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BoCAPS: Rapid screening of chemical races in *Botryococcus braunii* with direct PCR-CAPS

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ABSTRACT

The colony-forming microalga *Botryococcus braunii* produces petroleum-like hydrocarbons and is a promising source for biofuel production. *B. braunii* is classified into three chemical races (A, B, L) plus a tentative class (S), based on the structure of its hydrocarbons. The determination of hydrocarbon structure in a newly isolated strain requires several months for culturing and analysis. As potential for biofuel production differs among races, a rapid method for estimating race is needed for the effective bioprospecting. This study aimed to develop a DNA-based rapid estimation method (BoCAPS) of the chemical races of *B. braunii* and to test the feasibility of direct PCR from a single colony. A total of 247 wild strains were isolated from 29 ponds in Japan and Indonesia, their 18S rRNA sequences determined, and a phylogenetic tree constructed based on the sequences of standard strains with known chemical races. Polymorphisms between clades of the phylogenetic tree corresponding to chemical races were mapped, and a cleaved amplified polymorphic sequence (CAPS) marker was developed for race discrimination. Direct PCR was tested on a single colony as a template. We demonstrated that *Hae*III restriction of the PCR product enables race discrimination on agarose gels, and that direct PCR amplifies the 18S rRNA gene from a single colony without any pre-treatment. With BoCAPS, we investigated race compositions of the isolated strains and determined that the B-race was dominant (74 %). Neither the A-race nor S-race strains were isolated from tropical regions. In addition, we observed a few ponds in which minor races (L or A) were dominant and some ponds with a mixture of different races. The BoCAPS allows us to estimate chemical race without the time-consuming and expensive analysis of hydrocarbons and will be useful for high-throughput screening and ecological studies of wild populations.

1. Introduction

Microalgae are photosynthetic organisms that can efficiently convert solar energy into biomass and have the potential to become renewable and sustainable sources of food ingredients, bioactive medicinal products, and biofuels. Microalgal biomass contains valuable natural biologically active molecules, such as astaxanthin, carotenoids, polysaccharides, which can be used as pharmaceuticals [1] or health-promoting compounds [2]. Microalgae are considered as the most promising renewable feedstock for biofuel production and biorefineries [3]. Microalgal lipids and hydrocarbons can be converted into biodiesel. Bio-hydrogen from microalgae including cyanobacteria has attracted commercial awareness due to its potential as an alternative, reliable and renewable energy source [4]. Bio-ammonia production through pyrolysis and hydrothermal gasification of microalgal biomass has its promising prospects as a bio-production of energy carrier [5]. As microalgae have the ability of biosorption of nutrients, phosphorous, and carbon dioxide to perform photosynthesis, they can be utilized as a sustainable wastewater treatment [6], bioremediation of environmental pollutants [7], and atmospheric carbon mitigation [8], which contributes to effective biofuel production [9].

Botryococcus braunii is a colony-forming, freshwater microalga that has recently attracted considerable attention in the field of algal biofuel research, due to its ability to produce large quantities of petroleum-like hydrocarbons [10,11]. This species also has the distinctive feature of accumulating hydrocarbons in the extracellular space, and outside the

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cell wall, which has inspired researchers to develop techniques for "milking" the hydrocarbons without killing the algae [12–14]. Due to the development of sophisticated cultivation methods, such as high-density [15], mixotrophic [16,17], and attached cultivation [18], *B. braunii* has demonstrated one of the highest productivities of algal oil to date [19]. A high-density and continuous cultivation of *B. braunii* achieved a hydrocarbon productivity of 340 mg L^{-1} d^{-1} [15].

The bioprospecting of genetic resources and investigations on the natural ecologies of *B. braunii* will expand the possibilities of utilizing this alga as a biofuel producer. A drawback in utilizing *B. braunii* as a biofuel producer is its slow growth, but a recent large-scale screening of wild strains has identified novel fast-growing strains [19]. In addition to growth rate, a tolerance for strong light, large colony size, hydrocarbon content, and production of other valuable compounds, such as carotenoids [20,21], can be used as screening criteria to identify suitable bioresources of *B. braunii* from the natural environment. Furthermore, natural populations of *B. braunii* are normally present at low levels of abundance [22]; however, a few studies on the natural blooms of this alga have been reported [23–26]. Chiang et al. [26] noted the potential allelopathic effects of free fatty acids produced by *B. braunii*, but the underlying mechanisms for natural blooms of this slow-growing alga have not been fully elucidated. A clear understanding of the mechanisms governing the natural blooms of *B. braunii* would provide useful information for the development of outdoor mass cultivation [27,28].

To facilitate the bioprospecting and ecological exploration of *B. braunii*, this study aimed to develop a new DNA-based method for the rapid determination of the chemical races in *B. braunii*. This species of algae has been classified into three races (or chemotypes) according to the chemical structure of its hydrocarbons. Race A produces C_{25} to C_{31} odd-numbered n-alkadienes and alkatrienes, whereas race B produces polymethylated unsaturated triterpenes called botryococcenes (C_nH_{2n-10}, n = 30–37), and race L produces a single hydrocarbon $C_{40}H_{78}$, a tetraterpene named lycopadiene [29,30]. Furthermore, Kawachi et al. [31] proposed a new class of race S, comprised of epoxy-n-alkane and saturated n-alkane chains with carbon numbers 18 and 20. Each of the different hydrocarbon races of *B. braunii* has a unique biosynthetic pathway for hydrocarbon production [32,33] and different sized genomes [34]; therefore, they should be classified as different species. However, they are morphologically similar, and the classification of hydrocarbon race by morphology is not straightforward [35]. From the standpoint of bioprospecting, race B strains appear to have the greatest potential for biofuel production, due to their higher hydrocarbon content and productivity [11]. However, previous studies identified co-occurrences of different races in natural environments [22,36–38], which complicates the effective exploitation of only race B strains from the natural environment. From an ecological perspective, differences in habitat preferences between the races are of interest for further investigation.

The chemical races of *B. braunii* can be determined by Gas Chromatography–Mass Spectrometry (GC–MS) analysis of its hydrocarbons, but a substantial effort is required to prepare the samples, including isolation of the strain from natural sources of water, culturing it in an incubator for several months, harvesting the algae, and extracting the hydrocarbons. A simpler method with a smaller sample of the alga would be helpful in screening the large number of wild strains and for carrying out ecological studies of natural populations. Moutel et al. [39] developed a chemometrically-assisted GC–MS method for discriminating between the A and B races, which requires only small quantities of material (i.e., 1 mg of dried biomass), and a relatively straightforward sample preparation. This study aims to develop a DNA-based method capable of discriminating between all chemical races of *B. braunii* with a minimum quantity of sample (i.e., a single colony) and without sample preparation.

Hirano et al. developed a real-time Polymerase Chain Reaction (PCR) assay for specific detection of *B. braunii* race B strains by targeting a hydrocarbon biosynthesis gene, *SSL-3* [22], but this method cannot distinguish other races (A-, L-, S-races). Furthermore, the PCR-based assessment of the presence/absence of a gene contains a high risk of false-positive results. Therefore, this study focused on the universal gene 18S ribosomal RNA (rRNA) gene. Molecular phylogenetic studies have reported that the different chemical races belong to distinct clades in the phylogenetic tree of the 18S rRNA gene [22,31,34]. This finding suggests that the different chemical races could be determined by sequencing the 18S rRNA gene. To further reduce the time and cost of sequencing, this study aims to develop a cleaved amplified polymorphic sequence (CAPS) assay for the chemical race discrimination of *B. braunii*, based on the polymorphisms of the 18S rRNA gene between the races. Furthermore, we aim to test the feasibility of using direct PCR from a single colony without pretreatment, extraction, or purification of DNA. A recent study reported a simple direct PCR method for single cellular microalgae, such as *Chlorella* sp. [40], but to the best of our knowledge, direct PCR method has not yet been reported in the colonial microalga *B. braunii*. This study demonstrated the feasibility of direct PCR amplification of 18S rRNA gene from a single colony of *B. braunii* without any pre-treatment, and in combination with the CAPS assay a new estimation method of chemical races of *Botryococcus braunii* was developed and denominated as 'BoCAPS'.

2. Materials and methods

2.1. Isolation of wild strains

The algae in approximately 200 ponds from lowland areas in Japan (temperate climate) and in Indonesia (tropical climate) were surveyed from 2015 to 2021 by using a plankton net with a mesh size of 100 μm. Wild *B. braunii* was identified in approximately one pond per every 5–10 ponds in Japan and Indonesia [28]. A total of 247 wild strains were isolated from 18 ponds in Japan and 11 ponds in Indonesia (Fig. 1). As described by Kawamura et al. [28], a single colony of *B. braunii* was isolated by micropipette, transferred to a 10 mL glass tube containing AF-6 medium [41], and incubated at 25 °C under a 12-h light/12-h dark cycle using fluorescence lamps with a PPFD of 100 μmol m^{-2} s^{-1}. After one month, the colonies were transferred to 30 mL culture bottles filled with fresh AF-6 medium and incubated for an additional two months. The colonies were subsequently collected using a 10 μm filter membrane (JCWP04700, Merck), the filters were frozen with liquid nitrogen, and subsequently disrupted using a multi-bead shocker instrument (Yasui Kikai, Japan) at 3000 rpm for 30 s. DNA was extracted from the disrupted filter using a NucleoSpin Plant II (Macherey-Nagel, Germany) according to the manufacturer's protocol.

2.2. Construction of the molecular phylogenetic tree

The 18S ribosomal RNA sequences of 96 isolated strains were determined to estimate their phylogenetic relationship with previously described strains [22,31] and to classify them into clades of different chemical races. The sequences were deposited to DNA Data Bank of Japan (DDBJ), and the accession numbers are available from supplementary Table S1. As described by Hirano et al. [22], either specific primers (63F and 1818R) [31] or universal primers (EukF1 and EukR1) [42] were used to amplify 18S ribosomal RNA sequences with EmeraldAmp® PCR Master Mix (TaKaRa Bio, Japan) including 0.2 μM of primers and 1 ng of DNA template, using the following thermal cycler program: 2 min at 95 °C; 34 cycles of 95 °C/30 s, 55 °C/30 s, 72 °C/100 s; and 5 min at 72 °C. The PCR products obtained were directly sequenced. These sequences were aligned using the program ClustalW with additional reference sequences obtained from National Center for Biotechnology Information (NCBI). Neighbor-joining (NJ) analysis with the Tamura-Nei model of genetic distance was carried out using the sequence from *Choricystis* sp., the closest species to *Botryococcus* among the members of Trebouxiophyceae [31], as an outgroup. Bootstrap values of 1000 replicates were obtained using Geneious Prime®.

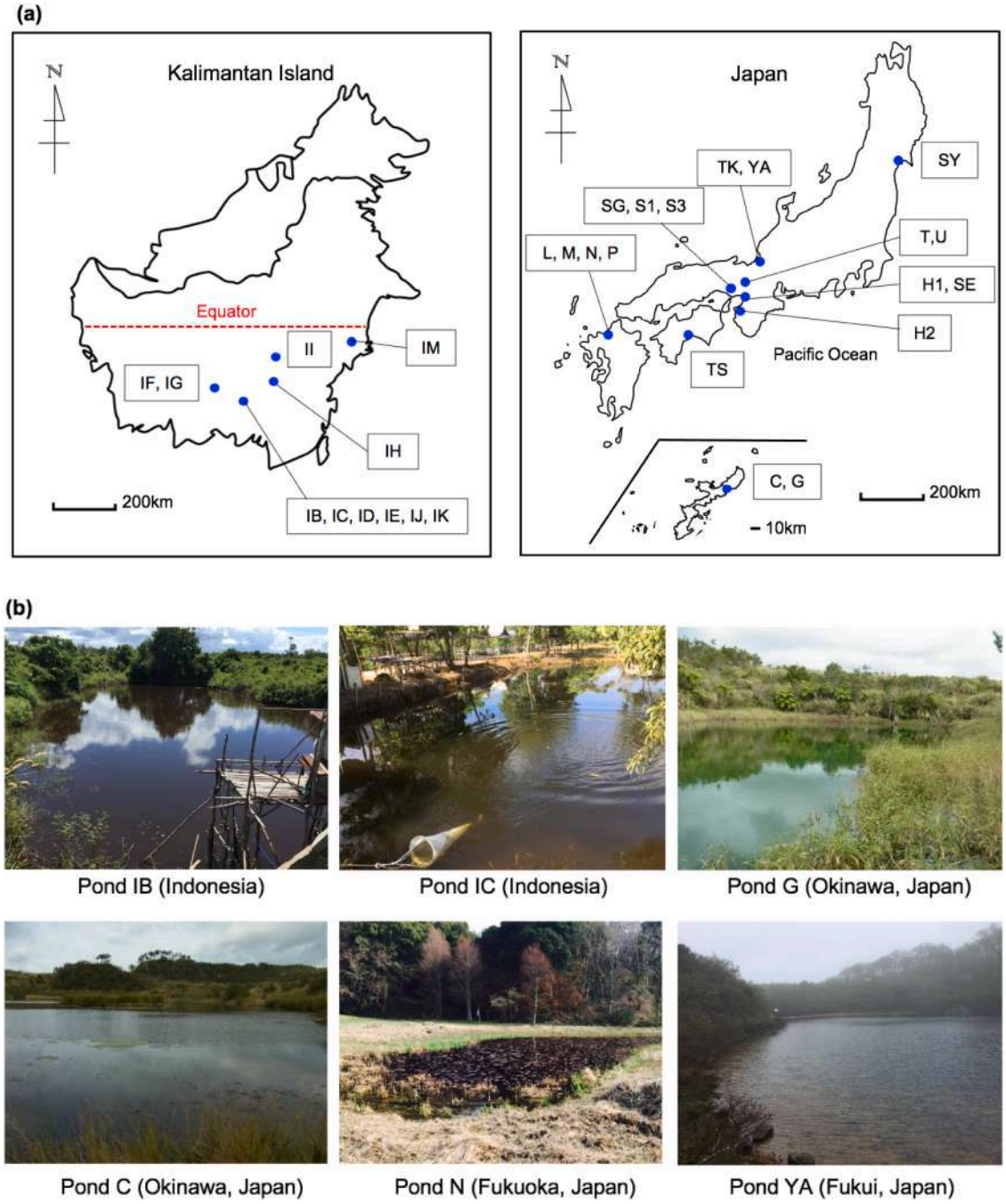


Fig. 1. Sampling points of wild *Botryococcus braunii* in Central Kalimantan, Indonesia, and Japan. (a) Map of the sampling locations. Palangka Raya (Pond IB, IC, ID, IE, IJ, IK); Pundu (IF, IG); Buntok (IH); Muara Teweh (II); Samarinda (IM); Okinawa (C, G); Fukuoka (L, M, N, P); Kochi (TS); Fukui (TK, YA); Wakayama (H2); Osaka (H1, SE); Kyoto (T, U); Miyagi (SY). (b) Pictures of some ponds. A total of 247 wild strains were isolated from the 29 ponds distributed from the tropical (Indonesia), subtropical (Okinawa), warm-temperate (Fukuoka, Kochi, Fukui TK, Wakayama, Osaka, Kyoto), and cool-temperate regions (Miyagi, Fukui YA).

2.3. Development of PCR-CAPS marker

The 18S rRNA sequences were aligned in each clade of the race, and consensus sequences were constructed. The number of sequences used to construct the consensus sequences included 15 strains for race A, 148 strains for race B, 19 strains for race L, and 17 strains for race S. These consensus sequences were subsequently compared between the races to identify variations specific to each race. Based on the sequence variations, CAPS markers were developed for race discrimination. The PCR reactions were performed using the EmeraldAmp® Max PCR Master Mix (Takara, Japan). Reactions of 10 μL were prepared, containing 5 μL of 2 × EmeraldAmp® Max PCR Master Mix, 1 μL of DNA template (1 ng/μL), 0.2 μL of each primer (10 μM), and ultrapure water. 18Sbot_F (5′-CAGCAGCCGCGGTAATTCC-3′) and 18Sbot_R (5′-CATTACTCCGGTCCCACAGAC-3′) primers were used. The amplification conditions were as follows: 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s. A total of 4 μL of the PCR products were mixed with 0.5 μL of *Hae*III (10 U/μL, New England Biolabs) and 0.5 μL of 10 × CutSmart buffer and incubated at 37 °C overnight. Restriction fragment length polymorphisms were visualized by 3 % agarose gel electrophoresis with a TAE buffer. For the discrimination of race L and race S, 18Sbot_F2 (5′-ACGTGCGTAAATCCCGACTTCT-3′) and 18Sbot_R2 (5′-

TGACACAGGGAGGTAGTGACA-3′) primers and the restriction enzyme *Bsp*1286I (New England Biolabs) were used.

2.4. Direct PCR method

The utility of direct PCR was tested for *B. braunii*. First, a method using algal biomass was tested. Algae were incubated in a 10 mL glass tube with AF-6 culture medium in a 25 °C incubator with 2 % CO_2 and 12-h illumination of 100 μmol m^{-2} s^{-1} per day [28]. When algal biomass increased to the point of visibility, (i) 200 μL of the algal culture was removed and mixed with the same volume of tris-ethylenediaminetetraacetic acid (EDTA) in a 2 mL tube. The mixture was crushed with a metal stone at 3000 rpm for 30 s by a bead-beating machine. (ii) The crushed mixture was incubated at 100 °C for 10 min, cooled on ice, and centrifuged at 20,000 ×*g* for 5 min at 4 °C. (iii) The intermediate water layer was transferred to a new tube as the "direct PCR template" and kept at 4 °C. PCR solutions of 10 μL each were prepared, containing 5 μL of 2 × EmeraldAmp® Max PCR Master Mix (TaKaRa, Japan), 1 μL of direct PCR template, 0.2 μL of each primer (10 μM), and ultrapure water. The 18Sbot_F and 18Sbot_R primers were used. The amplification conditions were as follows: 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s. A single colony

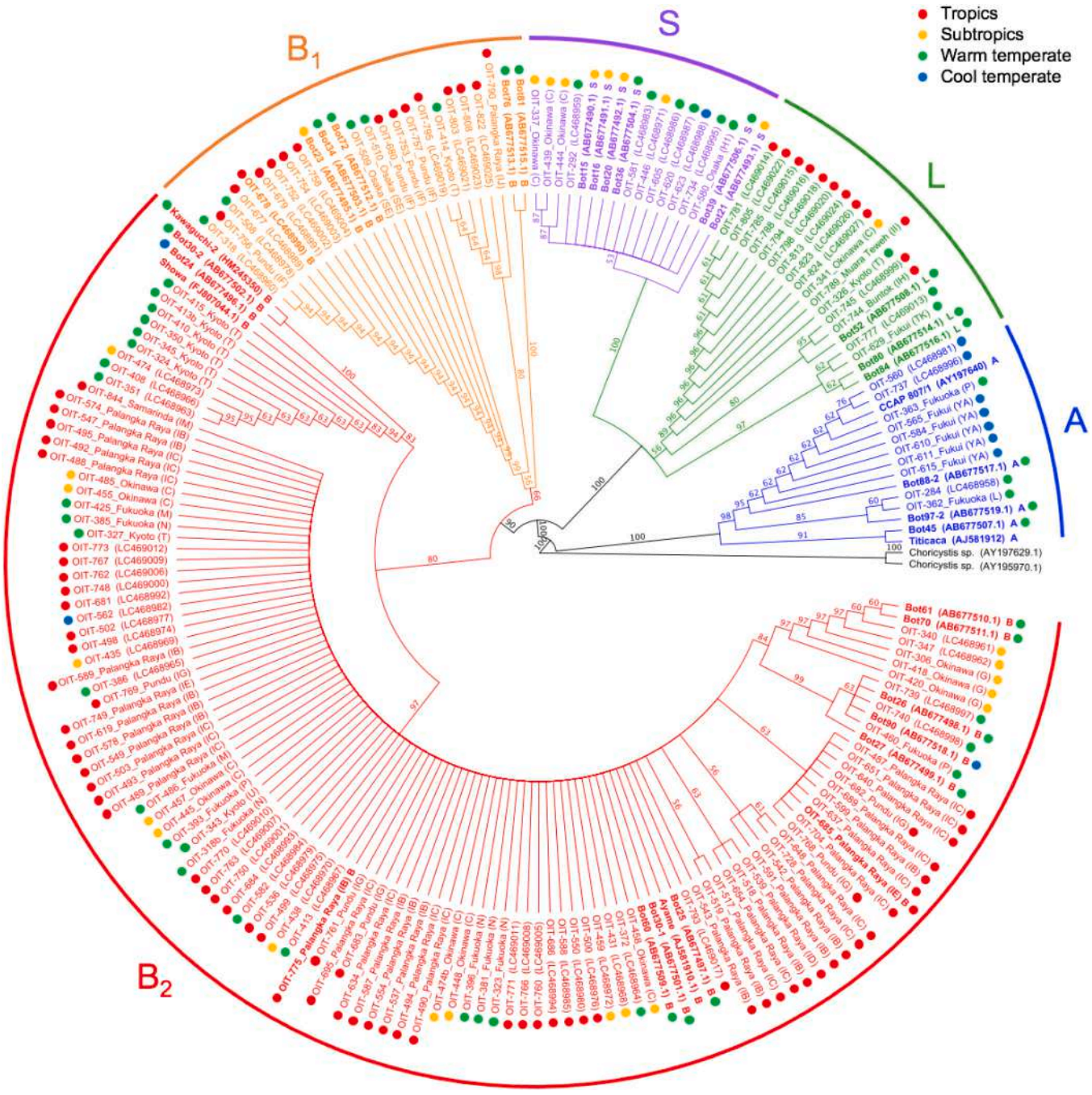


Fig. 2. Molecular phylogenetic tree of *Botryococcus braunii* wild strains based on 18S ribosomal RNA sequences. The tree is rooted on the branch between *Choricystis* and *Botryococcus*. Numbers around the internodes indicate bootstrap values in the neighborhood-joining analysis (1000 replications). New sequences obtained from 96 wild strains were combined with the data of a previous study [22]. Reference sequences with known chemical races are in bold with the classification of chemical race (A, B, L, S). The climate region of isolation sites is also indicated.

direct PCR was then tested. Instead of the 1 μL of direct PCR template, 1 μL of algal culture containing a single colony was manually taken up using an inverse microscope (×40) and directly placed into the PCR solution without the pretreatments of crushing or heating, and PCR was carried out under the same conditions as described above.

3. Results

3.1. Molecular phylogenetic tree

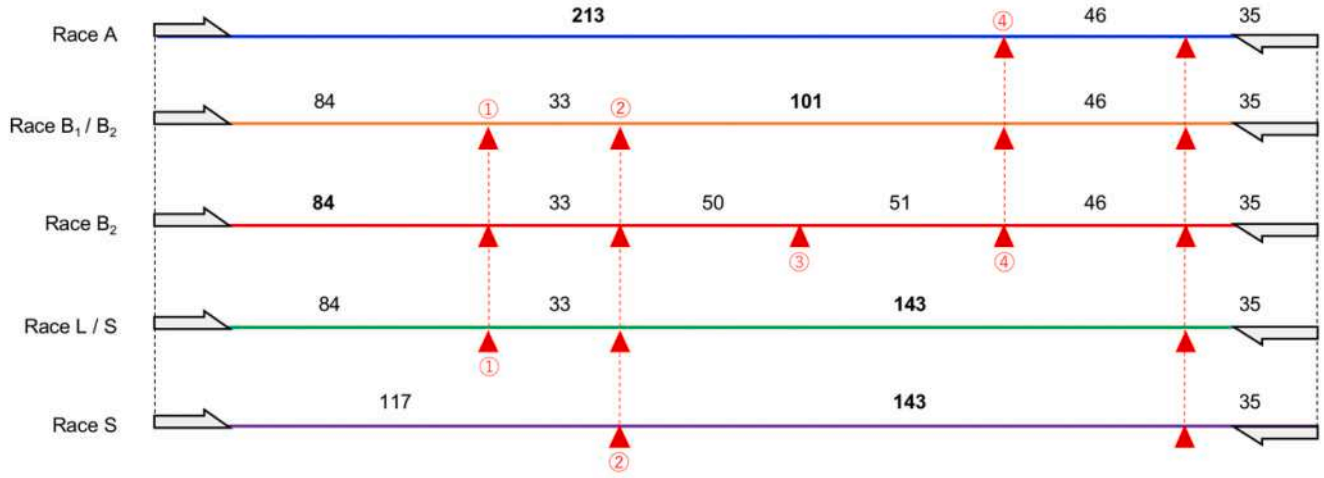
Fig. 2 shows a molecular phylogenetic tree of *B. braunii*, including 96

(a)

Polymorphism type		A/BLS	A/BLS	A/BLS	AL/(B)S	A/BLS	A/BLS	A(S)/BL	B/ALS	A/BLS	AB/SL	A/BLS	A/BLS	(B)/ALS	A/BLS	AB/SL	AB/SL	AB/SL
Position (bp)		89	100	125	138-143	199	358	513-516	528	546-549	559	566	569	593-596	602	628	630	632
Race	A	A	T	T	GGGYTT	T	A	GACC	A	AGCC	C	G	C	GGAC	G	A	A	T
	B	G	C	C	GGGCTY	G	C	GGCC	G	GGCC	C	A	T	GGMC	C	A	A	T
	L	G	C	C	GGGCTT	G	C	GGCC	A	GGCC	T	A	T	GGAC	C	G	C	C
	S	G	C	C	GGGCTC	G	C	GRCC	A	GGCC	T	A	T	GGAC	C	G	C	C
					Bsp1286I			HaeIII ①		HaeIII ②				HaeIII ③				

Polymorphism type		AB/SL	AB/SL	AB/SL	AB/SL	A/BLS	A/BLS	A/BLS	A/BLS	ABS/L	A/BLS	A/BLS	A/BLS	A/BLS	A/BLS	A/BLS	A/BLS
Position (bp)		639	643-646	658	660-661	677	929	1166	1218	1239	1260	1272-1274	1348	1415	1428	1458	1470
Race	A	A	GGCC	T	TG	A	T	C	A	C	T	AAT	T	T	A	T	A
	B	A	GGCC	T	TG	G	C	G	C	C	C	GGA	G	G	C	C	G
	L	C	GGCA	C	CT	G	C	G	C	T	C	GGA	G	G	C	C	G
	S	C	GGCA	C	CT	G	C	G	C	C	C	GGA	G	G	C	C	G
			HaeIII ④														

(b)



(c)

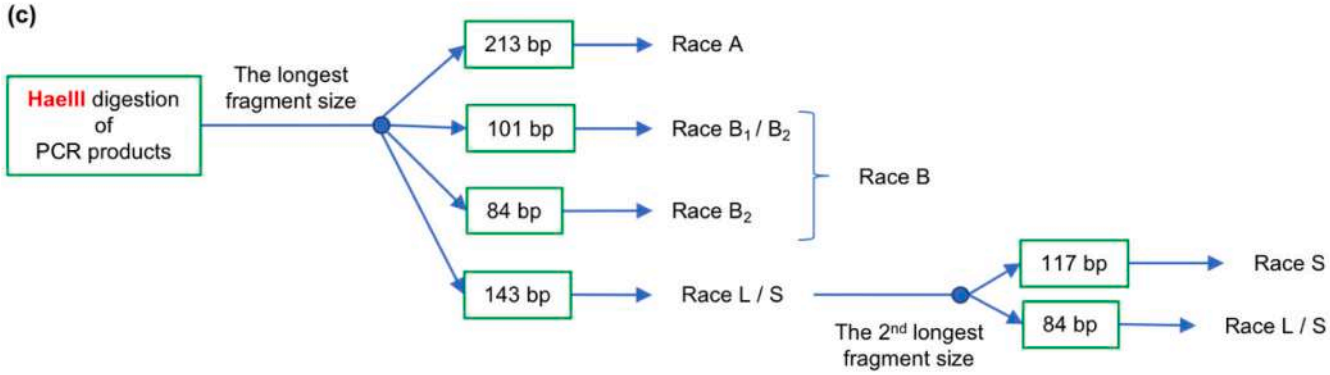


Fig. 3. The PCR-CAPS marker system for discrimination of hydrocarbon races in *Botryococcus braunii*. (a) Polymorphic sites of 18S rRNA sequences among the races. The polymorphism type indicates which races can be distinguished by the polymorphism. Restriction sites of *Hae*III (sites ①–④) and *Bsp*1286I are indicated. (b) PCR-CAPS marker system with *Hae*III digestion. Gray-boxed arrows indicate primers (18Sbot_F, 18Sbot_R). Numbers indicate the length of the fragment (bp). Triangles indicate restriction sites of *Hae*III. (c) Flow chart for race discrimination by the PCR-CAPS marker. Discrimination of race L and race S requires an additional PCR-CAPS marker targeting the restriction site of *Bsp*1286I enzyme with a different primer set.

wild strains isolated from temperate to tropical ponds with reference sequences obtained from NCBI. The four chemical races (A, B, L, and S) were classified into four major clades as previously reported [22,31], indicating that they are genetically distinct. In the B-race clade, two large sub-clades were formed and named as B_1 and B_2 clades. Our tropical strains were all classified into either the B- or L-race clade.

3.2. BoCAPS assay for race discrimination

Fig. 3 shows the outline of the PCR-CAPS marker systems that were developed. We focused on the *Hae*III restriction site polymorphisms in the 18S rRNA sequences (Fig. 3a, b). This PCR-CAPS marker can estimate the hydrocarbon races as either race A, race B, or race L/S. In the *Hae*III restriction site No. ③, there is a polymorphism within the race B. Almost all strains in the B_2 clade (n = 121) have the *Hae*III restriction site No. ③ (GGCC), whereas a few strains in the B_2 clade (n = 2), and all strains in the B_1 clade (n = 25) have no restriction site of *Hae*III (GGAC). This within-race polymorphism does not affect the race discrimination by the CAPS, because of the other consistent restriction sites polymorphisms between the races (Fig. 3c). In the *Hae*III restriction site No. ①, there is a polymorphism within the S-race clade. Some strains (n = 11) in the S-race clade have the sequences (GACC) that were not digested by *Hae*III, but other strains (n = 6) in the S-race clade and all strains (n = 19) in the L-race clade have the *Hae*III restriction site (GGCC). This within-clade polymorphism results in the variation in the second longest fragment size of the *Hae*III restriction (117 or 84 bp) in the S-race clade (Fig. 3c). The final distinction between race L and race S requires an additional PCR-CAPS test with different primer sets and a different enzyme (*Bsp*1286I).

The PCR-CAPS marker with *Hae*III enzyme worked well to distinguish the different hydrocarbon races on agarose gels (Fig. 4a). The visible differences in fragment lengths between the strains belonging to clades B_1 and B_2 were obscure, but both clades belong to race B and produce the same triterpene hydrocarbons. The second PCR-CAPS marker with *Bsp*1286I enzyme distinguished race L and race S (Fig. 4b).

3.3. Direct PCR method

Fig. 5 shows the results of direct PCR tests. All reactions using direct PCR templates that were prepared by crushing and heating algal cultures showed positive PCR amplifications regardless of race (Fig. 5a). Single-colony direct PCR also worked well and was combined with the PCR-CAPS marker described above (Fig. 5b). Therefore, this direct PCR-CAPS method (named as 'BoCAPS') was capable of estimating hydrocarbon races from a single colony of *B. braunii* without any pre-treatments for DNA extraction or purification.

3.4. Applicability in wild populations

To confirm the broad applicability of BoCAPS across a variety of strains, we carried out BoCAPS on additional 81 wild strains isolated from tropical (Indonesia) and temperate (Japan) regions and successfully estimated their races. Table 1 shows the race compositions of the isolated strains by their originated ponds and climate regions. The data includes 81 strains classified by BoCAPS, 96 strains classified by the sequencing of 18S rRNA (Fig. 2), and 70 strains classified by our previous study [22]. The B-race strains were dominant (n = 182, 74 %), the L-race strains were subdominant (n = 34, 14 %), and the compositions of A-race (n = 16, 7 %) and S-race strains (n = 15, 6 %) were small. However, there were a few ponds, such as SG and YA, where minor races (i.e., L or A) were dominant and no B-race strains were isolated. Neither A-race nor S-race strains were isolated from tropical regions.

4. Discussion

This study developed a DNA-based, simple method for estimating the chemical races found in *B. braunii* and termed the method as BoCAPS. The BoCAPS allows us to estimate chemical race without the time-consuming and expensive analysis of hydrocarbons and will be useful for high-throughput screening and ecological studies of wild populations. Because the BoCAPS assumes the consistency of the correspondence of chemical races with the major clades of 18S rRNA phylogenetic tree (Fig. 2), further confirmations of the genetic differentiation between chemical races are important to assure the accuracy of

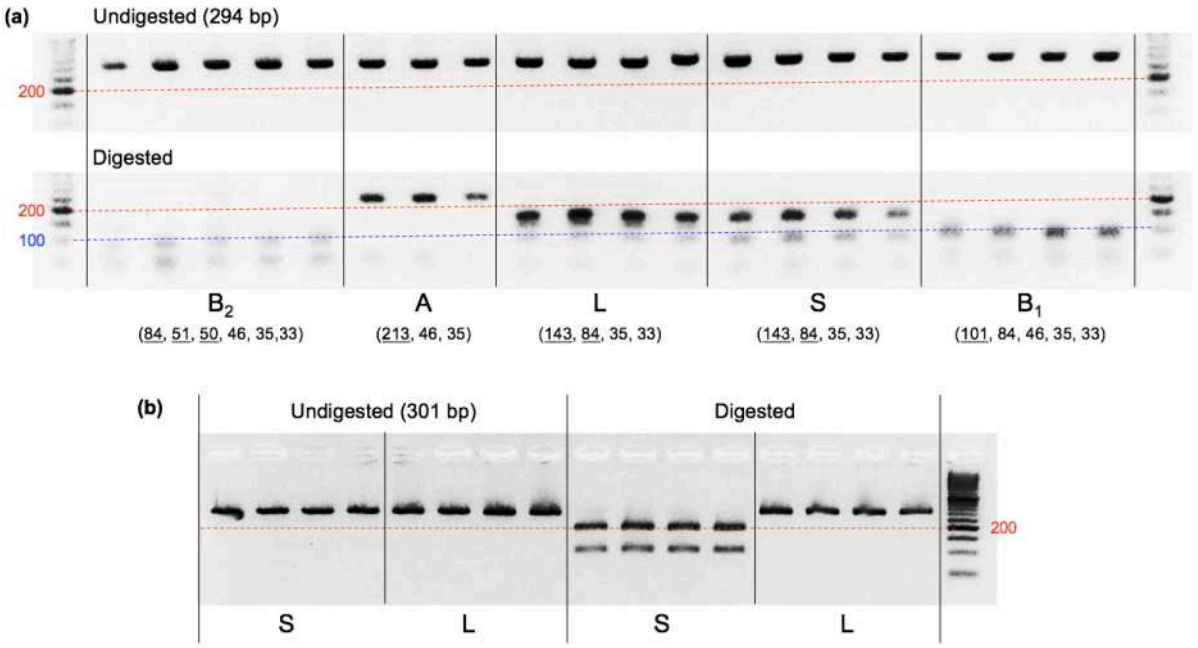


Fig. 4. Agarose gel images of the PCR-CAPS marker system for the discrimination of hydrocarbon races in *Botryococcus braunii*. Three to five strains per race were used to test the marker. The predicted fragment lengths are shown in parentheses with the visible bands underlined. (a) PCR-CAPS marker with *Hae*III enzyme, (b) PCR-CAPS marker with *Bsp*1286I enzyme.

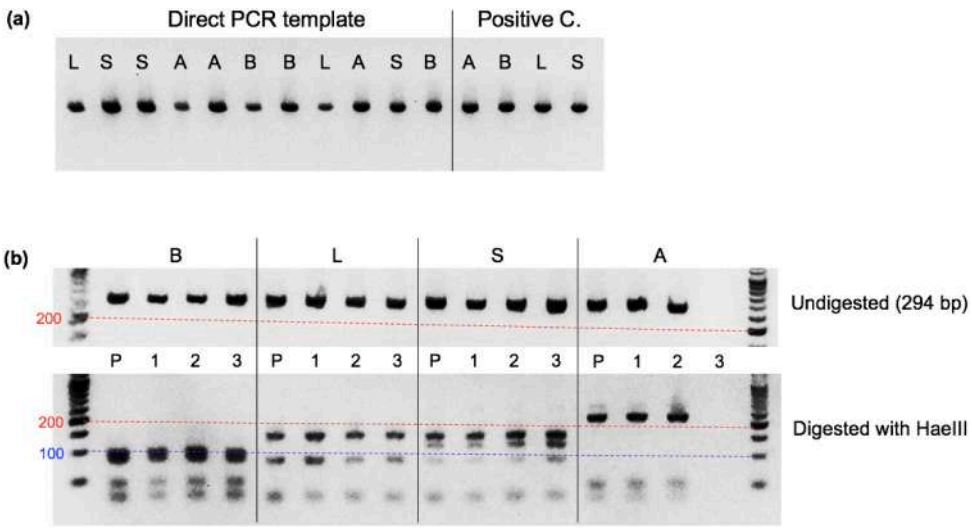


Fig. 5. Agarose gel images for the direct PCR test of *Botryococcus braunii*. (a) Direct PCR from algal culture after crushing and heating. Direct PCR templates were prepared from different strains and difference races. The positive control used DNA solutions prepared with a commercial DNA extraction kit. (b) Single-colony direct PCR test. P = positive control using the direct PCR template. The numbers 1–3 indicate three independent repetitions of single-colony direct PCR. After PCR amplification, the PCR products were digested with *Hae*III.

Table 1
Number of *Botryococcus braunii* wild strains estimated for their chemical races.

Pond ID[†]	Location	Climate region	Chemical race			
			A	B	L	S
IB	Palangka Raya, Indonesia	Tropics		20		
IC	Palangka Raya, Indonesia	Tropics		31		
IE	Palangka Raya, Indonesia	Tropics		9	1	
IF	Pundu, Indonesia	Tropics		10		
IG	Pundu, Indonesia	Tropics		14		
Others (ID, IJ, IK, II, IH, IM), Indonesia		Tropics		6	12	
Total (%, composition)			0	87	13	0
C	Okinawa, Japan	Subtropics		14	1	5
G	Okinawa, Japan	Subtropics		6		
Total (%, composition)			0	77	4	19
N	Fukuoka, Japan	Warm temperate		10		
SG	Hyogo, Japan	Warm temperate			14	
S3	Hyogo, Japan	Warm temperate		20		2
SE	Osaka, Japan	Warm temperate	4	18		1
T	Kyoto, Japan	Warm temperate		12	1	
Others (L, M, P, TS, S1, H1, H2, U, TK), Japan		Warm temperate	2	12	5	6
Total (%, composition)			0	54	41	5
SY	Miyagi, Japan	Cool temperate				1
YA	Fukui, Japan	Cool temperate	10			
Total (%, composition)			91	0	0	9

[†] See Fig. 1 for geographic location. In tropical and warm-temperate ponds, in which the numbers of strains were <10, these were grouped into "Others" for simplicity.

the method.

Previous screening studies of appropriate *B. braunii* strains for biofuel production adopted growth rate and hydrocarbon content as selection criteria [19,43–45]. Recently, Kleinert and Griehl provided a new screening procedure of suitable *B. braunii* strains for non-destructive in situ hydrocarbon extraction [46]. In addition to these criteria, a screening method for hydrocarbon structure (i.e., chemical race) is important, as it determines the heat-value of hydrocarbon. Our BoCAPS method is indirect but simple estimation method of hydrocarbon structure of *B. braunii* and is suitable for the screening procedure.

Although the extracellular matrix of hydrocarbon and polysaccharide was thought to prevent efficient DNA extraction from *B. braunii* colonies [47], we demonstrated that direct PCR from a single colony without any treatments for DNA extraction or purification is feasible, as reported in single cellular microalgae, such as *Chlorella* sp. [40]. The initial heating step of PCR appears to be enough to release algal DNA molecules from the *B. braunii* cells and inactivate algal enzymes. However, we did not quantify the amount of DNA released from the cell and the efficiency of PCR amplification. They might change with physiological conditions of algal cells and/or genotypes. To optimize the direct PCR method for *B. braunii*, further quantitative studies are required. Furthermore, we used a normal, low-cost Taq polymerase (EmeraldAmp® PCR Master Mix, TaKaRa Bio, Japan) for the direct PCR, but did not test other enzymes or PCR kits. In some enzymes or PCR kits, the direct PCR might not work well. We did not test whether this direct PCR method can successfully amplify other genes, such as single-copy genes in genomic DNA. The direct PCR amplification may be more difficult for single-copy genes than the multiple-copied gene 18S rRNA.

This study developed a CAPS method for race determination in order to reduce the costs and time needed for sequencing. The CAPS method requires nucleotide polymorphisms between the races, whereas the sequences should be well conserved within each race. In order to ensure universal applicability of our method, we determined 18S rRNA sequences for a total of 166 wild strains isolated from a wide geographic range that covered temperate zones and tropical regions. We combined the sequences with public data, analyzed the sequences of a total of 199 strains (Fig. 2), identified conserved polymorphic sites between the races, and developed the BoCAPS (Fig. 3). The large number of the sequences analyzed indicate a wide applicability of the BoCAPS. However, our sampling sites were concentrated in Japan and Indonesia, and the majority of analyzed strains is B-race strains (n = 148, 74 %). Thus, it is possible that there were other *B. braunii* strains with nucleotide sequences that could not be determined by our BoCAPS method.

With the aid of BoCAPS, we investigated race compositions of wild strains isolated from natural ponds distributed across temperate and tropical regions (Table 1), and the results suggest that (i) the B-race is a dominant race in both tropical and warm temperate climates; (ii) B- and L-races are found in both tropical and temperate regions, whereas A- and S-races were not isolated from tropical ponds; (iii) in several ponds different races coexisted, whereas in a few ponds only L- or A-race strains, were found, and B-race strains were not identified. The

dominance of B-race strains indicates that the bioprospecting of wild strains in these regions would likely have a high probability of finding B-race strains with a great potential for biofuel production. However, in ecological studies, caution is needed to interpret the result, because the race composition of our isolated strains may have been biased by the sampling method. We used a phytoplankton net with a 100 μm mesh, though it could have been a bit too large for the normal-sized colony of L-race strains [35].

Based on our observations (ii), the A-race is likely to favor relatively cool climates; however, due to the small number of samples collected from cool temperate regions, further studies are needed to test this hypothesis. Our observation (iii) suggests that the race composition could differ between ponds within the same climatic region. Previous environmental studies using hydrocarbons as biomarkers reported the co-occurrence of the three races (A, B, and L) [36,37]; however, our data suggest there might be some differences in environmental preferences between the races. Further studies investigating the relationship between race composition and environmental conditions, such as water quality, for example, will highlight the ecological differences between the races. Such insight is expected to provide useful information on the issues related to algal biofuel production: Under which types of environmental conditions would we find a wild strain possessing properties suitable for biofuel production? How can we successfully maintain outdoor mass production of *B. braunii*?

The L-race produces a longer-chain hydrocarbon with a higher heat value than that of the B-race but has the disadvantage of low hydrocarbon content [48]. However, our method enables the identification of the pond where the L-race is dominant, such as the pond SG (Table 1). The investment in efforts to isolate and screen such ponds might lead to the discovery of L-race strains with a high hydrocarbon content. The S-race was proposed by Kawachi et al. [31] as a new class of chemical race in *B. braunii*, although details of its ecophysiological properties have not yet been elucidated. The molecular phylogenetic tree indicates that the strains in the S-race clade have low genetic divergence and are phylogenetically close to the clade of L-race strains, suggesting that the strains in the S-race clade recently diverged from a strain of the L-race clade. Further studies are necessary to confirm the establishment of this new class of chemical race. Our BoCAPS method is helpful for obtaining appropriate materials for continued research in the strains of the S-race clade.

5. Conclusion

This study developed a simple PCR-based CAPS assay for the determination of chemical races of *B. braunii* and termed it BoCAPS. The BoCAPS substantially reduces the time and costs for screening of chemical races and only requires a simple thermal cycler and standard equipment for agarose gel electrophoresis. Therefore, the method will be useful as a first screening method of newly-isolated wild strains of *B. braunii* and contribute to the effective bioprospecting of the alga. It will be also helpful for the investigations of unknown ecological differences between chemical races. As this study mainly analyzed *B. braunii* strains in Japan and Indonesia, further studies with strains isolated from other regions are necessary to confirm the wide applicability of BoCAPS.

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CRediT authorship contribution statement

Koji Kawamura: Conceptualization, Funding acquisition, Supervision, Writing – original draft. **Suzune Nishikawa:** Methodology, Validation. **Kotaro Hirano:** Investigation. **Ardianor Ardianor:** Resources, Writing – original draft. **Rudy Agung Nugroho:** Resources, Writing – original draft.

Declaration of competing interest

The authors declare no conflict of interest.

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