



From a single cell to a molecular diagnostic assay

A study with southern African dinoflagellates



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Objectives

- i Genetic characterisation of toxic southern African dinoflagellates
- i Develop a single-cell sequencing method
- i Construct taxon-specific oligonucleotide probes and design a PCR-based lab assay
- i Test assay with lab products and seawater samples

Materials

Dinoflagellate Cultures

<i>Alexandrium catenella</i> (South Coast)	Marine and Coastal Mgmt
<i>Alexandrium catenella</i> (Yzerfontein)	î
<i>Karlodinium micrum</i> (Namibia) (prev. <i>G. galatheanum</i>)	î
<i>Akashiwo sanguinea</i> (LB5) (prev. <i>G. sanguineum</i>)	î
<i>Protoceratium reticulatum</i>	î
<i>Scrippsiella trochoidea</i>	î
<i>Alexandrium catenella</i> (CCMP 1598)	Provasoli-Guillard
<i>Karlodinium micrum</i> (CCMP 416) (prev. <i>G. galatheanum</i>)	î

Field Samples

Collected on Lambert's Bay (West coast) on March-April 2000 and 2001

Cells isolated from seawater samples (*Dinophysis acuminata*, *D. fortii*, *Ceratium furca*)

PP assemblages collected, microscopically examined and stored frozen for lab analysis

Single-cell Sequencing

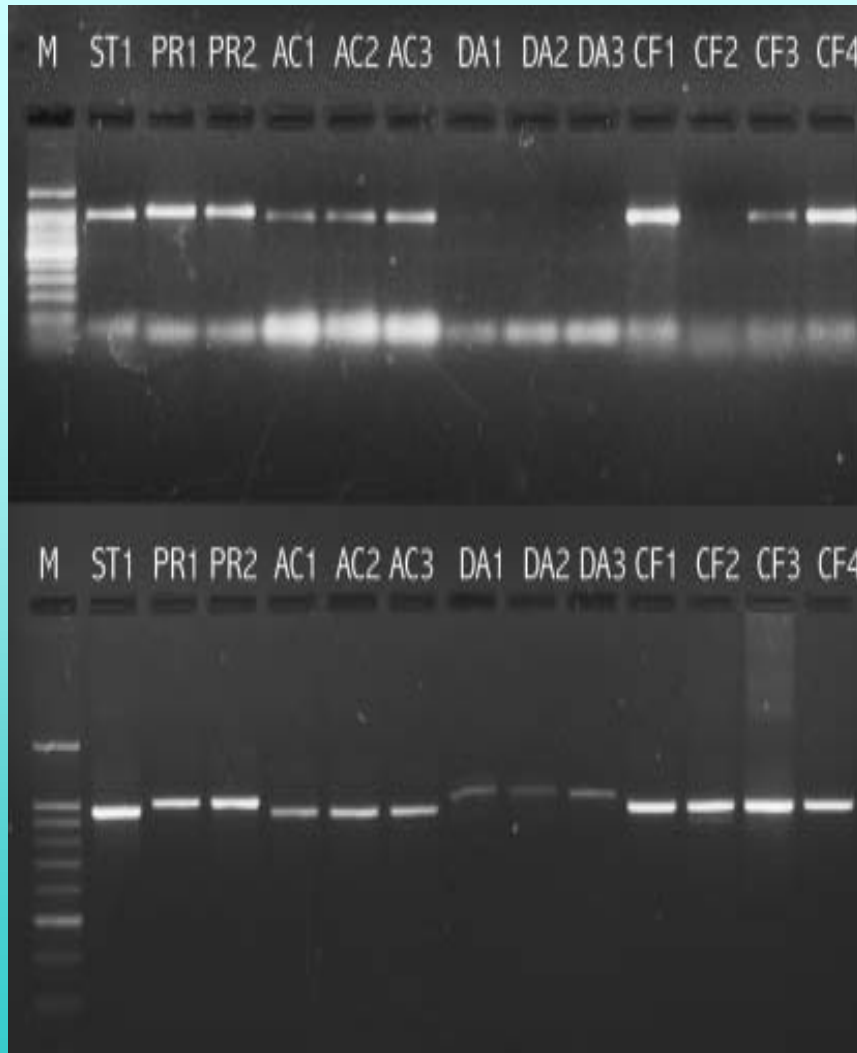
1. Single cells isolated from fresh and frozen samples, rinsed in GF/F-filtered seawater and stored in thin-walled PCR tubes with minimum medium (1-3 μ l)
2. PCR tubes with cells subjected to 3 freeze-thaw cycles (liquid N₂ ñ hot water bath, 30í each)
NB: More gentle lysis may be enough for fragile species
3. PCR mix added, initial amplification with rDNA primers (D1R-D3B for LSU ; ss12-ss936 for SSU)

Single-cell Sequencing

4. Visualisation with EthBr/UV, amplicon excised
NB: band excised even when not visible
5. Filtered-tip purification of amplicon
6. Use 1-5 µl elution for PCR re-amplification with same conditions (cycles reduced to 15)
7. Pool 2-3 re-amplification replicates and purify
8. Cycle-sequencing of both strands

Ruiz Sebastián, C. and C. O'Ryan (2001) Single-cell sequencing of dinoflagellate (Dinophyceae) nuclear ribosomal genes. *Molecular Ecology Notes* 1: 329-331.

Single-cell Amplification Results



S. trochoidea
P. reticulatum
A. catenella
D. acuminata
C. furca

Initial amplification

Re-amplification

← Partial LSU (~1050bp)
← Partial SSU (~950bp)

Single-cell Sequencing Results

- Sequence validation:
 - GenBank / BLASTn checks
 - Single-cell / culture comparisons
 - Single-cell replicates
- Spurious sequences:
 - 12.5 % sequences were not dinoflagellates
 - Basidiomycota (55%); Ascomycota (45%)
 - Cloning revealed rRNA from several species in amplicon
 - Hitchhikers? Symbionts? Parasites? Food?
 - Primers not specific enough?

Single-cell Sequencing Results

- Partial SSU (~950 bp) and LSU (~1050 bp; D1-D3)
- At least 2 single-cell replicates
- Species sequenced:
 - *A. catenella* (SC), *A. catenella* (Yzer), *A. catenella* (CCMP)
 - *P. reticulatum*
 - *S. trochoidea*
 - *D. acuminata*, *D. fortii*
 - *C. furca*
 - *A. sanguinea*, *K. micrum* (Namibia) and *K. micrum* (CCMP) (culture extraction using phenol-chloroform)

Sequence Analysis

- Multiple alignment (Clustal W) and preliminary phylogenetic analysis
- Are the *Alexandrium* isolates the same species?
 - No, two different species in *A. catenella* complex
 - *A. catenella* (south coast) → *A. tamarense* - *A. cohorticula*
 - *A. catenella* (Yzerfontein) → *A. fundyense*
- Is *K. micrum* (Namibia) the same as its North American conspecific?
 - No, < 95 % similarity, more similar to other gymnois
 - Probably an undescribed gymnodinoid (supporting evidence from ultrastructure and electron microscopy)

Identification of taxon-specific oligonucleotide probes

- Target taxa:
 - *Alexandrium* genus
 - *Dinophysis* genus
 - *P. reticulatum*
 - *K. micrum* (Namibia)
 - *A. sanguinea*

- Find candidate regions in rDNA:
 - Sequence alignment and examination
 - Taxon-specific

Identification of taxon-specific oligonucleotide probes

➤ Identify primer candidates

- Length, Δ , GC %, T_m , complementarity
- 3+ bp differences !!!
- GenBank / RDP checks

➤ 3 primer candidates identified per target taxon

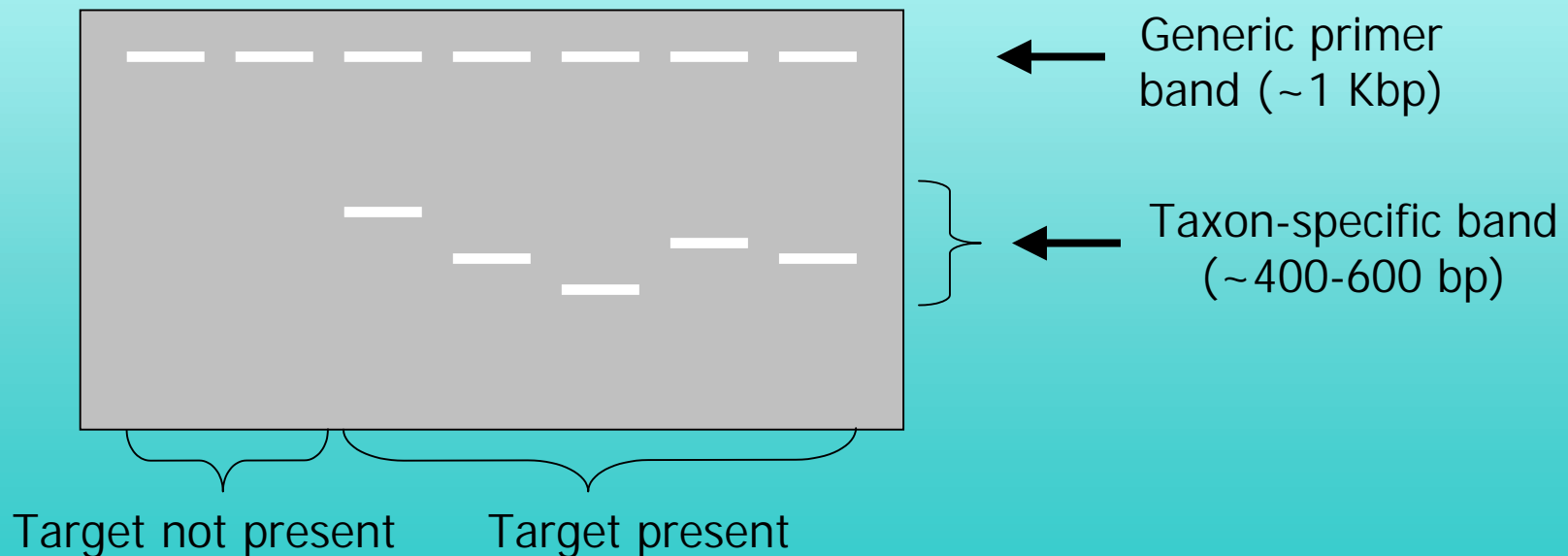
ALEXssu710	GCCTGAGCATTATCTTGAAAG
ALEXssu717	TGGCACAGCCTGAGCATT
ALEXssu757	CTGTGTGGTGTGTTATTGAGA
DINO439	CAAGGATTGGTTGCTGCCAC
DINO507	GTCTTACCTAGTGGGTCATTG
DINO632	TAAGCTTTAGTGTTTGTCTGGC
PROTO260	GGGATTTGAACGCAAACCTGT
PROTO399	GGGTGTATTTTCGCACGCAA
PROTO691	CACGACATGAAAATGATGGCC
SANG430	AGATTGTCGCATGCCAAGGC
SANG590	ACTCTGGGGACATGGATGCC
SANG675	CCTTGACTGTGAGCAGTGC
NAMI429	GCGCTACTGTGATTGCTCG
NAMI671	CTCATTGCCTGTGCTCGTCC
NAMI709	TCTCGTCGTCGCTTCGTGC

Design of a Triplex-PCR diagnostic assay

➤ Triplex-PCR assay:

- 2 generic primers (F, R) + 1 taxon-specific primer (F)
- Asymmetric PCR: tuning of primer concentrations

➤ Diagnosis:



Testing the Triplex-PCR diagnostic assay

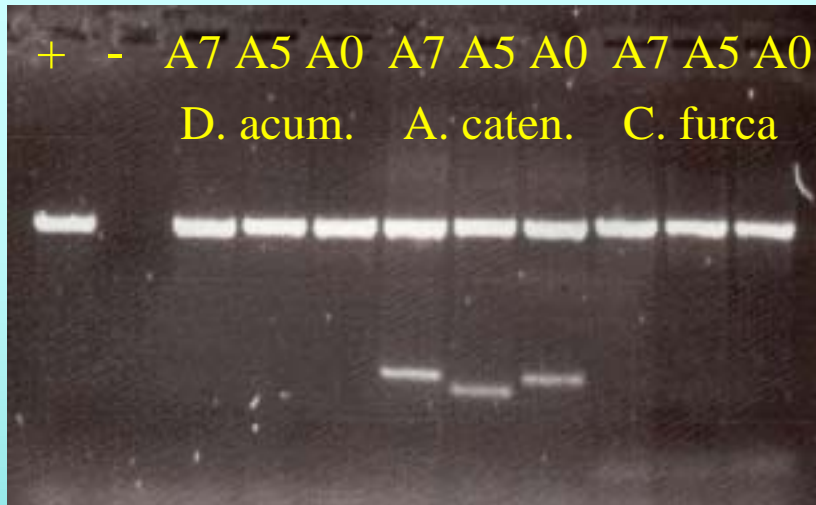
➤ Increasing complexity:

- PCR amplicon (probe specificity)
- Genomic DNA ()
- Mixed genomic DNA ()
- @ diff. concent. (sensitivity)
- Seawater samples (spec./field app.)

➤ Treatment of seawater samples:

- Gentle centrifugation, Freeze-thaw and Proteinase-K
- Use 1-3 ul as template source

Triplex-PCR Assay Results

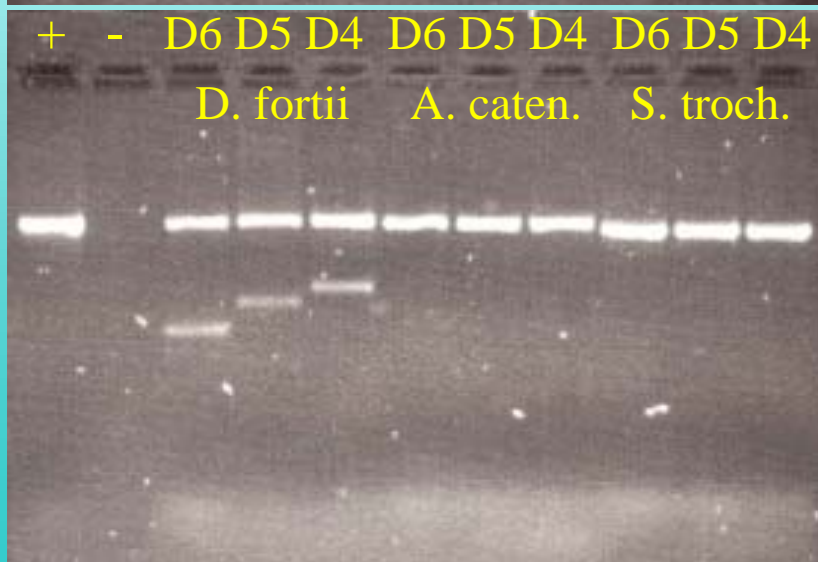


ALEXssu probes:

A7: ALEXssu717

A5: ALEXssu757

A0: ALEXssu710



DINO probes:

D4: DINO439

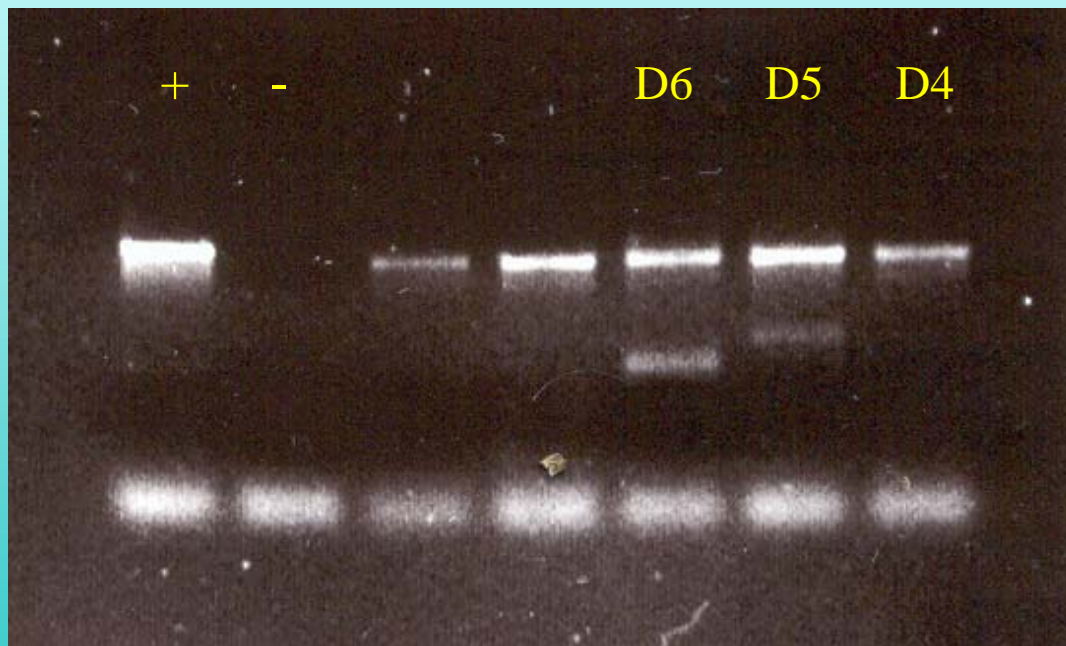
D5: DINO507

D6: DINO632

Template source: PCR amplicon

Triplex-PCR Assay Results

Seawater sample: assemblage containing *D. fortii*, *D. acuminata*



DINO probes

Conclusions

➤ Single-cell method:

- Efficient method to obtain rDNA sequences from fresh or frozen dinoflagellate single cells
- Product for sequencing can be obtained in 6–8 hr
- Simple and low-cost
- Sequence validation is very important

➤ Sequences:

- Obtained SSU and LSU rDNA partial sequences for 11 species
- Answered taxonomic questions

Conclusions

➤ Triplex-PCR diagnostic assay:

- Genus-specific probes for *Alexandrium* and *Dinophysis*
- Species-specific probes for *P. reticulatum*, *K. micrum* (Namibia) and *A. sanguinea*
- Probes highly specific
- Simple and robust assay
- Needs further validation with more seawater samples

Ongoing Work

- Test triplex-PCR assay with a broader range of seawater samples
- Assess diversity of *Alexandrium* and *Dinophysis* species on Southern African coasts

Thanks !

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