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Molecular markers indicate the phylogenetic identity of southern Brazilian sea asparagus: first record of *Salicornia neei* in Brazil

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Abstract

Molecular phylogenetic analyses based on ETS, ITS and *atpB-rbcL* spacer sequences assessed the phylogenetic status of the southern Brazil sea asparagus species of the genus *Salicornia* (Salicornioideae, Amaranthaceae). Accessions of Patos Lagoon estuary (32° S) were obtained from wild plants and two pure line lineages, selected from contrasting prostrate (BTH1) and decumbent (BTH2) ecomorphotypes found locally. Patos Lagoon wild plants, BTH1 and BTH2 f4 progenies showed 100% identical sequences for the *atpB-rbcL* and ITS spacers, only two mutations for ETS. Comparison of the sequences of these three markers with GenBank records confirmed the identity of Brazilian accessions as *Salicornia neei*. Maximum-likelihood phylogenetic analysis of ETS sequences indicated that the southern Brazilian accessions of *Salicornia* certainly are not close to any of the *Salicornia ambigua* accessions in GenBank, which are restricted to the northern hemisphere, nor are they related to any *Salicornia fruticosa*/*Salicornia perennis* clade accessions, which are also restricted to Eurasia. All above cited species have been wrongly applied to the southern Brazil sea asparagus.

Key words: DNA sequences, Salicornioideae, *Sarcocornia*, halophyte, salt marshes.

Resumo

Análises filogenéticas moleculares baseadas em sequências das regiões espaçadoras ETS, ITS and *atpB-rbcL* avaliaram o status filogenético da espécie de aspargo marinho do gênero *Salicornia* (Salicornioideae, Amaranthaceae) presente no sul do Brasil. Os acessos do estuário da Lagoa dos Patos (32° S) foram obtidos de uma população selvagem e de duas linhagens puras de aspargo marinho, selecionadas de ecomorfotipos contrastantes com forma de crescimento prostrada (BTH1) e decumbente (BTH2) encontrados localmente. Plantas selvagens do estuário da Lagoa dos Patos e as progênies f4 de BTH1 e BTH2 mostraram sequencias 100% idênticas para a região espaçadora *atpB-rbcL* e ITS, apenas duas mutações em ETS. A comparação das sequências desses três marcadores com registros do GenBank confirmou a identidade dos acessos como *Salicornia neei*. Análise filogenética pelo método de máxima verossimilhança das sequências ETS indicou que os acessos de *Salicornia* do sul do Brasil certamente não são semelhantes a nenhum acesso de *Salicornia ambigua* no GenBank, os quais possuem distribuição restrita ao hemisfério norte, e nem são relacionados com qualquer acesso do clado *Salicornia fruticosa*/*Salicornia perennis*, este último restrito a Eurásia. Todas as espécies citadas acima têm sido erroneamente consideradas como sinonímias para o aspargo marinho do sul do Brasil.

Palavras-chave: sequências de DNA, Salicornioideae, *Sarcocornia*, halófitas, marismas.

Introduction

The small succulent shrubs with leafless stems and branches of the genera *Salicornia* L. and *Sarcocornia* A.J.Scott (Salicornioideae, Amaranthaceae) are characterized as "extreme halophytes" based on their ability to thrive in seawater-flooded and hypersaline soils (Davy *et al.* 2006; Steffen *et al.* 2015). These halophytes have been consumed by humans for centuries, and to date are cultivated with a broad range of

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saline water sources and sold as 'sea asparagus' or 'samphire', particularly used as functional gourmet food, animal feed meal and oils for biodiesel (Ventura & Sagi 2013; Ventura *et al.* 2015; Costa & Herrera 2016).

The paucity of characters due to the strongly reduced morphology of *Salicornia* and *Sarcocornia*, the great phenotypic plasticity (Kadereit *et al.* 2006; Alonso & Crespo 2008; Doncato & Costa 2018) and high intrapopulation genetic diversity in addition to the formation of hybrids between sympatric species through polyploidy (Davy *et al.* 2006; Steffen *et al.* 2015) generate a notorious difficult taxonomy making it almost impossible for non-specialists to determine most species of these genera. Molecular makers have been used to clarify the phylogeny and biogeography of the subfamily Salicornioideae (Kadereit *et al.* 2006; Steffen *et al.* 2015; Piirainen *et al.* 2017). In a molecular phylogeny based on Internal Transcribed Spacer (ITS) and *atp*B-*rbc*L spacer (chloroplast DNA) sequences of *Sarcocornia* formed a monophyletic lineage together with the genus *Salicornia*, which is clearly separated from the genus *Arthrocnemum* (R.Br.) Moq. (Kadereit *et al.* 2006). External Transcribed Spacer (ETS) and *matK-trnK* intron (chloroplast DNA) sequence data showed that *Salicornia* is clearly nested in *Sarcocornia* and sister to a clade comprising American and Eurasian *Sarcocornia* (Kadereit *et al.* 2007; Piirainen *et al.* 2017). In the latest worldwide molecular phylogenetic treatment of Salicornioideae, Piirainen *et al.* (2017) showed that *Sarcocornia* is paraphyletic in relation to *Salicornia* and proposed a new taxonomic classification, merging of *Sarcocornia* in three subgenera under *Salicornia* affecting 19 new nomenclatural combinations and one replacement name. In the present study, we followed the nomenclature proposed in Piirainen *et al.* (2017).

In the most recent taxonomic classification of the genus *Salicornia* in South America based on morphological features, Alonso & Crespo (2008) accepted five perennial species of sea asparagus (originally cited as *Sarcocornia*) and cited the synonyms and types for the accepted taxa: *Salicornia ambigua* Michx., *S. andina* Phil., *S. magellanica* Phil., *S. neei* Lag. and *S. pulvinata* R.E.Fr. Alonso *et al.* (2017) described a sixth perennial species named *Salicornia cuscoensis* Gutte & G.K. Müll. found in high Andean saltmarshes in Peru, the identity of which is supported by differences in morphological characters and molecular analyses. Previously, Scott (1977) recognized only two species of perennial *Salicornia* in South America (*S. fruticosa* and *S. pulvinata*). Similar annual *Salicornia* plants, these two species have leafless succulent stems but distinctly unequal sized flowers in their cymes with a triangular arrangement.

Salicornia ambigua is the only species of sea asparagus listed for Brazil (Alonso & Crespo 2008). It was regarded as a synonym for *Salicornia gaudichaudiana* Moq., *Sarcocornia ambigua* (Michx.) M.A.Alonso & M.B.Crespo, the Mediterranean species *Sarcocornia fruticosa* (L.) A.J.Scott (Scott 1977), *Salicornia fruticosa* L. (BFG 2018) and *Salicornia perennis* Mill. (=*Sarcocornia perennis* (Miller) A.J.Scott) (Costa *et al.* 2006; Isacch *et al.* 2006; Davy *et al.* 2006) or the North American *Salicornia virginica* L. (Reitz 1961). Additionally, many plants referred to *Salicornia ambigua* Michx. and *Salicornia fruticosa* (=*Sarcocornia fruticosa*) on the coasts of Uruguay and NE Argentina are indistinguishable from southern Brazilian sea asparagus (Costa & Davy 1992; Davy *et al.* 2006; Isacch *et al.* 2006). Steffen *et al.* (2015) using molecular phylogenetic analyses based on ETS, *atp*B-*rbc*L and *rpl*32-*trn*L characterized *S. fruticosa* and *S. perennis* as exclusively Eurasian and partially supported Alonso & Crespo (2008) classification of South American *Salicornia*, but no accessions from the Brazilian coast was assessed.

Although both morphological (Scott 1977; Alonso & Crespo 2008) and molecular studies (Steffen *et al.* 2015; Piirainen *et al.* 2017) recognized the occurrence of *Salicornia ambigua* in Americas, they worked with specimens from distinct locations and poorly sampled the Brazilian coast. Alonso & Crespo (2008) recognized Brazilian plants as *S. ambigua* based on an over 45 years-old herbarium specimen with slender woody stems at the base, and long and narrow terminal inflorescence that produced small seeds with short appressed hairs mostly on the edge. Their sampling of *S. ambigua* pointed out the southernmost records to Mar del Plata in the Argentinian province of Buenos Aires (39° S), and they also examined specimens of this taxon collected in the Caribbean region of Dominican Republic (19° N). The original description of "*Salicornia ambigua*" was from plants from North American "Carolinas" (Michaux, 1803 sec. Alonso & Crespo 2008) and Steffen *et al.* (2015) highlighted that in Florida *S. ambigua* occurs in extensive tidal flats. The *S. ambigua* accessions analyzed in Steffen *et al.* (2015) were collected from the Caribbean region and the east Atlantic coast of U.S.A, and plants from Atlantic coast of Argentina turned out to belong to the *S. neei*-*S. pacifica*-*S. magellanica* lineage. Alonso & Crespo (2008) also sampled and listed *S. neei* and *S. magellanica* for the Atlantic coast of South America. *Salicornia magellanica* that would be endemic to southern Argentina and southern Chile, and the widely spread *S. neei* found southwards of Buenos Aires province and saline continental lowlands of Argentina (except for Strait of Magellan area), as well as in the Pacific coast of Chile and Peru. In their molecular study Steffen *et al.* (2015) recognized the phylogenetic affinity of these two species and their occurrence in the area described by Alonso & Crespo (2008).

The taxonomy and the geographic distribution of perennial *Salicornia* species in the Atlantic coast of South America are still controversial. The morphological identification of dried herbarium specimens can be very difficult, and the Brazilian coast represents a biogeographic gap in molecular studies of Salicornioideae in Americas. The Brazilian sea asparagus of the genus *Salicornia* shows a recent history of successful cultivation with saline water and shrimp farm effluent (Costa & Herrera 2016), high nutritional quality (Bertin *et al.* 2014; Costa *et al.* 2014) and chemical characteristics for biofuel production (D'Oca *et al.* 2012; Costa *et al.* 2014). However the designation of plants found in Brazil as *Salicornia ambigua* (=*Sarcocornia ambigua*) is uncertain. The proper taxonomic identification and the establishment genetically defined lines of the Brazilian sea asparagus of the genus *Salicornia* are necessary for the development of breeding programs that produce plants with desirable agricultural traits. Using molecular phylogenetic analyses based on ETS, ITS and *atpB*-*rbcL* spacer sequences, the present study is aimed at clarifying the phylogenetic status of the southern Brazil *Salicornia* population by comparison with GenBank records.

Materials and Methods

Plant material

The wild plant accessions of *Salicornia* were collected in the salt marsh of Pólvora Island (32º01' S, 52º06' W) at Patos Lagoon estuary (located in the state of Rio Grande do Sul, of the southern Brazil) and 8 weeks-old accessions of f4 progenies of the *Salicornia* lineages BTH1 and BTH2 were obtained from the germplasm of Laboratório de Biotecnologia de Halófitas (Instituto de Oceanografia, FURG, Rio Grande, RS, Brazil). These two lineages represent contrasting prostrate and decumbent plants with reddish and green colorations, respectively, obtained by a 5 year breeding program based on pure line selection from ecomorphotypes found in local estuarine habitats subject to distinctive flooding regimes and salt stresses (Freitas & Costa 2014; Doncato & Costa 2018). Since decumbent-prostrate growth forms found within different lineages of other *Salicornia* species might represent different stages of speciation (Steffen *et al.* 2015), the DNA analysis of BTH1 and BTH2 lineages was carried out in order to assess the range of genetic variation found in southern Brazil *Salicornia* populations. Vegetative stem segments harvested from the three accession groups (wild, BTH1 and BTH2) were separated *in situ*, placed in vials, frozen in liquid nitrogen, and kept at -80 ºC until DNA extraction.

DNA extraction and DNA sequences

Total DNA was extracted from 50 mg of frozen stem material using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following a conventional hexadecyl-trimethyl-ammonium bromide (CTAB) DNA extraction protocol (Doyle 1991). PCR was carried out in a T-Professional or T-Gradient Thermocycler (Biometra, Göttingen, Germany). We used the same primer sequences, PCR recipe and cycler programme for each marker applied by Steffen *et al.* (2015). We selected one variable chloroplast (cp) marker, *atpB-rbcL* spacer, and ITS and ETS ribosomal DNA markers as the most variable nuclear markers known for Salicornioideae (Kadereit *et al.* 2007, 2012; Steffen *et al.* 2015; Piirainen *et al.* 2017). For details of primer sequences see Kadereit *et al.* (2006) and Steffen *et al.* (2015). PCR products were checked on 1 % agarose gels and purified subsequently using the NucleoSpin Gel and PCR clean-up-Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's manual. DNA sequences were obtained using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in combination with the primers mentioned above following a purification step using an Illustra SephadexTM G-50 Fine DNA Grade (GE Healthcare, Little Chalfont, UK). DNA fragments were sequenced using an automatic capillary sequencer GA3130XL (Applied Biosystems) following the Sanger method. Forward and reverse sequences were edited and merged to consensus sequences, which then were aligned using

Sequencher 4.1.4 (Gene Codes Corp., Ann Arbor, MI, USA). All alignments were checked and corrected manually. ITS, atpB-rbcl spacer and ETS sequences of Brazilian accessions have been deposited in the GenBank database (accession numbers; BTH1= MG579955-MG579957; BTH2= MG579958-MG579960; Wild= MG579961- MG579963).

Data analysis

The chloroplast data set consisting of the *atpB-rbcL* spacer and the nuclear data set consisting of ITS (ITS-28S forward and ITS-18S reverse) and ETS sequences were analysed separately. Since the cp and ITS data were mostly uninformative among *Salicornia* samples from southern Brazil, phylogenetic analysis was performed using only ETS data. Maximum-likelihood (ML) phylogenetic analysis including bootstrapping was performed using MEGA7 (Kumar *et al.* 2016). The ETS analysis involved 46 nucleotide sequences. There were a total of 503 positions in the final dataset. Bootstrap support was calculated using ten random additions of taxa in 1000 bootstrap replicates and bootstrap reliability percentages of the clusters descending from each node of the tree were also calculated. We found T92 (substitution model parameters Tamura 3) with a Bayesian Information Criterion (BIC) score equal to 21142.9, transition/transversion bias (R) equal to 0.66, and nucleotide substitution rates ranging between 0.072 and 0.104 as the best evolutive model to our sequences.

Besides the 3 accessions from southern Brazil, we included DNA sequences of all coastal species of perennial *Salicornia* found in Americas, *S. perennis* and *S. fruticosa* (previously cited as synonyms for *S. ambigua*), South African *Salicornia* species (*S. natalensis*, *S. pillansii*, *S. tegetaria* and *S. capensis*) and, representative species of annual *Salicornia*, obtained from different studies through their GenBank records and available from Steffen *et al.* (2015). Representatives of *Microcnemum* Ung.-Sternb., *Arthrocaulon* Piirainen & G. Kadereit (~*Arthrocnemum* Moq.) and *Tecticornia*, were included as outgroups (Kadereit *et al.* 2006; Steffen *et al.* 2015). Altogether 46 accessions were included in the phylogenetic analysis representing approx. 10 species of perennial *Salicornia*, approx. 5 species of annual *Salicornia* and 4 outgroups (Tab. 1).

Results

Accessions of Patos Lagoon wild plant, BTH1 and BTH2 f4 progenies showed 100% identical sequences for *atpB-rbcL* and ITS spacers, which included 709 and 647 aligned nucleotide positions, respectively (Tab. 2). The *atpB-rbcL* spacer and ITS sequences are nearly identical to published sequences of *S. neei*/*S. magellanica* clade, with the highest score for *S. neei*.

Table 1 – List of sequenced accessions of external transcribed spacer (ETS) with lab code, voucher information, life form (p= perennial; a= annual) and GenBank accession numbers.

Taxon	Life form	GenBank	Voucher information (Collector; collection no.; deposited at; Country; State/Prov.; Locality)
Salicornia ambigua Michx.	p	EF433591	P. Teege; 05-US-004; MJG; USA; Massachusetts; Chatham
Salicornia ambigua Michx.	p	KM984569	Reznicek & Gregory; 27377; TRTE (5821); USA; Florida; Key Largo
Salicornia ambigua Michx.	p	KM984570	P.W. Ball; 94235; TRTE 70668; USA; Virginia; Gloucester County
Salicornia ambigua Michx.	p	KM984571	Shumway; 30896; CAS (990690); USA; New Jersey; Ocean City
Salicornia ambigua Michx.	p	KM984572	C.J. Rothfels; 2451; TRTE 67174; USA; North Carolina - Pender; Topsail Island
Salicornia ambigua Michx.	p	KM984573	C.J. Rothfels; 2449; TRTE 67175; USA; North Carolina; Pender, Surf City
Salicornia ambigua Michx.	p	KM984574	Luteyn; 2823; Duke 221694; USA; Florida, Gulf county, 5 miles south of Port St. Joe
Salicornia ambigua Michx.	p	KM984575	Luteyn; 2844; Duke 221663; USA; Florida; Taylor county, Keatonsbeach
Salicornia capensis (Moss) Piirainen & G.Kadereit	p	KM984583	L. Mucina; 081200/2; MJG; R.S.A; Western Cape; Darling
Salicornia capensis (Moss) Piirainen & G.Kadereit	p	KM984581	L. Mucina; 7200/5; MJG; R.S.A; Western Cape; Franskraal, Uilenkraalsmond

Taxon	Life form	GenBank	Voucher information (Collector; collection no.; deposited at; Country; State/Prov.; Locality)
Salicornia capensis (Moss) Piirainen & G.Kadereit	p	KM984582	S. Steffen & L. Mucina; 040406/12; MJG; R.S.A; Western Cape; Agulhas Plain, De Mond N.R., Heunignes Estuary
Salicornia capensis (Moss) Piirainen & G.Kadereit	p	KM984584	S. Steffen & L. Mucina; 140406/03; MJG; R.S.A; Western Cape; Darling, Tienie Versveld Flower Reserve, at the entrance to the reserve
Salicornia capensis (Moss) Piirainen & G.Kadereit	p	EF433594	L. Mucina 7202/2 (MJG, STEU); South Africa: Western Cape Prov., Gansbaai,Uilkraalsmond
Salicornia cf. *fruticosa* L.	p	KM984600	P. F. Maycock; 3.10.2009; TRTE 69389; Greece; Paros; SW of Nagussa
Salicornia cf. *fruticosa L.*	p	KF427921	Y. Ventura and M. Sagi; 23.05.2012 type VM; Israel; Ramat Negev, Red Sea area
Salicornia cf. *fruticosa L.*	p	KF427919	Y. Ventura and M. Sagi; 23.05.2012 type IS; Israel; Ramat Negev, inland salt flat
Salicornia magellanica Phil.	p	KM984608	Goodall; 2573; MICH 1215807; Argentina; s.n.; Tierra del Fuego, N.W. part of San Sebastian Bay
Salicornia magellanica Phil.	p	KM984609	S. Pfanzelt, E. Teneb & N. Bahamonde; 696; MJG; Chile; Region de Magallanes; San Juan, south of Punta Arenas
Salicornia natalensis Bunge *ex.* Ung.-Sternb. subsp. *natalensis*	p	KM984627	L. Mucina; 6937/1; MJG; R.S.A; Eastern Cape; Jeffery Bay, Paradise Beach
Salicornia natalensis Bunge ex. Ung.-Sternb. subsp. natalensis	p	KM984615	S. Steffen & V. Koecke; 090506/1; MJG; R.S.A; Eastern Cape; Boesmansriviermond, Bushmans River estuary
Salicornia neei Lag.	p	KM984628	Bartlett; 19902; MICH 1215815 ; Argentina; Buenos Aires; Pedro Luro, Laguna La Salada
Salicornia neei Lag.	p	KM984631	Schinini; 16576; F 1881258; Paraguay; Dept. Pte Hayes;
Salicornia neei Lag.	p	KM984633	Ricardi; 9126; B; Chile; Prov. Arauco; Márgenes del río Raqui
Salicornia neei Lag.	p	MG579957	C.S.B. Costa; chen 3336; BTH1 f4 progeny; FURG BTH Lab germoplam; Brazil; RS; Rio Grande; Patos Lagoon estuary.
Salicornia neei Lag.	p	MG579960	C.S.B. Costa; chen 3341; BTH2 f4 progeny; FURG BTH Lab germoplam; Brazil; RS; Rio Grande; Patos Lagoon estuary.
Salicornia neei Lag.	p	MG579963	C.S.B. Costa; chen 3346; FURG BTH Lab germoplam; Brazil; RS; Ilha da Pólvora; Patos Lagoon estuary.
Salicornia pacifica Standl.	p	KM984647	D. Valov; chen 2278; voucher info pending.
Salicornia pacifica Standl.	p	KM984644	R. Halse; 2905; UC/JEPS, OSC 166680; USA; Washington, Mason Co.; Mike's Beach Resort, about 10 miles north of Lilliwaup (2.5 miles north of Eldon) along U.S. Hwy. 101
Salicornia perennis Mill.	p	KM984656	G. Kadereit & J.W. Kadereit; 2006/32; MJG (041172); Spain; Alicante; El Hondo, Laguna del Fondo.
Salicornia perennis Mill.	p	KM984658	Lykke; 801; AAU; Senegal; Sine Saloum; Ile de Sangomar, Delta de Saloum National Park.
Salicornia pillansii (Moss) Piirainen & G.Kadereit	p	KM984674	S. Steffen & V. Koecke; 200406/08; MJG; R.S.A; Western Cape; Cape Town, Milnerton, Rietvlei Nature Reserve, close to parking area
Salicornia pillansii (Moss) Piirainen & G.Kadereit	p	KM984675	S. Steffen & L. Mucina; 030506/01; MJG; R.S.A; Western Cape; Witsand, Breede River estuary, near jetty
Salicornia pillansii (Moss) Piirainen & G.Kadereit	p	KM984676	L. Mucina; 6926/1; MJG; R.S.A; Eastern Cape; Addo Soutkloof
Salicornia pillansii (Moss) Piirainen & G.Kadereit	p	KM984677	Walter& Mucina; 5119; MJG; R.S.A; Western Cape; Cape Town, Milnerton, Rietvlei N.R.
Salicornia pillansii x *tegetaria*	p	KM984681	S. Steffen & L. Mucina; 140406/09; MJG; R.S.A; Western Cape; West Coast National Park, E of Seeberg Bird Hide

Taxon	Life form	GenBank	Voucher information (Collector; collection no.; deposited at; Country; State/Prov.; Locality)
Salicornia tegetaria x *natalensis*	p	KM984692	Walter& Mucina; 5107/8; MJG; R.S.A; Western Cape; Gansbaii, Franskraal
Salicornia bigelovii Torr.	a	KM984699	A. Juan; s.n.; MJG; Mexico; Baja California; s.n.
Salicornia borealis S.L.Wolff & Jefferies	a	EU000543	S. Wolff s.n. (TRT 243075, syntype); Canada, Manitoba, La Perouse Bay
Salicornia depressa Standley	a	EF433621	D. Stone; 2571; MJG; USA; California; Napa County, 7 min West of Vallejo
Salicornia europaea L.	a	EF433644	B. Böer s.n., 1996 (KAS); United Arab Emirates, Ras-al-Khaimah
Salicornia europaea L.	a	EF433643	P. Teege HK A_20 (MJG); chen HK A_20; Germany, North Sea Schleswig-Holstein, Büsum
Salicornia persica Akhani subsp. *persica*	a	EU000549	Moazzeni; 35040; MJG (040977); Iran; s.n.; Fars, Shiraz, Pol-e Fasa
Arthrocaulon macrostachyum (Moric.) Piirainen & G.Kadereit	p	KM984564	H. Freitag & N. Adigüzel; 28846; KAS, GAZI; Turkey; Seyhan Prov.; SE Adana
Microcnemum coralloides (Loscos & Pardo) Buen subsp. *coralloides*	a	EF433589	G. Kadereit; 2002/15; MJG; Spain; Aragón; Acaniz, Laguna de Guallar
Tecticornia australasica (Moq.) Paul G. Wilson	a	EF433590	S. Jacobs; 8685; NSW; Australia; Queensland; N of Townsville
Tecticornia indica (Willd.) K.A. Sheph. & Paul G. Wilson	p	EF433588	S. Jacobs; 9135; NSW; Australia; Queensland; Bowen

Table 2 – Number of aligned nucleotides, aligned position of detected mutations and mutated nucleotide sequences for three markers of southern Brazilian accessions of *Salicornia* (wild of the Pólvora Island, BTH1 and BTH2). Positions of variable nucleotides among accessions in italic and bold.

Molecular markers *	Number of aligned nucleotide	Mutated nucleotide positions	Accession / Mutated nucleotide sequence
AtpB-rbcL spacer	709	none	none
Internal transcribed spacer (ITS)	647	none	none
External transcribed spacer (ETS)	503	199	Wild - GGTATCGTTG***T***TGTCCCTCTAA
		199	BTH1 - GGTATCGTTG***G***TGTCCCTCTAA
		199	BTH2 - GGTATCGTTG***T***TGTCCCTCTAA
		239	Wild - ACATTTGTAA***T***AGGAGGTCG
		239	BTH1 - ACATTTGTAA***C***AGGAGGTCG
		239	BTH2 - ACATTTGTAA***C***AGGAGGTCG

* Lab codes and GenBank accession numbers of ITS, atpB-rbcl spacer and ETS sequences: Wild, chen 3346 (MG579961- MG579963); BTH1, chen 3336 (MG579955-MG579957); BTH2, chen 3341 (MG579958-MG579960).

In the ETS marker two mutations were found among the 503 aligned positions of southern Brazilian sequences. Accessions differed at nucleotide positions 199 and 239; BTH1 and BTH2 differed in one nucleotide and they had, respectively, 2 and 1 polymorphic nucleotides in relation to the wild accession (Tab. 2). ETS marker allows us to confirm the identity of Brazilian accessions as *S. neei*. The BLAST search in GenBank showed that accession KM984631 from Paraguay Charco or accession KM984633 from the margins of Raqui river estuary (south-central Chilean coast) are the closest matches (Fig. 1; Tab. 1).

The maximum likelihood tree of ETS sequences indicates that the coastal species of the *Salicornia* genus in Americas are mainly divided in two major sister clades showing a geographical signal in the phylogenetic relationships of the American species: East coast of North America clade with *S. ambigua*, and West-East coasts of South American clade with *S. neei*, *S. pacifica* and *S. magellanica*. Analysis of ETS sequences clearly distinguishes (bootstrap percentage = 100) between *S. ambigua* and *S. neei*/*S. pacifica*/*S. magellanica* clades at the molecular level (Fig. 1).

Discussion

Molecular markers confirm the identity of southern Brazilian accessions as *S. neei* and show (ETS) small differences among their nucleotides sequences. Previously ITS showed limited variability for other *Salicornia* species (Kadereit *et al.* 2006; de la Fuente *et al.* 2013), and Steffen *et al.* (2015) concluded that *atpB-rbcL* spacer data were mostly uninformative among closely related species of *Salicornia*. Nucleotide substitutions on southern Brazilian *Salicornia* accessions support the genetic base of prostrate and decumbent growths in pure lineages of local ecomorphotypes. It contrasts to accessions of erect and prostrate forms of *Salicornia perennans* from NW Kazakhstan that showed no differences between their ETS sequences (Kadereit *et al*. 2007). However, Doncato & Costa (2018) showed that BTH1 and BTH2 sea asparagus lineages had consistent differences in biometrics, production and mineral composition of their biomass under field trials, which were maintained between consecutive progenies of their plants. The analysis of AFLP variation suggested that intraspecific ecotypes might originate repeatedly among some tetraploid species of European *Salicornia* in adaptation to their specific environments (Teege *et al.* 2011). After their molecular analysis of *Salicornia*, Steffen *et al.* (2015) hypothesized that locally adapted prostrate/mat-forming and erect/decumbent ecotypes evolved independently due to selective forces that arise in ecological gradients such as salinity, frequency and duration of flooding.

Current records indicate *Salicornia* species names recognized in South America (Alonso & Crespo 2008; BFG 2018; Steffen *et al.* 2015), of which *S. neei* is not mentioned as having been collected in Brazil, or even at the Atlantic coast further north of the La Salada lagoon (Pedro Luro, Buenos Aires Province, Argentina; 39° S; Steffen *et al.* 2015). La Salada lagoon accession (KM984628) also shows highly similar ETS sequences to southern Brazilian accessions (Fig. 1; Tab. 1). Alonso & Crespo (2008) recorded accessions of *S. neei* in the lowlands of central-south Argentina, reaching the Atlantic coast at Valdés Peninsula (Chubut province; ~42° S). Recently Arce *et al.* (2016) reported *S. neei* as an important feeding resource for sheep rearing in saline steppes and marshlands of Patagonia. *Salicornia neei* presents a wide morphological variation in many parts of its distribution, which, according to Alonso & Crespo (2008), explains its separation across many taxa. For instance, decumbent and prostrate ecomorphotypes of *S. neei* were also reported in populations at the Raqui river estuary (Chile; Centro EULA 2011). The present genetic record extends the distribution limit of *S. neei* to the subtropical coast of Brazil.

The southern Brazilian accessions of sea asparagus certainly are not close to any of the *S. ambigua* accessions in GenBank, which are restricted to the Northern Hemisphere. As pointed out by Steffen *et al.* (2015), American perennial *Salicornia* (=*Sarcocornia*) is clearly monophyletic and most likely derived from Eurasian ancestors. The ancestral area of the crown node of the American clade was reconstructed to be located in the Northern Hemisphere (either North America or Eurasia or both). Although *S. fruticosa*/*S. perennis* clade accessions were widely distributed, along the entire Mediterranean region and even reaching the surrounds of the Dead Sea and the north coast of Africa, these taxa are not present in South America. Similarly, Kadereit *et al.* (2007) and Piirainen *et al.* (2017), in the ETS tree, annual *Salicornia* is sister to a clade comprising Eurasian and North American perennial *Salicornia*.

Further sampling of wild plants of sea asparagus and molecular evaluation of these accessions across the entire coast of Brazil and in the northern part of South America are required, in order to clarify the distribution limits of *S. ambigua* and *S. neei* in the Atlantic region. These samples should be obtained together with detailed morphological

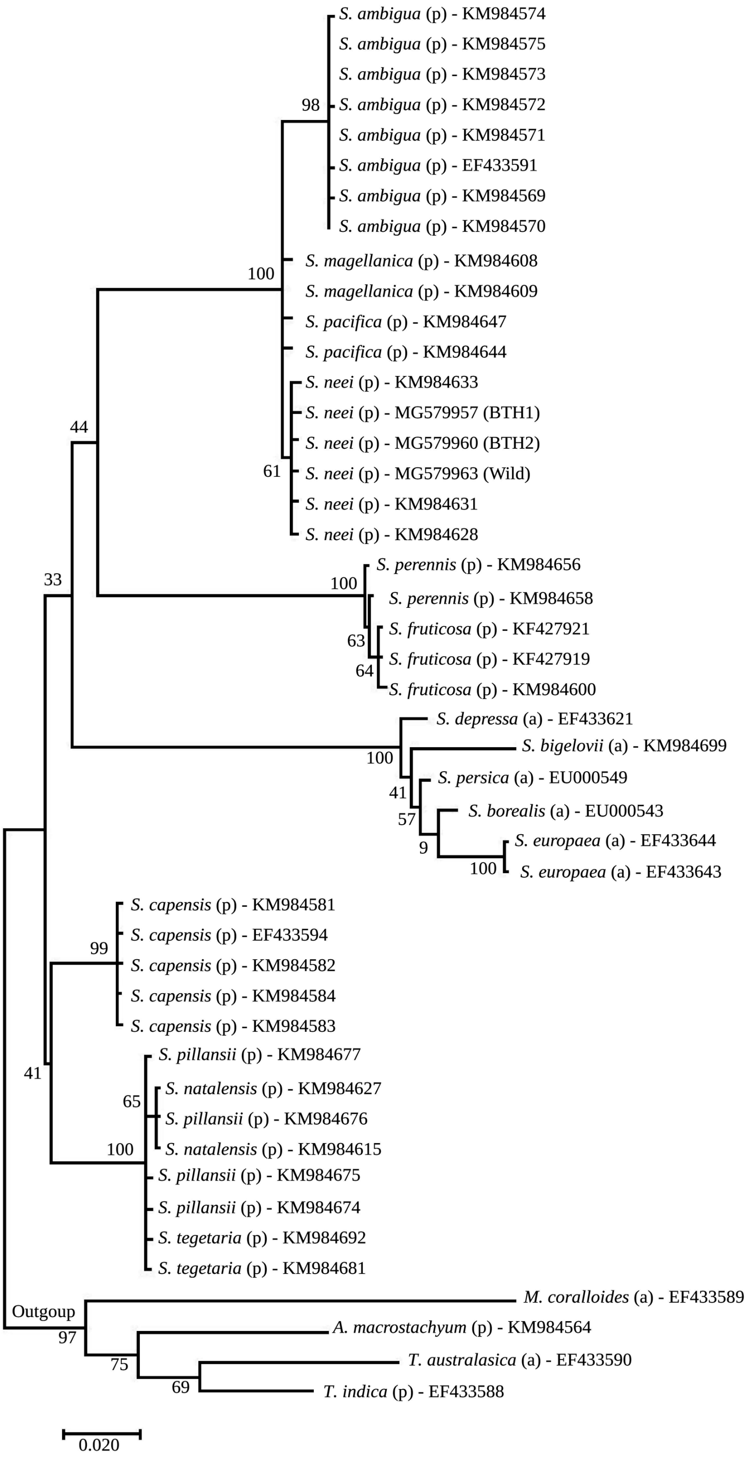

Figure 1 – Maximum likelihood tree based on 46 ETS sequences of *Salicornia* species; 3 ETS sequences are from accessions of southern Brazil obtained in this study (Wild, BTH1 and BTH2) and 43 ETS sequences from the NCBI database were included. Numbers near nodes are bootstrap values (in percentages) for the internal branches of the tree. The letters "a" and "p" indicate the annual and perennial habits of the species, respectively.

data (*i.e.*, growth habit, size of branches and inflorescence, spikes shape and seed indumentum) in order to support the taxonomic assignment. Also, more variable molecular markers using a next generation sequencing approach is needed to reveal the genetic diversification and phylogenetic relationships of *S. neei* and its close relatives.

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