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Short Communication

Determination of genetic diversity of two *Lessonia* species from the Atacama region using ISSR markers

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ABSTRACT. *Lessonia nigrescens* and *L. trabeculata* are economically important kelp species in northern Chile. Despite their considerable value, the genetic diversity of these kelp has received limited attention in the Atacama region. This study aimed to analyze genetic diversity, establish genetic relationships, and identify diagnostic DNA markers in the Atacama region's *L. nigrescens* and *L. trabeculata*. Both species' Inter-sequence simple repeat (ISSR) band patterns showed high reproducibility and reliability. Among 17 ISSRs, 161 polymorphic bands were identified in *Lessonia* spp. Parameters, such as percentage polymorphism, number of alleles per locus, effective number of alleles, and Shannon information index, indicated that *L. nigrescens* had slightly higher genetic diversity than *L. trabeculata*. The Unweighted pair group method with arithmetic average (UPGMA) cluster analysis revealed two main clusters: one containing *L. nigrescens* and the other containing *L. trabeculata*. Additionally, four species diagnostic ISSR markers were identified for *L. nigrescens* and *L. trabeculata*. The ISSR markers proved valuable in detecting genetic diversity in *L. nigrescens* and *L. trabeculata*. This information is particularly important for marine management strategies and ensuring successful conservation.

Keyword: *Lessonia trabeculata*; *Lessonia nigrescens*; ISSR markers; genetic diversity; species-diagnostic markers; Atacama region

Kelps, belonging to the order Laminariales, form the basis of many coastal temperate marine ecosystems (Nardelli et al. 2023). The genus *Lessonia*, an important kelp found in cool temperate waters of the southern hemisphere, currently comprises 11 recognized species (Nardelli et al. 2023). Among these, *L. nigrescens* Bory, 1826 and *L. trabeculata* Villouta & Santelices 1986 stand out as the main artisanal benthic resources in northern Chile, particularly in the Atacama region (Campos et al. 2021, Berrios et al. 2022). The economic and social importance of the genus *Lessonia* in Chile is underscored by its exportation to the hydrocolloid industry (Pérez-Araneda et al. 2020). The market demand has grown substantially in the last 15 years, shifting the extractive activity from the collection of dead plants to the intensive extraction of live individuals (Berrios et al. 2022). Accurate taxonomic identification of this natural resource is crucial to ensure traceability and validation (Pérez-Araneda et al. 2020). The discovery of cryptic species is uncommon

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within marine macroalgae (Tellier et al. 2011). For example, in the genus *Lessonia*, nuclear, mitochondrial, and chloroplast markers revealed two strongly divergent lineages within *L. nigrescens*, suggesting that the species contains two distinct phylogenetic species (Tellier et al. 2009). It shows the importance of studies using molecular markers widely used to evaluate species' genetic variation and identify the genotypes. Inter simple sequence repeat (ISSR), a PCR-based molecular marker, uses a single primer composed of di, tri, tetra, or pentanucleotide repetitions to amplify fragments between two successive, inversely oriented microsatellites (Zietkiewicz et al. 1994). ISSR techniques are simple, inexpensive, and do not require knowledge about the DNA sequence prior to application (Cui et al. 2017). They are quick and highly reproducible (Contreras et al. 2020). Despite its widespread use in various seaweed species for assessing genetic diversity, phylogenetic relationships (Cui et al. 2017), DNA-fingerprinting (Gupta et al. 2015, Alshehri et al. 2021), hybridization (Gupta et al. 2015), population studies (Jung-Kim et al. 2021), interspecific genetic variability (Safaei et al. 2016), and sex identification (Gu et al. 2014), ISSR markers have, until now, not been applied in genetic studies of *Lessonia* spp.

L. trabeculata and *L. nigrescens* coexist along the northern coast of Chile, with one inhabiting the rocky subtidal zone (up to 40 m deep) and the other inhabiting the rocky intertidal and shallow subtidal zone, respectively (Campos et al. 2021, Berrios et al. 2022). Consequently, their natural coexistence may have contributed to a certain level of interspecific genetic diversity, potentially including interspecific hybridization, which commonly occurs between closely related species. This study aimed first to assess the genetic diversity of *L. nigrescens* and *L. trabeculata*; second, to establish genetic relationships; and third, to identify diagnostic DNA markers using ISSR markers.

Fresh blade tissue (free of epiphytes) was collected from nine individuals of *L. nigrescens* and nine individuals of *L. trabeculata* (Fig. 1) along the coast of Punta Flamenquito located between 26°36'15.4"S, 70°42'15.6"W to 26°35'55.9"S, 70°42'04.7"W. Fragments of blade from each individual were collected along a 400 m transect. The "Asociación de Recolectores de Algas, Pescadores y Mariscadores de la Caleta Torres del Inca" support facilitated the sampling process, and a kelp specialist properly identified the samples. We selected blades without the reproductive section (sorus tissue) during the recollection. The collected samples were stored at 10°C in a portable cooler and at -80°C in the laboratory. DNA was isolated from the blades using the modified Cetyl-trimethylammonium bromide (CTAB) method (Contreras et al. 2021). The quality and concentration of the total DNA were assessed using a COLIBRI microvolume spectrophotometer (Titertek-Berthold, Pforzheim, Germany). DNA purity was evaluated based on the absorbance ratio at A260/A280, with a threshold of ≥1.8. The A260/A230 ratio served as a secondary measure of DNA purity, with an acceptance range between 2.0 and 2.2 (Aleksić et al. 2012). Additionally, the integrity of the genomic DNA was verified through agarose gel electrophoresis (0.7%).

We used 17 University of British Columbia (UBC) markers for the ISSR amplification: UBC807, UBC809, UBC810, UBC811, UBC823, UBC825, UBC835, UBC840, UBC842, UBC843, UBC848, UBC851, UBC859, UBC873, UBC880, ISSR001 and ISSR003 (Contreras & Tapia 2016, Cui et al. 2017). We selected the UBC markers that provided high numbers of fragments and better reproducibility. The 20 µL PCR solution consisted of 10 µL Master Mix SapphireAmp 2X (Takara, Clontech), 5 µL ISSR primer (5 µM), 1.5 µL genomic DNA (5 ng μL^{-1}), and 4.5 µL nuclease-free water. Amplifications were performed in a MultiGene Optimax thermal cycler (Labnet) using the following protocol: an initial step of 5 min at 94°C, 45 cycles of 30 s at 94°C, 45 s at 52°C and 2 min at 72°C, followed by a final extension step of 6 min at 72°C. The amplification products were separated by agarose gel electrophoresis (2%) and staining with GelRed™ (10,000X) at the ratio of 10 µL 100^{-1} mL of gel (with Tris-borate-EDTA at 0.5X). The electrophoresis run was performed at 100 V for 75 min. The gel was then placed in a UV transilluminator (Vilber Lourmat) and photographed with a digital camera (Canon, SX160 IS).

In each individual, the ISSR-PCR fragments were considered an independent locus, recorded as the presence (1) or absence (0) of bands. We obtained the total number of ISSR bands (TNB), the percentage of polymorphism bands at 99% (P), the number of different genotypes (NG), and the resolving power (Rp). The Rp of a primer was calculated as $Rp = \Sigma Ib$, where Ib (band information) takes the value of $1 - [2 \times (0.5 - P)]$, with P being the frequency of the lines the band contains (Prevost & Wilkinson 1999). The information content of each ISSR marker was calculated as $PIC_i = 2f_i \times (1 - f_i)$, where PIC_i is the polymorphic information content of marker 'i', f_i is the frequency of the amplified allele (band present), and $1-f_i$ is the frequency of the null allele (Roldán-Ruiz et al. 2000). A binary data matrix was constructed by Jaccard

Figure 1. Morphological features of *Lessonia nigrescens* and *L. trabeculata* from Atacama region, Chile.

similarity measures using the presence/absence of bands. The PAST program (Hammer et al. 2001) was used to construct the dendrogram, applying an Unweighted pair group method with an arithmetic average (UPGMA). Bootstrap support (BS) of 1000 replicates was calculated to test the robustness of the dendrogram. GenAlex 6.5 software (Peakall & Smouse 2012) was used to evaluate genetic diversity in each species. The parameters were the number of alleles per locus (Na), number of effective alleles (Ne), %P, Shannon information index (I), expected heterozygosity (He), and Nei's genetic distance. A non-parametric analysis of molecular variance (AMOVA) was performed using GenAlEx 6.5 software to evaluate the genetic variation among and within populations.

Seventeen ISSR primers produced 179 scorable bands for *L. trabeculata* and *L. nigrescens*, ranging from 150 to 2900 bp, of which 161 were polymorphic (89%) (Table 1). The average number of polymorphic bands per primer was 11. The percentage of polymorphic bands using 17 ISSR primers in both *Lessonia* species was notably high at 89% compared with the seaweed *Undaria pinnatifida* (Harvey) Suringar (Phaeophyceae, Laminariales), which exhibited 74% polymorphic bands (104 bands generated, of which 77 were polymorphic) using 18 ISSR primers (Wang et al. 2006). Even when compared to 17 varieties of the *Saccharina* gametophytes (Phaeophyceae, Laminariales), which displayed 97% polymorphic bands (262 bands generated, of which 256 were polymorphic) using 17 ISSR primers (Cui et al. 2017), our results remain noteworthy. The TNB varied between 7 (UBC825 and UBC873) and 15 (UBC835). The total NG per primer ranged from 3 (UBC825) to 18 (UBC840). The highest Rp value was observed with the UBC840 primer (8.5556), while the UBC843 primer exhibited the lowest value (4.2222). PIC values were calculated for each primer, with UBC851 showing the highest PIC value (0.4358) and UBC859 the lowest (0.2414) (Table 1). Thus, ISSR markers in both *Lessonia* species demonstrated a relatively high PIC (average = 0.3602), considering that the maximum PIC value for dominant markers is 0.5 (Chesnokov & Artemyeva 2015).

A UPGMA analysis, based on the ISSR markers, defined two main groups with robust bootstrap support (BS = 100), underscoring the high consistency and support of the clusters (Fig. 2). One group comprised the nine individuals of *L. nigrescens*. In contrast, the other group consisted of the nine individuals of *L. trabeculata*. Notably, within *L. nigrescens*, subclades formed by individuals Ln4-Ln5 and Ln2-Ln3 exhibited good bootstrap support (BS > 50). Similarly, in *L. trabeculata*, individuals Lt2-Lt1 and Lt3-Lt5 formed subclades with substantial bootstrap support (BS > 60) (Fig. 2). Examining the phylogenetic relationship of five *Lessonia* species based on *atp8/trnS* and *ITS1* sequences, described by Tellier et al. (2009), affirmed that *L. trabeculata* is the closest species to *L. nigrescens*.

Our ISSR results demonstrated high reproducibility and reliability in the band patterns of *L. nigrescens* and *L. trabeculata* (Fig. 3), consistent with another study using ISSR markers described by Contreras et al. (2020). Most ISSR markers showed different PCR patterns between *L. nigrescens* and *L. trabeculata* individuals. In Figure 3, we show electrophoresis patterns of three ISSR markers that have fragments exclusive to *L. nigrescens* (160 bp with the marker UBC848, 150 bp with UBC859, and 290 bp with UBC880) and one fragment exclusive to *L. trabeculata*, (270 bp with the marker UBC851). Additionally, we identified one fragment present in all *Lessonia* individuals (450 bp with the marker UBC823). Hence, this study revealed three species-diagnostic markers for *L. nigrescens*, one species-diagnostic marker for *L.*

Table 1. ISSR primer information using *Lessonia trabeculata* and *L. nigrescens*. The total number of bands (TNB), the number of polymorphic bands (NPB) at 99%, the percentage of polymorphic bands (P) at 99%, the number of different genotypes (NG), the resolving power (Rp), and the polymorphic information content (PIC) are shown.

Primer	Sequence	TNB	NPB (99%)	P (99%)	NG	Rp	PIC
ISSR001	$(GA)_6CC$	12	12	100	17	6.7778	0.3688
ISSR003	$(GCAGA)_3$	10	10	100	11	6.0000	0.3642
UBC807	$(AG)_8T$	13	13	100	13	7.4444	0.3509
UBC809	$(AG)_8G$	12	9	75	5	7.3333	0.3426
UBC810	$(GA)_8T$	12	12	100	17	6.0000	0.3549
UBC811	$(GA)_8C$	11	10	91	8	5.7778	0.3367
UBC823	$(TC)_8C$	9	8	89	11	4.8889	0.3512
UBC825	$(AC)_8T$	7	5	71	3	4.6667	0.3492
UBC835	$(AG)_8YC$	15	14	93	14	6.2222	0.2823
UBC840	$(GA)_8YT$	13	13	100	18	8.5556	0.3955
UBC842	$(GA)_8YG$	11	8	73	5	7.7778	0.3625
UBC843	$(CT)_8RA$	8	8	100	13	4.2222	0.3565
UBC848	$(CT)_8RG$	8	7	88	5	5.8889	0.3958
UBC851	$(GT)_8YC$	10	10	100	6	7.7778	0.4358
UBC859	$(TG)_8RC$	10	5	50	6	4.3333	0.2414
UBC873	$(GACA)_4$	7	7	100	5	6.0000	0.4815
UBC880	$GGA(GAG)_2AGGAGA$	11	10	91	10	6.5556	0.3541
TOTAL		179	161				
Average		11		89	10	6.2484	0.3602

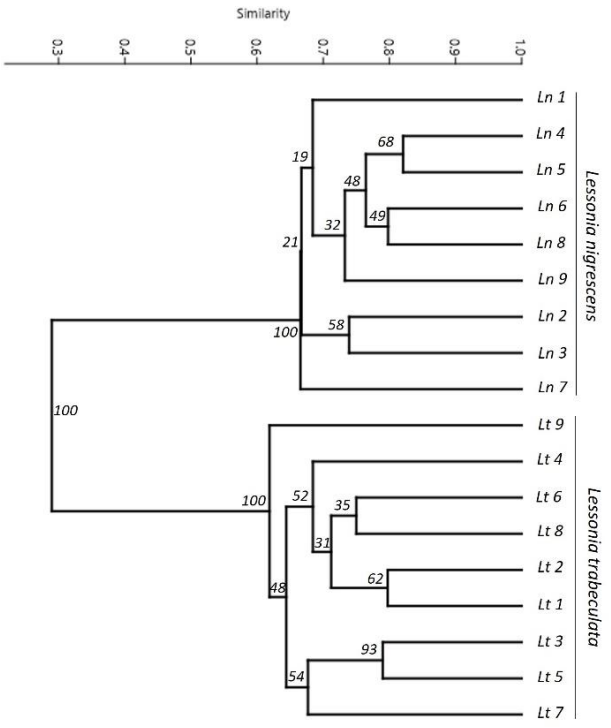

Figure 2. Cluster analysis shows the relationships between *Lessonia nigrescens* (Ln) and *L. trabeculata* (Lt) individuals. The tree was constructed using UPGMA cluster analysis using Jaccard similarity based on 179 markers obtained from ISSR. Bootstrap values are indicated as percentages on the branches of the tree.

trabeculata, and one genus-diagnostic marker for *Lessonia*. Recognizing the potential for further development, Sequence-characterized amplified region (SCAR) markers (El-Haggar et al. 2023) could be developed from these species-diagnostic ISSR markers to detect or distinguish between *L. nigrescens* and *L. trabeculata*.

The electrophoresis patterns of the UBC809 marker revealed some bands shared between *L. nigrescens* and *L. trabeculata* (Fig. 3). The PCR fragments from 900 to 2400 bp of the UBC809 marker observed in all individuals of *L. nigrescens* can be observed in some individuals of *L. trabeculata* (red arrow, Fig. 3). A similar observation was made with UBC807 and UBC810 markers. This phenomenon could potentially be attributed to introgression or hybridization events. According to the cluster analysis, individuals Ln7 and Lt9 are likely hybrids (Fig. 2). In Laminariales, successful hybridization in laboratory conditions between closely related species and members of different genera is well documented (Coyer et al. 1992). In brown algae, hybridization changes the genetic variability among and within populations through gene introgression (Murúa et al. 2020). These hybrids may contribute to the formation of novel evolutionary lineages (Bringloe et al. 2020).

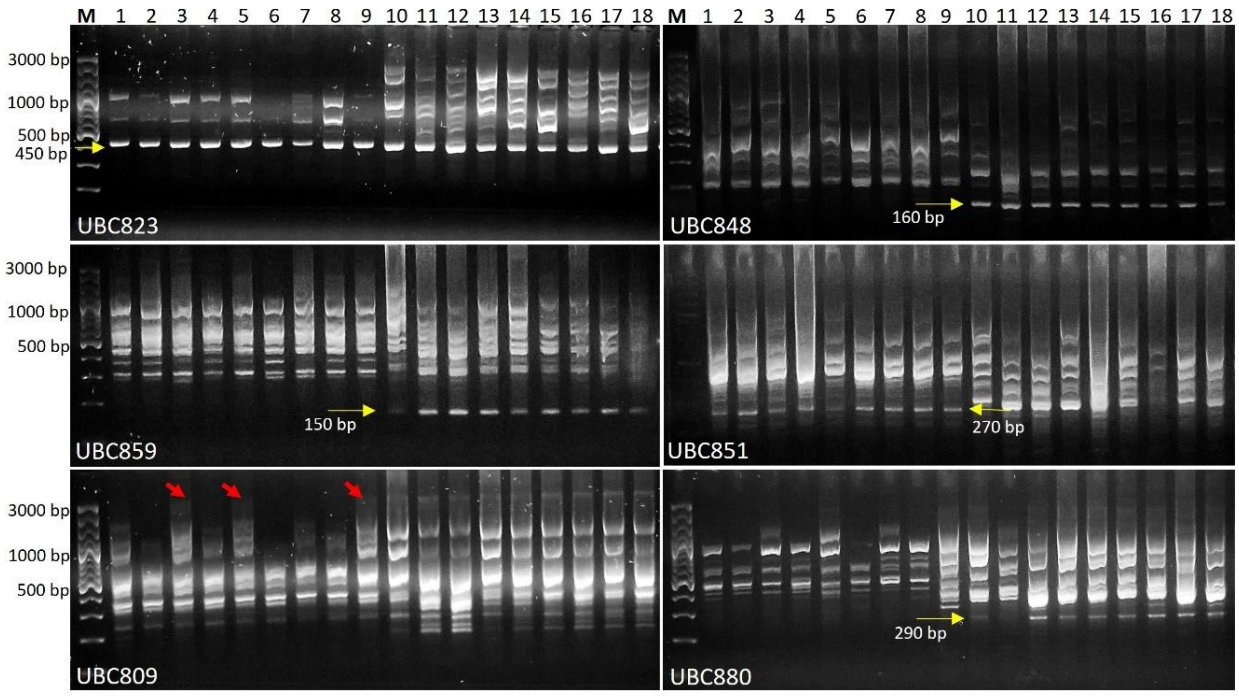

Figure 3. Example of electrophoresis patterns of six ISSR markers obtained from DNA samples of nine individuals of *Lessonia trabeculata* (lines 1-9) and nine *L. nigrescens* (10-18). The yellow arrows indicate the markers of genus-diagnostic (UBC823) and species-diagnostic (UBC848. UBC859. UBC851 and UBC 880). The red arrows indicate possible shared bands from 900 bp. M is a marker with a molecular weight between 100 and 3000 bp.

Nonetheless, the artificial genetic improvement of macroalgae through cross-hybridizations and selection carries potential risks, such as introducing invasive genotypes in wild populations if farmed individuals from aquaculture escape (Goecke et al. 2020). In seaweed, the ISSR profile has been useful to show species-specific bands, confirming the hybrid nature of a certain species (Gupta et al. 2015). Moreover, one of the advantages of molecular markers like ISSR is their ability to offer a high number of DNA markers (most non-coding regions), covering the whole genome. While, until this study, no hybrids have been detected in the *L. nigrescens* complex (Tellier et al. 2011), their presence has not been thoroughly evaluated in natural populations containing *L. nigrescens* and *L. trabeculata*. Genetic diversity changes can occur at the population (intra-specific) or the community (inter-specific) level in macroalgae and seagrasses, influencing growth, productivity, competition, and responses to environments, with ecosystem-wide consequences (Hu et al. 2020).

The genetic diversity parameters revealed a slightly higher level of genetic polymorphism in *L. nigrescens* (48.6%) than in *L. trabeculata* (47.5%) (Table 2). *L. nigrescens* also exhibited higher values for the number of alleles per locus (Na = 1.240), effective number of alleles (Ne = 1.324), Shannon information index (I = 0.272), and expected heterozygosity (He = 0.185), then *L. trabeculata* (Table 2).

The enhanced genetic diversity in *L. nigrescens*, as indicated by ISSR markers, could be attributed to two lineages identified in previous studies, particularly in the transition zone located in Aceituno-Choros (~29°S) (Tellier et al. 2009, 2011). Our sampling site at ~26°36'S is close to the known transition zone. The convergence of the two lineages of *L. nigrescens* may explain the observed higher genetic diversity in *L. nigrescens* than in *L. trabeculata*. It has been suggested that coalescence is a common process in kelp species from the central Chilean coast, including *Lessonia* spp. (González et al. 2014). Therefore, our samples collected (~26°36'S) may correspond to *L. berteroana* Montagne (northern lineage) as proposed by González et al. (2012), who identified two species within the *L. nigrescens* complex who identified two species within the *L. nigrescens* complex, *L. spicata* (Suhr) Santelices for the central lineage (central-south Chile, 29-41°S) and *L. berteroana* for the northern lineage [extending

Table 2. Genetic diversity parameters determined in *Lessonia nigrescens* (LNE) and *L. trabeculata* (LTR) based on ISSR markers. N: number of individuals; P: percentage of polymorphic bands; Na: number of alleles per locus; Ne: effective number of alleles per locus; I: Shannon index; He: expected heterozygosity. Standard deviations (in parentheses).

Species	N	Na	P(%)	Ne	I	He
L. trabeculata	9	1.173 (0.065)	47.5	1.254 (0.026)	0.231 (0.020)	0.152 (0.014)
L. nigrescens	9	1.240 (0.062)	48.6	1.324 (0.029)	0.272 (0.022)	0.185 (0.016)

from southern Peru (17°S) to central Chile (30°S)]. The high genetic diversity that we have found in *L. nigrescens* might be influenced by the prevalence of chimeras, i.e. more than 60% of *L. berteroana* individuals are chimeras in northern Chile (Rodríguez et al. 2014, Nardelli et al. 2023). Chimeras, genetically heteroge-neous individuals resulting from the fusion of different genotypes, are frequent in both *L. spicata* and *L. berteroana* (González et al. 2014, Nardelli et al. 2023), which could have contributed to the observed high genetic diversity in the *L. nigrescens* complex.

Nei's genetic distance between *L. nigrescens* and *L. trabeculata* was high (0.464), and in contrast with the low values (ranging from 0.023 to 0.242) found in *Sargassum liebmannii* J. Agardh populations in a study of intraspecific variation study using ISSR markers (Jung-Kim et al. 2021).

The AMOVA revealed significant molecular differences (P = 0.01) between both *Lessonia* spp., indicating that 65% of the total variance was due to genetic differentiation among populations and 35% genetic variability among individuals within popu-lations, underscoring a high genetic variability among both species. While macroalgal species generally exhibit low amounts of electrophoretically detectable genetic variation in natural populations (Sosa & Lindstrom 1999), our success in the genetic variability analysis is likely attributed to the employed DNA extraction method, ensuring higher purity, minimal contamination, and absence of PCR inhibitors, thereby enhancing PCR product amplification quality.

In conclusion, this study presents the first ISSR data for *L. nigrescens* and *L. trabeculata* to clarify their genetic diversity status. *L. nigrescens* demonstrated slightly higher genetic diversity than *L. trabeculata*. The presence of two main clades with strong support is consistent with clustering analyses observed in previous studies. Additionally, we identified four species-diagnostic ISSR markers for *L. nigrescens* and *L. trabeculata*, providing valuable tools for DNA fingerprinting techniques.

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