

# Outcrossing potential between 11 important genetically modified crops and the Chilean vascular flora

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## Summary

The potential impact of genetically modified (GM) crops on biodiversity is one of the main concerns in an environmental risk assessment (ERA). The likelihood of outcrossing and pollen-mediated gene flow from GM crops and non-GM crops are explained by the same principles and depend primarily on the biology of the species. We conducted a national-scale study of the likelihood of outcrossing between 11 GM crops and vascular plants in Chile by use of a systematized database that included cultivated, introduced and native plant species in Chile. The database included geographical distributions and key biological and agronomical characteristics for 3505 introduced, 4993 native and 257 cultivated (of which 11 were native and 246 were introduced) plant species. Out of the considered GM crops (cotton, soya bean, maize, grape, wheat, rice, sugar beet, alfalfa, canola, tomato and potato), only potato and tomato presented native relatives (66 species total). Introduced relative species showed that three GM groups were formed having: a) up to one introduced relative (cotton and soya bean), b) up to two (rice, grape, maize and wheat) and c) from two to seven (sugar beet, alfalfa, canola, tomato and potato). In particular, GM crops presenting introduced noncultivated relative species were canola (1 relative species), alfalfa (up to 4), rice (1), tomato (up to 2) and potato (up to 2). The outcrossing potential between species [OP; scaled from 'very low' (1) to 'very high' (5)] was developed, showing medium OPs (3) for GM–native relative interactions when they occurred, low (2) for GMs and introduced noncultivated and high (4) for the grape-*Vitis vinifera* GM–introduced cultivated interaction. This analytical tool might be useful for future ERA for unconfined GM crop release in Chile.

**Keywords:** vascular flora, genetically modified crops, outcrossing potential, native relatives, introduced relatives, Chile.

## Introduction

Genetically modified (GM) crops have been widely adopted, with approximately 181.5 million ha cultivated in 28 countries in 2014 (James, 2014). The possible impact of GM crops on landscapes where they are introduced has led to evaluation, measurement and modelling of pollen-mediated gene flow from GM crops to pollen recipients present among wild relatives and/or conventional agriculture (Wolt *et al.*, 2010). The movement of genetic material through pollen and seed arise from the same biological principles for GM and non-GM crops. Although specific genetic modifications might alter gene flow, the likelihood of pollen-mediated gene flow in a specific region or environment relies on the biology of a crop and the presence or absence of relative species. Once received, introgression of novel genes by recipients may lead to diverse effects, such as adventitious presence of transgenes, changes in competitiveness/fitness, increased or decreased invasiveness, and even population changes within the agricultural, feral or natural areas where GM crops are released (Andow and Zwahlen, 2006; Haygood *et al.*, 2003; Mallory-Smith and Zapiola, 2008; Warwick *et al.*, 2009). Additionally, important economic consequences in the marketplace, due to some biotech and nonbiotech traits introgressed into weedy species (Gressel, 2014) or non-GM cultivars (Redick,

2012), may result in adventitious presence of seed lots affecting the purity standards required.

Several factors affect hybridization frequency between GM crops (donors) and wild, weedy, and cultivated plant relatives (recipients). Among the most important are the extent of sexual compatibility, spatial isolation, relative density, synchrony of flowering, direction of the cross and the presence of pollen vectors. In this context, different systems were built, establishing gene flow indexes (GFI) (Flannery *et al.*, 2005) for species that establish numerical values according to their characteristics; for instance, the three principal crops in Ireland, *Lolium perenne*, *Beta vulgaris* spp. *Vulgaris* and *Brassica napus*, are the crop species that could require highlighted appropriate reproductive isolation measures to prevent pollen-mediated gene flow in that country. Additionally, botanical files (BFs) were made for 42 cultivated species in the Netherlands (de Vries *et al.*, 1992), supplying technical information about their use, origin, actual hybridization and/or crossing, and escapes from the field to nature of each of the considered species. These authors incorporated new codes for the elements dispersal of pollen (Dp), dispersal of reproductive plant parts (i.e. diaspores, Dd) and distribution frequency of wild relatives (Df). The numerical codes generated, and conclusions derived from them, indicated their potential ecological effects on the wild flora of the country,

establishing that about 25% of these 42 considered species could hybridize with wild relatives. Enhanced BFs were built later, using an additional factor, Dg, which refers to putative risks caused by the transgene in the hybrids generated after crossing (Jacot and Ammann, 1999), such as persistence of the hybrid because of alterations in seed dormancy or germination, competitiveness of vegetative parts and tolerance to biotic stresses, for the 30 most important crops in Switzerland. Results showed that some species, such as *Solanum lycopersicum*, *Solanum tuberosum* and *Zea mays*, do not appear to interbreed with wild species in the country, whereas others such as *Medicago sativa* and cereal crops (i.e. *Secale cereale*, *L. perenne*, *Triticum aestivum* and *Trifolium* spp.) might have substantial and wide-spread effects (as a result of interbreeding) on native Swiss flora.

Introgression of genes into populations of wild, weedy and cultivated species will occur only if the barriers of incompatibility, genetic instability and low hybrid pollen fertility are overcome (Warwick et al., 2009) and if the genes that are transferred do not decrease the fitness or competitiveness of the recipient population. Consequently, pollen-mediated gene flow from several crops poses some scientific considerations that are properly addressed in the problem formulation as conducted by the pipeline of an environmental risk assessment (ERA; Kuiper et al., 2001); for instance, wild relatives occurring in a region or country may be sexually compatible with introduced GM or non-GM crops. Knowing the spatial distribution of wild, weedy and cultivated relatives (presence/absence) and quantifying the potential for outcrossing before commercialization of GM crops allow regulatory agencies to assess the real risks of releasing a GM crop at confined or unconfined scales. This assessment can provide relevant information for the gene flow portion of a risk assessment for the release of a GM crop and if there is a likely harm arising from such gene flow, indicate whether mitigation measures are warranted.

An additional, often nonconsidered component of risk analysis involving pollen flow from GM crops to sexually compatible species is that even if cross-pollination between a GM crop and a native, sexually compatible species might occur, such hybrid would carry an homozygous allelic configuration which in most cases may not become expressed at the phenotypic level until an additional self or open pollination event occur leading to an homozygous allelic configuration.

Evaluation, measurement and modelling of the probability of outcrossing and its influence over wild, weedy and cultivated relatives should consider the particular conditions and aspects of every specific region. In the case of Chile, those aspects are referred to a geographically insulated region with wide climate heterogeneity and thus heterogeneity of plant biodiversity. There are almost 5100 species in Chile, out of which 51.5% are considered endemic (Marticorena, 1990). The main zone of endemism, recognized as a centre for diversification and speciation, is located between parallels 25° to 40° and the coastal string up to 19.5°. Different regions in Chile are the centre of origin for important cultivated species such as *S. tuberosum* sp. *Tuberosum* and *Phaseolus vulgaris*, and some wild relatives, such as *Fragaria chiloensis*, *Alstroemeria* spp. and *Bromus* spp., which can be found as crops in the same areas. Furthermore, few areas in some regions of Chile are considered a centre of diversity for cultivated species such as *Z. mays* spp. Additionally, about 2500 species have been classified as introduced, some of which became naturalized and, in some cases, weedy species (Espinoza, 1996; Matthei, 1995).

Although Chile currently lacks a regulatory framework enabling unconfined GM crops cultivation, it is a counter season country where confined activities such as seed multiplication and research and development (R&D) for GM plants are carried out at large scale by many private companies and academic institutions, leading to Chile's inclusion by the International Service for the Acquisition of Agri-biotech Applications (ISAAA) in the list of countries that have officially adopted GM crops (James, 2014). The main GM seeds produced in Chile are maize, soya beans and canola (James, 2014).

At a worldwide level, the main GM crops commercially available are maize, soya beans, canola and cotton; sugar beets and alfalfa are commercialized but at a much lower level based on total area planted. Genetically modified species, including potato, rice, tomato, grapes and wheat, are crops considered under the R&D stage. All of these crops would be of interest to Chilean farmers if they would bear biotech traits addressing the crop protection and/or environment issues of Chile's agriculture.

To gain insight from an ERA standpoint, we conducted a national-scale study of relationships and potential for outcrossing between 11 GM crops and the vascular Chilean flora. To achieve this, the occurrence, geographical distribution and some of the most important biological/agronomical characteristics of the wild, weedy and cultivated species occurring in the country were surveyed. Systematic botanical and agronomical information about Chilean vascular flora was organized and clustered into groups, leading to a basic comparative analysis that allowed the establishment of theoretical relationships among inter- and intraspecies groups. Thus, the likelihood of outcrossing between some key crops (GM or non-GM) and vascular Chilean flora, disregarding biosafety measures, was estimated. This effort contributes to the problem formulation as a first step in eventual ERAs for unconfined GM crop release in the country. The potential for pollen-mediated outcrossing following cultivation of the GM crop was evaluated, and informatics tools to assess that likelihood were developed. This is a screening approach to problem formulation that focuses on the likelihood for outcrossing to sexually compatible relatives of potential concern. This study does not deal with the potential risks of each specific biotech traits being introduced to crops.

## Results

### The local vascular flora composition and cultivated crops

A grand total of 8490 vascular species were computed, documented and incorporated as data sets for cultivated, native and introduced species, to be assessed for the likelihood of outcrossing with some specific crops (GM and non-GM). For cultivated species, that is those whose evolutionary process was influenced by humans to meet their needs, there were 257 species. The group of introduced species included 3505 species; these species are considered naturalized or exotic when they are not native to a given place or area and instead were accidentally or deliberately transported to this new location by human activity. Finally, the group of native species, those that occurred naturally in a given area, included 4993 species.

Based on genus and family relationships, of 257 cultivated species, just 11 (4.3%) were native, whereas the remaining 246 (95.7%) were introduced (Figure 1). In addition, the cultivated native group showed the 11 species with relationships at the genus level with 241 other native species (not shown). Two situations were observed in the cultivated introduced group: (i) a

group in which the species had no relatives (reaching up to 52 species, 21.2% of the total) and (ii) the remaining 194 cultivated introduced species (78.7% of the total) that had more than one relative (wild or cultivated), reaching up to more than 758 relatives in total.

### Genetically modified crops and the local vascular flora

The administrative division of Chile into 15 regions and their corresponding climate layers are depicted in Figure 2. Desert and steppe zones are located between north-top and north-central areas of the country (Arica-Parinacota to Coquimbo regions; north in Figure 2). Mediterranean-dry weather can be found in the area between Valparaíso and Bío-Bío (central in Figure 2), housing several highly productive transversal valleys. Mediterranean-rainy and cold-steppe conditions are found over the southern region (Araucanía to Magallanes Regions; south in Figure 2). GM seed production and R&D activities are located in the valleys between the Coquimbo and Araucanía regions and in addition in the northern top of the country (Arica-Parinacota; Arica city). Eleven GM crops considered relevant for Chilean agriculture were included in the present study, considering their availability in the market or due to their advanced stages in the precommercialization pipeline. The eventual interaction between these crops and the Chilean vascular flora, comprised of native and introduced species in the indicated areas, are summarized in Figure 2. Thus, nine of 11 GM crops analysed showed no native relatives in the country. Native relatives for these GM crops were found only for *S. lycopersicum* and *S. tuberosum*; these species were found in all the regions and had higher frequency of relative species (up to 12; 'N' in *x-axis* of frequency plots in Figure 2) along the steppe and Mediterranean-dry areas.

Introduced relative species showed that three GM groups were formed having: (i) up to one (cotton and soya bean), (ii) up to two (rice, grape, maize and wheat) and (iii) from two to seven (sugar beet, alfalfa, canola, tomato and potato) introduced relatives (Figure 2). In the case of introduced cultivated species (Figure 2, dark grey bars labelled 'I' (*x-axis*) in the frequency plots), the occurrence of relatives showed two groups of GM crops with: (i) up to two (sugar beet, cotton, rice, tomato, wheat, grape and maize) and (ii) up to five (canola and potato) relative species. The same analysis on introduced noncultivated species (light grey bars in frequency plots Figure 2) showed that, while six of 11 GM crops (sugar beet, cotton, soya bean, wheat, grape and maize) showed no presence of relatives, five GM species had relatives

from this group: canola (1 relative species), alfalfa (up to 4), rice (1), tomato (up to 2) and potato (up to 2).

To determine whether selected GM crops may interact by pollen-mediated gene flow with native and introduced relatives in Chile, the likelihood of outcrossing for these selected species on their relatives were translated into estimated values that summarized all of the agronomical, biological and botanical characteristics of the involved species. Outcrossing potential (OP) concepts/values in these relationships were ranked as 'very low' (OP = 1), 'low' (2), 'medium' (3), 'high' (4) or 'very high' (5) and were represented by geographical zones in the country (Figures 3–5). Analyses on the native species showed that all of the relatives of GM tomato and potato, either in the north (Figure 3), central (Figure 4) or south (Figure 5) areas of the country, reached an OP = 3. When GM crops under analysis involved relationships with introduced noncultivated species, the north area presented a maximum OP = 2 that corresponded to alfalfa and canola with their relatives (Figure 3); in the centre, the maximum OP = 3 was obtained for rice (Figure 4), and in the south region, tomato and its relatives showed OP = 3 (Figure 4).

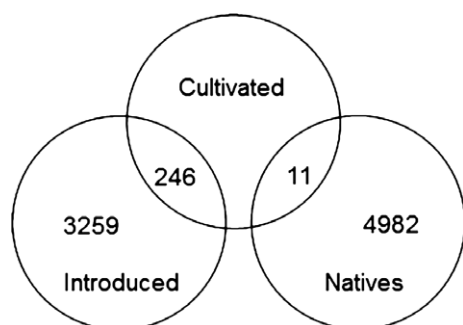
Higher OP values were deduced from GM crops and introduced cultivated relative species, which reached OP = 4 for grapes in the north (Figure 3), while in central Chile, OP reached up to 4 for sugar beet and grape (Figure 4); the same values were found for both species in some regions in the south (Figure 5).

None of the selected GM crops analysed in this study showed OP = 5 (very high) with Chilean vascular flora.

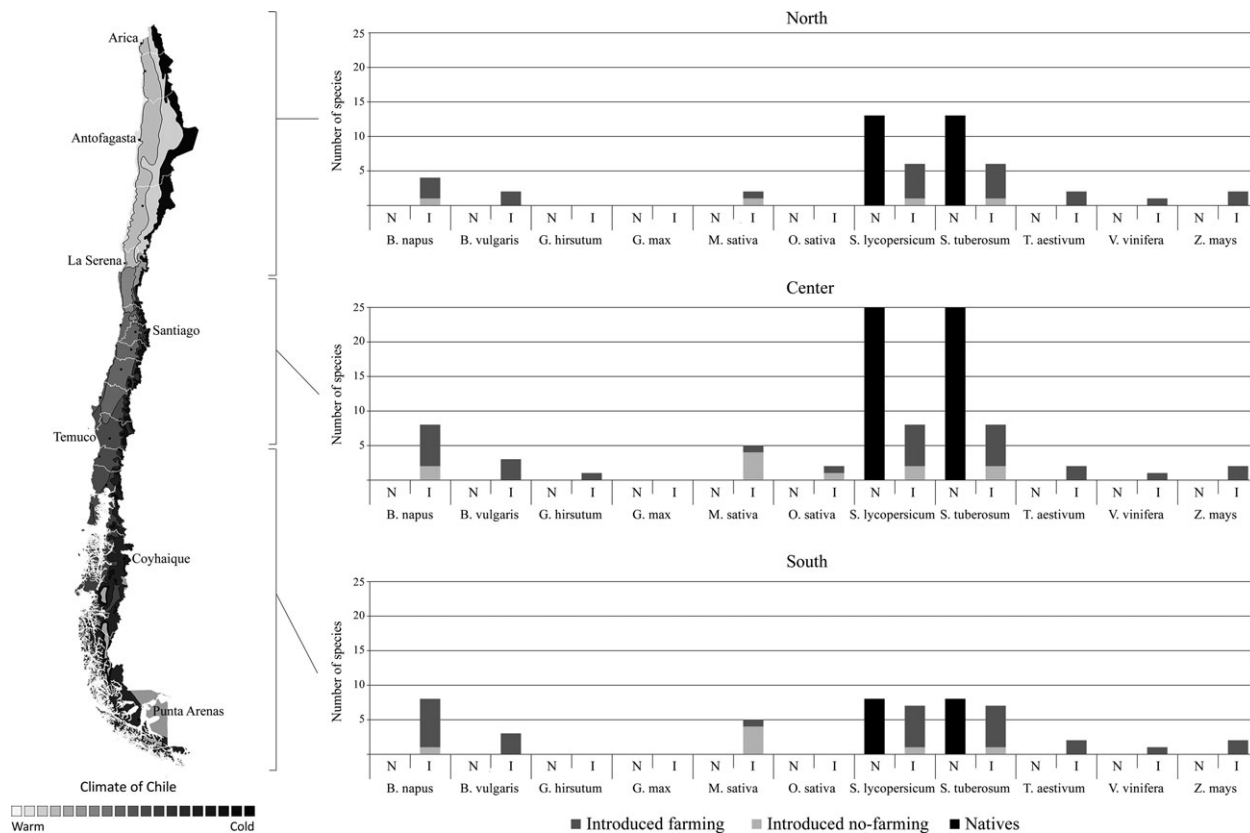
### Discussion

Building databases for the vascular plants allowed for the development of informatics tools enabling the organization and query of information about species occurrence, characteristics and dynamics. Several national or regional databases for vascular plants have been already built [Database of Vascular Plants of Canada (ABRS, 2009; Bilz *et al.*, 2011; Brouillet *et al.*, 2010)]; recent updates in the taxonomic knowledge in the South Cone species were noticeably improved by reviewed versions from this geographical area of South America (Zuloaga *et al.*, 2008), including Chile (Moreira-Muñoz, 2011). The present work used a novel database for Chilean vascular plants (publicly available at [www.flujogenico.cl](http://www.flujogenico.cl)) to infer botanical relationships between their species and 11 relevant GM crops. As primary results, composition and general relationships among groups of listed species were obtained. Our results also indicated that 20% of the native and introduced species have relationships (i.e. they are the same species or have genus correspondence) to cultivated species (824 of 4993 natives and 810 of 3505 introduced); these relationships were reduced into 2.6% of the native (132 of 4993) and 1.7% of the introduced (61 of 3505) species when these 11 GM crops were modelled.

Several approaches have been developed to model the potential impact of cultivation of certain GM species in a particular region (Arpaia *et al.*, 2014; Birch *et al.*, 2004; Charleston and Dicke, 2008; Conner *et al.*, 2003; Flannery *et al.*, 2005; Jacot and Ammann, 1999; Jesse and Obrycki, 2003; Knispel and McLachlan, 2010). As described, gene flow codes and BFs (Jacot and Ammann, 1999; de Vries *et al.*, 1992) are supported by Dp, Dd and Df indexes that consider geographically limited data (Conner *et al.*, 2003; Jacot and Ammann, 1999), and the addition of Dg (Jacot and Ammann, 1999) incorporates other characteristics, such as fitness of potential hybrids. Overall, these components



**Figure 1** General composition of vascular plants in Chile. The general composition of the vascular plants is depicted, divided into cultivated, introduced and native species, including the number of introduced and native species with agricultural use (shared numbers).



**Figure 2** Native and introduced relative species present in Chile for each of the 11 genetically modified (GM) crops under study. Species relationships, deduced from the database for each one of the GM crops, are shown, depending on the geographical (brackets) and political (white borders inside the map) regions of the country. Associated climate of each region is represented graphically for illustrative purposes. Bars labelled 'N' are natives and 'I' are introduced species.

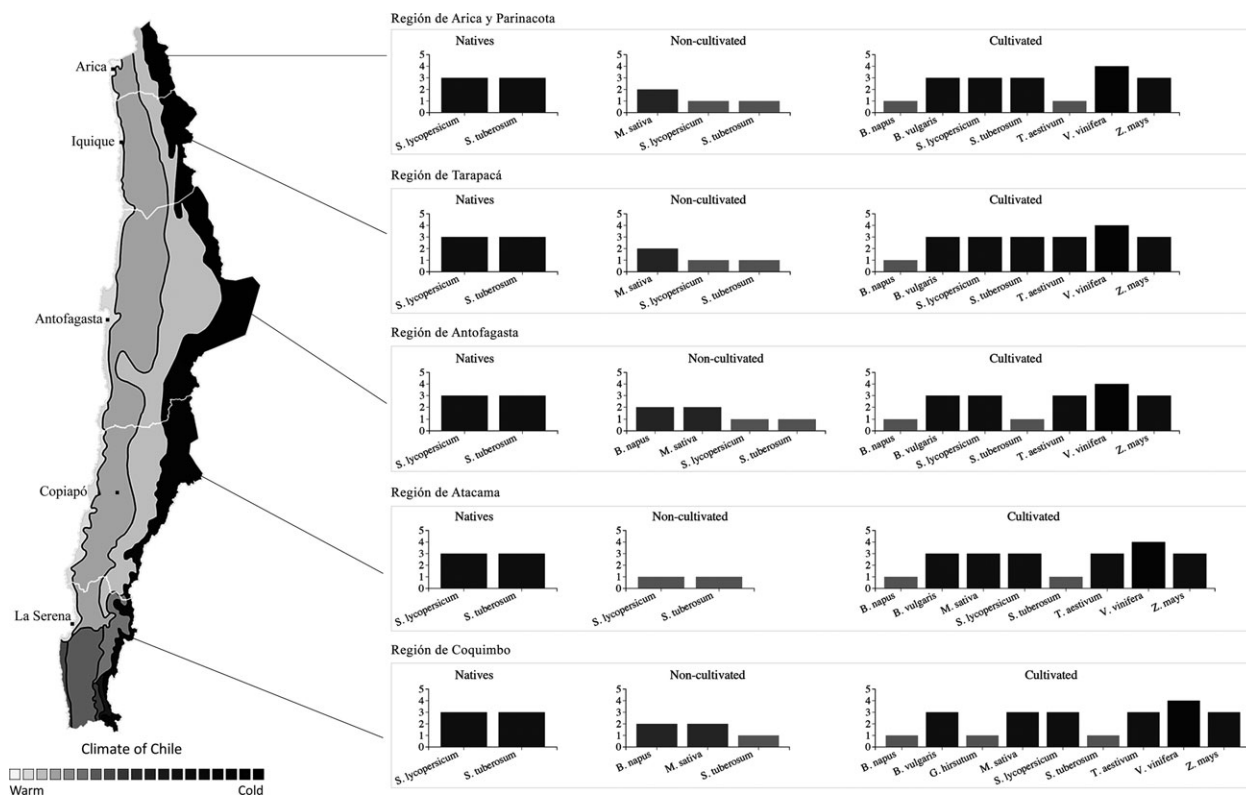
lead to build a more confident impact assessment (Arpaia *et al.*, 2014; EFSA 2010). Gene flow index values (Flannery *et al.*, 2005) translate, into numerical values, some group of characteristics (gene flow either due to pollen or to seeds from crops), including additional requirements, such as the susceptibility of pollen flow towards receptor plants or the ability of seeds from derived crops to form new individuals as volunteer or feral populations.

In the present work, we built an OP conceptual scale that summarizes and hierarchically orders most of those characteristics and translates them into a concept indicating the extent of possible coincidences (biological/geographical factors) for the interaction between two species. This effort could facilitate an *ex-ante* analysis in the case of potential unconfined release of GM crops in Chile, to figure out and develop problem formulations as performed in later ERA.

The tool reported here would contribute significantly to determine whether the environmental introduction of a biotech-derived plant, without requirements for reproductive (or physical) isolation or postharvest land use restrictions or monitoring for nearby related sexually compatible plant species, is possible or not. It is worth noting that, in the case of GM events for seed propagation and R&D initiatives, which are confined activities where the introduction into the environment of biotech-derived plants is stringently controlled through biosafety measures, the tool here reported could help to a lower extension because the likelihood of exposure to a hazard approaches zero.

This approach does not take into account other functional factors involved in species compatibility, such as ploidy levels (Birchler, 2014; Soltis *et al.*, 2004; Spooner *et al.*, 2010), self-incompatibility systems (Takayama and Isogai, 2005) and endosperm balance numbers (Carputo *et al.*, 1999). In our case, these factors were not included as information in the iterating Eqn 1 merely because of lack of knowledge in the art for several of the species listed. Due to this, several factors that influence gene flow (pollen-mediated), such as mating system, type of pollination, mode of seed dispersal and the particular characteristics of the habitat where the crops grow, are not homogeneously evaluated for all species and consequently their quantification is not accurate at this moment. As argued for Dp, Dd and Df, additional data referring to the competitive fitness of hybrids, chromosome reorganizations or possible gene rearrangements in the hybrids were not considered in our approach and represent key information which should be taken into account once they become available for all the plant species.

Whereas nine GM crops did not show native relatives within the country, native relatives were found in the cases of potato (*S. tuberosum*) and tomato (*S. lycopersicum*). Both GM potato and tomato crops and their relatives reached an OP 'medium' value, describing the possibility of a pollen-mediated interspecific cross due to species occurrence. Factors affecting cross-pollination in these species involve pollen characteristics such as stickiness and short-distance travel ability; additionally, flower architecture shows these species with petals opening flat out,



**Figure 3** Outcrossing potential (OP) for native and introduced relative species present in the North region of the country for each of the 11 genetically modified (GM) crops. Maximum OPs for relationships between GM crops (x-axis in the plots) and their corresponding related native, introduced cultivated, or introduced noncultivated species are shown, depending on political regions (names are indicated on the top of each plot). Scale for OP (y-axis) was 'very low' = 1, 'low' = 2, 'medium' = 3, 'high' = 4 and 'very high' = 5. Associated climate of each region is represented graphically for illustrative purposes. Absent species did not present relatives.

enabling nectar collection without stigma or anther contact by insects (Acquaah, 2007). Whereas in most plant species the pollen is exposed by anthers that open longitudinally, in tomato and potato, anthers are hollow tubes that open by small apical pores, making natural interspecific crosses highly difficult (Acquaah, 2007).

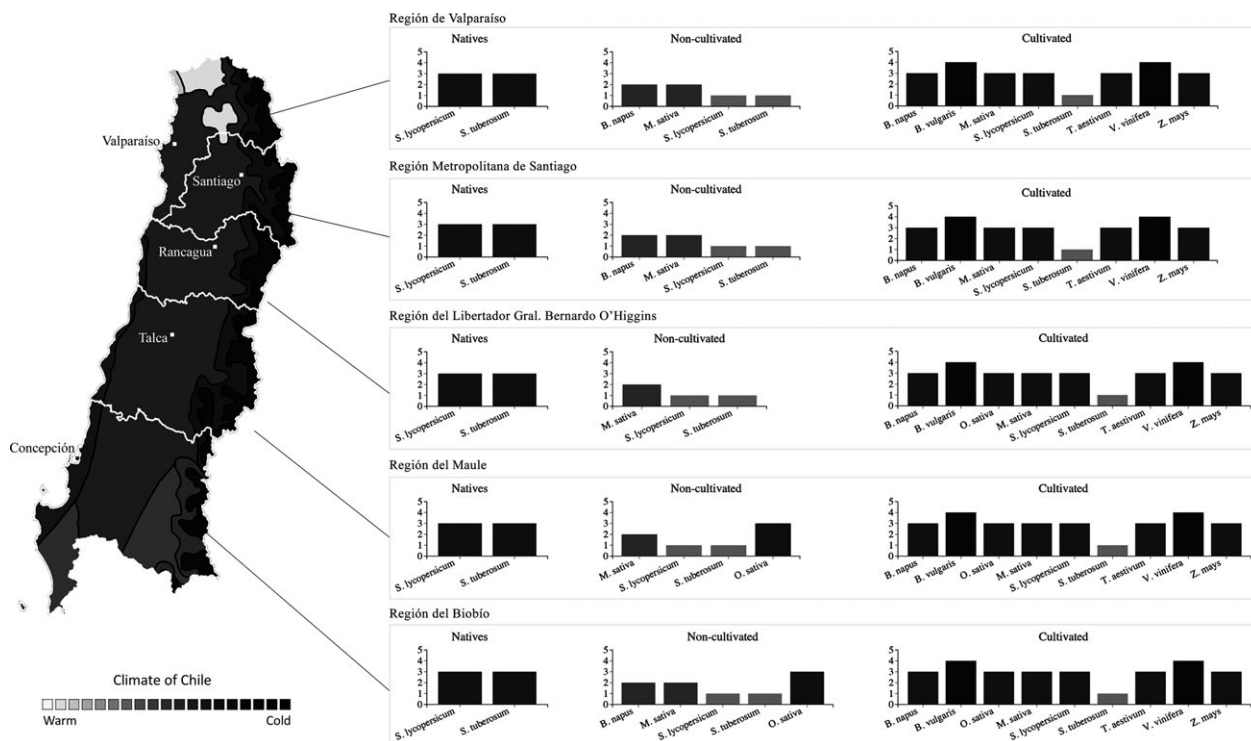
For potato, agricultural practices indicate that it is vegetatively propagated (using the 'eyes' of the tuber); thus, pollination is not a concern. In accordance with this, Ghislain *et al.* (2015) concluded, for potatoes in Peru, that the unintentional introduction of a transgene not under farmers' selection from a widely grown transgenic variety over a long period of time is unlikely to be detected. In the case of tomato, although wind and cultured insects provide sufficient pollen movement to produce commercially viable crops, domesticated cultivars were selected to maximize self-fertilization. Experimental studies with tomatoes have shown that pollinators act as 'buzz' agents that disrupt the anthers and force pollen out of the tubular anthers (Acquaah, 2007). These characteristics also apply to the unique, introduced noncultivated relative of *S. lycopersicum* occurring in the south region (Los Lagos Region, Figure 4) of the country.

Our results also indicated that the evaluated GM crops showing crossable relatives in the introduced noncultivated group, namely canola and alfalfa, had 'very low' or 'low' OP values. These species occur both as diploids and tetraploids, although tetraploid cultivars are more common (Brummer *et al.*, 1991), and the natural occurrence of gene transfer between these forms has been shown and named interploidy hybridization (Birchler, 2014;

Small and Jomphe, 1989). In addition, this process is equally efficient to that of the same ploidy level hybridization (Bingham and Saunders, 1974), leading to viable and competitive hybrids in alfalfa. These facts indicate that members of *M. sativa* complex (i.e. *M. sativa*, *Medicago falcata* and *Medicago varia*) can occur sympatrically in a landscape and, for this reason, they can naturally hybridize with each at both ploidy levels. In the present work, two species from the *M. sativa* complex member were found: *M. falcata* in the introduced noncultivated group (in addition to *Medicago turbinata*, *Medicago scutellata*, *Medicago polymorpha*, *Medicago orbicularis*, *Medicago minima*, *Medicago lupulina*, *Medicago hispida*, *Medicago echinus*, *Medicago denticulata*, *Medicago arborea* and *Medicago arabica*) and *M. sativa* L. in the cultivated introduced species. The interaction for these species was assigned as OP 'low' for the noncultivated and 'medium' in the case of cultivated lists, revealing possible implications for the confinement of GM events when these populations occur.

Feral alfalfa populations can persist in roadside habitats. However, timely mowing or regular targeted herbicide application will be effective in managing those and limit feral-population-mediated gene flow in alfalfa (Bagavathiannan *et al.*, 2012).

Canola is a self-fertile species whose pollen is dispersed by both wind and insects (Chifflet *et al.*, 2011; Liu *et al.*, 2013). Pollen-mediated gene flow in canola is affected by biological factors such as flowering time, genotype, insect type and movement, opening and position of flowers on plants, plant aggregation,



**Figure 4** Outcrossing potential (OP) for native and introduced relative species present in the Central region of the country for each of the 11 genetically modified (GM) crops. Maximum OPs for relationships between GM crops (x-axis in the plots) and their corresponding related native, introduced cultivated or introduced noncultivated species are shown, depending on the political regions (names are indicated on the top of each plot). Scale for OP (y-axis) was 'very low' = 1, 'low' = 2, 'medium' = 3, 'high' = 4 and 'very high' = 5. Associated climate of each region is represented graphically for illustrative purposes. Absent species did not present relatives.

interpatch distances, size of the source population and pollen contributions of all surrounding plants (Liu *et al.*, 2013). Although half of the pollen produced by an individual fell within 3 m of the plant (Lavigne *et al.*, 1998), effective fertilization of canola flowers ranges from 12% to 47% when plants are in close proximity in the field (Gulden *et al.*, 2008), decreasing up to about 1% at distances farther than 30 m (Hüsken and Dietz-Pfeilstetter, 2007).

In the Chilean case, interspecific crosses could be realized for both the not cultivated (*Brassica nigra*, *Brassica napobrassica*, *Brassica juncea* (L.) Coss., *Brassica alba*, *Brassica ruvo* and *Brassica rapa* L.) and the introduced cultivated (*Brassica oleracea* var. *acephala* L., *Brassica napus* L., *B. oleracea* L. var. *italica* Plenck, *B. oleracea* var. *gemmifera* DC., *B. oleracea* var. *capitata* L. and *B. oleracea* var. *botrytis* L.) relatives and rates of 3% of triploid individuals have been collected from commercial canola fields evidencing the *B. napus* × *B. rapa* hybridization (Prieto, 2006).

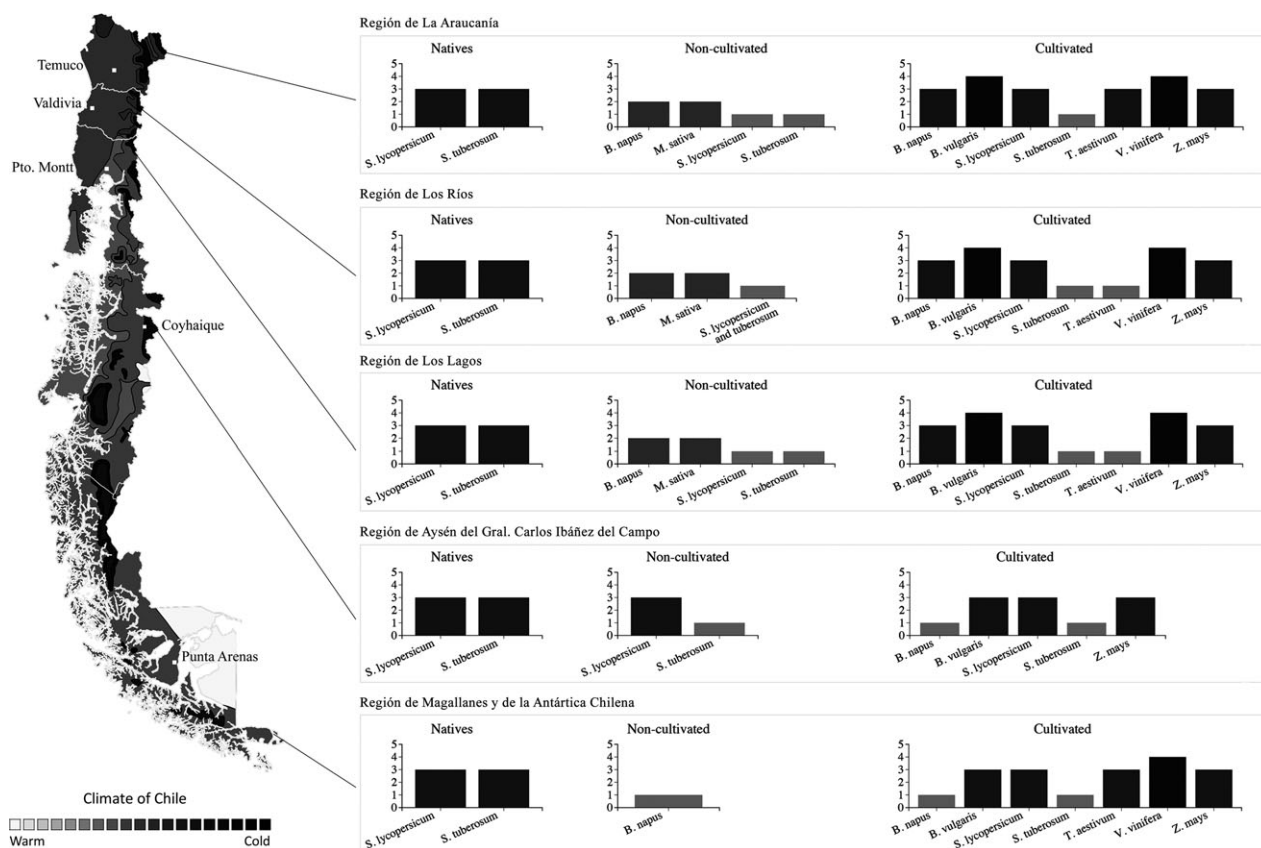
The appearance of volunteers may continue for a long time after the canola crop was grown and feral populations may appear (Liu *et al.*, 2013). Thus, crop rotation, rotating mode of action of herbicides, destroy volunteers, regular tillage, and adjacent or regional farming system organization could be effective to control volunteers. Further, male sterility, seed sterility, vegetative propagation (apomixes) and self-fertilization without opening of the flower (cleistogamy) also could help to avoid this phenomenon (Liu *et al.*, 2013).

The list of GM crops having only introduced cultivated species in the country included maize, sugar beet, wheat, rice and grape. In this group, the relationships among species reached OP values

up to 'high' for some of them, which describe this additional status of cultivated cores.

In maize, cross-fertilization between neighbouring maize fields is a biological source of gene flow observations between GM and non-GM plants as well as adventitious mixing of different maize varieties (Devos *et al.*, 2009; Sanvido *et al.*, 2008). Maize is a cross-pollinated crop, relying on wind for pollen dispersal. Furthermore, it is not able to survive as a feral population outside cropped areas due to its high degree of domestication. Other factors influencing cross-fertilization rates in maize cultivation are well studied and include, among others, the distance between fields, flowering synchrony, weather conditions, the relative positions of donor and receptor fields (with respect to dominant winds in the area), and the size and shape of fields (Devos *et al.*, 2009). Because of the difficulty in controlling some of these parameters, regulatory bodies from different countries decided to establish mandatory separation distances between GM and non-GM maize fields as the preferred single measure to limit cross-fertilization. It is important to note that for seed production, seed regulations in most countries require similar isolation measures.

As our results show, there are no cross-compatible wild relatives of maize in Chile, so there is no hazard to native species in this case. From an economic and market point of view, a separation distance of 40 m is sufficient to reduce admixture in maize cultivation below a threshold of 0.9% (Sanvido *et al.*, 2008). Moreover, pollen barriers consisting of non-GM maize, for example, reduce cross-fertilization rates more effectively than an isolation of the same distance with open ground or low-growing crops. With a maize barrier of 10–20 m, the remaining maize



**Figure 5** Outcrossing potential (OP) for native and introduced relative species present in the South region of the country for each of the 11 genetically modified (GM) crops. Maximum OPs for relationships between GM crops (x-axis in the plots) and their corresponding related native, introduced cultivated or introduced noncultivated species are shown, depending on the political regions (names are indicated on the top of each plot). Scale for OP (y-axis) was 'very low' = 1, 'low' = 2, 'medium' = 3, 'high' = 4 and 'very high' = 5. Associated climate of each region is represented graphically for illustrative purposes. Absent species did not present relatives.

harvest in the field rarely exceeds the threshold of 0.9% GM material (Messeguer *et al.*, 2006). Therefore, in order to avoid pollen-mediated gene flow from GM maize to introduced cultivated non-GM maize, short isolation distances may be applied in combination with other measures. For instance, buffer zones, discard zones or sowing maize at different dates, resulting in a difference in flowering periods, could be effective (Della Porta *et al.*, 2008; Devos *et al.*, 2009; Messeguer *et al.*, 2006; Riesgo *et al.*, 2010). Thus, the OP for GM maize and pollen-mediated gene flow to introduced cultivated relatives in Chile can be reduced towards zero if the appropriate reproductive isolation measures are in place.

Wheat is a predominantly self-pollinating species (de Vries, 1971) and has cross-pollination rates of 1%–2% for plants in close proximity (Griffin, 1987; Gustafson *et al.*, 2005; Martin, 1990). The cross-pollination rate considerably decreases with greater distance between pollen donor and recipient (Rieben *et al.*, 2011; de Vries, 1971). In wheat, fertilization usually occurs before the florets open, and although it is a wind-pollinated species, its pollen is relatively heavy and settles quickly compared to other grass species, making pollination with pollen from nearby fields unlikely (Rieben *et al.*, 2011). Thus, it has been suggested that pollen-mediated gene flow between GM and non-GM wheat might only be a concern if it occurs within fields, for example due to seed contamination (Rieben *et al.*, 2011). In this study, we identified a maximum OP of medium for GM

*T. aestivum* and introduced cultivated relatives along different regions in Chile. Thus, although wheat is the main crop cultivated in Chile with ca. 250 000 ha per year, the concerns for pollen-mediated gene flow in Chile would be not significant.

In rice, pollen-mediated gene flow to their wild and weedy relatives is currently one of the major concerns for ecologists. Rice is a self-compatible autogamous species that can lead to pollen-mediated outcrosses if short cultivation distances between individuals are applied between cultivars or subspecies, or when their flowering periods overlap. Pollen-mediated gene flow from GM to conventional rice cultivars was already reported, with percentages ranging from <0.2% between immediate neighbouring or mixed plants in a plot and up to <1% for more distant plots (Chun *et al.*, 2011; Jia *et al.*, 2007; Messeguer *et al.*, 2001, 2004; Rong *et al.*, 2005, 2007; Serrat *et al.*, 2013). Additionally, by the use of different experimental designs (i.e. adjacent plots, mixed plants or concentric circles) in which different pollen donor cultivars/ecotypes (i.e. glyphosate- or glufosinate-resistant red rice and the mutant imidazolon herbicide-resistant Clearfield™ rice) were used as pollen donors, the same <1% cross-pollination rate was concluded and the results emphasized a cultivar or ecotype influence (Chen *et al.*, 2004; Lentini and Espinoza, 2005; Messeguer *et al.*, 2004; Shivrain *et al.*, 2006, 2007). In the Chilean case, there are only introduced cultivated relatives for rice, so no environmental issues related to native species would arise from a potential unconfined GM rice release. Adventitious

presence and market conflicts may be addressed easily by implementing short isolation distances.

Sugar beet, *B. vulgaris*, is an obligate outbreeder with a complex self-incompatibility system (Maletsky and Weisman, 1978), and its pollen is easily transported by wind and insects (Archimowitsch, 1949; Free *et al.*, 1975). As sugar beet is a biennial crop grown for its root, it is not expected to flower during the first year of growth and, therefore, root-production areas are assumed to be exempted from such problems; however, a few volunteer plants were repeatedly reported in root-production fields, either induced by low temperature during spring growth (vernalization), or due to the presence of a bolting gene in a small percentage of the certified seeds sown (Darmency *et al.*, 2009). Flower fertilization of sugar beet plants up to 1000 m away from the pollen source was confirmed; however, a rapid decline in the crossing frequency was observed as the distance increased (Darmency *et al.*, 2009). According to our results, there are no native and introduced noncultivated relatives in Chile for sugar beet, so there is no likelihood of outcrossing in those cases. However, for introduced cultivated relatives, there are medium and high OPs along the country; those OPs do not consider biosafety measures. In order to avoid pollen-mediated gene flow from GM sugar beets to introduced cultivated relatives, and to diminish the probabilities of gene flow towards zero, the assurance of not flowering and the elimination of any volunteer plant may be necessary to handle this kind of crop.

Grapevines are of a clonal nature due to vegetative propagation, and the eventual use of seeds (mostly in breeding programs) presents problems associated with low germination due to a very tough seed coat. *Vitis vinifera* is principally wind pollinated (Huglin, 1998) and different flower types are described between wild (dioecious) and cultivated (hermaphroditic) vines (Caporali *et al.*, 2003). Recent work determining parentage and paternity-based estimation using nuclear single sequence repeats determined that both wild-type species and cultivars influence each other either in open landscapes (depending on the distance between individuals) or in closed environments (Di Vecchi-Staraz *et al.*, 2009). The fitness of hybrid seeds between cultivar and wild species was also confirmed by these technical approaches. In another work, Harst *et al.* (2009) transformed plants with the  $\beta$ -glucuronidase (GUS) gene from *Escherichia coli* with an intron sequence and followed GUS analysis of seedlings derived from a field assay between GM grape plots and neighbouring vineyards; these authors found an average cross-pollination rate of 2.7% at a distance of 20 m. Even though a high OP was acquired for grapes, as they are propagated primarily by cuttings, the likelihood of outcrossing due to an unconfined GM grape release in Chile does not generate environmental significant concerns.

It is worth noting that OP from non-GM crops were assessed as well. GM crops are not inherently riskier than non-GM crops (European Commission, 2010). Although specific genetic modifications might alter gene flow, according to our approach the probabilities and OP were the same to those obtained for GM crops (data not shown; see [www.flujogenico.cl](http://www.flujogenico.cl)).

The deployed system is a primary tool in which a maximum OP is obtained on the basis of species biogeography, reproductive biology, phenology, demography, hybridization and past introgression events. The list of biological parameters considered is relevant to outcrossing, but the proximity to agricultural areas could be an important factor, applying especially to noncultivated relatives. If the noncultivated relatives occur only in specific habitats that are unsuitable for agricultural production or for

cultivation of the related crop, outcrossing is likely to be minimal. Therefore, the obtained OP is a primary value describing a theoretical situation in which interaction between two species is formulated and it does not consider biosafety measures. These values will decrease depending on a specific environment and their components. From this starting point, other characteristics, such as distance between individuals, wind regimes, plot sizes, and biosafety measures, should be taken into account. Thus, this approach enables a risk assessor to rapidly dismiss the lowest risk species and instead focus on those that may present some actual OP. The approach indicates whether additional information should be sought to refine the problem formulation and whether outcrossing mitigation measures might be warranted for unconfined field trials or for cultivation if the consequences of outcrossing leading to gene introgression and therefore gene flow would be adverse in the context of defined protection goals. Consequently, the OP reported in this work and the approach for its deduction represent an *ex-ante* analysis on which regulators can start a decision process to evaluate unconfined releases for the different GM crops in Chile. The concept might also be applied elsewhere.

## Experimental procedures

### Lists of considered species

The vascular flora present in Chile was comprised by the entire wild flora (native and naturalized species); introduced and cultivated species for horticultural, ornamental, forest and medical uses were considered in this database ([www.flujogenico.cl](http://www.flujogenico.cl)). The 11 GM crops included in this study were commercially available species (maize, canola, soya bean, cotton, alfalfa and sugar beet) and those at final stages in the premarket pipeline (tomato, potato, wheat, rice and vine) (see <http://www.isa-aa.org/>). For each species, taxonomic data were standardized as used at Integrated Taxonomic Information System (ITIS, 2007) and by Germplasm Resources Information Network (GRIN, 2005).

### Native species

They are defined as those species that occurs naturally within a region, either evolving there or arriving and becoming established without human assistance. A revised list was built using as baseline list the 'Catálogo de la Flora Vascular de Chile' (Marticorena, 1990; Marticorena and Quezada, 1985). Its information was checked out and additionally completed by use of monographies, genus corrections and additional catalogues from bordering countries. The consulted materials were as follows: Barboza and Hunziker (1993), Barkworth (1990); Brako and Zarucchi (1993), Caro and Sánchez (1972), Dempster (1990), Domínguez and Elvebakk (2002), Everett and Jacobs (1990), Finot (2002), Jacobs and Everett (1997), King and Robinson (1987), Mabblerley (2002), Marticorena *et al.* (1998), Matthei *et al.* (1998), Mildner and Rogers (1978), Muñoz (1941), Muñoz (1983–1984), Muñoz (1990, 1995), Muñoz and Pinto (2003), Peñailillo (1996), Peñailillo (1998), Peñailillo (2002), Rojas (1997), Rosas (1983), Rossow (1986), Rua (1989), Rúgolo de Agrasar and Molina (1992), Taylor (1994, 1996a,b), Teillier (1996), Teillier and Taylor (1997), Tortosa (1983, 1989, 1992), Zuloaga and Morrone (1999) and Zuloaga *et al.* (1994, 2008). The status of Chile's rare, vulnerable, in danger and extinct plant species—known as 'state of conservation'—was obtained from Benoit (1989), Núñez *et al.* (1998) and Squeo *et al.* (2001).



### Introduced species

They are defined as those species that are not native to an ecosystem. As a backbone list, a previously reported sheet by Castro et al. (2005) was used and completed with reported information by Espinoza (1996), Marticorena and Quezada (1985), and Matthei (1995). The introduced species list had the following fields: taxonomic data, common name, origin or regions of diversity, geographical distribution in Chile, and cultivation category (horticultural, ornamental, forest, medicinal, weedy) and condition (naturalized or not).

### Cultivated species

This list was built using the 1997 Agriculture Censusing Report by Instituto Nacional de Estadísticas de Chile (INE, 2007). This base information was completed with specific searches for minor species, as described by Giaconi and Escaff (1999), from FAO (1996), López (1996), Sánchez-Monje (1981), Schilling (2001), Soto (1996) and some specific literature such as Core Classification version 99/2 (Eurocode2, 2000), Postharvest Technology—Research and Information Center (UC Davis, 2001. Properties and Recommended Conditions for Storage of Fresh Fruits and Vegetables. [http://postharvest.ucdavis.edu/Produce/Storage/span\\_c.shtml](http://postharvest.ucdavis.edu/Produce/Storage/span_c.shtml)) and Plant Scientific Names (Faucon, 2002). The list comprised information regarding taxonomic data, common name, condition (native, endemic, naturalized, introduced), cultivation category, life cycle, reproduction type [sexual (seed), asexual (bulb, cutting, apomixes)], landraces (presence or absence), type of pollination (autogamous, allogamous, parthenocarpic), pollinating agent (anemophilous, entomophilous, artificial), regions of diversity and range of geographical distribution in Chile.

### Genetically modified species

The 11 GM species were selected considering their commercial or advanced stage in the commercialization pipeline (see <http://www.gmo-compass.org/>). The GM list summarized the following characteristics: taxonomic data, common name, country of origin, state of development (under development, commercially available), countries of cultivation, gene flow information related with pollen-mediated gene flow, gene flow possibility mediated by seeds and seed bank capability (seed gene flow) of the species when the information was available (Kjellsson and Simonsen, 1994; Kjellsson et al., 1997).

### Comparative computing between lists

Lists were recorded on individual Excel (Microsoft) sheets (cultivated, introduced, natives or GM). Design of macros in the MS-Excel environment, and the procedures indicated therein, allowed for the development of a basic searching script based on *taxa* coincidences, able to compare species, genus or family names among sheets. Conspecific individuals, species belonging to the same genus and genus related within a family were cross-referred as putative candidate couples for cross-pollination.

### Ranking for putative donor–recipient couples

#### *Outcrossing potential associated with a specific species (OPS)*

The relative potential for a given species to receive cross-pollination was obtained by the assignment of a numeric value associated with each species that theoretically summarizes all

the annotated characteristics. The total amount of computed characteristics present in one species was obtained using the information included on each list (cultivated, natives and introduced). These lists included specific and relevant 'characteristics' grouped into 'types', as indicated in Table 1. As an example, in the cultivated list, the reproduction ('type') of a species could be classified as sexual or asexual ('characteristics'). A formula for the quantification of the accumulated characteristics per species was established by definition of an OP associated with the type of characteristics (OPT) (Eqn 1). The OPT value corresponds to a summation of each independent 'i' characteristic ( $C_i$ ) (for instance in cultivated species: agricultural, ornamental, forest or weed) belonging to a 'type' (in cultivated: origin). By the use of a scale from 1 to 5 (1 = very low, 2 = low, 3 = medium, 4 = high and 5 = very high), each  $C_i$  acquired a value depending on its hypothetical relevance (see Table 1, column 'Impact'), which was also weighted by its relative importance  $P_i$ , which is an arbitrary factor that represented the hierarchy among characteristics. This summation was normalized by the product between the average of relative importances ( $\bar{P}_C$ ), the total number of present characteristics ( $N_C$ ) and the maximum value used in the  $C_i$  scale (which was 5 in this work)

**Table 1** Selected species and type of specific characteristics considered for the database

List	Type	Characteristic	Impact*
Cultivated species	Origin	Endemic	Very high
		Introduced	Very low
		Naturalized	Medium
		Native	High
		Cultivation	Agricultural
	Cultivation	Ornamental	Very low
		Weed	Very high
		Forest	Very low
	Landrace	Landrace	High
		Reproducción	Sexual
	Life cycle	Asexual	High
		Annual	Medium
		Bi-annual	Medium
	Pollination	Perennial	Low
		Autogamous	Very low
Allogamous		High	
Pollinating agent	Entomophilous	Medium	
	Anemophilous	Low	
	Artificial	Very low	
	Conservation	Extinct	Very low
Native species	Conservation	In danger	Very high
		Vulnerable	High
		Rare	Medium
		Endemism	Low
		Endemism	Low
Introduced species	Cultivation	Agricultural	Low
		Ornamental	Very low
		Forest	Very low
		Weed	Very high
	Naturalization	Medical	Low
		Naturalized	High

\*Weighting of each characteristic used in this work as described by Muñoz et al. (2004).

**Table 2** Conversion of outcrossing potential (OP) values into concepts

OP value (%)	Concept
0–20	Very low
>20 to 40	Low
>40 to 60	Medium
>60 to 80	High
>80 to 100	Very high

**Table 3** Example of outcrossing potential associated with the type of characteristics (OPT) calculation for *Brassica rapa* as pollen receptor

$P_t$	Type	Characteristic	$P_c$	Value ( $C_i$ )	Eqn 1	OPT=
1	Cultivation	Weed	1	5	$\frac{1 \times 5}{1 \times 1 \times 5}$	1
1	Naturalization	Naturalized	1	4	$\frac{1 \times 4}{1 \times 1 \times 5}$	0.8

$$OPT = \frac{\sum_{i=1}^{N_c} P_i \times C_i}{\bar{P}_c \times N_c \times C_{max}} \quad (1)$$

For OPS calculation (Eqn 2), each OPT value was weighted for the relevance among the 'j' types and the summation of these values was normalized by the product between the average relevance value of present types ( $\bar{P}_t$ ) and the total number of considered types ( $N_t$ ).

$$OPS = \frac{\sum_{j=1}^{N_t} P_j \times OPT_j}{\bar{P}_t \times N_t} \quad (2)$$

Factors  $P_i$  and  $P_j$  consider special circumstances or compensate for a specific condition that can be relevant for the assessment. In the present work, both  $P_i$  and  $P_j$  were always assigned with value = 1, that is all weighted equally.

#### Potential of pollen sources

Two different patterns of gene flow were incorporated into the listed (cultivated and GM) species, which were considered as eventual donors:

**Pollen-mediated.** The potential risk of gene flow (RGF) was defined as a species-specific value between 1 and 5, referring to a weighted risk of pollen-mediated gene flow, considering reported data about con- and interspecific hybridizations. In the present work, any report describing even minimal degrees of hybridization were scored as very high risk (a value of 5) (Kjellsson et al., 1997).

**Seed-mediated.** The RGF also incorporated previous art about data describing seed banks and seed viability under natural conditions for a given species. The numeric scale was as follows: an average value of medium risk (a value of 3) was used when data were not reported; <5 years of seed bank occurrence was judged as a value of 4; and over 5 years was considered a very high risk factor (a value of 5) (Kjellsson and Simonsen, 1994).

#### General index for donor–recipient couples

A summarizing factor, defined as a general weighted OP for a specific situation between two species (donor/recipient), was built based on the combination of OPS and RGF, using Eqn 3:

$$OP = (OPS \times RGF \times 50)/5 + 50 \times ES \quad (3)$$

The OP is represented as a percentage, directly related with a risk degree assigned to an interaction couple. Equivalence of the species (ES, yes = 1 or no = 0) was also incorporated as an additional risk (conspecific hybridization), immediately reflected by a minimum OP value of 50% (arbitrary value). The translation of OP values into a concept scale was as indicated by Table 2.

#### Assigned scenario and ranking of the found candidate couples

The degree of importance for each one of the listed characteristics (i.e. weighting of characteristics  $C_i$  and  $P_i$ ) specifically regarding the Chilean scenario was summarized in Table 1. Also none of the types (i.e.  $P_j$ , Table 1) were defined as heavier than the other (i.e. each  $P = 1$  and average  $P = 1$ ). These impact factors were adopted from a previously defined scenario during a national-scale study referring the status of the local vascular flora, carried out by the Chilean National Environmental Committee (CONAMA; Muñoz et al., 2004).

#### Example of calculation

*Brassica napus* is a cultivated crop that presents introduced and naturalized relatives in the country, among which is found *B. rapa*. Table 3 summarizes C and P values for the receptor *B. rapa* and the assumed local scenario. The deduced OPTs came from the use of  $N_t = 2$  (five characteristics for cultivation: agricultural, ornamental, forest, weed and medical; one for the type naturalization: naturalized).

$$OPS = \frac{1 \times 1 + 1 \times 0.8}{1 \times 2} = 0.9$$

When *B. napus* is considered as an impacting crop (either GM or non-GM), its OP (Eqn 3) is filled with values corresponding to a high risk of gene flow (i.e. RGF = 5) broadly reported as pollen-mediated gene flow (FitzJohn et al., 2007). Because it is compared with *B. rapa* (an introduced species), with no equivalence of the species, Eqn 3 becomes:

$$OP = \frac{5 * 0.9 * 50}{5} + 50 * 0 = 45(\text{Medium})$$

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#### Conflict of interest

Authors declare they have no competing interests as defined by the Journal, or other interests that might be perceived to

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