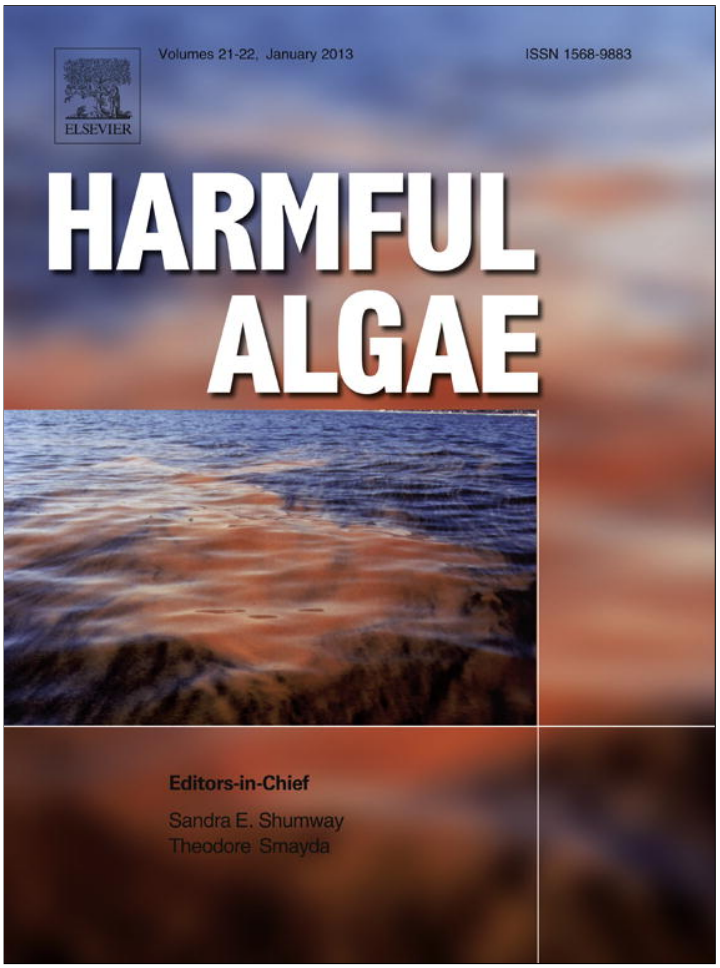

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Genetic diversity among clonal isolates of *Karenia brevis* as measured with microsatellite markers

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ABSTRACT

Karenia brevis is the major harmful bloom-forming dinoflagellate in the Gulf of Mexico yet little is known about the intraspecific genetic diversity of this species. Here we describe nine new microsatellite markers and, combined with nine previously described microsatellites, use them to genotype 40 cultured isolates of *K. brevis*. Genetic diversity identified from cultured isolates was compared with the genetic diversity identified from two field samples to assess how well the current cultures represent the field population. Thirty-nine unique haplotypes were identified from 40 cultured isolates of *K. brevis* using 18 microsatellite markers. Genetic diversity was similar between cultured isolates and the two field samples. The success of 18 microsatellite markers to distinguish individual isolates supports the use of microsatellites as a genetic tool for diagnostic identification of cultured isolates of *K. brevis*.

1. Introduction

The major, harmful bloom-forming species in the Gulf of Mexico is *Karenia brevis*, an unarmored dinoflagellate responsible for large fish kills and for respiratory problems in humans living near the coast (Steidinger et al., 1998). Factors influencing bloom dynamics of *K. brevis*, in particular bloom initiation, are not well understood. Prior studies have shown that considerable physiological variation exists among clones of *K. brevis* but little work has been done to identify plausible genetic variation (Loret et al., 2002; Brown et al., 2006; McKay et al., 2006; Errera et al., 2010). Because blooms of dinoflagellates result from accumulations of haploid vegetative cells that reproduce by binary fission, it might be expected that genetic diversity would be low within a bloom. In fact, in a number of bloom-forming dinoflagellates, high levels of genetic diversity have been observed using highly polymorphic markers (e.g. microsatellites; Nagai et al., 2007, 2009; Alpermann et al., 2009; Erdner et al., 2011). A more detailed understanding of genetic diversity, within and among blooms of *K. brevis*, is needed so that the dynamics of toxic blooms of *K. brevis* can be described and links to environmental factors investigated.

Here we focus on the use of microsatellite markers to identify genetic diversity among cultured isolates of *K. brevis*. Nine microsatellite markers currently exist for *K. brevis* (Renshaw et al., 2006). In this study, we first developed new microsatellite markers to combine with previously identified microsatellites for *K. brevis* in order to obtain a better estimate of the genetic diversity in cultured isolates of *K. brevis*. Cultures of *K. brevis* have proven very difficult to establish; consequently, relatively few isolates are available for study (Table S1). Because of the difficulty encountered in culturing *K. brevis*, isolates that survive the culturing process may have reduced genetic diversity due to possible culturing biases. Inaccurate representation of the field population becomes important when the results from experiments on cultured isolates of *K. brevis* are extrapolated to field populations. To obtain an estimate of how well the current isolates represent the field population, genotypes from single cells of *K. brevis* were compared with genotypes from cultured strains.

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The goal of the present study is to describe the genetic variation among cultured strains of *K. brevis*. Clonal cultures of *K. brevis* available from several different laboratories were examined to address the following questions:

1) Are strains currently in culture representative of the genetic diversity present in field populations?
2) Can microsatellite markers provide a diagnostic tool to differentiate clonal cultures?

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2. Materials and methods

2.1. DNA isolation

Cell pellets for 40 clonal cultures were obtained by centrifugation (10,000 × *g* for 15 min.) of 1.7 mL Eppendorf tubes (VWR; Radnor, PA, USA) each containing approximately 1.5 mL of a dense culture. The resulting supernatant was removed and discarded. Genomic DNA from the cell pellet was extracted using the cetyl trimethylammonium bromide (CTAB) buffer extraction method described by Doyle and Doyle (1990). The 40 strains have been isolated over a span of 50+ years; most were isolated from water samples taken from the Gulf of Mexico. Collection information for each culture can be found in Table S1.

2.2. Microsatellite development

Two approaches were taken to develop additional microsatellite markers. In the first approach, expressed sequence tag (EST) sequences from *K. brevis* were downloaded from GenBank and imported into Sequencher (v4.2; Gene Codes, Ann Arbor, MI, USA). Sequences not containing a microsatellite motif were removed from the dataset. The remaining sequences were visually inspected and sequences lacking suitable flanking regions at both ends of the microsatellite motif were removed. Sequences with identical microsatellite motifs were then aligned and manually inspected to eliminate duplicate sequences.

The second approach identified microsatellite markers from an enriched genomic DNA library, using the method of Renshaw et al. (2006). Primers for all microsatellite markers were developed using Primer3 (http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky, 2000).

All microsatellite markers (EST-based and those obtained from genomic DNA) were tested using DNA from four different strains of *K. brevis*; those markers producing a product consisting of a single visible band after gel electrophoresis (2% agarose) were further tested using DNA from all remaining cultures. Polymorphic microsatellites were visually identified by gel electrophoresis (4% agarose). The forward primer of each identified polymorphic microsatellite was labeled with a fluorescent label from Applied Biosystems standard dye filter set D (Applied Biosystems; Foster City, CA, USA). All polymorphic microsatellite markers were tested further using DNA from a wide variety of dinoflagellate species (*Alexandrium monilatum*: CAAE 106; *Crypthecodinium* sp.; *Scrippsiella* sp.; *Karlodinium micrum*: CCMP1974, CCMP2282, CCMP415; *Karenia mikimotoi*: C21 [isolated in 2001 from Corpus Christi Bay, Texas]; *K. papilionacea*: CAWD91; *K. bidigitata*: CAWD92; *K. selliformis*: CAWD79; *Oxyrrhis marina*; *Pfiesteria* spp.: CCMP2301, CCMP2362; and *Pseudopfiesteria* sp.: CCMP2089) to confirm specificity of the primers.

2.3. Allele sizing

Each culture was genotyped by polymerase chain reaction amplification (10 μL reaction) of each microsatellite from CTAB extracted DNA. Allele sizing was conducted by running the resulting product on a 5% polyacrylamide gel (Long Ranger Singel Pack, Cambrex Bio Science Rockland, Rockland, ME, USA) and ABI Prism 377 genetic analyzer (Applied Biosystems). Gels were analyzed with Genescan 3.1.2 (Applied Biosystems) and alleles scored in Genotyper version 2.5 (Applied Biosystems).

2.4. Genetic analysis

For each microsatellite, number of alleles, allelic ranges, and estimates of gene diversity (*H*; Nei, 1973) were calculated using PopGene v1.32 (Yeh and Boyle, 1997). Unbiased estimates of gene diversity were calculated according to Nei (1987) to account for the small sample sizes from each location. Tests for genotypic disequilibrium between pairs of loci were run in Genepop v1.2 (Raymond and Rousset, 1995) and Bonferroni correction for multiple tests was applied following the method of Rice (1989).

Diversity results from all cultures were compared to those obtained from two different field samples. One field sample was taken during a bloom off the west coast of Florida (26.555°N, −82.477°W; 2006) and the other sample taken from a bloom in Corpus Christi Bay in Texas (2005). Single cells were isolated from both field samples and genotyped with five microsatellites (*Kbr*5, *Kbr*7, *Kbr*8, *Kbr*9, *Kbr*10) following the method of Henrichs et al. (2008). Briefly, single cells were isolated into individual 0.2 mL PCR tubes and subjected to three rounds of freeze/thaw to lyse cells. This was followed by two rounds of PCR amplification, a multiplexed first round PCR and a simplexed second round PCR with fluorescently labeled forward primers. To account for the difference in sample size between cultures and field samples, allelic richness estimates for the field samples were calculated by rarefaction following the method of El Mousadik and Petit (1996). For the rarefaction calculation, the sample size for each microsatellite was equivalent to the number of strains of *K. brevis* producing an allele for that microsatellite.

2.5. Diagnostic test

To test the utility of microsatellite markers for diagnostic strain confirmation, cell pellets were obtained from several laboratories studying *K. brevis*. The CCFWC268 strain was obtained from six different laboratories and the CCMP718 strain was obtained once from one laboratory and three times (2000, 2004, 2007) from the National Center for Marine Algae and Microbiota (NCMA). The CCFWC266 and CCFWC267 strains were obtained once from each of three different laboratories (2003, 2004, 2006) and the CCFWC256 strain was obtained twice (2006, 2009) from the Culture Collection at the Florida Fish and Wildlife Conservation Commission. Genotype information was obtained from CTAB extracted DNA of each strain received as described above and the resulting PCR products were analyzed as detailed above.

3. Results

3.1. New microsatellite markers

Nine new microsatellite markers were identified, bringing the total number of microsatellite markers from *K. brevis* to 18. Primer sequences and allelic ranges for the nine new microsatellites can be found in Table S2. Of the nine, only one microsatellite produced an allele in all 40 strains of *K. brevis*. The remaining eight each failed to amplify in at least one strain, in spite of repeated amplification attempts, which suggested the presence of null alleles. Two microsatellites (*Kbr*12, *Kbr*14) each failed to amplify in 13 strains, though the strains with possible null alleles differed between the two microsatellites. Of the six remaining microsatellites, five amplified successfully in 39 strains and one amplified successfully in 38 strains. None of the microsatellites produced observable bands after gel electrophoresis (2% agarose) when tested with extracted DNA of other plankton species, which confirmed the specificity of the primers to *K. brevis*.

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3.2. K. brevis *haplotypes*

Based on the 18 microsatellite loci, 39 unique haplotypes were identified among the 40 strains of *K. brevis* (~97%). Haplotype

Table 1
Microsatellite allele sizes for 40 cultures of *Karenia brevis*.

Culture	Locus																
	*Kbr*1	*Kbr*3	*Kbr*4	*Kbr*5	*Kbr*6	*Kbr*7	*Kbr*8	*Kbr*9	*Kbr*10	*Kbr*11	*Kbr*12	*Kbr*13	*Kbr*14	*Kbr*15	*Kbr*16	*Kbr*17	*Kbr*18	*Kbr*19
CCFWC121	258	274	278	190	231	261	146	161	173	184		203		202	175	254	133	116
CCFWC122	249	235	266	182	223	261	138	161	177	182		221	322	206	167		113	116
CCFWC123	261	235	266	184	225	261	134	161	175	184	207	212		204	171	248	113	118
CCFWC124	249	253	276	190	225	261	130	158	175	172	204	215	314	212	169	258	109	128
CCFWC125	252	241	262	190	223	261	136	164	189	178		218		202	165	258	115	134
CCFWC126	261	235	268	182	225	261	126	161	173	172	210	218		202	173	264		122
CCFWC127	258	241	266	182	223	255	136	158	173	181		224	302	204	167	274	117	118
CCFWC128	258	250	268	188	223	261	132	161	171	178	213	245	282	204	171	260	125	118
CCFWC129	258	241	270	188	225	261	134	161	173	184	207	206	318	212	175	256	131	118
CCFWC130	252	238	270	188	223	261	134	164	179	181	207	218	334	196	167	270	115	124
CCFWC250	246	247	270	188	221	261	134	161	169	178		206	330	210	171	262	109	
CCFWC251	255/258	235/247	264/274	190	223	261	128/132	161	171/175	181	207	203/206		206	173	262/276	111/117	116
CCFWC252	258	235	266	188	225	261	132	161	175	181			330	202	169	252	133	122
CCFWC253	258	235	268	184	225	261	144	161	169	178		221	338	204	175	270	143	124
CCFWC254	264	256	268	186	225	261	132	161	175	178		218		208		262	119	126
CCFWC256	258	232/247	264	190	224	258/261	128/138	158/164	173	178	207	206/221	314/322	204	169	260	125	122
CCFWC257	258	232	264	182	229	261	144	158	175	172	207	206			175	280	109	128
CCFWC258	261	238	276	182	221	261	108	161	173	172	201	227	310	210	179	258	123	118
CCFWC259	258	235	264	182	219	261	144	161	171	178		206	294	202	167	254	123	116
CCFWC260	258	235	264	182	219	261	144	161	171	178		206	294	202	167	254	123	116
CCFWC261	255	247	272	188	223	258	146	164	179	181	207	206		206	169	262	111	116
CCFWC262	246	232	272	188	225	258	146	164	179	181		203	278	206	169	270	127	122
CCFWC263	258	232	270	190	221	261	132	161	173	184	207	206	318	206		260	129	116
CCFWC265	264	238	268	190	225	258	138	167	171	178		221	262	202	169	262	123	122
CCFWC266	258	235	264	190	221	261	128	161	175	178	207	203		204	173	276	117	128
CCFWC267	255	229	264	182	225	261	146	161	177	178	207	221	294	206	173	266	115	118
CCFWC268	267	235	266	186	221	261	377	164	173	178	207	215		204	167	254	111	118
CCFWC269	249	229	264	184	221	261	132	164	181	178	207	200		206	167	262	115	128
CCMP2228	258	235	266	190	221	261	134	161	179	184	207	200	306	208	165	258	109	122
CCMP2229	249	244	264	180	221	261	134	161		178		227		200	165	256	111	122
CCMP2281	258	235	270	182	219	261	130	161	173	184	207	200	322	202	167	256	121	116
CCMP2820	261	235	268	184	221	261	134	161	171	178	204	209	282	198	171	256	137	120
CCMP718	261	235	252	184	219	261	128	161	181	181	204	212	294	204	167	262	137	130
EPA JR	258	256	268	190	221	264		161	173	175	204	200	302	208	165	262	135	118
NBK	252	232	272	182	221	261	134	161	177	175	207	236	318	208	173	260	113	118
NOAA-1	255	238	270	188	223	261	136	161	173	181	207	206	314	200	171	260	129	120
NSP3[a]	249	235	266	190	221	252	138	161	175	169	207	224		206	165	262	111	118
SP1	264	235	272	182	221	261	128	164	177	181	207	200	290	204	167	248	115	118
SP2	258	238	266	182	223	261	132	161	175	181	207	215	290	206	169	264	127	120
SP3[b]	264	235	270	186	223	258	134	161	173	184	204	212	294	204	167	262	137	120

Blanks indicate no amplification product was observed.
[a] Named NTSP3 in Henrichs et al. (2008).
[b] Named TSP3 in Henrichs et al. (2008).

information for all 40 strains is summarized in Table 1. Two identical haplotypes were identified from strains established from the same bloom and these strains could have originated from clonal cells. Two strains (CCFWC251, CCFWC256) repeatedly produced two observable bands at each of several loci (Table 1). The two bands were from 3 to 15 bp apart and were clearly defined when viewed on agarose or polyacrylamide gels. Both cultures produced two bands at three loci (*Kbr*3, *Kbr*8, *Kbr*13). Additionally, CCFWC251 produced two bands at *Kbr*1, *Kbr*4, *Kbr*10, *Kbr*17, *Kbr*18 and CCFWC256 produced two bands at *Kbr*7, *Kbr*9, *Kbr*14. To confirm the observed pattern of two bands, single cells were isolated from CCFWC256 and genotyped at four microsatellites (*Kbr*8, *Kbr*9, *Kbr*13, *Kbr*14). The isolated single cells produced two bands for the four microsatellites tested, though some cells occasionally produced a single band for one microsatellite, presumably due to amplification failure in the first round of PCR, while the remaining microsatellites produced two bands. The sizes of the bands produced from single cell analysis were equivalent to the sizes observed from extracted culture DNA. To reduce the chance of bias in the analyses from arbitrarily picking one allele to represent the strain, the microsatellites producing two bands were coded as missing data in the two strains.

3.3. Genetic analysis

The number of different alleles identified for each microsatellite ranged from four to 16 and unbiased estimates of gene diversity (all 40 strains combined) ranged from 0.323 to 0.945 (0.775 ± 0.170 [mean $\pm$ SD]; Table 2). Tests for genotypic disequilibrium among cultures resulted in 11 pairs of loci exhibiting significant disequilibrium ($P < 0.05$); however, none remained significant after Bonferroni correction (data not shown). A total of 288 single cells (192 from Florida; 96 from Texas) of *K. brevis* from field samples collected during blooms were genotyped to compare with the cultures. From these, 192 cells (107 from Florida; 85 from Texas) produced an allele at three or more microsatellites (out of the five that were amplified) including 41 cells producing an allele at all five microsatellites. The number of different alleles identified from field populations ranged from five to 13 and unbiased estimates of gene diversity ranged from 0.280 to 0.843 (0.650 ± 0.216 [mean $\pm$ SD]; Table 3), comparable to the values identified from cultured strains for those same five loci (N_A: 4–11; H: 0.323–0.891; 0.657 ± 0.225 [mean $\pm$ SD]; Table 2). The total number of different alleles identified from field samples was higher than the total identified from all cultured strains of *K. brevis* for four of the five microsatellites (*Kbr*5, *Kbr*8, *Kbr*9, *Kbr*10). Estimates of allelic richness (calculated by rarefaction) from field samples were higher than those obtained from cultured strains for two microsatellites (*Kbr*5, *Kbr*10), lower in one microsatellite (*Kbr*7), and approximately the same at the remaining two microsatellites (*Kbr*8, *Kbr*9; Tables 2 and 3).

Table 2
Summary of microsatellite information for cultures of *Karenia brevis*.

Locus	n	N_A	H	Allelic range
*Kbr*1	39	8	0.794	246–267
*Kbr*3	37	10	0.787	229–256
*Kbr*4	39	9	0.858	252–278
*Kbr*5	40	6	0.792	180–190
*Kbr*6	40	6	0.764	219–231
*Kbr*7	39	5	0.323	252–264
*Kbr*8	37	11	0.891	108–377
*Kbr*9	39	4	0.457	158–167
*Kbr*10	37	7	0.823	169–181
*Kbr*11	39	6	0.760	169–184
*Kbr*12	27	5	0.484	201–213
*Kbr*13	37	12	0.908	200–245
*Kbr*14	26	14	0.942	262–338
*Kbr*15	39	9	0.841	196–212
*Kbr*16	38	7	0.841	165–179
*Kbr*17	38	13	0.902	248–280
*Kbr*18	38	16	0.945	109–143
*Kbr*19	39	9	0.842	116–134

n: number of cells; N_A: number of alleles; H: unbiased estimates of gene diversity.

Table 3
Summary of microsatellite information for field cells of *Karenia brevis*.

Locus	n	N_A	H	Allelic range
*Kbr*5	162	10 (7.1)	0.772	178–198
*Kbr*7	176	4 (2.1)	0.280	258–267
*Kbr*8	116	13 (10.7)	0.843	108–148
*Kbr*9	173	5 (3.9)	0.531	110–167
*Kbr*10	119	12 (8.8)	0.823	167–213

n: number of cells; N_A: number of alleles, numbers in parentheses represent allelic richness estimates calculated by rarefaction to the number of cultures for each locus; H: unbiased estimates of gene diversity.

3.4. Diagnostic test

The genotype results produced for CCFWC268 (also known as the "Wilson" clone) obtained from all five laboratories were identical. The same result (identical genotype) was observed for all samples of the strains CCMP718, CCFWC256, CCFWC266, and CCFWC267. No new alleles were identified at any microsatellite locus for any strain.

4. Discussion

The nine new microsatellite markers identified in this study have doubled the number of available microsatellite markers for *K. brevis*. For studies investigating population genetic structure, increasing the number of loci (and/or alleles) can provide increased power to detect genetic divergence among populations than would be obtained from increased numbers of individuals (Kalinowski, 2002, 2005; Landguth et al., 2012). The addition of four microsatellites derived from ESTs provides a glimpse of the genetic variation present at presumably adaptive loci. Though selection may be acting on adaptive loci, potentially causing them to diverge based on local pressures, these loci can still provide insight about genetic divergence (Landguth and Balkenhol, 2012). Among the nine new microsatellites, two of the microsatellites derived from ESTs had the lowest number of alleles (5, 6 for *Kbr*11and *Kbr*12, respectively) and contained trinucleotide repeat motifs. Surprisingly, the remaining two EST microsatellite markers consisted of dinucleotide repeat motifs, which would cause a frame-shift to occur each time a single repeat is added, and had higher numbers of alleles. It appears at least one of the dinucleotide repeats is present in a protein coding gene. The sequence for the *Kbr*18 locus produced one hit from the BLAST nucleotide database indicating the microsatellite sequence is within a sequence coding for "replication protein A large subunit" (Genbank acc. no: JF491176; Brunelle and Van Dolah, 2011). *Kbr*18 produced the highest number of alleles among the cultures but it remains unknown if differences in allele size for this microsatellite have any effect on expression levels among cultures. The other three EST microsatellite markers did not produce any significant BLAST hits and it is unknown whether these sequences are truly coding sequences. Though the microsatellites derived from EST sequences are presumably not neutral markers, the four markers may be useful in future work investigating adaptive characteristics.

The small number of cultured isolates of *K. brevis* from Texas precluded an analysis of genetic divergence between Florida isolates and Texas isolates. The number of different alleles identified and the estimates of gene diversity are comparable to

previous genetic work on dinoflagellates. Nagai et al. (2004) described 13 microsatellite markers from *A. tamarense* with gene diversity estimates between 0.632 and 0.974. Nagai et al. (2007) identified an increased number of alleles (between seven and 42) at nine microsatellite markers when tested on 500 clonal cultures (Nagai et al., 2007) and compared to the 20 originally tested by Nagai et al. (2004). A similar result was observed in the present study of *K. brevis*. Four of the five microsatellites amplified from both cultures and field samples had a greater number of alleles in field samples but this result is likely due to the higher number of genotyped individuals. It is unlikely that 40 clonal cultures would contain all the alleles present in field populations of *K. brevis*. Similar estimates of gene diversity between field samples (unexposed to possible culturing biases) and cultures, along with the high number of unique genotypes among the cultures, suggest the clonal cultures currently being grown and studied incorporate much of the diversity present in the field. However, for two (*Kbr*5, *Kbr*8) of the five microsatellites (*Kbr*5, *Kbr*7, *Kbr*8, *Kbr*9, *Kbr*10), the most frequent allele identified from the field samples differed from the most frequent allele identified from cultures (*Kbr*5 shown in Fig. 1). Reduced genetic diversity for the four microsatellite markers derived from EST sequences could be an indication of culturing bias. Possible biases introduced by the culturing process may have resulted in cultures that, while diverse, are not an accurate representation of the populations present in the field but this aspect requires additional investigation and ideally, an increased number of cultures.

The two cases (out of 40) where two bands, presumed to be two alleles, were identified from a single microsatellite marker, even in single cells of a clonal culture, could be the result of a gene duplication event or indicate the presence of diploid cells in these strains. The possibility of a gene duplication event cannot be ruled out however, it is unlikely that multiple loci from a single strain would undergo gene duplication events while other strains had none. Diploid cells of *K. brevis* are difficult to distinguish from haploid vegetative cells (Walker, 1982). Further investigation quantifying the DNA content of the two cultures producing two alleles at some loci is needed to support or reject the possibility of a diploid culture.

As a diagnostic tool, microsatellite genotyping will also help to eliminate confusion in identifying different isolates. Difficulties in interpreting experimental results may arise if experiments are conducted with different strains. Two strains of *K. brevis* isolated from Florida in 1953 (CCFWC268, CCMP718) are often confused with each other in the literature. Although both are from Wilson's laboratory, they are in fact genetically distinct isolates based on the observed differences in allele size at 12 of the 18 microsatellites (Table 1). This result is consistent with observed physiological differences (e.g. growth rates differ between these two strains; Brown et al., 2006; Errera et al., 2010). It is unknown whether these two strains are products of separate single cell isolations or the result of the same culture being separated for a long period of time (accumulating mutations along the way). The lack of observed genetic variation among the replicates obtained from multiple labs, including one strain (CCMP718) obtained three different times from the same culturing facility over a seven year period, coupled with the physiological variation, supports the notion that the two strains are the products of separate single cell isolations. It must be noted that the possibility of accumulated mutations within separated cultures derived from the same single cell cannot be ruled out. Future studies utilizing one or both of these cultures should positively identify the cultures before making comparisons with previous work, especially those studies investigating physiological differences. The confirmation of each strain from several laboratories supports the use of microsatellite markers as a diagnostic tool for researchers who wish to confirm the identity of their strains.

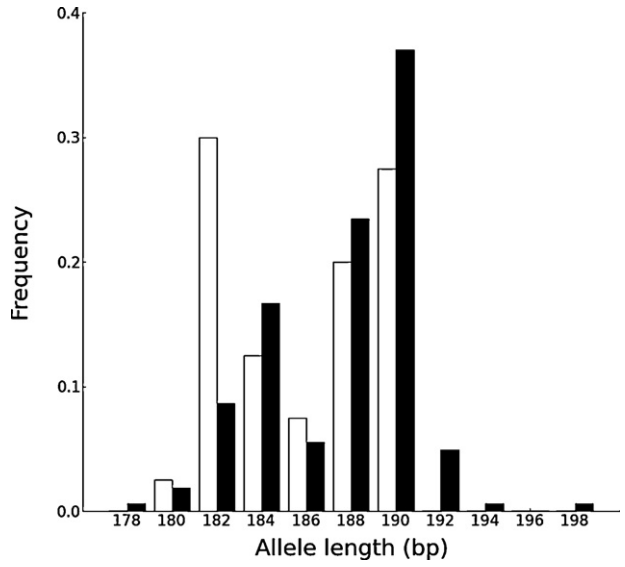

Fig. 1. Allele frequency distribution for microsatellite marker *Kbr*5 from cultures (white) and field cells (black).

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