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. (1998) and Brenan et al. (2014) and to the ten *L. ochroleuca* loci previously developed by Coelho et al. (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.

Introduction

Kelp forests are mainly formed by large brown algae of the order Laminariales, present along rocky shores of temperate to polar regions and occupy approximately 36% of the world's coastlines (Jayathilake 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 1813*, L. hyperborea* (Gunnerus) Foslie 1885 *and L. ochroleuca* Bachelot Pylaie 1824) being partially sympatric along the NE Atlantic coast (Bartsch et al. 2008; Araújo et al. 2016); while the other one (*L. rodriguezii* Bornet 1888) 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 1967, Marins et al. 2014) and the Namibian and South African coasts (*L. pallida* Greville 1848, 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; King et al. 2019; Evankow 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 in 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 400bp.

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 microsatellites 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 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 were amplified with labelled 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 30s, annealing at 68°C for 30s (-1°C per cycle - touchdown to 58°C), extension at 72°C for 30s, 30 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s, extension at 72°C for 30s, 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, LoIVVIV-28, Coelho et al. 2014), giving a total of 40 microsatellite markers. Exceptionally, a new reverse primer Ld1_124_R2 (5'-CACTCTGCCCGCTGACC-3') was designed for the locus Ld1_124 (using the contig n°10005) 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 (*Na*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), 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). 99 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; Madison, USA), 0.5 µM of each primer (Table 2), 2 mM $MgCl_2$, 150 µM dNTPs (Thermo Fisher Scientific Inc., USA), 0.35 U GoTaq® Flexi Polymerase (Promega; Madison, USA). DNA amplifications were carried out in a BioRad 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 30s, 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 amplified (including 4 di-, 13 tri-, 12 tetra- and 8 pentanucleotide repeats, Supp. Table 3) and were retained for further analyses. 20 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).

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 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 3A, 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 3B). 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 3B) and dropped to 0% when considering all sister species (Table 3B). 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 4 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 4) 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 4). 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 grey in Table 4). Therefore, the number of polymorphic loci at least doubled in the three first species (17 more polymorphic loci for *L. digitata*, 11 for *L. hyperborea*, 9 for *L. ochroleuca*) compared to 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 4) 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). The level of polymorphism was highly variable among the 30 retained loci for *L. digitata*, with the number of alleles (*Na*) ranging from 2 to 16 with a mean value of 5.89, the expected heterozygosity (*He*) 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 4). However, a non-negligible number of markers remained informative as illustrated by the PIC values 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; Vranken et al. 2021; Reynes 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 principe, 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 (10615) to investigative neutral genetic structure of the kelp species *Undaria pinatifida* (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; King et al. 2019; Evankow 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 2019) and *Agarophyton* (Huanel 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 2006, *Saccorhiza polyschides* (Lightfoot) Batters 1902), 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 Leclerc 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 gametophotypes 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 amplified, probably because the amount of DNA present in the gametophytes was negligible compared to those of the sporophyte.

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Consent for publication

All authors read and approved the manuscript for the publication in Journal of Applied Phycology.

Conflict of interest

The authors declare that they have no conflicts of interest concerning the present article.

Availability of data

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

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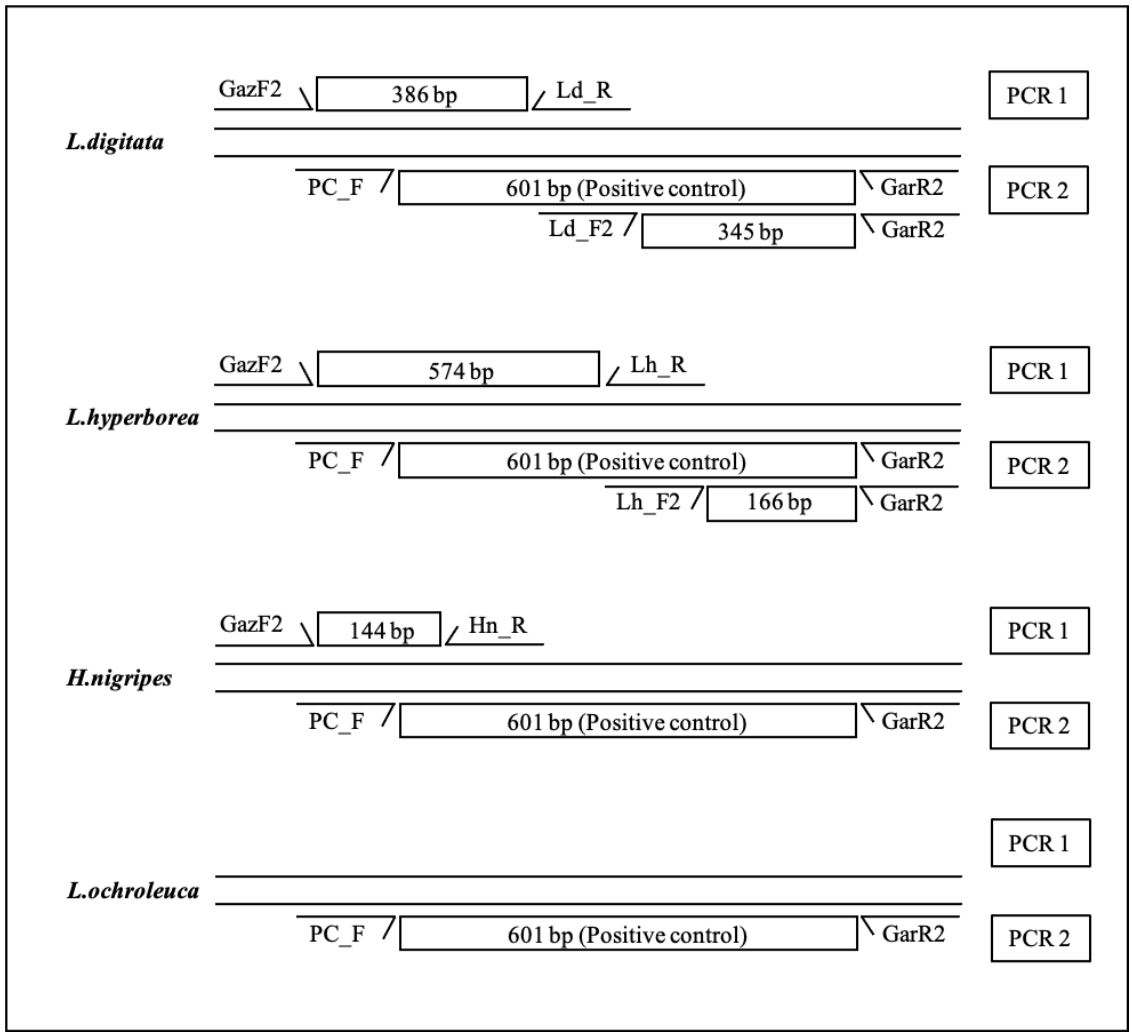


Fig. 1 Annealing sites and expected fragment sizes for the five *Laminaria* species using the rapid identification method. PCR products were pooled from PCR 1 and PCR 2 and include the positive internal control amplification product (601 bp), two fragments for *L. digitata* (345 bp and 386 bp) and *L.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

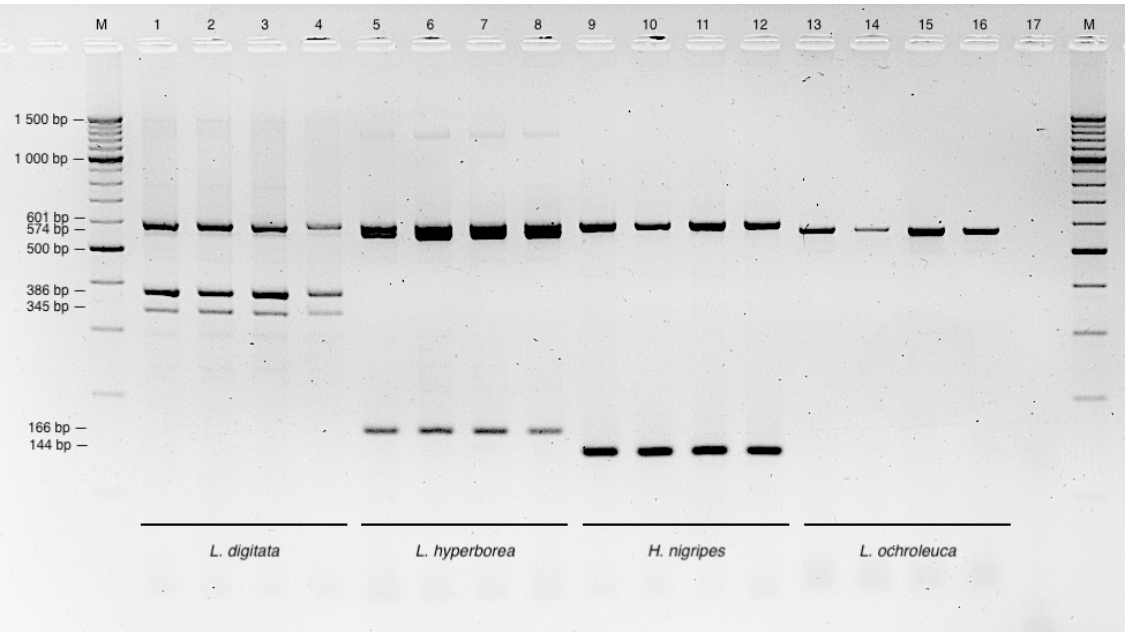


Fig. 2 Gel electrophoresis of PCR amplicons were pooled from PCR 1 and PCR 2 using the rapid species identification tool. (1 – 4) *L. digitata* samples; (5 – 8) *L. hyperborea* samples; (9 – 12) *H. nigripes* samples and (13 – 16) *L. ochroleuca* samples. (17) pooled negative control and (M) 100 bp Ladder.

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.

								L. digitata		*L. hyperborea*		*L. ochroleuca*		*L. rodriguezii*		*L. pallida*	
Locus	Source	Contigs no.	Primers sequences (5'-3')	Repeat motif	Dye	*Ta* (°C)	$MgCl_2$ (mM)	*Na*	Size range (bp)	*Na*	Size range (bp)	*Na*	Size range (bp)	*Na*	Size range (bp)	*Na*	Size range (bp)
Ld19_003	This study	1453	F: ATTCCGTGGTTGTGAGAGAGA R: CCGATACGAGAGACAGCACTT	$(GA)_{30}$	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: TGGGTGGTGTGTTGGATGT R: GGCAGTCGTAGGTATCGGTT	$(AC)_{30}$	PET	68 ↓ 58	2.0	12	97 - 185	NA		NA		NA		NA	
Ld19_005	This study	5613	F: GGCTGATAGACGGAGACAGAG R: GCAGTAGAAGGAGTGGACGAA	$(GA)_{47}$	NED	68 ↓ 58	2.0	16	243 - 379	NA		NA		6	262 - 302	NA	
Ld19_021	This study	17056	F: CGTCAATGTGGTATGGTATGG R: ACTCGTAACCTGGGCGATTT	$(TATGG)_{24}$	VIC	68 ↓ 58	2.0	7	235 - 300	4	205 - 220	NA		NA		NA	
Ld19_025	This study	52036	F: ATTCCACCATTCCATTCCATC R: GGAGGTTGTGACGCCTTTC	$(ATCCC)_{46}$	FAM	68 ↓ 58	2.0	9	282 - 387	NA		NA		5	185 - 240	3	98 - 138
Ld19_027	This study	2628	F: ACGGCACAGGTTAGCAGTT R: ATAGTCTTCCGCCTTTCGCT	$(TACCA)_{42}$	PET	68 ↓ 58	2.0	5	271 - 311	NA		NA		NA		NA	
Ld19_032	This study	1519	F: TTTGCTTTGATGATACTGCGA R: GTGGGTGAGTGGGTTGGTT	$(TACTT)_{3}$	NED	68 ↓ 58	2.0	3	387 - 397	5	390 - 415	NA		4	370 - 395	2	390 - 405
Ld19_033	This study	8512	F: ACGAGGACGAAGGTAGACTGG R: GACGGAAGGTAGGCTCTGG	$(GCAG)_{17}$	NED	68 ↓ 58	1.0	16	103 - 179	9	147 - 171	1	129	8	137 - 169	NA	
Ld19_034	This study	3470	F: CCCAGTCAAGAAATCAACCAA R: GCTTTGCTCCGTGCCTCT	$(TCAA)_{17}$	VIC	68 ↓ 58	2.0	4	128 - 172	1	144	1	102	1	118	3	94 - 110
Ld19_038	This study	16041	F: TTGTTGGTTTGTTTGTCTGTTTG R: GAATCCTCGCTTTCGTTGTTC	$(TGTT)_{15}$	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: CTATCTGTCTGTCCGTCCGTC R: CGTAAGGCTCTCGCAATCA	$(TCTG)_{23}$	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: ATCAACCACAGACGGACAGAC R: CTGCTGACACCCTTACCCTC	$(ACAG)_{47}$	NED	68 ↓ 58	1.0	7	269 - 309	7	118 - 190	3	250 - 288	6	259 - 287	NA	
Ld19_053	This study	25504	F: CCAACCTACTACGACGAACCA R: GATAGACAGATAGACAGCCAGCC	$(CTGT)_{47}$	PET	68 ↓ 58	2.0	6	271 - 291	9	192 - 264	7	283 - 315	NA		6	205 - 237
Ld19_067	This study	8496	F: CGCAGTAGGATAGCAGAGGAG R: CAGCAGCAGTAGAAACAGCAC	$(CTG)_{26}$	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: CGGAGATGAGGGTATGTGAGA R: ATTTCTTGGTGGTTACAGCGA	$(CAT)_{25}$	NED	68 ↓ 58	1.0	12	103 - 163	4	112 - 127	3	94 - 109	4	120 - 129	NA	
Ld19_071	This study	70523	F: ACTCTCCTCACCACCACCATC R: CCCTAAGCCTCAAACACTAA	$(CCA)_{25}$	PET	68 ↓ 58	2.0	8	120 - 147	NA		NA		NA		NA	
Ld19_074	This study	28415	F: CTGTCCTCTTTGCCATCGT R: CGCTTCATTGCTGTTCCTTT	$(GTG)_{42}$	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: CGAAACAGCAACAACAGCAA R: GTCGTGGTCGTCGTGGTC	$(AAC)_{40}$	FAM	68 ↓ 58	2.0	7	274 - 358	NA		3	252 - 279	4	265 - 286	NA	
Ld19_080	This study	29473	F: GCTGCTACTGCTCCTACTGCT R: GTGGGTCCTGATTACTATGCC	$(CTA)_{37}$	PET	68 ↓ 58	2.0	11	144 - 231	NA		NA		NA		NA	
Ld19_082	This study	28987	F: ACACCAACAACACCAACAACA R: CGGACGAAATGAAATGAAAGA	$(CAC)_{71}$	FAM	68 ↓ 58	1.0	4	271 - 289	7	254 - 278	4	203 - 266	NA		3	204 - 243
CN466672	Brennan 2014	12546	F: CTCCAACGATCCGCCTTG R: GGTCGCTTTCTTTCGTTGC	$(AAC)_{18}$	FAM	58	2.5	9	81 - 114	3	98 - 107	2	87 - 90	5	83 - 101	2	87 - 90
CN467658	Brennan 2014	5588	F: CTCTTCCGTCGACCTTGTTC R: GGTCGAGGCGATTTTTCATA	$(AGC)_{11}$	PET	58	2.5	10	197 - 248	2	212 - 215	1	204	1	219	1	204

AW401303	Brennan 2014	59231	F: TTCGAACCCAGTATCTACCTCGT R: CCGAGTACAGGCGGCAAC	complex (TGC)	NED	58	2.5	10	252 - 303	NA		NA		NA		NA	
Ld1_124	Billot 1998	10005	F: ATCTACATTACTGAATGCTCTTCGG R: CACTCTGCCCGCTGACC	$(TAG)_9$	VIC	60	2.5	6	109 - 130	NA		NA		2	124 - 127	NA	
Ld2_148	Billot 1998	7906	F: TGGCGTGTTCCCTGATATG R: TTTCTTGTCTAGGCCTCTCTGG	$(CAA)_{18}$	PET	60	2.5	9	168 - 204	6	206 - 248	2	174 - 192	6	196 - 228	4	170 - 190
Ld2_158	Billot 1998	4964	F: CGACTAGAAGGGAGCGAGAA R: CGTTTTTGCGCCTAACGTAT	$(AC)_{11}$	NED	60	2.5	7	199 - 244	2	221 - 228	NA		2	199 - 215	NA	
Ld2_167	Billot 1998	506	F: CGGACTCGATTTTAGCGATGGG R: TCGGAAGCACGTGTTCTGTAT	$(CAA)_{11}$	VIC	60	2.5	8	152 - 191	8	146 - 167	1	90	NA		NA	
Ld2_371	Billot 1998	74542	F: TACATGCCTCGTGTCTTTTGTGCG R: AGGAAAAAGCGGTGCAGTATTA	$(TTG)_{16}$	NED	60	2.5	10	117 - 180	NA		1	90	2	199 - 215	NA	
Ld2_531	Billot 1998	22811	F: TACTAACCCATTGTTTGTTGTGC R: CGACGTGGTCCTTGTTCTCATC	$(ATT)_8$	FAM	60	2.5	5	200 - 242	1	200	NA		NA		NA	
Ld2_704	Billot 1998	6665	F: GTCACAAGTAGGTGTTAAAAG R: CTGTGGTGTCTTTCGTCTCC	$(TC)_{23}$	PET	60	2.5	5	274 - 294	10	320 - 426	1	306	1	286	3	308 - 312
Lo4_24	Coelho 2014	8468	F: AACGCTGTGGGTACAT R: CATTTTCTTTACCGCTATC	$(TC)_9$	PET	50	2.0	2	222 - 224	3	212 - 218	3	220 - 230	1	210	1	206
LoIVVIV_10	Coelho 2014	4477	F: TTCAACAGCGGAGGTTTAGG R: GTGGTGGGTGGAGGTAGTGT	$(GCT)_{15}$	VIC	60	1.5	4	137 - 218	NA		10	184 - 232	3	140 - 165	NA	
LoIVVIV_13	Coelho 2014	2387	F: ACACAATTTGCCGTCCTTTC R: AGCTCATGCTGCACATTGTC	$(AAT)_{20}$	VIC	60	1.5	3	153 - 162	1	160	7	184 - 229	NA		NA	
LoIVVIV_15	Coelho 2014	52998	F: AGAATCAGGACGGACACTGG R: TCCGCGACTTATTCCCTCTA	$(AAC)_{21}$	FAM	60	1.5	6	152 - 197	6	152 - 173	5	188 - 215	4	170 - 182	4	179 - 210
LoIVVIV_16	Coelho 2014	10622	F: TTGTCAGATGGCAAATGGAA R: TGGAGTGGATAAGGGAGCAC	$(AAC)_{17}$	PET	60	1.5	3	192 - 219	7	205 - 238	5	211 - 235	3	201 - 213	3	210 - 222
LoIVVIV_17	Coelho 2014	6887	F: ATACTCCTGGGCCGGTTACT R: CGAGGGTGGGAGTAGTGGT	$(ACC)_{16}$	VIC	60	2.0	6	159 - 195	5	156 - 180	3	197 - 203	1	180	1	180
LoIVVIV_23	Coelho 2014	4727	F: AGAAAAAGCCTGCCGTGAC R: CAGCCTGGAGCTTTCGAT	$(AAC)_{14}$	NED	60	2.0	5	304 - 316	6	196 - 223	7	134 - 158	3	129 - 140	5	364 - 376
LoIVVIV_24	Coelho 2014	19992	F: AAGTTTCGTCTCCGTTTCCTC R: TTAACGTTCGTGCACCGTAG	$(GTT)_{17}$	FAM	60	2.0	5	307 - 328	6	198 - 289	7	327 - 363	1	295	5	292 - 323
LoIVVIV_27	Coelho 2014	56090	F: GACTGCCCCTTTGATTTTGA R: AATGGTGTCTGACTTGGGTTG	$(CTGT)_{11}$	PET	60	2.0	2	201 - 205	2	201 - 205	2	235 - 239	2	206 - 210	4	206 - 236
LoIVVIV_28	Coelho 2014	51886	F: CACCAAGCTTGATTGGTTGA R: TCGTGAGGTTATCGTGGTGA	$(ATCCC)_{16}$	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; *Ta*, annealing temperature (start T (°C) ↓ final T (°C)) and $MgCl_2$ concentration used for PCR (mM).
Na number of alleles, observed size range in base pairs (bp). NA = No Amplification.

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: GGATGACCAAARAACCAAAA
Ld_R: CTGAATACCACTAAGGGGG	Ld_F2: GACGGTCTACCCCCCCC
Lh_R: AGCGATAACAGTAACAAGAAAG	Lh_F2: GGTCTGTCTTAATCACAGCTTTC
Hn_R: CATTGTATAACTGGTGATTACCTCC	PC_F: GTCCTGGAAATCAATTTTTAGGAGG

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

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

Table 3B Percentage of microsatellite markers with a higher allelic size in the focal species compared to the sister species.

Compared species	Ratio	%
Lh/Ld	4/19	21%
Lo/Ld	5/18	30%
Lr/Ld	3/20	15%
Lp/Ld	4/14	29%
(Lh+Lo+Lr+Lp)/Ld *	0/10	0%

Ld: L. digitata, Lh: L. hyperborea, Lo: L. ochroleuca, Lr: L. rodriguezii and *Lp: L. pallida.*
*In this comparison, we took into account only the markers that amplified in all sister species.

Table 4 Genetic diversity at each polymorphic microsatellite locus in the five *Laminaria* species.

	L.digitata							*L.hyperborea*							*L.ochroleuca*						
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	0.088	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[$]	0.860
LoIVVIV_13		32	1						30	1					0.093	16	5	0.563	0.700	0.201	0.638
LoIVVIV_15	0	30	2	0.267	0.235	-0.137	0.204	0.280*	29	6	0.310	0.561	0.451[$]	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**

Table 4 Continued

Locus	*L.rodriguezii*							*L.pallida*						
	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				
AW401303														
Mean								**0.154**	**11.0**	**2.00**	**0.364**	**0.519**	**0.310**	**0.373**
Ld1_124	0	16	2	0.125	0.121	-0.034	0.110							
Ld2_148	0	16	4	0.500	0.427	-0.176	0.387	0.059	10	4	0.600	0.711	0.163	0.619
Ld2_158	0.035	14	2	0.429	0.476	0.103	0.354							
Ld2_167														
Ld2_371	0.035	14	2	0.429	0.476	0.103	0.354							
Ld2_531														
Ld2_704		16	1					0.149	5[£]	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	0.077	14	3	0.429	0.519	0.179	0.427							
LoIVVIV_13														
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	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	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	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 £
Na number of alleles per locus, *He* expected heterozygosity (sensu Nei 1978), *Ho* observed heterozygosity obtained with GENEPOP.
Fis estimate of deviation from random mating obtained with GENEPOP (Rousset 2008). F_{IS} values significantly different from zero are shown with $
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 grey (i.e., polymorphic, that amplified in more than 80% of the individuals, no-significant *Nf*, no-significant F_{IS}).
The mean values over loci were calculated over locus retained as suitable polymorphic markers.
No-colored cells in the table correspond to locus non retained for polymorphism.

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

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Table S1 Geographical location of the populations used in the study and sample size (n) including the wide scale and the single population sampling.

Species	Populations	n	Latitude (N)	Longitude (W)
Laminaria digitata	Stubhallet, Norway*	13	79.2013	11.7767
	Hansneset, Norway*	25	79.0111	12.1900
	Hvammur, Iceland	1	65.4827	-22.4008
	Tjarno, Sweden	1	58.8811	11.1056
	Clachan, Scotland	1	56.3171	-5.5832
	Frederiksborg, Denmark	1	56.0436	12.6267
	Londonderry, Irland	1	55.2525	7.6186
	Helgoland, Germany	1	54.1779	7.8926
	Treaddur, Wales	1	53.2786	-4.6236
	Wissant, France	1	50.9036	1.6712
	Plymouth, United Kingdom	1	50.3150	-4.0836
	Roscoff, France	**32**	**48.7275**	**-4.0079**
	Saint Malo, France	1	48.6967	-1.9186
	Ouessant, France	1	48.4506	-5.0958
	Quiberon, France	1	47.4700	-3.0914
	Halifax, Canada	1	44.4936	-63.9186
	Bar Harbor, USA	1	44.3894	-68.1977
Laminaria hyberborea	Forsøl, Norway	1	70.7214	23.8131
	Maskar, Sweden	1	58.8539	10.9772
	Lon Liath, Scotland	1	56.9828	-5.8433
	Helgoland, Germany	1	54.1779	7.8926
	Pembrokeshire, Wales	1	51.6850	-5.1138
	County Cork, Irland	1	51.4739	-9.5124
	La Hague, France	1	49.6900	-1.9365
	Roscoff, France	**32**	**48.7289**	**-4.0079**
	Landéda, France	1	48.6158	-4.6037
	Camaret-sur-Mer, France	1	48.2881	-4.6000
	Quiberon, France	1	47.4700	-3.0914
	Le Croisic, France	1	47.2536	-2.6350
	Moledo, Portugal	1	41.8349	-8.8763
Laminaria ochroleuca	Ile-de-Bréhat, France	1	48.8330	-3.0077
	Ile pigued, France	1	48.7329	-3.9704
	Landéda, France	**16**	**48.6158**	**-4.6037**
	Ile-Molène, France	1	48.3936	-4.9527
	Le Conquet, France	1	48.3582	-4.7812
	Ile-de-Sein, France	1	48.0326	-4.8348
	Ile-des-Glénan, France	1	47.7213	-4.0000
	São Bartolomeu do Mar, Portugal	1	41.5716	-8.7997
	Ericeira, Portugal	1	38.9619	-9.4205
	El Jadida, Morocco	1	33.2519	-8.4969
	Essaouira, Morocco	1	31.4952	-9.7902
Laminaria rodriguezii	Cap Camarat, France	2	43.1666	6.7333
	Banc Magaud_1, France	**16**	**43.0333**	**6.5833**
	Banc Magaud_2, France	2	43.0333	6.5833
	Bonifacio, France	2	41.3491	9.3166
Laminaria pallida	**Swakopmund, Namibia**	**11**	**-22.6724**	**14.5221**
Hedophyllum nigripes	Spitzbergen, Norway	8	78.9480	11.8849
	Stubhallet, Norway*	5	79.2013	11.7767
	Hansneset, Norway*	9	79.0111	12.1900

The populations in bold correspond to those used to examine the within populations polymorphism.
The populations with * correspond to those used to test the primer combinations on specimens from the same site in which L. digitata and H. nigripes species co-occurred in sympatry.

Table S2 Removed contigs from the draft assembled genome that contained published markers

Locus	Contigs no.	Study
Ld1_124	10005	Billot et al 1998
Ld2_148	7906	Billot et al 1998
Ld2_158	4964	Billot et al 1998
Ld2_167	506	Billot et al 1998
Ld2_225	45649	Billot et al 1998
Ld2_357	48816	Billot et al 1998
Ld2_371	74542	Billot et al 1998
Ld2_520	79664	Billot et al 1998
Ld2_531	22811	Billot et al 1998
Ld2_704	6665	Billot et al 1998
Lo4_24	8468	Coelho et al 2014
LoIVVIV_10	4477	Coelho et al 2014
LoIVVIV_13	2387	Coelho et al 2014
LoIVVIV_15	52998	Coelho et al 2014
LoIVVIV_16	10622	Coelho et al 2014
LoIVVIV_17	6887	Coelho et al 2014
LoIVVIV_23	4727	Coelho et al 2014
LoIVVIV_24	19992	Coelho et al 2014
LoIVVIV_27	56090	Coelho et al 2014
LoIVVIV_28	51886	Coelho et al 2014
AW401303	59231	Brennan et al 2014
CN466672	12546	Brennan et al 2014
CN467658	5588	Brennan et al 2014

Table S3 Steps involved in the selection of *L. digitata* microsatellite markers, with the percentage of loci with specific motif types (Di-, Tri-, Tetra- and Pentanucleotide) associated to each step.

Reduction step	Di	Tri	Tetra	Penta	Number of retained loci
SPUTNIK output	33,861 (11.1%)	151,588 (49.6%)	54,408 (17.8%)	65,789 (21.5%)	305 646
Primer3 output	30,900 (11.3%)	138,604 (50.7%)	49,062 (18%)	54,665 (20%)	273 231
Unique loci with "N" base removed	8,792 (15.7%)	25,672 (45.8%)	10,353 (18.5%)	11,217 (20%)	56 034
Final selection (highest number of repeats)	16 (17.7%)	32 (33.3%)	32 (33.3%)	16 (17.7%)	96
Positive amplification	4 (10.8%)	13 (35.1%)	12 (32.4%)	8 (21.6%)	37
PCR product of the expected size and polymorphic over species range distribution	3 (15%)	7 (35%)	6 (30%)	4 (20%)	20
Rate of success to obtain polymorphic marker	3/16 (18.8%)	7/32 (21.9%)	6/32 (18.8%)	4/16 (25%)	20/96 (20.8%)

The percentage of loci with specific motif types is also presented for each step of the selection process.
Di, loci with dinucleotide repeat motifs; Tri, loci with trinucleotide repeat motifs; Tetra, loci with tetranucleotide repeat motifs and Penta, loci with pentanucleotide repeat motifs.

Table S4 Technical information concerning the 17 microsatellite primers selected from *L. digitata* genome, which were not retained because they were monomorphic (*Na*=1)

Locus	Source	Contigs no.	Primers sequences (5'-3')	Repeat motif	Dye	*Ta* (°C)	$MgCl_2$ (mM)	*Na*	Size range (bp)
Ld19_007	This study	24030	F: GTGTTGGTGTTGATGCGAAG R: ACAGATACAGGCGGGACAAA	$(TC)_{45}$	VIC	68 ↓ 58	2.0	1	167
Ld19_018	This study	70736	F: GGTCCAGTCAAAGCACGG R: CTTATGTCGCCCAGCCTCT	$(AAGGC)_{16}$	VIC	68 ↓ 58	2.0	1	148
Ld19_022	This study	34069	F: GACACAACCCAACCCAACC R: GCCTAATAACACGGGCTCA	$(AACAC)_{24}$	NED	68 ↓ 58	2.0	1	196
Ld19_026	This study	17071	F: CGTGTTGTGTTATGCTGTGTTG R: CGTTATGTCGGTCCTTCACCT	$(TTGTG)_{42}$	PET	68 ↓ 58	2.0	1	320
Ld19_029	This study	22583	F: CAAACCATACCATACCACACCA R: GATGTTCCACGACACCTTCAC	$(ACCAT)_{65}$	FAM	68 ↓ 58	2.0	1	381
Ld19_035	This study	65710	F: AGTGGTGTGGTGGTTAGATGG R: GAGGGTAGGGAGGGATGG	$(TCCC)_{16}$	NED	68 ↓ 58	2.0	1	143
Ld19_040	This study	15190	F: CACCAAACCCAAGACAGGTAA R: CGTAAACCGTAAACTGGCGTAA	$(GTCC)_4$	NED	68 ↓ 58	2.0	1	152
Ld19_041	This study	523	F: CTTGGGTTTCTTGCTTGGTTT R: GCGAGTGAGTGAGTGAGT	$(TGCT)_{26}$	PET	68 ↓ 58	2.0	1	193
Ld19_045	This study	28907	F: GATGGATGGATGGATGGGT R: TAGGCGACCAAGGAGTAACA	$(GATT)_{24}$	PET	68 ↓ 58	2.0	1	196
Ld19_046	This study	51189	F: CGTGGAACTAACGCTTGCC R: CGAAACGATGAGAGCAGGT	$(ACCC)_{24}$	PET	68 ↓ 58	2.0	1	159
Ld19_050	This study	45250	F: CTGGAGACGGGAGCGAGA R: GCGTGATTTGCTTACGAG	$(TTGA)_{52}$	FAM	68 ↓ 58	2.0	1	286
Ld19_068	This study	4762	F: ATTGGCGGTGGCGGTGATG R: GCAGCAAGAGCAGCAAGAG	$(GGT)_{26}$	PET	68 ↓ 58	2.0	1	149
Ld19_070	This study	4834	F: CAACAACACCACAACCATCAC R: ACGGGAAATACAGCAGGACA	$(CAC)_{25}$	VIC	68 ↓ 58	2.0	1	139
Ld19_075	This study	1337	F: CTGCTGCTGTTGCTGTTG R: CTCTCGGAACGACGAACGA	$(AGT)_{41}$	FAM	68 ↓ 58	2.0	1	197
Ld19_079	This study	24310	F: ATCTGCTTCTGTGGCGAG R: TTCGGACAACTACAATGAGGG	$(CAC)_{38}$	VIC	68 ↓ 58	2.0	1	175
Ld19_085	This study	40040	F: TCGTGGGTCCTATTTACTATTT R: ATTACTGTGGGTGTGGCAG	$(CTA)_{69}$	VIC	68 ↓ 58	2.0	1	273
Ld19_088	This study	15109	F: TACTGGACCTCACGATTGGTT R: CTGCTTCTACTCCTGCTGCC	$(GCA)_4$	VIC	68 ↓ 58	2.0	1	225

F, forward primer (labeled with fluorescent dye); R, reverse primer; *Ta*, annealing temperature (start T (°C) ↓ final T (°C)) and $MgCl_2$ concentration used for PCR (mM).
Na number of alleles, observed size range in base pairs (bp).

Table S5 Expected sizes of PCR products for rapid species identification

	PCR 1 (bp)	PCR 2 (bp)	PCR 2 (bp)
L. digitata	386	345	601
L. hyperborea	574	166	601
H. nigripes	144	-	601
L. ochroleuca	-	-	601

Expected size fragment in base pairs (bp) with positive control.