Title:

ThermoPhyl: a software tool for selecting phylogenetically optimized conventional and quantitative-PCR taxon-targeted assays for use with complex samples.

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Abstract

The ability to specifically and sensitively target genotypes of interest is critical for the success of many PCR-based analyses of environmental or clinical samples that contain multiple templates. Next-generation sequence data clearly show that such samples can harbour hundreds to thousands of operational taxonomic units; a richness which precludes the manual evaluation of candidate assay specificity and sensitivity using multiple sequence alignments. To solve this problem we have developed and validated a free software tool which automates the identification of PCR assays targeting specific genotypes in complex samples. ThermoPhyl uses user-defined target and non-target sequence databases to assess the phylogenetic sensitivity and specificity of thermodynamically optimised candidate assays derived from primer design software packages. ThermoPhyl takes its name from its central premise of testing **Thermo**dynamically optimal assays for **Phyl**ogenetic specificity and sensitivity and can be used for two primer (traditional PCR) or two primers with an internal probe (e.g. TaqMan® qPCR) applications and potentially for oligonucleotide probes. Here we describe the use of ThermoPhyl for traditional PCR and qPCR assays. PCR assays selected using ThermoPhyl were validated using 454 pyrosequencing of a traditional specific PCR assay and with a set of four genotype-specific qPCR assays applied to estuarine sediment samples.

Introduction

A basic task for many environmental and clinical researchers is to target a specific genotype in a complex sample to understand, for example, the abundance of a particular taxon or the expression of a particular gene. For conventional PCR, users have traditionally designed PCR assays manually, starting with a visual comparison of alignments of multiple sequences. This approach is at best laborious and, even when a phylogenetically optimal assay (i.e. maximally sensitive and specific) can be identified, empirical tests are time-consuming and can still produce poor PCR results. Software is available that can facilitate primer and probe design and analysis but none of these completely meet the needs of designing PCR assays to detect target clades specifically. In particular, there is no software presently available to the community that can assess the probe and primer sets required for quantitative-PCR (qPCR) assays using an internal probe, the most accurate and specific qPCR technique. Given the wealth of sequence data rapidly accumulating from high-throughput sequencing and the diversity such methods are detecting in environmental and clinical samples (Sogin *et al.*, 2006; Acosta-Martinez *et al.*, 2008; Andersson *et al.*, 2008; Biddle *et al.*, 2008; Dowd *et al.*, 2008; Hamady *et al.*, 2008), it is clear that high-throughput PCR primer and probe design approaches are required to specifically and sensitively target genotypes in a complex sample.

The software we describe here, ThermoPhyl, exploits the output of proprietary software that can produce large numbers of thermodynamically optimised candidate assays for PCR and qPCR (e.g. PrimerExpress (Applied Biosystems (ABI), Warrington, UK), BatchPrimer3 (You *et al.*, 2008)) and subsequently assessing each individual assay for sensitivity (proportion of target group perfectly matched) and specificity (number of non-target organisms perfectly matched) to user-defined target and non-target sequences in a local database. ThermoPhyl is built around a simple pattern-matching script and is designed for applications in which a user wishes to target a taxonomic group of interest in a complex sample. The name ThermoPhyl derives from its central goal which is to test thermodynamically optimal PCR assays for phylogenetic sensitivity and specificity to arrive at an assay which is both **thermo**dynamically and **phyl**ogenetically optimal. ThermoPhyl is designed to analyse a very large number of candidate assays simultaneously and to facilitate primer and probe set choice by providing an output that clearly summarises the specificity and sensitivity of each candidate assay.

ThermoPhyl complements several software tools currently available such as NCBI Primer Blast, probeCheck (Loy *et al.*, 2008) and ARB (Ludwig *et al.*, 2004) which meet a range of user needs related to determining the phylogenetic specificity of targeted primers and probes. ThermoPhyl performs the high-throughput assessments of assays with one (e.g. FISH), two (conventional PCR), or three (e.g. TaqMan® qPCR) oligonucleotides, which no other available software can perform. Additionally, ThermoPhyl is installed locally which allows users to utilize personal databases and fully control processing speed and throughput. ThermoPhyl can efficiently harness the power of large datasets by rapidly performing a large number of comparisons which are summarized in output sorted by specificity and sensitivity.

In this paper, we describe the rationale behind and the use of ThermoPhyl, compare it to other commonly used primer PCR primer and probe design and assessment programmes and validate its use in the design of both conventional PCR and qPCR assays. For conventional PCR we used ThermoPhyl to select a PCR primer set to amplify the *mcrA* gene of the methanogenic archaeal genus *Methanosaeta* and experimentally tested its specificity and sensitivity using pyrosequencing. For qPCR we used ThermoPhyl to select four *Desulfobulbus* genotype-specific qPCR assays and compared the data produced by this analysis with existing data. The program, a user's manual, and a training dataset are made freely available to the research community at: http://go.warwick.ac.uk/thermophyl.

Materials and Methods

Program validation: Traditional PCR primer set

Sequence Database and Target Group Definition. Using *mcr*A sequences from the functional gene pipeline/repository (http://fungene.cme.msu.edu/), a database was constructed in ARB (Ludwig *et al.*, 2004). Based on maximum-likelihood phylogenetic reconstructions, a monophyletic clade containing 19 *Methanosaeta* sequences, including sequences from the characterised three isolates *M. concilii*, *M. harundinacea*, and *M. thermophila* was identified as a target group. BatchPrimer3 (You *et al.*, 2008) was used with default parameters to design 50 primer pairs for each of these target sequences with amplicon lengths set between 400 and 500 bp.

After comparison with the target sequences two primer pairs ranked highly by ThermoPhyl, F1-1044 (5'-CTACATGTCCGGYGGTGTC-3') and R1-1507 (5'-TAGTTRGCGCCYCTCAKCTC-3'), and F2-1060 (5'-GTCGGWTTCACMCAGTACGC-3') and R2-1470 (5'-TGCCCTCGTCKGACTGGTA-3'), were chosen for empirical testing after the inclusion of several degeneracies based on a manual comparison to the sequence database.

Primer assessments. Primer specificity, sensitivity, and amplification efficiency were evaluated empirically using genomic DNA from *M. concilii* (DSM6752), *M. harundinacea* (DSM 17206), *M. thermophila* (DSM4774), *Methanosarcina mazei* (DSM2053), *Methanosarcina acetivorans* (DSM2834), and environmental DNA from sediment in the Colne Estuary, U.K. DNA was extracted from actively growing cultures using the DNAeasy Blood and Tissue kit (Qiagen, Crawley, UK) and environmental DNA was extracted from sediment samples representing marine and freshwater conditions (Sites 1 and 10 in Oakley *et al.* (2010)) as previously described (Purdy *et al.*, 1996; Purdy, 2005). The first round of PCR was performed in 25 µl volumes containing 1X EpiCentre FailSafe Master Mix G (EpiCentre, Madison WI, USA), 600 nM each primer F1 and R1, and 1.25 U EpiCentre FailSafe Enzyme Mix. Touchdown PCR was performed with an initial denaturation of 96°C for 2 min followed by 10 cycles of 94°C for 30 sec, 52°C deg (-1°C/cycle) for 30 sec, 72°C for 40 sec, followed by 22 cycles with 42°C annealing. Because of low amounts of

Methanosaeta DNA present in the environmental samples, 1 µl of these PCR products was used in a second round of PCR containing 1X Promega PCR buffer, 2.5 mM $MgCl_2$, 10 µg BSA, 600 nM each primer F2 and R2, 200 nM dNTPs, and 1 U Promega Taq polymerase. Thermal cycling consisted of 96°C for 2 min, 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 40 sec, and a final extension at 72°C for 10 min. Triplicate PCRs from each of three biological replicates (sediment samples taken within 50 cm at each site (Hawkins & Purdy, 2007)) were cleaned using the QiaQuick Gel Extraction kit (Qiagen) and pooled after normalization based on quantification of PCR products using QuantIt PicoGreen (Invitrogen, Paisley, UK) as per manufacturers instructions and fluorescence measured with a Perkin-Elmer Wallac Victor^2 1420 plate reader.

Pyrosequencing methods. Pyrosequencing was performed at Research and Testing Laboratory (Lubbock, Texas, USA: http://www.researchandtesting.com) using tagged amplicon methods similar to those described previously (Dowd *et al.*, 2008) modified for titanium chemistry (Roche, Indianapolis, USA). In short, concatamer primers were synthesized using the construct "5' 454TitaniumLinkerA-tag-primer 3'" where the 454 linker A was biotin labelled and based upon Roche amplicon sequencing Titanium Linker A, the tag was a random 10mer (GC content 40-60%) and utilized to bin out sequences resulting from a specific sample and the primers used for this study. The reverse concatenate was in the format "5' 454TitaniumLinkerB-primer" where the primer was the appropriate reverse primer for the reaction. 20 cycles of PCR were utilized (94°C for 30 sec, 50°C for 30 sec, 72°C for 40 sec) with a final extension at 72°C for 10 min to incorporate the linkers and tag. Pyrosequencing based upon titanium bulk sequencing methods was utilized based upon manufacturers' protocols by introducing the amplicon into the steps in the protocol following library creation. A 200 flow Titanium sequencing run was performed according the Roche protocols with amplicon signal processing. Following the sequencing and image processing, the sequences were binned out based upon tag sequence into individual multi-fasta files and used for data analysis.

Pyrosequence data analysis. Raw sequence data were edited using a series of custom Perl and Bioperl scripts which performed the following initial steps: Trimming of pyrosequencing tag sequences, removal of sequences with one or more ambiguous base calls, and removal of sequences shorter than 410 bp. Sequences were screened for the presence of both forward and reverse primer sequences and then translated in all three forward frames and screened for the presence of a conserved motif ('VGF') within the forward primer region; translated sequences without stop codons and no more than one unknown amino acid passed the screen and leading and trailing nucleotides were trimmed to complete codons in the appropriate frame. A total of 1745 sequences from site 10 (out of 7661) and 4517 sequences from site 1 (out of 10874) passed all screens. Minimum, median, and maximum sequence lengths were 411, 415, and 419 bp respectively.

To determine the identity of sequences, a Blastp analysis was performed by querying each translated sequence against a custom database of 44 *mcr*A reference sequences from pure cultures including all known *Methanosaeta* strains. The only changes to default Blastp parameters were the use of soft-masking (-F "m S") to enable filtering for low-complexity subsequences during the word seeding phase but not the extension phase of the Blastp algorithm.

Sequences were aligned with Muscle (Edgar, 2004) invoked from a Bioperl shell which first appended an anchoring oligo sequence (5'-ACCACACAAAAACCCACA-3') to both the 5' and 3' ends of the alignment and then randomly split all sequences into subsets of 1000 sequences and aligned these first to each other, then to a single reference sequence from *M. concilii* (AF313802). Each alignment of 1000 sequences was then appended to the previous, and finally, the entire alignment aligned as a profile to the reference sequence. Alterations to default Muscle parameters were gap-open and gap-extend penalties of -500. This alignment strategy was arrived at empirically by optimization of a training data set of pyrosequencing data derived from a clonal *M. concilii* sample and resulted in significantly increased accuracy and reduced computational time (ca. 1 hr vs. 66 hrs) relative to default command line invocation of Muscle (data not shown).

Distance matrices were made with Phylip (Felsenstein, 1989) using the Kimura 2-parameter model and sequences were grouped into operational taxonomic units (OTUs) using the furthest-neighbour method of DOTUR (Schloss & Handelsman, 2005). Perl was used to generate a custom import filter to incorporate

sequences into ARB (Ludwig *et al.*, 2004) including a data table of sequence membership for each OTU. Phylogenetic trees were built with maximum-likelihood algorithms in ARB using representative sequences from each site for the 27 OTUs identified by a 20% sequence dissimilarity cut-off conservatively based on a pairwise nucleotide difference between *M. concilii* and *M. harundinacea* of 25% for the amplicon region.

Program validation: qPCR methods

Probe and primer sets were designed using PrimerExpress (ABI) which outputs Taqman probe and primer sets that should function effectively using ABI's standard qPCR conditions. Multiple potential assays were derived from all of the target sequences that were available and all candidate assays tested using ThermoPhyl.

Quantitative-PCR assays were performed using Applied Biosystems TaqMan® gene expression master mix with MGB probes as described previously (Oakley *et al.*, 2010). Primer and probe sequences for assays M, Mh, FW1, and FW2 are listed in Table 2. In brief, PCR conditions were as per manufacturer recommendations on an ABI 7000 or ABI 7500. Each assay was optimized with titrations of primer and probe and the sensitivity and specificity validated with plasmids from representative target and closely related non-target clones. Optimized assays contained 300 nM (600 nM for FW2) each of the forward primer and reverse primers, 200 nM MGB probe and 1X ABI GeneExpression Master Mix in 25 µl reactions. Assays were considered valid when all no-template controls were negative, calibration-curve R^2 values were >98%, and amplification efficiency was between 90% and 115%. All replicates from each sampling site were run on a single plate for consistency. Assays have been utilised and analysed previously in Oakley *et al.* (2010).

Results and Discussion

Program structure and function

Conventional methods of PCR primer design for the analysis of complex communities rely on an initial visual comparison of sequence alignments and then a painstaking comparison of potential primers. While programs like ARB and Primer Blast can perform these functions and do produce good PCR primers and probes performing comparisons between different primer sets is laborious and does not guarantee success. Furthermore, designing a phylogenetically coherent, thermodynamically optimised Taqman-like qPCR assay is simply not possible in any of the presently available programs. Primer design programs can produce large numbers of thermodynamically optimised primer (Primer3, www.sourceforge.net) or primer/probe (PrimerExpress, ABI) sets but only do so for individual sequences and so cannot produce assays that specifically target only the members of a clearly defined clade without the need for very laborious comparisons of individual candidate assays. ThermoPhyl fills this gap by comparing the output of high quality primer design software to sequence data derived from a coherent phylogeny. A flow diagram showing the steps required to use ThermoPhyl is given in Figure 1. ThermoPhyl is a simple pattern-matching Perl script that compares primers and probes to two user-defined datasets, the “target group” and the “non-target group”. For any number of possible primer/probe sets ThermoPhyl determines assay sensitivity, that is how many of the “target group” are a perfect match for each primer/probe set, and specificity, how many of the “non-target group” are a perfect match for the primer/probe sets, for each individual assay. ThermoPhyl then outputs a “sorted” assay file detailing assays in order of the highest sensitivity and specificity first (see figure 2a). It also outputs a “raw-data file” showing which members of the “target group” and “non-target group” matched with each assay (Figure 2b). From this output it is possible to determine whether the addition of degeneracies in the primers might improve assay sensitivity, although such changes may adversely affect the assay as a whole.

Program requirements

ThermoPhyl, a freeware Perl script programme (download Perl from http://www.activestate.com/activeperl), is a simple matching programme that runs on both windows and Unix machines. In tests on moderately fast WinXP machines (e.g. 2 GHz Pentium CPU with 3 Gb of RAM), testing 5,000 candidate qPCR assays against a database with 5,000 taxa, makes the required 25 million comparisons in about 2.5 hrs. However, most users will have many fewer comparisons than this and for most applications ThermoPhyl generally produces output in seconds to minutes.

To run ThermoPhyl, three input files are required. First, a fasta file which contains all of the desired target and non-target sequences, all with unique names, thought to be present in the samples of interest. This file should contain as many representative sequences as possible (typically 100 to 50,000 depending on the application) to maximize confidence in distinguishing between target and non-target groups. Because many public databases (e.g. GreenGenes (DeSantis *et al.*, 2006) and Silva (Pruesse *et al.*, 2007) for 16S rRNA genes) now contain many very similar sequences; users may want to reduce these databases to representative sequences.

The second file required is a text file containing only the names of the target sequences. The names must correspond exactly to those in the fasta file above and should be unique, such as a GenBank accession number.

Finally, a list of candidate assays based on the target sequences must be provided. These can be produced by a number of primer design programs. For traditional PCR, BatchPrimer3 (You *et al.*, 2008), http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi, can provide specific candidate assays for a number of different target sequences from a single fasta file and is a good high-throughput solution to creating candidate assays to test. For qPCR, software such as ABI's PrimerExpress can quickly generate a list of candidate assays to test and so allow the use of standardised protocols for qPCR. Using these approaches, we have typically generated 50 candidate assays per target sequence which are then compiled into a single tab-delimited text file, the candidate-assay file.

Comparison of ThermoPhyl to other primer analysis programmes

Several programmes have been developed to allow users to design or assess probes and primers to determine whether they are specific and sensitive. However,

ThermoPhyl was designed when it became apparent that there was no software available that was designed to assess whether a Taqman-like qPCR probe/primer set was specific and sensitive. To highlight the differences and similarities between these programmes ThermoPhyl is compared to several other commonly used programmes in Table 1. The primary advantages ThermoPhyl has over all the other programmes is that it is the only programme that is capable of assessing qPCR probe/primer sets and that is designed to assess very many candidate assays at one time. The other programmes all have additional limitations which makes ThermoPhyl a valuable tool in the design of probes and primers. One of ARB's strengths is its oligonucleotide probe design capability, which it does with reference to its sequence database and allows the user to visually assess the newly designed probes. However, ARB's PCR primer design is much more limited. It utilises just a target single sequence to produce candidate assays and offers no simple possibility of assessing these primer sets against the database. Primer Blast also designs PCR primer sets (using Primer3) and, like ARB, this is presently limited to using a single target sequence. Primer sets are then compared to a choice of databases, including the whole nucleotide database but with a strong recommendation to use non-redundant databases such as Refseq RNA. The user can set the target clade, however, this is then entirely dependent on the GenBank taxonomy to define the target clade and there is no option to use a user-defined database. Primrose does allow the use of user defined databases, although it is designed to work with the RDP database. Its major drawbacks are that it only designs oligonucleotides not PCR primer sets and each oligonucleotide needs to be individually assessed against the database to determine exactly what it matches, which is time-consuming. Therefore, after a search for potential oligonucleotides potential primer sets will need to be further assessed for thermodynamic suitability. Finally probeCheck is designed to assess previously designed oligonucleotides but does so individually and not as a primer set. Therefore, ThermoPhyl, along with programmes such as PrimerExpress and BatchPrimer3, allows the very rapid design and assessment of very many thermodynamically optimised PCR and qPCR assays against any dataset that the user chooses.

Validation of ThermoPhyl-selected PCR and qPCR assays

Validation of a ThermoPhyl selected conventional PCR assay. As the first empirical test of ThermoPhyl, conventional PCR primers specifically targeting the α-subunit of the methyl-coenzyme M reductase gene (*mcrA*) of *Methanosaeta* were selected following the scheme set out in Figure 1. Over 700 candidate primers sets were designed using BatchPrimer3 from a range of available *Methanosaeta mcrA* sequences from both isolates and environmental clone sequences. As this particular taxon generally represents a small proportion of DNA in the estuarine sediments analysed here a nested PCR approach was adopted and so two primer sets were selected. After routine manual optimization of thermal cycling conditions, the primers selected by ThermoPhyl produced a strong single band from both genomic DNA prepared from the three available *Methanosaeta* isolates (*M. concilii*, *M. harundinacea* and *M. thermophila*) and environmental DNA preparations but did not amplify DNA from the closely related *Methanosarcina mazei* or *Methanosarcina acetivorans* (Figure 3).

Using these nested primer sets *Methanosaeta*-specific PCR products were amplified from DNA extracted directly from the two contrasting environmental sediment samples (marine dominated Site 1 and freshwater-dominated Site 10) from the River Colne, Essex, UK (Hawkins & Purdy, 2007; Oakley *et al.*, 2010). These amplicons were analysed using only a small proportion (~1%) of a 454 pyrosequence read. After screening to remove poor quality sequences as described above/below 6,262 high-quality sequences remained (4,517 sequences from Site 1 and 1,745 sequences from Site 10). These sequences were checked using a local Blastp analysis to a database of 44 *mcrA* sequences and across the 2 sampling sites 99.9% (6,257/6,262) of sequences were most closely related to *Methanosaeta* (Table 1). Rarefaction analysis of these sequences showed that, at a sequence dissimilarity of 20% (the difference between the *mcrA* genes of the two mesophilic *Methanosaeta* isolates, *M. concilii* and *M. harundinacea*, is 25% therefore 20% is a reasonable species-level definition) the *Methanosaeta* community at both sites have been completely sampled (Figure 4). Pyrosequencing of these environmental amplicons also revealed extensive novel diversity within the *Methanosaeta* clade (Figure 5). Twenty-seven OTUs were defined at a 20% cut-off, all falling within the *Methanosaeta* clade, yet many clearly represent novel lineages affiliated with

Methanosaeta. Therefore, these nested PCR primer sets are both specific and sensitive and show the value of using ThermoPhyl in primer selection.

Validation of ThermoPhyl selected qPCR assays. The second validation test was to use ThermoPhyl to select Taqman qPCR primer and probe sets, designed using PrimerExpress (ABI), that targeted four *Desulfobulbus* genotypes detected in the Colne estuary, UK (data presented previously in Oakley *et al.*, 2010). The four *Desulfobulbus* clades were selected for qPCR analysis because they exhibited a differential distribution along the estuary based on an initial DGGE analysis, a distribution that was subsequently supported by clone sequence data (Figure 6). Candidate assays were designed to target each of the four genotypes, specificity and sensitivity of these assays was determined using ThermoPhyl and the best assays selected. Data from these four assays supported our previous data showing all four genotypes have a restricted distribution along the estuary (Figure 6) indicating that the assays were targeting the correct genotypes. However, this data does not prove that the assays are specific. It can be reasoned that a "good" qPCR assay should produce PCR products that, if sequenced and analysed phylogenetically, should produce a monophyletic clade, with the caveat that as qPCR assays usually produce very short fragments the resultant trees are unlikely to be very robust. Therefore, we cloned and sequenced ~12 amplicons for each assay and all four assays produce monophyletic groups after sequence analysis (data not shown). Therefore, ThermoPhyl was successful in selecting highly specific and sensitive qPCR primers and probes from a large number of thermodynamically optimised candidate assays.

These two validation tests show that ThermoPhyl is capable of analysing large numbers of potential PCR and qPCR assays for specificity and sensitivity using a user-defined sequence database and thus will allow the user to make a phylogenetically informed choice about which primer sets to use for a specific target group. This is particularly powerful with qPCR assays as no presently available phylogenetic program is capable of assessing the validity of even a single qPCR primer/probe set let alone many hundreds of candidate assays. Therefore while ThermoPhyl is in itself a simple pattern-matching program it fills a gap in the available software by linking a wholly user-defined dataset to powerful PCR and qPCR primer design software.

Potential pitfalls and specific recommendations

To use ThermoPhyl effectively, target groups must form a natural phylogenetic group. Prior to using ThermoPhyl sequences should be properly placed in some sort of a phylogenetic tree to evaluate this and to designate target and non-target sequences in a way which reflects the evolutionary history of the gene in question. If the target sequences do not form a coherent phylogenetic group, it will be difficult to design an accurate assay, although it is possible that different sequence data (e.g. another gene) for the same taxa could still be used in such a case.

Additionally, the more sequence data available for both target- and non-target-groups, the better. The strength of ThermoPhyl, in fact its central goal, is to summarize a very large number of comparisons to arrive at a single 'best' assay. However, users should be aware that some genes or clades may prove more challenging than others, especially if the targeted gene is highly variable or does not carry a strong phylogenetic signal. Additional guidance is provided in the user's manual, and common questions are listed in the FAQ, both accessible via the ThermoPhyl website.

While ThermoPhyl can perform the most laborious aspects of selecting primer sets it is necessary for the user to engage with the ThermoPhyl output to determine how well the "best" assays suit their purpose. We have found that using ThermoPhyl's output within programmes such as ARB can rapidly confirm the potential value of a primer set and highlight where degenerate bases could improve sensitivity without unduly compromising specificity, although this is not recommended for qPCR probe and primer sets unless absolutely necessary.

Conclusions

ThermoPhyl can utilize large sequence datasets now commonly available to identify phylogenetically specific and sensitive assays for traditional and quantitative PCR. ThermoPhyl is run locally on a user's computer, avoiding constraints of internet data transmission, and allowing for customized, personal databases. ThermoPhyl can provide a high-throughput data-driven solution to the problem of targeted assay design in complex samples and is made available free to the research community at: http://go.warwick.ac.uk/thermophyl/.

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References

Acosta-Martinez V, Dowd S, Sun Y & Allen V (2008) Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. *Soil Biol Biochem* **40**: 2762-2770.

Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P & Engstrand L (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* **3**: e2836.

Biddle JF, Fitz-Gibbon S, Schuster SC, Brenchley JE & House CH (2008) Metagenomic signatures of the Peru Margin subseafloor biosphere show a genetically distinct environment. *Proc Nat Acad Sci USA* **105**: 10583-10588.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P & Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**: 5069-5072.

Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA & Wolcott RD (2008) Survey of bacterial diversity in chronic wounds using Pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiol* **8**: 43.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucl Acids Res* **32**: 1792-1797.

Felsenstein J (1989) PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* **5**: 164-166.

Hamady M, Walker JJ, Harris JK, Gold NJ & Knight R (2008) Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. *Nat Meth* **5**: 235-237.

Hawkins RJ & Purdy KJ (2007) Genotypic distribution of an indigenous model microorganism along an estuarine gradient. *FEMS Microbiol Ecol* **62**: 187-194.

Loy A, Arnold R, Tischler P, Rattei T, Wagner M & Horn M (2008) probeCheck - a central resource for evaluating oligonucleotide probe coverage and specificity. *Environ Microbiol* **10**: 2894-2898.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucl Acids Res* **32**: 1363-1371.

Oakley BB, Carbonero F, van der Gast CJ, Hawkins RJ & Purdy KJ (2010) Evolutionary divergence and biogeography of sympatric niche-differentiated bacterial populations. *ISME J* **4**: 488-497.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucl Acids Res* **35**: 7188-7196.

Purdy KJ (2005) Nucleic acid recovery from complex environmental samples. *Meth Enzymol* **397**: 271-292.

Purdy KJ, Embley TM, Takii S & Nedwell DB (1996) Rapid extraction of DNA and rRNA from sediments by a novel hydroxyapatite spin-column method. *Appl Environ Microbiol* **62**: 3905-3907.

Schloss PD & Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71**: 1501-1506.

Sogin ML, Morrison HG, Huber JA, Mark Welch D, Huse SM, Neal PR, Arrieta JM & Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc Nat Acad Sci USA* **103**: 12115-12120.

You FM, Huo NX, Gu YQ, Luo MC, Ma YQ, Hane D, Lazo GR, Dvorak J & Anderson OD (2008) BatchPrimer3: A high throughput web application for PCR and sequencing primer design. *BMC Bioinfomatics* **9**: 253.

Table legends

Table 1. Comparison of ThermoPhyl to four other commonly used oligonucleotide analysis programmes.

Table 2. Summary of the local Blastp analysis of the *Methanosaeta mcrA* sequence data. Pyrosequence data were analyzed by Blastp run locally on a custom database containing 44 pure culture *mcr*A sequences, including all *Methanosaeta* strains. The only changes to default parameters were the use of soft-masking (-F "m S") to enable filtering for low-complexity subsequences during the word seeding phase but not the extension phase of the Blastp algorithm.

Table 3. *Desulfobulbus* clade-specific qPCR assay primer and probe sequences (Oakley *et al.*, 2010) selected using ThermoPhyl.

Figure legends

Figure 1. Flow diagram showing how ThermoPhyl is used in comparison to existing standard practice.

Figure 2. Output files from ThermoPhyl (text files opened in Excel). **a**. Results sorted according to the highest number of "target group" hits for each individual. The F1/R1 primers have been move to the top of table and highlighted in grey. **b**. Raw data, showing how each assay accumulates matches with target and non-target sequences. The F1/R1 primers have been move to the top of table and highlighted in grey.

Figure 3. PCR amplification of *Methanosaeta mcrA* gene from Colne estuary sediment using the primers F2 and R2 as described in the text. Lanes 1) *M. concilii*, 2) *M. harundinacea*, 3) *M. thermophila*, 4) *Methanosarcina mazei*, 5) *Methanosarcina acetivorans*, 6) Site 1 sediment DNA, 7) Site 10 sediment DNA, and 8) no-template control.

Figure 4. Rarefaction analysis of *Methanosaeta mcrA* pyrosequence data with OTUs defined at 10%, 15%, 20%, and 25% sequence dissimilarity from **a.** Site 1 and **b.** Site 10. Curves marked with * were saturated for OTU definitions. Sequence dissimilarity between *M. concilii* and *M. harundinacea* is 25% for the amplicon region.

Figure 5. Phylogenetic representation of *mcr*A sequence diversity recovered by ThermoPhyl-generated primers. The tree is a maximum-likelihood phylogenetic reconstruction based on alignment of nucleotides restricted to the amplicon region. Sequences are labelled with either S1 or S10 indicating whether they are from Site 1 or Site 10 respectively and those shown are representatives of the 27 OTUs defined as described in the text.

Figure 6. Identity and distributions of *Desulfobulbus*-affiliated *dsr*B ecotypes (adapted from Figure 3 in Oakley *et al.*, 2010). a**.** Phylogenetic positions of the four assayed clades within *Desulfobulbus*. Tree was reconstructed from amino acid informed DNA alignment using the maximum-likelihood algorithm AxML. **b.** Distributions of these four genotypes across the estuary as assessed by DGGE. Values represent peak heights normalized within each lane to control for loading differences. **c.** Distributions of four genotypes as assessed by clade-specific qPCR assays. Values represent means of three biological replicates (error bars = 1 SEM).

Characteristics	Programmes				
	THERMOPHYL	ARB[1]	PRIMER-BLAST	PRIMROSE[2]	PROBECHECK[3]
Types of assay					
qPCR probe/primer sets	**YES**	no	no	no	n/a
Standard PCR	**YES**	**YES**	**YES**	no	n/a
Oligonucleotide	**YES**	**YES**	no	**YES**	**YES**
Design primers/oligos	no	**YES**	**YES**	**YES**	no
N°. of sequences assays designed against	User defined (external program)	User defined (oligos) 1 (primers)	1	User defined	User defined (external program)
Assessment					
Phylogenetic basis	**YES**	**YES** (oligos) no (primers)	**YES**	**YES**	**YES**
Tree interface	No	**YES**	no	no	no
Databases	User defined	16/23S	RNA ref Seq	User defined	16S
N°. of target sequences assessed	User defined	User defined (oligos) 1 (primers)	1	User defined	n/a
Target/non-target definition	**YES**	**YES**	**YES**	**YES**	**YES** (target)
Define non-target hits	**YES**	**YES** (oligo) no (primers)	no	**YES**	**YES**
Degeneracies	no	**YES**	no	**YES**	**YES**
N°. of assays assessed	No limit[4]	User defined (Oligo) 0 (PCR)	≤2000	n/a	10
Output	Text	Text	Graphic html	Primrose specific	Text
Local/Remote	Local	Local	Remote	Local	Remote
Reference	This study				

[1]Arb designs and compares oligonucleotide probes with reference to the whole database but does not do so for PCR primer sets, which are designed with reference to a single sequence only and the subsequent primer sets cannot be directly compared against the database.

[2]Primrose designs and assesses single oligonucleotides not PCR primers sets

[3]probeCheck is designed to check single probe specificity.

[4]Limit is based on processing time, which depends of the speed of the local computer, the number of candidate assays and the sizes of the target and non-target databases.

Table 1.

Top BLASTP hit	Site 1	Site 10
Targets		
Methanosaeta concilii AF313802	2563	1674
Methanosaeta harundinacea AY970348	1825	13
Methanosaeta concilii VeAc9 AF313803	115	148
Methanosaeta thermophila PT gb|ABK14360.1	9	10
Non-targets		
Methanothermobacter marburgensis X07794	2	
Methanothermus fervidus J03375	1	
Methanococcus jannaschii mrtA U67465	1	
Methanococcoides burtonii U22234	1	
Total	4517	1745

Table 2. Summary of the local Blastp analysis of the *Methanosaeta mcrA* sequence data. Pyrosequence data were analyzed by Blastp run locally on a custom database containing 44 pure culture *mcr*A sequences, including all *Methanosaeta* strains. The only changes to default parameters were the use of soft-masking (-F "m S") to enable filtering for low-complexity subsequences during the word seeding phase but not the extension phase of the Blastp algorithm

Assay	Forward Primer	Reverse Primer	Probe	Position
M	TGATTGACCACACCCGTATTACC	GCCGTTCACCTCAGCCTTAG	ATCTCTGCTTGTCCGCTC	673-783
Mh	CGCTGTTCATGCTTCCGATA	GATCGATCATCGGCGGTTT	CCTCGGTGTGCATCG	620-681
FW1	TCGCCATTCTCGGTATCCAT	CCGGTGATCCGGTCGTT	CAAACCGCCGATGAT	640-704
FW2	CCGGTTAAGGCGGTTATGG	CGCCGGCAAGGTCATG	TGATCTGTTCGAGTATTTTGGTT	595-653

Table 3. *Desulfobulbus* clade-specific qPCR assay primer and probe sequences (Oakley *et al.*, 2010) selected using ThermoPhyl (all 5'-3').

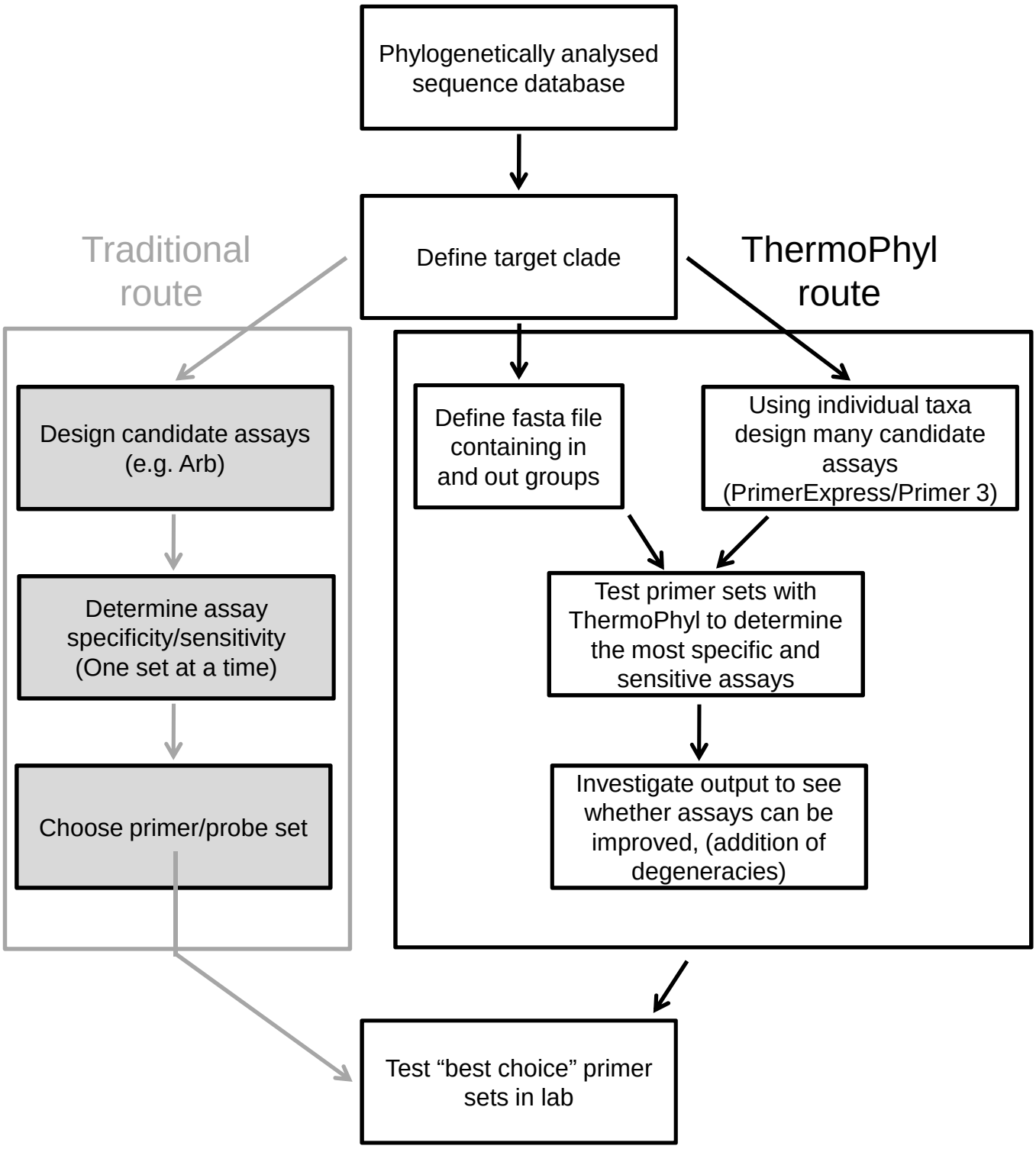

Figure 1. Flow diagram showing how ThermoPhyl is used in comparison to existing standard practice.

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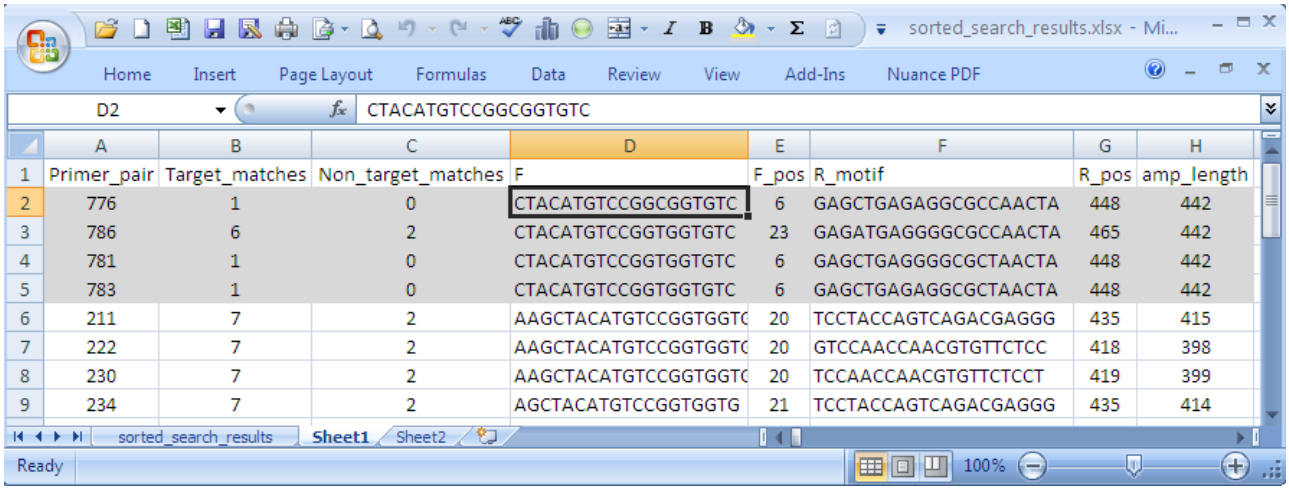

sorted_search_results.xlsx - Mi...

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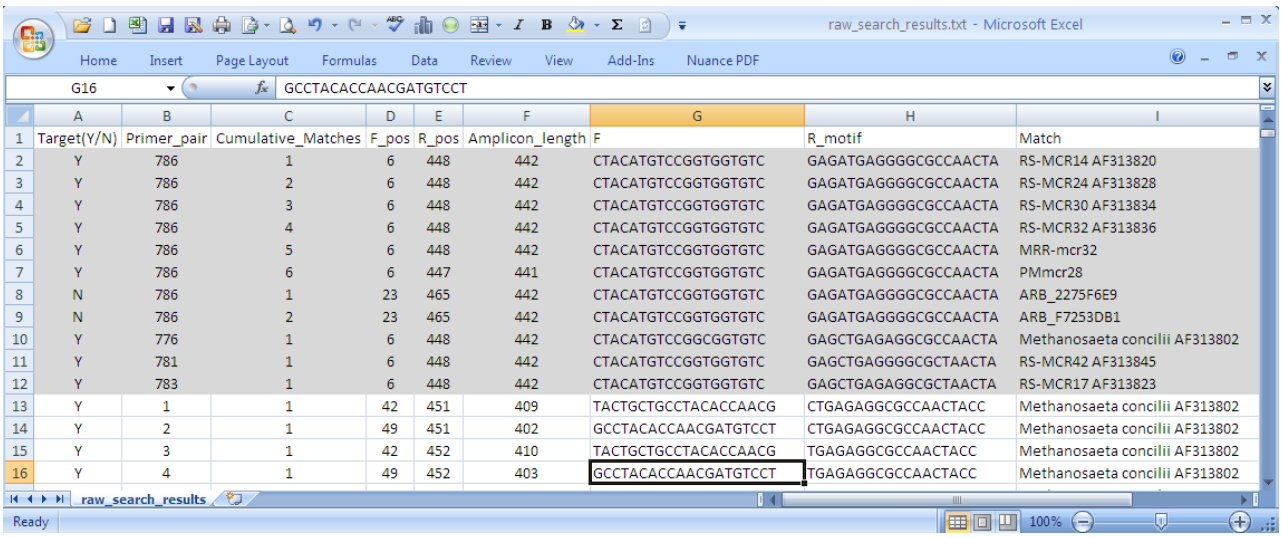
D2 CTACATGTCCGGCGGTGTC

	A	B	C	D	E	F	G	H
1	Primer_pair	Target_matches	Non_target_matches	F	F_pos	R_motif	R_pos	amp_length
2	776	1	0	CTACATGTCCGGCGGTGTC	6	GAGCTGAGAGGCGCCAACTA	448	442
3	786	6	2	CTACATGTCCGGTGGTGTC	23	GAGATGAGGGGCGCCAACTA	465	442
4	781	1	0	CTACATGTCCGGTGGTGTC	6	GAGCTGAGGGGCGCTAACTA	448	442
5	783	1	0	CTACATGTCCGGTGGTGTC	6	GAGCTGAGAGGCGCTAACTA	448	442
6	211	7	2	AAGCTACATGTCCGGTGGTC	20	TCCTACCAGTCAGACGAGGG	435	415
7	222	7	2	AAGCTACATGTCCGGTGGTC	20	GTCCAACCAACGTGTTCTCC	418	398
8	230	7	2	AAGCTACATGTCCGGTGGTC	20	TCCAACCAACGTGTTCTCCT	419	399
9	234	7	2	AGCTACATGTCCGGTGGTG	21	TCCTACCAGTCAGACGAGGG	435	414

sorted_search_results Sheet1 Sheet2

Ready 100%

b. “raw_search_results”



raw_search_results.txt - Microsoft Excel

Home Insert Page Layout Formulas Data Review View Add-Ins Nuance PDF

G16 GCCTACACCAACGATGTCCT

	A	B	C	D	E	F	G	H	I
1	Target(Y/N)	Primer_pair	Cumulative_Matches	F_pos	R_pos	Amplicon_length	F	R_motif	Match
2	Y	786	1	6	448	442	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	RS-MCR14 AF313820
3	Y	786	2	6	448	442	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	RS-MCR24 AF313828
4	Y	786	3	6	448	442	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	RS-MCR30 AF313834
5	Y	786	4	6	448	442	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	RS-MCR32 AF313836
6	Y	786	5	6	448	442	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	MRR-mcr32
7	Y	786	6	6	447	441	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	PMmcr28
8	N	786	1	23	465	442	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	ARB_2275F6E9
9	N	786	2	23	465	442	CTACATGTCCGGTGGTGTC	GAGATGAGGGGCGCCAACTA	ARB_F7253DB1
10	Y	776	1	6	448	442	CTACATGTCCGGCGGTGTC	GAGCTGAGAGGCGCCAACTA	Methanosaeta concilii AF313802
11	Y	781	1	6	448	442	CTACATGTCCGGTGGTGTC	GAGCTGAGGGGCGCTAACTA	RS-MCR42 AF313845
12	Y	783	1	6	448	442	CTACATGTCCGGTGGTGTC	GAGCTGAGAGGCGCTAACTA	RS-MCR17 AF313823
13	Y	1	1	42	451	409	TACTGCTGCCTACACCAACG	CTGAGAGGCGCCAACTACC	Methanosaeta concilii AF313802
14	Y	2	1	49	451	402	GCCTACACCAACGATGTCCT	CTGAGAGGCGCCAACTACC	Methanosaeta concilii AF313802
15	Y	3	1	42	452	410	TACTGCTGCCTACACCAACG	TGAGAGGCGCCAACTACC	Methanosaeta concilii AF313802
16	Y	4	1	49	452	403	GCCTACACCAACGATGTCCT	TGAGAGGCGCCAACTACC	Methanosaeta concilii AF313802

raw_search_results

Ready 100%

Figure 2. Output files from ThermoPhyl (txt files opened in Excel). **a**. Results sorted according to the highest number of “target group” hits for each individual assays. The F1/R1 primers have been move to the top of table and highlighted in grey. **b**. Raw data, showing how each assay accumulates matches with target and non-target sequences. The F1/R1 primers have been move to the top of table and highlighted in grey.

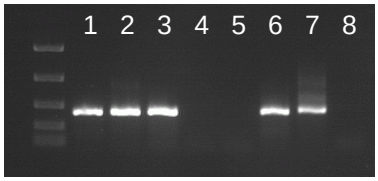
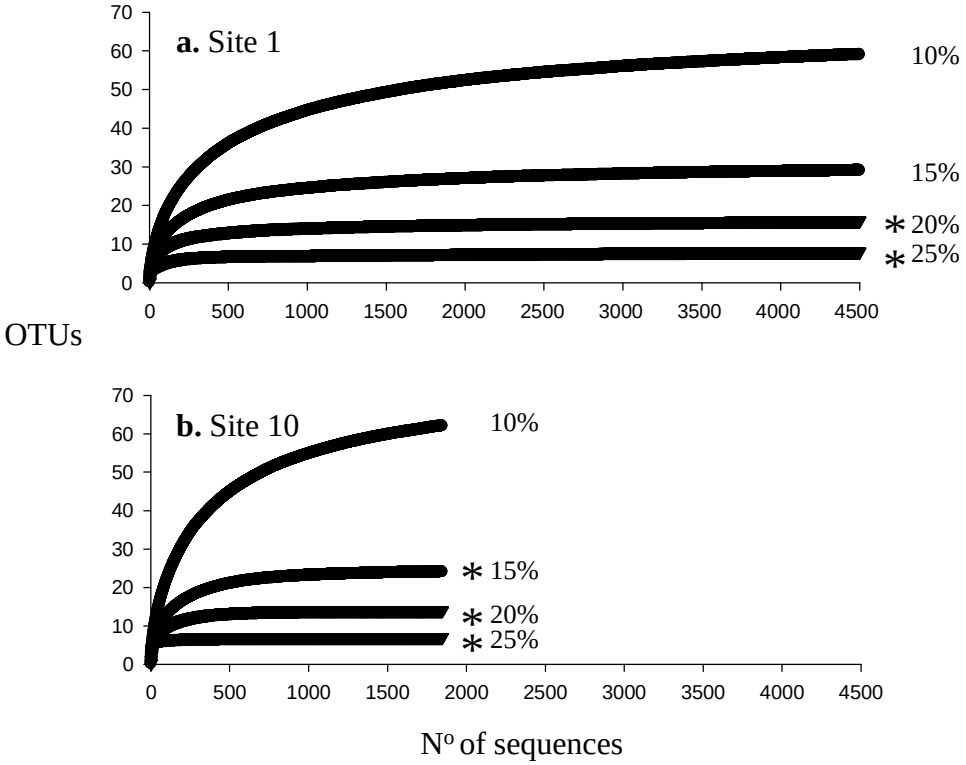


Figure 3. PCR amplification of *Methanosaeta mcrA* genes from Colne estuary sediment using the primers F2 and R2 as described in the text. Lanes 1) *M. concilii*, 2) *M. harundinacea*, 3) *M. thermophila*, 4) *Methanosarcina mazei*, 5) *Methanosarcina acetivorans*, 6) Site 1 sediment DNA, 7) Site 10 sediment DNA, and 8) no-template control.



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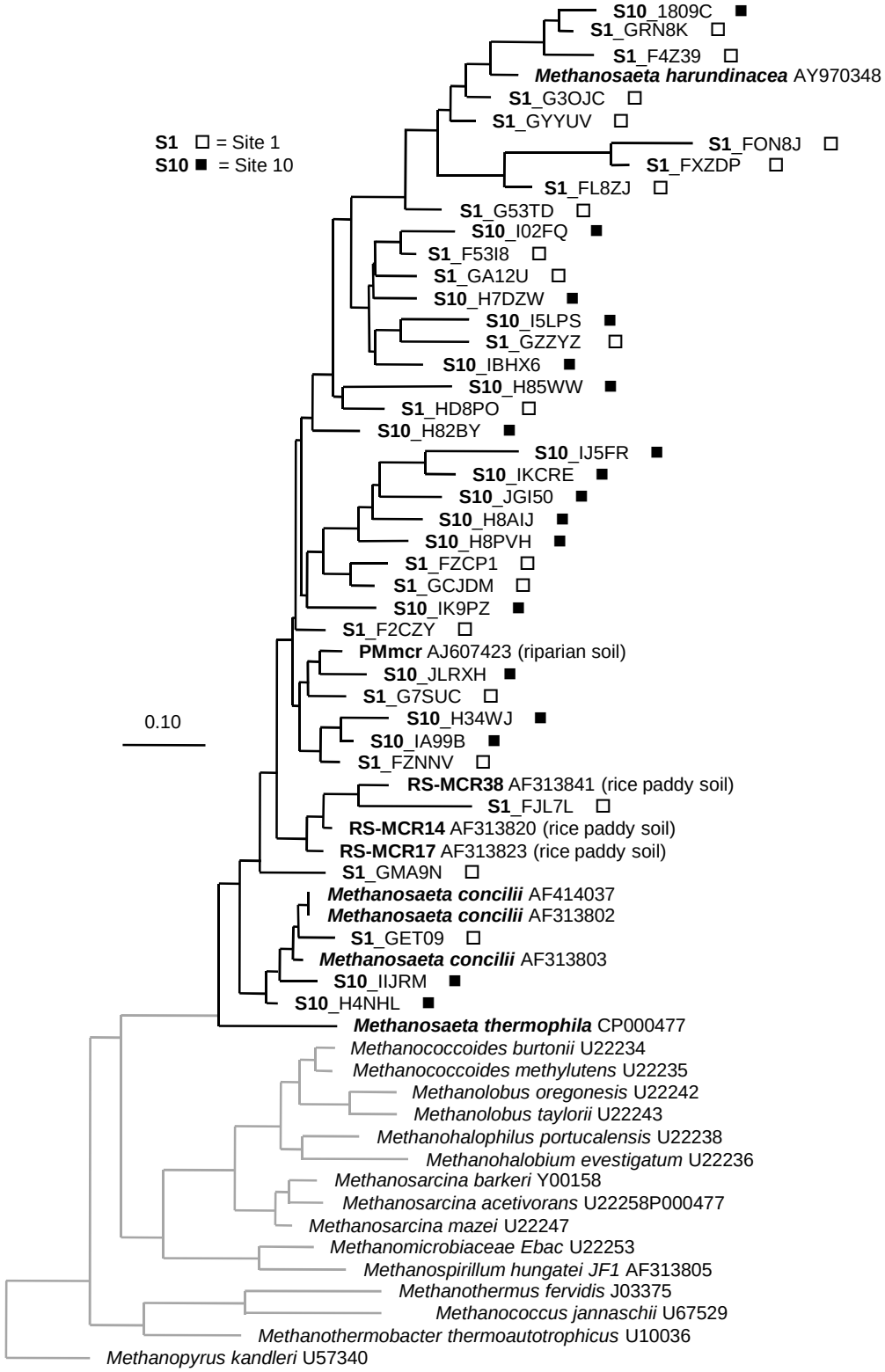

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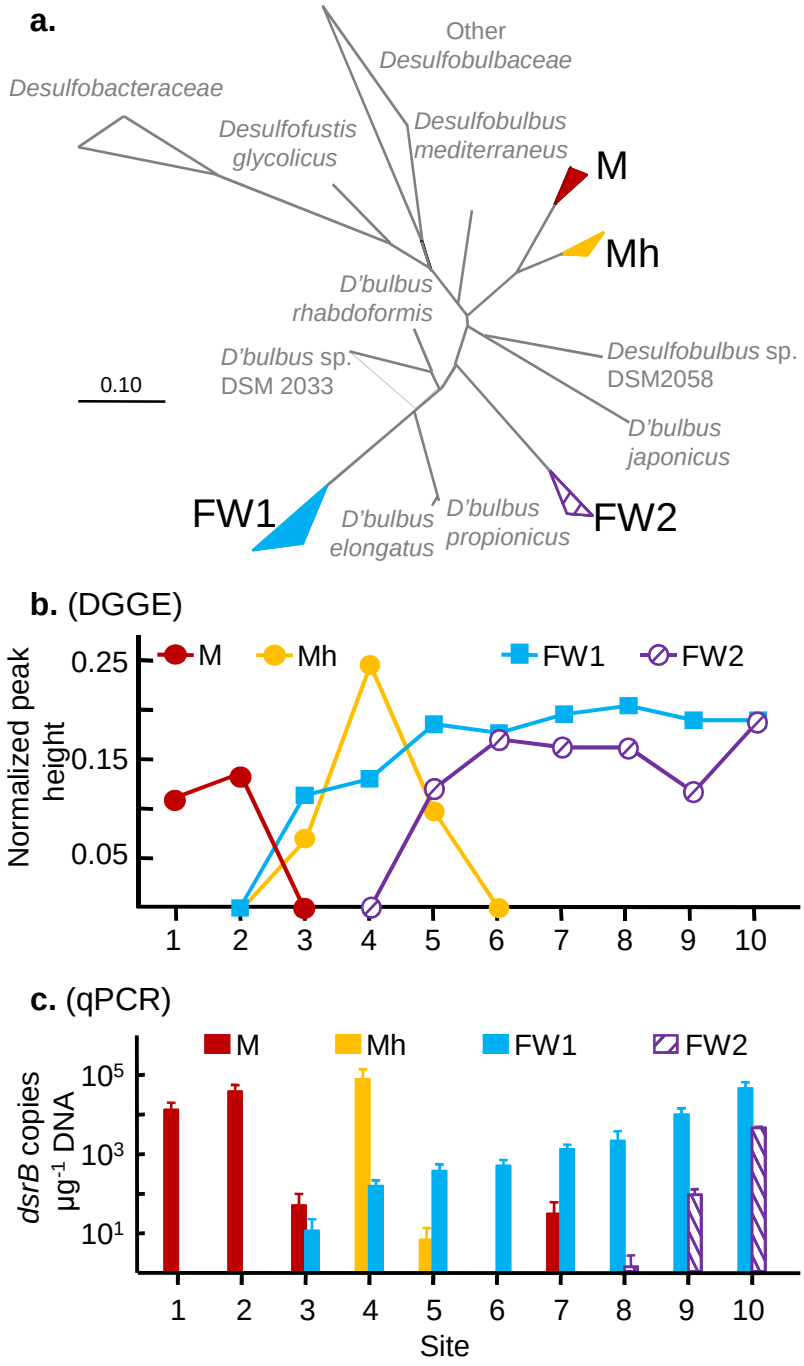

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