Global Ecology and Conservation 3 (2015) 461–476

Contents lists available at ScienceDirect

Global Ecology and Conservation

journal homepage: www.elsevier.com/locate/gecco

Original research article

In situ conservation of wild potato germplasm in Argentina: Example and possibilities

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ARTICLE INFO

Article history:
Received 30 December 2014
Received in revised form 29 January 2015
Accepted 29 January 2015
Available online 9 February 2015

Keywords:
Crop wild relatives
Genetic reserves
Management plan
Protected Areas
Solanum kurtzianum, *Solanum* section *Petota*

ABSTRACT

The potato is the third most important food crop worldwide and in situ conservation of its wild relatives is considered an urgent priority. Although regions of the Americas with high wild potato species richness have been identified, the need to identify specific sites for establishing genetic reserves is still pending. Matching distribution data of Argentinean wild potato species to existing protected areas (PA), two priority sites were identified. The creation of genetic reserves in these two PA would make possible to preserve populations of species that have been successfully incorporated into the crop and are listed in the global priority Crop Wild Relative inventory. While the presence of target species in PA could ensure a passive conservation, in situ conservation programs require to actively intervene in selected areas. From a field study performed on populations of the wild potato *Solanum kurtzianum* naturally growing in a PA, the Villavicencio Natural Reserve (Mendoza province), a baseline with distribution, biotic interactions, sprouting behavior, population dynamics, AFLP and pollen viability data was established. Based on a systematic work in this Reserve we have generated a working protocol to be implemented at national and regional levels for the in situ conservation of potato wild relatives.

1. Introduction

Nowadays it is undeniable that conservation and sustainable use of biological diversity is of critical importance for meeting the food, health and other needs of the ever-growing human population. Concerns about the considerable reduction of biodiversity due to human activities was one of the factors that led to the celebration of the Convention on Biological Diversity (CBD) in 1992, whose objectives are the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising from the utilization of genetic resources.

From all sources of living organisms, the conservation of plant genetic resources for food and agriculture (PGRFA), which represent the basis of global food security, has occupied a prominent position in the efforts undertaken by the international community. The PGRFA comprise, chronologically ordered, the diversity of genetic material contained in: (i) the crop wild relatives (CWR), which include crop progenitors and their closely related, (ii) landraces or traditional varieties, and (iii) modern cultivars. The crops tend to contain limited genetic diversity compared with their wild relatives, as a product of the

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http://dx.doi.org/10.1016/j.gecco.2015.01.009

successive bottlenecks imposed by the ancestral domestication, about 10 000 years ago and, then, by the successful application over the past century of modern plant breeding methodologies that have produced the high-yielding crop varieties (Tanksley and McCouch, 1997). For any crop, a broad genetic base from which new varieties can be developed is increasingly required to meet the ever changing and often unpredictable needs, and the opportunities and challenges of the future. CWR offer large and diverse gene pools to address these demands, providing plant breeders with potentially useful traits, such as pest and disease resistance, abiotic stress tolerance, quality and cytoplasmic male sterility (Hajjar and Hodgkin, 2007).

Although Nicolai Vavilov documented in the 1920s and 1930s the potential of CWR as gene donors in plant breeding programs (Vavilov, 1949), his collections consisted mainly of cultivated varieties, with a relatively poor representation of wild species (Zohary, 1970). As a consequence of an alarming deterioration of natural resources, agricultural scientists stressed CWR as a target group for conservation in the 1970s (Frankel and Bennett, 1970; Frankel and Hawkes, 1975), and currently, in the face of climate change, there are argument to consider them as the most important component of PGRFA. Taking actions to conserve CWR diversity is no longer an option but an urgent priority (Ford-Lloyd et al., 2011; Maxted et al., 2012; Vincent et al., 2013).

There are two fundamental approaches for the conservation of plant genetic resources: ex situ and in situ (Maxted et al., 1997a). The ex situ conservation method that has historically dominated the preservation actions around the world and involves exploration, collection and maintenance of plant genetic materials outside the native habitat, exhibit limitations for the effective conservation of CWR germplasm (Meilleur and Hodgkin, 2004; Maxted and Kell, 2009). The in situ conservation of CWR, first proposed in the 1970s (Frankel, 1970; Jain, 1975), took relevance by the late 1980s in response to the unsatisfactory progress in conserving these irreplaceable resources for agriculture. At the present time, the adoption of a complementary strategy that incorporates both ex situ and in situ techniques is considered an ideal scenario for conserving the gene poll of target species (Maxted et al., 2007).

According to the CBD (1992), in situ conservation is the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings. One particular advantage of in situ conservation is that it allows the maintenance of evolving populations in their natural habitats, permitting the preservation of gene frequencies and the generation of genetic variability during the dynamic and permanent interaction of target populations with biotic and abiotic factors. The great long term challenge of in situ conservation of CWR is the creation of genetic reserves, which involves the location, designation, management and monitoring of target wild populations in their natural habitats to maintain their genetic diversity (Maxted et al., 1997b).

For the designation of a genetic reserve a series of prioritizations should be established, among which there is the selection of crop gene pools, the selection of target species and the selection of target sites (Maxted and Kell, 2009). A criterion for the selection of priority crop gene pool is to consider the most socio-economically important global food crops. According to their contribution to food security and interdependence within a multilateral system, the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) (FAO, 2009) has identified in its Annex 1 the major importance crop, and this Annex has been used to elaborate an approach for the systematic conservation of the global CWR diversity (Maxted and Kell, 2009; Maxted et al., 2012). For selection of target species, a prioritization could be based on the degree of relationship among the crop and their wild relatives. The Gene Pool (GP) concept (Harlan and de Wet, 1971), which classifies wild relatives into three gene pools according to the feasibility of incorporating their genes by sexual crossing into the crop, is often used to select priority species. The highest priority CWR are typically those in GP1B (directly crossable wild or weedy forms of the crop) and GP2 (less closely related species from which gene transfer to the crop is possible but with some manipulations) (Maxted et al., 2012). Respect to the selection of target sites, the establishment of CWR genetic reserves within already existing protected areas (PA) is largely accepted as the most efficient approach (Heywood and Dulloo, 2006; Maxted and Kell, 2009; Hunter and Heywood, 2011; Maxted et al., 2012). The PA are geographically defined areas designated, regulated and supported by legal and institutional structures to achieve specific conservation objectives, and are the cornerstone of conservation efforts in virtually all countries (CBD, 1992). One strategy to identify the most suitable PA in which to locate genetic reserves of target CWR, is matching the geographic distribution data of these species with the network of PA in a given country or region (Maxted et al., 2012).

The potato, *Solanum tuberosum* L., has been presented as an excellent case study for the importance of CWR germplasm utilization in addressing global food security needs (Jansky et al., 2013). Potato annual production has remained above 300 million metric tons during the 2003–2012 period, which ranks this crop in the fifth place worldwide behind sugar cane, maize, wheat and rice (FAOSTAT, 2014). In addition, the potato ranked third in food supply behind wheat and rice, with an average of 33.01 kg/capita/yr in the 2002–2011 decade; and it is the fourth crop worldwide in terms of protein supply, behind wheat, rice and maize, averaging 1.45 g/capita/day during the years 2002–2011 (FAOSTAT, 2014). From this data related to food and agriculture, obtained for ca. 200 countries, it is clear why the potato is identified as a crop of major importance for food security and included in the Annex 1 of the ITPGRFA. More than 200 wild potato species have been described and grouped in section *Petota*, which occur in the Americas from south-western United States to central Argentina and Chile, Uruguay, Paraguay and Brazil (Hawkes, 1990). The number of species has been reduced in recent years (Spooner and Salas, 2006; Spooner, 2009). However, a consensus in the classification of potato species has not yet been reached (Camadro et al., 2012). In the present work the Hawkes (1990) treatment was used. Potato CWR are found in a wide variety of habitats with the following ranges of ecogeographic distribution: (i) from see level to 4697 m in altitude; (ii) from 96 to 3601 mm in annual precipitations; and (iii) from 5.6 to 22.1 °C in annual average temperature (Hijmans et al., 2002). Over 150 years ago the late blight epiphytia in Ireland precipitated the use of wild potato germplasm in breeding programs (Ross, 1966; Hawkes, 1990).

Afterwards, potato CWR have been successfully used to introduce bacteria, nematode, insect and virus resistance; improve culinary and good processing qualities, enhanced yield and tolerance to abiotic factors (Ross, 1966; Plaisted and Hoopes, 1989; Bradshaw and Ramsay, 2005; Bradshaw, 2009). Nowadays, the use of CWR is acknowledged as prominent in potato breeding (Maxted et al., 2012).

Based on the background information presented, the in situ conservation of potato CWR have been placed firmly on the international agenda (Maxted and Kell, 2009; Maxted et al., 2012; FAO, 2013; Vincent et al., 2013). Here we address two approaches concerning the recommendations to establish programs for in situ conservation of CWR. First, we identify PA from Argentina with high priority to establish genetic reserves of potato CWR. Northern Argentina has been identified as a region with high potato species richness (Hijmans et al., 2002) and in our country there are more 360 PA covering an area of 18.936 million ha, which is equivalent to nearly 7% of the national territory (Burkart, 2006; PAB, 2007). Second, we present practical strategies concerning the monitoring of natural populations of the wild potato species *Solanum kurtzianum* Bitt. et Wittm. growing in a protected area, the Villavicencio Natural Reserve (VNR). Based on a systematic work in this Reserve since 2006, we have generated a working protocol that would justify its implementation at national and regional levels for the in situ conservation of wild potato species.

2. Material and methods

A combination of desk-based and field-based research was conducted in this study for the identification and prioritization of specific sites to be designated as genetic reserves in Argentina and to develop a protocol to actively intervene in selected sites in order to ensure long-term potato wild relatives conservation, respectively.

2.1. Desk-based research

2.1.1. Identification of predicted potato CWR found in the protected areas from Argentina

Using the software ArcView GIS 3.2 (Environmental Systems Research Institute, Inc. 1999) the geographic information of accessions of wild potato species from the Potato and Forages Germplasm Bank of Instituto Nacional de Tecnología Agropecuaria, Balcarce, Argentina (PFGB-INTA), was matched against the GIS data set of existing network PA in Argentina, which is available from the Federal System of Protected Areas, National Secretary of Environment and Sustainable Development (http://www.ambiente.gov.ar/?idseccion=153). Then, a literature revision was performed for known uses in crop improvement and desirable characteristics of wild potato species with predicted presence in Argentinean PA.

2.2. Field-based research

We confirm that the permits were obtained from the VNR and the Directorate of Renewable Natural Resources for performing a field-based research in a provincial Protected Area.

2.2.1. Site description

The VNR, located 30 km northwest from Mendoza city, department of Las Heras, has an area of 72,000 ha and an altitude range from 700 to 3000 m a.s.l. in which the Monte, Cardonal and Puna phytogeographical provinces are represented (Dalmasso et al., 1999). The name of Natural Reserve was officially granted in 2000 by the Directorate of Renewable Natural Resources, an agency of the Ministry of Environment of the Government of Mendoza province. According to the provincial law No. 6045 of Natural Protected Areas, the VNR is categorized as "Private Voluntary Nature Reserve of Multiple Use".

2.2.2. Species description

Solanum kurtzianum is probably the wild potato from Argentina best adapted to dry environments (Hawkes and Hjerting, 1969). It is a diploid species ($2n = 2x = 24$) with an outcrossing breeding system found in Catamarca, La Rioja, Mendoza and San Juan provinces, growing naturally in drier valleys and hillsides of the western and north-western Argentine Andes. It is the only wild potato species that grows in the VNR.

2.2.3. Field observations

Since 2006 we explore systematically the VNR for the presence of *S. kurtzianum* along the whole altitudinal range (700–3000 m a.s.l.).

2.2.3.1. Population census. Because during 2006 and 2007 we observed that plants of *S. kurtzianum* kept on emerging until March, this month was chosen to perform the population census. In order to monitor demographically selected populations, five areas of 16 m^2 (4 m $\times$ 4 m) were delimited and geo-referenced at different sites within the VNR in 2007. The number of plants within these squares was set by direct counting in the second half of March of the years 2007, 2008, 2010, 2011, 2012, 2013 and 2014. In 2011, three new areas of 16 m^2 were incorporated and the same procedure was performed in 2011, 2012, 2013 and 2014 (Table 1).

Table 1
Solanum kurtzianum populations and studies performed within the Villavicencio Natural Reserve.

Origen (accessibility)[a]	Population	Localization	Altitude (m a.s.l.)	AFLP analysis (sample number)	Population census	Sprouting behavior	Pollen viability
Quebrada Hornillos (remote)	1667QH1	S32°30′51.84″ WO69°0′34.14″	1667	Yes (3)	No	No	No
	1713QH2	S32°30′52.45″ WO69°0′33.26″	1713	Yes (3)	No	No	No
	1910QH3	S32°30′5.85″ WO69°1′11.49″	1910	Yes (3)	No	No	No
	1910QH4	S32°30′6.95″ WO69°1′12.00″	1910	Yes (3)	Yes	No	Yes
	1910QH5	S32°30′5.18″ WO69°1′19.99″	2000	Yes (3)	No	Yes[b]	No
	2000QH6	S32°30′5.00″ WO69°1′22.00″	2000	Yes (3)	Yes	No	Yes
	2000QH7	S32°30′5.00″ WO69°1′26.00″	2100	Yes (4)	No	No	No
Caracoles (easy, near to Provincial Route 52)	1800A	S32°31′33.42″ WO69°0′37.44″	1800	Yes (3)	No	No	No
	1800B	S32°31′33.42″ WO69°0′37.92″	1800	Yes (3)	No	No	No
	1800C	S32°31′33.18″ WO69°0′38.04″	1800	Yes (3)	No	No	No
	1800D	S32°31′32.45″ WO69°0′38.48″	1800	Yes (3)	No	No	No
	1800F	S32°31′32.63″ WO69°0′39.24″	1800	Yes (3)	No	No	No
	2000A	S32°31′58.44″ WO69°1′15.72″	2090	Yes (3)	No	No	No
	2000B	S32°31′59.46″ WO69°1′15.96″	2108	Yes (3)	Yes	Yes[c]	Yes
	2000C	S32°32′0.60″ WO69°1′16.44″	2123	Yes (3)	No	No	No
	2000D	S32.53423 WO69.02194	2069	Yes (3)	No	No	No
	2000E	S32°31′57.76″ WO69°1′14.05″	2000	Yes (3)	No	No	No
	2166A	S32°32′6.87″ WO69°1′18.65″	2166	Yes (4)	No	Yes[b]	No
	2166B	S32°32′7.02″ WO69°1′28.33″	2166	Yes (2)	No	No	No
	2166C	S32°32′4.99″ WO69°1′31.98″	2166	Yes (3)	No	Yes[b]	Yes
	2166D	S32°32′5.11″ WO69°1′26.86″	2166	Yes (3)	No	No	No
	2166E	S32°32′6.06″ WO69°1′20.65″	2166	Yes (3)	No	No	No
Quebrada del Toro (easy, near to Provincial Route 52)	1240QT	S32°31′50.06″ WO68°56′52.36″	1240	No	Yes	Yes[b],[c]	Yes
	1258QT	S32°32′5.16″ WO68°56′59.19″	1258	No	Yes	Yes[c]	Yes
	1262QT	S32°32′5.17″ WO68°56′59.90″	1262	No	Yes	No	No
	1266QT	S32°32′5.33″ WO68°57′2.07″	1266	No	Yes	Yes[c]	No
	1275QT	S32°32′17.85″ WO68°57′11.79″	1275	No	Yes	Yes[c]	No

[a] According to Bamberg et al. (2010).
[b] Evaluated in genotypes cultivated in pots under experimental conditions.
[c] Evaluated in natural populations.

Simple regression analysis was performed to assess the relationship between plant number per population and monthly precipitation (from December in year $n-1$ to March of year n) using Statgraphics Centurion® software (Version 16.0,

Statpoint Inc., Herndon, VA, USA). Precipitation data were provided by the National Meteorological Service (Meteorological Information Center) from the nearest meteorological station, El Plumerillo, located approximately 40 km from the VNR.

2.2.3.2. Tuber sprouting behavior in natural populations. During the last week of 2011 and 2013 five sites randomly selected, in which populations of *S. kurtzianum* had been previously identified, were excavated to search tubers formed in the previous growing season (Table 1). At each site, three holes of approximately 30 × 30 × 30 cm were excavated spaced about 2 m from each other. The found tubers were counted and, by visual inspection classified as sprouted (i.e. visible growth of the apical bud) and non-sprouted tubers. Then, the percentage of tubers in each class was established for each of the five evaluated populations.

2.2.3.3. Pollen viability. This evaluation was performed in six populations in March of 2014 (Table 1). We removed two open flowers from 10 randomly taken plants per population. Pollen samples of each plant were stained with acetocarmine solution (0.2 g carmine and 100 ml 45% glacial acetic acid in distilled water) and observed under an optical microscope (100 X). More than 300 grains were counted in at least four randomly taken visual fields. Pollen grains that were fully stained, plump and with well-defined contours were considered as viable, whereas those that were poorly (or not) stained, and/or irregularly shaped were considered as unviable. Average pollen viability was calculated for each evaluated population.

2.2.4. Tuber sprouting behavior in an experimental assay

Botanical seeds from four populations were collected. To break dormancy, the seeds were pre-soaking with gibberellic acid at 1500 ppm for 24 h and germinated in Petri dishes (Table 1). Two genotypes per population were transplanted into pots and cultivated under uniform conditions in an insect-proof screen house. Tubers produced in each pot were harvested from mature plants and stored at 4 °C for 30 days. Then, the tubers were maintained at 24 °C in dark and evaluated for sprouting 60 days later. For each genotype, the percentage of sprouted and non-sprouted tubers was established.

2.2.5. Molecular analysis

We used AFLP (Amplified Fragment Length Polymorphism) molecular markers to generate a fragment database for *S. kurtzianum* populations distributed in the VNR which represent a baseline for monitoring the genetic variability over time.

2.2.5.1. Plant material. In March 2011, 22 natural populations of *S. kurtzianum* distributed at different altitudes within the VNR were sampled. The collection sites were geo-referenced using a global positioning system (GPS) device. In turn, according to the sampling strategy described by Bamberg and co-workers (2010), samples were collected in two types of sites: (a) "easy" (Caracoles), where populations were sampled near to Provincial Route 52, and (b) "remote" (Quebrada Hornillos), where populations were found along a trekking of about 1 h. Each sample consisted of a pool of apical leaves of four different plants. Leaves were collected in plastic bags which were labeled and preserved at 4 °C in a portable cooler until return to the laboratory. In each population surveyed, between two and four samples were collected, thus between eight and 16 plants per population were sampled (Table 1).

2.2.5.2. DNA extraction and AFLP analysis. Once in the laboratory, the samples were frozen with liquid nitrogen and ground with a mortar. Subsequently, about 15 mg of the obtained leaf plant powder were placed into two 1.5 ml eppendorf tubes (i.e. two replicates for each sample) and then stored in freezer at −80 °C. For DNA extraction the protocol described in Cara et al. (2014) was followed. DNA concentration was determined using a spectrophotometer (GeneQuant RNA/DNA Calculator; Pharmacia Biotech, Cambridge, England) and then, the samples were diluted to a concentration of 50 ng. μL^{-1} for use in AFLP analysis. The AFLP analysis was performed essentially as described by Vos et al. (1995), using EcoRI and MseI endonucleases as the rare and frequent cutters, respectively. For the detection we used fluorescent labeled selective EcoRI + 3 primers. A total of six EcoRI + 3/MseI+ 3 primer pairs were first screened for selective amplification in a preliminary study conducted on eight random samples from the 22 studied populations. The following two selective primer pairs were chosen for providing the most reliable and consistently scoreable AFLP fragments: FAM − EcoRI + ACG/ MseI + CAA and FAM − EcoRI + ACA/ MseI + CAA. The fluorescent amplified AFLP fragments were separated on an ABI PRISM 3130 DNA sequencer and the presence/absence of each fragment in each sample was scored manually by visualizing electrophoregrams with GeneMapper 3.7 software following Cara and co-workers specifications (2014).

2.2.5.3. Data analyses. For each sample, fragments with different patterns on replicates were excluded from analysis as possible methodological artifacts. To estimate the fragment sizing variation, two independent capillary electrophoresis (i.e. two independent injections) and presence/absence fragment analysis were performed for the full set of samples (each sample with its replicate). The variation rate of a given fragment was calculated as the difference between the largest and the smallest sizing (in bp) of the fragment, considering all samples in which the fragment was detected and both injections. The total number of AFLP fragments was computed for each population. Then, the AFLP fragments were categorized as given below: (i) unique fragments, present in only one population; (ii) rare fragments, shared by two to four populations; and (iii) frequent fragments, shared by five or more populations. Using the software Statgraphics Centurion®, a multinomial logistic regression analysis was performed to check for differences among populations in the proportion of unique and rare fragments taking the proportion of frequent fragments as a comparison group. Principal coordinates analyses (PCoA) was

performed with NTSYSpc software version 2.10t (Rohlf, 1992) to determine relationships among samples based on genetic polymorphism. Clusters were generated from genetic similarity matrices obtained with the Dice coefficient (Sneath and Sokal, 1973).

2.2.5.4. AFLP fragment database. The results of the previous analyses were used to build a database consisting of: (i) the variation rate of each analyzed fragment and the category in which it was classified (unique, rare or frequent), and (ii) the presence of each fragment in each of the analyzed populations.

3. Results and discussion

3.1. Identification and prioritization of Argentinean protected areas for in situ conservation of potato wild relatives

According to the matching of geographic information of wild potato species accessions from the PFGB-INTA against the existing Argentinean network of PA, 17 species of *Solanum* section *Petota* could be found in 19 PA distributed in ten Argentinean provinces (Fig. 1 and Table 2). The PA with higher species richness were Las Yungas Biosphere Reserve (10 species), Laguna de los Pozuelos Natural Monument (six species) and Los Cardones National Park (five species) (Table 2). Las Yungas Biosphere Reserve includes five core zones: Baritú National Park, Laguna Pintascayo Provincial Park and the Nogalar de los Toldos National Reserve, in Salta province; and Calilegua National Park and Potrero de Yala Provincial Park in Jujuy province. Interesting, data analysis showed that none of the PFGB-INTA recorded accessions fall within these core zones. Although proximity indicates that these five core zones may contain wild populations of the 10 species predicted in the Biosphere Reserve, the most promising areas for the creation of genetic reserve, based on species richness, would be Laguna de los Pozuelos Natural Monument and Los Cardones National Park. The Jujuy and Salta provinces had already been identified as American regions with high wild potato species richness, along with central Mexican highlands (México and Michoacán states), a small area in central Ecuador (Chimborazo province); a stretch from northern to central Peru (in Ancash, southern Cajamarca, La Libertad and Lima departments); southern Peru (in Cusco department) and central Bolivia (in Cochabamba, Chuquisaca and Potosí and to a lesser extent La Paz and Tarija departments) (Hijmans et al., 2002). From the point of view of conserving maximum species diversity, Maxted and Kell (2009) considered these regions as priority sites to be targeted for genetic reserve conservation. Furthermore to the presence of a given species in PA, additional evidence can be used as arguments to support the establishment of a genetic reserve for in situ conservation of target taxa, like as their potential use for crop improvement or threatened status (Vincent et al., 2013). Out of the 17 species with predicted presence in Argentinean PA, 14 are listed in the global priority crop wild relative inventory, seven have been incorporated in modern cultivars and in all of them one or several characters the importance for breeding have been described (Table 3). The creation of genetic reserves in Laguna de los Pozuelos Natural Monument and Los Cardones National Park would make possible the in situ conservation of a gene pool that is considered as priority by the international community. On the other hand, although the information presented in Table 3 indicates that one or more genotypes of a given taxonomic species have been source of desirable genes for breeding, further research is needed to evaluate the sexual compatibility among the genotypes growing in these PA with the cultivated potato.

Although the results obtained indicate that 17 wild potato species has been collected in the PA throughout Argentina, there is an obvious need to confirm the actual existence of the target taxon populations within these sites. This would be the first aim to achieve in areas considered as priorities in this work. Future expeditions to Laguna de los Pozuelos Natural Monument and Los Cardones National Park must be taken into consideration that the accession of the PFGB-INTA were classified under the Taxonomic Species Concept according to their resemblance to holotypes, a human construction that could be poorly reflecting what is actually occurring in sites with high wild potato species richness: the presence of introgressed populations and hybrid swarms formed by interspecific hybridization (Masuelli et al., 2009; Camadro, 2012; Camadro et al., 2012; Cara et al., 2013; Ispizúa et al., 2014).

In potato, a recent article provides guidelines to direct in situ conservation efforts to priority areas in Bolivia (Cadima et al., 2014). While inventory, site selection activities and the theory of design, establishment, management and monitoring of CWR diversity in genetic reserves have especially increased, full practical implementation remains limited (for revision see Meilleur and Hodgkin, 2004 and Maxted et al., 2011). Following, we present results obtained from active monitoring and detailed surveys of a wild potato species *S. kurtzianum* growing in a PA: the VNR.

3.2. Field observations and sprouting behavior

During the nine years of exploration in the VNR (2006–2014), the population in the lower altitude was found at 1082 m a.s.l (S32°43′47″, WO68°55′54″), while the highest one was found at 2393 m a.s.l (S3231′9.60″S, WO69°2′11.10″). Over this period, the most conspicuous pest detected was *Epicauta spp.*, affecting populations growing between 1082 and 1667 m a.s.l. Damages caused by adults consist of plant defoliation. A common observation was to find a gradation level of damage within the same population, ranging from completely defoliated mature plants (with compound leaf), mature plants attacked in basal leaves but with undamaged apical leaves and immature plants (without compound leaf) without any injury (Fig. 2). While injured but not totally defoliated mature plants could represent tolerant genotypes to defoliation by *Epicauta spp.*, our observations indicate an escape strategy as a mechanism of resistance to this pest. The tuber sprouting is an asynchronous

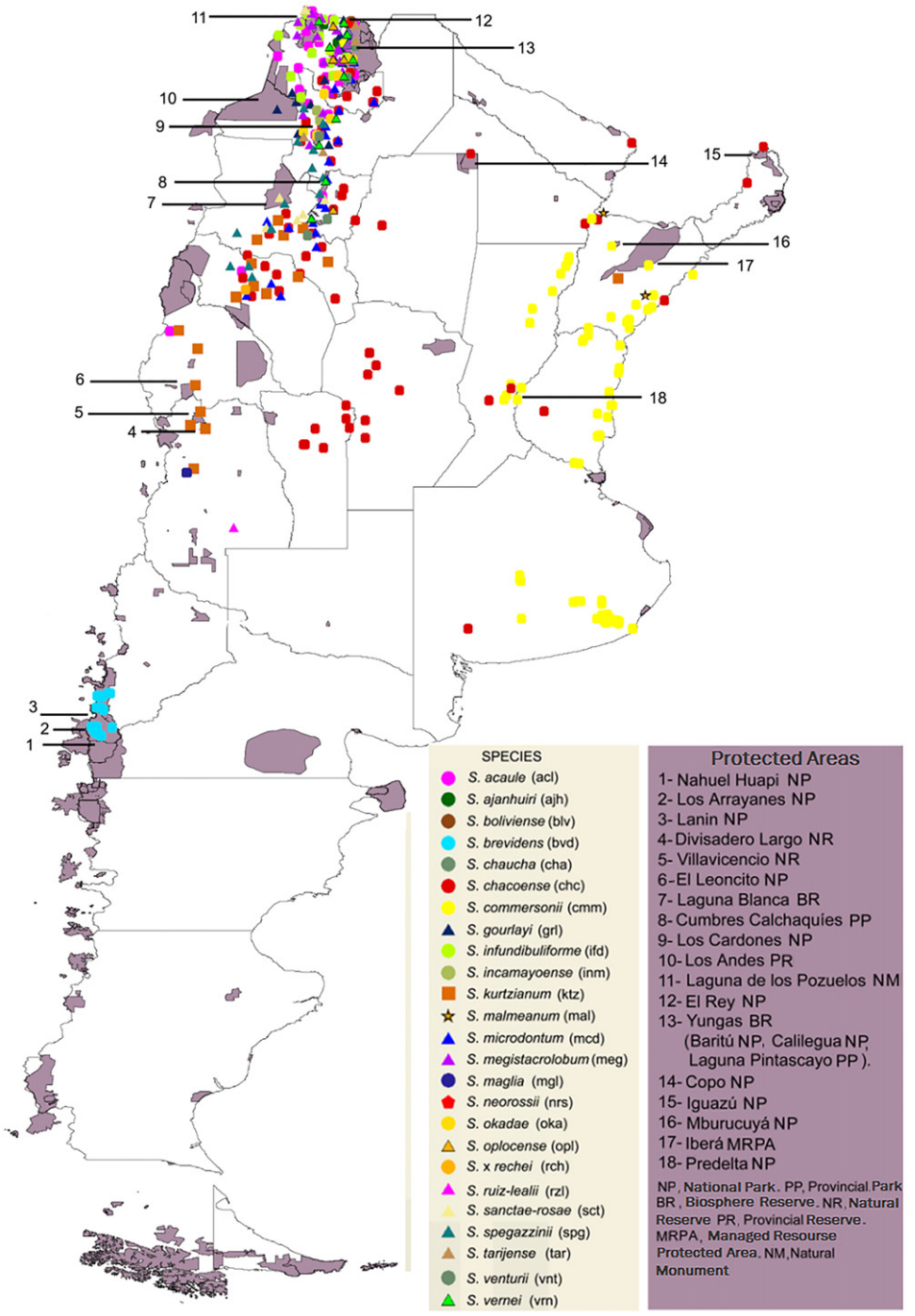


Fig. 1. Distribution map of wild potato species and Protected Areas from Argentina.

process (Figs. 2 and 3). This phenomenon allows the presence in the same population and at the same time of plants at various developmental stages, including both, plants that emerge when the pest incidence decreases, as well as plants with a degree of development that, although severely injured, produce new tubers ensuring the persistence of these genotypes for the next growing season.

The tuber sprouting was quantified in five natural populations the last week from 2011 and 2013 (Fig. 3(a) and Table 4). The obtained results confirm the asynchronicity of this process: in all populations sprouted and non-sprouted tubers were found. The percentage of sprouted tubers varied from 38 to 75 and from 33 to 70 in 2011 and 2013, respectively (Table 4). Furthermore, the tuber sprouting percentage of genotypes cultivated in experimental conditions was evaluated 90 days

Table 2
Predicted presence of wild potato species in Protected Areas from Argentina.

Province	Protected area	Creation year	Wild potato species (*Solanum*, section *Petota*)
Catamarca	Laguna Blanca Biosphere Reserve	1979	*S. sanctae-rosae*, *S. spegazzinii*
Corrientes	Mburucuyá National Park	2001	*S. commersonii*
	Iberá Managed Resources Protected Area	1982	*S. commersonii*
Entre Ríos	Predelta National Park	1991	*S. chacoense*, *S. commersonii*
Jujuy	Laguna de los Pozuelos Natural Monument	1980	*S. acaule*
			S. infundibuliforme
			S. megistacrolobum
			S. oplocense
			S. sanctae-rosae
			S. vernei
Jujuy/Salta	Yungas Biosphere Reserve	2002	*S. acaule*
			S. boliviense
			S. chacoense
			S. gourlayi
			S. microdontum
			S. megistacrolobum
			S. okadae
			S. tarijense
			S. venturii
			S. vernei
Mendoza	Divisadero Largo Natural Reserve	1983	*S. kurtzianum*
	Villavicencio Natural Reserve	2000	*S. kurtzianum*
Misiones	Iguazú National Park	1934	*S. chacoense*
Neuquén	Los Arrayanes National Park	1971	*S. brevidens*
	Lanin National Park	1937	*S. brevidens*
Neuquén/ Río Negro	Nahuel Huapi National Park	1934	*S. brevidens*
Salta	Los Cardones National Park	1996	*S. acaule*
			S. gourlayi
			S. megistacrolobum
			S. spegazzinii
			S. vernei
	Los Andes Provincial Reserve	1980	*S. acaule*
			S. gourlayi
			S. infundibuliforme
	El Rey National Park	1948	*S. microdontum*
San Juan	El Leoncito National Park	2002	*S. kurtzianum*
Santiago del Estero	Copo National Park	2000	*S. chacoense*
Tucumán	Cumbres Calchaquíes Provincial Park	1965	*S. microdontum*, *S. vernei*

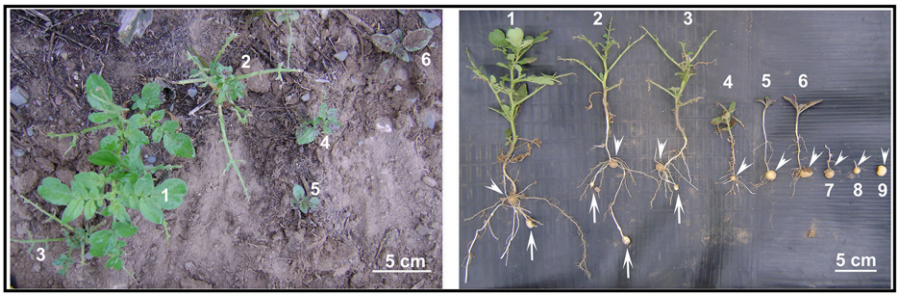


Fig. 2. A population of *Solanum kurtzianum* affected by *Epicauta spp*. Population as it was found (left panel) and after digging up this site to explore the underground parts of the observed plants (right panel). Consecutive numbers from 1 to 9 indicate plants and tubers found. Note that when the site was excavated, three additional tubers were found, two of them sprouted (numbers 7 and 8) and one non-sprouted (number 9). Plant 1, mature plant attacked by *Epicauta spp*. in basal leaves but with undamaged apical leaves. Plants 2 and 3, completely defoliated mature plants. Plants 4–6, immature plants (without compound leaf) without any injury. Arrows heads, mother tubers from which plants were originated. Arrows, new tubers, formed during the growing season.

post-harvest (Fig. 3(b) and Table 5). For all analyzed genotypes we observed that tubers formed during the same growing season and in the same pot showed an asynchronicity in sprouting. In a previous work we demonstrated that in *S. kurtzianum* populations there may be more than one plant of the same genotype (Marfil and Masuelli, 2014). The results presented here point out that in natural population plants of the same genotype could emerge at different time during the growing season. Because in the evaluated population tubers with sprouts up to 4 cm were found, future survey should begin in early November for get more precise data on the beginning of sprouting. Annual monitoring of sprouting would be useful to correlate

Table 3
Criteria for prioritizing in situ conservation of wild potato species with predicted presence in protected areas from Argentina.

Solanum species	Presence in the global priority CWR inventory[a]	Presence in modern cultivar pedigrees[b]	Potential breeding use	Reference
S. acaule	Yes	Yes	–Virus resistance	–Ross (1979), Ross (1986), Ruiz de Galarreta et al. (1998), Bradshaw et al. (2006)
			–Pest resistance	–Ross (1986)
			–Frost resistance	–Hawkes et al. (2000)
S. boliviense	Yes	No	–Virus resistance	–Ruiz de Galarreta et al. (1998)
			–Resistance to *Fusarium*	–Lynch et al. (2003)
S. brevidens	No	No	–Potato leaf roll virus resistance	–Jones (1979)
S. chacoense	Yes	Yes	–Virus resistance	–Bradshaw et al. (2006)
			–Virus and pest resistance	–Ross (1986)
			–Resistance to cold induced sweetening	–Jansky et al. (2011)
			–Resistance to Verticillium wilt	–Frost et al. (2006)
			–Tuber dry matter content	–Santini et al. (2000)
S. commersonii	Yes	Yes	–Virus resistance	–Ruiz de Galarreta et al. (1998)
S. gourlayi	No	No	–Virus resistance	–Ruiz de Galarreta et al. (1998)
S. infundibuliforme	Yes	No	–Virus resistance	–Ruiz de Galarreta et al. (1998)
S. kurtzianum	Yes	Yes	–Cyst nematode resistance	–Bradshaw and Ramsay (2005)
			–Virus resistance	–Ruiz de Galarreta et al. (1998)
S. megistacrolobum	Yes	No	–Virus resistance	–Ruiz de Galarreta et al. (1998)
			–Frost resistance	–Hawkes et al. (2000)
S. microdontum	Yes	Yes	–Resistance to *Phytophthora infestans*	–Zoteyeva et al. (2012)
S. oplocense	Yes	No	–Cyst nematode resistance	–Bradshaw and Ramsay (2005)
S. okadae	Yes	No	–Resistance to insects	–Pelletier et al. (2001)
S. sanctae-rosae	Yes	No	–Virus resistance	–Ruiz de Galarreta et al. (1998)
S. spegazzinii	Yes	Yes	–Virus resistance	–Ruiz de Galarreta et al. (1998)
S. tarijense	No	No	–Resistance to cold induced sweetening	–Jansky et al. (2011)
			–Resistance to Verticillium wilt.	–Frost et al. (2006)
			–Virus resistance	–Ruiz de Galarreta et al. (1998), Ortega and Carrasco (2005)
S. venturii	Yes	No	–Virus resistance	–Ruiz de Galarreta et al. (1998)
S. vernei	Yes	Yes	–Cyst nematode resistance	–Ross (1979), Bradshaw and Ramsay (2005)
			–Virus and pest resistance	–Ross (1986)
			–PVY resistance	–Ortega and Carrasco (2005)

[a] According to results presented by Vincent et al. (2013).
[b] According to results presented by Plaisted and Hoopes (1989).

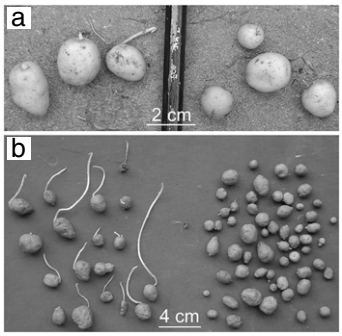


Fig. 3. Asynchronous sprouting behavior of *Solanum kurtzianum*. (a) Sprouted tubers (left) and non-sprouted tubers (right) documented in a natural population the last week of 2011. (b) Sprouted tubers (left) and non-sprouted tubers (right) from a selected genotype cultivated in pot under experimental conditions. All tubers were harvested from the same pot and evaluated 90 days post-harvest.

phenological changes in natural populations with environmental variables such as temperature and rainfall, aspects that have not yet been addressed in wild potato species.

In potato plants, during their tuber lifecycle, tuber formation can be divided into several developmental stages involving stolon induction and initiation, tuberization, dormancy and tuber sprouting (Ewing and Struik, 1992). Since the extent of these processes has significant economic importance, much attention is given to their study in the cultivated species, *S.*

Table 4
Number of sprouted and non-sprouted tubers in five natural *Solanum kurtzianum* populations evaluated during the last week of 2011 and 2013.

Population	Year			
	2011		2013	
	Sprouted (%)	Non-sprouted (%)	Sprouted (%)	Non-sprouted (%)
1240QT	12 (60)	8 (40)	11 (50)	11 (50)
1258QT	17 (59)	12 (41)	12 (60)	8 (40)
1266QT	8 (67)	4 (33)	7 (58)	5 (42)
1275QT	8 (38)	13 (62)	6 (33)	12 (67)
2000B	6 (75)	2 (25)	7 (70)	3 (30)

Table 5
Number of sprouted and non-sprouted tuber in eight genotypes of *Solanum kurtzianum* cultivated under experimental conditions.

Population	Genotype	Sprouted (%)	Non-sprouted (%)
2166A	1	13 (32.5)	27 (67.5)
	2	12 (40)	18 (60)
2166C	1	18 (23)	59 (77)
	2	23 (45)	28 (55)
1910QH5	1	25 (38)	40 (62)
	2	34 (47)	39 (53)
1240QT	1	64 (73)	24 (27)
	2	13 (23)	43 (77)

tuberosum. It has been documented that different processes occur simultaneously in an individual plant and that the whole sequences of events, from stolon induction to sprouting, are asynchronous (Vreugdenhil and Struik, 1989; Claassens and Vreugdenhil, 2000). However, the asynchronicity of the potato tuber lifecycle stages in nature has been overlooked. Disease escape is particularly useful in natural ecosystems (Nelson, 1973) and the presence of plants in the same population at different development stages (the dispersion in time) is a factor that promotes this strategy (Agrios, 1980). The escape strategy mediated by the asynchronous sprouting undoubtedly plays a decisive role in *S. kurtzianum* populations and could represent a common strategy in wild potato species. Significant intra-accession (i.e. plants within populations) variability in resistance to several pests and diseases has been reported for dozens of wild potato species (Pérez et al., 2001; Jansky et al., 2006, 2008, 2009; Spooner et al., 2009). These results indicate that in nature, resistant plants to pest and disease grow in association with susceptible ones. In addition, the dispersion in time would let wild potato populations survive to adverse abiotic conditions, such as seasonal water availability or extreme temperatures. Populations with the capacity of plastic response when exposed to environment experiencing short-term fluctuations (biotic and abiotic) may be expected to retain more genetic variability than would be the case in its absence (Bennett, 1970). This prediction has been widely confirmed in several wild potato species: in addition to the variability in the response to pest and disease cited above, molecular marker analyses shown that the higher percentages of genetic variation was within accessions or populations (Bedogni and Camadro, 2009; Erazzú et al., 2009; Marfil and Masuelli, 2014). This biological scenario represents both, an obstacle and a challenge in the evaluation and selection of materials to supply breeders (Spooner et al., 2009), and an opportunity to develop and strengthen an efficient strategy for the conservation of these genetic resources: in situ conservation in a single area (a genetic reserve) would already preserve a large portion of the total variability of wild potato species (Marfil and Masuelli, 2014).

3.3. Population census

Analyzing the population size we document periodic cycles of abundance (Fig. 4). Although large fluctuations were observed, the populations were not locally extirpated from none of the analyzed sites. In the year 2010 no growing plants (except in the population QT1262, where one plant was found) were observed on sites where previous years several plants had been counted. We excavated these sites and found sprouted tubers in which the sprout growth was supplanted by the formation of new tubers (data not shown). This observation indicates that the *S. kurtzianum* tubers can persist underground for almost one year and originate a new plant under favorable environmental conditions. In addition, the permanence through the time of all evaluated populations point out that in the VNR the human activities, like tourism or road maintenance, do not represent threats for *S. kurtzianum* population abundance. Simple correlation analysis between the plant number per population and precipitation revealed that total monthly precipitation in February had the most marked effect on the population size of *S. kurtzianum* at the VNR (Table 6). These results are relevant for planning collect activities for the ex situ conservation of *S. kurtzianum*. Optimal collections years could be predicted by evaluating the February rainfall. The three peaks of plant abundance observed in the years 2008, 2011 and 2014 (Fig. 4) were recorded with February total precipitations of 45.3, 109.9 and 141.5 mm, respectively. In addition, the population dynamic reported here establishes a baseline for monitoring demographic changes over time. On the other hand, it is important to highlight that the number

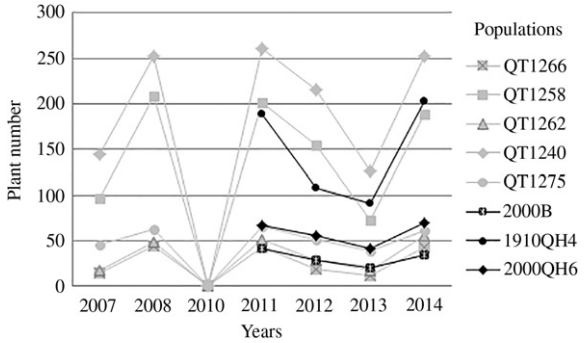


Fig. 4. Fluctuations in plant number of *Solanum kurtzianum* populations at the Villavicencio Natural Reserve. In 2009 the populations were not surveyed. The populations, 2000B, 1910QH4 and 2000QH6 (in black) were censused since 2011.

Table 6
Simple correlations between plant number of *Solanum kurtzianum* populations at Villavicencio Natural Reserve and total monthly precipitation (mm).

Population	December[c]	January	February	March
QT1266[a]	0.231	0.450	0.825**	0.182
QT1258[a]	0.029	0.481	0.741*	0.193
QT1262[a]	0.239	0.351	0.872**	0.088
QT1240[a]	0.023	0.424	0.745*	0.179
QT1275[a]	0.005	0.451	0.688*	0.310
2000B[b]	0.731	−0.048	0.806	0.229
1910QH4[b]	0.804	−0.447	0.993**	0.378
2000QH6[b]	0.636	−0.483	0.960**	0.105

[a] Evaluated in 2007, 2008, 2010, 2011, 2012, 2013 and 2014.
[b] Evaluated from 2011 to 2014.
[c] For the year $n - 1$.
* $P < 0.10$.
** $P < 0.05$.

of counted plants could not be representative of the number of tubers in the evaluated populations, since observed plants might originate from seeds or be propagules from stolons.

3.4. AFLP and pollen viability analyses

We used AFLP markers to generate a baseline for monitoring whether genetic erosion is occurring, and if so, to access a precise estimation of this phenomenon. The reproducibility of AFLP data in our analysis was higher than 95%: out of the 225 amplified AFLP fragments, 11 were not considered in posterior analyses because of inconsistences in the amplification between replicates. In the 22 analyzed populations, the total number of amplified AFLP fragments varied among 109 and 143. The fragments classified as frequent (which varied from 105 to 132 between populations) outnumber by far the other two kinds of fragments, followed by rare (varied from 1 to 11 among populations) and unique (varied from 0 to 4 among populations) (Fig. 5). The presence of rare or unique fragments in populations could be adopted as a criterion for targeting specific populations for conservation (Bamberg et al., 2010). However, no statistical differences among populations in the proportion of rare ($p = 0.517$) or unique ($p = 0.881$) fragments were observed. The total number and the relative proportion of unique, rare and frequent AFLP fragments establish a baseline that may be used for future monitoring the genetic variability in the evaluated populations, as well as in new populations incorporated into the analyses. In addition, an AFLP fragments database was elaborated (Appendix A, Table A1, Table A2). We detected that out of 214 analyzed fragments 126 (58%) showed differences higher than 0.5 bp when the two injections of the full set of samples were compared. Because fragments with differences higher than ±0.5 bp are assigned to different loci by the GeneMapper program, the differences between injections represent a problem for using the database for direct comparison of presence/absence of each fragment in the evaluated populations at different time points. This problem can be solved by maintaining standard samples to incorporate into future comparisons. In our laboratory we keep in a freezer at −80 °C one sample (and its replicate) of each analyzed population (i.e. DNA and digestion, ligation and pre-amplification reactions). In future comparisons, in addition to the sampling performed, these 22 samples must be incorporated into the analysis. The use of AFLP markers to generate a baseline for monitoring genetic diversity and its limitations have been described in *Brassica* species in the UK (Watson-Jones et al., 2006). Here, we present an alternative on how such data could be used and propose a recommendation: in the designation of genetic reserves it would be advisable to consider the participation of a research institution to generate a

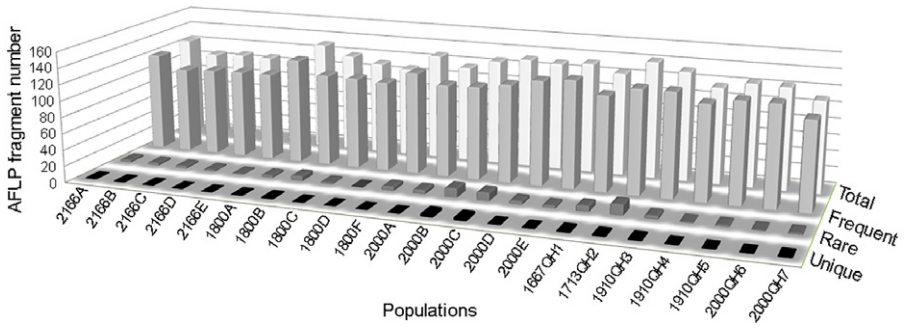


Fig. 5. Number of total, frequent, rare and unique AFLP fragments amplified in 22 *Solanum kurtzianum* populations using two primer combinations.

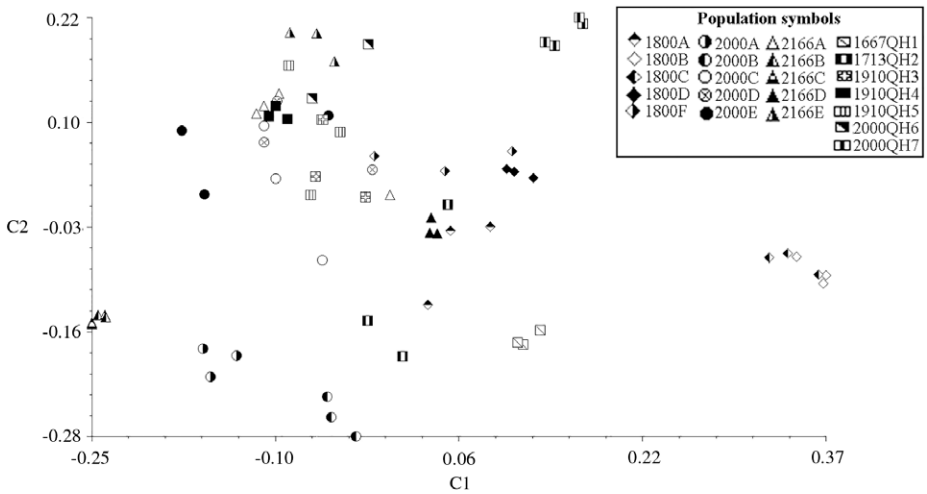


Fig. 6. Principal coordinate analysis plots of 22 *Solanum kurtzianum* populations based on Dice coefficient.

AFLP database as here presented, and the commitment to conserve and maintain standard samples for detailed analysis of presence/absence of specific fragments through time.

No obvious geographic pattern was observed on clustering obtained from the PCoA analysis (Fig. 6). Although there was an apparent separation of the populations 1667QH1, 2000QH7, 1800B, 1800C, 2000A, 2000B and 2166B, not all samples from the same origin (i.e. Caracoles or Quebrada Hornillos) grouped together and, in addition, samples and populations of different origin overlap each other. The overlap between populations observed in the PCoA plot and the high proportion of frequent AFLP fragments indicate enough gene flow among *S. kurtzianum* populations within the VNR to prevent genetic isolation and population differentiation. In addition, these results indicate that it would be advisable to focus the monitoring on populations that are distributed adjacent to roads, which are accessible with vehicle without dispensing time and effort to undertake long treks to access remote populations. In a previous work performed in the VNR we showed that the genetic variability of *S. kurtzianum* is mainly present among plants within populations, observed high proportion of plump seeds per fruit, demonstrated the seed dispersal by storm water channels and identified and calculated the foraging distance of four insect pollinators that visit the flowers of *S. kurtzianum* (Marfil and Masuelli, 2014). Here we present a new contribution regarding the reproductive biology of *S. kurtzianum* populations within the VNR: pollen stainability was taken as a measure of male fertility. Of the 60 evaluated plants, all exhibited pollen viability percentages above 60%, with a dispersion range from 63.6 to 98.5%. The average pollen viability per populations varied from 64.8% to 94.3% (Table 7). From these results it can be inferred that, in addition to other ways of gene flow, virtually all plants of *S. kurtzianum* within the VNR have the potential to share their genes via pollen flow, and in addition, suggest the feasibility of incorporating this germplasm into breeding programs. However, additional studies are needed to establish the sexual compatibility between the genotypes of *S. kurtzianum* from the VNR with the cultivated potato.

3.5. Education

Since 2011 we have been working jointly under the supervision and with the logistical support provided by managers and park rangers of the VNR. In 2014 we conducted a workshop for managers and park rangers of this Reserve, for provincial natural resource managers and for general public. The objective of the workshop was to highlight the value of the PGRFA, to

Table 7
Average percentage of pollen viability evaluated by 2% acetocarmine staining and calculated by averaging the percentages recorded for ten individual plants per population. In brackets minimum and maximum values founded in each population are indicated.

Population	Pollen viability percentage
2166C	64.8 (63.6–66)
1910QH4	89.5 (76.3–98.5)
2000QH6	91.2 (87.2–96.6)
2000B	91.6 (85.1–96.6)
1240QT	94.3 (91.2–97.1)
1258QT	79.6 (69.7–93.4)

raise awareness about the importance of their conservation and to strengthen the bond and partnership with the managers and park rangers of the VNR. We presented the background, results and perspectives that are part of this manuscript and that can be summarized in the following agenda of activities that were developed during two days in the VNR:

- Conservation strategies of plant genetic resources. Ex situ conservation and role of genebanks. In situ conservation and role of PA.
- CWR definition. The essential role of potato for the global food security. Distribution and characteristics of potato CWR.
- The wild potato species as an experimental model in the biological sciences: genetic and epigenetic variability and phenotypic plasticity.
- Field work: recognition of natural populations of *S. kurtzianum*. Botanical description, reproductive strategies and biotic and abiotic interactions of this species.

In 2014 we started a project to study the acclimation of *S. kurtzianum* plants along an altitudinal range within the VNR. For it purpose, experimental gardens at 1200 m a.s.l and 2200 m a.s.l were established with the collaboration of the park rangers. In turn, park rangers had a vital task of daily monitoring of experimental gardens. It is noteworthy that the development of this project includes the monitoring of crop plants at different altitude in successive years, so the prospects to continue strengthening our partnership are promising. On the other hand, we think that the joint work developed in the VNR is a prominent example that may further enhance the public perception of this PA and would help to ensure longer-term site security.

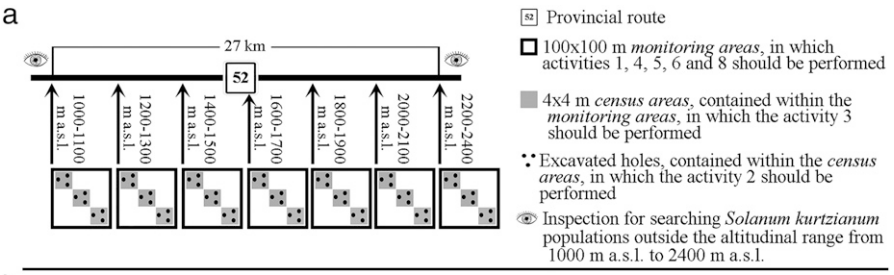
4. Conclusions

While the first step is to register the presence of predicted species in the priority PA identified in this work, we strongly advice to fit this activity within a general framework aimed to clarify the evolutionary relationships among the wild potato species. Lack of phylogenetic structure has been documented for South American wild potato species (Spooner and Castillo, 1997; Jacobs et al., 2008) and field experimentation focused on the reproductive biology and breeding relations among spontaneous populations will provide for a better understanding of the morphological and molecular variability encountered in the group (Camadro, 2012; Camadro et al., 2012). This strategy will ensure the long-term conservation of populations and the ultimate purpose of the conservation of genetic resources: its effective use in breeding. This recommendation could be useful to implement in all those sites that have been described to possess high species richness.

In the VNR the spatial scale of interest for monitoring target populations was identified. Our proposal is to work on selected populations near to Provincial route 52. One of the features of the VNR is that a short journey for this route leads to sites with a large difference in altitude: at a distance of 27 km in vehicle, it ascends from 1000 m a.s.l. to 2400 m a.s.l., the whole altitudinal distribution range of *S. kurtzianum* observed so far in this Reserve. In Fig. 7 a five year Management Plan is presented. The implementation of this plan should involve the selection of seven *monitoring areas* covering the full range of altitudinal distribution of *S. kurtzianu*m for continuous and exhaustive monitoring of populations there distributed. Three of these seven areas could be established on sites over which we have worked (two in Caracoles and one in Quebrada del Toro, see Table 1). Within each *monitoring areas*, three *census areas* should be delimited. Within each *census areas*, three holes should be excavated each year for monitoring the sprouting behavior. The Management Plan includes Park ranger training for two years to perform specific tasks. According to previous recommendation (Camadro, 2012), inspections for biotic and abiotic interactions will allow identifying germplasm to be conserved ex situ and facilitate their use by breeders: (i) fruits or tubers of mature plants affected by *Epicauta spp.* but not totally defoliated will be collected and sent to the PFGB-INTA tagged as "apparently tolerant to *Epicauta*", (ii) fruits or tubers of mature plants observed in dry seasons will be collected and sent to the PFGB-INTA tagged as "apparently tolerant to water stress", (iii) we hope to detect and characterize presence of other pest or disease in the population distributed within the *monitoring areas* and if some plants were not affected they will be collected as described above. As it is important that the general public should become aware of this activity, a demonstrative garden of *S. kurtzianum* with relevant information will be established in the Interpretive Center of the VNR.

The results obtained from the activities 1 to 7 (see Fig. 7) during five years will be compared with the baseline established in this work and previous (Marfil and Masuelli, 2014) respect to distributional range, population size, sprouting behavior, pollen and seed viability and biotic and abiotic interactions. From this comparison we will decide if it is necessary to

a



b

Activity	Periodicity/Period	Time required per period	Compatibility	Responsible personnel
1-Inspection for biotic and abiotic interactions	Annually/Last week of January, February and March	Three days	Compatible with activities 3,4, 5, 6 and 7	Researchers and park rangers the first two years, park rangers after the second year
2-Inspection for sprouting behaviour	Annually/First week of November, December and January	Three days	Exclusive activity	Researchers
3-Population census	Annually/Last week of March	One day	Compatible with activities 1,4, 5, 6 and 7	Researchers and park rangers the first two years, park rangers after the second year
4-Inspection for flower collection and pollen viability studies	Annually/Last week of February and March	Two weeks (considering field and laboratory activities)	Compatible with activities 1, 3, 5, 6 and 7	Researchers
5-Inspection for fruit collection and seed viability studies	Annually/Last week of February and March	Two weeks (considering field and laboratory activities)	Compatible with activities 1, 3, 4, 6 and 7	Researchers
6-Inspection for collect seeds and tubers to send to the Potato and Forages Germplasm Bank	Annually/Last week of February and March	One day	Compatible with activities 1, 3, 4, 5 and 7	Researchers
7- Inspection outside the altitudinal range from 1000 to 2400 m a.s.l.	Annually/From January to march	Unknown, intimately related to the activity of park rangers who continually explore the Reserve	Compatible with activities 1, 3, 4, 5 and 6	Park rangers (mainly) and researchers
8-AFLP analysis	If necessary, depending of results obtained from activities 1 to 6	Six months	Exclusive activity	Researchers

Fig. 7. A five years Management Plan for the in situ conservation of *Solanum kurtzianum* at the Villavicencio Natural Reserve. (a) Schematic representation (not to scale) of seven monitoring areas that must be distributed along the whole altitudinal distribution range of *S. kurtzianum* observed so far in this Reserve. (b) Schedule and details of the activities that should be performed.

perform an AFLP analysis (activity 8, see Fig. 7) for monitoring the genetic variability and will allow the reformulation and/or improvement of the Management Plan for the next five years. With the results obtained in this study and the proposed Management Plan our goal is to obtain a legislation of the VNR as a genetic reserve of *S. kurtzianum*.

Acknowledgments

This work was funded by Secretaría de Ciencia, Técnica y Posgrado de la Universidad Nacional de Cuyo proyectos bienales 2011–2013 number 06/A514 and Proyectos de Investigación Plurianuales, 2011–2013 CONICET PIP number 00245. We are grateful to Silvina Giudicci, the manager of the VNR, and the Villavicencio Natural Reserve's Park Ranger Team for their assistance in the field-based research, and Marcelo Alberto (Dpto. de Biomatemática y Fisicoquímica, Facultad de Ciencias Agrarias, UNCuyo) for valuable assistance with statistical analyzes.

Appendix A. Supplementary data

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.gecco.2015.01.009.

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