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Rapid diagnostic PCR method for identification of the genera *Sarcocornia* and *Salicornia*

Método de PCR de diagnóstico rápido para identificación de los géneros Sarcocornia y Salicornia

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ABSTRACT
Plants that belong to different genera sometimes may present close morphological similarity and cannot be distinguished phenotypically by non-specialists. The aim of this study was to develop a simple diagnostic PCR for the identification of plants of *Sarcocornia* and *Salicornia* and to test this new procedure to identify 82 samples of *Sarcocornia neii* from coastal and valleys of the Atacama region of Chile. Six primer pairs were designed from ETS sequences of the genera *Sarcocornia* and *Salicornia* and evaluated for the identification of both genera. Primers with a mismatch in the 3' nucleotide indicate the site of the SNP. Four primer pairs (SALI2F-2R, SALI3F-4R, SARCO1F-1R and SARCO3F-3R) were selected to develop an efficient and simple diagnostic PCR for the identification of *Sarcocornia* and *Salicornia*. The results show that with this method is possible to identify *Sarcocornia* and *Salicornia*. This method may be useful as an approach for genetic traceability of conserved products (sea asparagus). This work provides an applicable and efficient method using only DNA, PCR and electrophoresis.
Key words: *Sarcocornia*, PCR, allele-specific primer, SNP.

RESUMEN
Las plantas que pertenecen a diferentes géneros, a veces, pueden presentar fuertes similitudes morfológicas y pueden no ser identificadas fenotípicamente por no especialistas. El objetivo de este trabajo fue desarrollar una PCR diagnóstico para la identificación de plantas de los géneros Sarcocornia *y* Salicornia *y probar este nuevo procedimiento para identificar 82 muestras de* Sarcocornia neii *distribuidas en la costa y valles de la región de Atacama. Se diseñó seis parejas de cebadores a partir de secuencias ETS de los géneros* Sarcocornia *y* Salicornia*, y se evaluó la identificación de cada género. Los cebadores contienen un desajuste en el nucleótido 3' que corresponde al sitio del SNP. Se seleccionaron cuatro pares de cebadores (SALI2F-2R, SALI3F-4R, SARCO1F-1R y SARCO3F-3R) para desarrollar una PCR de diagnóstico eficiente y simple para la identificación de los géneros* Sarcocornia *y* Salicornia*. El resultado muestra que con este método se puede identificar los géneros* Sarcocornia *y* Salicornia*. Adicionalmente, este método puede ser útil como propuesta para la trazabilidad genética de productos conservados (espárragos de mar). Este trabajo proporciona una aplicación eficiente, utilizando sólo ADN, PCR y electroforesis.*
Palabras clave*:* Sarcocornia*, PCR, primer específicos de alelos, SNP.*

Introduction

The genus *Sarcocornia* was established by Scott (1978), who separated it from *Salicornia* L. and *Arthrocnemum* Moq on the basis of morphological characteristics. Currently, *Sarcocornia* includes about 28 species of perennial succulent halophytes distributed worldwide (Steffen *et al*., 2015). Since Scott's publication, the validity of the distinction of these genera has been questioned. Taxonomic studies of *Salicornia* in South America initially indicated the presence of this genus in Chile (Gunckel, 1978) and Peru (Gutte and Muller, 1985) as well as other countries; however, Scott (1978) concluded that these plants belong to the genus *Sarcocornia*. Scott (1978) defined two species in the South American continent as *Sarcocornia fruticosa* and *Sarcocornia pulvinata*. Later, Alonso and Crespo (2008) redefined

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Sarcocornia individuals in the American continent, recognizing four new species and confirming one of those established by Scott (1978), named *S. neei*, *S. ambigua*, *S. andina*, *S. magellanica* and *S. pulvinata*.

Sarcocornia is described as a pioneer plant in marine environments, and is characterized by extreme salt tolerance (Davy *et al*., 2006). In the coastal Pacific, halophilic communities of *Sarcocornia* grow in saline coastal marshes of Chile, coastal regions of the south Pacific slope of Peru (San Martín *et al*., 2006; Montesinos-Tubée, 2012), and saltmarshes of the Andes of northern Chile (Faúndez and Macaya, 1997). Due to their high salt tolerance, both *Salicornia* and *Sarcocornia* possess significant potential as a production model for arid and saline environments (Katschnig *et al*., 2013); they have by high nutritional value and high biomass yield in field conditions (Ventura and Sagi, 2013). *Salicornia* is presently cultivated commercially for human consumption in Israel; it has been introduced to the European market as a vegetable with leafless shoots resembling green asparagus, and is in great demand in gourmet kitchens due to its high mineral and antioxidant content (Ventura *et al*., 2011; Lu *et al*., 2010). In aquaculture production, a diet of *Salicornia bigelovii* flour supplied to juveniles of the blue shrimp *L. stylirostris* in intensive production systems has produced positive results in terms of growth and survival, and offers a low-cost alternative to more expensive fish- and corn-based flour (Acosta-Ruiz *et al*., 2011).

Salicornia and *Sarcocornia* show marked morphological similarity; they are distinguished phenotypically by inflorescence characteristics and life form (Steffen *et al*., 2015). However, according to studies on the basis of morphological characteristics by Judd and Ferguson (1999) including flower arrangement and growth habit, these features alone are insufficient to distinguish the genera confidently. Molecular markers could serve as a modern approach for identification of these plants.

Many types of molecular techniques have been used to identify plants of a wide range of varieties, species and genera, including RFLP, AFLP, RAPD and ASP-PCR (allele-specific primer PCR). Among these, allele-specific primer PCR by agarose gel electrophoresis is recognized as an efficient approach for cultivar identification (Soleimani *et al*., 2003). Likewise, diagnostic PCR using specific primers offers a cost-effective alternative for molecular identification of specific plant taxa. SNPs (single nucleotide polymorphisms) can be detected using allele-specific PCR primers designed in such a manner that the 3' nucleotide of a primer corresponds to the site of the SNP (Ugozzoli and Wallace, 1991). This technique allows preferential amplification of one allele relative to another on account of the primers being complementary to the site of DNA (Ugozzoli & Wallace 1991). Using this technique, SNPs from ETS sequences of *Salicornia* and *Sarcocornia* (Steffen *et al*., 2015) could potentially be applied to discriminate between genera.

The goal of this study was to design allele-specific primers for amplification of short fragments from ETS sequences of *Sarcocornia* and *Salicornia*, and to evaluate an efficient diagnostic PCR to discriminate *Salicornia* and *Sarcocornia* using samples from the Atacama, Coquimbo and Los Lagos regions of Chile and *Salicornia* control samples.

Materials and Methods

Plant Materials

Eighty two *Sarcocornia neii* plants from the Atacama Region and seven *S. neii* plants from the Coquimbo Region were sampled during 2016. Plants were selected randomly and located by GPS as shown in Table 1. Two DNA samples of *Salicornia europaea,* supplied by Dr. Dirk Albach (Institut für Biologie und Umweltwissenschaften, Carl von Ossietzky-Universität, Germany), were used as positive *Salicornia* controls. *Salicornia* 1 control (ecotype 1) and *Salicornia* 2 control (ecotype 2) were collected by Dr. Albach near mudflats and low salt marshes in Spiekeroog Island (Germany), respectively. Two DNA samples of *Sarcocornia perennis* from Spain and two DNA samples of *Sarcocornia neii* from Puerto Mont (41º25'19.31''S 72º53'42''W, Los Lagos region) were used as a positive *Sarcocornia* control. *Sarcocornia* plants used as DNA controls were identified morphologically according to Scott's approach. All samples were processed and analyzed in the CRIDESAT Research Center, University of Atacama.

Plant DNA Extraction

Genomic DNA from eighty nine plants was extracted by a modified CTAB method following the procedure described by Doyle and Doyle (1987). Five grams of stem material were placed in sterile mortars

Table 1: Origin, location and samples collected in the Atacama and Coquimbo regions of Chile.

Samples	Latitude (ºS)	Longitude (ºW)	Altitude (m)	Samples	Latitude (ºS)	Longitude (ºW)	Altitude (m)
RCOP02	27°19'22.9"S	70°50'39.2"W	60	CLTO54	27°49'49.2"S	71°05'12.3"W	4
RCOP03	27°19'22.8"S	70°50'38.9"W	61	CLTO55	27°49'49.0"S	71°05'12.4"W	4
RCOP04	27°19'23.0"S	70°50'39.3"W	56	CLTO56	27°49'48.7"S	71°05'11.6"W	3
RCOP05	27°19'23.3"S	70°50'39.8"W	58	CLTO57	27°49'48.7"S	71°05'11.1"W	2
PVCO08	27°12'31.1"S	70°57'05.9"W	1	CLTO58	27°49'48.4"S	71°05'10.9"W	4
PVCO09	27°12'31.6"S	70°57'05.3"W	1	CLTO59	27°49'48.2"S	71°05'10.4"W	2
PVCO10	27°12'32.5"S	70°57'03.5"W	1	CLTO60	27°49'48.4"S	71°05'10.2"W	1
PVCO11	27°12'33.4"S	70°57'02.2"W	1	CLTO61	27°49'47.5"S	71°05'09.8"W	3
PVCO12	27°12'35.3"S	70°57'01.3"W	1	CLTO62	27°49'47.4"S	71°05'09.6"W	2
DECO13	27°16'39.6"S	70°56'32.6"W	0	CLTO63	27°49'54.6"S	71°05'11.5"W	7
DECO14	27°16'39.9"S	70°56'32.4"W	0	CLTO64	27°49'53.8"S	71°05'11.8"W	4
DECO15	27°16'40.1"S	70°56'32.2"W	0	CABJ65	28°05'16.1"S	71°08'27.3"W	6
DECO16	27°16'40.3"S	70°56'32.0"W	0	CABJ66	28°05'15.9"S	71°08'27.7"W	4
DECO17	27°16'40.6"S	70°56'31.6"W	0	CABJ67	28°05'16.0"S	71°08'27.9"W	3
DECO18	27°16'41.1"S	70°56'31.1"W	0	CABJ68	28°05'16.4"S	71°08'28.4"W	4
DECO19	27°16'41.5"S	70°56'30.9"W	0	CABJ69	28°05'16.7"S	71°08'29.8"W	5
DECO20	27°16'42.1"S	70°56'30.7"W	0	CABJ70	28°05'16.7"S	71°08'30.4"W	5
DECO21	27°16'42.7"S	70°56'30.2"W	0	CABJ71	28°05'16.7"S	71°08'31.9"W	3
DECO22	27°16'43.4"S	70°56'29.0"W	0	CABJ72	28°05'15.9"S	71°08'32.8"W	3
DECO23	27°16'57.4"S	70°56'16.4"W	0	CABJ73	28°05'15.2"S	71°08'32.8"W	5
DECO24	27°16'57.4"S	70°56'16.5"W	0	CABJ74	28°05'14.2"S	71°08'32.8"W	7
DECO25	27°17'22.6"S	70°56'02.8"W	0	CABJ75	28°05'13.4"S	71°08'33.4"W	5
DECO26	27°17'22.7"S	70°56'02.9"W	0	CABJ76	28°05'13.3"S	71°08'33.6"W	2
DECO27	27°17'29.0"S	70°56'00.0"W	0	CABJ77	28°05'12.4"S	71°08'33.4"W	4
DECO28	27°17'29.2"S	70°56'00.0"W	0	CABJ78	28°05'12.1"S	71°08'33.7"W	4
DECO29	27°17'38.0"S	70°55'56.2"W	0	CABJ79	28°05'11.3"S	71°08'34.0"W	3
DECO30	27°17'38.2"S	70°55'56.2"W	0	PBTO81	27°54'01.8"S	70°57'05.3"W	151
DECO31	27°17'55.2"S	70°55'48.8"W	0	PBTO82	27°54'01.7"S	70°57'05.2"W	151
DECO32	27°17'55.3"S	70°55'49.0"W	0	PBTO83	27°54'01.4"S	70°56'55.6"W	154
DECO33	27°18'12.5"S	70°55'43.8"W	0	PBTO84	27°54'01.8"S	70°56'55.4"W	154
DECO34	27°18'12.7"S	70°55'43.8"W	0	PBTO85	27°54'02.1"S	70°56'54.8"W	153
DECO37	27°19'03.4"S	70°55'06.8"W	4	PBTO86	27°54'02.2"S	70°56'54.5"W	152
DECO38	27°19'03.5"S	70°55'07.0"W	4	PBTO87	27°54'02.2"S	70°56'54.6"W	152
DECO39	27°19'03.7"S	70°55'07.1"W	5	VAHS88	28°27'55.0"S	71°12'23.8"W	8
DECO43	27°19'15.8"S	70°55'04.0"W	8	VAHS89	28°27'54.9"S	71°12'23.8"W	8
DECO44	27°19'15.7"S	70°55'04.0"W	7	VAHS90	28°27'54.8"S	71°12'23.4"W	9
DECO45	27°19'16.0"S	70°55'04.1"W	6	VAHS91	28°27'54.9"S	71°12'23.0"W	10
CLTO46	27°49'50.5"S	71°05'15.1"W	2	VAHS92	28°27'55.1"S	71°12'22.9"W	10
CLTO47	27°49'50.5"S	71°05'14.8"W	1	LASE93	29°57'40.4"S	71°19'19.4"W	3
CLTO48	27°49'50.5"S	71°05'14.4"W	1	LASE94	29°57'40.3"S	71°19'18.8"W	3
CLTO49	27°49'50.3"S	71°05'14.0"W	2	LASE95	29°57'40.5"S	71°19'21.7"W	4
CLTO50	27°49'50.3"S	71°05'13.6"W	3	LASE96	29°57'40.3"S	71°19'22.9"W	4
CLTO51	27°49'50.3"S	71°05'13.2"W	2	LASE97	29°57'39.9"S	71°19'11.2"W	4
CLTO52	27°49'49.8"S	71°05'12.6"W	2	LASE98	29°57'41.1"S	71°19'13.1"W	6
CLTO53	27°49'49.4"S	71°05'12.5"W	3	LASE99	29°57'40.9"S	71°19'14.6"W	7

at -80 ºC for a period of 12 h, and a fine powder produced. About 500 mg of powder was placed in 2 mL tubes and mixed with 850 µL of preheated (65 ºC) extraction buffer [100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB], supplemented with 0.7% β-mercaptoethanol and 3% PVP-40. Samples were vortexed vigorously 10 s and incubated 1 h at 65 ºC. Mixes were inverted three times during the incubation. The aqueous phase was recovered by centrifuging at 14000 rpm for 10 min. The recovered volume was mixed with an equal volume of chloroform-isoamyl alcohol (24:1). The mix was inverted gently over a period of 2 min. Centrifugation at 14000 rpm for 10 min was repeated. The upper phase was transferred to a new tube and treated with 5 µL RNase A (100 µg/mL) at 37 ºC for 30 min. The extraction was mixed with two-thirds volume isopropanol at -20 ºC. The mix was inverted gently thirty times and incubated on ice for 40 min. Centrifugation at 14000 rpm for 10 min was repeated. The supernatant was discarded and a DNA pellet was obtained, to which a washing solution of 600 µL of 70% ethanol and 10 mM ammonium acetate was added; this was followed by centrifuging for 2 min at 14000 rpm. The wash was repeated twice. The washing solution was discarded and the DNA dried for 15 min at RT. To elute DNA, 20 µL TE was added and incubated overnight at 4 °C. Quality and concentration of total DNA were verified by Colibri Microvolume Spectrophotometer (Titertek Berthold, Germany) at 260, 280 and 230 nm, and genomic DNA integrity checked on 0.7% agarose gels.

Specific primer design

Specific primers were designed using ETS (external transcribed spacer) sequences of ribosomal RNA from nine *Salicornia* species and eight *Sarcocornia* species previously obtained by Kadereit *et al*. (2007). The accession numbers of these 17 ETS sequences were obtained from GenBank (Figure 1). The sequences were aligned by MEGA 7.0 software and ClustalW as shown in Figure 1. Oligo® software was used to design three specific primer pairs each for *Salicornia* and *Sarcocornia* (Table 2) from conserved SNP. These six potentially genus-specific primer pairs were examined for their presence in other plant species available in GenBank (BlastN option, http://www.ncbi.nlm.nih.gov/).

PCR optimization

DNA control samples were used as a template to investigate analytical sensitivity. Serial DNA concentrations were prepared for each PCR reaction at 1 ng, 5 ng, 10 ng, 20 ng and 30 ng to establish the sensitivity limit of the PCR assay. PCR reactions were performed in 12 µL final volume containing (1ng to 30 ng) DNA, 0.85 µL each primer (5 µM) and 6 µL DreamTaq PCR Master Mix 2X (Thermo Scientific). Amplifications were carried out in Swift Max Pro (Esco) and MultiGene Optimax (Labnet) thermal cyclers with the following program: an initial step of 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 54 °C and 2 min at 72 °C, followed by a final extension step of 7 min at 72 °C. To determine the optimum annealing temperature (Ta), a gradient PCR was performed using DNA control samples from the *Sarcocornia* control (from Puerto Montt), and *Salicornia* 1 and 2 controls. The PCR mix and program were identical to those indicated above, but with optimized DNA concentration and a different Ta. The Ta were 50 °C, 50.7 °C, 51.5 °C, 54 °C, 57.2 °C, 59.2 °C and 60 °C in order to obtain the optimum temperature at which only the template DNA of *Sarcocornia* or *Salicornia* would be amplified. All PCR products were visualized on 1.5% TBE agarose gels.

Multiplex PCR and allele-specific PCR

To demonstrate that diagnostic PCR works well with specific *Sarcocornia* and *Salicornia* DNA primer pairs, a primer pair from plastid *trn*L (UAA) intron (~110 pb) was used as a positive plant control in the same PCR reaction. The multiplex PCR to amplify plastid *trn*L (UAA) intron and the ETS region from *Salicornia* and *Sarcocornia* were performed under conditions similar to those used for PCR optimization, except for the primer concentrations: 1 µL of each primer SALI or SARCO to 5 µM and 0.7 µL of each *trnL* primer to 5 µM (F: 5'GGGCAATCCTGAGCCAA 3' R: 5' CCATTGAGTCTCTGCACCTATC 3'; Taberlet *et al*., 2006). The amplification of allele-specific diagnostic PCR was performed using 3 primers simultaneously in a reaction tube with genomic DNA from *Sarcocornia* and *Salicornia* controls. Diagnostic PCR reactions were performed in 16 µL final volume containing 20 ng DNA, 0.55 µL SARCO3F primer, 1.3 µL SARCO1R, 0.55 µL SARCO3R (all primers

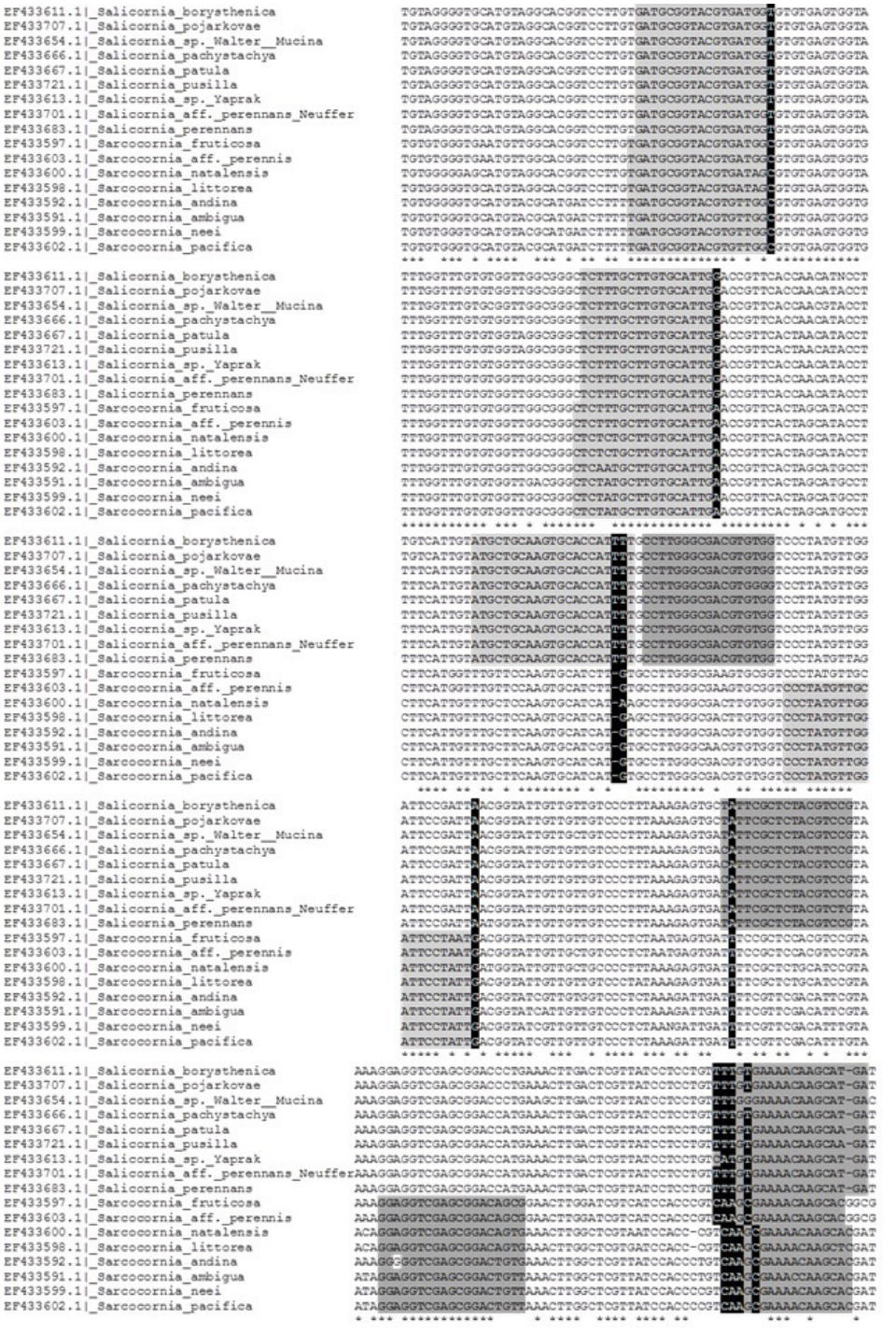

Figure 1: Position of specific primers in the ETS (external transcribed spacer) sequences of ribosomal RNA of nine *Salicornia* and eight *Sarcocornia* available in GenBank. Forward primer was colored in light grey and reverse primer in dark grey. Primers with SNP in position 3' were shown in white letters.

Table 2: Sequences, temperature and SNP information of the primers designed.

Primer name	Sequence (5'-3')	Target taxa	Tm °C	Length of base pair	SNP	Length of PCR product
SALI1F	GATGCGGTACGTGATGG**T**	*Salicornia sp.*	54.3	18	T/C	138
SALI1R	CCACACGTCGCCCAAGG			17	--	
SALI2F	TCTTTGCTTGTGCATTG**G**	*Salicornia sp.*	51.9	18	G/A	155
SALI2R	CGGACGTAGAGCGAATA			17	--	
SALI3F	ATGCTGCAAGTGCACCAT**TT**T	*Salicornia sp.*	52.3	21	T/G;T/-	176
SALI4R	TCATGCTTGTTTTC**ACAAA**			19	T/C;T/A	
SARCO1F	CTCTATGCTTGTGCATTG**A**	*Sarcocornia sp.*	52.5	19	A/G	221
SARCO1R	GTGCTTGTTTTC**GCTTG**			17	C/T;A/T	
SARCO2F	TGATGCGGTACGTGTTGG**C**	*Sarcocornia sp.*	55.9	19	C/T	232
SARCO2R	AACAGTCCGCTCGACCTCC			19	--	
SARCO3F	CCCTATGTTGGATTCCTATT**G**	*Sarcocornia sp.*	52.3	21	G/A	243
SARCO3R	CATCCATCATCAGCGTAC (*)			18	--	

(*) sequence not shown in Figure 1

at 5 µM), and 8 µL DreamTaq PCR Master Mix 2X (Thermo Scientific). Amplifications were carried out in Swift Max Pro (Esco) and MultiGene Optimax (Labnet) thermal cyclers, with the following program: an initial step of 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 2 min at 72 °C, followed by a final extension step of 7 min at 72 °C. All PCR assays were repeated three times and PCR products visualized on 1.5% TBE agarose gels.

Results and Discussion

Various genetic techniques have been used to authenticate differences between different plant cultivars, varieties, species and genera. Recently the application of diverse molecular approaches has allowed the validation of plant species that previously, due to the difficulty in identifying plants based on morphological characteristics, were considered to be of questionable taxonomic status (Nybom *et al*., 2014). Systematic establishment of phylogenetic relationships in *Salicornia* and *Sarcocornia* using morphological markers is difficult due to the lack of or significant reduction of principal structures such as leaves and flowers (Kadereit *et al*., 2006). Morphological characteristics such as perennial habitat and similarity in flower size were the main basis of the argument to distinguish *Sarcocornia* from *Salicornia* (Scott, 1978). However, there are species such as *Sarcocornia natalensis* and *Sarcocornia freitagii* containing short-lived and herbaceous perennials. Therefore, the traditional characteristics used to identify these genera are currently insufficient (Steffen *et al*., 2015).

In this study, six primer pairs were designed to evaluate allele-specific amplifications from *Salicornia* and *Sarcocornia*. For this, the 3' end base of the forward or reverse primer was positioned strictly on the SNP. Prior to plant identification, two PCR optimization tests were performed using control DNA from each genus. The results of testing for PCR sensitivity showed good amplification of the expected fragments using samples containing between 1 and 20 ng per DNA/PCR reaction (Figure 2). Therefore, to ensure the effectiveness of the majority of PCR assays it was decided to employ a final volume of 15 ng of DNA/14 µL PCR.

To evaluate the effect of annealing temperature (Ta), *Salicornia* and *Sarcocornia* control DNA was amplified using six primer pairs, using a Ta between 50 °C and 60 °C. The results of the diagnostic PCR based on the SALI1F-SALI1R primer pair indicated the presence of a well-defined fragment of expected size and high intensity in *Salicornia* controls 1 and 2, whereas the same robust fragment was observed in the *Sarcocornia* control at all annealing temperatures (Figure 3). In contrast, correct amplifications were observed in both *Salicornia* controls with the SALI2F-SALI2R primer pair at almost all annealing temperatures, while the *Sarcocornia* control showed no amplification of fragments at different annealing temperatures. Secondary PCR products with SALI2F-SALI2R were observed in the *Salicornia* 1 control, however, these PCR products disappeared at Ta greater than 57.2 °C. The *Salicornia* controls of the SALI3F-SALI4R primer pair resulted in good amplification of fragments at all annealing temperatures, but not in the *Sarcocornia* control, which showed no amplification (Figure 3).

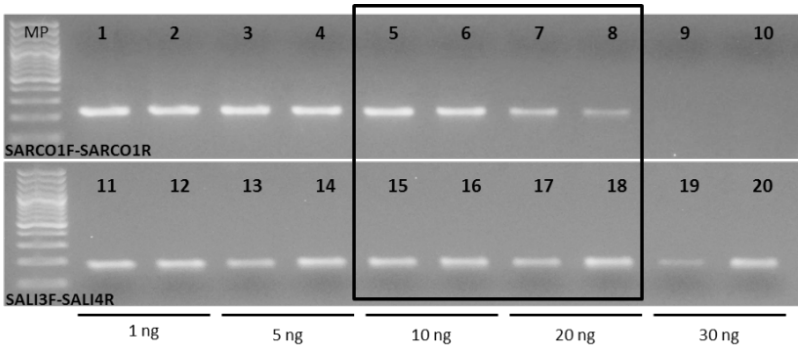

Figure 2: Agarose gel electrophoresis of PCR products obtained from the sensitivity test (1 ng to 30 ng) of primer pair SARCO1F-SARCO1R and SALI3F-SALI4R. Lanes 1, 3, 5, 7 and 9 = *Sarcocornia* control; 2, 4, 6, 8 and 10 = DECO19; 11, 13, 15, 17 and 19 = *Salicornia* 2 control; and 12, 14, 16, 18 and 20 = *Salicornia* 1 control. MP: 100 bp DNA ladder.

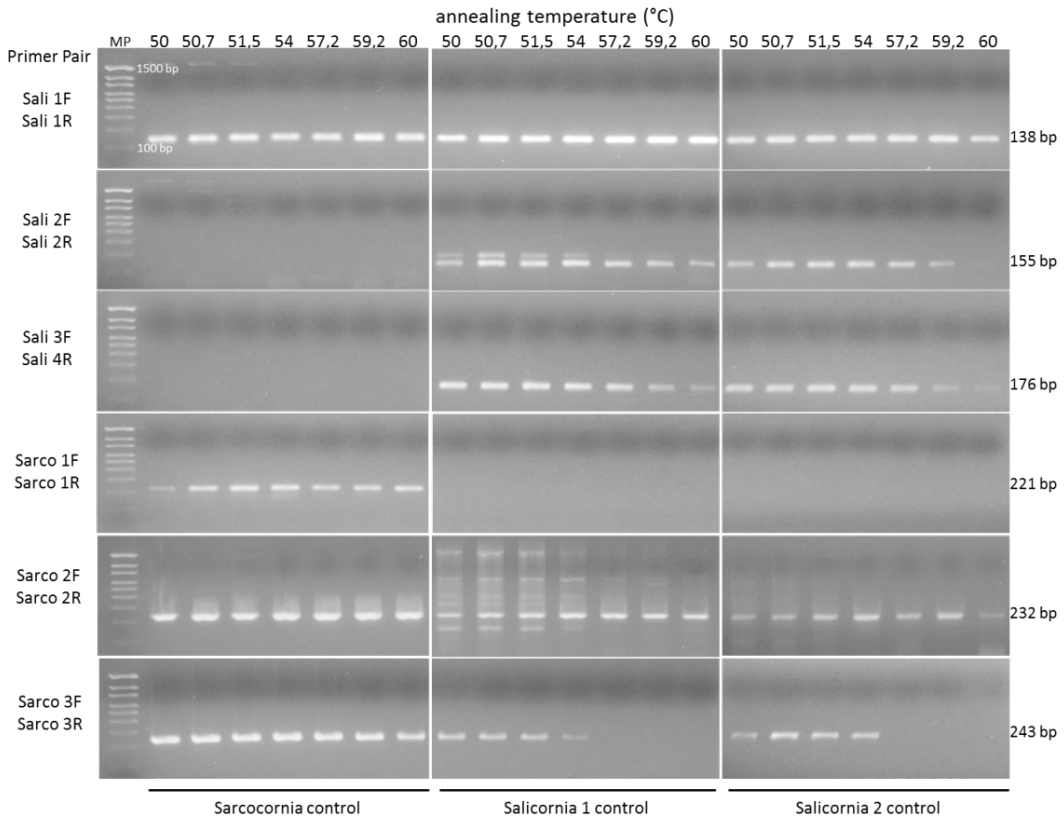

Figure 3: Electrophoresis of DNA amplified fragments obtained from annealing temperature (Ta) tests with *Sarcocornia* control (*Sarcocornia* Puerto Montt), *Salicornia* 1 control and Salicornia 2 control, using primer pair SALI1F-SALI1R (138 bp), SALI2F-SALI2R (155 bp), SALI3F-SALI4R (176 bp), SARCO1F-SARCO1R (221 bp), SARCO2F-SARCO2R (232 bp) and SARCO3F-SARCO3R (243 bp). MP: 100 bp DNA ladder.

PCR based on the SARCO1-SARCO1F primer pair indicated the presence of a fragment of expected size in the *Sarcocornia* control, but no fragment was observed at any annealing temperature for *Salicornia* controls 1 and 2 (Figure 3). Amplification of fragments in both *Salicornia* and *Sarcocornia* controls was detected at different annealing temperatures using the SARCO2F-SARCO2R primer pair. Unlike previous primer pairs, the SARCO3F-SARCO3R primer pair showed good amplification of PCR fragments based on the *Sarcocornia* control at different annealing temperatures, although the *Salicornia* controls also showed fragment amplification at a temperature of 54 °C; amplification was not observed at temperatures over 57.2 °C (Figure 3).

Finally, two primer pairs (SALI1F-SALI1R and SARCO2F-SARCO2R) were discarded since they produced amplification in controls of both *Sarcocornia* and *Salicornia*. Each of these primer pairs had one SNP in the 3' end position of the forward primer. However, in the SARCO2F-SARCO2R primer pair differences in amplification between *Sarcocornia* and *Salicornia* controls were observed at annealing temperatures of up to 68 °C, whereas no amplification differences were observed with the SALI1F -SALI1R primer pair at annealing temperatures of up to 72 °C (data not shown). The reason for this is that in most cases a single base pair change at the 3' end is not a sufficient basis for reliable discrimination (Kwok *et al*., 1994); in fact, this is the main reason that allele-specific primer techniques are not widely used. Otherwise, despite having one SNP in the 3' position on the forward primer, SALI2F-SALI2R and SARCO3F-SARCO3R showed a good match in controls of the genera. However, large genus differences were demonstrated efficiently with SARCO1F-SARCO1R and SALI3F-SALI4R compared to other primer pairs. These primer pairs performed optimally because three consecutive SNPs from the 3' position were considered in the process of primer design. The high specificity of primer pairs SARCO1F-SARCO1R and SALI3F-SALI4R for genus detection is due to an increase in the number of SNP targets which were designed, such as forward and reverse primers.

To evaluate PCR amplification with a positive control fragment (*trnL* intron), primers selected to identify *Sarcocornia* and *Salicornia* were tested in a multiplex PCR assay including the *trnL* primer pair in the same reaction. The results indicated that the SARCO1F-SARCO1R and SARCO3F-SARCO3R primer pairs produced amplification of PCR products in *Sarcocornia* samples, whereas no amplification was observed in *Salicornia* controls. Likewise, the SALI2F-SALI2R and SALI3F-SALI4R primer pairs resulted in amplification in the *Salicornia* controls, but not in the *Sarcocornia* control. The *trnL* positive control primer pair was observed in all DNA samples mentioned above in a single band at ~110 pb (Figure 4A).

In another test, the diagnostic PCR by allele-specific primers (SARCO3F, SARCO1R and

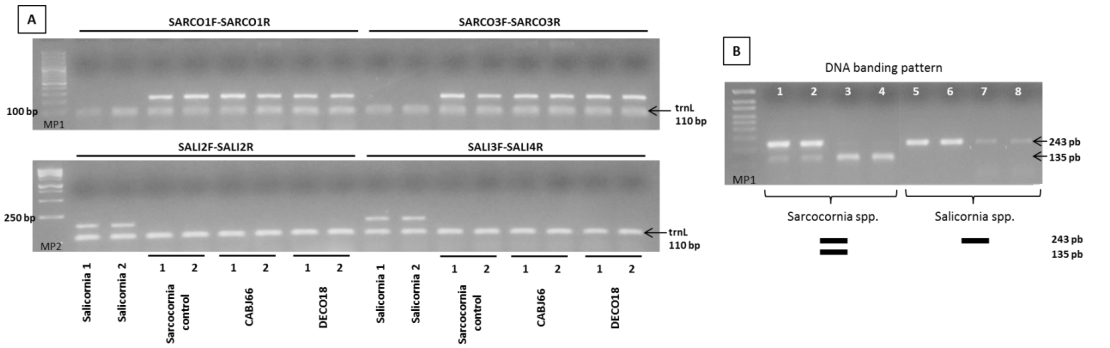

Figure 4: Example of diagnostic PCR with *Salicornia* and *Sarcocornia*. A) Electrophoresis of DNA amplified fragments obtained from *Salicornia* 1 control, *Salicornia* 2 control, *Sarcocornia* control (Puerto Montt), CABJ66 and DECO18 by means of multiplex PCR with a primer set of the *trnL* region and primer pairs SARCO1F-SARCO1R (Ta=54°C), SARCO3F-SARCO3R (Ta=57°C), SALI2F-SALI2R (54°C) and SALI3F-SALI4R (Ta=54°C). B) Agarose gel electrophoresis of amplified fragments with two replicates from *Sarcocornia* controls (1, 2 = *Sarcocornia* of Puerto Montt; 3, 4 = Sarcocornia of Spain) and *Salicornia* controls (5, 6 = *Salicornia* 2 control; 7, 8 = *Salicornia* 1 control) and with three primers used for allele-specific PCR (SARCO3F, SARCO1R and SARCO3F). MP1: 100 bp DNA ladder and MP2: 1 kb DNA ladder.

SARCO3F) were used to amplify *Sarcocornia* and *Salicornia* DNA in a simultaneous reaction. The result confirmed the presence of one DNA banding pattern for *Sarcocornia* and one DNA banding pattern for *Salicornia*: the former pattern with bands of 243 and 135 bp and the latter with only one band of 243 bp (Figure 4B). However, diagnostic PCR based on Spanish *Sarcocornia* DNA produced a weak 234 bp band and a strong 135 bp band. The DNA of *Sarcocornia* showed a band of 135 bp, while this was not observed in *Salicornia*. In brief, using these specific primer pairs in a PCR reaction followed by agarose gel visualization offers a reliable method for identification of *Sarcocornia* and *Salicornia*; additionally, the detection and validation of SNPs from ETS sequences of these genera, described by Kadereit *et al*. 2007, was confirmed.

Using primer pairs appropriate for the identification of both genera, we tested 82 samples from the Atacama region, seven samples from the Coquimbo region, and two samples each from the Los Lagos region and from Spain. The results showed positive amplification in all plants (82) from the Atacama region using the SARCO1F-1R and SARCO3F-3R primer pairs, however two DNA samples (DECO23 and DECO32) did not show amplification, and one sample (DECO21) showed two PCR fragments using the SARCO3F-3R primer pair (Table 3). The DNA samples from the Coquimbo region, the Los Lagos region and Spain had positive amplification using the SARCO1F-1R and SARCO3F-3R primer pairs (Table 3). None of the DNA samples from any of the regions demonstrated amplification with the control primer SALI 2F-2R and SALI 3F-4R, although only the CABJ74 sample showed one PCR fragment similar to the expected size with SALI2F-2R. The results of the present study are consistent with the report of Alonso and Crespo (2008), indicating the predominance of *Sarcocornia* in Chile; this suggests that the approach is efficiently designed for the detection of plants of this genus. In addition, we believe that the method could theoretically be applied in other parts of the world, since the primer pairs were designed from representative *Sarcocornia* ETS sequences of three continents.

In a further step, this PCR diagnostic method was applied using *Sarcocornia* tissue from commercial products in which clear identification of genus (data not shown) was possible. This discrimination method could therefore potentially be useful for market quality control purposes, particularly given that distinctions on the basis of morphological characteristics (flower) and growth habit (area) are obviously not feasible in the case of final packaged products (sea asparagus). The potential for a molecular approach in this context is of particular interest considering the differences in nutritional and agronomic characteristics of the genera; *Sarcocornia* has a saltier taste than *Salicornia,* verified by an elevated EC (electrical conductivity) value in shoots immersed in seawater (Ventura *et al*., 2011), and although both possess high total lipid content relative to other plants using culture in seawater, the *Salicornia* ecotype exceeds *Sarcocornia* in terms of total fatty acids and omega-3 percentage. Furthermore, the annual *Salicornia* ecotype has higher yields than perennial *Sarcocornia* ecotypes (Ventura *et al*., 2011). In summary, a PCR-based genotyping method has been developed to discriminate between *Salicornia* and *Sarcocornia* using only DNA, PCR and electrophoresis.

Conclusion

In this study, a simple and reliable method through four specific-primers (SALI2F-2R, SALI3F-4R, SARCO1F-1R and SARCO3F-3R), multiplex PCR and allele specific-primers has been developed to discriminate between *Salicornia* and *Sarcocornia*.

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Table 3: Summary of the results of positive (+) and negative (-) PCR amplifications with different primer pairs on each sample.

	Primer pair					Primer pair			
Sample	SARCO 1F SARCO 1R (Ta=54°C)	SARCO 3F SARCO 3R (Ta=57.2°C)	SALI 2F SALI 2R (Ta=54°C)	SALI 3F SALI 4R (Ta=54°C)	Sample	SARCO 1F SARCO 1R (Ta=54°C)	SARCO 3F SARCO 3R (Ta=57.2°C)	SALI 2F SALI 2R (Ta=54°C)	SALI 3F SALI 4R (Ta=54°C)
RCOP02	+	+	-	-	CLTO55	+	+	-	-
RCOP03	+	+	-	-	CLTO56	+	+	-	-
RCOP04	+	+	-	-	CLTO57	+	+	-	-
RCOP05	+	+	-	-	CLTO58	+	+	-	-
PVCO08	+	+	-	-	CLTO59	+	+	-	-
PVCO09	+	+	-	-	CLTO60	+	+	-	-
PVCO10	+	+	-	-	CLTO61	+	+	-	-
PVCO11	+	+	-	-	CLTO62	+	+	-	-
PVCO12	+	+	-	-	CLTO63	+	+	-	-
DECO13	+	+	-	-	CLTO64	+	+	-	-
DECO14	+	+	-	-	CABJ65	+	+	-	-
DECO15	+	+	-	-	CABJ66	+	+	-	-
DECO16	+	+	-	-	CABJ67	+	+	-	-
DECO17	+	+	-	-	CABJ68	+	+	-	-
DECO18	+	+	-	-	CABJ69	+	+	-	-
DECO19	+	+	-	-	CABJ70	+	+	-	-
DECO20	+	+	-	-	CABJ71	+	+	-	-
DECO21	+	+(*)	-	-	CABJ72	+	+	-	-
DECO22	+	+	-	-	CABJ73	+	+	-	-
DECO23	+	-	-	-	CABJ74	+	+	+(**)	-
DECO24	+	+	-	-	CABJ75	+	+	-	-
DECO25	+	+	-	-	CABJ76	+	+	-	-
DECO26	+	+	-	-	CABJ77	+	+	-	-
DECO27	+	+	-	-	CABJ78	+	+	-	-
DECO28	+	+	-	-	CABJ79	+	+	-	-
DECO29	+	+	-	-	PBTO81	+	+	-	-
DECO30	+	+	-	-	PBTO82	+	+	-	-
DECO31	+	+	-	-	PBTO83	+	+	-	-

Continuación Table 3:

	Primer pair					Primer pair			
Sample	SARCO 1F SARCO 1R (Ta=54°C)	SARCO 3F SARCO 3R (Ta=57.2°C)	SALI 2F SALI 2R (Ta=54°C)	SALI 3F SALI 4R (Ta=54°C)	Sample	SARCO 1F SARCO 1R (Ta=54°C)	SARCO 3F SARCO 3R (Ta=57.2°C)	SALI 2F SALI 2R (Ta=54°C)	SALI 3F SALI 4R (Ta=54°C)
DECO32	+	-	-	-	PBTO84	+	+	-	-
DECO33	+	+	-	-	PBTO85	+	+	-	-
DECO34	+	+	-	-	PBTO86	+	+	-	-
DECO37	+	+	-	-	PBTO87	+	+	-	-
DECO38	+	+	-	-	VAHS88	+	+	-	-
DECO39	+	+	-	-	VAHS89	+	+	-	-
DECO43	+	+	-	-	VAHS90	+	+	-	-
DECO44	+	+	-	-	VAHS91	+	+	-	-
DECO45	+	+	-	-	VAHS92	+	+	-	-
CLTO47	+	+	-	-	LASE93	+	+	-	-
CLTO48	+	+	-	-	LASE94	+	+	-	-
CLTO49	+	+	-	-	LASE95	+	+	-	-
CLTO50	+	+	-	-	LASE96	+	+	-	-
CLTO51	+	+	-	-	LASE97	+	+	-	-
CLTO52	+	+	-	-	LASE98	+	+	-	-
CLTO53	+	+	-	-	LASE99	+	+	-	-
CLTO54	+	+	-	-					
Spain Sarcocornia1	+	+	-	-	Sarcocornia Puerto Mo.	+	+	-	-
Spain Sarcocornia2	+	+	-	-	Sarcocornia Puerto Mo.	+	+	-	-
Salicornia 1 control	-	-	+	+	Salicornia 2 control	-	-	+	+

(*) two fragments amplified

(**) one fragment amplified similar to expected size.

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