Genet Resour Crop Evol
DOI 10.1007/s10722-015-0357-9

RESEARCH ARTICLE

Genetic diversity of Bolivian wild potato germplasm: changes during ex situ conservation management and comparisons with resampled in situ populations

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Received: 17 June 2015 / Accepted: 8 December 2015

Abstract Potato wild relatives are important sources of novel variation for the genetic improvement of the cultivated potato. Consequently, many natural populations have been sampled and were deposited as accessions in gene banks around the world. Here we investigate to what extent the genetic variation of Bolivian wild potato species is maintained under gene bank conditions and how this diversity relates to that of current in situ populations. For this purpose, materials from seven potato species were screened for microsatellite variation. Genetic changes between different generations of ex situ germplasm were not observed for *Solanum leptophyes* and *S. megistacrolobum*, but were detected for *S. neocardenasii* and *S. okadae*, while each of the species *S. acaule*, *S. avilesii* and *S. berthaultii* showed stability in some cases and genetic change in others. The observed changes were ascribed to genetic drift and contamination resulting from human error during regeneration. Re-collected populations of six of the studied species showed highly significant genetic differences with the ex situ accessions that, apart from changes during ex situ maintenance, are most likely to be attributed to sampling effects during collecting and in situ genetic changes over time. The implications of the results for ex situ and in situ conservation strategies of wild potato species are discussed.

Keywords Bolivia · Crop wild relatives · Genetic variation · Genetic resources management · Potato

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Introduction

The wild relatives of potato are an important reservoir of genetic resources for crop improvement. Wild species have been used, in particular, as sources of resistance to pests and diseases caused by bacteria, fungi, insects, nematodes and viruses, and to abiotic stress such as drought and frost (Bradshaw and Ramsay 2005; Bradshaw et al. 2006; Coleman 2008; Estrada Ramos 2000; Gabriel 2010; Gabriel et al. 2007; Hijmans et al. 2003; Jacobs et al. 2010; Jansky et al. 2008; Spooner et al. 2009).

Wild tuber-bearing *Solanum* species are found in the Americas from the southwestern United States to

Published online: 19 January 2016

central Argentina and Chile (Hijmans and Spooner 2001; Hijmans et al. 2002). Potato genetic resources have been collected extensively in the past and these collections are conserved worldwide in at least 23 gene banks (GCDT 2015). Due to taxonomic reconsiderations, the number of species has been gradually reduced from 232 (Hawkes 1990) to 188 (Spooner and Salas 2006) to around 100 (Spooner et al. 2014). However, the resulting synonymy has not yet been fully implemented by all gene banks. The Centre for Genetic Resources, the Netherlands (CGN) maintains a potato collection of 2700 accessions comprising 127 accepted species names (CGN 2014). Part of this potato collection was originally collected in Bolivia. The earliest Bolivian material mentioned in the CGN database was collected in 1941, while the most recent materials were added in 2004. The total collection of Bolivian wild potato material was repatriated in 2004 to join the potato collection of the National Gene Bank of Andean Tubers and Roots, maintained by PROINPA (Cochabamba, Bolivia).

Gene banks are concerned with the maintenance of the genetic variation of crop genetic resources. Figure 1 shows the potential factors influencing genetic variation under ex situ and in situ conditions. Ex situ management includes the multiplication of seeds from the original sample collected from the field (G0), representing the source for the next generation (G1), which in its turn may be the source of later regenerations (G2, etc.). The frequency of regenerations may vary between accessions, depending on seed depletion and loss of viability. As a result of regeneration the genetic variation of an accession may be affected by unintentional selection, mutation, genetic drift and gene flow, the latter either through seed contamination or unwanted cross pollination between different accessions (Bacchetta et al. 2008; Bamberg and Del Rio 2006; Rao et al. 2007).

How the genetic variation of gene bank accessions relates to that of the current in situ populations depends not only on the influences experienced during ex situ maintenance, but also on the collecting procedures used to sample the original in situ populations and the processes that in situ populations may have experienced since they were sampled. Whether representative samples were collected for ex situ storage depends on the sample size, the type of material sampled (e.g. seeds, tubers or whole plants) and the extent of spatial variation at the sampled location. After collecting for ex situ storage, in situ populations may have experienced mutation, natural selection, genetic drift and gene flow (Fig. 1). Moreover, there is a growing concern that in situ genetic variation may be negatively influenced through

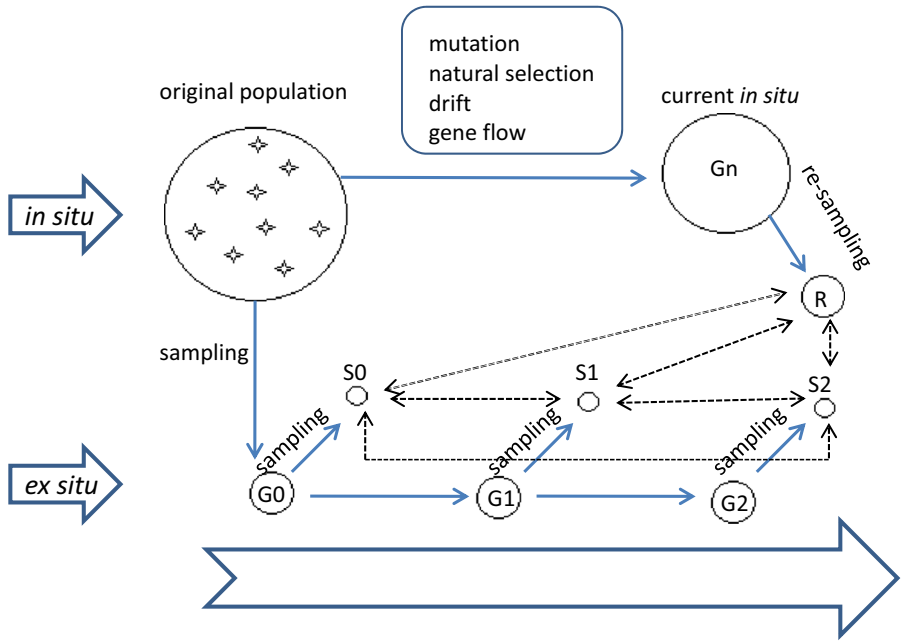

Fig. 1 Schematic representation of the possible factors influencing population genetic variation under in situ and ex situ conditions over time. G0 the original collection, G1–G2 the different regeneration cycles, S0–S2 the samples taken from G0–G2, R the re-sampling of the in situ population. Pairwise comparisons are indicated by *dashed arrows*

changes in environmental conditions, for example as a result of climate change (Jarvis et al. 2008, 2010).

Extensive discussions have been conducted on the issue whether ex situ conservation in gene banks or in situ conservation in their natural habitat is the most appropriate method to preserve wild species (Maxted et al. 1997, 2008; Scarascia-Mugnozza and Perrino 2002). It has been argued that crop wild relatives are best preserved under in situ conditions because of the potential to evolve and adapt to changing environmental conditions resulting in novel variation that may be exploited in crop improvement (Dulloo et al. 2010). However, it is clear that ex situ collections are of enormous importance as they generally provide well-identified material that is immediately available for the identification of useful traits and for utilization in plant breeding. Furthermore, ex situ collections may serve as safety back-ups in case in situ populations go extinct.

In the present study, microsatellite markers were used to investigate genetic changes in ex situ conserved wild potato species, while these accessions were also compared to in situ populations recently re-collected at the same sites as the original collections. Microsatellites, also known as simple sequence repeat (SSR) markers, were chosen for this study because of their high resolving power, high reproducibility and simplicity of use (Kantartzi 2013). Previously, microsatellites have been used in potato to study phylogeny (Ghislain et al. 2009a; Merino Méndez 2006), taxonomy (Lara-Cabrera and Spooner 2005; Spooner et al. 2007), genetic diversity (De Haan et al. 2010; Ghislain et al. 2004, 2009b) and disease resistance (Ghislain et al. 2001). Results from our microsatellite study are discussed within the context of the future conservation management of Bolivian wild potato species.

Materials and methods

Study material

This study included the wild potato species *Solanum acaule* Bitter, *S. avilesii* Hawkes et Hjerting, *S. berthaultii* Hawkes, *S. leptophyes* Bitter, *S. megistacrolobum* Bitter, *S. neocardenasii* Hawkes et Hjerting and *S. okadae* Hawkes et Hjerting, comprising a total of 12 accessions represented by 37 seed samples (Table 1). Species names in the study followed the taxonomy used by CGN, notwithstanding the recently proposed synonymy questioning the distinction of the species *S. leptophyes* and *S. avilesii* (both belonging to the *Solanum brevicaule*-complex), and suggesting the reduction of *S. megistacrolobum* to a synonym of *S. boliviense* Dunal in DC (Spooner et al. 2014). The present study is not affected by these taxonomic issues because our research addresses intra-species comparisons, focusing on the analysis of genetic diversity between ex situ regenerations and between ex situ and in situ populations of the same accession.

All study accessions were acquired from CGN. Its potato collection is the continuation of the Dutch-German Potato Collection, which was established in 1974 as the Braunschweig Genetic Resources Center (BGRC) constituting a merger of the national collections of Germany and the Netherlands (Lange 1976). Some of the samples used in this study originated from the German Erwin Bauer Sortiment, the collection of the University of Birmingham or the Dutch Wageningse Aardappel Collectie, from which only very limited details on the rejuvenation history are available.

To rejuvenate accessions, CGN aims to obtain 20–25 plants per accession. In general, 60 botanical seeds are sown to obtain sufficient numbers of plants, but a higher number of seeds is used in case of expected low germination. If necessary, these plants are first tested for quarantine diseases by the Dutch Plant Protection Service and then grown in a greenhouse or transplanted into the field in isolated plots. When no fruits are produced by natural pollination in field plots, hand-pollination is carried out. In the greenhouse, plants are hand-pollinated with bulked pollen mixtures from all the flowering plants of the accession. Per accession a similar number of berries is harvested per plant and subsequently bulked for seed extraction. A similar procedure is used in subsequent seed multiplications, either using the original seed-lot or the previous generation as parents. This methodology is commonly used by gene banks to regenerate wild potato species (Salas et al. 2008).

For the present study, accessions were selected based on their provenance from Bolivia, the availability of several regenerations of the same accession and the availability of sufficient documentation to enable resampling of the original collecting site. Three types of material were examined in the study (Fig. 1): gene bank seed samples (S0) from the originally collected

Table 1 Study accessions, maintained at CGN and denoted by BGRC code

Species	BGRC	Origin	G0	G1	G2	R
S. acaule	07973	Potosí-Sucre	1971	1972		2008
	07976	Tiwanacu	1971	1972	1990; 1994	2010[c]
	15473	Tiwanacu	1959	1976		2010[c]
S. avilesii	31184	Vallegrande-Pucara	1980	1981; 1985	1991[b]	2008[d]; 2010[e]
	31185	Vallegrande-Pucara	1980	1981; 1988		2008[d]; 2010[e]
	31186	Vallegrande-Pucara	1980	1981; 1988		2008[d]; 2010[e]
S. berthaultii	10063[a]	Cerro San Pedro	1959	1960		2009
S. leptophyes	08222	Potosí-Oruro	1971	1972	1998	2008
S. megistacrolobum	08234	Toralapa	1971	1973	2001	2010
	27115	Koari	1980	1991		2010
S. neocardenasii	28001	Vallegrande-Mataral	1980	1983		2008; 2010
S. okadae	27040	Quime-Licoma	1980	1989	1992	2010[f]

Year of the original collection is indicated as G0, while the first and second regeneration year is denoted as G1 and G2, respectively. Column R indicates the year in which the original location was resampled. The underlined years indicate the material that was used in the present study

[a] Also material available from 1962 (originating from either G0 or G1), 1975 (possibly originating from a mixture of samples from the period 1959–1962) and 1986 (originating from the 1975 sample)

[b] Originating from G1 of 1981

[c, d, e] Same codes indicate identical recollections

[f] Re-collection was lost

material (G0), seed samples (S1, S2) from ex situ regenerations (G1 and G2) and seeds from the recent resampling of the original location of the accession (R). Unfortunately, for none of the studied accessions complete generation series (G0 as well as G1 and G2) were available. For some accessions multiple samples were available per regeneration, these were compared to check whether differences between regenerations from the same material occurred. The re-collections were obtained in the period 2008–2010 through resampling of the original location, or as close as possible to this location. Unfortunately, the newly collected material of *S. okadae* did not survive. The accessions are denoted by the former BGRC code because some were not yet assigned a CGN accession number (Table 1). More detailed accession and locality information is presented by Cadima Fuentes (2014).

Molecular analyses

DNA extraction

In 2009, 25 individuals of each of 24 populations were raised from true seed and planted in a greenhouse at PROINPA, Bolivia. Additionally, 13 populations were grown by CGN in 2010 in Wageningen, the Netherlands. In a number of cases less than 25 individuals could be sampled. Leaves collected in the Netherlands were dehydrated using silica gel and sent to PROINPA for DNA extraction. Collected leaves were ground in liquid nitrogen, and approximately 100 mg per ground tissue sample was stored at −20 °C until DNA extraction. DNA was extracted from young leaves using the protocol of Doyle and Doyle (1990) with slight modifications (Ghislain et al. 1999). DNA concentration was estimated on 1 % agarose gels using a size ladder of 200–10,000 bp (SmartLadder, Eurogentec) as a reference, and SYBR Green for visualization.

Selection of microsatellite markers

For an initial pre-screening, 30 microsatellites were selected based on high polymorphic information content and a high quality of amplicons, as determined by clarity and reproducibility, reported in SSR fingerprinting studies of cultivated potato (Feingold et al. 2005; Ghislain et al. 2009b; Milbourne et al. 1998).

The selected microsatellites were tested in the laboratory of PROINPA with a group of seven randomly chosen plants of the species *S. acaule*, *S. avilesii*, *S. berthaultii*, *S. leptophyes* and *S. neocardenasii* to check their amplification in wild potato species. No pre-screening was carried out for *S. megistacrolobum* and *S. okadae*, because it was decided to include these species at a later stage in the study when the markers had been already selected. Polymerase chain reactions (PCR) were performed in 15 µL volumes containing 20 ng DNA, 1.5 µL 1× PCR buffer (15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris–HCl 1 M pH 8.3), 0.2 mM of each dNTP, 1 pmol/µL of forward and reverse primer, and 1 U of Taq polymerase. PCR reactions were carried out in a MJ Research PTC 100 thermocycler using the following thermal profile: one initial denaturation step of 5 min at 94 °C; 35 cycles, each of 1 min at 94 °C, 30 s at optimal annealing temperature depending on the microsatellite and 30 s at 72 °C; 1 final extension step of 5 min at 72 °C. Amplification products were checked on 1.8 % agarose gels using SYBR green for staining. The microsatellites STG0001, STG0010, STG0016, STG0025, STI0001, STI0004, STI0014, STI0030, STI0032, STI0033, STM0019, STM0037, STM1052, STM1053, STM1106, STM5114 and STPoAc58 were selected based on the quality of the amplified products and were used to screen the 37 study populations. In addition, microsatellites STI0012 and STM1064 were selected for the screening of *S. leptophyes* only. Details about the markers, such as repeat motif, primer sequences and annealing temperatures, can be obtained from Ghislain et al. (2009b).

Microsatellite genotyping

Microsatellite genotyping was performed at the Genotyping Services Laboratory of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Patancheru, India. The M13 primer sequence 5′CACGACGTTGTAAAACGAC3′ was used as a pigtail extension at the 5′ end of each forward primer (Oetting et al. 1995). PCR was performed in 5 µL reaction volumes with 5 ng of DNA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1× PCR buffer, 0.006 pM of M13-tailed forward primer, 0.09 pM of M13-tailed forward primer labelled with either 6-Fam, Vic, Ned or Pet (Applied Biosystems), 0.09 pM of reverse primers and 0.1 U of *Taq* DNA polymerase (SibEnzyme Ltd., Russia). A GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, USA) was used to carry out PCR according to the following thermal profile: one initial denaturation step of 3 min at 94 °C; 10 cycles, each of 1 min at 94 °C, 1 min at 61 °C (annealing temperature reduced by 1 °C after each cycle) and 1 min extension at 72 °C; 4 cycles, each of 1 min at 94 °C, 1 min annealing at 54 °C and 1 min extension at 72 °C; final extension step of 10 min at 72 °C. Based on the expected size of the amplicons, PCR products were multiplexed together with an internal size standard (GeneScan™ 500 LIZ® from Applied Biosystems) and size fragments were separated by capillary electrophoresis using an ABI 3730*xl* Genetic Analyzer (Applied Biosystems, USA). Electrophoresis data were analysed using the software package Genemapper (Applied Biosystems, USA) and fragment sizes were scored in base pairs (bp) relative to the migration of the internal size standard.

Data analysis

Microsatellite peak patterns were visually inspected for clarity and consistency using the Peak Scanner™ Software v1.0 (Life Technologies). Ambiguous microsatellite scores were removed from the dataset, which for some marker/species combinations resulted in disregarding the entire data. In most cases, the samples showed one or two alleles per microsatellite. In cases where three or four unambiguous microsatellite peaks were observed, more than two alleles were recorded. The number of analysed microsatellites per species ranged from 13 in *S. berthaultii* to 17 in *S. acaule* and *S. okadae*. The number of analyzed individuals was 694, ranging from 35 for *S. okadae* to 159 for *S. avilesii*. More detailed microsatellite information, including observed allele sizes and number of alleles, can be obtained from Cadima Fuentes (2014).

Microsatellite data were transformed to binary data by denoting the presence of an allele as one and the absence as zero. The binary data were used to calculate Jaccard's similarity coefficient between individuals. Subsequently, these were used to perform a UPGMA cluster analysis. Relationships between individuals were graphically represented by phenograms, both for the entire data set and for each species separately. All cluster analyses were performed using NTSYS-pc 2.10 (Rohlf 2000).

Genetic differences between two samples were tested for statistical significance by means of randomization tests (Sokal and Rohlf 1981). For this purpose, the binary microsatellite data were used to calculate the mean Jaccard similarity value between all pairs of individuals from the two samples. The individuals of the two samples were then pooled and two simulated samples with a size equal to that of the original sample were constructed by randomly drawing individuals with replacement from the pooled set. Subsequently, the mean Jaccard similarity value between the two simulated samples was calculated. This procedure was repeated 10,000 times, and the proportion of simulated similarity values smaller or equal to the original similarity value was recorded. This value was denoted by *P*, representing the probability of erroneously rejecting the null hypothesis of genetic uniformity. Statistical analyses, using a tailor-made program written in Turbo Pascal, were carried out per microsatellite locus as well as for the combined set of markers.

Results

Genetic diversity within and between species

Among the 694 examined plants, a total of 236 alleles were identified for the set of 19 microsatellites. The observed number of alleles differed among the studied species, ranging from 57 for *S. okadae* to 110 for *S. avilesii*. STM0037 was found to be the most polymorphic microsatellite, with the observed number of alleles ranging from 7 for *S. acaule* to 16 for *S. neocardenasii*.

Although developed for cultivated potato, the selected microsatellites were able to distinguish the seven wild potato species, as a UPGMA cluster analysis grouped the 694 examined individuals into seven main clusters in accordance with the species designations (results not shown). The representatives of the series *Tuberosa*, i.e. *S. avilesii*, *S. berthaultii*, *S. leptophyes*, *S. neocardenasii* and *S. okadae*, grouped together in a large cluster, separate from a second cluster formed by *S. acaule* and *S. megistacrolobum.* Within the cluster of the series *Tuberosa* the two members of the so-called 'brevicaule-complex', i.e. *S. avilesii* and *S. leptophyes* (Alvarez et al. 2008; Van den Berg et al. 1998), formed a subgroup. Thus, the topology of the phenogram is in accordance with the taxonomic classification adopted by CGN for the investigated *Solanum* species.

Effects of regeneration on gene bank accessions

Based on mean Jaccard similarity values, significant genetic differences were observed between the samples of the subsequent generations of *S. neocardenasii* and *S. okadae*. No significant effects of regeneration on the genetic diversity of accessions were observed for *S. leptophyes* and *S. megistacrolobum*. For each of the species *S. acaule*, *S. avilesii* and *S. berthaultii* genetic differences between generations were observed in some cases, but other cases showed genetic stability. The comparisons between the multiple samples from the same generation also differed in their statistical significance (Table 2). Private alleles were observed in all comparisons, with the exception of S1(1972)/S2(1990) of BGRC07976 of *S. acaule* and S1(1972)/S2(1998) of BGRC08222 of *S. leptophyes*. Even when no significant differences were observed, the number of private alleles could be relatively high, such as in the case of the two generations of BGRC08234 of *S. megistacrolobum* with 18 and 13 private alleles, respectively (Table 2). The number of shared alleles exceeded the number of private alleles in all comparisons.

Genetic diversity between ex situ and in situ populations

For all comparisons of each of the studied species, genetic differences between ex situ and in situ populations were statistically significant (Table 3). Observed differences between ex situ samples and in situ populations were most pronounced for *S. leptophyes*, showing significant effects for the two comparisons at 13 and 12 microsatellite loci, respectively. Compared to the comparisons between ex situ samples, the number of private alleles was considerably higher for the ex situ/in situ comparisons, in *S. avilesii* even exceeding the number of shared alleles in several cases. No consistent pattern of either the ex situ or in situ material showing higher numbers of private alleles could be observed (Table 3).

The substantial genetic differences between the samples of the gene bank accessions and the resampled populations at the origin locations were reflected

Table 2 Pairwise comparison of microsatellite differences between samples of gene bank accessions

Species	BGRC	Comparison	Private alleles	Shared alleles	Jaccard	P^b
S. acaule	07976	S1(1972)–S2(1990)	0–0	31	0.939	0.413
	07976	S1(1972)–S2(1994)	6–1	25	0.869	0.000***
	07976	S2(1990)–S2(1994)	6–1	25	0.861	0.001***
	15473	S0(1959)–S1(1976)	5–2	30	0.918	0.099
S. avilesii	31184	S1(1981)–S1(1985)	6–14	33	0.462	0.098
	31184	S1(1981)–S2(1991)	9–15	30	0.373	0.000***
	31184	S1(1985)–S2(1991)	17–15	30	0.395	0.012*
	31185	S1(1981)–S1(1988)	5–1	35	0.532	0.011*
	31186	S1(1981)–S1(1988)	2–11	35	0.503	0.298
S. berthaultii[a]	10063	S0(1959)–S1(1960)	9–9	26	0.489	0.017*
	10063	S1(1960)–Sx(1975)	9–12	26	0.527	0.097
	10063	Sx(1975)–Sx(1986)	3–9	35	0.515	0.331
S. leptophyes	08222	S1(1972)–S2(1998)	3–0	49	0.472	0.058
S. megistacrolobum	08234	S1(1973)–S2(2001)	18–13	44	0.401	0.126
S. neocardenasii	28001	S0(1980)–S1(1983)	10–13	28	0.637	0.013*
S. okadae	27040	S1(1989)–S2(1992)	3–5	49	0.437	0.012*

Samples are denoted by their generation number ('x' when unknown) with the production year given between parentheses. The number of private alleles per sample as well as the number of shared alleles is presented for each comparison. Jaccard denotes the mean similarity value of all pairwise comparisons between individuals from two samples, while the corresponding probability of erroneously rejecting the null hypothesis of genetic homogeneity is presented by P

[a] Sample comparisons not presented in the table showed significant differences in all cases

[b] Significance level: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$

by the clustering pattern observed in the species-specific UPGMA phenograms. Separate clusters were observed for the genetically different *S. acaule* gene bank accessions BGRC07976 and BGRC15473, both collected at the ruins of Tiwanacu (Fig. 2). The re-collected material from Tiwanacu appeared to fall into two subgroups, each clustering with the material of one of the two original accessions.

Discussion

The continuous demand for increasing agricultural productivity requires new sources of genetic variation. In this context, gene banks play an important role by making germplasm available for present and future users. The maintenance of the genetic diversity of gene bank accessions is the main challenge during ex situ conservation (Rao et al. 2007). This genetic diversity may be compromised when gene bank accessions are regenerated to produce new seed stocks (Börner et al. 2000; Chebotar et al. 2003; Van Hintum et al. 2007). Several processes may affect the genetic diversity of accessions during regeneration, including genetic drift, selection and gene flow (Fig. 1), while also handling errors may occur. The extent to which gene bank accessions still represent the diversity occurring in situ depends on several factors, including the sampling procedure applied in the natural populations and the genetic changes that have occurred in situ as well as ex situ (Del Rio et al. 1997b). In the present study, genetic changes in gene bank accessions of Bolivian wild potato species were examined, while also a comparison was made with in situ re-collections.

Genetic change in accessions during ex situ conservation

As genetic drift is expected to affect all loci to more or less the same extent, selection is generally inferred from a strong differentiation at specific loci, while

Table 3 Pairwise comparison of microsatellite differences between ex situ samples of gene bank accessions and resampled in situ populations

Species	BGRC	Comparison[a]	Private alleles	Shared alleles	Jaccard	P[b]
S. acaule	07973	S1(1972)–R(2008)	12–7	31	0.654	0.000***
	07976	S1(1972)–R(2010)	2–12	29	0.782	0.000***
	15473	S0(1959)–R(2010)	3–9	32	0.760	0.000***
S. avilesii	31184	S1(1981)–R(2008)	10–49	29	0.259	0.000***
	31184	S1(1981)–R(2010)	16–26	23	0.240	0.000***
	31185	S1(1981)–R(2008)	7–45	33	0.254	0.000***
	31185	S1(1981)–R(2010)	16–25	24	0.242	0.000***
	31186	S1(1981)–R(2008)	7–48	30	0.261	0.000***
	31186	S1(1981)–R(2010)	12–24	25	0.291	0.000***
S. berthaultii	10063	S0(1959)–R(2009)	10–10	25	0.440	0.000***
S. leptophyes	08222	S1(1972)–R(2008)	22–15	30	0.302	0.000***
S. megistacrolobum	08234	S1(1973)–R(2010)	26–20	36	0.359	0.003***
	27115	S1(1991)–R(2010)	30–9	31	0.312	0.000***
S. neocardenasii	28001	S0(1980)–R(2008)	13–18	25	0.499	0.000***
	28001	S0(1980)–R(2010)	17–7	21	0.541	0.000***

The samples are denoted by "S" plus the number of the corresponding generation, with the production year of the generation given between parentheses. In situ populations are denoted by "R" with the year of resampling between parentheses. The number of private alleles per sample as well as the number of shared alleles is presented for each comparison. Jaccard denotes the mean similarity value of all pairwise comparisons between individuals from different samples, while the corresponding probability of erroneously rejecting the null hypothesis of genetic homogeneity is presented by P

[a] Comparisons are presented for the oldest gene bank sample of an accession. Comparisons for gene bank samples not presented in the table showed significant differences in all cases

[b] Significance level: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$

other loci exhibit no or smaller changes (e.g. Van Hintum et al. 2007). The present study did not provide clear support for the influence of selection as the observed significant changes did not systematically involve the same loci. However, it cannot be ruled out that selection plays a role as it may operate on genes that are unlinked to the marker loci used in this study (Ghislain et al. 2009b). As multiple private alleles were observed in nearly all comparisons, it seems more likely that the examined accessions have been affected by genetic drift. Depending on the extent of genetic drift, accessions may change significantly during a single regeneration or through the accumulating effects of multiple regenerations. The extent of genetic drift increases when population size is reduced. In several cases where significant genetic changes were observed, the regeneration history indicated a reduced population size. For example, the regeneration of *S. okadae* in 1992 was performed in a greenhouse in autumn. Compared with summer conditions the plants grew under reduced light intensity and shorter day length. As a result, only 15 plants flowered and fruits could be collected from only 14 plants. For *S. neocardenasii*, generation G1 was obtained from only 13 plants. Furthermore, in both cases plants may have produced a disproportional quantity of seeds. In seed bags intended to be used for future regeneration, each regenerated plant is represented with the same number of seeds, but such balanced bulks are not made for "user bags", which were used for the current study. In the case of *S. leptophyes* and *S. megistacrolobum* the regeneration history did not indicate protocol deviations, while seeds were harvested from 17 and 21 plants, respectively, during regeneration. In both cases, no significant changes were observed.

In addition to genetic drift also handling errors have most probably contributed to the observed genetic changes. For example, fixations for different alleles were observed in successive generations of accession

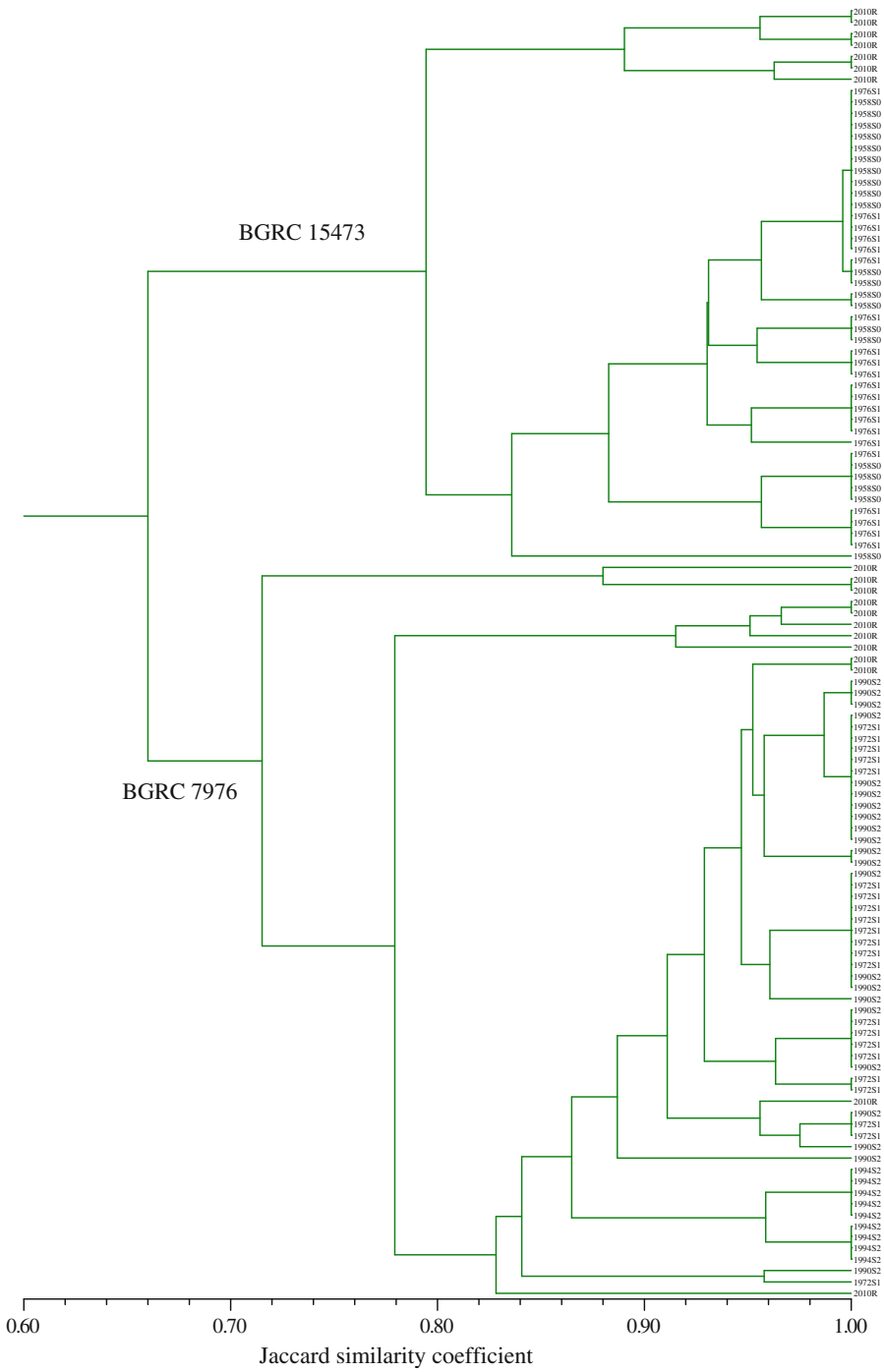

Fig. 2 UPGMA phenogram of the *Solanum acaule* samples collected in the Tiwanacu ruins in the Department La Paz, based on 17 microsatellite markers. Sample codes are a combination of the year of origin (see Table 1) and sample origin (see Fig. 1)

BGRC7976 of *S. acaule*, which are unlikely to be explained by genetic effects but rather point towards a substitution of accessions. In *S. avilesii*, the G2 generation of accession BGRC31184 differed significantly from the two G1 samples. Surprisingly, the G2 generation of BGRC31184 was not significantly different from the populations of BGRC31185, suggesting that generation G2 of BGRC31184 was erroneously derived from BGRC31185. This is supported by a UPGMA analysis, showing that samples of

generation G2 of BGRC31184 are intermixed with those of G1 of BGRC31185 (data not shown).

With optimal regenerations one would not expect large differences between regenerations from the same material. However, two of the four comparisons between multiple samples of the same regeneration showed significant differences (Table 2), indicating that genetic drift can play a role even when sampling the same material twice.

Compared to autogamous species, the level of genetic variation in populations of allogamous species is generally much higher. Therefore, populations of outbreeders are more prone to genetic changes during regeneration (Bamberg and Del Rio 2004; Chebotar et al. 2003), although earlier studies on the genetic diversity of time-series generations showed no significant changes both in selfing and outcrossing potato species (Del Rio et al. 1997a). In the self-pollinating wheat, no genetic changes were observed in accessions after long-term maintenance in the German gene bank (Börner et al. 2000). The single autogamous species included in the present study was *S. acaule*. Apart from the comparisons involving G2 of BGRC7976, which seemed to involve a substitution of accessions, no significant genetic changes were observed for *S. acaule*.

Comparisons between ex situ generations and in situ re-collections

In the present study genetic differences between ex situ conserved accessions and in situ re-collected material were significant in all comparisons. Apart from genetic changes during gene bank maintenance, sampling effects at the original collecting site have most likely contributed to the genetic differences observed between the ex situ and in situ material. In *S. acaule*, the observed differences were most likely caused by the differential sampling of a heterogeneous population. The two examined gene bank accessions were collected from the same location (ruins of Tiwanacu), respectively in 1971 and 1959. Our SSR data revealed significant differences between the two ex situ accessions (data not shown) and the existence of two subgroups within the re-collected material from Tiwanacu, each clustering with the material of one of the two original accessions (Fig. 2). Apparently, BGRC15473 and BGRC7976 represent genetically different subpopulations from the Tiwanacu area, while the recollected material from 2010 included both subpopulations. Previous studies support the finding that *S. acaule* may display wide genetic variation within a limited area (McGregor et al. 2002; Van Treuren et al. 2004). In those studies, BGRC7976, collected from the Tiwanacu ruins in the La Paz Department appeared very similar to most of the other *S. acaule* accessions collected from the same department, whereas BGRC15473, also originating from the Tiwanacu ruins, clustered with *S. acaule* accessions collected in southern Bolivia (e.g. BGRC7973 from the Potosi Department) and northern Argentina. These results show how the diversity collected from a genetically heterogeneous area depends on the sampling procedures.

In *S. avilesii*, material recollected in 2008 and 2010 captured many alleles that were not observed in the ex situ samples. Although it cannot be ruled out that alleles may have been lost during ex situ regeneration, the scale of resampling may have been an important underlying cause. Also in *S. avilesii*, the original area may have been resampled to a larger extent, as exact coordinates of the geographic origin of the gene bank accessions were missing. Strong genetic differentiation over short geographic distances was also observed for *S. megistacrolobum*, not only between the collecting sites at Toralapa and Koari, which are only 3 km apart, but also within the Koari location (data not shown). In such cases, variation in collecting methods can easily result in genetically different samples. For *S. berthaultii* and *S. megistacrolobum* tubers instead of seeds were collected during the recent expeditions, which explains the finding of identical SSR genotypes for these outcrossing species. This difference in sampling method also explains the lower levels of variation found for these species in the in situ populations compared to the ex situ samples. The difference in the number of sampled genotypes has most likely contributed to the significant differences observed between the in situ and ex situ material of these species.

It can be expected that natural populations will experience genetic change over time. However, to infer that genetic differences between ex situ and recently collected in situ samples are due to developments in natural populations requires the availability of the original seed sample (G0), while also the influence of sampling effects has to be ruled out (Fig. 1). Unfortunately, this appeared difficult in the present study, because G0 was not available for all accessions and

detailed original collecting data were largely missing. Also Del Rio et al. (1997b) were unable to ascribe the observed differences between ex situ and in situ materials of the wild potato species *S. jamesii* Torr. and *S. fendleri* A. Gray to either in situ genetic changes or sampling effects. In the present study, in situ developments were observed that may have contributed to genetic changes in natural populations. For example, Cerro San Pedro, the type locality of *S. berthaultii*, was an area of natural vegetation, while more recently this population has suffered from habitat destruction caused by ongoing urbanization as it is situated within the city of Cochabamba. Reduction of the population size since 1959 may have strongly reduced the level of genetic variation, which may have contributed to the observed differences with the ex situ samples.

Genetic differences between ex situ and in situ samples were most pronounced for *S. leptophyes*, as significant differences were observed for nearly all investigated markers, while also fixations for different alleles were found. Several explanations may account for these findings. First, the recollecting may not have been performed exactly at the same location as the original material and substantial spatial variation may occur within the area. Second, the in situ population may have been strongly disturbed by the effects of agricultural activities during the last 40 years which may have led to the loss of alleles and the introduction of other alleles by gene flow (hybridization) from nearby diploid cultivated potatoes. Third, although the taxonomic identity of the recollected material was confirmed by the curator of the wild potato gene bank of CIP, the pronounced SSR differences between in situ and ex situ populations may indicate the existence of different species with similar morphological characteristics. Different taxonomic authorities repeatedly mentioned the difficulty in discerning species of the "brevicaule complex" (Van den Berg et al. 1998) and recent studies have already agreed in the recognition of only two species within this complex, one representing a northern group and another a southern group with great variability, to which *S. leptophyes* belongs (Jacobs et al. 2008, 2011; Spooner et al. 2014).

Implications for conservation

In the present study, private alleles were observed in nearly all comparisons of ex situ samples, indicating that alleles may easily be lost during regeneration. The probability of losing genetic variation by genetic drift depends on the frequency of alleles in the population and the effective population size during regeneration (Chebotar et al. 2003; Crossa 1995). The latter is determined, amongst others, by the number of plants used for seed production. The higher the number of plants, the higher the probability that alleles are maintained in the population. For perennial allogamous species, it has been suggested to use at least 100 plants for regeneration (Johnson et al. 2002). Substantial lower numbers of plants have been used to regenerate accessions of the outbreeding potato species examined in the present study. Therefore, increasing the size of the regeneration population can be expected to reduce the effects of genetic drift. In practice, capacity limitations often drive gene banks to accept suboptimal regeneration sizes. Inevitably, this will cause the loss of alleles, in particular those occurring at low frequency. However, genetic drift in individual accessions may leave the level of genetic diversity in the total set of accessions relatively unaffected if the accessions become fixed for different alleles. This issue is analogous to the Single Large Or Several Small (SLOSS) debate (Simberloff and Abele 1982), addressing the conservation dilemma whether a single large or several small populations are a better means of conserving biodiversity. Regarding the conservation of genetic diversity it has been suggested based on population genetic theory that several small populations are the optimal solution (Gliddon and Goudet 1994). In case of capacity limitations, gene banks may therefore prefer to allocate available resources to small regenerations of a substantial number of accessions, rather than to large regenerations of a small set of accessions.

Deviations from the commonly used regeneration protocol and handling errors during ex situ maintenance contributed to the loss of genetic diversity of the examined accessions. These effects can be minimized by introducing commonly agreed minimum standards (Dulloo et al. 2008; FAO 2013; FAO/IPGRI 1994) for the regeneration of potato germplasm and by operating according to an international quality management system. Such a system should include detailed documentation on operating procedures and the independent monitoring of practices in order to increase efficiency and effectiveness.

Our study suggests the existence of extensive geographical and temporal genetic variation for the examined wild potato species. Therefore, material

collected for ex situ conservation may represent only a small fraction of the available genetic diversity. The representativeness of the genetic diversity in ex situ collections may be improved by wider sampling of the distribution area and resampling of earlier collecting sites. Species that are under-represented in gene banks and/or are threatened in situ, such as *S. avilesii* and *S. neocardenasii*, should be prioritized in order to keep ex situ collections manageable. In addition, a broader conservation strategy (at national or regional level) should be developed for the in situ conservation of crop wild relatives, placing emphasis on endemic species that show restricted distribution, such as *S. avilesii* and *S. neocardenasii*. This will provide a complementary strategy for preserving both the genetic diversity already present in gene banks and the diversity that continues to evolve under in situ conditions.

Acknowledgments The research described in this paper was part of the Ph.D. study of X.C., which was conducted under the auspices of the Graduate School of Experimental Plant Sciences, and was funded by the Netherlands organization for international cooperation NUFFIC. The contribution of R.v.T. was carried out in the framework of the Fundamental Research Program on Sustainable Agriculture (KB-12-005.03-003), which is funded by the Dutch Ministry of Economic Affairs.

Compliance with ethical standards

The authors declare that this manuscript has been prepared according to the ethical standards as formulated by Genetic Resources and Crop Evolution.

Conflict of interest The authors declare that they have no conflict of interest.

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