Development of a Hierarchical Oligonucleotide Primer Extension Assay for the Qualitative and Quantitative Analysis of *Cylindrospermopsis raciborskii* Subspecies in Freshwater

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A newly-developed molecular method, hierarchical oligonucleotide primer extension (HOPE), was used to analyze various groups within the species *Cylindrospermopsis raciborskii.* PCR-amplified internally transcribed spacer sequences of 16S-23S from *C. raciborskii* in reservoir samples of Taiwan and Kinmen were examined. One of eight sequencers in the clone libraries was closely related to strains obtained from the European continent, while the others, designated of Taiwan (TW) type, belonged to a novel group. Optimized HOPE analyses showed that *C. raciborskii* distributed in different reservoirs with a relative abundance of 0.5% to 76.4% in the cyanobacterial communities. They further detected the concurrence of three *C. raciborskii* subpopulations, in which European and TW groups were predominant. The TW sequencers accounted for greater than 87.5% of *C. raciborskii* in the reservoirs Taihu, Yangmin, Jinsha, and Mudan, while this decreased to 55.4–58.1%, accompanied by a proportional increase of the European group, in reservoirs Lantan and Renyi. These findings revealed the complex subspecies structure within *C. raciborskii* and the subspecies dynamics associated with geographic locations.

**Key words:** HOPE, relative abundance, *Cylindrospermopsis raciborskii*

* C. raciborskii is an invasive cyanobacterial species, originally discovered in tropical regions, but currently found even at high latitudes (16). In view of its adaptability to freshwater ecosystems and in certain strains, ability to produce cyanotoxins (8, 12, 19), the prevalence of *C. raciborskii* in inland waters is increasingly important in the management of water quality.

Previous studies suggested the rapid dispersal of *C. raciborskii* from tropical to temperate habitats to result from a collective effect of broad tolerance to environmental conditions and a warming climate (2). Analyses of ribosomal internally transcribed spacer (ITS1) fragments of the 16S-23S rRNA gene have revealed that the phylogenetic distribution of *C. raciborskii* strains from the Australian, African, American and European continents was geographically dependent (5, 6, 19). However, although it was postulated that the expanding European strains could originate from the Eurasian continent (5), genetic information from the *C. raciborskii* counterpart in the Asian area to support the hypothesis is not available.

The qualitative and quantitative analysis of *C. raciborskii* populations is often performed through microscopic observation. The PCR technique also allows for specific detection and quantification (3, 4, 20, 23). Nevertheless, these two approaches can not identify *C. raciborskii* at the subspecies level. To gain a better ecological insight and study population dynamics, methods of rapidly detecting, typing, or quantifying the *C. raciborskii* population must be developed.

Recently, a new method called hierarchical oligonucleotide primer extension (HOPE) was developed to qualitatively and quantitatively profile the microbial populations in complex ecosystems (24). The rationale of the HOPE technique is based on single-base primer extension, whereby DNA polymerase specifically incorporates single fluorescent deoxynucleotides at the 3’ end of unlabeled hierarchical primers upon the formation of duplexes with targeted DNA strands. The resulting fluorescently labeled primers are then analyzed using a capillary-based four-color DNA sequencer, thus providing phylogenetic information or typing of the microbial population. For quantification, the numerical relationship between the concentration or fluorescence intensity of any two hierarchical primers, referred to as the calibration factor (CF), can be obtained using reference targets at a high primer-to-template ratio. On normalization to the CF value, the relative abundance of a specific microbial target at a lower rank with respect to that at a higher hierarchy in environmental samples can be determined accordingly. The method has excellent specificity, sensitivity and single mismatch discrimination as well as a multiplexing capability and flexibility (24).

The goal of this study was to come up with a HOPE assay to rapidly analyze the various subspecies of *C. raciborskii* and their relative abundance. To achieve this, a culture-independent approach was used. First, 16S-ITS1-23S sequences of *C. raciborskii* in Asia (Taiwan) were retrieved by cloning and sequencing to improve our understanding of phylogeography. Then, a HOPE assay with newly designed hierarchical primers was established, validated and used to profile the numerical distribution of various *C. raciborskii* groups.
among reservoirs in Taiwan.

Materials and Methods

Reservoir samples and C. raciborskii strains

Water samples (500 mL) were collected at a depth of 1 m from the eutrophic reservoirs Renyi (23°28’N, 120°30’E), Lantan (23°28’N, 120°28’E) and Mudan (22°08’N, 120°47’E) in Taiwan and the eutrophic reservoirs Tailu (24°26’N, 118°25’E), Yangmin (24°27’N, 118°25’E), and Jinsha (24°29’N, 118°24’E) in Kinmen during 2006–2008. The cyanobacterial cells in these samples were harvested by filtering through a 0.45-μm polycarbonate membrane, and stored at −20°C prior to further analysis. The American type C. raciborskii strain LB2897 was purchased from The Culture Collection of Algae at the University of Texas at Austin, and the Australian type C. raciborskii strain 020J was obtained from the Australian Water Quality Centre.

Phylogenetic analysis of C. raciborskii populations

A modified protocol, integrating bead beating, lysozyme and SDS/proteinase digestion, and phenol-chloroform extraction (14), was used to extract genomic DNA from C. raciborskii strains and reservoir samples. A mixture (50 μL) of 1–10 ng of DNA extract, 1×PCR buffer, 0.5 U of ExTaq (Takara Bio, Otsu, Japan), 200 μM of dNTP (dATP, dTTP, dCTP, dGTP), and 20 μM each of primers 62F and 30R (Table 1) was prepared for amplification. PCR was performed in a Thermocycler (Biometra GmbH, Goettingen, Germany) with denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 120 s, and a final extension at 72°C for 5 min. The products were checked by electrophoresis using agarose, then subjected to TOPO TA cloning and blue-white screening according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Positive clones with the correct insert were sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit, followed by capillary electrophoresis in an ABI PRISM® 3100 genetic analyzer according to the manufacturer’s directions (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the clones, excluding the singleton clones in the libraries, as well as those of previous studies (5, 6) from the NCBI database were aligned using the ClustalW program provided in BioEdit (7) and manually corrected. Phylogenetic trees were then constructed with 1,000 rounds of bootstrapping based on the neighbor-joining, maximum-parsimony, and minimum-evolution methods using the MEGA4 software package (21).

Oligonucleotide primer

Primers for the HOPE analysis were designed by the ARB package (15) to target the 16S rRNA gene or ITS1 sequence at either strand with sequence lengths in the range of 18 to 24 nucleotide (nt) (Table 1). If necessary, a homologous (dA) or non-homologous (dATGC) sequence was added to the 5′ end of the primer to change its mobility, allowing for an effective separation by capillary electrophoresis. To ensure the specificity of group-specific primers in the HOPE analysis, the mismatch positions in the sequences of non-targets were located near or at the primer’s 3′ terminus (25). The oligonucleotide primers ≤30 nt were synthesized with OPC purification by Mission Biotech, Taiwan, while those >30 nt were of HPLC grade obtained from Integrated DNA Technologies (Corvalal, IA, USA). The concentrations of oligonucleotide primers were determined spectrophotometrically using a NanoVue™ spectrophotometer (Fisher Scientific, Uppsala, Sweden).

Hierarchical oligonucleotide primer extension analysis

C. raciborskii-related or Cyanobacteria-related DNA amplicons were individually obtained by PCR amplification with the primer set 62F-30R under operational conditions described previously or with the primer set CYA359F-1492R (Table 1) under conditions of 30 cycles of 95°C for 40 s, 55°C for 40 s, and 72°C for 90 s. Prior to the HOPE analysis, the PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). The HOPE reaction was conducted in a solution of 5 μL, containing 1.5 μM each of the unlabeled primers, 5 fmol of the purified PCR amplicons, and 2.5 μL of the premix from the SNAPshot<sup>®</sup> multiplex kit (Applied Biosystems). The SNAPshot premix consisted of AmpliTaq polymerase, salt, buffer and fluorescently labeled deoxy-nucleotides (dROX-ddATP, dTAMRA-ddCTP, dR110-ddGTP and dG-ddTTP). The single-base extension reaction was conducted in a Thermocycler (Biometra) with 20 cycles of 96°C for 10 s, 60°C for 30 s and 72°C for 15 s. Then, 1U of shrimp alkaline phosphatase (Roche Applied Science, Penzberg, Germany) was added and the reaction mixture was incubated at 37°C for 60 min to clean-up unincorporated dye-terminators. This was followed by thermal inactivation of the enzymatic reaction at 75°C for 10 min. Next, the products were subjected to capillary electrophoresis using a

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Table 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Sequence (5’-3’)</th>
<th>Specificity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ddNTP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Used for</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>62F</td>
<td>16S</td>
<td>CGAACGGGATGCTTACATC</td>
<td>C. raciborskii</td>
<td>—</td>
<td>PCR</td>
<td>This study</td>
</tr>
<tr>
<td>30R</td>
<td>23S</td>
<td>CTTGCCTCTGTGCTCAG</td>
<td>Cyanobacteria</td>
<td>—</td>
<td>PCR</td>
<td>(22)</td>
</tr>
<tr>
<td>CYA359F</td>
<td>16S</td>
<td>GGGGAATYTTCGCAATGG</td>
<td>Cyanobacteria</td>
<td>—</td>
<td>PCR</td>
<td>(18)</td>
</tr>
<tr>
<td>1492R</td>
<td>16S</td>
<td>GGTYATCTTGTTCAGCTT</td>
<td>Prokaryote</td>
<td>—</td>
<td>PCR</td>
<td>(13)</td>
</tr>
<tr>
<td>U1390</td>
<td>16S</td>
<td>(ATGC)&lt;sub&gt;30&lt;/sub&gt;</td>
<td>Bacteria</td>
<td>A</td>
<td>HOPE (Set_A)</td>
<td>Modified from (26)</td>
</tr>
<tr>
<td>16S1273</td>
<td>16S</td>
<td>(A)&lt;sub&gt;30&lt;/sub&gt;</td>
<td>C. raciborskii</td>
<td>G</td>
<td>HOPE (Set_A/Set_D)</td>
<td>This study</td>
</tr>
<tr>
<td>T4TWEU</td>
<td>ITS1-L</td>
<td>CTTGTCGCAAGGATGCAG</td>
<td>C. raciborskii</td>
<td>C</td>
<td>HOPE (Set_B)</td>
<td>This study</td>
</tr>
<tr>
<td>T4EU</td>
<td>ITS1-L</td>
<td>CTAACCTCACATTAGAAG</td>
<td>EU group</td>
<td>A</td>
<td>HOPE (Set_B)</td>
<td>This study</td>
</tr>
<tr>
<td>T4TWS</td>
<td>ITS1-L</td>
<td>CTAACCTCACATTAGTAGT</td>
<td>TW &amp; AS/AF groups</td>
<td>T</td>
<td>HOPE (Set_B)</td>
<td>This study</td>
</tr>
<tr>
<td>T6TWEU</td>
<td>ITS1-L</td>
<td>CCTAAATGGGTCTGAGCCA</td>
<td>C. raciborskii</td>
<td>C</td>
<td>HOPE (Set_C)</td>
<td>This study</td>
</tr>
<tr>
<td>T6EU</td>
<td>ITS1-L</td>
<td>CTAGGGTCTTCCATGTTAGT</td>
<td>EU groups</td>
<td>T</td>
<td>HOPE (Set_C)</td>
<td>This study</td>
</tr>
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<td>T6TW</td>
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<td>This study</td>
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<td>ITS1</td>
<td>GACCACTTGGCTTCATTCTTCT</td>
<td>EU&amp;TW groups</td>
<td>G</td>
<td>HOPE (Set_D)</td>
<td>This study</td>
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<tr>
<td>IT5292</td>
<td>ITS1</td>
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<td>AM/AF&amp;AS/TW groups</td>
<td>C</td>
<td>HOPE (Set_D)</td>
<td>This study</td>
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<tr>
<td>ITS54</td>
<td>ITS1</td>
<td>CGCACGAGGCTGATGCTT</td>
<td>EU groups</td>
<td>A</td>
<td>HOPE (Set_D)</td>
<td>This study</td>
</tr>
<tr>
<td>ITS270</td>
<td>ITS1-L</td>
<td>TCTACTGCTGCTGATGCTAC</td>
<td>Some TW groups</td>
<td>T</td>
<td>HOPE (Set_D)</td>
<td>This study</td>
</tr>
<tr>
<td>T6TWEU</td>
<td>ITS1-L</td>
<td>GGTCTGCGGCGCTATTAGC</td>
<td>C. raciborskii</td>
<td>qPCR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> AF, AM, AS, EU, and TW, the groups found in the African, American, Australian, and European continents and in Taiwan.

<sup>b</sup> extended ddNTP (ddATP, ddTTP, ddCTP, ddGTP)
ABI PRISM® 3130 genetic analyzer (Applied Biosystems) with the POP-7™ matrix as described previously (9). Fluorescence data were automatically collected and subsequently analyzed by the GeneMapper (Applied Biosystems). In this study, the HOPE experiment was carried out with 2–5 replicates to ensure reproducibility.

SYBR Green real-time PCR
Quantitative PCR (qPCR) was carried out in triplicate in a LightCycler (Roche Applied Science). The reaction solution (10 μL) contained 4 mM of MgCl₂, 7.5 μM of oligonucleotide primers (Table 1), 1 μL of FastStart DNA Master SYBR green 1 premix consisting of Taq polymerase, dNTP and SYBR green 1 (Roche Applied Science), and the template DNA. The reaction profile as follows: preheating at 95°C for 10 min, quantification including 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, and a melting curve analysis from 72°C to 95°C increasing at a rate of 0.2°C s⁻¹. Fluorescence intensity was monitored at a wavelength of 530 nm during PCR amplification. The clone RYL23 for the primer set T6TWEUf–T6TW and the clone RYL25 for the primer sets T6TWEUf–T6EU and T6TWEUf–T6TWEU (Table 1) were used as reference templates to determine linearity between the copy number and the cycle threshold (Cₜ) from 10⁻¹⁰ copies of template with a series of 10-fold dilutions.

Calculation of abundance and data processing
For relative abundance derived from the SYBR-qPCR method, the absolute numbers of ITS1 copies of specific and total C. raciborskii targets were determined separately, and then converted to percentages, as shown in equation (i):

\[ Relative\ abundance\ of\ the\ target\ (%) = \frac{C_t}{C_{total}} \times 100\% \]  

(i)

Cₜ represents the copy number of the ITS1 fragment of C. raciborskii group A in the sample. C_{total} represents the copy number of the ITS1 fragment of the total C. raciborskii population in the sample.

Relative abundance determined by the HOPE method was calculated based on a formula modified from a previous study (24). The relative abundance of a specific C. raciborskii group with respect to the total C. raciborskii population in the sample can be obtained according to the following equation (ii):

\[ Relative\ abundance\ of\ the\ target\ (%) = \frac{I_s}{I_{max} \times CF_{s,T}} \times 100\% \]  

(ii)

Iₜ represents the fluorescence intensity (peak area) of the group-specific primer extended in the HOPE assay and I_s represents the fluorescence intensity of the C. raciborskii-specific primer. The calibration factor CF_{s,T} is the ratio of fluorescence intensity of the extended group-specific primer with respect to that of the extended C. raciborskii-specific primer, signals for which were obtained using perfect-match reference clones.

Results
PCR amplification of 16S-ITS1-23S rRNA
To analyze the C. raciborskii subpopulations, a C. raciborskii-specific PCR was carried out. As shown in Table 1, the forward primer 62F was designed to target the front of the 16S rRNA gene of C. raciborskii with the reverse primer 30R targeting the front of the 23S rRNA gene of Cyanobacteria (22). Amplification with the primer set specifically retrieved the target fragments, which contained nearly a full-length 16S, all of ITS1 and part of the 23S rRNA gene of C. raciborskii (Fig. 1). The results showed two fragments in the amplified products with sizes of about 1,869 bp and 1,712 bp. The amplicons were then used for further analyses.

Phylogenetic analysis
Two reservoir samples, one from reservoir Renyi in Taiwan and the other from reservoir Taihu in Kinmen, were used for the construction of cloning libraries. Twenty-six and 33 clones with a large fragment of ITS1 (ITS1-L), and 20 and 23 clones with a small fragment of ITS1 (ITS1-S) were obtained from the clone libraries of Renyi and Taihu, respectively, for sequencing. The ITS1-L and ITS1-S sequences possessed 392–393 bp and 235 bp, respectively. The difference in length between ITS1-L and ITS1-S was the sequence conserved for rRNA^{5′} and tRNA^{5′} molecules (5). Among the 102 clones, twenty five with a nearly full-length (1,423–1,427 bp) 16S rRNA sequence were obtained. A homology-based analysis suggested that all were closely affiliated with the species C. raciborskii with sequence identity of 98–100%, suggesting the efficacy of the selective PCR amplification.

To further study the phylogenetic diversity of the C. raciborskii population at the subspecies or strain level, a total of cloned 59 ITS1-L sequences were analyzed. The results
showed that all were closely related to the ITS1-L sequences from members of *C. raciborskii*. Among the 59 ITS1-L sequences, eight clones (one from the Renyi sample and seven from the Taihu sample) present as single copies in the clone libraries were excluded from the phylogenetic analysis. The remaining 51 were classified into eight unique sequence types or sequevars each with at least two copies in the clone libraries. The sequevars obtained in this study together with those from previous studies (5, 6) were subjected to a phylogenetic analysis.

Fig. 2 shows the phylogenetic tree constructed with the *C. raciborskii*-related ITS1-L sequences. The phylogenetic inference showed that the *C. raciborskii* strains from the same continents were closely associated. It was found that one abundant sequevar, RYL25, accounting for 56% of clones in the library of the Renyi sample, was closely related to relatives from the European continent. The other seven sequevars, which were highly divergent, were not assigned to any known *C. raciborskii* strain currently available, and thus designated of the Taiwan (TW) type. Of these, the sequevars THL106 and THL18 appeared predominantly in reservoir Taihu. In addition, as shown in Fig. 2, three sequevars (RYL25, RYL78, and RYL19) from the Renyi sample and three (THL106, THL28 and THL45) from the Taihu sample seemed to be reservoir-dependent, while two sequevars, RYL23 and THL18, were detected in both samples. As a result, the phylogenetic analysis of ITS1-L sequences clearly suggested that diverse *C. raciborskii* strains inhabited the reservoirs Renyi and Taihu.

**HOPE primer design**

A total of 12 HOPE primers were designed to specifically detect *C. raciborskii* and assembled into four sets in the HOPE experiments, as shown in Table 1. Set_A had two primers with specificity for the 16S rRNA gene sequences of *C. raciborskii* (primer 16S1273) and all cyanobacterial populations (primer U1390). Set_B and Set_C each consisted of three primers targeting only ITS1-L sequences, one specific to all types of *C. raciborskii*, one specific to the Australian/African and TW types, and another specific to the European type (Table 1 and Fig. 1). The amplicons of *C. raciborskii*-related and *Cyanobacteria*-related targets from the reservoir samples were individually obtained by the corresponding PCR amplifications. Accordingly, the relative abundance of the 16S rRNA gene of the *C. raciborskii* population with respect to total cyanobacterial populations can be determined by a HOPE analysis with Set_A. The percentages of ITS1-L sequences of the individual *C. raciborskii* groups (species-level) targeted by the specific primers in the total population targeted by the species-level primer can be determined by a HOPE analysis with Set_B and Set_C. Additionally, five primers (Set_D) were used to optimize the experimental conditions. Besides 16S1273, three primers (ITS378, ITS292 and ITS54) targeted the ITS1 sequences of specific *C. raciborskii* populations and one primer (ITS270) detected the ITS1-L sequences of *C. raciborskii* strains found on the American continent. The primers ITS378 and ITS292 can be extended with different fluorescent dye-terminators depending on the target (Table 1).

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**Fig. 2.** A neighbor-joining phylogenetic tree was constructed based on the sequences of 16S-23S ITS1 (large fragment) from *Cylindrospermopsis raciborskii* strains in the NCBI database and the clones that were obtained in this study from reservoirs Taihu (TH) and Renyi (RY). Brackets indicate the source of the strain or number of identical sequences in the clone library. Bootstrap values (• >90%, ○ >70%, ■ >60%) for neighbor-joining, minimum-evolution and maximum-parsimony methods are given at the nodes. The specificity of the primers used in the HOPE analysis is shown at the right of the tree.
Optimization of the HOPE analysis

First, the extension efficiency of the oligonucleotide primers under different annealing temperatures and the separation of multiple primers in a tube in the capillary electrophoresis were evaluated using perfect match (PM) amplicons obtained from the reference strains and clones. Next, the actual specificity of the single-base extension of individual primers was validated against the amplicons with a mismatch (MM). The results showed that all primers used in this study correctly extended with the same nucleotide as predicted in silico for the PM references, while the subspecies-level primers displayed no extension for the MM templates under the experimental conditions. Though their melting temperatures varied, the primers that were arranged in a tube could be effectively extended without comprising the specificity at an annealing temperature of 60°C. The labeled primers could be effectively separated and correctly identified based on the color of the extended dye-terminators in the capillary electrophoresis.

The fluorescence intensity obtained with the ABI PRISM 3130 genetic analyzer was further assessed with the four primers in Set_D and three PM references (clones RYL23, RYL25, and THL45). The experiments were carried out in duplicate individually with three cloned DNA templates on different days. As shown in Table 2, the fluorescence intensity in terms of the peak area for primers ITS378 (a ddGTP extended), ITS292 (a ddCTP extended), ITS292 (a ddTTP extended) and ITS54 with respect to primer 16S1273 was highly consistent (R = 0.99), suggesting that the DNA templates and run-to-run errors were negligible. Hence, the use of the fluorescence intensity as CF values allows for a reproducible quantitative analysis.

Relative abundance of C. raciborskii groups in modeled samples as determined by HOPE

To test accuracy, three samples were prepared with known copies of ITS1-L (clone RYL23 and RYL25) and ITS1-S (clone THS15) fragments and then subjected to the HOPE analysis using Set_B and Set_C. The samples RY2325a, RY2325b and RY2325c were prepared with clones RYL23 and RYL25 equally at the respective copies of 4×10^5, 2×10^6 and 1×10^6. Then, the mixtures were mixed with 2×10^6, 6×10^6 and 8×10^6 copies of clone THS15, respectively. The RYL25 or RYL23 clone, which represented the European and TW types, respectively, accounted for 50% of ITS1-L fragments in the three samples, while the ITS1-S fragment that can not be detected by the primers had no influence on the relative abundance of ITS1-L fragments. The resulting data showed that the HOPE analysis with primers of both Set_B and Set_C could accurately and reproducibly detect the relative abundance of specific ITS1-L targets in the model samples. As shown in Table 3, the relative abundances of clone RYL23 (TW type) and RYL25 (European type) in the ITS1-L pools was 47.5–57.4% and 44.2–51.8% (SD<9.8%), respectively with coverage of around 96.5–102.7%. Also, little significant difference occurred between the data sets obtained with the two sets of primers (Student’s t-test, P=0.7918).

Relative abundance of C. raciborskii groups in reservoir samples as determined by HOPE and SYBR-qPCR

The HOPE assay with Set_B and Set_C was then used to investigate the relative abundance of specific C. raciborskii populations in environmental samples from the reservoirs Taihu and Renyi. As shown in Table 3, for the sample RY0406 from the reservoir Renyi, the HOPE analyses with both primer sets that the European-related sequevars accounted for 95.3–96.5% (SD<6.5%) in relative abundance, which was slightly higher than that detected with Set_C by about 11.1%. Unlike the distribution in the reservoir Renyi, the relative abundance of the European-related C. raciborskii group was much lower than the percentage of the African/Australian/TW-related counterparts in the reservoir Taihu. For the two samples, TH0418 and TH0509, from the reservoir Taihu, it was observed with both primer sets that the European-related sequevars occupied a low fraction (3.8–3.9%, SD<0.3%) in the total C. raciborskii population. The relative abundance of African/Australian/TW-related groups detected with Set_B accounted for 66.5±2.8%, which was slightly higher than that detected with Set_C by about 11.1%.

Table 2. Fluorescence intensity of the dye-terminator-tagged primers analyzed by capillary electrophoresis

<table>
<thead>
<tr>
<th>Template</th>
<th>Intensity of ITS1-specific primers with respect to primer 16S1273 (average percentage ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITS378 (G)</td>
</tr>
<tr>
<td>RY23 (n=5)</td>
<td>86.8±2.8</td>
</tr>
<tr>
<td>TH45 (n=4)</td>
<td>86.0±4.1</td>
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<tr>
<td>RY25 (n=4)</td>
<td>87.3±1.0</td>
</tr>
</tbody>
</table>

ND, not detected

Table 3. Relative abundance of specific ITS1-L fragments related to Cylindrospermopsis raciborskii populations in modeled and environmental samples analyzed by HOPE and qPCR

<table>
<thead>
<tr>
<th>Samples</th>
<th>HOPE, Set B</th>
<th>HOPE, Set C</th>
<th>SYBR-qPCR</th>
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<tbody>
<tr>
<td></td>
<td>TW</td>
<td>EU</td>
<td>Sum</td>
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<tr>
<td>RY2325a</td>
<td>48.9±2.3</td>
<td>47.6±1.4</td>
<td>96.5</td>
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<tr>
<td>RY2325b</td>
<td>52.3±2.4</td>
<td>44.2±1.0</td>
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<tr>
<td>RY2325c</td>
<td>57.4±6.2</td>
<td>45.3±6.4</td>
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<tr>
<td>TH0418</td>
<td>66.5±2.8</td>
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</tr>
<tr>
<td>TH0509</td>
<td>96.5±6.3</td>
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<td>100.3</td>
</tr>
</tbody>
</table>

Average±standard deviation (n=3–5)
For comparison, the DNA amplicons used for the HOPE analysis were properly diluted and subjected to quantitation using the SYBR-qPCR method. For the SYBR-qPCR experiment, one forward primer specific to *C. raciborskii* ITS1-L sequences (i.e., T6TWEUf, Table 1) was designed to pair individually with the three reverse primers in Set C. As a result, the relative abundance of African/Australian/TW and European-related groups in the total *C. raciborskii* population could be calculated in accordance with the copy numbers of corresponding ITS1-L fragments measured independently [see Materials and Methods, equation (i)]. As shown in Table 3, the resulting data showed that the relative abundance of African/Australian/TW- and European-related *C. raciborskii* groups was 47.5±4.1% and 45.9±4.8% with a sum of 93.4%, very close to the results obtained using the HOPE assay. For the two samples from the reservoir Taihu, 63.2–63.6% (SD<5.6%) and 4.1–4.4% (SD<0.6%) in relative abundance were accounted for the population sizes of the African/Australian/TW- and European-related *C. raciborskii* groups, respectively. The detection of relative abundance using the SYBR-qPCR and HOPE methods coincided well with each other, except that the SYBR-qPCR method detected a lower percentage for the African/Australian/TW-related *C. raciborskii* group in the reservoir Taihu by about 23.9–25.4%, as compared to the HOPE analysis. This result suggests excellent performance of HOPE with fewer primers than needed in qPCR for determining the relative abundance of microbial targets in a community.

**Qualitative and quantitative HOPE analysis of specific *C. raciborskii* groups in the reservoir samples**

Another two and seven DNA samples having the *C. raciborskii* cells observed in water were collected from eutrophic reservoirs in Taiwan and Kinmen from 2006 to 2008. These samples were then subjected to PCR amplification to retrieve total 16S rRNA gene fragments of *Cyanobacteria* and 16S-ITS1-23S rRNA gene fragments of *C. raciborskii*, respectively. Subsequently, the purified PCR amplicons were analyzed by optimized HOPE assays.

It was noted that based on the ITS1-L sequences, the primer to discriminate the TW-related sequevars from the Australian/African group was unavailable. However, one polymorphic primer (ITS378) targeting the ITS1 sequences could be designed with a ddGTP extended for the Eurasian groups and with a ddATP extended for the Australian/African groups (Table 1). The result of genotyping with primer ITS378 suggested that majority of the *C. raciborskii* populations in all samples tested were associated with the Eurasian types. However, a very small fraction of *C. raciborskii* populations (Fig. 3) might be associated with the Australian/African strains. In addition, testing with the primer ITS270 validated the absence of American strains in the samples tested in this study.

Further, HOPE with Set_B was used to profile the relative abundances of ITS1-L sequences related to the European and Australian/African/TW groups with respect to the total *C. raciborskii* population, while the primers in Set_A would detect the relative abundance of the 16S RNA gene related to the species *C. raciborskii* with respect to the total cyanobacterial population. As shown in Fig. 4a, the species *C. raciborskii* grew in a broad range of relative abundance in the cyanobacterial community. The percentages of *C. raciborskii*-related 16S rRNA gene fragments ranged from 0.5±0.3% to 3.7±0.3% with respect to total cyanobacterial 16S rRNA gene fragments in the samples of Taihu in Kinmen, which shared the approximate abundances observed in
the reservoirs Mudan and Lantan (2.4–2.7%, SD<1.2%) in Taiwan. Moreover, the *C. raciborskii* population proliferated in a similar abundance (3.5±1.0%) in the winter of 2007 and increased to a higher percentage (24.1±1.4%) in the spring of 2008 in the reservoir Jinsha in Kinmen. In a nearby reservoir Yangmin, it was observed that the species thrived with a percentage of up to 62–76.4% (SD<6.5–7.7%) in the total cyanobacterial population during the period. These findings suggested that the size of the *C. raciborskii* population varied greatly in the cyanobacterial communities and could be the predominant cyanobacterial species in the reservoirs.

As shown in Fig. 4b, the HOPE analysis further revealed the distinct distribution of *C. raciborskii* subspecies in the reservoirs. The European and Australian/African/TW groups detected by primers T4EU and T4TW accounted for about <6.2% (SD<0.8%) and 95.0–100.8% (SD<6.5%) of the total *C. raciborskii* population, respectively in reservoirs such as Taihu, Yangmin, and Jinsha in Kinmen as well as in the reservoir Mudan in Taiwan. On the other hand, the percentage of European-related sequevars in the reservoir Lantan in Taiwan was 45.8±0.2%, slightly lower than that (58.1±1.1%) of the *C. raciborskii* groups associated with the Australian/African/TW relatives. This shared a similar profile to that observed for the sample from the reservoir Renyi (Table 3).

**Discussion**

The HOPE method showed excellent sensitivity and specificity for detecting the relative abundance of multiple 16S rRNA gene fragments in PCR amplicons (24). The approach has been applied to the qualitative and quantitative analyses of functionally important indicators such as *Bacteroides*, *Parabacteroides* and *Bifidobacterium* spp. in faeces and domestic wastewater, and successfully discriminated faecal contamination of human, swine and bovine origins (9–11). Also, this technique was used for the genotyping of various *Dehalococcoides* groups in groundwater samples (17). So far, the HOPE method has solely been applied to the 16S rRNA gene as a biomarker for the analysis of microbial community structures at the species level or higher. In this study, we further developed the HOPE assay to study cyanobacterial communities and demonstrated that it was able to correctly profile the ITS1-L fragments of various *C. raciborskii* groups and to accurately detect the relative abundance of corresponding targets at the subspecies level.

A number of hypotheses have been proposed to elucidate the observed phylogeography and dispersal of *C. raciborskii* populations worldwide (1, 5, 16, 19). Gugger and co-workers (5) observed that *C. raciborskii* populations were expanding from warm areas towards higher latitudes on the European continent. Based on the ITS1-L sequence between 16S-23S rRNA genes, which was considered an ideal biomarker for grouping the *C. raciborskii* strains, it was hypothesized that the origin of the spreading European strains could be warm areas on the Eurasian continent. In this study, the result of the phylogenetic analysis was consistent with Gugger’s observation that the strains or sequevars obtained on each continent clustered together in the phylogenetic tree. In particular, an interesting finding showed that one abundant *C. raciborskii* sequevar (RYL25) was phylogenetically affiliated with the cluster originating from the European continent. Although no corresponding ITS1-L sequence was identified in the reservoir Taihu, the HOPE analysis suggested that the sequevar similar to the European strains commonly inhabited the reservoirs. Consequently, given the island geography at the east edge of the Eurasian continent, this result, for the first time, provides corroborative support of the genetic homogeneity between the European and Asian *C. raciborskii* populations.

In addition to the relatives of the European strains, the result suggested that at least another seven *C. raciborskii*-like sequevars were present in the reservoirs Renyi and Taihu. As shown in Fig. 2, the designated TW sequevars were hardly affiliated with known strains and were branched distantly from one another as well. These two *C. raciborskii*-like groups occupied the majority of the clone libraries of ITS1-L sequences. On the other hand, despite a lack of the relevant sequence found in the clone libraries, the HOPE genotyping with primer ITS378 could still qualitatively detect the *C. raciborskii* group associated with the African/Australian strains (Fig. 3). This clearly suggested the prevailing sensitivity, as the HOPE method could detect a microbial population down to 0.1–0.5% of relative abundance in a community without difficulty (24). However, the concurrence of European and African/Australian *C. raciborskii* sequevars at the same location may be in contradiction to our understanding of the phylogeography of the *C. raciborskii* strains (5). To clarify this, African/Australian-related sequevars or strains obtained outside the African/Australian continents would be needed. Overall, through the culture-independent approach, the results clearly suggested a high degree of subspecies diversity within the *C. raciborskii* population in the reservoirs in Kinmen and Taiwan.

The population size varied substantially among the three *C. raciborskii* groups and differed geographically, too. As shown in Fig. 3, it was reasonably assumed that the numerical contribution of African/Australian sequevars to the size of the total *C. raciborskii* population might be negligible due to very low percentages. As a result, the sum of population size of the European- and TW-related sequevars could roughly complement the total *C. raciborskii* population. As shown in Fig. 4b, the profile of *C. raciborskii*-like TW sequevars was distributed in high percentages, often reaching over 95.0% of the total *C. raciborskii* population. It was noted that this numerical predominance was relatively stable across the four reservoirs (Taihu, Yangmin, Jinsha and Mudan) and was independent of the size of species *C. raciborskii* in cyanobacterial communities. Likely, the stable predominance might be attributed to the high degree of sequevar richness in the ecosystems, since at least seven relevant sequevars were retrieved in this study. Although only one European *C. raciborskii* sequevar was found in the clone libraries, the population still proliferated with a size near the abundance of the TW counterparts in the reservoirs Renyi and Lantan. Hence, the two groups appeared to compete strongly with each other, resulting in the varied population sizes. Yet, it is not clear why the water environment of reservoirs Renyi and Lantan favored the growth of European strains. Conditions like water temperature and light requirement may not be satisfactory to explain...
its success, because the reservoir Mudan is located at a lower latitude but the *C. raciborskii* population shared a subspecies structure similar to those distributed in Kinmen. To further interpret the ecological dominance and the competition of *C. raciborskii* groups in a single reservoir, more studies regarding physiology and toxin production, as well as seasonal dynamics with a link to water quality should be implemented.

The performance of HOPE and SYBR-qPCR methods in analyzing the relative abundance of specific targets in the actual samples was compared. Technically, quantification of the SYBR-qPCR method would require one additional primer, as compared with the HOPE method in this study. It was observed that a discrepancy occurred for the relative abundance of the *C. raciborskii*-like TW sequevars in the reservoir Taihu, in which values lower by 23.9–25.4% were determined using SYBR-qPCR than using HOPE (Table 3). The inefficacy of SYBR-qPCR was likely ascribed to the specificity of the forward primer and a stringent thermal condition (annealing temperature 60°C) applied in the SYBR-qPCR experiment, as well as the highly varied characteristics of TW-related ITS1-L sequences. Other than this, the results in fact showed that the relative abundances determined by the HOPE and SYBR-qPCR methods were highly consistent (Table 3). Since a quantitative analysis based on the qPCR approach would require standard curves to be established, it would be very time-consuming and labor-intensive for determining relative abundances of multiple targets in environmental samples. This weakness actually underlined the niche of the HOPE approach with high-multiplexing strength in a microbial community analysis.

In conclusion, the culture-independent approach demonstrated in this study elaborately looked into the diversity and geographic distribution of *C. raciborskii* subspecies in a quantitative manner. With the advantages of being high-throughput, flexible, rapid and easy-to-perform, the HOPE assay can be used to monitor various *C. raciborskii* populations in water ecosystems on a temporal or geographical basis for future studies.

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**References**


