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Species separation within the *Lessonia nigrescens* complex (Phaeophyceae, Laminariales) is mirrored by ecophysiological traits

Abstract: *Lessonia nigrescens* used to be an abundant kelp species along the Chilean coast, but recent molecular studies revealed the existence of a *L. nigrescens* species complex, which includes the two cryptic species *Lessonia berteroa* and *Lessonia spicata*. Since these species have different distributions (16°S–30°S for *L. berteroa* and 29°S–42°S for *L. spicata*), they experience differences in environmental conditions, such as solar irradiance, seawater temperature and air exposure during low tide. This study tested to what extent the genetic distinctness of each of the two species [identified by a mitochondrial marker (*atp8/trnS*)] is reflected by ecophysiological traits (total lipids, fatty acid composition, phlorotannins, pigments and variable chlorophyll *a* fluorescence of PSII) in response to the respective environmental conditions, prevailing along the latitudinal gradient. We studied algal individuals from eight populations (27°S–32°S,

including the species overlapping zone). Phlorotannins, pigments and Chl *a* fluorescence of PSII were most crucial for species-specific adaptations at the respective growth sites, whereas changes in total lipids and fatty acid compositions were negligible. Hence, species differentiation within the *L. nigrescens* complex is also manifested at the ecophysiological level. These findings may help to predict kelp responses towards future environmental changes.

Keywords: Chile; fatty acid composition; *Lessonia nigrescens* complex; phlorotannins; photosynthetic pigments.

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Introduction

Members of the kelp genus *Lessonia* (Bory de Saint-Vincent, 1825; Lessoniaceae, Laminariales) are distributed in the Pacific and Atlantic Oceans of the Southern Hemisphere. Along the coasts of Chile, Peru, Tasmania, New Zealand and the sub-Antarctic islands (Nelson 2005), *Lessonia* represents an ecosystem engineer (*sensu* Jones et al. 1994) and forms extensive kelp beds of significant ecological and economic importance (Steneck et al. 2002). These kelp beds exhibit high primary production rates (Tala and Edding 2007), provide three-dimensional habitats, refuge and shelter, food sources and nursery grounds for associated invertebrates and fishes (e.g., Santelices et al. 1980, Villouta and Santelices 1984), and modify water motion (Santelices and Ojeda 1984). Additionally, *Lessonia* is harvested commercially for alginate extraction and high-quality feed for abalone cultures (Vásquez 2008).

Along the Pacific coast of South America, *Lessonia nigrescens* used to be one of the major representatives of the genus *Lessonia* (Searles 1978). Recently, molecular work

by Tellier et al. (2009) revealed that *L. nigrescens* is actually a species complex, which includes two cryptic species (*Lessonia berteroa* Montagne (1842) and *Lessonia spicata* (Suhr) Santelices; as renamed by González et al. 2012). In their study, Tellier et al. (2009) detected that, among other characteristics, *L. berteroa* and *L. spicata* vary by the size of the intergenic spacer *atp8/trnS*, a mitochondrial marker previously used for phylogenetic studies (Voisin et al. 2005, Tellier et al. 2009). Since the size of this marker is unique for each of the two species, species identification can be performed easily via nucleotide electrophoresis, hence without sequencing (Tellier et al. 2011c).

Both cryptic species inhabit the middle to low intertidal zones of wave-exposed rocky shores (Santelices et al. 1980), but they show contrasting latitudinal distribution ranges along the southeast Pacific coast, with *L. berteroa* occurring from 16°S to 30°S and *L. spicata* from 29°S to 42°S (Tellier et al. 2009). In the zone between 29°S and 30°S, the two species spatially overlap in strict parapatry, so that a mosaic of monospecific populations either of *L. berteroa* or *L. spicata* can be observed. This strict geographic segregation of the two species is accompanied by a complete absence of interspecific gene flow and consequently the lack of hybridization (reproductive isolation; Tellier et al. 2011a). The location of the overlapping zone corresponds to a biogeographic transition zone at 30°S, which represents a margin for numerous marine organisms with low dispersal potentials (Camus 2001, Haye et al. 2014) and is characterized by changes in their recruitment patterns (e.g., of some Phaeophyceae; Meneses and Santelices 2000). Nonetheless, the distribution of species with a higher dispersal potential, such as the gastropod *Concholepas concholepas*, is less affected by the transition zone. Those species cross the transition zone without any changes in abundance (Broitman et al. 2001, Cárdenas et al. 2009). Until now the cause for this biogeographic transition zone located at 30°S is not entirely resolved but is presumably the outcome of a combination of ancient and present-day oceanographic features (e.g., breaks in eddy kinetic energy, equatorward wind stress and upwelling regimes; Hormazabal et al. 2004, Thiel et al. 2007).

Since the two cryptic species cover contrasting distribution ranges, they experience differences in environmental conditions, for example, in seawater temperatures. Consequently, the species may differ in their tolerances with respect to temperature (Martínez 1999, Oppliger et al. 2011, 2012). For example, Martínez (1999) found that young sporophytes of the *L. nigrescens* complex sampled at 20°S grow better at higher temperatures (19°C, 12 days of incubation) compared to those sampled at 40°S. Similarly, gametophytes of *L. berteroa* can tolerate higher temperatures

(20°C, 25 days of incubation) than those of *L. spicata* (Oppliger et al. 2012). In the latter study, further temperature-related differences in life history strategies were detected between the species. At increased temperatures, *L. berteroa* displays a shorter haploid phase, whereas *L. spicata* shows an extended haploid phase with remarkable vegetative growth. The different temperature optima for the two species are apparently related to the species' geographic origin and play an important role in the adaptation to the prevailing local seawater temperatures. Besides genetic differences and contrasting tolerance ranges, these species vary in very few morphological characteristics. Based on the external morphology, for example, individuals of *L. spicata* are shorter and show more dichotomies than those of *L. berteroa*. Internally, blades of *L. spicata* are composed of smaller and higher amounts of cortex cells as well as more filaments in the medulla compared to those of *L. berteroa* (González et al. 2012). However, since those morphological differences are solely based on relative traits, we suggest that the two species are only completely distinguishable from each other by genetic identification, especially within the overlapping zone.

The objective of this study was to test to what extent the genetic identity of the two cryptic species within the *L. nigrescens* complex is reflected by ecophysiological traits. To check for ecophysiological differences, sporophytes of *L. berteroa* and *L. spicata* were collected at eight locations (27°S–32°S) along the coast of northern-central Chile. Thereby, algal individuals were selected from within the overlapping zone and from within the monospecific core zone of each species. Ecophysiological differences and potential adaptive traits at their specific growth sites were addressed by measurements of total lipids, phlorotannins, pigments and variable chlorophyll (Chl) *a* fluorescence of photosystem II (PSII). Further, variations in fatty acid composition were studied for the first time. More specifically, we tackled the question of whether observed ecophysiological differences are based on variations in genetics (species differentiation, identified by a mitochondrial marker (*atp8/trnS*)) or whether they are impacted by environmental conditions prevailing along the latitudinal gradient.

Materials and methods

Algal material and sampling sites

Individuals of *Lessonia* spp. (without holdfast) were detached at low tide from the middle to low intertidal

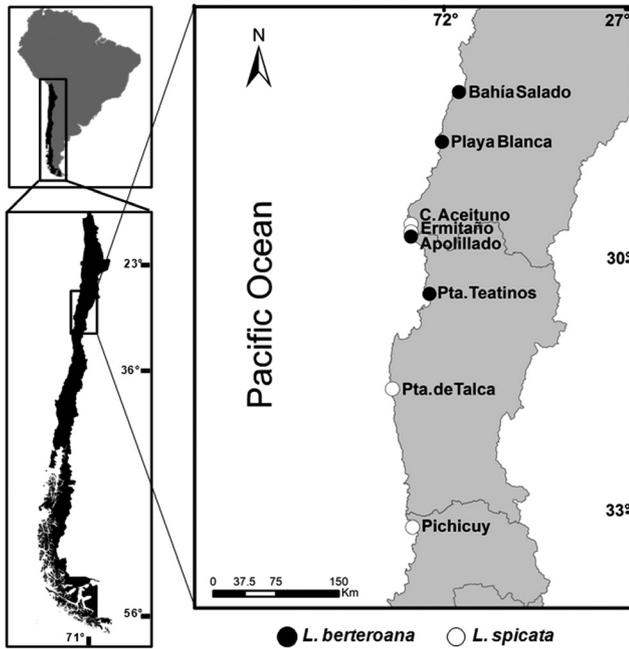


Figure 1: *Lessonia berteroaana* and *Lessonia spicata*: map of Chile indicating the sampling sites along the northern-central coast at which algal individuals were collected. Species were identified using the mitochondrial marker *atp8/trnS*. See Table 1 for further details.

of rocky shores at eight locations along the coast of northern-central Chile (Figure 1 and Table 1) under comparable solar irradiance conditions during January to February 2012. At each sampling location, at least ten individuals of similar size and weight were collected in order to minimize differences in the physiological status due to size/age effects. After collection, algal individuals were kept in darkness and were immediately transported in coolers with seawater at ambient water temperature to the

marine laboratory at Universidad Católica del Norte (UCN) in Coquimbo, Chile (29°57'S, 71°20'W), where they were stored overnight in large outdoor flow-through seawater tanks (approximately 2000 l).

Species identification

Although the two cryptic species (*Lessonia berteroaana* and *Lessonia spicata*) existing within the *Lessonia nigrescens* complex have largely disjoint geographic distributions, their distribution ranges overlap between 29°S and 30°S. Within this overlapping zone, *L. berteroaana* and *L. spicata* form a mosaic of monospecific populations (Tellier et al. 2011b). In order to distinguish between the species, molecular characterization was conducted for a subset of individuals: ten individuals for the locations within the overlapping zone (ACE, ERM, APO, TEA) and five individuals for the locations located north and south of this zone (BSA, PBL, PTAL, PCH; Table 1).

Species identification was done using the mitochondrial intergenic spacer *atp8/trnS*, a species-diagnostic marker previously used for similar purposes (Tellier et al. 2011a). Based on the length polymorphism of this marker, species were identified through nucleotide electrophoresis, and a subset of 16 individuals was sequenced to check the accuracy of the method. DNA extraction, PCR amplification, electrophoresis migration and DNA sequencing were performed according to Tellier et al. (2009, 2011c). Thereby, it was confirmed that individuals collected at the locations BSA, PBL, APO and TEA belonged to the species *L. berteroaana*, whereas individuals sampled at the locations ACE, ERM, PTAL and PCH were representatives of *L. spicata* (Table 1).

Table 1: *Lessonia berteroaana* and *Lessonia spicata*: sampling sites along the coast of northern-central Chile at which algal individuals were collected.

Sampling site	Abbreviation	Species	Latitude (S)	Longitude (W)	Sampling date (2012)
Bahía Salado	BSA	<i>L. berteroaana</i>	27°39'	70°58'	February 01
Playa Blanca	PBL	<i>L. berteroaana</i>	28°11'	71°09'	February 01
Cañaral de Aceituno	ACE	<i>L. spicata</i>	29°04'	71°29'	February 21
Ermitaño	ERM	<i>L. spicata</i>	29°09'	71°29'	February 21
Apolillado	APO	<i>L. berteroaana</i>	29°12'	71°29'	February 13
Punta Teatinos	TEA	<i>L. berteroaana</i>	29°49'	71°17'	February 22
Punta de Talca	PTAL	<i>L. spicata</i>	30°50'	71°41'	January 26
Pichicuy	PCH	<i>L. spicata</i>	32°19'	71°28'	January 31

For each site, the abbreviated site name, geographic coordinates and sampling date are given. Species were identified using the mitochondrial marker *atp8/trnS*.

Ecophysiological analyses

To test for differences in the ecophysiological status and potential adaptive traits at the specific growth sites of *L. berteriana* and *L. spicata*, the following response variables were measured from five individuals per sampling location: total lipid content, fatty acid composition, phlorotannin and pigment content and variable Chl *a* fluorescence. In the morning (09:00, local time), four vegetative blades of each individual were gently cleaned of epibionts. Algal discs (1.5 cm diameter, hereafter subsamples) were cut from the four blades with a cork borer and haphazardly selected for measurements of the different response variables. Measurements of variable Chl *a* fluorescence were carried out immediately, whereas subsamples for the other physiological analyses were shock-frozen in liquid nitrogen and stored at -80°C for later processing. Subsamples for species identification were dried in plastic bags filled with silica gel until DNA extraction (see above).

Total lipid content and fatty acid composition

The algal subsamples were lyophilized for 48 h and pulverized at 1500 rpm for 1 min with liquid nitrogen in a homogenizer (Mikro-Dismembrator, Typ U, B. Braun Biotech International GmbH, Melsungen, Germany). Total lipids were extracted in dichloromethane:methanol (2:1 per volume; Merck, Darmstadt, Germany) following the methods described by Folch et al. (1957) and Bligh and Dyer (1959). Extracts were mixed and ultrasonicated, and total lipid contents were determined gravimetrically after Hagen (2000). For the analysis of fatty acid composition, aliquots of the algal extracts were taken. Fatty acids were converted to their methyl ester derivatives (FAMES) by transesterification with methanol (Merck, Darmstadt, Germany) containing 3% concentrated sulphuric acid (Merck, Darmstadt, Germany) for 4 h at 80°C. After extracting the FAMES three times with hexane (Merck, Darmstadt, Germany), their composition was analyzed using a HP 6890 gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a DB-FFAP column (60 m length, 0.25 mm inner diameter, 0.25 µm film thickness; Agilent Technologies, Waldbronn, Germany) operated with temperature programming according to the method of Kattner and Fricke (1986). FAMES were identified by comparing their retention times with those derived from standards of known composition. Individual fatty acids were presented as mass percentage of total fatty acids. Based on the individual fatty acid composition, they were grouped according to their degree of saturation.

Phlorotannins

The total soluble phlorotannin content was determined using the Folin-Ciocalteu method described in Cruces et al. (2012). Purified phloroglucinol (Sigma-Aldrich, Seelze, Germany) was used as standard. Algal subsamples were lyophilized for 24 h and pulverized at 4 m s⁻¹ for 20 s in a high-speed benchtop homogenizer (FastPrep®-24; MP Biomedicals, Solon, OH, USA). Soluble phlorotannins from subsamples (approximately 10 mg dry weight) were extracted in 1 ml of 70% acetone (Merck, Darmstadt, Germany) for 24 h at 4°C under shaking. After centrifugation (10 min, 4°C, 2500 g), 50 µl of the supernatant was mixed with 250 µl of deionized water, 200 µl of 20% sodium carbonate (NaCO₃; Sigma-Aldrich, Steinheim, Germany) and 100 µl of 2N Folin-Ciocalteu reagent (Sigma-Aldrich, Steinheim, Germany). After 45 min of incubation at room temperature in the dark and centrifugation (3 min, room temperature, 2000 g), the absorbance was read at 730 nm using a microplate reader (FLUOstar OPTIMA; BMG Labtech GmbH, Ortenberg, Germany). Subsamples were measured in triplicate. Soluble phlorotannin contents were expressed as micrograms per milligram dry weight.

Pigments

Pigment determination was performed by reversed-phase HPLC. Algal subsamples were lyophilized for 24 h and pulverized at 4 m s⁻¹ for 20 s in a high-speed benchtop homogenizer. Pigments from subsamples (approximately 40 mg dry weight) were extracted in 1 ml of ice-cold 90% acetone (Merck, Darmstadt, Germany) for 24 h at -20°C in the dark. After centrifugation (5 min, 4°C, 13,000 g) and filtration through a 45-µm nylon syringe filter (Nalgene®, Nalge Nunc International, Rochester, NY, USA), HPLC analysis was performed on a LaChromElite® system equipped with a chilled autosampler L-2200 and a DAD detector L-2450 (VWR-Hitachi International GmbH, Darmstadt, Germany). A Spherisorb® ODS-2 column (25 cm×4.6 mm, 5 µm particle size; Waters, Milford, MA, USA) with a LiChropher® 100-RP-18 guard cartridge was used for the separation of pigments, applying a gradient according to Wright et al. (1991). Peaks were detected at 440 nm and identified as well as quantified by co-chromatography with standards for Chl *a* and *c*, fucoxanthin (Fuc), β-carotene (β-caro), violaxanthin, antheraxanthin and zeaxanthin (DHI Lab Products, Hørsholm, Denmark) using the software EZChrom Elite ver. 3.1.3. (Agilent Technologies, Santa Clara, CA, USA). Pigment contents were expressed as micrograms per milligram dry weight. The de-epoxidation

state (DPS) of the xanthophyll cycle was calculated as described in Colombo-Pallotta et al. (2006).

Chl *a* fluorescence

In vivo variable Chl *a* fluorescence of PSII was measured with a pulse amplitude-modulated fluorometer (Diving-PAM; Walz, Effeltrich, Germany). The maximum quantum yield (F_v/F_m) was determined in dark-adapted (5 min) algal subsamples. Electron transport rates (ETR) were estimated from rapid photosynthesis versus irradiance curves (P-E curves). Algal samples were irradiated with a series of stepwise increasing actinic irradiances (approximately 150–2150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 30-s intervals, provided by a halogen lamp (Schreiber et al. 1994). Subsequently, the photosynthetic capacity (ETR_{max} , maximum electron transport rate), the photosynthetic efficiency (α , initial linear slope) and the saturating irradiance (E_k) were defined by P-E curve fitting after Jassby and Platt (1976). We are aware that the reliability of P-E parameters derived from a Diving-PAM is hampered by potential shifts in the emission spectra during recording. However, those limitations have been accepted, as we only took account of the relative difference in the species from different sampling sites.

Statistical analysis

A one-factorial analysis of variance (one-way ANOVA) and an independent Student's *t* test were carried out to evaluate differences in the ecophysiological status (lipids, fatty acids, phlorotannins, pigments, variable Chl *a* fluorescence of PSII) of algal individuals between the different locations and between the algal species. When the ANOVA revealed significant differences, a post hoc Tukey's honest significant difference (HSD) test was applied. For Student's *t* test comparisons along the full latitudinal range (27°39'S–32°19'S), individual values of all four sampling sites from one species were pooled and tested against the corresponding pooled values from the other species (BSA, PBL, APO and TEA for *L. berteroana* and ACE, ERM, PTAL and PCH for *L. spicata*). For Student's *t* test comparisons within the overlapping zone (29°04'S–29°49'S), individual values of the two sampling sites from one species were pooled and tested against the corresponding pooled values from the other species (APO and TEA for *L. berteroana* and ACE and ERM for *L. spicata*). Prior to all statistical analyses, percentage data were arcsin-transformed. Further, all data were tested for normality and homogeneity of variances, using Kolmogorov-Smirnov's test and Levene's test, respectively. Non-normal and/or heterogeneous data were

log-transformed. The software PASW Statistics 18 (SPSS; Armonk, NY, USA) was used for statistical analyses. Critical significance levels of 5% were applied.

Results

Overall, total lipid contents and saturation states of fatty acids such as sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA), sum of polyunsaturated fatty acids (PUFA) and saturated/unsaturated fatty acid ratio (sat/unsat FA) of algal samples did not differ by species (Table 2) or sampling location (data not shown).

In general, 13 different fatty acids (four saturated and nine unsaturated fatty acids) were detected in the algal samples (Table 2); 16:0 (approximately 21.8% of total fatty acids) was the most abundant saturated fatty acid and

Table 2: *Lessonia berteroana* and *Lessonia spicata*: fatty acid compositions, sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA), sum of polyunsaturated fatty acids (PUFA) (mass% of total fatty acids), saturated/unsaturated fatty acid ratio (Sat/unsat FA) and total lipid content (% of dw) of algal samples collected during January to February 2012.

Fatty acid	Species	
	<i>L. berteroana</i>	<i>L. spicata</i>
14:0	4.0±0.2	4.1±0.1
16:0*	22.7±0.7	20.8±0.4
16:1(n-7)	4.3±0.2	5.1±0.1
18:0	1.1±0.2	0.9±0.1
18:1(n-9)	17.5±1.0	14.8±0.6
18:2(n-6)	6.0±0.2	6.7±0.2
18:3(n-6)	1.0±0.1	1.4±0.1
18:3(n-3)	4.6±0.3	4.3±0.2
18:4(n-3)	8.0±0.5	8.4±0.4
20:0	1.1±0.0	0.8±0.0
20:4(n-6)	18.5±0.6	20.6±0.3
20:4(n-3)	0.9±0.1	1.0±0.1
20:5(n-3)	8.0±0.5	7.7±0.4
SFA	29.6±0.8	27.5±0.5
MUFA	22.3±0.9	20.6±0.6
PUFA	48.1±1.6	51.9±1.0
Sat/unsat FA	0.4±0.0	0.4±0.0
Total lipids	2.04±0.09	1.97±0.08

The nomenclature of fatty acids (a:b(n-x)) is defined as follows: a=no. of C-atoms (chain length), b=no. of double bonds and (n-x)=position of first double bond relative to the methyl-end. For each species, data are given as pooled values from all four sampling sites (BSA, PBL, APO and TEA for *L. berteroana* and ACE, ERM, PTAL and PCH for *L. spicata*). See Table 1 for site name abbreviations. Table shows means±SEM (n=20). *Indicate significant differences between species along the full latitudinal range (independent Student's *t* test, $p < 0.05$).

20:4(n-6) (approximately 19.5% of total fatty acids) the dominant unsaturated fatty acid. Other principal fatty acids were 16:1(n-7), 18:1(n-9), 18:2(n-6), 18:4(n-3) and 20:5(n-3). In both species, fatty acid compositions did not show clear differences with respect to geographic latitude (data not shown); only 18:2(n-6) increased with increasing latitude (BSA: $5.7 \pm 0.1\%$ of total fatty acids and PCH: $7.8 \pm 0.3\%$ of total fatty acids; $p < 0.001$). Fatty acid compositions also did not differ between the two species, except for 16:0. *Lessonia berteroa* exhibited significantly higher contents of 16:0 than *Lessonia spicata*. This species-specific difference could be recognized along the full latitudinal range ($p = 0.045$; Table 2) and within the overlapping zone (*L. berteroa*: $24.1 \pm 0.8\%$ of total fatty acids and *L. spicata*: $21.1 \pm 0.3\%$ of total fatty acids; $p = 0.020$; data not shown).

The calculation of soluble phlorotannin contents and pigment concentrations on a dry weight and Chl *a* basis led to very similar results. Therefore, we decided to use dry weight as a reference for both physiological parameters. Along the full latitudinal range, soluble phlorotannin contents tended to be higher in *L. berteroa* compared to *L. spicata*, with the highest amounts present in individuals of *L. berteroa* from PBL and APO (42.1 ± 6.4 and $47.0 \pm 5.8 \mu\text{g mg}^{-1}$ dw, respectively) and the lowest in *L. spicata* from ERM and PTAL (9.4 ± 2.7 and $8.1 \pm 1.8 \mu\text{g mg}^{-1}$ dw, respectively; Figure 2). Due to high data variability, the t test comparison between the species did not confirm this difference as being significant. However, within the overlapping zone, *L. berteroa* had more than three times as much soluble phlorotannin as *L. spicata* ($p = 0.003$).

Pigment composition of algal samples did not differ clearly in terms of geographic latitude (data not shown).

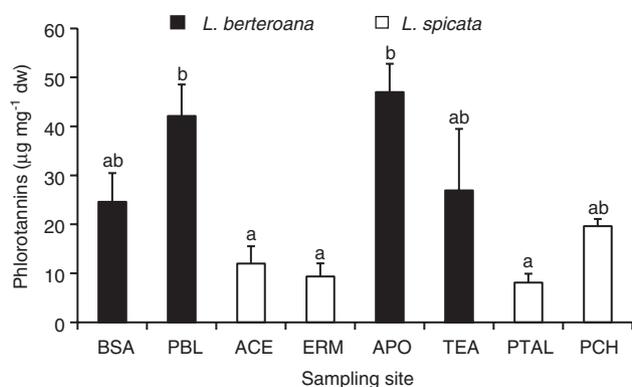


Figure 2: *Lessonia berteroa* and *Lessonia spicata*: soluble phlorotannin contents ($\mu\text{g mg}^{-1}$ dw) of algal samples collected at eight different sampling sites during January to February 2012. See Table 1 for site name abbreviations. Bars are means \pm SEM ($n = 5$). Different letters (a and b) indicate differences among sampling sites that are significant at $p = 0.05$ (one-way ANOVA followed by a post hoc Tukey's HSD test).

Only the molar ratios of fucoxanthin+Chl *c* to Chl *a* (Fuc+Chl *c* Chl *a*⁻¹) were significantly higher at lower latitudes (BSA: 0.39 ± 0.01 and PBL: 0.43 ± 0.01) compared to higher latitudes (TEA: 0.35 ± 0.01 , PTAL: 0.36 ± 0.01 and PCH: 0.36 ± 0 ; $p < 0.001$). However, there were significant differences between the species for the majority of the pigments. The contents of Chl *a* and *c*, Fuc and Fuc+Chl *c* Chl *a*⁻¹ were higher in *L. berteroa* than in *L. spicata*. These species-specific differences could be observed along the full latitudinal range (Chl *a*: $p = 0.004$, Chl *c*: $p = 0.001$, Fuc: $p < 0.001$ and Fuc+Chl *c* Chl *a*⁻¹: $p = 0.011$; Table 3) as well as within the overlapping zone, with the exception of Fuc+Chl *c* Chl *a*⁻¹ (Chl *a*: $p = 0.003$, Chl *c*: $p = 0.005$ and Fuc: $p = 0.002$; Table 3). No significant differences between the two species were observed with respect to β -carotene contents (Table 3). The pool size of the xanthophyll cycle pigments (VAZ) was larger in *L. berteroa* than in *L. spicata* both along the full latitudinal range ($p = 0.001$; Figure 3A) and within the overlapping zone (*L. berteroa*: 0.18 ± 0.01 and *L. spicata*: 0.14 ± 0.01 , $p = 0.001$). The DPS, in contrast, showed significant differences with respect to geographic latitude, with highest values in individuals from PBL and lowest in those from TEA ($p < 0.001$; Figure 3B).

Maximum quantum yields of algal samples were within the typical range reported for non-stressed brown

Table 3: *Lessonia berteroa* and *Lessonia spicata*: pigment concentrations ($\mu\text{g mg}^{-1}$ dw) and molar ratio of Fuc+Chl *c* to Chl *a* (Fuc+Chl *c* Chl *a*⁻¹) of algal samples collected during January to February 2012.

Pigment	Species (full latitudinal range)		Species (overlapping zone)	
	<i>L. berteroa</i>	<i>L. spicata</i>	<i>L. berteroa</i>	<i>L. spicata</i>
Chl <i>a</i> *	1.57 ± 0.07	1.29 ± 0.06	1.54 ± 0.07	1.20 ± 0.06
Chl <i>c</i> *	0.11 ± 0.01	0.09 ± 0.00	0.11 ± 0.00	0.09 ± 0.00
Fuc*	0.49 ± 0.02	0.38 ± 0.02	0.69 ± 0.04	0.36 ± 0.02
β -caro	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.01	0.08 ± 0.00
Fuc+Chl <i>c</i>	0.38 ± 0.01	0.37 ± 0.00	0.37 ± 0.00	0.37 ± 0.00
Chl <i>a</i> ^{-1**}				

Along the full latitudinal range ($27^{\circ}39'S - 32^{\circ}19'S$), data for each species are given as pooled values from all four sampling sites (BSA, PBL, APO and TEA for *L. berteroa* and ACE, ERM, PTAL and PCH for *L. spicata*). Within the overlapping zone ($29^{\circ}04'S - 29^{\circ}49'S$), data for each species are given as pooled values from the two sampling sites (APO and TEA for *L. berteroa* and ACE and ERM for *L. spicata*). See Table 1 for site name abbreviations. Table shows means \pm SEM ($n = 20$ for full latitudinal range and $n = 10$ for overlapping zone). Asterisks indicate significant differences between species (*in full latitudinal range and overlapping zone, **only in full latitudinal range; independent Student's t test, $p < 0.05$). Fuc, fucoxanthin; β -caro, β -carotene.

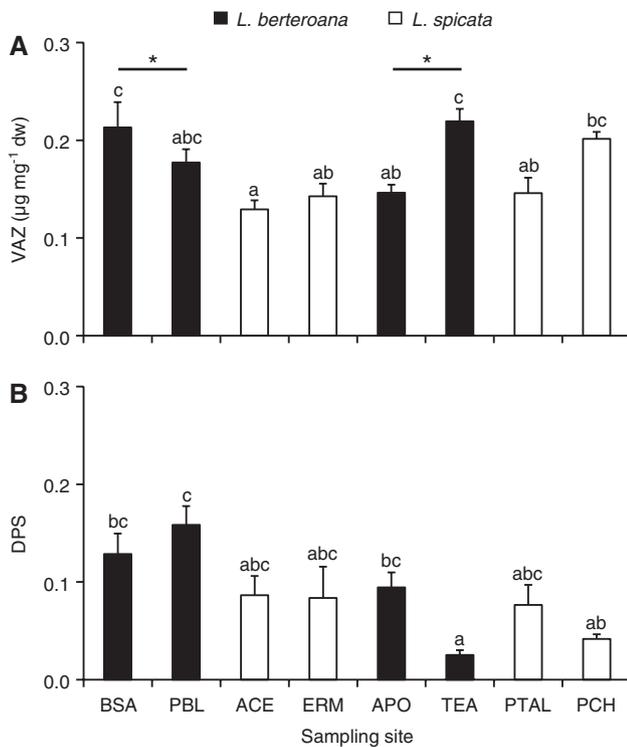


Figure 3: *Lessonia berteroaana* and *Lessonia spicata*: (A) xanthophyll cycle pigment pools (VAZ, $\mu\text{g mg}^{-1} \text{ dw}$) and (B) de-epoxidation states of xanthophyll cycle (DPS) of algal samples collected at eight different sampling sites during January to February 2012. See Table 1 for site name abbreviations. Bars are means \pm SEM (n=5). Different letters (a, b and c) indicate differences among sampling sites that are significant at $p=0.05$ (one-way ANOVA followed by a post hoc Tukey's HSD test). For Student's t test comparisons, individual values of all four sampling sites from *L. berteroaana* (BSA, PBL, APO and TEA) were pooled and tested against the corresponding pooled values from *L. spicata* (ACE, ERM, PTAL and PCH). Asterisks indicate species-specific differences between these pooled data that are significant at $p=0.05$.

algae (data not shown; Büchel and Wilhelm 1993). As for the photosynthetic pigments, the parameters of variable Chl *a* fluorescence of PSII exhibited no clear trends with latitude (data not shown). However, ETR_{max} and E_k showed significant species-specific differences along the full latitudinal range, with 30% and 20% higher values in *L. berteroaana* than in *L. spicata*, respectively (ETR_{max} : $p=0.005$, E_k *L. berteroaana*: $480\pm 21 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and *L. spicata*: $396\pm 21 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$; $p=0.008$; Figure 4). The same pattern was observed within the overlapping zone (ETR_{max} : $p<0.001$, E_k *L. berteroaana*: $546\pm 18 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and *L. spicata*: $457\pm 17 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$; $p=0.001$; data not shown). In contrast, α did not differ significantly between the species (*L. berteroaana*: $0.273\pm 0.006 \mu\text{mol e}^{-} \text{ m}^{-2} \text{ s}^{-1} (\mu\text{mol photons m}^{-2} \text{ s}^{-1})^{-1}$ and *L. spicata*: $0.256\pm 0.007 \mu\text{mol e}^{-} \text{ m}^{-2} \text{ s}^{-1} (\mu\text{mol photons m}^{-2} \text{ s}^{-1})^{-1}$).

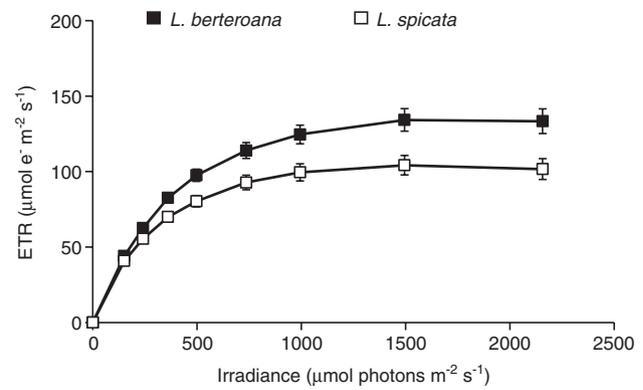


Figure 4: *Lessonia berteroaana* and *Lessonia spicata*: photosynthesis versus irradiance curves (P-E curves) of algal samples collected during January to February 2012. Curves are based on estimations of electron transport rates (ETR, $\mu\text{mol e}^{-} \text{ m}^{-2} \text{ s}^{-1}$). For each species, data are given as pooled values from all four sampling sites (BSA, PBL, APO and TEA for *L. berteroaana* and ACE, ERM, PTAL and PCH for *L. spicata*; site name abbreviations as in Table 1). Data points are means \pm SEM (n=20). Species-specific differences in maximum electron transport rates (ETR_{max}) and saturating irradiances [E_k ; defined by P-E curve fitting after Jassby and Platt (1976)] were found that are significant at $p=0.05$.

Discussion

Overall, our results reveal that the two cryptic species within the *Lessonia nigrescens* complex exhibit differences in many of the ecophysiological characteristics (phlorotannins, pigments, Chl *a* fluorescence of PSII) tested. These differences were not only found along the full latitudinal range (approximately 520 km of coastline) but also within the narrow overlapping zone (approximately 80 km of coastline), in which populations of *Lessonia berteroaana* and *Lessonia spicata* grow in close proximity. Therefore, we propose that the observed ecophysiological differences might be considered as genetically determined species-specific differences, which are not masked by responses to the respective environmental settings along the latitudinal gradient.

The Chilean coast is affected by the Humboldt Current System, and changes in several abiotic parameters followed a latitudinal gradient (Thiel et al. 2007). Generally, solar irradiance and sea surface temperature (SST) decrease with increasing latitude (Broitman et al. 2001, Hernández et al. 2012). However, temperature conditions along the Chilean coast can be highly heterogeneous due to persistent upwelling of cold nutrient-rich subsurface waters at certain locations. Further, the intensity of air exposure during a tidal cycle decreases towards higher latitudes, as a result of the decline in SST and rise in relative humidity

(Thiel et al. 2007, López-Cristoffanini et al. 2013). During these periods of air exposure, intertidal kelps experience numerous stress factors, like high levels of photosynthetically active radiation (PAR), UV, temperature and salinity as well as desiccation and nutrient limitation (Davison and Pearson 1996). Thus, due to their contrasting latitudinal distribution along the Chilean coast (Tellier et al. 2009), *L. berteriana* and *L. spicata* experience differences in environmental conditions. Overall, *L. berteriana* is exposed to higher solar irradiance and SST as well as longer air exposure during a tidal cycle compared to *L. spicata*. Mean annual as well as monthly maximum and minimum seawater temperatures can differ strongly between extreme localities along the distributional range of the *L. nigrescens* species complex (Martínez 1999, Oppliger et al. 2011). Of course, variations in environmental conditions are more pronounced along the total distributional range of the two cryptic species as compared to the much smaller full latitudinal range (27°39'S–32°19'S) tested in our study. Nonetheless, it was previously shown that differences in abiotic forcings were also detectable within comparable latitudinal gradients along the Chilean coast (Broitman et al. 2001, Oppliger et al. 2011, 2012, Tellier et al. 2011b). For example, Tellier et al. (2011b) found a difference of 5°C between the weekly mean SST (1982–2008) at 25°S and 35°S. López-Cristoffanini et al. (2013) reported that the weekly mean SST ranged from 14 to 20°C at 26°S (Pan de Azúcar) and from 13 to 18°C at 29°S (ERM and APO) as well as at 33°S (Las Cruces). Furthermore, due to the local upwelling region of Punta Lengua de Vaca (approximately 30/31°S), the *Lessonia* populations at the two southernmost locations (PTAL and PCH) of the full latitudinal range are thought to be continuously exposed to colder and more nutrient-rich waters, whereas populations at the more northern locations (BSA to TEA) may be temporarily exposed to warmer waters with less nutrients. However, local hydrodynamic features may further modulate temperature conditions on a smaller scale (Thiel et al. 2007, Tapia et al. 2014). The global horizontal irradiance (2003–2012) tended to decrease from BSA to PCH by about 5% (Explorador de energía solar, <http://walker.dgf.uchile.cl/Explorador/Solar2/>).

The species-specific differences in ecophysiological characteristics may allow *L. berteriana* and *L. spicata* to survive and grow at the prevailing abiotic conditions at their respective growth sites. Both cryptic species followed the well-known pattern of photosynthetic acclimation to various solar irradiances along their distributional range (Reiskind et al. 1989, Marquardt et al. 2010), displaying higher values of ETR_{max} and E_k in *L. berteriana* than in *L. spicata*. This species-specific adaptation enables photosynthesis of *L. spicata* to be saturated already at lower

irradiances. With respect to photosynthetic pigments, species-specific differences were found for Chl *a* and *c* as well as Fuc. However, *L. berteriana* exhibited higher concentrations of those pigments than *L. spicata*, which is contradictory to the expected and typical photoacclimatory adjustments with reduced relative amounts of antenna pigments in high light environments (e.g., Wheeler 1980, Smith and Melis 1987). Why both cryptic *Lessonia* species reacted contrarily to this expectation remains unresolved. Further, VAZ displayed species-specific differences, whereas DPS showed differences with respect to geographic latitude. The xanthophyll cycle is known to play a key role in the dissipation of excess light energy via non-photochemical quenching and thus in the protection of the photosynthetic apparatus against photodamage. Thereby, adjustments of the pigment pool size (VAZ) were considered to be long-term responses that mirror the protective activity of the xanthophyll cycle (Pfündel and Bilger 1994). We propose that the enhanced VAZ in *L. berteriana* compared to *L. spicata* forms part of the acclimation response to higher solar irradiances at its growth sites. Colombo-Palotta et al. (2006) described the same acclimation response with respect to VAZ in highly irradiance-exposed surface blades of the giant kelp *Macrocystis pyrifera*. In contrast, adjustments of the rates of xanthophyll cycle pigment conversion (DPS) were defined as short-term responses (Pfündel and Bilger 1994), potentially caused by prevailing changes in solar irradiance along the latitudinal gradient of the Chilean coast. The DPS, however, showed significant differences with respect to geographic latitude, with highest values in individuals from PBL and lowest in those from TEA ($p < 0.001$; Figure 3B).

As for the pigments, soluble phlorotannin contents displayed species-specific differences. Phlorotannins are phenolic compounds with several putative secondary functions such as herbivore deterrence, scavenging of reactive oxygen species (ROS) and screening against potentially harmful UV radiation (Koivikko et al. 2005). Since *L. berteriana* experiences higher levels of UV and possibly enhanced ROS formation, both in the water column and in the air during low tide, we suggest that larger amounts of phlorotannins mirror the species' latitudinal spread. Similar results were found by Cruces et al. (2012), who detected enhanced phlorotannin contents in *L. spicata* under high PAR and especially under high UV conditions. Additionally, larger amounts of phlorotannins might allow *L. berteriana* to tolerate the potentially higher grazing pressure, which was found to increase at lower latitudes (Broitman et al. 2001).

Overall, total lipid contents in *L. berteriana* and *L. spicata* were relatively low (approximately 2% of dw).

This agrees with a study on five macroalgal species by Herbeteau et al. (1997), who also propose that very low total lipid levels appear to be characteristic for plants living in marine environments. However, Westermeier and Gómez (1996) determined total lipid contents of around 0.4% of dw for fronds of the *L. nigrescens* complex. To our knowledge, fatty acid compositions of the two cryptic species are described for the first time in the present study, but differences in abiotic conditions within the contrasting distribution ranges of the two species were hardly reflected by their fatty acid compositions. The fatty acid composition is an important determinant of membrane fluidity, which is essential to maintain photosynthetic functions (e.g., re-integration of *de novo* synthesized D1 reaction centre proteins; Becker et al. 2010 and references therein). Previous studies have shown that differences in abiotic conditions such as light and temperature may result in changes of the fatty acid composition and metabolism of macroalgae (e.g., Pettitt et al. 1989, Khotimchenko and Yakovleva 2005, Becker et al. 2010). Nonetheless, according to our results, adjustments of fatty acid composition seem to play a negligible role in the adaptive processes in the two cryptic species of the *L. nigrescens* complex.

Conclusion

The results of the present study confirm that species differentiation within the *Lessonia nigrescens* complex is also manifested at the biochemical level. Of all parameters tested, phlorotannins, pigments and Chl *a* fluorescence of PSII seem to be the most crucial for species-specific adaptations to the prevailing abiotic conditions at the respective growth sites, whereas differences in total lipids and fatty acid compositions were non-existent between the two species (exception fatty acid 16:0). These new findings with respect to the ecophysiology of *Lessonia berteriana* and *Lessonia spicata* might help to explain their differences in tolerance to temperature and air exposure (Martínez 1999, Oppliger et al. 2011, 2012, López-Cristoffanini et al. 2013) or to predict their responses to abiotic stresses (e.g., rise of SST during El Niño Southern Oscillation events; Martínez et al. 2003). To test whether our findings are also valid for more marginal *Lessonia* populations, future studies should extend the characterization of ecophysiological characteristics to the total distributional range of both cryptic species. Furthermore, gene expression studies are suggested, which will allow in-depth insights into physiological implications of speciation processes along environmental gradients.

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