



Development of tools to rapidly identify cryptic species and characterize their genetic diversity in different European kelp species

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Abstract

Marine ecosystems formed by kelp forests are severely threatened by global change and local coastline disturbances in many regions. In order to take appropriate conservation, mitigation, and restoration actions, it is crucial to identify the most diverse populations which could serve as a “reservoir” of genetic diversity. This requires the development of specific tools, such as microsatellite markers to investigate the level and spatial distribution of genetic diversity. Here, we tested new polymorphic microsatellite loci from the genome of the kelp, *Laminaria digitata*, and tested them for cross-amplification and polymorphism in four closely related congeneric species (*Laminaria hyperborea*, *Laminaria ochroleuca*, *Laminaria rodriguezii*, and *Laminaria pallida*). Adding these 20 new microsatellite loci to the ten *L. digitata* loci previously developed by Billot et al. (Mol Ecol 7:1778–1780, 1998) and Brenan et al. (J R Soc Interface 11:1–12, 2014) and to the ten *L. ochroleuca* loci previously developed by Coelho et al. (Conserv Genet Resource 6:949–950, 2014), we retained a total of 30 polymorphic loci for *L. digitata*, 21 for *L. hyperborea*, 16 for *L. ochroleuca*, 18 for *L. rodriguezii*, and 12 for *L. pallida*. These markers have been tested for the first time in the last two species. As predicted, the proportion of markers that cross-amplified between species decreased with increasing genetic distance. In addition, as problems of species identification were reported in this family, mainly between *L. digitata* and *Hedophyllum nigripes*, but also between *L. digitata*, *L. hyperborea*, and *L. ochroleuca* in areas where their range distributions overlap, we report a rapid PCR identification method based on species-specific COI mitochondrial primers that allows these four species of kelp to be rapidly distinguished.

Keywords Genetic diversity · Microsatellite markers · Cross-amplification · Species identification · Brown seaweed

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Introduction

Kelp forests are mainly formed by large brown algae of the order Laminariales, are present along rocky shores of temperate to polar regions and occupy approximately 36% of the world's coastlines (Jayatilake and Costello 2021). They build major biogenic habitats that are one of the most diverse and productive ecosystems (Steneck et al. 2002; Bolton 2010; Krumhansl et al. 2016; Teagle et al. 2017) but are increasingly prone to decline due to climate change (e.g., Wernberg et al. 2012; Smale 2020) and anthropogenic pressures, including sedimentation and turbidity, as well as direct exploitation, such as for alginate (Teagle et al. 2017). A wealth of studies has revealed that kelp populations can be extremely structured in space and that genetic diversity is not evenly distributed across the species ranges. Instead, populations can be considerably differentiated and genetic diversity

concentrated in refugial areas of disproportionate conservation value, e.g., where species were able to persist across past glacial cycles (Assis et al. 2018; Neiva et al. 2020). In the context of the management of genetic resources, it is crucial to identify the most diverse populations which could serve as a “reservoir” of genetic diversity in order to inform appropriate conservation and restoration actions (Valero et al. 2011; Wernberg et al. 2018).

Within the order Laminariales, species of the *Laminaria* genus occur mainly in the northern Hemisphere on both Atlantic and Pacific coasts (Bartsch et al. 2008). The recent phylogeny of Rothman et al. (2017) grouped the six Atlantic and the Mediterranean species of this genus within the same clade. Some of these species are distributed in the northern Hemisphere with three of them (*L. digitata* (Hudson) J.V.Lamouroux, *L. hyperborea* (Gunnerus) Foslie, and *L. ochroleuca* Bachelot Pylaie) being partially sympatric along the NE Atlantic coast (Bartsch et al. 2008; Araújo et al. 2016), while the other one (*L. rodriguezii* Bornet) is a deep-water Mediterranean-endemic (Ballesteros 2006; Araújo et al. 2016). *L. ochroleuca* has also been observed in deep waters in some parts of its distribution (Araújo et al. 2016; Assis et al. 2018). The other part of the clade is constituted by two species occurring in Southern Atlantic, along a restricted part of the Brazilian coast (the deep-water *L. abyssalis* AB Joly & EC Oliveira; Marins et al. 2014) and the Namibian and South African coasts (*L. pallida* Greville; Rothman et al. 2017). Molecular tools can be useful to estimate the conservation status of a species (Soulé and Mills 1992; Allendorf and Ryman 2002), in addition to the demographic history and the evolutionary and conservation value of distinct populations. In the genus *Laminaria*, ten microsatellite markers have been specifically developed for *L. digitata* (Billot et al. 1998; Brennan et al. 2014) and an additional ten for *L. ochroleuca* (Coelho et al. 2014). These genetic markers cross-amplified across these two species as well as for *L. hyperborea* and were used for phylogeography and connectivity studies (Billot et al. 2003; Valero et al. 2011; Couceiro et al. 2013; Robuchon et al. 2014; Assis et al. 2018; Evankow et al. 2019; King et al. 2019; Liesner et al. 2020; Neiva et al. 2020; Schoenrock et al. 2020), but no microsatellite markers have been developed for the other species of the same genus.

Another challenge in *Laminaria* genus that can be overcome by the use of molecular tools is field misidentification of cryptic species (species that are problematic to identify based on morphological criteria), which can lead to inaccurate estimation of population connectivity and dynamics and therefore can have deleterious consequences on their conservation (Saunders 2005; Longtin and Saunders 2015). Taxonomic confusions based on morphological traits were reported between species of this family with overlapping range distributions (e.g., between *L. digitata* and *L.*

hyperborea, Schoenrock K. M., Krueger-Hadfield S. A., Robuchon M., pers. comm.), especially between *L. digitata* and *Hedophyllum nigripes* (J.Agardh) Starko, S.C.Lindstrom & Martone 2019 (as *Saccharina groenlandica*, Longtin and Saunders 2015; as *Saccharina nigripes*, Longtin and Saunders 2016; as *Hedophyllum nigripes*, Neiva et al. 2020). These problems are also frequent when dealing with juvenile stages. In these situations, DNA-based methods, such as DNA barcoding of the mitochondrial cytochrome c oxidase subunit I gene (COI hereafter), have been proven especially useful to uncover and/or confirm the identity of cryptic species where classical taxonomy were reported problematic (McDevit and Saunders 2009). Indeed, molecular barcoding was reported to be the only way to distinguish *L. digitata* from *H. nigripes* in the European Arctic where both species share the same habitat (Fredriksen et al. 2019). However, a faster and cheaper method is still needed, particularly when the ID of a large number of samples needs to be quickly confirmed, such as when selecting individuals for physiological experiments.

In this study, we developed new microsatellite markers based on the genome of *L. digitata* and tested them for transferability (cross-amplification and polymorphism) in four other *Laminaria* species (*L. hyperborea*, *L. ochroleuca*, *L. rodriguezii*, and *L. pallida*). In parallel, we developed a cheap and rapid PCR species identification method based on species-specific primers for the COI gene to avoid misidentification between sister species and with *H. nigripes*, a species which is frequently mistaken for *Laminaria* species.

Materials and methods

Sample collection and DNA extraction

Two types of sampling were used in this paper: (1) “wide scale sampling” for which we used one to several individuals sampled from different populations over the entire range distribution of the different species; (2) “single population sampling” for which we used individuals coming from a unique population. The wide scale sampling was designed to capture the extent of variability over the entire range of the species, in order to characterize new microsatellite markers in *L. digitata* and study their transferability in four sister species. When possible, a minimum of ten localities per species was retained, from which a single individual per locality was used (giving a total of 11 to 15 individuals for *L. digitata*, *L. hyperborea*, and *L. ochroleuca*). We had access to only four localities for *L. rodriguezii*; therefore, two individuals were analyzed from each locality (giving a total of eight individuals). For *L. pallida* as we had only access to samples from a single locality, 11 individuals were used, giving a total of 58 individuals across the species (Supp. Table 1). The single

population sampling was used to assess the polymorphism within a population with a minimum of 11 to 32 individuals for each species (giving a total of 107 individuals, Supp. Table 1).

The molecular tool developed to identify species (called “species identification molecular tool” hereafter) is based on mtDNA and has been developed from the wide scale sampling dataset for *L. digitata*, *L. hyperborea*, and *L. ochroleuca* to which we have added *H. nigripes* (eight individuals from four localities, Supp. Table 1), a species that is morphologically difficult to distinguish from *L. digitata*.

For all samples, genomic DNA was extracted from 5 to 10 mg of dry tissue using the Nucleospin 96 plant kit (Macherey–Nagel, Germany). The extraction was performed according to the manufacturer’s instructions except that samples were left in the PL1 lysis buffer at 65 °C for 15 min rather than 30 min. The extracted DNA was eluted in 200 µL of the supplied elution buffer.

Microsatellite loci screening and primer design

A total of 81,223 *L. digitata* unique contigs obtained from the draft assembled genome of a male gametophyte (Lami-Digitata_Contigs_V1.fa) of the Phaeoexplorer project (unpublished, M. Cock pers. comm.) were screened to identify and remove contigs containing microsatellite markers that had been previously published (Supp. Table 2). These represented a total of 23 contigs associated with previously developed microsatellite loci for *L. digitata* (Billot et al. 1998; Brennan et al. 2014) and *L. ochroleuca* (Coelho et al. 2014). A total of 81,200 unique contigs were screened for di-, tri-, tetra-, and pentanucleotide microsatellite motifs using the SPUTNIK program (<http://abajian.net/sputnik/>), which uses a recursive algorithm to identify repeated patterns of nucleotides with a length ranging between two and five. PCR amplification primers were designed using Primer3 (Rozen and Skaletsky 2000) for regions with a flanking sequence of sufficient length on either side of the repeated motif. In-house scripts were used to specify the primer selection conditions, which used default values for all parameters except the optimum melting temperature, which was set to 60 °C (range 57–62 °C), and the expected product size which was set at 400 bp.

In order to cost-effectively test these primers for amplification, we reduced the number of selected microsatellite regions to 96 (the capacity of a microtiter plate), by employing the following criteria: we first eliminated duplicate regions and all regions containing an unknown base (N) within the repeated motifs (56,034 microsatellite regions remaining); then, the number of microsatellite regions was further reduced to 96 by selecting the ones with the highest number of repeats for each of the four motif categories (see Supp. Table 3 for details).

Amplification trials of microsatellite loci

The 96 primer pairs were tested for amplification using DNA from 15 *L. digitata* individuals (Supp. Table 1). Amplifications were carried out in 10 µL reaction volume with 2 µL of DNA template diluted to 1:100 and following the instructions from Guzinski et al. (2016). The PCR products labeled with the four different colors were pooled (24 pools in total) and diluted 1:10 with water. Next, 2 µL of the diluted PCR product pool was added to 10 µL of loading buffer made up of 0.5 µL of the SM594 size standard (Mauger et al. 2012) and 9.5 µL of Hi-Di formamide, denatured at 95 °C for 3 min, and run in an ABI 3130 XL capillary sequencer (Applied Biosystems, USA). Genotypes were scored manually in Genemapper version 4.0 (Applied Biosystems). No product was amplified from 59 of the initial 96 primer pairs for any of the 15 individuals despite several amplification attempts (Supp. Table 3). In addition, 17 other primer pairs were monomorphic, and therefore, further analyses were conducted on the remaining 20 polymorphic markers (Supp. Tables 3 and 4).

Transferability of microsatellite loci and investigation of the levels of polymorphism

Samples from the wide scale sampling dataset (15 *L. digitata*; 13 *L. hyperborea*; 11 *L. ochroleuca*; 8 *L. rodriguezii*; and 11 *L. pallida*, Supp. Table 1) were genotyped for the 20 retained polymorphic primers to assess polymorphism and cross-amplification. Each of the 20 loci was amplified with labeled primers in the same manner as described for the amplification trials, except the addition of 0.3 µM of forward fluorescently labeled primer (Eurofins Genomic, Germany) and 0.3 µM of reverse primer (see Table 1 for primer information). Amplifications were carried out in the following conditions: initial denaturation at 95 °C for 5 min, 10 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 30 s (−1 °C per cycle—touchdown to 58 °C), extension at 72 °C for 30 s, 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, followed by a final extension of 72 °C for 10 min. Genotyping were carried out in the same manner as described for the amplification trials.

In addition to these 20 newly developed polymorphic microsatellite loci, we also screened all samples for the microsatellites previously developed for *L. digitata* from a nuclear library (Ld1_124, Ld2_148, Ld2_158, Ld2_167, Ld2_371, Ld2_531, Ld2_704, Billot et al. 1998), from an Expressed Sequence Tag (EST) library (AW401303, CN466672, CN467658, Brennan et al. 2014) and the ones developed for *L. ochroleuca* from a nuclear library (Lo4-24, LoIVVIV-10, LoIVVIV-13, LoIVVIV-15, LoIVVIV-16, LoIVVIV-17, LoIVVIV-23, LoIVVIV-24, LoIVVIV-27,

Table 1 Technical information concerning the 40 microsatellite primers selected for this study (primer sequence, repeat motif, dye, annealing temperature) and characteristics of the amplicons (*Na* and observed size range) for each of the five *Laminaria* species

Locus	Source	Contigs no	Primers sequences (5'-3')	Repeat motif	Dye	<i>T_a</i> (°C)	MgCl ₂ (mM)	<i>L. digitata</i>		<i>L. hyperborea</i>		<i>L. ochroleuca</i>		<i>L. rodriguezii</i>		<i>L. pallida</i>	
								<i>Na</i>	Size range (bp)	<i>Na</i>	Size range (bp)	<i>Na</i>	Size range (bp)	<i>Na</i>	Size range (bp)	<i>Na</i>	Size range (bp)
Ld19_003	This study	1453	F: ATTCCGTGGTTGTGA GAGAGA R: CCGATACGAGAGACA GCACCTT	(GA) ₃₀	VIC	68 ↓ 58	2.0	5	141–165	6	147–163	3	153–210	3	146–202	1	153
Ld19_004	This study	28527	F: TGGGTGGTGTGTGG ATGT R: GGCAGTCGTAGGTAT CGGTT	(AC) ₃₀	PET	68 ↓ 58	2.0	12	97–185	NA	NA	NA	NA	NA	NA	NA	NA
Ld19_005	This study	5613	F: GGCTGATAGACGGAG ACAGAG R: GCAGTAGAAGGAGTG GACGAA	(GA) ₄₇	NED	68 ↓ 58	2.0	16	243–379	NA	NA	NA	NA	6	262–302	NA	NA
Ld19_021	This study	17056	F: CGTCAATGTGGTATG GTATGG R: ACTCGTAACCTGGC GATTT	(TATGG) ₂₄	VIC	68 ↓ 58	2.0	7	235–300	4	205–220	NA	NA	NA	NA	NA	NA
Ld19_025	This study	52036	F: ATTCCACCATTCAT TCCATC R: GGAGTTGTGACGCC TTTC	(ATCC) ₄₆	FAM	68 ↓ 58	2.0	9	282–387	NA	NA	NA	NA	5	185–240	3	98–138
Ld19_027	This study	2628	F: ACGGCACAGGTTAGC AGTT R: ATAGCTTCCGCCCTT TCGCT	(TACCA) ₁₂	PET	68 ↓ 58	2.0	5	271–311	NA	NA	NA	NA	NA	NA	NA	NA
Ld19_032	This study	1519	F: TTTGCTTTGATGATACTG CGA R: GTGGGTGAGTGGGTT GGTT	(TACTT) ₃	NED	68 ↓ 58	2.0	3	387–397	5	390–415	NA	NA	4	370–395	2	390–405
Ld19_033	This study	8512	F: ACGAGGACGAAGGTA GACTGG R: GACGGAAGGTAGGCT CTGG	(GCAG) ₁₇	NED	68 ↓ 58	1.0	16	103–179	9	147–171	1	129	8	137–169	NA	NA
Ld19_034	This study	3470	F: CCCAGTCAAGAAATC AACCA R: GCTTTGCTCCGTGCCCTCT	(TCAA) ₁₇	VIC	68 ↓ 58	2.0	4	128–172	1	144	1	102	1	118	3	94–110
Ld19_038	This study	16041	F: TTGTTGGTTTGTGTGTCT GTTTG R: GAATCCTCTCGCTTTCG TTGTTC	(TGTT) ₁₅	FAM	68 ↓ 58	2.0	7	123–159	7	124–144	8	100–184	6	112–184	7	109–167
Ld19_047	This study	13132	F: CTATCTGTCTGTCCG TCCGTC R: CGTAAAGGCTCTCGCA ATCA	(TCTG) ₂₃	VIC	68 ↓ 58	2.0	4	172–186	3	174–182	9	86–130	8	106–126	6	145–197
Ld19_052	This study	13699	F: ATCAACCACAGACGG ACAGAC R: CTGCTGACACCTTA CCCTC	(ACAG) ₄₇	NED	68 ↓ 58	1.0	7	269–309	7	118–190	3	250–288	6	259–287	NA	NA

Table 1 (continued)

								<i>L. digitata</i>	<i>L. hyperborea</i>	<i>L. ochroleuca</i>	<i>L. rodriguezii</i>	<i>L. pallida</i>				
Ld19_053	This study	25504	F: CCAACCTACTACGAC GAACCA R: GATAGACAGATAGAC AGCCAGCC	PET	68 ↓ 58	2.0	6	271–291	9	192–264	7	283–315	NA	6	205–237	
Ld19_067	This study	8496	F: CGCAGTAGGATAGCA GAGGAG R: CAGCAGCAGTAGAAA CAGCAC	FAM	68 ↓ 58	2.0	4	145–187	6	151–178	2	136–178	2	173–197	5	132–225
Ld19_069	This study	16361	F: CGGAGATGAGGGTAT GTGAGA R: ATTCTTGGTGGTTA CAGCGA	NED	68 ↓ 58	1.0	12	103–163	4	112–127	3	94–109	4	120–129	NA	NA
Ld19_071	This study	70523	F: ACTCTCTCACCACC ACCATC R: CCTAAGCCTCAAAC ACTAA	PET	68 ↓ 58	2.0	8	120–147	NA	NA	NA	NA	NA	NA	NA	NA
Ld19_074	This study	28415	F: CTGTCCTCTTTGCCA TCGT R: CGCTTCATTGCTGTT CCTTT	NED	68 ↓ 58	2.0	2	199–214	7	130–190	5	156–207	5	169–220	2	204–216
Ld19_076	This study	4142	F: CGAAACAGCAACAAC AGCAA R: GTCGTGGTCGTCGTG GTC	FAM	68 ↓ 58	2.0	7	274–358	NA	NA	3	252–279	4	265—286	NA	NA
Ld19_080	This study	29473	F: GCTGCTACTGCTCCT ACTGCT R: GTGGGTCTCTGATTAC TATGCC	PET	68 ↓ 58	2.0	11	144–231	NA	NA	NA	NA	NA	NA	NA	NA
Ld19_082	This study	28987	F: ACACCAACAACACCA ACAACA R: CGGACGAAATGAAAT GAAAGA	FAM	68 ↓ 58	1.0	4	271–289	7	254–278	4	203–266	NA	3	204–243	NA
CN466672	Brennan 2014	12546	F: CTCACAAGATCCGCCTTG R: GGTCTGCTTCTTTTCG TTGC	FAM	58	2.5	9	81–114	3	98–107	2	87–90	5	83–101	2	87–90
CN467658	Brennan 2014	5588	F: CTCCTCCGTCGACCT TGTTTC R: GGTCGAGGCGATTTT TCATA	PET	58	2.5	10	197–248	2	212–215	1	204	1	219	1	204
AW401303	Brennan 2014	59231	F: TTCGAACCCAGTATCTAC CTCGT R: CCGAGTACAGGCGGC AAC	NED	58	2.5	10	252–303	NA	NA	NA	NA	NA	NA	NA	NA
Ld1_124	Billot 1998	10005	F: ATCTACATTACTGAATGC TCTTCGG R: CACTCTGCCCGCTGACC	VIC	60	2.5	6	109–130	NA	NA	NA	NA	2	124–127	NA	NA

Table 1 (continued)

									<i>L. digitata</i>	<i>L. hyperborea</i>	<i>L. ochroleuca</i>	<i>L. rodriguezii</i>	<i>L. pallida</i>		
Ld2_148	Billot 1998	7906	F: TGCGTGTTCCTGA TATG R: TTTCCTGTCTAGGCCTCT CTGG	(CAA) ₁₈	PET	60	2.5	9	168–204	6	206–248	2	174–192	4	170–190
Ld2_158	Billot 1998	4964	F: CGACTAGAGGGAGC GAGAA R: CGTTTITGCGCCTAA CGTAT	(AC) ₁₁	NED	60	2.5	7	199–244	2	221–228	NA	NA	2	199–215
Ld2_167	Billot 1998	506	F: CGGACTCGATTTAGCGA TGGG R: TCGGAAGCACGTGTT CTGTAT	(CAA) ₁₁	VIC	60	2.5	8	152–191	8	146–167	1	90	NA	NA
Ld2_371	Billot 1998	74542	F: TACATGCTCTGTCTTT TGTGCG R: AGGAAAAGCGGTGC AGTATTA	(TTG) ₁₆	NED	60	2.5	10	117–180	NA	NA	1	90	2	199–215
Ld2_531	Billot 1998	22811	F: TACTAACCCATTTTGT TGTGC R: CGACGTGGTCTTGTCT CATC	(ATT) ₈	FAM	60	2.5	5	200–242	1	200	NA	NA	NA	NA
Ld2_704	Billot 1998	6665	F: GTCACAAGTAGTGT TAAAAG R: CTGTGGTGTCTTTTCG TCTCC	(TC) ₂₃	PET	60	2.5	5	274–294	10	320–426	1	306	1	286
Lo4_24	Coelho 2014	8468	F: AACGCTGTGGGTACAT R: CATTTCTTTACCGC TATC	(TC) ₉	PET	50	2.0	2	222–224	3	212–218	3	220–230	1	210
LoIVVIV_10	Coelho 2014	4477	F: TTCAACAGCGAGGT TTAGG R: GTGGTGGGTGGAGGT AGTGT	(GCT) ₁₅	VIC	60	1.5	4	137–218	NA	NA	10	184–232	3	140–165
LoIVVIV_13	Coelho 2014	2387	F: ACACAATTGCCGTC CTTTC R: AGCTCATGCTGCACA TTGTC	(AAT) ₂₀	VIC	60	1.5	3	153–162	1	160	7	184–229	NA	NA
LoIVVIV_15	Coelho 2014	52998	F: AGAATCAGGACGGAC ACTGG R: TCCGCGACTTATTC CTCTA	(AAC) ₂₁	FAM	60	1.5	6	152–197	6	152–173	5	188–215	4	170–182
LoIVVIV_16	Coelho 2014	10622	F: TTGTCAGATGGCAA TGGAA R: TGGAGTGGATAAGGG AGCAC	(AAC) ₁₇	PET	60	1.5	3	192–219	7	205–238	5	211–235	3	201–213
LoIVVIV_17	Coelho 2014	6887	F: ATACTCTGGGCCGG TTACT R: CGAGGTGGGAGTAG TGGT	(ACC) ₁₆	VIC	60	2.0	6	159–195	5	156–180	3	197–203	1	180

Table 1 (continued)

								<i>L. digitata</i>	<i>L. hyperborea</i>	<i>L. ochroleuca</i>	<i>L. rodriguezii</i>	<i>L. pallida</i>					
LoVVIV_23	Coelho 2014	4727	F: AGAAAAAGCCTGCCG TGAC R: CAGCCTGGAGCTTTC GAT	(AAC) ₁₄	NED	60	2.0	5	304–316	6	196–223	7	134–158	3	129–140	5	364–376
LoVVIV_24	Coelho 2014	19992	F: AAGTTTCGTCTCCGT TTCCTC R: TTAACGTTCTGTCAC CGTAG	(GTT) ₁₇	FAM	60	2.0	5	307–328	6	198–289	7	327–363	1	295	5	292–323
LoVVIV_27	Coelho 2014	56090	F: GACTGCCCTTTTGAT TTTGA R: AATGGTGTCTGACTT GGGTTG	(CTGT) ₁₁	PET	60	2.0	2	201–205	2	201–205	2	235–239	2	206–210	4	206–236
LoVVIV_28	Coelho 2014	51886	F: CACCAAGCTTGATTG GTTGA R: TCGTGAGGTTATCGT GGTGA	(ATCC) ₁₆	NED	60	2.0	5	198–242	7	223–259	3	226–234	1	187	2	194–210

F forward primer (labeled with fluorescent dye), *R* reverse primer, *T_a* annealing temperature (start *T* (°C) ↓ final *T* (°C)) and MgCl₂ concentration used for PCR (mM)

N_a number of alleles, observed size range in base pairs (bp). *NA* no amplification

LoIVVIV-28, Coelho et al. 2014), giving a total of 40 microsatellite markers. Exceptionally, a new reverse primer Ld1_124_R2 (5'-CACTCTGCCCCGCTGACC-3') was designed for the locus Ld1_124 (using the contig n°10,005) to increase the size of the amplification products (+ 185 bp) compared to the ones obtained with the primer pairs described in Billot et al. (1998). The purpose of this increase was to facilitate scoring in GENEMAPPER v4.0 (Applied Biosystems). Genotyping was conducted using the same protocol as described in the section “Amplification trials of microsatellite loci.”

Genetic analyses

In order to compare the levels of polymorphism between the set of markers, the 40 markers were amplified in each species using the single population sampling dataset (11 to 32 individuals per populations, Supp. Table 1). Prior to the genetic analysis, we tested for null alleles using MICRO-CHECKER (Van Oosterhout et al. 2004). GENEPOP v4.7.5 (Rousset 2008) was used to test for linkage disequilibrium (global test employing Fisher's method, the following Markov chain parameters were used: 100,000 for dememorization, 1000 batches and 50,000 iterations per batch). The same software was used to obtain the number of observed alleles per locus (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), the within-population deviation from Hardy–Weinberg equilibrium (F_{IS}), and its significance. The polymorphic information content (PIC) was calculated with CERVUS (Marshall et al. 1998).

Development of a rapid species identification molecular tool

A fragment of the mitochondrial COI gene (COI-5P) was obtained for *L. digitata*, *L. hyperborea*, *L. ochroleuca*, and *H. nigripes* using the primers GAZ_F2 and GAZ_R2 (Lane et al. 2007). Ninety-nine individuals (53 *L. digitata*, 13 *L. hyperborea*, 11 *L. ochroleuca*, and 22 *H. nigripes*, Supp. Table 1) were sequenced, and four consensus sequences (one per species) were generated using CODONCODEALIGNER (<https://www.codoncode.com/>). New species-specific primers were designed to amplify shorter fragments using the AMPLIFX program (<https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr/>). These primers were Ld_F2 and Ld_R for *L. digitata*, Lh_F2 and

Lh_R for *L. hyperborea*, Hn_R for *H. nigripes*, and PC_F for positive control of PCR (Table 2 and Fig. 1).

In order to test whether the primers could be used to distinguish the four species, two multiplex PCRs containing four primers each were generated PCR1 and PCR2 (Table 2), and the expected sizes for each species were obtained using AMPLIFX. PCR were carried out in 10 µL reaction volumes containing 2 µL of DNA template diluted 1:100, 1X Green GoTaq Flexi Buffer (Promega, USA), 0.5 µM of each primer (Table 2), 2 mM MgCl₂, 150 µM dNTPs (Thermo Fisher Scientific Inc., USA), and 0.35 U GoTaq Flexi Polymerase (Promega). DNA amplifications were carried out in a Bio-Rad DNA Engine Peltier Thermal Cycler under the following conditions: initial denaturation phase at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. For each sample, the two multiplex PCRs were pooled, and amplified fragments were visualized under UV light after electrophoresis on 3% (p/w) agarose gels stained with ethidium bromide (Fig. 2).

Results

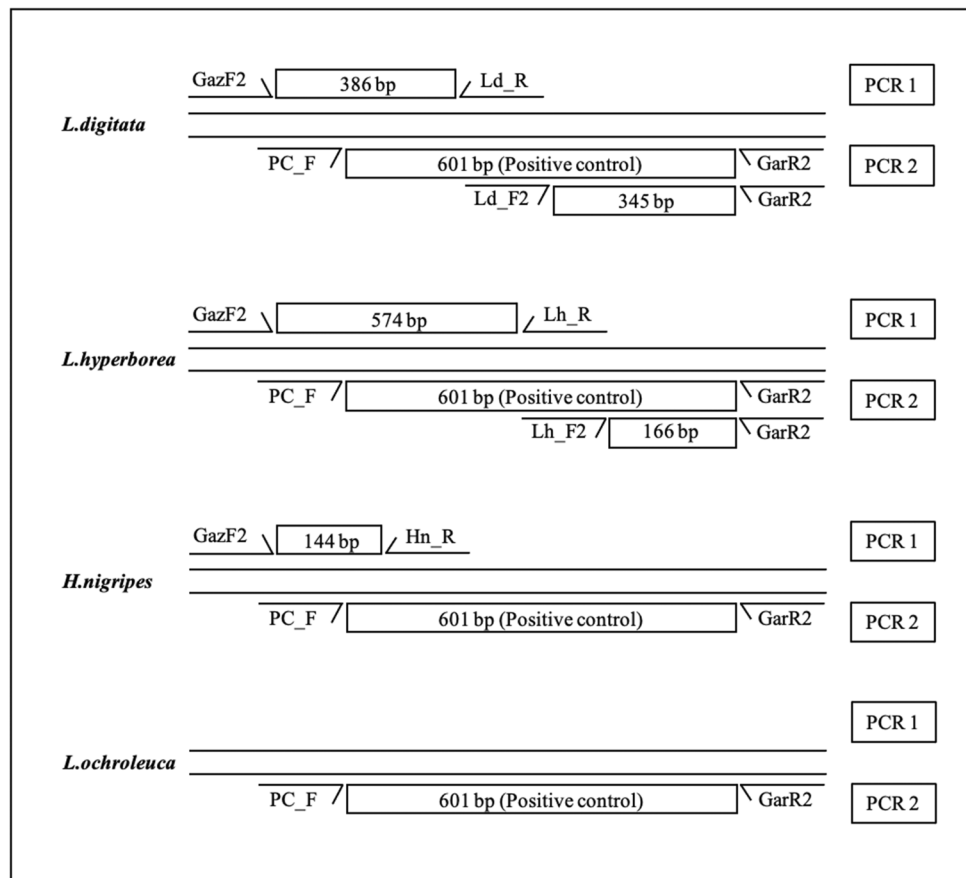
Development of microsatellites

In the genome of *L. digitata*, 305,646 microsatellite motifs were found among the 81,200 unique contigs. The microsatellites contained 33,861 (11.1%) di-, 151,588 (49.6%) tri-, 54,408 (17.8%) tetra-, and 65,798 (21.5%) pentanucleotide motifs (Supp. Table 3). After choosing regions with flanking sequences of sufficient length and giving a product size of 400 bp, we obtained 273,231 primer pairs corresponding to 30,900 (11.3%) di-, 138,604 (50.7%) tri-, 49,062 (18%) tetra-, and 52,665 (20%) pentanucleotide repeated motifs (Primer3 output, Supp. Table 3). Among the 96 primer pairs tested, 37 were amplified (including 4 di-, 13 tri-, 12 tetra-, and 8 pentanucleotide repeats, Supp. Table 3) and were retained for further analyses. Twenty out of these 37 putative loci (including 7 tri-, 6 tetra-, 4 penta-, and 3 dinucleotide repeats, Supp. Table 3) produced PCR products of the expected size and were polymorphic. Therefore, the rate of success for each of the four motif categories in the acquisition of polymorphic microsatellites was 18.8% for di-, 21.9% for tri-, 18.8% for tetra-, and 25.0% for pentanucleotide repeats (Supp. Table 3).

Table 2 Primers sequence (5'-3') and multiplex composition of PCR 1 and PCR 2 used for rapid identification molecular tool

PCR 1	PCR 2
GAZ_F2: CCAACCAYAAAGATATWGGTAC	GAZ_R2: GGATGACCAAAARAACCAAAA
Ld_R: CTGAATACCACTAAGGGGG	Ld_F2: GACGGTCTACCCCCCCC
Lh_R: AGCGATAACAGTAACAAGAAA	Lh_F2: GGTCTGTCTTAATCACAGCTTTC
Hn_R: CATTGTATAACTGGTGATTACCTCC	PC_F: GTCCTGGAAATCAATTTTtaggagg

Fig. 1 Annealing sites and expected fragment sizes for the five *Laminaria* species using the rapid identification method. PCR products were pooled from PCR 1 and PCR 2 and include the positive internal control amplification product (601 bp), two fragments for *L. digitata* (345 bp and 386 bp) and *L. hyperborea* (574 bp and 166 bp), one fragment for *H. nigripes* (144 bp), and none for *L. ochroleuca*



Transferability assessment

All the loci analyzed in this study (20 microsatellites markers developed in this study, in addition to the 20 previously published ones, see “Materials and methods” section) were selected for their amplification success and their polymorphism (in the case of the new microsatellites markers) in the focal species (*L. digitata*). Twenty-nine of these 40 markers were successfully amplified in *L. hyperborea*, 28 in *L. ochroleuca*, 29 in *L. rodriguezii*, and 22 in *L. pallida* and 18 markers amplified across all species (Table 1). The proportion of microsatellite markers that cross-amplified decreased with increasing phylogenetic distance from *L. digitata* (Table 3, ranked according to Rothman et al. 2017 phylogeny when considering the 30 markers developed from *L. digitata* genome). In addition, we tested the ascertainment bias hypothesis which stipulates that the median allele length of microsatellites is longer in the species from which the markers were derived (Ellegren et al. 1995). For this purpose, we computed the proportion of markers associated with a higher allelic size in the focal species compared to each sister species taken individually and when taken altogether (Table 4). The percentage of markers which showed a higher allelic size in *L. digitata*

never exceeded 50%, as expected under the ascertainment bias hypothesis, and ranged from 15 to 30% (corresponding to *L. rodriguezii* and *L. ochroleuca*, respectively, Table 4) and dropped to 0% when considering all sister species (Table 4). Therefore, our results do not seem to support this hypothesis.

Genetic diversity across species

Null allele frequency, genetic diversity indices, and estimates of departure from random mating (F_{IS}) are given in Table 5 for each of the 40 loci and species. However, among the loci that correctly amplified, several markers (10 in *L. digitata*, 8 in *L. hyperborea*, 12 in *L. ochroleuca*, 11 in *L. rodriguezii*, and 10 in *L. pallida*, Table 5) were discarded because they appeared to be either monomorphic within populations, amplified in less than 80% of the individuals, showed a significant frequency of null alleles, or showed an extreme departure from random mating in *L. digitata* suggesting non-Mendelian inheritance ($F_{IS} = -0.83$, Table 5). Overall, from our study, 30 markers appear to be useful for *L. digitata*, 21 for *L. hyperborea*, 16 for *L. ochroleuca*, 18 for *L. rodriguezii*, and 12 for *L. pallida* (corresponds to the markers highlighted in light gray in Table 5). Therefore, the number of polymorphic loci at least doubled in the three first

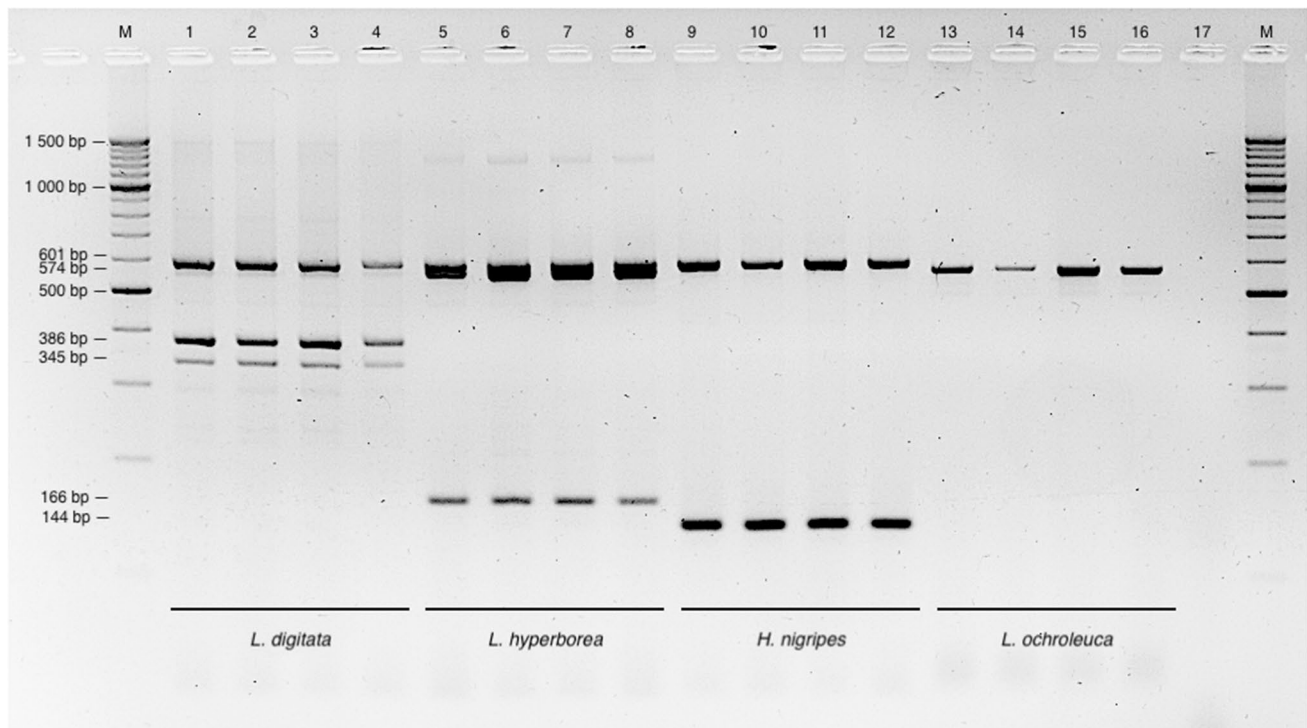


Fig. 2 Gel electrophoresis of PCR amplicons was pooled from PCR 1 and PCR 2 using the rapid species identification tool. (1–4) *L. digitata* samples; (5–8) *L. hyperborea* samples; (9–12) *H. nigripes* sam-

ples; and (13–16) *L. ochroleuca* samples. (17) pooled negative control and (M) 100 bp ladder

species (17 more polymorphic loci for *L. digitata*, 11 for *L. hyperborea*, 9 for *L. ochroleuca*) compared to the previous studies. In addition, a minimum of 12 new polymorphic loci was developed for *L. rodriguezii* and *L. pallida*, for which no microsatellite markers were available before this study.

For *L. digitata*, the within-population genetic diversity indices of newly developed markers (Table 5) were on average similar to those previously developed from the *L. digitata* genome (markers from Billot et al. 1998 and Brennan et al. 2014) and were one and a half to two times higher than the ones developed from *L. ochroleuca* genome (markers from Coelho et al. 2014). It should be noted that seven out of the 10 markers developed from *L. ochroleuca* could not be used in our *L. digitata* population, either because of the significant frequency of null alleles, the highly significant deviation from random mating (locus LOIVVIV_10, LOIVVIV_16, LOIVVIV_24, LOIVVIV_28), or because they were monomorphic in the studied population (Lo4_24, LOIVVIV_13, LOIVVIV_27).

Table 3 Percentage of primers defined based on the *L. digitata* genome that amplified in each of the sister *Laminaria* species

Compared species	Ratio	%
<i>Lh/Ld</i>	20/30	67%
<i>Lo/Ld</i>	18/30	60%
<i>Lr/Ld</i>	20/30	67%
<i>Lp/Ld</i>	14/30	47%

The level of polymorphism was highly variable among the 30 retained loci for *L. digitata*, with the number of alleles (N_a) ranging from 2 to 16 with a mean value of 5.89, the expected heterozygosity (H_e) from 0.092 to 0.910 with a mean value of 0.581 and PIC from 0.089 to 0.868 with a mean value of 0.537, while the departure from random mating (F_{IS}) was less variable among loci ranging from -0.264 to 0.305 with a mean value of 0.048.

For all sister species, we observed a slight decrease in genetic diversity with increasing genetic distance from *L. digitata* (whichever the diversity index and the set of markers, Table 5). However, a non-negligible number of markers remained informative as illustrated by the PIC values

Table 4 Percentage of microsatellite markers with a higher allelic size in the focal species compared to the sister species

Compared species	Ratio	%
<i>Lh/Ld</i>	4/19	21%
<i>Lo/Ld</i>	5/18	30%
<i>Lr/Ld</i>	3/20	15%
<i>Lp/Ld</i>	4/14	28%
$(Lh + Lo + Lr + Lp)/Ld$ *	0/10	0%

Ld *L. digitata*, *Lh* *L. hyperborea*, *Lo* *L. ochroleuca*, *Lr* *L. rodriguezii*, *Lp* *L. pallida*

*In this comparison, we took into account only the markers that amplified in all sister species

Table 5 Genetic diversity at each polymorphic microsatellite locus in the five *Laminaria* species

Locus	<i>L. digitata</i>						<i>L. hyperborea</i>						<i>L. ochroleuca</i>								
	Nf	n	Na	Ho	He	F _{IS}	PIC	Nf	n	Na	Ho	He	F _{IS}	PIC	Nf	n	Na	Ho	He	F _{IS}	PIC
Ld19_003	0.088	32	7	0.625	0.757	0.177	0.708	0.098	32	10	0.719	0.888	0.193	0.861	0.097	16	9	0.688	0.863	0.209	0.817
Ld19_004	0.199*	32	7	0.500	0.761	0.347 ^{\$}	0.713														
Ld19_005	0.225*	30	28	0.600	0.965	0.382 ^{\$}	0.947														
Ld19_021	0	32	11	0.844	0.847	0.004	0.818	0.037	27	7	0.593	0.651	0.091	0.595							
Ld19_025	0.021	32	12	0.844	0.894	0.057	0.868														
Ld19_027	0	32	4	0.594	0.472	-0.264	0.423														
Ld19_032	0	32	3	0.969	0.536	-0.830 ^{\$}	0.418	0.053	31	5	0.516	0.583	0.116	0.521							
Ld19_033	0.015	32	13	0.844	0.883	0.045	0.857	0.072	28	11	0.750	0.882	0.152	0.852		16	1				
Ld19_034	0	32	3	0.125	0.121	-0.033	0.116		32	1						16	1				
Ld19_038	0	32	7	0.844	0.790	-0.070	0.742	0.055	32	9	0.688	0.779	0.119	0.741	0	14	5	0.571	0.524	-0.095	0.473
Ld19_047	0	32	6	0.719	0.719	0.001	0.665	0.239*	31	5	0.161	0.267	0.400 ^{\$}	0.253	0	16	2	0.063	0.063	0.000	0.059
Ld19_052	0.045	32	7	0.656	0.729	0.101	0.672	0.001	31	6	0.613	0.625	0.019	0.538	0.031	15	8	0.733	0.807	0.094	0.753
Ld19_053	0	32	3	0.281	0.255	-0.103	0.237	0.067	32	16	0.719	0.835	0.141	0.807	0.127*	15	11	0.667	0.890	0.257 ^{\$}	0.845
Ld19_067	0	32	6	0.469	0.398	-0.182	0.365	0.168	32	4	0.125	0.178	0.301	0.170	0	16	4	0.375	0.333	-0.132	0.299
Ld19_069	0	32	16	0.938	0.870	-0.080	0.845	0	31	7	0.710	0.638	-0.114	0.592	0	16	7	0.688	0.633	-0.089	0.588
Ld19_071	0	32	7	0.438	0.394	-0.111	0.358														
Ld19_074	0	32	4	0.094	0.092	-0.016	0.089	0.102	32	5	0.188	0.234	0.200	0.223	0	16	5	0.250	0.238	-0.053	0.224
Ld19_076	0.065	31	10	0.677	0.785	0.139	0.742								0.157	13	2	0.154	0.271	0.442	0.226
Ld19_080	0.012	30	10	0.667	0.694	0.040	0.632														
Ld19_082	0.101	32	9	0.313	0.388	0.198	0.373	0.030	32	7	0.656	0.707	0.073	0.662	0	16	7	0.750	0.752	0.003	0.689
Mean	0.020	31.8	7.94	0.587	0.593	-0.006	0.559	0.062	30.9	7.91	0.571	0.636	0.117	0.597	0.032	15.3	5.44	0.475	0.498	0.042	0.459
CN466672	0.077	32	8	0.656	0.778	0.158	0.734	0.132	32	5	0.156	0.207	0.248	0.199	0.329*	16	4	0.313	0.639	0.519 ^{\$}	0.559
CN467658	0.066	32	5	0.531	0.616	0.140	0.555	0	32	2	0.063	0.062	-0.016	0.059		16	1				
AW401303	0.170	30	4	0.333	0.477	0.305	0.429														
Mean	0.104	31.3	5.67	0.507	0.624	0.201	0.573	0.066	32.0	3.50	0.110	0.135	0.116	0.129							
Ld1_124	0.080	28	6	0.500	0.597	0.166	0.553														
Ld2_148	0.060	32	7	0.688	0.788	0.129	0.747	0.085	29	5	0.655	0.790	0.173	0.740		16	1				
Ld2_158	0.037	28	4	0.571	0.626	0.089	0.556	0.124	29	4	0.172	0.225	0.237	0.212							
Ld2_167	0	32	7	0.750	0.752	0.003	0.702	0.099	29	7	0.655	0.813	0.197	0.771		16	1				
Ld2_371	0.044	32	13	0.813	0.901	0.099	0.877									12 [£]	1				
Ld2_531	0.004	32	4	0.688	0.704	0.024	0.637		29	1											
Ld2_704	0.088	32	3	0.375	0.454	0.177	0.371	0.308*	29	13	0.414	0.796	0.485 ^{\$}	0.765		15	1				
Mean	0.045	30.9	6.29	0.626	0.689	0.098	0.634	0.103	29.0	5.33	0.494	0.609	0.202	0.574							
Lo4_24		32	1					-0.038	29	3	0.172	0.163	-0.061	0.151	0	16	2	0.125	0.121	-0.034	0.110
LoIVVIV_10	0.741*	18 [£]	5	0.111	0.768	0.859 ^{\$}	0.709								0.399*	16	10	0.375	0.901	0.592 ^{\$}	0.860
LoIVVIV_13		32	1						30	1					0.093	16	5	0.563	0.700	0.201	0.638

Table 5 (continued)

<i>L. digitata</i>												<i>L. hyperborea</i>						<i>L. ochroleuca</i>					
Locus	Nf	n	Na	Ho	He	F _{IS}	PIC	Nf	n	Na	Ho	He	F _{IS}	PIC	Nf	n	Na	Ho	He	F _{IS}	PIC		
LoIVVIV_15	0	30	2	0.267	0.235	-0.137	0.204	0.280*	29	6	0.310	0.561	0.451 [§]	0.516	0	16	4	0.688	0.603	-0.146	0.496		
LoIVVIV_16	0	32	2	1.000	0.508	-1.000 [§]	0.375	0.293*	28	7	0.429	0.797	0.467 [§]	0.755	0	16	5	0.375	0.341	-0.104	0.317		
LoIVVIV_17	0	32	2	0.313	0.268	-0.170	0.229	0	29	5	0.690	0.673	-0.026	0.608	0	16	2	0.125	0.121	-0.034	0.110		
LoIVVIV_23	0	32	7	0.750	0.756	0.008	0.710	0.060	29	4	0.621	0.712	0.130	0.645	0.182	16	2	0.313	0.466	0.336	0.349		
LoIVVIV_24	0.349*	32	3	0.188	0.395	0.529 [§]	0.334	0.031	29	7	0.655	0.710	0.078	0.641	0	15	4	0.600	0.561	-0.072	0.495		
LoIVVIV_27		32	1						32	1						16	1						
LoIVVIV_28	0.757*	32	2	0.063	0.458	0.866 [§]	0.349	0.107	29	5	0.552	0.696	0.210	0.632	0.143	16	4	0.438	0.603	0.281	0.496		
Mean	0	31.3	3.67	0.443	0.420	-0.100	0.381	0.032	29.0	4.80	0.538	0.591	0.066	0.535	0.052	15.9	3.50	0.403	0.440	0.054	0.376		
Global mean	0.042	31.3	5.89	0.541	0.581	0.048	0.537	0.066	30.2	5.39	0.428	0.493	0.125	0.459	0.042	15.6	4.47	0.439	0.469	0.048	0.418		

<i>L. rodriguezii</i>												<i>L. pallida</i>									
Locus	Nf	n	Na	Ho	He	F _{IS}	PIC	Nf	n	Na	Ho	He	F _{IS}	PIC	Nf	n	Na	Ho	He	F _{IS}	PIC
Ld19_003	0	16	2	0.125	0.121	-0.034	0.110		11	1											
Ld19_004																					
Ld19_005	0.695*	16	4	0.125	0.718	0.831 [§]	0.636														
Ld19_021																					
Ld19_025	0.036	16	4	0.625	0.694	0.102	0.612	0	11	3	0.455	0.385	-0.190	0.326							
Ld19_027																					
Ld19_032	0.030	16	3	0.625	0.685	0.091	0.590	0	8 [£]	2	0.625	0.458	-0.400	0.337							
Ld19_033	0.456*	14	5	0.286	0.794	0.649 [§]	0.726														
Ld19_034		16	1					0.227	10	3	0.400	0.668	0.415	0.559							
Ld19_038	0	16	5	0.813	0.732	-0.114	0.661	0	10	7	0.600	0.584	-0.029	0.536							
Ld19_047	0.180*	16	6	0.500	0.742	0.333 [§]	0.680	0.342*	9	6	0.333	0.719	0.551 [§]	0.641							
Ld19_052	0	14	5	0.786	0.754	-0.044	0.682														
Ld19_053								0	7 [£]	6	0.857	0.791	-0.091	0.701							
Ld19_067	0.068	16	2	0.375	0.444	0.159	0.337	0	9	5	0.444	0.405	-0.103	0.368							
Ld19_069	0	16	4	0.750	0.669	-0.125	0.582														
Ld19_071																					
Ld19_074	0	16	5	0.875	0.726	-0.214	0.657	0	7 [£]	2	0.857	0.527	-0.714	0.370							
Ld19_076	0	14	3	0.714	0.688	-0.040	0.589														
Ld19_080																					
Ld19_082								0	7 [£]	3	0.571	0.473	-0.231	0.386							
Mean	0.011	15.6	3.67	0.632	0.613	-0.024	0.536	0.057	10.0	4.50	0.475	0.511	0.023	0.447							
CN466672	0	16	4	0.875	0.597	-0.489 [§]	0.510	0.154	11	2	0.364	0.519	0.310	0.373							
CN467658		16	1						11	1											

Table 5 (continued)

L. rodriquezii							L. pallida							
Locus	Nf	n	Na	Ho	He	F _{IS}	PIC	Nf	n	Na	Ho	He	F _{IS}	PIC
AW401303														
Mean	0	16	2	0.125	0.121	-0.034	0.110	0.154	11.0	2.00	0.364	0.519	0.310	0.373
Ld1_124														
Ld1_124	0	16	4	0.500	0.427	-0.176	0.387	0.059	10	4	0.600	0.711	0.163	0.619
Ld2_148														
Ld2_148	0.035	14	2	0.429	0.476	0.103	0.354							
Ld2_167														
Ld2_371														
Ld2_371	0.035	14	2	0.429	0.476	0.103	0.354							
Ld2_531														
Ld2_704		16	1					0.149	5 ^f	3	0.400	0.600	0.360	0.466
Mean	0.018	15.0	2.50	0.371	0.375	-0.001	0.301	0.059	10.0	4.00	0.600	0.711	0.163	0.619
Lo4_24		16	1						11	1				
LoIVVIV_10														
LoIVVIV_10	0.077	14	3	0.429	0.519	0.179	0.427							
LoIVVIV_13														
LoIVVIV_15														
LoIVVIV_15	0	16	4	0.625	0.556	-0.128	0.483	0	9	4	0.778	0.673	-0.167	0.565
LoIVVIV_16														
LoIVVIV_16	0.289	16	3	0.125	0.234	0.474	0.215	0	10	3	0.300	0.279	-0.080	0.247
LoIVVIV_17		16	1						10	1				
LoIVVIV_23														
LoIVVIV_23	0.172	16	3	0.375	0.548	0.323	0.468	0	11	5	0.818	0.658	-0.259	0.581
LoIVVIV_24		16	1					0.107	11	5	0.364	0.472	0.238	0.428
LoIVVIV_27														
LoIVVIV_27	0	16	2	0.500	0.484	-0.034	0.359	0.093	11	4	0.545	0.688	0.216	0.606
LoIVVIV_28		16	1					0.120	11	2	0.364	0.485	0.259	0.356
Mean	0.108	15.6	3.00	0.411	0.468	0.163	0.390	0.053	10.5	3.83	0.528	0.543	0.035	0.464
Global mean	0.045	15.4	3.06	0.471	0.485	0.046	0.409	0.081	10.4	3.58	0.492	0.571	0.133	0.476

Null allele frequency (*Nf*) obtained with MICROCHECKER (Van Oosterhout et al. 2004), locus showing significant frequencies of null alleles are shown with *

n number of genotyped individuals

Locus in which successful amplification was obtained in less than 80% of the individuals are shown by ^f

Na number of alleles per locus, *He* expected heterozygosity (sensu Nei 1978), *Ho* observed heterozygosity obtained with GENEPPOP

F_{IS} estimate of deviation from random mating obtained with GENEPPOP (Rousset 2008), *F_{IS}* values significantly different from zero are shown with ^s

PIC polymorphic information content obtained with CERVUS software (Marshall et al. 1998)

Locus retained as suitable polymorphic markers for future studies are shown in cells colored in light gray (i.e., polymorphic, that amplified in more than 80% of the individuals, no-significant *Nf*, no-significant *F_{IS}*)

In bold cells in the table correspond to locus retained for polymorphism

In italic the mean values over loci were calculated over locus retained as suitable polymorphic markers

being higher than 0.5, as well as the expected and observed heterozygosity.

Rapid species identification

Amplification of COI using the two sets of species-specific multiplex PCR primers provided direct identification of the four species of North-Atlantic kelps (*L. digitata*, *L. hyperborea*, *L. ochroleuca*, and *H. nigripes*) with 100% accuracy (Fig. 2). All the combination of primers (GAZ_F2/Ld_R/Lh_R/Hn_R and GAZ_R2/Ld_F2/Lh_F2/PC_F) showed positive internal control amplification products (601 bp). Multiplex PCR amplification produced the expected sizes and number of fragments for each species: two fragments for *L. digitata* (345 bp and 386 bp) and for *L. hyperborea* (574 bp and 166 bp), one fragment for *H. nigripes* (144 bp), and none for *L. ochroleuca* (Fig. 2 and Supp. Table 5).

Discussion

In this study, we developed highly informative microsatellite markers for five closely related species of the genus *Laminaria*, in addition to a molecular tool for rapid identification of morphologically similar species. We will first discuss the technical aspects and possible phylogenetic bias of microsatellite development and then focus on the potential use of the new markers.

Characteristics of microsatellites and transferability between species

With the increasing number of sequenced genomes, in silico mining of simple sequence repeats (SSR) from genome sequence databases has been widely used for microsatellite detection in plants and animals (see references in Wang et al. 2019). This method is less time-consuming and more effective than earlier methods based on the construction and screening of microsatellite enriched library (Zane et al. 2002). The occurrence and the frequency of different types of motifs among microsatellites seem to be characteristic to species, which could be among distantly or closely related species (see for review in Wang et al. 2019; Zhu et al. 2021). In the present study, trinucleotide repeats were found to be the most abundant motif (49.6%), but the low number of sequenced genomes in brown seaweed is still not sufficient to characterize the distribution and motif across species (Zhu et al. 2021), or even to explore their putative biological functions. The most common class of polymorphic marker contained trinucleotide motifs for each sister species (33–50% of the polymorphic markers), except for *L. pallida* for which polymorphic markers with tetranucleotide motifs were the most abundant (44% of the polymorphic

markers). Dinucleotide and pentanucleotide repeats were the least abundant type of polymorphic marker for all sister species (0–17% for dinucleotide and 0–22% for pentanucleotide). However, microsatellite loci constituted by three to five repeated motifs are easier to score than markers with dinucleotide repeats, because the latter show “stutter” bands (multiple PCR products from the same fragment that are typically shorter by one or a few repeats than the full-length product, Chambers and MacAvoy 2000), possibly leading to technical bias.

The ability to effectively transfer microsatellite markers across taxa has been successfully demonstrated in many species (see for review in plants: Ellis and Burke 2007; in animals: Hutter et al. 1998; and in kelps: Liu et al. 2012; Coelho et al. 2014; Zhang et al. 2015). The results of many studies have clearly indicated that EST-derived microsatellite markers have a higher transfer rate compared to those developed from anonymous nuclear DNA regions, due to greater DNA sequence conservation in transcribed versus non-coding regions. Cross-amplification success rate is correlated with phylogenetic distance and is, predictably, highest among closely related congeners, but it has been exceptionally reported across multiple genera within a family (see for review Kalia et al. 2011). In our study, we mostly tested cross-amplification for anonymous nuclear-derived microsatellites (27 loci) since only three EST-derived markers were available. The level of transferability within *Laminaria* was quite high, ranging from 47 to 67% and, as expected, decreased proportionally to the genetic distance between *L. digitata* and the other *Laminaria* species. Consequently, the use of microsatellites developed by cross-amplification from other taxa can possibly lead to various types of bias. Moreover, several studies using cross-species amplification techniques have demonstrated that microsatellite alleles are longer and more variable in the species from which they were derived (see references in Hutter et al. 1998). This observation could result from an ascertainment bias in the selection of clones when sequencing because the screening conditions in the focus species favor the identification of clones with long repeat units (Ellegren et al. 1995) and the presence of polymorphism. This bias in allele size was not confirmed in this study, but we did find a slight decrease in genetic diversity with an increase in the genetic distance with *L. digitata*.

Potential uses of the new developed microsatellites and species identification markers

Microsatellites are among the most frequently used markers to study intraspecific genetic diversity in population genetics, conservation genetics, and evolutionary studies. They also represent a molecular tool of great interest for applied seaweed research (see for review Brakel et al. 2021), such

as when identifying genetic resources in wild populations, or assessing the level of connectivity between populations for seaweed aquaculture.

In this study, for the first time, we defined 12 polymorphic loci for the South African *L. pallida* and 18 for the rare Mediterranean-endemic *L. rodriguezii*. This could be particularly interesting for *L. pallida*, which has not been investigated so far despite its ecological importance and biogeographical significance. For *L. rodriguezii*, genetic diversity within populations was until now only investigated using more than 4000 SNPs (Reynes et al. 2021). These technological advances in next-generation sequencing (NGS) are becoming available for studying population genetics in non-model organisms including kelps, such as reduced-representation sequencing (RRS) (Rad-seq SNPs: Guzinski et al. 2018, 2020; Reynes et al., 2021; Vranken et al. 2021) and whole genome sequencing (WGS, Graf et al. 2021). In contrast with microsatellite loci that inform about patterns of genetic variation that result from neutral or stochastic processes; these new population genomic approaches characterized by a better covering of the genome allow to search for loci associated with coding regions and can be used to quantify adaptive variation and inform about functional deterministic processes such as selection. In addition, RRS and WGS were shown to increase power and allow, in principle, for clearer detection and higher resolution of neutral genetic structure by increasing the number of markers compared to microsatellites. In particular cases, a limited number of highly polymorphic microsatellites (10) can perform as well as thousands of SNPs (10,615) to investigate neutral genetic structure of the kelp species *Undaria pinnatifida* (Guzinski et al. 2018), though with some advantages including rapid genotyping without the need to introduce strong bioinformatic background.

In *L. rodriguezii*, Reynes et al. (2021) demonstrated that SNPs markers were indeed highly efficient to investigate partial clonality under a population genomic approach and identified a particularly high level of genetic differentiation in comparison to other kelps. Nevertheless, the use of SNPs from RRS is far more expensive compared to microsatellite genotyping, and their study was restricted to a rather small number of individuals ($N=43$). Therefore, the 18 newly developed microsatellite loci in this species can still be seen as a good alternative to extend genetic structure analysis to a larger sampling size but also to perform comparative analyses between the five kelp species, as the same loci can be used across species.

In the three North Atlantic kelps, seven to 15 microsatellites were already available and mainly used to reconstruct their phylogeography and to analyze the level of connectivity in relation to life history traits and environmental factors (Billot et al. 2003; Valero et al. 2011; Couceiro et al. 2013; Robuchon et al. 2014; Assis et al. 2018; Evankow

et al. 2019; King et al. 2019; Liesner et al. 2020; Neiva et al. 2020; Schoenrock et al. 2020). The number of polymorphic loci at least doubled in these species (17 new polymorphic loci out of 30 for *L. digitata*, 11 out of 21 for *L. hyperborea*, and 9 out of 16 for *L. ochroleuca*), significantly improving the power of detection of genetic diversity and differentiation. These new markers should lead to a better estimation of the pattern of intraspecific genetic diversity and may also open up the possibility of carrying out studies that require higher individual discrimination power, such as parentage assignment studies. Consequently, the new markers will not only help to further elucidate the origins and demographic history of the NW Atlantic populations (e.g., Neiva et al. 2020) but also the species' adaptation potential, which is an increasingly important factor for sustainable conservation, exploitation of wild resources and to implement cultivation. In this context, the newly developed markers can be seen as a step towards expanding the repertoire of genetic toolsets, while we wait for higher resolution options to become more accessible.

Finally, we have presented species-specific primers based on the mitochondrial COI gene in order to discriminate *L. digitata*, *L. hyperborea*, *L. ochroleuca*, and *H. nigripes* based on the rapid species identification method developed in *Brachionus* (Seudre et al. 2020) and *Agarophyton* (Huanal et al. 2020). The multiplex PCR amplification of the specific primers produced fragments of different sizes for each species, and the specific primers were tested in a small panel of other co-occurring and potentially confounding kelp species (*Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders, *Saccorhiza polyschides* (Lightfoot) Batters), confirming the sensitivity of the approach, and they never produced band patterns similar to *L. digitata*, *L. hyperborea*, and *H. nigripes*. This method does not require Sanger sequencing and therefore is much faster and cheaper. This method should avoid taxonomic confusions and could be particularly helpful when sampling in areas where species range distributions overlap, particularly along the Arctic and the NW Atlantic where *L. digitata* and *H. nigripes* often occur in mixed stands. This rapid method of correctly assigning individuals to species is essential for the design of appropriate management and conservation of these kelp species. As many cryptic species are constantly discovered in algae (Leliaert and Clerck 2017), the ability of conservation and management programs to correctly distinguish species is an issue that can be easily addressed with the rapid identification method proposed here. In the field of speciation and biogeography, the correct identification of sympatric morphologically similar species may change our understanding of the history of algal lineage diversification and also allow the identification of recent introductions. Another useful application includes pre-screening of samples for non-genetic studies, such as physiological

experiments dealing with species-specific responses to thermal stress, where fast and cheap identification of dozens of samples may be a necessity. The identification of juvenile stages can also be problematic but critical in the context of ecologic and monitoring studies. Another challenging question in the ecology of kelp is to better understand the role of the microscopic gametophytes in the field. Recently, Bringloe et al. (2018) have successfully used a PCR identification method based on species-specific nuclear ITS of the ribosomal cistron primers of *Alaria esculenta* and *L. digitata* to detect the presence of gametophytes of these two kelps species on different red algae hosts. We did not test if the rapid identification method developed in our paper could also be used to detect the occurrence of gametophytes, but we suppose that this method should be very efficient when gametophyte detection is done on phylogenetically distant host species as in Bringloe et al. (2018). On the other hand, in order to detect the presence of gametophytes on a sporophyte of these four kelp species, it would be necessary to modify the method in order not to mix the primers of the four species. Indeed, our method shows that we never identify more than a single species even if the target sporophytes were sampled in sites where the different species co-occurred in sympatry (see for example the two Norway sites: Stubhallet and Hansneset, in which 52 individuals where sampled, 18 and 34, respectively, in each site, resulting in the identification of 38 *L. digitata* and 14 *H. nigripes* sporophytes, Supp. Table 1). Even though the gametophytes of the two sympatric species were potentially present on sporophytes, only the primers of the target sporophyte were amplified, probably because the amount of DNA present in the gametophytes was negligible compared to those of the sporophyte.

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Data availability All data generated during this study are openly available in a Dryad Digital Repository (<https://doi.org/10.5061/dryad.612jm643j>).

Declarations

Conflict of interest The authors declare no competing interests.

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