

Article type : Regular Manuscript

## Epigenetic consequences of interploidal hybridisation in synthetic and natural interspecific potato hybrids

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Received: 9 October 2018

Accepted: 17 January 2019

**Key words:** aneuploidy, crop wild relatives, DNA methylation, introgression, *Solanum* section *Petota*, *Solanum kurtzianum*, *Solanum microdontum*, *Solanum x rechei*

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### Summary

- Interploidal hybridisation can generate changes in plant chromosome numbers, which might exert effects additional to the expected due to genome merger *per se* (*i.e.*, genetic, epigenetic and phenotypic novelties).

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/nph.15706

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- Wild potatoes are suitable to address this question in an evolutionary context. To this end, we performed genetic (AFLP and SSR), epigenetic (MSAP), and cytological comparisons in: i) natural populations of the diploid cytotype of the hybrid taxonomic species *Solanum x rechei* ( $2n=2x, 3x$ ) and its parental species, the triploid cytotype of *Solanum microdontum* ( $2n=2x, 3x$ ) and *Solanum kurtzianum* ( $2n=2x$ ); and ii) newly synthesised intraploidal ( $2x \times 2x$ ) and interploidal ( $3x \times 2x$ ) *S. microdontum*  $\times$  *S. kurtzianum* hybrids.
  - Aneuploidy was detected in *S. x rechei* and the synthetic interploidal progeny; this phenomenon might have originated the significantly higher number of methylation changes observed in the interploidal vs. the intraploidal hybrids. The wide epigenetic variability induced by interploidal hybridisation is consistent with the novel epigenetic pattern established in *S. x rechei* compared to its parental species in nature.
  - These results suggest that aneuploid potato lineages can persist throughout the short term, and possibly medium term, and that differences in parental ploidy resulting in aneuploidy are an additional source of epigenetic variation.

### Introduction

McClintock (1984) proposed that the genome is a highly sensitive cell organ that can monitor -and respond to- unanticipated types of ‘genome shock’. One of these “shocks” is interspecific hybridisation, which can trigger dramatic genome restructuring and the setting of new contexts for gene expression. Interspecific hybridisation and genome multiplication (polyploidy) have played major roles in generating plant biodiversity, either separately or, more frequently, in combination upon allopolyploidisation (Dobzhansky, 1941; Doyle *et al.*, 2008; Soltis & Soltis, 2009). Thus, significant attention has been paid to the participation of genetic and epigenetic changes in diverse genomic reactions to both genome merging and genome multiplication (Chen, 2007; Doyle *et al.*, 2008).

Wild potatoes (*Solanum*, section *Petota*) provide an ideal scenario to explore the role of hybridisation and polyploidisation in the diversification of the group, which form a euploid series with somatic chromosome numbers of  $2n=2x=24$  up to  $2n=6x=72$  ( $x=12$ ). Wild potatoes are adapted to a wide variety of environments from Southern USA in North America to Chiloé Island in South America, where they naturally grow following the Andes and extending to the East into Argentina, Uruguay, Paraguay and Brazil (Hawkes, 1990). Internal breeding barriers, if present, can be incomplete, thus, hybridisation can occur within and between ploidy levels and populations in areas of sympatry (Camadro *et al.*, 2012; Spooner *et al.*, 2014).

Homoploid hybridisation (between species of the same ploidy) has been proposed as the main mechanism involved in the origin and evolution of diploid potatoes (Masuelli *et al.*, 2009), and genetic and epigenetic changes in response to interspecific hybridisation have been documented

(Marfil *et al.*, 2006). Moreover, it has been suggested that polyploidy has played an important role in environmental differentiation and range expansion of this group (Hijmans *et al.*, 2007).

Both homoploid hybridisation and polyploidisation have received considerable attention. In this regard, Stupar *et al.* (2007) reported phenotypic differences in diploids *vs.* their synthetic autotetraploids, and differential expression between ploidy groups in around 10% of 9,000 genes assayed with a potato cDNA microarray. Aversano *et al.* (2013, 2014) reported only subtle changes in newly synthesized autotetraploids *vs.* their diploid parents in phenotypic, genetic, epigenetic and transcriptomic studies. Moreover, Fasano *et al.* (2016) detected mostly stochastic changes in the transcriptome and metabolome of autotetraploids, and proposed that a nucleotide precursor pool imbalance triggers a genomic shock conducting to this stochastic response. Notwithstanding, the connection between interploidal hybridisation and the creation of genetic and epigenetic diversity in wild potatoes has been poorly addressed.

It is difficult to ascertain if either genome merger or genome multiplication is prominent over the other. However, from the sympatric geographic distribution of wild potato populations of various ploidies and incomplete breeding barriers, it can be inferred that the two mechanisms are not mutually exclusive and, probably, have simultaneously occurred in the evolution of the group.

Experiments with natural and synthetic allopolyploids have made focus on the evolutionary significance of hybridisation and genome multiplication. As examples, it has been demonstrated that hybridisation *per se* triggered pronounced epigenetic changes in *Brassica*, *Gossypium*, *Senecio*, *Spartina* and *Arabidopsis* (Salmon *et al.*, 2005; Albertin *et al.*, 2006; Wang *et al.*, 2006; Flagel *et al.*, 2008; Hegarty *et al.*, 2011), and that genome merging may have had a more profound effect than genome dosage in these species. However, no consistent evidences on the variability generated by genome duplication have been obtained with synthetic autopolyploids. In fact, in a synthetic autopolyploid (1x, 2x, and 4x) series of the cultivated diploid potato *Solanum phureja* J. & B., Stupar *et al.* (2007) observed positive correlations between cell size and organ thickness with ploidy level, and subtle expression changes in a substantial percentage of genes upon ploidy alterations. Notwithstanding, in comparing diploids and synthetic autotetraploids of the wild diploid potatoes *Solanum commersonii* D. and *Solanum bulbocastanum* D., no clear superiority of the latter was observed for leaf thickness and area, although tetraploids had subtle DNA methylation modifications and strong alterations in the expression patterns of eight important cell cycle-regulatory genes (Aversano *et al.*, 2013). Results from genomic restructuring comparisons between allopolyploids and autopolyploids suggest that hybridisation in potatoes is an additional genomic shock (Marfil *et al.*, 2018).

Among wild potatoes from Argentina, the hybrid species *Solanum x rechei* H. & H. (2n=2x=24 and 2n=3x=36) offers excellent opportunities for studying the effects of changes in chromosome number and hybridisation/introgression on the genome, and the ecologically and evolutionary significance of these phenomena. *S. x rechei* has been only found growing in sympatry

with its wild progenitors, *Solanum kurtzianum* B. & W. ( $2n=2x=24$ ; 2 EBN) and *Solanum microdontum* B. ( $2n=2x=24$ ; 2 EBN and  $2n=3x=36$ ), within the limits of their geographic range (Okada & Hawkes, 1978; Cara *et al.*, 2013). Although most *S. microdontum* populations are diploid, triploid populations have been recorded at the southern end of their range in Argentina (Okada & Hawkes, 1978; Okada, 1981; this work), where they can hybridise with *S. kurtzianum*. Based on this observation, Okada and Hawkes (1978) proposed that triploid *S. microdontum* cytotypes were involved in the origin of *S. x rechei*.

In meiosis, triploid potatoes may form trivalents, bivalents and univalents; *i.e.*, average reported configurations are  $3.75 \text{ III} + 9.11 \text{ II} + 6.39 \text{ I}$  in cultivated *S. juzepczukii* B. (Hawkes, 1962) and  $4.85 \text{ III} + 9.05 \text{ II} + 3.45 \text{ I}$  in synthetic *S. commersonii* x *S. gourlayi* H. hybrids (Masuelli & Camadro, 1992). Therefore, triploids form mainly unbalanced gametes with reduced viability (unless meiotic restitution mechanisms operate). Notwithstanding, aneuploids have been obtained by fertilisation of unbalanced female gametes from triploid *S. commersonii* x *S. gourlayi* hybrids with male gametes of various ploidies (Masuelli & Camadro, 1992). The importance of interploidal hybridisation in evolutionary processes in higher plants is well documented (Chapman & Abbott, 2010; Ricca *et al.*, 2011; Moraes *et al.*, 2013). Differences in ploidy level between parental species and aneuploid formation could be additional sources of genetic and epigenetic variability (de la Casa-Esperón & Sapienza, 2003; Bean *et al.*, 2004; Henry *et al.*, 2005).

Previously, we demonstrated that a novel epigenetic pattern was established in *S. x rechei* in comparison to the parental genomes (*S. microdontum* and *S. kurtzianum*), a phenomenon that could influence the phenotypic plasticity and adaptation of hybrids to new environments (Cara *et al.*, 2013). In the present work we aim to answer whether differences in ploidy of the parental species can induce changes additional to the expected due to hybridisation *per se*. For this purpose, we quantified and compared genetic and epigenetic changes between synthetic intraploidal and interploidal *S. microdontum* x *S. kurtzianum* hybrids (Fig. 1). The other key question addressed was whether genomic rearrangements resulting from experimental hybridisation can be identified in natural hybrids. To this end, we incorporated genotypes of *S. x rechei* collected from natural populations into the analyses, and explored the differences and similarities between synthetic and natural hybrid lineages.

## Materials and methods

### Plant materials

They consisted of: i) 81 genotypes of natural sympatric populations classified as *S. kurtzianum* (30), *S. microdontum* (18) and *S. x rechei* (33), collected as tubers in an area of hybridisation in Chilecito, La Rioja, Argentina ( $29^{\circ}10'S$ ,  $67^{\circ}40'W$ ), and ii) 11 synthetic interspecific hybrids: six derived from intraploidal  $2x \text{ } S. \text{ kurtzianum} \times 2x \text{ } S. \text{ microdontum}$  crosses (hereinafter, “DxD”) and five derived from one interploidal  $3x \text{ } S. \text{ microdontum} \times 2x \text{ } S. \text{ kurtzianum}$  cross

(hereinafter, “TxD”). Only reciprocal DxD crosses were performed because the interploidal ones were only successful when *S. microdontum* was used as the female parent, due its low pollen viability (Supporting Information Table S1). Three plants obtained from an intraploidal 2x *S. microdontum* x 2x *S. microdontum* cross were used as controls (Table 1, Fig. 1 and Supporting Information Table S1). Tubers collected in nature (one per genotype) were planted in pots; seeds obtained from the artificial crosses were germinated in Petri dishes and the derived seedlings were transplanted into pots. Plants were grown following a complete randomised design, in an insect-proof screenhouse under natural conditions of temperature and photoperiod (S 33°0', W 68°52', 942 m a.s.l.) from November 2009 to February 2010. Water and fertilizer were applied as needed.

### **AFLP and MSAP analyses**

DNA extraction and quantification were performed following Cara *et al.* (2013). Twenty-seven genotypes were included in the genetic and epigenetic analyses: two of *S. kurtzianum* and three of *S. microdontum* (parental genotypes), eight of *S. x rechei*, three intraspecific controls, five TxD hybrids, and six DxD hybrids. To assess genetic variability, an AFLP analysis was performed by using enzymes *EcoRI* and *MseI* according to Vos *et al.* (1995). Methylation sensitive amplified polymorphism (MSAP) markers were used to estimate epigenetic variability following Xiong *et al.* (1999), with slight modifications. The MSAP technique is an adaptation of the original AFLP technique, with substitution of the frequent cutter enzyme *MseI* by the isoschizomers *HpaII* and *MspI*, whereas the rare cutter remains unchanged. *HpaII* and *MspI* recognise the same tetranucleotide restriction site (5'-CCGG), but have different sensitivities to cytosine methylation. For both techniques, fluorescent labelled *EcoRI*+3 primers were used in the selective amplification. The fluorescent amplified fragments were separated on an ABI PRISM 3130 DNA sequencer (Applied Biosystems) and allele scoring was performed on the resulting electronic profiles by using GeneMapper version 3.7 (Applied Biosystems) according to Cara *et al.* (2014).

### **Genetic and epigenetic variability in the offspring of artificial crosses**

Amplified patterns observed in the AFLP analysis were transformed into a binary matrix by scoring fragments as either present (1) or absent (0). For the MSAP analysis, patterns of presence/absence between the *EcoRI/HpaII* and the *EcoRI/MspI* digests were codified from 0 to 3 (Fig. S1, upper panel); then, this codification was converted into a binary matrix for either presence (1) or absence (0) of patterns 3, 2, or 1. Pattern 0 was not codified in the binary matrix (Fig S1, lower panel) because the absence of fragments in *EcoRI/HpaII* and *EcoRI/MspI* digests might be due to either methylation of external cytosines or variations in the nucleotide sequence.

To calculate the error rate of the AFLP and MSAP techniques, two restriction, ligation, preamplification and amplification reactions were performed in parallel -starting from the same DNA samples- in a subset of seven genotypes.

Distance matrices were generated from AFLP and MSAP binary matrices with Dice coefficient. Genetic and epigenetic variability among groups (*i.e.* DxD and TxD hybrids, and controls) were compared by ANOVA. To detect potential biases in the analysis due to differences in the sample size of each group, subsamples of three individuals (minimum sample size) were generated from the original samples, and means and standard deviations were calculated (Supporting Information Table S2). Subsequent analyses were based on the original samples because non-significant differences between the original and subsampled data sets were detected ( $P > 0.05$ ).

### **Inheritance of methylation patterns in synthetic hybrids**

The methylation status of each locus was analysed in both parents and offspring with MSAP markers to detect if it remained unchanged (Fig. S2). Because patterns 2 and 3 can lead to ambiguous interpretation (Fulneček & Kovařík, 2014), they were pooled and considered as methylated loci. In addition, the absence of fragments in the hybrids (pattern 0) was not considered because it could have resulted from segregation (Marfil *et al.*, 2006) given that the parental species are highly heterozygous. Therefore, the analysis was focused on new fragments. For any given locus, if the hybrid presented the same pattern -other than 0- as either one or the other parent, the locus was considered “unchanged” (Fig S2). If the pattern of the hybrid differed from the pattern of both parents, two types of changes were distinguished: hypermethylation and hypomethylation. The change from pattern 1 in the parents to patterns 2 or 3 in the hybrid was considered hypermethylation. Changes from patterns 2 or 3 in the parents to pattern 1 in the hybrid and from pattern 0 in the parents to patterns 1, 2 or 3 in the hybrid were considered hypomethylation. ANOVAs were conducted to compare the number of each type of methylation change in hybrids and controls.

### **Novel genetic and epigenetic patterns in synthetic hybrids also present in the natural hybrid *S. x rechei***

To evaluate if eventual genome and/or methylation restructuring in the synthetic hybrids were also present in natural *S. x rechei*, eight genotypes of this species were analysed with the same AFLP and MSAP primers. The amplification patterns observed in the evaluated natural hybrids were compared to the patterns obtained in the synthetic hybrids and their parental genotypes. First, fragments present in the synthetic hybrids were categorized as *S. microdontum* or *S. kurtzianum* species-specifics. In addition, the presence novel amplification fragments in the synthetic hybrids (*i.e.* present in the hybrids but absent in the genotypes of the parental species *S. microdontum* and *S. kurtzianum*) was assessed in the natural hybrids. Novel fragments were classified as *S. x rechei* species-specific if they were also present in at least one of the *S. x rechei* evaluated genotypes or as novel if they were only observed in the synthetic hybrids (Fig. S3).

### **Ploidy analysis**

Chromosome numbers were determined in root-tips -harvested from plants growing in 250 ml pots in a growth chamber with a photoperiod of 16-8 h light/dark at 24° C- pretreated with 8-hydroxyquinoline 0.29 g.l<sup>-1</sup> for 5 h at 14° C, fixed in ethanol-acetic acid (3:1, v/v) for 24 h at room temperature, and stored in 70% ethanol at 4° C until use. For cytological observations, fixed root-tips were rinsed with water, treated with Pectinex SP ULTRA ® for 45 min at 37° C, and stained on glass slides with 1% (w/v) orcein. Ten to 20 mitotic metaphases were analysed per genotype.

### **SSR analysis**

Additional evidences on chromosome numbers were obtained with co-dominant SSR molecular markers. Each genotype was confirmed as being either diploid (maximum: two alleles/locus) or polyploid (three or more alleles/locus). In genotypes identified as near-diploid aneuploids (*i.e.*  $2n=2x+1$ ,  $2n=2x+2$ , etc.), the detection of three or more alleles/locus was considered indication of the presence of extra chromosome(s). Twenty-four EST-derived SSR markers mapping in the 12 potato chromosomes were pre-screened. A final set of 15 pairs of SSR primers (Table S3) - mapping in 10 chromosomes and producing reliable amplification patterns- were assayed in four genotypes of *S. microdontum*, four of *S. kurtzianum* and eight of *S. x rechei* (all of them collected in nature), and in nine synthetic hybrids, five TxD and four DxD. Primer sequences and PCR amplification protocols were according to Feingold *et al.* (2005). Amplified products were separated by electrophoresis in 6% denaturing polyacrylamide gels, and visualised by silver staining (see Marfil *et al.*, 2011).

### **NewHybrids analysis**

Based on plant morphology, *S. x rechei* has been classified as a hybrid taxonomic species. As such, it can be either reproductively isolated from its progenitors or undergoing an evolutionary process which could eventually lead to differentiation of a new species or introgression into its parental species. To clarify this point, an analysis was performed with AFLP data previously generated with six AFLP primer combinations in 78 genotypes from natural populations (Cara *et al.*, 2013). These genotypes had been classified as either *S. kurtzianum* (30), *S. microdontum* (18), or *S. x rechei* (30) according to morphological phenotypes. NewHybrids version 1.1 Beta3 (Anderson & Thompson, 2002) was employed for the analysis. This software implements a Markov Chain Monte Carlo algorithm to compute the posterior probabilities that an individual belongs to each of six genotypic classes: species 1, species 2, filial (F<sub>1</sub>, F<sub>2</sub>) and backcross (BC<sub>1</sub>, BC<sub>2</sub>) generations. No linkage or Hardy-Weinberg disequilibrium in the parental species is assumed in *n* generations before the sampling event. Any disequilibrium in the mixed population is considered to arise from the mixture of the two species and their hybrids (Anderson & Thompson, 2002). Individual genotypes were assigned to either a taxonomic species or one class. The default genotype categories were used



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for first and second crossing generations, and uniform priors were chosen for both allele frequencies and mixing proportions to down-weight the influence of alleles which might be rare in one species and absent in the other, following the software author's recommendation. We ran 10,000 iterations as a burn-in period, followed by 100,000 MCMC repetitions. Stability was tested in five independent runs.

## Results

### Genetic changes induced by hybridisation

A total of 492 loci were evaluated with six AFLP primer combinations. The error rate -based on seven replicated samples- was 3.4%. In analysing five (TxD) *S. microdontum* CMM/5 x *S. kurtzianum* LR3/4 hybrids, 384 fragments were found. Among these, 153 (39.8%) were shared by both, parents and hybrids. *S. kurtzianum* presented 93 species-specific fragments, 82 of which were also present in the hybrids. *S. microdontum* presented 129 species-specific fragments, 123 of which were also present in the hybrids. Nine fragments (2.3%) observed in the hybrids were absent in the parental species (Fig. 2a).

In six (DxD) *S. microdontum* 4631/8 x *S. kurtzianum* 4548/1 hybrids, a total of 371 fragments were analysed, 125 of which were present in both parents and, with one exception, in the hybrids. The number of species-specific fragments was 112 in *S. kurtzianum* and 118 in *S. microdontum* of which, respectively, 101 and 108 were also present in the hybrids. Sixteen fragments (4.3%) were found exclusively in the hybrids (Fig. 2b). No differences were observed when the AFLP fragment distribution was compared in the reciprocal DxD hybrids (data not shown).

In the 2x *S. microdontum* x 2x *S. microdontum* control cross, 267 fragments were analysed, two of which (0.75%) were observed exclusively in the progeny (Fig. 2c).

### Extensive alteration in methylation patterns associated with interploidal hybrid formation

Six primer combinations were used to evaluate 298 loci, six of which were monomorphic. By considering the methylation status of each locus, 463 epialleles were identified. The error rate -based on seven replicated samples- was 2.6%. In the TxD hybrids (Fig. 3a), 235 epialleles were analysed; of these, 42 (17.9%) were observed in both parents, and all but two of them were observed in the hybrids. Regarding the number of species-specific alleles, *S. kurtzianum* presented 55 and *S. microdontum* 75, of which 41 and 65, respectively, were present in the hybrids. In the hybrids, 63 (26.8%) unique epialleles were detected, 41 of which were unequivocally methylation changes because the parental species presented fragments for the same epiloci, but with different methylation patterns. For the other 22 epiloci, no fragments were observed in the parental species; this could



indicate either a change in the methylation status in the hybrids (if sites were completely methylated in the parents) or a genetic change.

In analysing the DxD hybrids (Fig. 3b), 205 epialleles were identified, 41 of which (20%) were present in both parents and hybrids. *S. kurtzianum* exhibited 65 exclusive methylation patterns and *S. microdontum* 64; of these, 52 and 53, respectively, were present in the hybrids. Thirty-five novel epialleles (17.1%) were observed in the hybrids; 27 of them were unequivocally methylation changes because the parental species displayed a different methylation pattern for the same loci. Regarding the other eight loci, no fragments were present in the parental species.

When the progeny of the control cross was epigenetically compared to the parental genotypes, a total of 132 patterns were generated with six primer combinations. Four of the analysed patterns (3%) were found exclusively in the progeny (Fig. 3c).

### **Genetic and epigenetic variability in the offspring of the artificial crosses**

Two types of comparisons were performed: i) within each group, genetic variability was compared to epigenetic variability, and ii) among groups, genetic and epigenetic variabilities were compared. In the progeny of the control cross, no differences were observed between the genetic and epigenetic variabilities calculated by using Dice coefficient (Fig. 4). On the contrary, the epigenetic variability was significantly higher than the genetic variability in the TxD and DxD hybrids. On the other hand, when among-groups comparisons were performed, both the genetic and the epigenetic variabilities in the interspecific hybrids were higher than in the progeny of the control cross, except when the genetic variabilities of the DxD hybrids and the latter were compared. In addition, the epigenetic variability of the TxD hybrids was higher than the observed in the DxD ones (Fig. 4).

### **Inheritance of methylation patterns in synthetic hybrids**

There were statistical differences in all evaluated types of change between the hybrids and the control cross (Fig. 5). The TxD hybrids presented significantly higher number of hypomethylation changes than the DxD ones, with averages of 11.6 and 5.0 hypomethylations/genotype, respectively. On the other hand, the DxD hybrids presented significantly more hypermethylations than the TxD ones, with averages of 8.3 and 6.6 hypermethylations/genotype, respectively. The average total number of changes was 14.0 and 18.8 changes/genotype for the DxD and TxD hybrids, respectively; with statistical differences.

### **Novel genetic and epigenetic patterns in synthetic hybrids also present in the natural hybrid *S. x rechei***

To explore differences and similarities between synthetic and natural hybrid lineages, we quantified the number of AFLP and MSAP fragments that were novel in the synthetic hybrids

(presents in the hybrids but absent in the parental genotypes) and verified the presence of these in the natural hybrids, which presumably derived from the same parental species combination.

In the synthetic hybrids, 4.0% to 17.0% of the AFLP fragments were not present in either parent. Among these fragments, 1.7% to 8.2% were *S. x rechei* species-specific and 2.0% to 11.6% were only observed in the synthetic hybrids and, thus, classified as novel (Fig. 6a).

When analysing the distribution of novel methylation patterns in the synthetic hybrids, 29.5% to 47.9% of them were not present in either parent. Among these patterns, 2.2% to 8.9% were *S. x rechei* species-specific and 22.0% to 31.1% were exclusive of the synthetic DxD hybrids (Fig. 6b, upper panel). In the TxD hybrids, 6.1% to 13.8% of the patterns were *S. x rechei* species-specific and 23.8% to 37.5% of them were exclusive of them (Fig. 6b, lower panel).

### Chromosome numbers

All the analysed TxD hybrids were aneuploid, with chromosome numbers ranging from  $2n=2x+2=26$  to  $2n=3x-4=32$  (Table 2, Fig. 7). On the other hand, the four analysed DxD hybrids were euploid, with  $2n=2x=24$ .

In addition, the chromosome numbers of seven *S. x rechei* genotypes collected in the hybridisation area were euploid ( $2n=2x=24$ ) and two were aneuploid ( $2n=2x+1=25$ ) (Table 2, Fig. 7).

### SSR analysis

The polymorphism generated for five SSR loci enabled the distinction of more than two alleles in the tested genotypes (Table S3). In the amplifications of the StI021 locus (chromosome 6), three alleles were observed in four *S. x rechei* genotypes (GV02/1, GV09A/1, GV09B/3 and GV11/1) (Fig. S4). In one of these genotypes (GV02/1), three alleles were also observed at the StI057 locus (chromosome 9). In all five TxD hybrids, more than two alleles were observed in at least one of the five polymorphic SSR loci. In the triploid cytotype of *S. microdontum*, three alleles were detected for two loci. In the four DxD hybrids and their diploid parental species, one or two alleles were observed at the same SSR loci (Fig. S4).

### Genetic identity of the natural hybrid *Solanum x rechei*

All 30 *S. kurtzianum* genotypes and 16 out of the 18 *S. microdontum* genotypes were identified as purebred with posterior probabilities  $> 0.9$  (Fig. 8). The remaining two *S. microdontum* genotypes were intermediate between a *S. microdontum* backcross and a *S. microdontum* purebred.

Out of 30 analysed genotypes classified as *S. x rechei* on the basis of molecular and morphological data, 22 (73.3%) and one (3.3%) were identified, respectively, as F<sub>1</sub> hybrids and *S. kurtzianum* backcrosses, with posterior probabilities  $> 0.9$  (Fig. 8). The remaining seven *S. x rechei* genotypes presented less than 0.9 probability of belonging to one of the classes and, thus, remained

unclassified. Five of these seven genotypes (16.6%) were intermediate between a *S. kurtzianum* backcross and a *S. kurtzianum* purebred, one (3.3%) was intermediate between an F<sub>1</sub> and an F<sub>2</sub>, and another one (3.3%) was an admixture of F<sub>1</sub>, F<sub>2</sub> and a *S. kurtzianum* backcross.

## Discussion

### DNA methylation changes in synthetic populations

The comparison between euploid and aneuploid genotypes derived from the same interploidal crosses could provide data relevant to understanding the contributions of aneuploidy and hybridisation to genome restructuring. In this work, however, we only obtained aneuploid genotypes from the assayed parental combinations. Notwithstanding, the MSAP analyses revealed genomic instability in the progeny of the interspecific hybrids, in accordance with the previously reported in wild potatoes (Marfil *et al.*, 2006). MSAP markers are considered appropriate for monitoring inheritance or variation of parental methylation patterns in hybrid progenies (Madlung *et al.*, 2002; Marfil *et al.*, 2006; Zhang *et al.*, 2007). We demonstrated that the TxD hybrids [*S. microdontum* (2n=3x=36) x *S. kurtzianum* (2n=2x=24)] presented significantly higher numbers of methylation changes than the DxD ones [*S. microdontum* (2n=2x=24) x *S. kurtzianum* (2n=2x=24) and its reciprocal] and, moreover, larger epigenetic variability among their siblings. These results could be explained by assuming: i) genetic differences between the diploid and triploid *S. microdontum* cytotypes, which could have possibly affected nuclear-cytoplasmic interactions, or ii) aneuploidy in the TxD hybrid progeny. In a previous work (Cara *et al.*, 2013), we demonstrated that there were no apparent genetic or epigenetic differences between diploid and triploid *S. microdontum* cytotypes. Since the same genotypes were used in the present work, the first alternative can be excluded. Although we cannot rule out potential differences in the cytoplasmic genomes of the two *S. microdontum* cytotypes, the results obtained with the reciprocal DxD hybrids allowed us to hypothesize that the role of nuclear-cytoplasmic interactions has not been significant in the changes. The presence of aneuploid genotypes in the TxD progeny is undoubtedly the most likely alternative to explain the differences observed between intraploidal and interploidal hybrids. Furthermore, by comparing interploidal vs. intraploidal hybrids, we can hypothesise that differences in parental ploidy resulting in aneuploid progeny is an additional source of epigenetic variation. Among the five interploidal hybrids, no correlation was found between the number of extra or missing chromosomes and the number of novel AFLP fragments and MSAP patterns (results not shown). Although a larger number of progeny may be required to obtain conclusive evidence; the magnitude of the observed genome reprogramming is likely to be more closely linked with the specific extra or missing chromosome type/s than with the number of chromosomes (Henry *et al.*, 2010).

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Epigenetic mechanisms can operate in response to aneuploidy. The results of a trisomic tobacco line study in which a transgene locus was carried on the tripled chromosome offered the first evidence on possible mechanisms of chromosome and epigenetic instability in plants with unbalanced genomes (Papp *et al.*, 1996). Comai *et al.* (2005) have considered that aneuploidy is relevant because it can trigger a syndrome of epigenetic and genomic instability. Moreover, results obtained from aneuploid lines of common wheat (*Triticum aestivum* L.) and their derived euploid progenies revealed heritable alterations in DNA methylation patterns as a result of aneuploidy (Gao *et al.*, 2016). Since the past decade, the role of increased chromosomal and epigenetic instability induced by aneuploidy in human diseases has also drawn special attention (Matzke *et al.*, 2003; Davidsson, 2014).

### **Aneuploidy in natural populations**

There is no information in the literature regarding the occurrence of aneuploidy in natural wild potato populations, except for Hawkes' (1990) statement that the phenomenon was unknown in that plant group. However, our results indicate that interploidal hybridisation can generate aneuploids, which can be successfully established in nature. Similar results were obtained in natural tetraploid populations of *Tragopogon mirus* and *T. miscellus* (Lim *et al.*, 2008), in which extensive aneuploidy and karyotypic variation among individuals, populations and even siblings were observed, indicating that chromosomal changes can rapidly occur following polyploidisation. In *Epidendrum* spp. the presence of aneuploid hybrids has been attributed to interploidal hybridisation in sympatric areas (Moraes *et al.*, 2013). Our results revealed that interploidal hybridisation in wild potatoes is a source of chromosome number variation, and allow us to hypothesise that some triploid cytotypes must be sufficiently fertile to produce advanced generation hybrid progeny. In fact, working with synthetic polyploid potato hybrids, Carputo *et al.* (2003) and Iovene *et al.* (2004) demonstrated that tolerance to aneuploidy was evident in the phenotype of pentaploid–tetraploid progenies, which exhibited neither morphological anomalies nor decreasing vigour, two features that are common in aneuploids of other plant groups. These and our results refute the proposal that individuals with unbalanced chromosomes cannot persist in natural populations and give rise to progeny. In the present work, two of the eight *S. x rechei* genotypes analysed (22%) were aneuploids, in contrast to Okada and Hawkes's (1978) report of only euploid cytotypes in five analysed plants (four diploid and one triploid). These differences could be due to sample size. On the other hand, results similar to ours were obtained by the same authors in the synthesis of the natural hybrid through artificial triploid *S. microdontum* x diploid *S. kurtzianum* crosses. From a progeny of 33 plants, they characterised four (12 %) as diploid and 29 (88%) as aneuploid, with chromosome numbers varying between 25 and 37. Similarly, aneuploids with 25 to 31 chromosomes were obtained by Masuelli and Camadro (1992) in crosses between one triploid *S. commersonii* x diploid *S. gourlayi* hybrid and the diploid species *S. infundibuliforme*, *S. gourlayi* and *S. commersonii*. In our work, all the analysed interploidal hybrids were aneuploid, with

26 to 32 chromosomes. In meiosis of triploid and hexaploid potato hybrids, the tendency of chromosomes to clump together in the equatorial plate at metaphase I (Masuelli *et al.*, 1995) and to migrate as a group to the poles in anaphase I, rather than to be distributed at random (Masuelli & Camadro, 1992), may explain the near-triploidy (32 chromosomes) or near-diploidy (25, 26, 27 chromosomes) determined in the analysed hybrids. Moreover, Carputo *et al.* (2003), in studying experimental *S. commersonii*-*S. tuberosum* progenies derived from pentaploid–tetraploid crosses, observed progeny means for chromosome numbers ranging from  $50 \pm 0.5$  to  $54 \pm 0.7$ , with most genotypes (70%) exhibiting low aneuploid levels (48 to 53 chromosomes), which corresponded to near-tetraploidy to near-pentaploidy. Normal seed development requires both normal embryo and endosperm development. The latter, in turn, would depend on the balance between genetic factors provided by the female and male parent, respectively, to this tissue (Johnston *et al.*, 1980). Thus, only gametes with low aneuploid levels will provide for these features. In our work, it is feasible that eggs with  $n=14$ , 15 or 20 chromosomes from triploid *S. microdontum* were fertilized by *S. kurtzianum* male gametes with  $n=12$  chromosomes, giving origin to normal plants because both embryo and endosperm were genetically balanced. In experimental conditions, Okada and Hawkes (1978) observed that diploid *S. microdontum* x *S. kurtzianum* hybrids grew faster and more vigorously than their aneuploid siblings, and proposed that these differences could explain the absence of aneuploidy in the hybridisation area. The presence of aneuploid genotypes of *S. x rechei* in natural populations described herein leads us to formulate new hypotheses concerning the establishment and perpetuation of aneuploids in nature (see below).

In *A. thaliana*, the response to triploidy was analysed in interploidal crosses between diploid Col-0 and two tetraploids, one of them natural (Wa-1) and the other synthesised from Col-0 (Henry *et al.*, 2005). The authors demonstrated that the *A. thaliana* triploids were male and female fertile, producing a swarm of aneuploids. The subsequent sexual propagation of the progeny resulted in diploid and tetraploid cohorts. Thus, it was inferred that *A. thaliana* triploids could readily form tetraploids, functioning as bridges between euploid types. Moreover, in amphidiploid plants derived from intergeneric hybridisation between two species with identical chromosome number but different genomes, *Raphanus sativus* L. and *Brassica alboglabra* Bailey, intensive genetic changes were observed in the  $F_4$  to  $F_{10}$  generations, which led to mixopolyploid formation (Li *et al.*, 2010). The mixopolyploidy observed in the  $F_4$  to the  $F_8$  generations gradually reverted to euploidy in more advanced generations, via neo-chromosome formation or chromosome elimination. Therefore, the authors suggested that the stability of the  $F_{10}$  generation was a result of epigenetic regulation.

It is feasible that in *S. x rechei* populations -as in other natural wild potato populations with mixoploidy- the transition from aneuploidy to euploidy could be operating. We observed lower percentages of aneuploid genotypes in the natural vs. the synthetic interploid hybrids. Thus, we consider that the proposed transition is probably occurring by selective mechanisms operating either

in mitosis, during asexual reproduction, and/or in meiosis, if gametes produced by the aneuploids can provide for the required genetic balance in both embryo and endosperm, as previously discussed.

### **Synthetic vs. natural hybrids**

The observation that novel AFLP and MSAP patterns in synthetic hybrids were also present in the natural hybrid taxonomic species *S. x rechei* is an indication that there are DNA sequences in the genome of tuberous *Solanum* that are highly susceptible to restructuring in response to hybridisation. In newly synthesised *Brassica napus* allotetraploids, immediate and directed non-additive changes in gene expression were observed by Albertin *et al.* (2006), who suggested that the early steps of allopolyploidisation repatterning were controlled by non-stochastic mechanisms. Epigenetic modifications could be additional sources of inheritable variability which, in turn, would be available for selection (Rapp & Wendel, 2005). The persistence of novel epigenetic patterns in wild populations of *S. x rechei* could be explained by the two types of reproduction -asexual and sexual- available to potatoes. In the NewHybrids analysis, genotypes classified as *S. x rechei* according to morphological phenotypes were identified as F<sub>1</sub> hybrids or backcrosses to *S. kurtzianum*. Since this germplasm was collected as tubers, it can be inferred that the newly established epigenetic patterns in *S. x rechei* were maintained at least for one (or for several) generations of asexual reproduction of the F<sub>1</sub> hybrids. This scenario implies a mitotic inheritance of epigenetic patterns in potato. On the other hand, the persistence of epigenetic patterns in backcross progenies requires a transgenerational inheritance of this type of patterns established after hybridisation. The importance of such differentiation (mitotic vs. trans-generational inheritance) would be clarified by analysing the novel patterns observed in segregating generations (F<sub>2</sub> or BC<sub>1</sub>) of synthetic F<sub>1</sub> hybrids.

The variability observed in the SSR analysis could be useful for distinguishing diploids (maximum: two alleles/locus) from polysomic aneuploids (minimum: > two alleles/locus). In accordance with the mitotic chromosome number, three alleles from chromosome six were amplified in the aneuploid genotypes GV09A/1 and GV09B/3. However, three alleles were also observed in the euploid genotypes GV02/1 and GV11/1, with  $2n=2x=24$ , which could be the result of genomic restructuring. The evidences of genomic restructuring observed in the SSR analysis were consistent with the AFLP results, in which high variability for the number of novel alleles was observed in the interploidal hybrids. But not only the presence of extra chromosomes could be the cause of chromosome structural instability. As an example, in chromosome 5 of *Arabidopsis* trisomics, truncated derivatives of the triplicated chromosome were regularly observed, being the triplicated chromosome vulnerable to breakage, particularly in the vicinity of repetitive regions (Huettel *et al.*, 2008). Interooidal hybridisation in our model led to a broad range of chromosome variation, both in number and structure. These chromosomal disorders could be the principal cause of the wide epigenetic variability generated in the interploidal synthetic hybrids and established in natural *S. x rechei*.



## Implications of interploidal hybridisation and aneuploidy in potato evolution

The wide epigenetic variability induced by interploidal hybridisation described in this work is consistent with previous results revealing that a novel epigenetic pattern was rapidly established in natural *S. x rechei* regarding its parental species, *S. microdontum* and *S. kurtzianum* (Cara *et al.*, 2013). The presence of aneuploid genotypes in both the natural and the synthetic interploidal hybrids suggests that changes in chromosome number might play a key role in the generation of variability.

In plants and animals, aneuploidy has been associated with negative consequences on gametophytes (in the former), gametes (in both) and progenies, and with the initiation of pathways leading to developmental disorders and inviability of individuals (Khush, 1973; Matzke *et al.*, 2003). In *S. x rechei*, male sterility, flower abortion and floral abnormalities ranging from slight changes in corolla shape and symmetry up to severe alterations in floral cycles -such as rudimentary stamens and petals-, and homoeotic transformations have been described (Okada & Hawkes, 1978; Cara *et al.*, 2013). Almost all of the previously described morphological phenotypes were reproduced in synthetic triploid *S. microdontum* x diploid *S. kurtzianum* hybrids, with the addition of leaf abnormalities (Okada & Hawkes, 1978). A crucial aspect to understand potato diversification is to consider the consequences of the two alternative modes of reproduction -sexual by seeds and asexual by stolons and tubers- on the generation of genetic variability and fitness (Camadro *et al.*, 2012). When plants of spontaneous potato populations are unable to reproduce sexually (*i.e.*, they do not flower, or internal breeding barriers are expressed), it is highly likely that they would reproduce asexually, allowing the survival of adapted genotypes (Marfil *et al.*, 2015). In comparing individual plants of the natural hybrid species and its parents, it was observed that those of *S. x rechei* stood out for their vigour and, often, outperformed the ones of *S. kurtzianum* and *S. microdontum* in height, foliage, and tuber size and number (Okada & Hawkes, 1978; Cara *et al.*, 2013). Additionally, Cara *et al.* (2013) reported that morphological variability had a higher correlation with epigenetic variability than with genetic variability, suggesting that the former might influence the phenotypic plasticity and adaptation of the hybrid to new environments. In our study, the occurrence of aneuploidy -as a product of interploidal crosses- would induce a genomic shock that generates wide epigenetic variability leading to phenotypic changes. Although initially completely or partially sterile -depending on the polysomic chromosome(s) or region(s)- aneuploids would be able to persist in nature by asexual reproduction, allowing phenotypic and chromosomal stabilisation and, even, the eventual transition to euploidy.

Our results reveal that natural hybrids include mostly F<sub>1</sub>s but also some inferred backcross individuals, indicating that hybridisation and possibly longer-term introgression is occurring in regions of sympatry. The empiric data generated in our work could be representative of an ongoing phenomenon in other wild potatoes as well, and could account for the observed natural variability. We consider that this introgressive hybridisation hypothesis deserves to be addressed in order to resolve historical taxonomic conflicts in this plant group (see Camadro, 2012; Camadro *et al.*, 2012).



## Acknowledgments

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica and Universidad Nacional de Cuyo, Argentina. We are grateful to Juan Urdampilleta (Instituto Multidisciplinario de Biología Vegetal, CONICET–UNCórdoba) and Carolina Kozub (IBAM–CONICET–UNCuyo) for valuable technical assistance with chromosome preparations.

## Author contribution

R.W.M. and C.F.M. planned and design the research. N.C. performed the molecular and data analyses. M.S.F. obtained the artificial hybrids and performed the cytological experiments. E.L.C. contributed to the cytological analysis. C.F.M. drafted the manuscript. N.C., R.W.M. and E.L.C. contributed in writing the final manuscript.

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## Tables

**Table 1.** Plant material and performed analyses.

Species	Population Code	Type of analysis			
		Molecular AFLP/MSAP <sup>b</sup>	Chromosome numbers	NewHybrids <sup>c</sup>	
<i>Solanum kurtzianum</i>	LR1	-	+ (1)	+ (1)	
	LR2	-	+ (1)	+ (3)	
	LR3	+ (1)	+ (1)	+ (3)	
	CG	-	-	+ (3)	
	CMK	-	-	+ (6)	
	4544	-	-	+ (2)	
	4548	+ (1)	-	+ (3)	
	4505	-	-	+ (3)	
	4549	-	-	+ (3)	
	4552	-	-	+ (3)	
	<i>Solanum microdontum</i>	GV07	-	+ (1)	+ (6)
		CMM	+ (1)	+ (3)	+ (4)
4631		+ (2)	-	+ (5)	
5902		-	-	+ (3)	
<i>Solanum x rechei</i>	GV02	+ (1)	+ (1)	+ (3)	
	GV04	+ (1)	+ (1)	+ (3)	
	GV05	-	-	+ (2)	
	GV08	+ (1)	+ (2)	+ (5)	
	GV09	+ (2)	+ (2)	+ (6)	
	GV11	+ (1)	+ (1)	+ (1)	
	GV12	+ (1)	+ (1)	+ (3)	
	NN	+ (1)	-	+ (4)	
	CMR	-	+ (1)	+ (3)	
	4574	-	-	+ (2)	
	4583	-	-	+ (1)	
<sup>a</sup> DxD hybrids	kxm	+ (2)	+ (2)	-	
	mxk	+ (4)	+ (2)	-	
<sup>a</sup> TxD hybrids	8	+ (5)	+ (5)	-	
Control cross	mxm	+ (3)	-	-	

<sup>a</sup> Synthetic intraploidal (DxD) and interploidal (TxD) *Solanum microdontum* x *S. kurtzianum* hybrids.

<sup>b</sup> +, Included in the analysis; -, not included. In parenthesis, number of analysed genotypes. <sup>c</sup> Carried out with amplified fragment length polymorphism (AFLP) data obtained by Cara *et al.* (2013). MSAP, methylation-sensitive amplified polymorphism.

**Table 2.** Somatic chromosome number of individual plants from natural populations of *Solanum kurtzianum*, *S. microdontum* and *S. x rechei* and in synthetic hybrids obtained from controlled *S. microdontum* x *S. kurtzianum* crosses.

Plant material	Population/Genotype	Somatic chromosome number
<i>Solanum kurtzianum</i>	LR2/3	$2n = 2x = 24$
	LR3/4	$2n = 2x = 24$
	CG/1	$2n = 2x = 24$
<i>Solanum microdontum</i>	CMM/2	$2n = 3x = 36$
	CMM/5	$2n = 3x = 36$
	CMM/6	$2n = 3x = 36$
	GV07/4	$2n = 3x = 36$
<i>Solanum x rechei</i>	CMR/2	$2n = 2x = 24$
	GV02/1	$2n = 2x = 24$
	GV04/3	$2n = 2x = 24$
	GV08/4	$2n = 2x = 24$
	GV09A/1	$2n = 2x+1 = 25$
	GV09B/3	$2n = 2x+1 = 25$
	GV11/1	$2n = 2x = 24$
	GV12/2	$2n = 2x = 24$
<i>S. microdontum</i> (2n=3x=36) x <i>S. kurtzianum</i> (2n=2x=24) TxD hybrids	8.1	$2n = 2x+3 = 27$
	8.2	$2n = 2x+3 = 27$
	8.4	$2n = 2x+2 = 26$
	8.5	$2n = 2x +3= 27$
	8.8	$2n = 3x-4 = 32$
<i>S. microdontum</i> (2n=2x=24) x <i>S. kurtzianum</i> (2n=2x=24) DxD hybrids	kxm 1	$2n = 2x = 24$
	kxm 3	$2n = 2x = 24$
	mxk 1	$2n = 2x = 24$
	mxk 7	$2n = 2x = 24$



## Figure legends

**Figure 1. Experimental model for studying dynamic genomic responses to hybridisation.** Natural populations of wild potatoes classified as *Solanum kurtzianum*, *S. microdontum* and *S. x rechei* according to morphological phenotypes were collected in areas of sympatry. Synthetic populations were generated through controlled sexual crosses between the parental species *S. kurtzianum* and *S. microdontum*.

**Figure 2. Amplified fragment length polymorphism (AFLP) analysis of *Solanum kurtzianum*, *S. microdontum*, synthetic interspecific hybrids and intraspecific control cross.** Venn diagrams showing number and distribution of AFLP fragments in interploidal (TxD, a) and intraploidal (DxD, b) *S. microdontum* x *S. kurtzianum* synthetic hybrids, and in *S. microdontum* x *S. microdontum* control cross (c). Total number of amplified fragments (loci) in parental genotypes and hybrids are indicated in parenthesis.

**Figure 3. Methylation-sensitive amplified polymorphism (MSAP) analysis of *Solanum kurtzianum*, *S. microdontum*, synthetic interspecific hybrids and intraspecific control cross.** Venn diagrams showing number and distribution of methylation patterns (epialleles) inferred from MSAP fragments in (a) interploidal (TxD) and (b) intraploidal (DxD) *S. microdontum* x *S. kurtzianum* synthetic hybrids, and in the *S. microdontum* x *S. microdontum* control cross (c). Total number of epialleles detected in parental species and hybrids are indicated in parenthesis.

**Figure 4. Genetic and epigenetic variability originated in interploidal and intraploidal *Solanum microdontum* x *S. kurtzianum* hybrids and in the *S. microdontum* x *S. microdontum* control cross.** Within-group genetic and epigenetic distances, based on amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) molecular markers, respectively. Values are means  $\pm$ SE. Different letters indicate significant differences among the six bars ( $P < 0.01$ ).

**Figure 5. DNA methylation changes in the progeny of interploidal (TxD) and intraploidal (DxD) *Solanum microdontum* x *S. kurtzianum* hybrids and in the *S. microdontum* x *S. microdontum* control cross respect to the parental genotypes.** Two types of methylation changes are distinguished: hypomethylation and hypermethylation. Values are means  $\pm$ SE. Different letters indicate significant differences between bars within a type of methylation change ( $P < 0.01$ ).

**Figure 6. Genomic restructuring observed in synthetic interspecific hybrids, also present in natural populations of the hybrid taxonomic species *Solanum x rechei*.** Proportion of novel fragments and species-specific fragments of *S. kurtzianum*, *S. microdontum* and *S. x rechei* in interploidal (TxD) and intraploidal (DxD) synthetic hybrids for amplified fragment length polymorphism (AFLP) (a) and methylation-sensitive amplified polymorphism (MSAP) (b).

\*Aneuploid genotypes determined by chromosome counting.

**Figure 7. Mitotic metaphases in root-tip cells of natural and synthetic genotypes.** Parental species: (a) *Solanum microdontum* ( $2n=3x=36$ ) and (b) *S. kurtzianum* ( $2n=2x=24$ ). Natural and synthetic interploidal hybrids: *S. microdontum* ( $2n=3x=36$ ) x *S. kurtzianum* ( $2n=2x=24$ ): (c) aneuploid genotype (GV09A/1) of the natural hybrid species *Solanum x rechei* ( $2n=2x+1=25$ ), (d) aneuploid interploidal synthetic hybrid (8.4) ( $2n=2x+2=26$ ) (d). Bars, 5  $\mu\text{m}$ .

**Figure 8. Hybrid assignment performed with NewHybrids using AFLP data.** Each vertical bar represents an individual. Colours indicate the probability of belonging to either *Solanum kurtzianum* (ktz), *S. microdontum* (mcd) or a hybrid class ( $F_1$ ,  $F_2$ , BC to ktz or BC to mcd). At the bottom, species classification based on morphological and molecular data (Cara *et al.* 2013). \*, Aneuploid genotypes determined by chromosome counting;  $\Delta$ , chromosome number not evaluated.

### Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1.** Analysis of the epigenetic variability generated in the offspring of controlled crosses using methylation-sensitive amplified polymorphism (MSAP) markers.

**Fig. S2.** Inheritance of methylation patterns in synthetic hybrids.

**Fig. S3.** Genetic and epigenetic comparisons among synthetic and natural interspecific hybrids.

**Fig. S4.** Number of alleles detected for five SSR loci in plants from natural populations of *Solanum microdontum*, *Solanum kurtzianum*, the hybrid species *Solanum x rechei* and in synthetic hybrids obtained from *S. microdontum* x *S. kurtzianum* crosses.

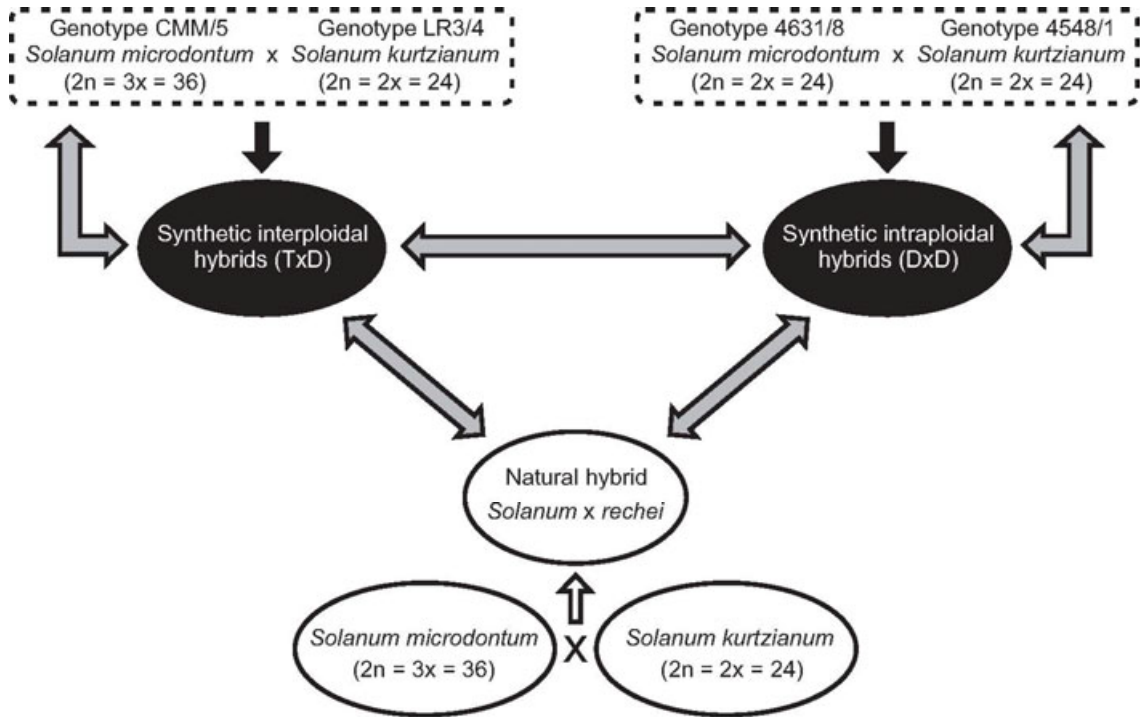
**Table S1.** Number of pollinated flowers, fruits and viable seeds per fruit in interspecific and control crosses.

**Table S2.** Mean distances based on Dice coefficient for resampled groups to determine unequal sample bias.

**Table S3.** Results of 15 simple sequence repeat (SSR) primers.

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**References**

- ↓
Controlled sexual crosses.
- ⇓
Cross that originated *Solanum x rechei* in nature.
- ↔
Performed comparisons: amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), methylation sensitive amplified polymorphism (MSAP) patterns and chromosome number.
- Synthetic populations.
- Natural populations.
- ⋯⋯
Genotypes used for controlled crosses.

