taken from up to 40 individual plants per site, stored separately and dried in silica gel in tea filter bags. When possible, plants were sampled at a distance of 2 m between individuals so as to minimize kinship. The leaf samples were processed in a standardized manner for molecular analysis in the Julius Kühn-Institut.

Laboratory analysis

Genomic DNA was extracted from dried (20 mg) leaf material after vigorous homogenization in a mixer-mill disruptor according to the modified CTAB protocol (Saghai-Maroof et al. 1984). DNA amplification was carried out in a total volume of 10 µL. The fluorescent dye labelling of PCR products in one reaction was performed with three primers. The PCR mix contained 25 ng template DNA, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.05 µM of a sequence-specific forward primer with M13 tail at its 5’ end, 0.17 µM of a sequence-specific reverse primer, 0.035 µM of the universal fluorescent-labelled (dye: D2, D3, D4) M13 primer and 0.5 U Taq DNA polymerase. Thus, a multiplexing could be performed depending on the marker. A touch-down PCR profile was generally used as described by Nachtigall et al. (2016). The microsatellite analysis was conducted using a capillary electrophoresis GenomeLab^{TM} GeXP Genetic Analysis System (Sciex). Fragment sizes were determined and documented in a database developed by Enders (2010). The analysis of a probe was once repeated in case of the absence of a fragment.

Table 1 Identifiers and geographic origin of the occurrences

Population identified	Origin country FAO ISO3 code	Location of the site	Site code	Region code used in Fig. 1	Legend used in Figs. 3 and 4, and Supplement 1a and 2	Legend used in Figs. 2, 5 and 6, and Supplement 1b and 3
	P. patellaris					
AZO2403151630	ESP	Murcia	AZO	IBE	1	–
BAL2104150900	ESP	Almería	BAL	IBE	2	–
CV.SV.BG-1	CPV	Cape Verde, São Vicente Island	**BG**	CAP	3	–
CGO3103151000	ESP	Málaga	**CGO**	IBE	4	–
CLM0707151601	PRT	Madeira	**CL**	MAD	5	–
CNE2303151030	ESP	Murcia	CNE	IBE	6	–
COL2005151000	ESP	Castellón	COL	IBE	7	–
CTM0707151415	PRT	Madeira	**CT**	MAD	8	–
FM-1	PRT	Setúbal	**FM**	IBE	9	–
FXM0707151719	PRT	Madeira	FX	MAD	10	–
H	ESP	La Gomera	H	CAN	11	–
HS	ESP	El Hierro	**HS**	CAN	12	–
MOR0903151000	ESP	Alicante	MOR	IBE	13	–
PCA3003151000	ESP	Málaga	PCA	IBE	14	–
PPL	ESP	La Palma	PPL	CAN	15	–
PS-1	PRT	Faro	PS	IBE	16	–
PSM2704151936	PRT	Madeira	PS32	MAD	17	–
CV.SV.RSJ-1	CPV	Cape Verde, São Vicente Island	**RSJ**	CAP	18	–
S	ESP	La Gomera	S	CAN	19	–
STE2104151800	ESP	Almería	STE	IBE	20	–
TES	ESP	Tenerife	**TES**	CAN	21	–
TLG	ESP	Tenerife	TLG	CAN	22	–
TLS	ESP	Tenerife	TLS	CAN	23	–
TPA	ESP	Tenerife	TPA	CAN	24	–
TPH0604151200	ESP	Tenerife	TPH	CAN	25	–
TPM	ESP	Tenerife	TPM	CAN	26	–
	P. procumbens/P. webbiana					
–	ESP	Gran Canaria	**PWGI**	–	–	1
TAL	ESP	Tenerife	TAL	–	–	2
TBA	ESP	Tenerife	TBA	–	–	3
TGA	ESP	Tenerife	TGA	–	–	4
TPC	ESP	Tenerife	TPC	–	–	5
TPH0604151144	ESP	Tenerife	**TPH**	–	–	6
TPS	ESP	Tenerife	**TPS**	–	–	7

The descriptor “population identifier” and “site code” was used in the final report of the GeDiPa project (Frese et al. 2017b) and is listed here as a reference. Site codes printed in bold letter indicate the locality of recommended genetic reserves

If the fragment remained absent, the allele was recorded as a null allele.

Twenty-six occurrences of *P. patellaris* with a total number of 581 individuals were analysed using 24 markers. Furthermore, six occurrences of *P. procumbens* plus one *P. webbiana* summing up to a total number of 172 plants were analysed with 22 markers.

The complete data sets were published separately as open access files (Nachtigall et al. 2018).

Statistical analysis

The microsatellite markers used in the present study were all derived from genomic sequences of diploid *P. procumbens* (Nachtigall et al. 2016). Statistical procedures for diploid species were therefore employed in subsequent statistical analyses. The ALLELE procedure of SAS (version 9.3) was used to calculate the number of alleles per SSR locus over all occurrences and the polymorphic information content of the SSR loci. For each occurrence/SSR marker combination the deviation from the Hardy–Weinberg principle (HWP) was tested with the Chi^2-Test and the result either indicated as HWE (in equilibrium) or HWD (in disequilibrium). Factorial analysis (Software Darwin, Perrier and Jacquemound-Collet 2006) was applied to identify variation patterns in the *P. patellaris* as well as *P. procumbens/P. webbiana* dataset and to learn if the variation patterns agree with the geographic origin of the individuals. All data including null alleles were used.

Nei's measure of diversity (Nei 1973) as well as F-statistics (Weir and Cockerham 1984) and derived measures have been widely used to illustrate genetic relationships among populations. These measures reflect only the differences in the relative frequencies of the trait states and do not consider quantitative differences in trait states. To overcome this problem, Gregorius et al. (2003) developed the difference measure Δ that is based on both frequency and trait differences. The measure Δ has a number of advantages compared to commonly used measures as explained and comprehensively discussed by Gillet and Gregorius (2008), Gillet (2013) and Gregorius et al. (2014). It allows inter alia the analysis of differentiation among occurrences at different levels of genic integration. In addition, the genetic distance measure Δ is free of assumptions such as the presence of the HWE in panmictic ideal populations and its subpopulations.

For a specific trait and a pair of populations, the pairwise genetic distance (Δ) equals the minimal extent to which the genetic types (e.g., alleles of a gene) *of individuals in one population must be altered to obtain the composition of genetic types in the other* (Gillet and Gregorius 2008). The measure Δ can be used to calculate the complementary compositional differentiation within a set of several populations whereby Δ_j is the contribution of the jth population to genetic differentiation. It is the genetic distance of the jth population to the pooled remainder ("the complement"). Δ_{SD} quantifies the average degree to which all populations differ from their complements. This approach is applicable to co-dominant marker data sets (Gillet 2013).

The genetic distance measure Δ considers trait differences (Gregorius et al. 2003), i.e. fragment size in case of the *Patellifolia* research project. As null alleles are coded with 999, which would represent the largest possible fragment size, all individuals having a null allele at any of the loci were excluded from the dataset prior to genetic analysis. The software DifferInt (Gillet 2013) was used to calculate the pairwise genetic distance Δ between two occurrences as well as Δ_j and Δ_{SD}. The matrix of Δ values was loaded into DARwin to construct an UPGMA tree illustrating the genetic relationships between occurrences. Distance and differentiation measures as well as the UPGMA tree were obtained for three levels of genic integration, namely the gene pool (GP) level, i.e. all alleles at one or more loci and the single-locus (SL) level (single-locus genotypes). These two are characterized by locus and allelic state. The highest level of genic integration is the multi-locus (ML) level. The multi-locus genotypes (individual plants) are characterized by the allelic states at all loci (Gillet 2013).

Ten thousand new data sets were generated by random permutation of all genes (alleles) at each locus among the individuals within each occurrence. In a second permutation analysis all individuals together with their multi-locus types were randomly permuted among the populations. The results of the permutation analyses were used to test hypothesis on forces involved in differentiations.

Results

Descriptive genetic parameter

Within the total set of *P. patellaris* marker data (581 plants, 24 marker; 27,936 data points) 434 null alleles were detected. The number of null alleles found in the *P. patellaris* material decreased to 390 after exclusion of JKIPat02 and JKIPat03, the marker loci which did not function well in *P. procumbens/P. webbiana*. Only 82 null alleles were recorded for *P. procumbens/P. webbiana* (172 plants, 22 marker; 7568 data points). If calculated over the 22 markers commonly used for analyzing both groups of material, 1.52% of null alleles were observed for *P. patellaris* as compared to 1.08% in *P. procumbens/P. webbiana.* Marker loci JKIPat06, JKIPat07, JKIPat10, JKIPat21, JKIPat22, JKIPat24, JKIPat25, and JKIPat26 produced no null alleles in *P. procumbens/P. webbiana* at all. All markers were derived from a *P. procumbens* sequence (Nachtigall et al. 2016) which explains the lower percentage of null alleles observed in the set of *P. procumbens/P. webbiana* plants.

The range of allele size described by Nachtigall et al. (2016), the observed range, the number of alleles at each locus and the polymorphic information content values (PIC) are presented in Table 2. In *P. patellaris* the number of alleles ranged from 4 to 29, the PIC-value from 0.2722 to 0.8711, the observed heterozygosity from 0.0223 to 0.9742 and the expected heterozygosity from 0.2900 to 0.8823. Within the set of *P. procumbens/P. webbiana* the number of alleles ranged from 4 to 24, the PIC-value from 0.3289 to 0.9047, the observed heterozygosity from 0.2500 to 0.8663 and the expected heterozygosity from 0.3905 to 0.9112. In total 359 alleles with an average of 15 alleles per locus were detected in *P. patellaris* and 264 alleles with an average of 12 alleles per locus were observed in the smaller set of *P. procumbens/P. webbiana*.

In *P. patellaris* the average percentage of invariant "occurrence/marker" combinations was 15.5%. The percentage of combinations in HWE ranged between 0.0 and 58.3% with an overall average of 14.6%. The overall average of all combinations in HWD was 69.4%. Contrary to *P. patellaris* all "occurrence/marker" combinations of *P. procumbens/P. webbiana* were variable. The percentage of marker loci per occurrence showing HWE ranged between 31.8 and 59.1% and the overall average of all "occurrence/marker" combinations in HWE or HWD was 50.6% and 49.4%, respectively (Supplement 1a, b). Because all of the *Patellifolia* samples used in this study did not match the criteria of random mating plant groups in HWE, the measure Δ was chosen for statistical analysis.

Structures of phenotypic diversity

The results of the factorial analysis of the *P. patellaris* data set (581 individuals × 24 markers) are presented in Fig. 1. The x-axis explains 28.2% and the y-axis 6.8% of the variation. The individuals were assigned to the four geographic groups (Table 1) that are isolated by the Atlantic Ocean by several hundreds of kilometres, i.e. the Iberian Peninsula, Madeira, the Canary Islands and Cape Verde Islands. The distribution pattern of individuals in the scatter plot coincides well with the geographic origin of the sampled plants. The material from the Iberian Peninsula is separated into plants sampled in Portugal and a larger group distributed from South to East Spain. Plants from Tenerife, La Palma and La Gomera form a swarm, whereas the samples from El Hierro form a separate cloud. The individuals from Madeira are interspersed with those from Tenerife, La Gomera and La Palma. Finally, the individuals from Cape Verde Islands form a distinct group but weakly separated from that composed by individuals from Tenerife, La Gomera, La Palma and Madeira.

Figure 2 displays the results for *P. procumbens/P. webbiana* (172 individual × 22 markers). The y-axis explains 11.6% and the x-axis 6.1% of the total variation. As observed for *P. patellaris*, the patterns of *P. procumbens/P. webbiana* coincide well with the geographic origin of the sampled individuals. The dots located in the left part of the figure represent *P. webbiana* individuals collected in 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 (occurrences no. 2, 3, 7 in Fig. 2). Occurrence 6, also located on the northern coastline, forms a separate group as do the groups from north-eastern (occurrence

Table 2 Motif, reported (Nachtigall et al. 2016) and observed allele range, number of alleles per marker loci (NoA), polymorphic information content (PIC), observed heterozygosity (H_o) and expected heterozygosity (H_e)

Locus	Motif	Range reported	Range observed	NoA	PIC	H_o	H_e	Range observed	NoA	PIC	H_o	H_e
			P. patellaris					*P. procumbens/P. webbiana*				
JKIPat01	$(GA)_8$	170–191	162–220	13	0.6973	0.0309	0.7361	186–200	8	0.7547	0.2558	0.7858
JKIPat02	$(CA)_8$	196–227	163–256	13	0.3905	0.2027	0.3997	*				
JKIPat03	$(TG)_{19}$	198–231	208–254	26	0.8486	0.9038	0.8622	*				
JKIPat04	$(CT)_8$	216–228	221–249	15	0.8711	0.7612	0.8823	233–249	8	0.5771	0.4477	0.6207
JKIPat05	$(GT)_8$ $(GA)_{16}$	181–232	197–263	11	0.5500	0.6804	0.5885	224–256	16	0.7478	0.8314	0.7734
JKIPat06	$(AG)_{24}$	161–197	132–226	20	0.6690	0.7801	0.6981	178–225	24	0.9047	0.6395	0.9112
JKIPat07	$(TC)_8$	173–179	168–290	14	0.3835	0.4966	0.4481	187–197	7	0.6902	0.5523	0.7298
JKIPat08	$(AG)_8$	183–193	194–227	12	0.3760	0.0223	0.3867	191–211	10	0.5942	0.3023	0.6270
JKIPat10	$(CA)_6$	164–169	172–223	10	0.2722	0.0361	0.2900	181–189	4	0.5627	0.4012	0.6335
JKIPat11	$(TC)_{11}$	157–184	142–214	25	0.7938	0.2010	0.8143	159–224	22	0.7463	0.2500	0.7595
JKIPat12	$(AG)_9$	165–191	180–217	14	0.6510	0.8797	0.6867	182–231	20	0.8725	0.5116	0.8824
JKIPat13	$(CA)_2$ $(GA)_8$	151–179	166–211	25	0.8551	0.9364	0.8683	169–217	18	0.8875	0.4826	0.8951
JKIPat14	$(AC)_8$	219–223	218–262	16	0.7117	0.1890	0.7380	214–261	16	0.6623	0.4884	0.6846
JKIPat15	$(GT)_{10}$	174–192	174–232	29	0.8194	0.9742	0.8378	190–210	10	0.8349	0.5988	0.8509
JKIPat17	$(CT)_{14}$	188–202	203–217	9	0.5086	0.0344	0.5872	148–217	11	0.7119	0.3140	0.7310
JKIPat18	$(TG)_8$ $(AG)_4$	177–191	167–235	11	0.4885	0.0790	0.5781	195–226	10	0.6651	0.5407	0.6963
JKIPat19	$(GGAT)_8$	203–258	182–284	16	0.7015	0.9450	0.7277	203–279	14	0.8705	0.7384	0.8818
JKIPat20	$(TCTT)_9$	231–256	236–264	9	0.6920	0.0979	0.7366	228–297	17	0.7714	0.6279	0.7950
JKIPat21	$(GGAA)_7$	175–195	173–230	13	0.7552	0.8969	0.7884	202–218	4	0.3289	0.4128	0.3905
JKIPat22	$(GAAA)_3$	130–148	147–165	4	0.3898	0.6443	0.4784	147–216	6	0.5449	0.8663	0.6203
JKIPat23	$(TA)_8$ $(GATA)_5$	185–224	167–319	12	0.4479	0.0275	0.4735	190–278	14	0.8429	0.4012	0.8588
JKIPat24	$(ATTC)_7$	182–214	208–232	9	0.4102	0.4897	0.4769	207–233	9	0.7317	0.6919	0.7683
JKIPat25	$(GTGA)_9$	179–195	177–310	12	0.7258	0.7199	0.7631	191–211	6	0.6973	0.4826	0.7419
JKIPat26	$(GA)_9$ $(TAGA)_3$	159–191	156–273	21	0.6542	0.7973	0.6827	156–184	10	0.4757	0.5233	0.5690

The number of individuals used to calculate the observed marker features is 581 for *P. patellaris* and 172 for *P. procumbens/P. webbiana*. *In *P. procumbens/P. webbiana* JKIPat02 and JKIPat03 caused too many irregular laboratory results and were therefore excluded from further analysis

no. 4) and south-western Tenerife (occurrence no. 5 in Fig. 2).

Analysis of genetic diversity

The number of individuals in the *P. patellaris* data set decreased from 581 to 458 and in the data set of *P. procumbens/P. webbiana* from 172 to 145 after exclusion of individuals having a null allele at any of the marker loci.

P. patellaris

The results of the permutation analysis for *P. patellaris* are presented in Table 3. The Δ_{SD} values within the 95% confidence interval generated by 10,000 permutations are significantly lower (p =0.0018) than the observed Δ_{SD} value of 0.4862 at the mean single-locus level. This result indicates that the association of alleles within single loci depends on the type of alleles present in the occurrence and that the occurrences

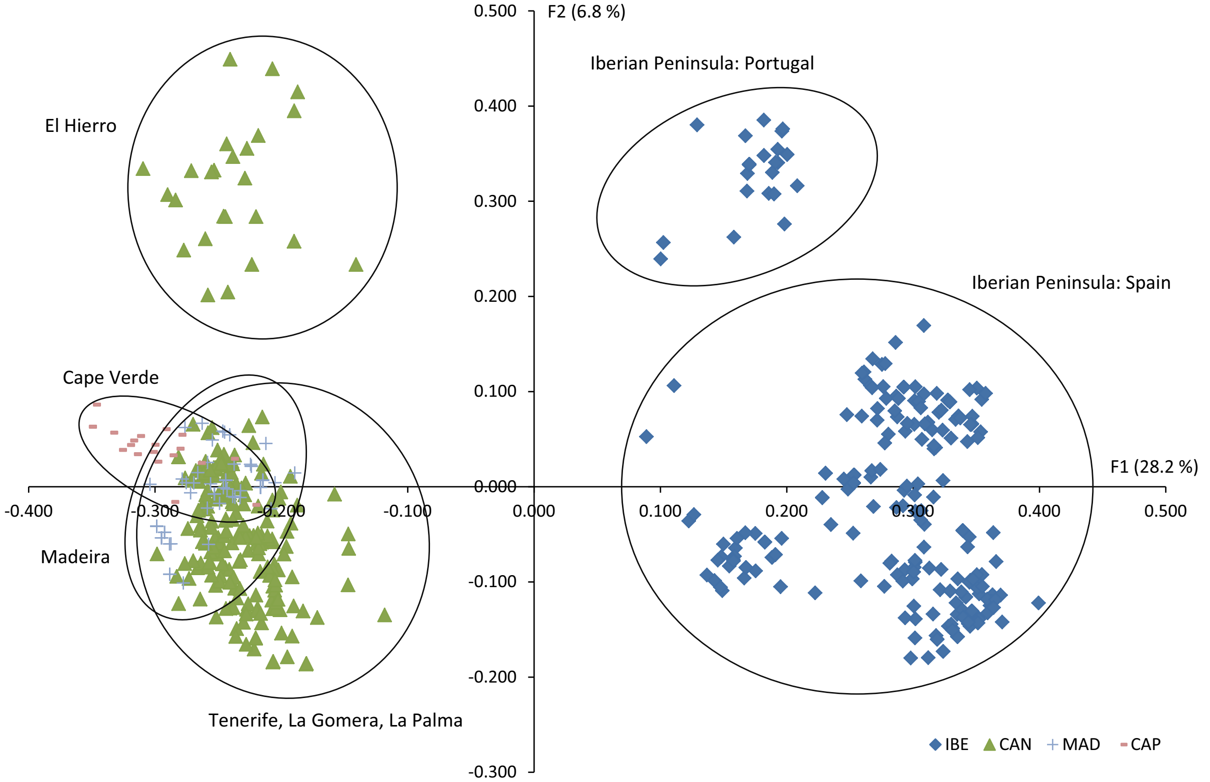

Fig. 1 Factorial analysis for *P. patellaris*. Factorial coordinates were calculated from dissimilarity values. IBE = Iberian Peninsula, two occurrences from Portugal, eight occurrences from Spain; CAN = Canary Islands, Spain, ten occurrences; MAD = Madeira, Portugal, four occurrences; CAP = Cape Verde Republic, two occurrences

differ significantly in the form of homologous association of genes. In other words, the Δ_{SD} can increase only if a fraction of individual loci within single occurrences higher than the observed would be heterozygous and would allow the generation of new single-locus genotypes through permutation. The genes were also randomly combined to multi-locus types and the observed distance compared to the Δ_{SD} values generated by 10,000 permutations. The Δ_{SD} values within the 95% confidence interval are significantly higher (p =1.0) than the observed Δ_{SD} value of 0.5144 at the multi-locus level. It indicates that forces restrict the formation of new multi-locus genotypes within the observed occurrence.

Self-fertilisation appears to take place at each locality at a higher rate than expected by chance. In addition, within the set of 458 individuals, 360 distinct multi-locus genotypes were observed. Individual genotypes were represented twice up to eightfold (a total of 98 duplicated genotypes) indicating preferential self-fertilization in *P. patellaris*.

To test the hypothesis that individuals associate with occurrences independently of their genetic type at a given level of integration, individuals were randomly permuted among the occurrences. The Δ_{SD} values are significantly lower than the observed ones at all integration levels. The difference between the maximal Δ_{SD} generated by permutation and the observed Δ_{SD} is high (gene pool level: 0.2684, mean single-locus level: 0.2639, multi-locus level: 0.2007) for all integration levels. This finding indicates a combined effect of genes and their associations on differentiation (Gillet 2013).

The pairwise genetic distance (Δ) ranged between 0.1783–0.9157 (gene pool level), 0.1901–0.9184 (mean single-locus level) and 0.2481–0.9178 (multi-locus level). The differences between occurrences are indeed pronounced as visualised by the UPGMA trees presented in Supplement 2. The trees reflect the

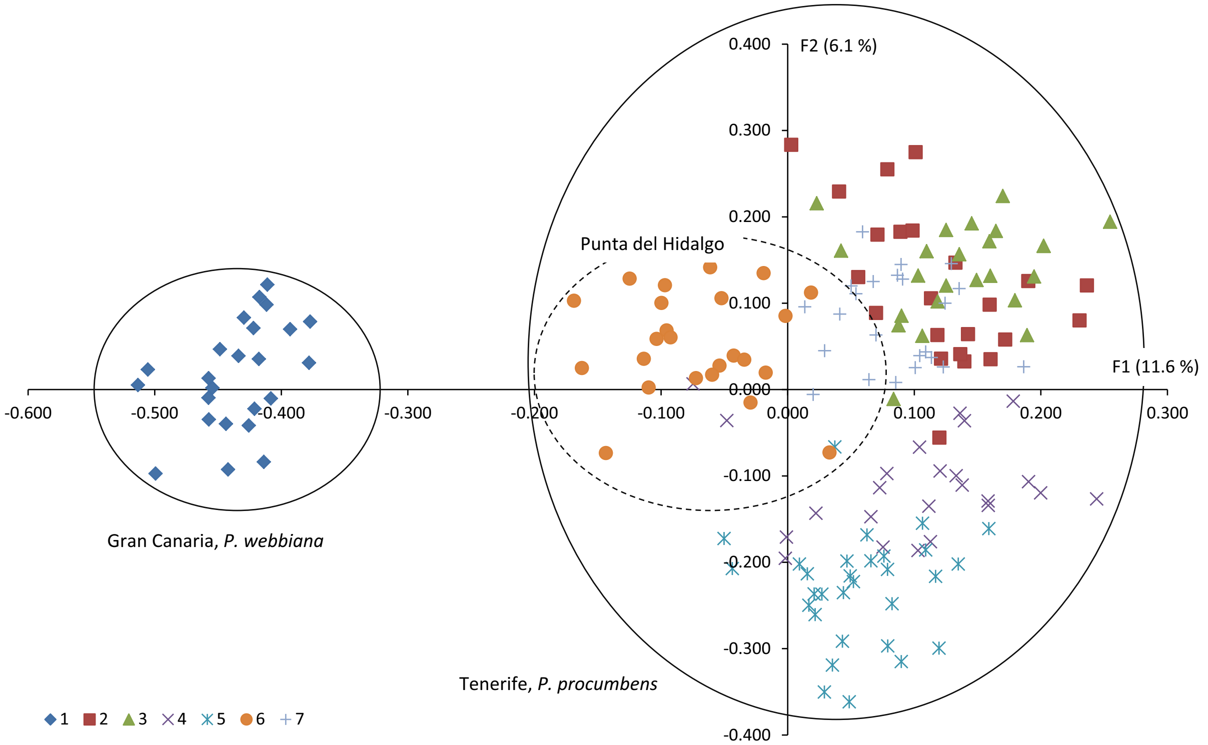

Fig. 2 Factorial analysis for *P. procumbens* and *P. webbiana*. Factorial coordinates were calculated from dissimilarity values. Numbers shown next to the symbols refer to occurrence no. 1–7 (see Table 1)

matrices of pairwise genetic distance calculated for the gene pool, mean single-locus and multi-locus level of genic integration.

Figure 3a–c illustrates the differentiation within the set of *P. patellaris* occurrences. The mean complementary compositional differentiation increased from the gene pool ($\Delta_{SD} = 0.472$; 100%), the mean single-locus ($\Delta_{SD} = 0.486$; 103%) to the multi-locus level ($\Delta_{SD} = 0.514$; 109%). At the gene pool level occurrence no. 12 (El Hierro) surmounts the mean differentiation, i.e. the genetic diversity contained in the remaining pooled 25 occurrences (marked by the circle) while occurrence no. 21, located in Tenerife, best represents the genetic diversity of its complement. Between these two extremes the remaining occurrences are sorted clockwise according to their Δ_j values in decreasing order.

Occurrences representing the complement perfectly are characterised by $\Delta_j = 0$ while occurrences sharing none of the genetic types with the complement in common are indicated by $\Delta_j = 1$.

As shown in Fig. 3a–c the ranking of occurrences changes with increasing level of genic integration. To test that the changed ranking is not just a random effect, the same set of random permutations of genes within populations was used to calculate the covariation of contributions to differentiation of occurrences between integration levels. High and positive covariation of Δ_j values for all pairs of integration levels show that no form of association completely overturns the ranking prescribed by the gene pool (Gillet and Gregorius 2008). The results are given in the lower part of Table 3. The covariation is strong, positive and significant.

P. procumbens/P. webbiana

The alleles in *P. procumbens/P. webbiana* are arranged to single-locus types and to multi-locus types at random as indicated by the values of $p = 0.2642$ and $p = 0.2375$, respectively (Table 4). This type of genic association can be explained by mainly random combination of alleles to genotypes and the mainly random combination of single loci to multi-locus genotypes. The set of 145 analysed plants was composed of 144 distinct multi-locus types.

Table 3 *P. patellaris*. Permutation analysis of alleles over individuals within occurrences, of all individual genotypes among occurrences and the covariation of differentiation

Permutations	Observed Δ_{SD} values	Min	0.95 confidence interval	Max	*p* value
Alleles over individuals within occurrences					
Gene pool level	0.4719		Not affected		
Mean single locus genotypes	0.4862	0.4827	0.4836; 0.4857	0.4866	0.0018 **->
Multilocus genotypes	0.5144	0.5485	0.5503; 0.5529	0.5543	1.0000 **<-
All individual genotypes between occurrences					
Gene pool level	0.4719	0.1561	0.1804; 0.2042	0.2035	0.0000 **->
Mean single locus genotypes	0.4862	0.1725	0.1804; 0.2042	0.2223	0.0000 **->
Multilocus genotypes	0.5144	0.2635	0.2729; 0.2968	0.3137	0.0000 **->
Covariation of differentiation	Observed covariation value				
Gene pool versus mean single locus level	1	Undefined	******; ******	undefined	1.0000 **<-
Gene pool versus multilocus level	0.9974	0.9970	******; ******	undefined	1.0000 **<-
Mean single locus versus multilocus level	0.9977	0.9900	0.9927; 0.9970	0.9987	0.0039 **->

The values are based on 10,000 permutations for all 24 loci. The observed Δ_{SD} value is shown for the three integration levels. The calculated minimum and maximum Δ_{SD} values, the 95% confidence interval as well as the permutation (*p*) values are shown. The symbols **-> (upper part of the distribution) and **<- (lower part of the distribution) indicate that fewer than 1% and more than 99% of the permutations yielded Δ_{SD} values that are significantly equal or greater than the observed distance. **$p \leq 0.01$

DifferInt generates a list of distinct multi-locus types which are labelled with a sequence number. Except for sequence number 53, the genotype of plant number 18 and 19 of occurrence no. 3 growing next to each other at the collecting site Bajamar in Tenerife, all of the multi-locus types represent a unique arrangement of the alleles. Thus, it appears that outbreeding is the preferential mode of reproduction of *P. procumbens/ P. webbiana*.

The individuals associate with occurrences independently of their genetic type, as already described for *P. patellaris*. The Δ_{SD} values generated by permutation are significantly lower than the observed ones at all integration levels. The difference between the maximal Δ_{SD} generated by permutation and the observed Δ_{SD} is high (gene pool level: 0.2077, mean single-locus level: 0.1836, multi-locus level: 0.0984) for all integration levels. This finding indicates a combined effect of genes and their associations on differentiation.

The pairwise genetic distances (Δ) ranged between 0.3498 and 0.6881 at the gene pool level, between 0.3696 and 0.6983 at the mean single-locus level, and between 0.5114 and 0.7528 at the multi-locus level. The cluster diagrams representing the genetic distances at all three levels of genic integration are given in Supplement 3.

The complementary compositional differentiation values Δ_j varied from 0.3349 to 0.5907 (gene pool level), from 0.3702 to 0.6030 (mean single-locus level) and from 0.5557 to 0.7110 (multi-locus level). With increasing level of integration, the mean differentiation increased from the gene pool ($\Delta_{SD} = 0.4258$; 100%), the mean single-locus ($\Delta_{SD} = 0.4477$; 105%) to the multi-locus level ($\Delta_{SD} = 0.6049$; 142%). At all levels occurrence no. 7 in Tenerife represents the composition of its complement best, while occurrence no 1 in Gran Canaria followed by occurrence no. 6 in Tenerife deviate stronger from their complements (Fig. 4a–c).

The strong and significant covariation for all pairs of integration levels show that the ranking of occurrence does not change with increasing level of genic integration. The differentiation snails (Fig. 4) illustrate this finding.

Discussion

Although plant breeding researchers and conservation biologists belong to different scientific communities

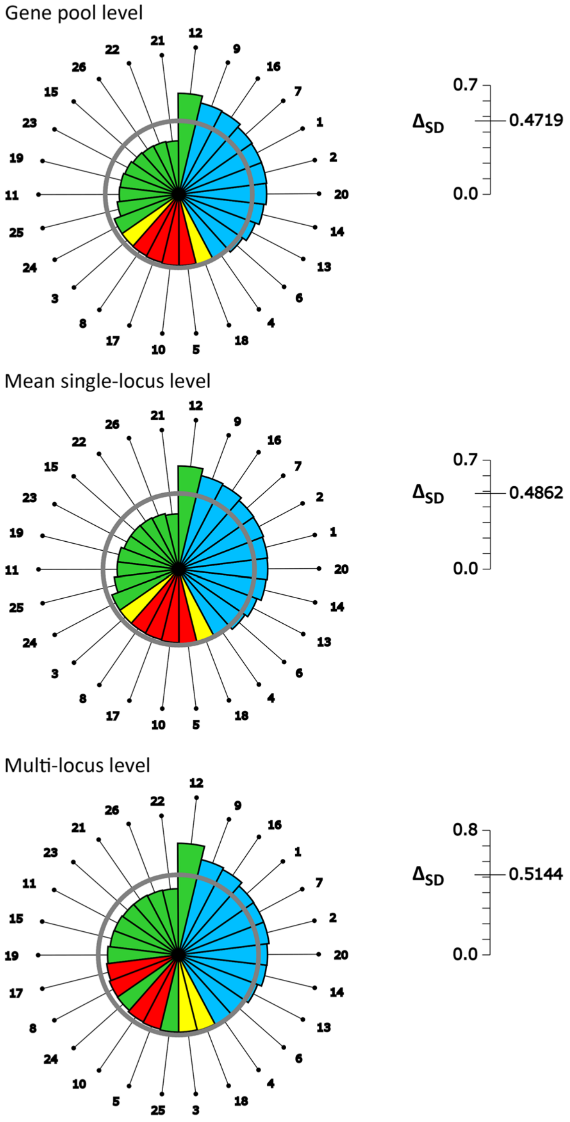

Fig. 3 Snail diagrams showing the differentiation between occurrences of *P. patellaris* at the genepool (**a**), mean single-locus (**b**) and multi-locus (**c**) level. The geographic origin of the occurrences is depicted by colours (green: Canaries; blue: Iberian Peninsula; yellow: Cape Verde; red: Madeira). For more detail on occurrence numbers see Table 1. A sector illustrates one of the occurrences. The radius of its sector equals the contribution of this occurrence to differentiation. The sectors are arranged according to the radius length clockwise in decreasing order starting with the largest radius at 12:00 h. The grey circle (e.g. Figure 3) is equal to the weighted mean of the sector radii and marks Δ_{SD}. The Δ_{SD}-value is also shown on the bar next to the snail graph

they share a common interest: the conservation of CWR. Plant breeders need to know to what extent anthropogenic climate change induced genetic erosion within CWR (Parmesan and Hanley 2015; Aguirre-Gutiérrez et al. 2017) will gradually diminish resources for breeding. Conservation biologists need to understand how loss of biological diversity including the intraspecific diversity will affect ecosystem functions. Both communities are interested to halt the loss of genetic diversity. The establishment of a network of genetic reserves for crop wild relatives was already described by nature conservationists as a new and useful module of species conservation programmes, as each established network will alleviate pressure on species that are not yet threatened (Zehm and Weber 2013).

This study has been implemented to provide species conservation agencies with information on localities suited for the establishment of genetic reserves. The selection of MAWP is still a challenge to the supporters of the genetic reserve conservation approach as the data basis required to take informed decisions is only slowly developing. It concerns the availability of comprehensive European inventories of georeferenced occurrences of CWR and information on the geographic patterns of genetic diversity of a species as well. The geographic patterns of genetic diversity are the result of the interplay between the species breeding system, demographic processes such as extinction and re-colonization events, and environmental factors which determine the survival and reproduction rate of genotypes best adapted to local conditions (see reviews of Eriksson et al. 1993; Manel et al. 2003).

Breeding system

The measure Δ was used to test assumptions on the mode of reproduction of *Patellifolia* species. Frese et al. (2017a) interpreted the results of the statistical analysis of SSR data of 10 *P. patellaris* occurrences as the effect of self-fertilisation. The result of the permutation test using 26 occurrences (Table 3) further substantiates the assumption. Within the set the mean differentiation increases from the gene pool to multi-locus level of genic integration by 9%, only. It indicates that mainly self-fertilization resulting in homologous gene associations determines the differentiation. Several characteristics of inbreeders summarized by Jain (1975) also apply to *P. patellaris*. The population size is often small, and the colonizing ability is high as can be deduced from the very large

Table 4 *P. procumbens/P. webbiana.* Permutation analysis of alleles over individuals within occurrences, of all individual genotypes among occurrences and the covariation of differentiation

Permutations	Observed Δ_{SD} values	Min	0.95 confidence interval	Max	p value
Alleles over individuals within occurrences					
Gene pool level	0.4258		not affected		
Mean single locus genotypes	0.4477	0.4425	0.4446; 0.4493	0.4519	0.2642 ns
Multilocus genotypes	0.6049	0.5972	0.6004; 0.6069	0.6094	0.2375 ns
All individual genotypes between occurrences					
Gene pool level	0.4258	0.1669	0.1760; 0.2020	0.2181	0.0000 **->
Mean single locus genotypes	0.4477	0.2183	0.2270; 0.2492	0.2641	0.0000 **->
Multilocus genotypes	0.6049	0.4749	0.4803; 0.4964	0.5065	0.0000 **->
Covariation of differentiation	Observed covariation value				
Gene pool versus mean single locus level	1	0.9991	1; 1	1	0.0000 **->
Gene pool versus multilocus level	1	0.9970	0.9992; 1	1	0.0000 **->
Mean single locus versus multilocus level	1	0.9960	0.9995; 1	1	0.0000 **->

The values are based on 10,000 permutations for all 22 loci. The observed Δ_{SD} value is shown for the three integration levels. The calculated minimum and maximum Δ_{SD} values, the 95% confidence interval as well as the permutation (p) values are shown. The symbols **-> (upper part of the distribution) and **<- (lower part of the distribution) indicate that fewer than 1% and more than 99% of the permutations yielded Δ_{SD} values are significantly equal or greater than the observed distance. **$p \leq 0.01$; ns = not significant

distribution area compared to *P. procumbens/P. webbiana*. However, only crossing experiments will reveal to what extent *P. patellaris* reproduces by self-fertilization.

The statistical results presented in Table 4 underpin the observations of Frese (2002b) who crossed typical *P. webbiana* with typical *P. procumbens* plants using two phenotypically male sterile *P. webbiana* plants and succeeded to produce several hundreds of seeds. Within the set of plant material described in this paper cross-fertilization appears to be playing an important role in reproduction. The permutation of alleles over individuals within occurrences yielded non-significant permutation values suggesting that non-homologous gene association have a stronger impact on differentiation patterns than homologous gene associations. In addition, the large increase in the mean differentiation, by 42% from the gene pool to the multi-locus integration level, further confirms the assumption of preferential cross-fertilization within the *P. procumbens/P. webbiana* occurrences. The Δ_j values (multi-locus level) of the individual occurrences are high and deviate only slightly from the radius $\Delta_{SD} = 0.6049$. It shows that each of the occurrences has a specific genotypic composition and only moderately represents its complement. In fact, only a single multi-locus genotype was duplicated in the set of *P. procumbens/P. webbiana* plants as opposed to 98 duplicated genotypes in the set of 458 *P. patellaris* plants. This is a diversity pattern of a cross-fertilizing species.

Some characteristics of outbreeders apply to *P. procumbens/P. webbiana*. According to Jain (1975) outbreeders tend to form large, continuous populations characterized by a limited population turnover and the colonizing ability is less than that of inbreeding species. At least in Northwest Tenerife, *P. procumbens* forms large, more or less continuous and permanent populations. At the large geographic scale (countries) *P. procumbens* has a limited distribution compared to *P. patellaris* which is a striking finding. Because all *Patellifolia* species have very similar seed morphology and share similar habitats, one would expect they would have equal chance to spread over long distances. This is obviously not the case for *P. procumbens* and certainly not the case for *P. webbiana*. The latter mainly occurs on La Isleta (Gran Canaria), i.e. the distribution area of this taxon is extremely small.

Diversity patterns, genetic differentiation and conservation planning

Recommendations for conservation planning based on genetic criteria include the risk of being

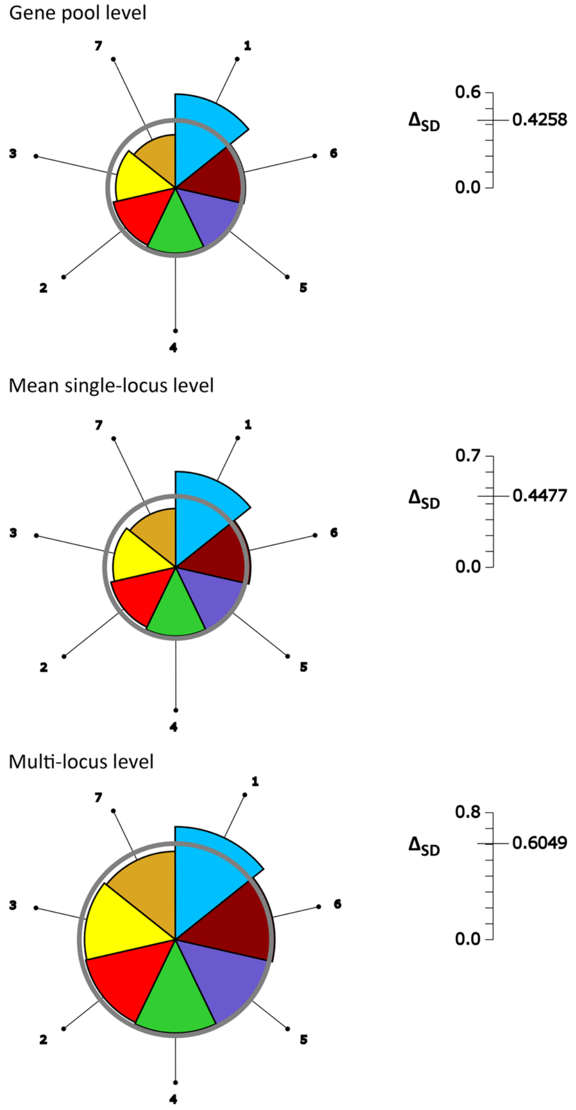

Fig. 4 Snail diagrams showing the differentiation between occurrences of *P. procumbens/P. webbiana* at the genepool (**a**), mean single-locus (**b**) and multi-locus (**c**) level. Contrary to Fig. 3 the colours have no specific meaning. A sector illustrates one of the occurrences. The radius of its sector equals the contribution of this occurrence to differentiation. The sectors are arranged according to the radius length clockwise starting with the largest radius to the right of 12:00 h. The grey circle is equal to the weighted mean of the sector radii and marks Δ_{SD}. The Δ_{SD}-value is shown on the bar next to the snail graph. Occurrence number 1 was sampled on Gran Canaria, occurrences number 2–7 were sampled on Tenerife. For more detail on occurrence numbers see Table 1

misinterpreted. Any recommendation in favour of a specific occurrence only means that this plant group is, at the best of our current knowledge, particularly suited to maintain the adaptability of a species. The recommendation cannot be understood as a categorisation of occurrences into indispensable and dispensable populations. *Patellifolia* populations are first and foremost part of the ecosystem and should be protected wherever they naturally occur. Genetic analyses can only assist identifying those populations within the distribution area which are, according to the current state of knowledge, of particular importance and which deserve specific attention by conservation agencies.

The factorial analysis revealed diversity distribution patterns which agree well with the geographic origin of the *P. patellaris* (Fig. 1) and P. *procumbens*/*P. webbiana* samples (Fig. 2). *P. patellaris* samples from the Iberian Peninsula are subdivided into (1) an Eastern and (2) small Western group. The occurrence samples from the (3) Canary Islands overlap with the exception of the (4) El Hierro occurrence and form a larger swarm. Plants from (5) Madeira are interspersed into the swarm while the material from (6) Cape Verde is more separated. A set of MAWP should at least contain one occurrence of these six groups to capture genetic variation of adaptive traits which cannot be measured with the applied set of SSR marker.

In short-lived *Patellifolia* species the bulk of seeds produced at a location at the end of a season represents the gene pool of the respective occurrence and is the conservation unit. The following discussion concerns therefore the gene pool differentiation patterns. The search of genetic reserves localities can start with a pair of occurrences: one with the highest and one with the lowest Δ_j value. The latter represents the distribution of genetic types in the complement best. The occurrence with the highest Δ_j value is of particular interest as it points to an unusual genetic composition. This occurrence may have specific adaptive potential or interesting traits. Additional criteria, such as the likelihood that the minimum quality standards for genetic resources conservation (Iriondo et al. 2012) can be guaranteed by the responsible genetic reserve manager, can modify the ranking of occurrences during the decision-making process.

P. patellaris

On the Iberian Peninsula the size of *P. patellaris* occurrences is alarmingly small (Frese et al. 2017b) and if the occurrences get lost we will lose a relevant

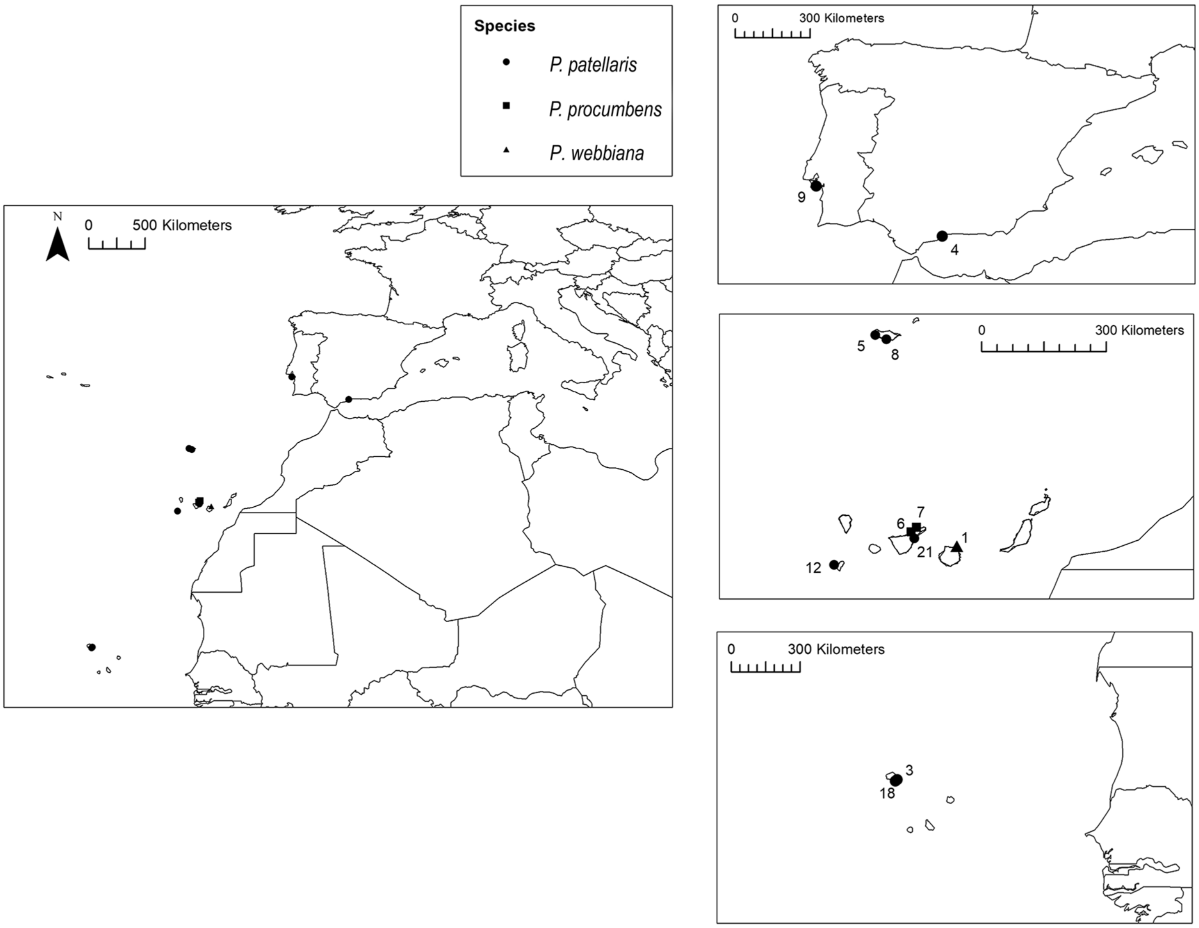

Fig. 5 The maps show the locations recommended genetic reserves sites. Numbers indicate either occurrence of *P. patellaris* or *P. procumbens/P. webbiana* as listed in Table 1. An overview map is displayed on the left. Right part from top to bottom: Iberian Peninsula, Madeira and Canary Islands, Cape Verde Islands

fraction of the genetic diversity. If the conditions do not allow the conservation of all populations within the distribution area, genetic criteria can help taking the best possible decision. When the criterion "lowest/highest Δ_j value" is applied region-wise eight occurrences of *P. patellaris* qualify for proposing MAWP for the following areas: occurrence no. 9 and 4 (E and W Iberian Peninsula, respectively), occurrence no. 5 and 8 (Madeira), occurrence no. 18 and 3 (Cape Verde), and occurrence no. 12 and 21 (Canary Islands; gene pool level, Fig. 3a). The localities of the occurrences are shown in Fig. 5.

P. procumbens/P. webbiana

At Punta del Hidalgo (TPH), in Tenerife, *P. procumbens* forms plant canopies with few interstratified *P. webbiana*-like and *P. patellaris* plants. There is therefore the chance for interspecific crosses between the taxa. Interspecific crosses actually seem to happen as can be concluded from two observations. The *P. procumbens* material sampled at TPH (occurrence no. 6) clusters next to occurrence no. 1, i.e. *P. webbiana* sampled on Gran Canaria, indicating relatedness between occurrence no. 6 and 1 (Supplement 3). In fact, the genetic distance between occurrence no. 6 and 1 is $\Delta = 0.6047$ and the lowest compared to the remaining five *P. procumbens* occurrences sampled on Tenerife. The community of *Patellifolia* plants at TPH is not only distinguished by a high morphological variation (Frese et al. 2017b) but also shows cytogenetic variation. At this site diploid, triploid and tetraploid plants were observed. The results indicate that interspecific hybridization generates novel genetic variation within the genus, which is positive for the species survival (Castro et al. 2017).

Occurrence no. 1 (*P. webbiana*) showed the highest Δ_j value (Fig. 4a) which can be explained by its almost unique composition at five of the 22 SSR loci (details not presented). In this particular case occurrence no. 1 is evidently a MAWP. *P. procumbens* occurrence no. 6 and 7 showed, respectively the largest and smallest distance to the complement and are also recommended as MAWP. Last not least, *Beta macrocarpa* is present at the TPH site (occurrence no. 6) which makes the site a candidate for the establishment of a multispecies genetic reserve. The locations of the occurrences are shown in Fig. 5.

This paper presents the first comprehensive study on patterns of genetic diversity of *Patellifolia*. The focus was set on the identification of a number of occurrences thought best suited to maintain the genetic diversity of the species within the European distribution area. Additional analysis will help to better understand the type and intensity of mechanisms generating fine-scale (Lara-Romero et al. 2016) and large-scale spatial genetic structures. With its scattered distribution in a large geographic area, research on the migration histories and genetic relationships between occurrences is not only a scientifically fascinating subject but also of relevance as to the identification of further localities for a future implementation of a comprehensive *Patellifolia* genetic reserve network.

It is necessary at this point to emphasize that, not solely for *Patellifolia*, conceptual and procedural tasks call for completion. *P. patellaris* occurrence no. 9 from West Portugal is a good example for pending issues. If the quality criteria for MAWP as defined by Maxted et al. (2015) are strictly applied, *P. patellaris* occurrence no. 9 from West Portugal would not qualify for the establishment of a genetic reserve due to the very low number of individuals. In Portugal the species is very rare, highly endangered and is thus a candidate for urgent species conservation actions. If the plant genetic resources conservation community does not recommend a genetic reserve site for occurrence no. 9 and if the nature conservation agencies are not aware of the conservation status of the species no actions will be taken at all. This case exemplarily underpins the need for systematic collaboration and information exchange between the plant genetic resources conservation and species conservation community, as well as with legislative authorities, which can be facilitated by the working group “Wild Species in Genetic Reserves” of the European Cooperative Programme for Plant Genetic Resources (ECPGR). Concepts for in situ conservation of CWR in Europe have been published (Maxted et al. 2015) and research gains momentum. However, the actual implementation is lagging behind and waiting the support of adequate public policies.

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Authors’ contribution LF coordinated the work, performed the statistical analyses and drafted the paper. MN supervised the laboratory work, documented and processed the raw data. JMI, MLRT, MCD, and MÂAPC organised and conducted the collection trips. All persons contributed to the writing of the paper.

Compliance with ethical standards

The research work complies with ethical standards.

Conflict of interest The authors have no conflicts of interest to declare.

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