Development of Field Implemented Fillet Identification (FIFI) for Coral Reef Fin Fish

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Fisheries Research and Development Corporation

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Non-technical summary

2005/011 Development of Field Implemented Fillet Identification (FIFI) for Coral Reef Fin Fish

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OBJECTIVES:

1. To investigate the possible development of a rapid, simple and inexpensive method to indicate the presence of target mitochondrial DNA sequences from restricted and protected coral reef finfish species; and if successful.

2. To optimise and validate this method as a field test to detect the presence of, and to identify, restricted species of coral reef finfish species from samples that have had phenotypic markers removed (such as fillets), and to confirm that cross-reactivity with unrestricted species will not occur. This will form the basis of FIFI (Field Implemented Fillet Identification), and subsequently be used by fisheries officers as a compliance tool for the Fisheries (Coral Reef Fin Fish) Management Plan.

3. To workshop and demonstrate FIFI in order to train and familiarise fisheries officers and any other interested parties in its use (extension).

4. To use media coverage to create public awareness of FIFI and deter non-compliance with fishing regulations.

5. To investigate the most appropriate scientific communication of FIFI technology based upon the optimum procedures used (from objectives 1 and 2) and any current patents held on those procedures. This may include either publication or commercialisation.

OUTCOMES ACHIEVED TO DATE

This project describes DNA probes specific to species of coral reef finfish that are noted as protected or restricted. Whilst it was the aim of the project to produce a field test utilising these probes in boat-side species identification test, this was not achieved. There remains a great potential for application of the work to several platforms that result from international multi-disciplinary investigations into hand-held devices.

NON TECHNICAL SUMMARY:

The coral reef finfish fishery has been increasingly targeted for commercial and recreational exploitation over the last 25 years. Legislation has been introduced to limit the impacts upon the sustainability of this natural resource. Queensland Boating and Fisheries Patrol Officers can confiscate fish suspected of being caught in breach of the legislation. Identity of fish that have had phenotypic markers removed is currently determined using laboratory-based genetic testing. This project investigates the potential to use genetic probes to construct a simple "dipstick" test that can provide a presumptive identification of species from fish tissue.

A number of techniques were assessed and compared, along with some in-house designed methods that included a novel method of coating a plastic dipstick that showed great promise. However, validation with respect to reproducibility showed the method was not sufficiently robust or reliable in its current form to perform as a precursor for potential legal action. Re-optimisation of the method did not improve this adequately.
Whilst, not achieving its overall aim, this project has identified species–specific probe sequences that may be applied to recently described hand-held devices that employ multiple scientific strategies from the fields of physics, chemistry and biology.

KEYWORDS: Coral reef finfish, dipstick, species-specific probes.

ACKNOWLEDGMENTS

This project was funded by FRDC, with in-kind contributions from Biosecurity Queensland and Queensland Boating and Fisheries Patrol. In kind contributions included the prior intellectual property associated with genetic sequence of coral reef finfish, laboratory/bench space, and the time contribution by the principal investigator.

BACKGROUND

The coral reef finfish fishery has been increasingly targeted for commercial and recreational exploitation over the last 20 years. It has been reported that within the Queensland commercial sector, the harvest rose dramatically from 2,034 tonnes in 1988 (Williams, 2002) to 4,831 tonnes in 2001 (Fisheries (Coral Reef Fin Fish) Management Plan 2003). Some individual species and groups have been increasingly targeted at an alarming rate. For example, the harvest of coral trout species rose from 818 t in to 2,094 t, the emperor group from 419 t to 743 t and the red throat emperor species from 711 t to 941. Such increases put the sustainable future of this fishery resource, and associated ecosystems, in jeopardy.

The Commonwealth's Environment Protection and Biodiversity Conservation Act 1999 stated that all Australian export fisheries and those fisheries that have the potential to affect protected species must be assessed to ensure they are managed to a sustainable level that will ensure resource availability in the future. As a result of subsequent reports such as "Queensland's fisheries resources: current condition and recent trends" (Williams, 2002), the Queensland Government introduced the Fisheries (Coral Reef Fin Fish) Management Plan 2003 (hereafter termed "the Plan"). This legislation is concerned with ensured sustainability of coral reef fisheries resources on the Queensland coast. The Plan includes size and possession (bag) limits on a number of species (hereafter termed "restricted species"), particularly those identified by Williams (2002) as harvested at an alarmingly increasing rate over the last 20 years. Bag and size limits apply to recreational fishers and charter boat operators. Size limits and a quota management regime apply to the commercial fishing sector. The limits were established based on allowing at least half of the fish in a population to reach reproductive maturity and spawn before they can be harvested. In addition, some species have been designated as protected and are not permitted to be taken. These include Maori wrasse, Barramundi cod, Potato cod, Queensland grouper, Red bass, Chinaman fish and Paddletail. Restricted species of coral reef finfish have a minimum size limit of 25 cm unless the legislation states otherwise. Alternative size limits apply to tuskfish, cod, red throat emperor, long nose emperor, spangled emperor, red emperor, gropers, coral trouts, and nannygai.

In addition to the size limits, the Plan introduced new possession limits of five per species unless specified. For example, the coral trout possession limit is seven in total for all coral trout species, and nannygai is nine in total for all species. The Plan also includes a combined limit of 20 coral reef fish in total. The quota management regime prescribed a quota limit for commercial catches of coral trout, red throat emperor and other reef fish. There are some fish taken by commercial fishers that are not subject to quota restrictions. Queensland boating and fisheries compliance Officers are required to ensure that quota limits are not exceeded or that "quota fish" are not misrepresented as "non-quota fish".

The majority of the fish species included in the Plan can be recognised by experienced persons based on phenotypic characteristics, and the Plan includes prohibitions to allow for this where restricted species of coral reef fin fish on board a boat must be either whole or gilled and gutted. However, the regulations apply only to those species included in the Plan. The ability of fisheries officers to ensure the integrity of the quota system has been severely hampered by a decision to allow commercial fishers to fillet fish at sea. A permit to fillet does not exclude a fisher from the size and possession limits. Officers are unable to identify which species of fish a particular fillet may be and therefore are unable to ensure that the quota provisions are being complied with. Hence, it becomes impossible to prove or disprove compliance with the legislation if these fishers fillet their catch and are in possession of a valid permit or claim the fillets were from an unrestricted species.

In 2001, a CSIRO publication, Australian Seafood – Domestic Species (Yearsley *et al.*, 2001) included a chapter of protein profiling for species differentiation (Chapter 9, Ward *et al*.). This was part of a FRDC funded project that commenced in 1994, and suggested that protein fingerprinting could serve as a field test for fillet identification. The process involved electrophoresis, a method of passing high voltage electricity through a bath of liquid buffer surrounding a solidified toxic polyacrylamide gel matrix. Samples are loaded into fine wells in the gel matrix and the electrical current passes through, separating proteins according to charge and size. When complete, the gel is stained to visualise the position of the protein fragments and comparative markers. Operators estimate the sizes of the protein fragments and compare their results with published profiles included in the book. There are some safety concerns with the application of this method on potentially turbulent boat, as this method was designed primarily for land-based laboratories (R Ward, CSIRO, *personal communication*). Safety concerns aside, the major disadvantage to using this method for identification of coral reef finfish is the lack of discriminatory power. Protein profiles are ideal for differentiating between the broader groups of animals, but lack the ability to discriminate between related species. In addition, this method requires each fillet or sample to be tested individually as mixed profiles cannot be identified.

Later advancement in technology made it possible to study DNA as easily, if not more so, as proteins. DNA sequences are far more discriminatory than protein profiles. The enhanced discriminatory power of DNA compared to proteins was acknowledged by Ward *et al* (2001), who stated that the protein profiling must be confirmed by DNA sequence analysis prior to the implementation of any legal proceedings.

As a result of these difficulties the Fisheries Resources groups of Queensland Primary Industries and Fisheries (QPIF) funded the support costs for the creation of a database of mitochondrial DNA sequences from 16 of the restricted species, representing the key species in the fishery. This work was carried out at the Tropical and Aquatic Animal Health Laboratory in Townsville (Biosecurity Queensland, QPIF). The database was completed in June 2004 and included three mitochondrial gene partial sequences for each species, based upon three to seven samples of each species. It was observed that there are species differences within parts of the gene sequences, and that the presence of certain sequence "motifs" were indicative of species identification. Other regions of the genes are common to all species tested. Closely related fish species had more closely matched DNA sequences than those that are less related. The purpose of the database was to serve as an objective source of comparison for sequence data obtained from samples seized in the event of suspected non-compliance with the Plan. Validation of this method of identification has shown it to be highly discriminatory, objective and very successful at species identification, based on a blind trial where samples were tested without the identity of the sample being revealed to the laboratory. However, the cost of the analyses makes it prohibitory for spot-checking or routine use, particularly as each fillet or sample requires testing individually. Identification through sequence analysis can be carried out only by experienced personnel with specialised equipment, and takes a number of days to perform. Hence, it is likely that this method will only be used in the event of obvious non-compliance where legal evidence is required. The Fisheries Resources and Biosecurity Queensland groups therefore identified a requirement for a discriminatory test that is quick and easy to use, relatively cheap when compared to the sequencing analysis, and is safe to perform in the field by boating patrol officers.

Also in May 2004, Prof. Hebert at University of Guelph in Canada commenced the Barcode for Life program. The program intends to accumulate data from a single mitochondrial gene for every living organism on earth over the next 20 years as a taxonomic aid. Superficially, this initiative appears to replicate in part the coral reef finfish database that was developed by QPIF, but this is not the case. The Barcode for Life program is collecting data from a single gene from single sample of as many species as possible. Because of the huge amount of work involved, replicate testing is not being applied (pers. comm. Prof Dan Heath, University of Windsor, Canada). For these reasons the basis of the science in the Barcode program has been questioned (see The New York Times Dec. 14, 2004, and Moritz and Cicero, for examples). The concerns were based upon the inability for single genes to differentiate closely related species, which was

observed also in the QPIF coral reef finfish database. Supporters for the Barcode program have already made claims that what was previously considered to be a single species is actually a collection of ten species, and that closely related species may actually be a single species, when taxonomists claim that there is no phenotypical, morphological or other biological basis for these claims. In summary, the Barcode for Life program remains an unproven theory and much more conceptual research is required before universal acceptance by the scientific and public communities. Moreover, according to Prof Dan Heath (*pers. comm.*), who is involved in the program, the Barcode data is intended as a research and taxonomic tool rather than to have the integrity level required for legal proceedings.

Given the current concerns, the apparent lack of data integrity, and the anticipated time before data is accumulated, the Barcode project is not considered further in this report. The Barcode data does not provide for determination of intra-species sequence variation, and therefore does not provide for identification of legally acceptable species-specific probes. The project described herein seeks to solve an immediate and current need by Fisheries Resources, QBFP in particular, to monitor compliance with the Plan. Data of high replication and integrity of multiple genes from the target fish species has already been accumulated with consideration for legal applications. This will provide a sound basis for using that data in the field.

SUMMARY OF NEED

The need to manage the growth of the coral reef fishery to prevent depletion of this natural resource, and its associated ecosystems, has been clearly identified and legislation has been put in place under the Fisheries (Coral Reef Fin Fish) Management Plan 2003. To ensure compliance, Officers must be able to identify the fish they are inspecting, and currently there is no suitable test to identify fish fillets in the field and to confirm that commercial and recreational fishers comply with quota and possession limits, thus assuring sustainability of the resource.

Recent research by the Principal Investigator has shown that DNA sequencing can identify coral reef finfish to a species level even when visible markers have been removed (such as through filleting). However, DNA sequencing is complex and expensive making it unsuitable for screening large numbers of fillets. Hence there is a need for a tool that can identify fish species, be rapidly and easily used at sea, and is sufficiently discriminatory to differentiate between closely related coral reef finfish. Public awareness of the availability of such a tool will deter non-compliance.

SUMMARY OF OBJECTIVES

1. To investigate the possible development of a rapid, simple and inexpensive method to indicate the presence of target mitochondrial DNA sequences from restricted and protected coral reef finfish species; and if successful,
2. To optimise and validate this method as a field test to detect the presence of, and to identify, restricted species of coral reef finfish species from samples that have had phenotypic markers removed (such as fillets), and to confirm that cross-reactivity with unrestricted species will not occur. This will form the basis of FIFI (Field Implemented Fillet Identification), and subsequently be used by fisheries officers as a compliance tool for the Fisheries (Coral Reef Fin Fish) Management Plan;
3. To workshop and demonstrate FIFI in order to train and familiarise fisheries officers and any other interested parties in its use (extension);
4. To use media coverage to create public awareness of FIFI and deter non-compliance with fishing regulations;
5. To investigate the most appropriate scientific communication of FIFI technology based upon the optimum procedures used (from objectives 1 and 2) and any current patents held on those procedures. This may include either publication or commercialisation.

BENEFITS OF A SUCCESSFUL OUTCOME

1. Sustainability

A successful outcome will contribute to the overall sustainability of coral reef finfish populations as described in the Fisheries (Coral Reef Fin Fish) Management Plan 2003. The principal beneficiaries of sustainability are fishers both current and in the future, and the community in general.

2. Compliance monitoring

A successful outcome will enhance compliance monitoring through a simple and cost effective method (FIFI). Current methods require seizure and removal of suspected non-compliances for laboratory testing which can take time and is expensive, whether using preliminary protein profiling (Ward *et al*. 2001), or DNA sequence analysis (QPIF, 2004). The planned outcome is a quicker compliance tool that can be performed by fisheries officers in the field. Only samples giving a positive result will need to be confiscated for mtDNA sequence confirmation if legal proceedings will take place. Such a method is likely to be more widely accepted by fishing patrol officers, fisheries resource management and complying fishers alike.

FIFI has been designed according to specifications and needs of the Queensland Boating and Fisheries Patrol (QBFP). The Plan specifies quotas or bag limits for restricted species and has designated protected species as no-take, where it is prohibited to remove the fish from the fishery. All other species are simply labelled as "other" and no limits apply. The monitoring of the quota/no-take regulations is severely hampered by the filleting process. Fishers can apply for licences to fillet their catch, a process that renders many species unidentifiable to the patrol officers. Under the Plan, fishers are required to store fillets of restricted species separately from the other species. However, QBFP experience shows that some fishers have concealed restricted species fillets under batches of those designated as other, and when buried in ice this is difficult to detect (R Grimley, Regional Manager North, QBFP). Similarly other tricks have been known to take place, such as sending the restricted species fillets back to shore in a dinghy prior to the advised time of arrival of the primary fishing vessel (R Koch, District Manager, Cairns, QBFP).

A successful outcome would be used by the patrol officers in routine surveillance/random testing, to pursue advice that non-compliance may be occurring by a named vessel, or in any other suspicious circumstances. QBFP are permitted to board any vessel fishing in Queensland waters to inspect fish catches. FIFI would be integrated into the inspection process where officers will remove slivers of fillets, take bores through frozen blocks, or any other appropriate samples. The samples would be applied to FIFI on board the QBFP vessel, and it is anticipated results would take no longer than 1-2 hours. Negative results would incur no further action, with potential damage to the fillets being negligible. Positive results would indicate presumptive identification of the target species and QBFP will use their discretion and standard protocols to determine whether to confiscate some or part of the fishers catch. If it is indicated that more than 5% of the catch is unlawful, the officers have the right to seize the entire catch, if less than 5% then they can only remove the suspect fillets. Confiscated fillets will be treated as any other seizure by QBFP according to their standard procedure. Fillets are placed in tamperproof bags and labelled accordingly. A standard QBFP chain of evidence commences and all temporary storage is secure. QBFP transports and delivers the samples to the laboratory where the samples are signed for on the QBFP chain of evidence form and a laboratory chain commences. In the laboratory all samples are stored in locked boxes in secure areas. DNA sequences of the samples are tested in the laboratory according to the methods previously implemented and validated in previous projects. When completed the samples are collected by QBFP and the chain of evidence returns to the patrol, samples are further stored at QBFP secure premises. The patrol determines if prosecution is applicable by taking the sequencing data into account along with other facts about that case such as numbers of unlawful fillets, circumstances etc.

3. Deterrent

The presence of, and knowledge of, FIFI is highly likely to act as a deterrent against non-

compliance by fishers. At the present time, while time consuming and expensive tools are used for compliance checks, fishers may be tempted to keep a catch over the limitations of the Plan with the knowledge that the chances of being caught are small. As a deterrent, the project would benefit the community in general.

4. Consumer confidence

If successful, FIFI could be used by seafood marketers or retailers to confirm the authenticity of their products, and justify prices charged for high quality genuine reef fish.

METHODS/RESULTS/DISCUSSION

The progression of the project to each stage is dependent upon the acquired results and conclusion from previous stages. Hence each component of the project is described separately with a summary, method, results and conclusion.

OBJECTIVE 1: To investigate the possible development of a rapid, simple and inexpensive method to indicate the presence of target mitochondrial DNA sequences from restricted and protected coral reef finfish species

1.1 Comparing the performance of DNA extraction methods and their suitability for field-based application and probe-capture techniques

Summary

Rationale: DNA is intracellular and therefore needs to be released to be accessible to subsequent reaction. The majority of DNA extraction methods that produce high yields of pure nucleic acids require specialised equipment, toxic chemistry and/or long time periods. There are, however, a number of "rapid extraction" kits on the market and a number of rapid methods described in the literature. This project requires a method that produces high yields of DNA from fish muscle as rapidly as possible without the use of toxic chemicals or expensive specialised equipment.

Experimental design: Commercial products and "manual" methods were compared with a proven laboratory method (Roche High-Pure commercial kit) for yield, purity, time, ease of use and cost. All methods were performed and compared using fillet tissue from the same sample. As the methods reportedly extract genomic DNA, the extracts were checked for the presence of intact mtDNA through established PCRs. PCR involves the hybridisation of primers to template and was an indication of quality for the intended use.

Performance indicators: Comparative data with respect to quality, quantity, time and ease of use, and cost for DNA extraction methods.

Methods

a) High-Pure PCR Template Preparation Kit (Roche Applied Science, Castle Hill, NSW. Cat. No. 11796828001*)*

In this method, tissue was ground and the cells were lysed using proteinase K and chaotropic salts to inactivate released nucleases. Nucleic acids bound to glass fibre filters in a proprietary single-use plastic column, while other cellular components were removed by centrifugation. The bound nucleic acids were purified by washing the filter. Finally, the nucleic acids were released from the filter with a low-salt buffer and collected by centrifugation. This commercial product required a heating block and a microcentrifuge with a minimum capacity of 12,000 *rpm*.

This method was used according to the manufacturers instructions, with the minor modification that ~50 mg tissue was ground prior to cell lysis using a sterile wooden toothpick in a conical microfuge tube, in place of the recommended shredding by scalpel. This modification was made in recognition of the safety risks that would arise from using and disposing of scalpels in the field.

This method was used to provide a reference for other methods.

b) Dynabeads Magnetic bead separation (Dynal, Invitrogen, VIC)

Approximately 50 mg tissue was ground in 200 µL suspended Dynabeads with a sterile wooden toothpick and incubated at ambient temperature for 2 mins for lysis to occur. The tube was placed in a proprietary magnetic stand to draw the beads to a tight pellet and the liquid was removed with a pipette. The tube was removed from the magnet and the beads were washed with proprietary washing buffer. Liquid was removed using the same magnetic capture of the beads, and the washing process was repeated. The beads were mixed with 50 µL elution buffer using a pipetting action. The liquid containing DNA was removed into a clean tube, using the magnetic capture to remove the beads.

c) EDNA HiSpEx™ Tissue Kit (Fisher Biotec, Subiaco, WA. Cat. No. ET-100*)*

This method extracted denatured DNA from tissues without the requirement of centrifugation. Approximately 50 mg tissue was ground treated with proprietary reagents according to manufacturers instructions and heated to 95^{o}C in a heating block.

This method was included in the experimental comparison as it provided denatured DNA that will be required for subsequent probe capture, and it appeared quick and simple to perform with minimal equipment and consumable plasticware.

d) Lyse-N-Go™ (Pierce, via Quantum Scientific, Murrarie, QLD. Cat. No. 78882*)*

This method extracted DNA from tissue using the addition of a proprietary solution to ground tissue followed by a series of heating and cooling steps to lyse cells and denature endogenous enzymes. For application to field use, a heated block and an ice brick were used in the place of the recommended thermal cycler.

This method was included as it appeared simple, with only a single reagent and minimal specialised equipment.

e) QuickExtract™ DNA Extraction Solution (Epicentre Biotechnologies, via Astral Scientific, Gymie, NSW. Cat. No. EPBQ0916S*)*

This method extracted DNA from tissue also using a proprietary solution followed by single heating and cooling steps to lyse cells and denature endogenous enzymes. For application to field use, a heated block and an ice brick were used in the place of the recommended thermal cycler.

This method was included as it appeared simple, with only a single reagent and minimal specialised equipment.

f) Generation® Capture (Gentra Systems, via Progenz, NSW. Cat. No. GC-0050*)*

This method used the spin-column method similar to that of the Roche commercial product. The method was included as the columns were reported by the manufacturer to be effective with lower centrifugation speeds that could be accomplished with the provision of a light, small and cost-effective mini-centrifuge that could be part of the probe capture kit set-up.

g) Tissue boiling (Valsecchi, 1998)

Approximately 50 mg tissue was homogenised in 200 µL lysis solution, using a sterile wooden toothpick. Lysis solution consisted of 1% Triton-X 100, 20 mM Tris-HCl (pH8) and 2 mM EDTA. The tops of the tubes were pierced and the mixture was boiled in a heating block for 12 to 15 minutes.

This method was used for its low cost and simplicity. It was anticipated that the solution would provide a crude extract with limited shelf-life. This would not, however, be of prime concern for the field application.

h) Alkaline lysis (Oakey, 1997)

Approximately 50 mg tissue was homogenised in 200 µL saline, using a sterile wooden toothpick. An equal volume of 50 mM sodium hydroxide solution was added and mixed

well using repeated tube inversion. Mix was heated to 95°C for 15 mins in heating block and neutralised with 1M Tris-HCl (pH7.0) at rate of 4 uL per 25 uL NaOH added.

This method was used for its low cost and simplicity. It was anticipated that the solution would provide a crude extract with limited shelf-life. This would not, however, be of prime concern for the field application.

i) Rapid enzymatic digestion (McClive and Sinclair, 2001)

Approximately 50 mg tissue was homogenised in 100 µL digestion buffer, using a sterile wooden toothpick. Digestion buffer consisted of 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.3), 1 mg/mL gelatine, 0.45% Nonidet40, 0.45% Tween 20 and 200 µg/mL proteinase K. This digestion buffer was prepared previously and frozen in 100 µL aliquots. Homogenates were heated to 55 °C for 5 minutes in a heating block, and then raised to 95°C for 10 mins to denature the enzyme.

This method was used for its low cost and simplicity. It was anticipated that the solution would provide a crude extract with limited shelf-life, although may provide higher quality material than the alkaline lysis method. This would not, however, be of prime concern for the field application.

To conserve previously stored samples of coral-reef finfish, these methods were used in the first instance with tissue excised from a fillet of *Lates calcarifer* (barramundi) discarded from another research project. For each method, performance duration and subjective notes regarding ease of use were recorded.

Suitability of extracts for DNA hybridisation

a) Polymerase chain reaction

To indicate suitability for hybridisation (through primer annealing) and downstream processes using a tried-and-tested procedure, all extracts were used as template material in a universal 16S rDNA PCR reported by Palumbi *et al* (1991). The PCR reaction consisted of 1X polymerase buffer (MBI Fermentas, via Quantum Scientific, Murrarie, QLD), 1.5 mM magnesium chloride, 1 U *Taq* polymerase (MBI Fermentas), 200 µM each dNTP's, 10 µg bovine serum albumin, 5 pmol each of primers 16Sar-L (5'- `cgc ctg ttt atc aaa aac at` -3') and 16Sbr-H (5'- `ccg gtc tga act cag atc acg t` -3'), 5 µL extracted DNA or DNA suspension, and a volume balance to 50 µL of sterile nuclease-free water. Reactions were cycled at 94°C for five mins; 40 X (94°C / 30 sec; 45°C / 30 sec; 72°C / 45 sec); and a final extension of 72°C for three mins. A DNA-free negative control was included. All reactions were resolved using 1.5% agarose gel electrophoresis and ethidium bromide staining. The expected amplicon was 550 bp as determined by comparison with a 100 bp DNA ladder (MBI Fermentas).

Those methods that appeared to suit the purpose were applied to nine species of coral-reef finfish samples archived from a previous project and that had been preserved in a solution of 70% ethanol 30% glycerol. The 16S rDNA PCR was applied to these also to confirm that optimal methods were successful with other species of finfish.

b) Dot-blot membrane hybridisation

Labelled probes were generated from a 16s rDNA oligonucleotide designed to be universal for all fish species (see Experimental procedures 2). Oligonucleotides were labelled at the 3' end with digoxygenin tail with a DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Applied Science, product code 03 353 583 910). Labelling was performed and evaluated according to the manufacturers instructions.

Hybond positively charged nylon membrane was cut to fit a BioRad dot-blot apparatus. Approximately 200 µL 0.4 mol l^{-1} sodium hydroxide were added to the wells and drawn through the membrane with vacuum using a water pump. DNA extracted with optimal methods (above) from *Lates calcarifer* were applied in 300 µL aliquots to replicate wells of the apparatus, drawn through the membrane, further treated with 100 µL alkali to maintain denaturation, incubated for two minutes at room temperature and finally all the liquid was drawn through the membrane. The membrane was removed from the apparatus and dried at 80°C for two hours.

Membranes were incubated with 50 mL hybridisation buffer containing 5 X SSC[1], 1% blocking agent (Roche Applied Science), 0.1% N-laurylsarcosine and 0.02% sodium dodecyl sulphate (SDS) at 42°C for two hours in a Hybaid rotisserie hybridisation oven. The buffer was discarded and replaced with 36 mL fresh hybridisation buffer containing approx 15 ng mL^{-1} labelled oligonucleotide (8 µL) that had been denatured by boiling for ten minutes and placed on ice. Membranes were incubated with the probe at 42°C overnight in the rotisseries oven. Hybridisation was followed by a series of high stringency washes at 42°C. The washes consisted of twice for five mins in 2X SSC containing 0.1% SDS; twice for 15 mins in 0.5X SSC containing 0.1% SDS.

Hybridisation was detected using anti-digoxygenin alkaline phosphatase conjugate (Roche Applied Sciences) and chromogenic reagents nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-indolyl phosphate (BCIP) (Roche Applied Sciences) according to the manufacturers instructions.

Those DNA methods that were shown to support hybridisation of the 16S rDNA probe were used in similar experiments using ten species of coral reef finfish.

Results and Observations

All methods initially were used and optimised through extraction of DNA from a fillet of *Lates calcarifer* and were compared with respect to ease of use, time, necessary equipment and cost and purity of the resulting DNA. The results are described in Table 1.

The 16S rDNA PCR amplified the expected amplicon of approximately 550 bp from *Lates calcarifer* in addition to the nine coral reef finfish specimens tested. All DNA extraction methods yielded DNA of amplifiable quality although the volumes of extracts needed to obtain a robust and visible product using agarose gel electrophoresis varied according to the method. The results are shown in Table 2.

The 16S rDNA oligonucleotide probe designed to be universal for all fish species hybridised to DNA extracted with the High Pure method for all species tested with the dot-blot method, as shown by chromogenic reaction. The other extraction methods did not all support probe hybridisation, as shown in Table 2. Methods that were shown to be unsuitable for downstream purposes, or did not support PCR as well as the other methods, were not tested with all species and were excluded from the project prior to testing. These results indicate that the tissue boiling method yielded the most consistent results, with extracts from all species showing a visible hybridisation signal. Using the same volume of extract for direct comparative purposes, the other three methods yielded insufficient material to visibly hybridise to all species.

[1] 20X SSC stock: 3M Sodium chloride, 0.3M trisodium citrate (pH7.0)

Table 1. Comparison of DNA extraction methods

Key to methods: 1 = High Pure commercial kit; 2 = Dynabeads; 3 = EDNA HiSpEx™; 4 = Lyse-N-Go™; 5 = QuickExtract™; 6 = Generation® Capture; 7 = Tissue boiling; 8 = Alkaline lysis; 9 = Rapid enzymatic digestion

	Method number (refer to Table key)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Minimum time taken to perform extraction from ~50 mg tissue (total duration in minutes)	60	25	25	5	10	25	5	20	20
Minimum time taken to perform extraction from ~50 mg tissue ("hands-on" duration in minutes)	20	10	5	2	3	15	2	5	5
Number of heating blocks needed	2	1	1	1	2	1	1	1	2
Number of variable volume piston-action pipettors needed (max vol)	2 (1mL, 200µL)	1 (1mL)	1 (1mL)	1 (1mL)	1 (1mL)	1 (1mL)	1 (1mL)	2 (1mL, 200µL)	1 (1mL)
Requirement for other specialised equipment	+[1]	+[2]	-	-	-	+[3]	-	-	-
Necessity for electricity supply (number of sockets)	+ (3)	+ (1)	+ (1)	+ (1)	+ (2)	+ (2)	+ (1)	+ (1)	+ (2)
Subjective ease-of-use (1 = difficult/fiddly, 5 = simple)	1	1	3	5	2	1	5	4	2
Cost of materials/reagents per extraction (AUD, 2005)	5	2	2	4	5.5	4	<1	<1	<2

1: High-speed micro-centrifuge (with up to 13,000 x g)
2: Proprietary magnetic stand for immobilisation of magnetic beads, re-usable
3: Micro-centrifuge, high speed not necessary (can be performed with speeds of ~5000 x g)

Table 2. Comparison of DNA extraction methods for fit-for-purpose in this project (probe hybridisation)

Key to amplification scores: + = visible amplicon; ++ = strongly visible amplicon; - = no visible amplicon; weak = barely visible with agarose gel electrophoresis; NT = not tested (method excluded before this step of the experiment)

Key to hybridisation scores: + = clear chromogenic reaction; - = no visible chromogenic reaction; NT = not tested (method excluded before this step of the experiment)

Key to species used as DNA source: a = *Lates calcarifer*; b = *Plectropomus maculates*; c = *Cromileptes altivelis*; d = *Lutjanus erythropterus*; e = *Plectropomus laevis*; f = *Plectropomus areolatus*; g = *Plectropomus leopardus*; h = *Lutjanus sebae*; i = *Lethrinus olivaceus*; j = *Lethrinus nebulus*; k = *Scomberomorus commerson*

		Method number (refer to Table 1 key)								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Were the extracts of sufficient purity/quality for amplification of 16S rDNA as an indicator of oligonucleotide binding and presence/ absence of annealing inhibitors?	a(1µL) a(2µL)	++ NT	weak ++	++ NT	weak weak	- +	++ NT	++ NT	++ NT	weak weak
	b(1µL)	++	-	-	++	-	NT	+	++	weak
	c(1µL)	++	+	++	++	-	NT	++	++	weak
	d(1µL)	++	NT	+	++	-	NT	++	++	NT
	e(1µL)	++	NT	-	+	NT	NT	++	++	NT
	f(1µL)	++	NT	-	-	NT	NT	+	weak	NT
	g(1µL)	++	NT	+	+	NT	NT	++	+	NT
	h(1µL)	++	NT	++	+	NT	NT	++	++	NT
	i(1µL)	++	NT	+	+	NT	NT	++	++	NT
	j(1µL)	++	NT	-	+	NT	NT	+	weak	NT
Were the extracts of sufficient purity/quality for hybridisation of a 16S rDNA probe when DNA was immobilised?	a	+	NT	+	+	NT	NT	+	+	NT
	b	+	NT	+	+	NT	NT	+	+	NT
	c	+	NT	weak	+	NT	NT	+	weak	NT
	d	+	NT	weak	+	NT	NT	+	weak	NT
	e	weak	NT	+	+	NT	NT	+	weak	NT
	f	+	NT	+	-	NT	NT	+	weak	NT
	g	+	NT	weak	+	NT	NT	+	weak	NT
	h	+	NT	-	+	NT	NT	+	+	NT
	i	weak	NT	weak	weak	NT	NT	weak	weak	NT
	j	+	NT	+	-	NT	NT	+	weak	NT
	k	+	NT	+	+	NT	NT	+	+	NT

Discussion

All assayed extraction methods yielded DNA with the capacity to bind oligonucleotides, although some apparently had higher yields and/or quality than others as seen by the volumes of template required for robust amplification. Hybridisation to immobilised DNA was also shown to be successful for the extraction methods that performed best with PCR. For the optimum quality the methods can be ranked into three groups: those that provided DNA for optimum reactivity (the High Pure kit, HiSpEx™, tissue boiling and alkaline lysis); those that gave a lower yield or retained endogenous inhibiting agents (Dynabeads and QuickExtract™); and those that have inferior quality (Lyse-N-Go™ and rapid enzymatic digestion).

The HighPure kit was included as a known standard with which to compare the other methods. It is unsuitable for a field test as it requires laboratory equipment such as a high-speed microfuge and multiple power outlets. The Generation Capture kit was eliminated from the application to the field test for similar reasons. Of the remaining optimal methods, the tissue boiling and alkaline lysis were selected for on-going work as a result of their reduced cost and ease of use. Tissue boiling was the fastest method and the simplest to perform and will be used in preference to alkaline lysis in the following experimental procedures. If it becomes necessary to consider alternative techniques

resulting from unanticipated problems with this method, other methods such as Lyse 'n' Go could be re-evaluated using higher volumes in the hybridisation reactions, or similar re-optimisation techniques.

1.2. Determination and optimisation of specific and generic (universal) DNA probe sequences for probe capture of specific coral reef finfish

Summary

Rationale: Mitochondrial DNA (mtDNA) sequences have been obtained for the targeted fish species, with partial 12S, 16S and cytochrome B sequences determined at a consensus level for replicate samples (n=3-7) of each species. These sequences were determined within a previous QPIF (QBFP) funded project. Within the 12S and 16S genes apparent species-specific sequences have been identified, abutted by an apparent conserved sequence. This phase of the project was intended to confirm that DNA probes designed from these regions hybridised to the target DNA with the expected specificity.

Experimental design: Probes were identified from these mtDNA sequences. A conserved probe for all species, and species-specific probes, were synthesised commercially, and labelled with digoxygenin (DIG) by adding a 3' tail. These were hybridised using ideal conditions with conventional membrane dot-blot procedures and digoxygenin (DIG) labelling/detection methods. In addition to verifying the specificity of the probes, these initial experiments were intended to determine optimum hybridisation temperatures for the probes. At least one proven conserved probe was re-synthesised with a biotin label.

Performance indicators: Identification of probe sequences and corresponding hybridisation temperatures for generic fish (mtDNA capture probes) and for each species (specific identification probes).

Methods

It was determined by QBFP officers that the required set of detection probes would include:

a) Fish subject to quota/bag size:

- *Plectropomus* spp. (coral trouts) targeting *Pl. maculates*, Pl. leopardus*, Pl. laevis* and Pl. areolatus**
- Species specific *Pl. laevis* (chinese footballer)*
- *Variola louti** and *V. albimarginata** (coronation trout and lyretail)
- Species specific *Lethrinus miniatus* (Red throat emperor)*
- *Lethrinus* spp. to target the emperors including red throat, longnosed, spangled*
- *Lutjanus* spp. to target red emperor*, smallmouth nannygai*, largemouth nannygai*, mangrove jack* (snappers)*
- Species specific *Scomberomorus commerson* (Spanish mackerel)*

b) Protected species / no-take

- Species specific *Cromoleptes altivelis* (Barramundi cod)*
- Species specific *Cheilinus undulates* (Humphead maori wrasse)*
- Species specific *Lutjanus gibbus* (Paddletail / red snapper)
- Species specific *Epinephelus tukala* (Potato cod)
- Species specific *Epinephelus lanceolatus* (Queensland grouper)
- Species specific *Lutjanus boha* (Red bass)
- Species specific *Symphorus nematophorus* (Chinaman fish)

Those species marked with * had been previously included in a QPIF/QBFP mitochondrial DNA sequence database where replicate 16S rDNA, 12S rDNA and Cytochrome b partial gene sequence had been determined for 3-7 individuals that had been confirmed to the species level of identity.

Alignment of these sequences indicated that species specific regions could be identified within the positions 88 to 136 and 267 to 324 of the partial 12S and 16S gene sequences, respectively, as defined by the Palumbi primers. In addition, positions 214 to

230 of the *Variola* spp. 12S sequence appeared specific to this genus and positions 412 to 435 of the *Pl. laevis* 16S sequence appeared specific to this species. The partial Cytochrome b sequences were not considered to be suitable for this project as polymorphism between species was noted at the degenerate third base of codons rather than in a block region as described for the ribosomal genes.

The above analysis was used to examine these potential variable sites for species-specific DNA probes. It was the aim to assess regions within these variable sites for maximum specificity while retaining a common annealing temperature. This process included:

- Alignment with all samples previously tested within the targeted group/species;
- Alignment with other samples of other groups/species previously tested;
- Determination of virtual melting temperatures of probe sequences, and adjustment of length to achieve common melting temperatures without affecting specificity;
- Alignment with species previously untested but included in Genbank, using BLAST searches;
- Examine and eliminate probe sequences with obvious hairpin or stuttering.

The potential probes identified by this process were synthesised as unlabelled oligonucleotides by Proligo (Lismore, NSW). All probes were labelled with a 3' digoxygenin tail as described above. Labelled probes were assessed for specificity and optimum hybridisation temperature and time initially using the dot-blot technique described above with DNA extracted using the tissue boiling method from seven genera of coral reef finfish included in the QPIF database. Muscle samples from all species in the database had been preserved in 70% ethanol: 30% glycerol. Specific probes were labelled with a 3' tail of digoxygenin using a commercial kit (DIG Oligonucleotide Tailing Kit, 2nd Generation, Roche Applied Science, product code 03 353 583 910). Labelled probe concentration was conducted as recommended by the manufacturer. DIG-labelled probe hybridisation was detected as described above.

The QPIF sequence database indicated that there were conserved regions for potential universal-species probes within the 12S and 16S partial regions. To allow for DNA degradation and/or sheering that may occur during the extraction process, conserved regions were examined that were closely located to the species-specific regions. Hence, potential universal probes corresponded to 12S positions 66 to 86, 166 to 199, 230 to 260 and 328 to 360, and to 16S positions 98-132, 228-259, 472-498 and 526-602. As for the specific probes these potential universal probes were similarly examined, synthesised and tested. These universal probes were labelled with biotin as opposed to digoxygenin. Biotinylation was conducted during oligonucleotide synthesis by the service provider (Proligo, Lismore, NSW). In preparation for the different strategies of the proposed dipstick technique, universal probes were designed to have similar melting temperatures to the specific probes, and another set to have melting temperatures below those of the specific probes.

As described in the results, the NBT/BCIP chromogenic detection of specific probe binding was unsuccessful and an alternative method was used to determine specificity. The alternative method used streptavidin coated plates to bind the biotinylated universal probes pre-bound to target DNA, and the DIG-labelled specific probes were applied to the bound complex. Target DNA was prepared by PCR to amplify the 16S region described by Palumbi *et al* (1991). Amplicons were purified using a commercial kit (QIAquick PCR purification, QIAgen, VIC), eluted in 50 µL water and diluted with a further 50 µL water. Amplified material was used to assess probes to reduce potential variables and/or sensitivity-induced false negatives from the DNA extraction processes.

Variable concentrations of biotinylated universal probe 16S-1 were added to 100 µL 1.5X hybridisation buffer and denatured at 95^{0}C for 5 mins Similarly denatured target amplicon (50 µL) was added prior to placing in 3X washed streptavidin coated plates. Plates were incubated at variable hybridisation temperatures for one hour before

washing three times in 0.5 X SSCT[2], Variable concentrations of DIG-labelled specific probe was added to 1 X hybridisation solution, denatured, added to plates and incubated for a further one hour before washing four times in 2 X SSCT+1% sodium dodecyl sulphate and once with phosphate buffered saline (PBS). Hybridisation was detected with anti-DIG Fab fragments conjugated to horseradish peroxidase (HRP, Roche Biosciences) as variable concentrations in a solution of 100 mM Tris-HCl, 150 mM sodium chloride and 3% blocking agent (Roche Biosciences). The antibody solution was added to the plate, incubated for one hour and washed three times in 2 X SSCT. The chromogenic agent tetramethylbenzidine (TMB) was added (100 µL per well) and a chromogenic reaction was detected using a photospectrometer at a wavelength of 620 nm. Following initial testing it was noted that positive reactions could be observed by eye and this was used thereafter unless it was deemed beneficial for a more accurate comparison.

Results

Tables 3a and 3b show the results of the theoretical examination of the potential species-specific probes identified from the variable regions of 12S and 16S partial sequences. For each of the species that required probes, and for which mitochondrial sequence has been determined, Tables 3a and 3b show that theoretical probes could be designed that were species or genus specific and had a melting temperature of 61^{o}C +/- 3.

Table 4 shows the results of theoretical examination of the potential universal probes, with melting temperatures similar to the specific probes and with a melting temperature of approximately 10^{o}C lower than the probes. The PCR primers described by Palumbi *et al* (1991) are included as potential universal probes, to be considered as "back-ups".

The probes selected for testing using the dot-blot technique are listed in Table 5. This table lists also the melting temperatures determined empirically by the manufacturer. It can be seen that there is some variation between this temperature and that determined using a predictive internet source.

The dot-blot technique showed the biotinylated universal probes 16S-1 and 16S-3 hybridised to the seven tested genera of coral-reef finfish at a number of hybridisation temperatures, hybridisation signal intensity increased as the hybridisation temperature increased towards the melting temperature of the probe sequence. The 12S universal probes were less successful. These results are summarised in Table 6.

The dot-blot technique using chromogenic detection of DIG-labelled specific probes was not successful. Signals were weak or hidden by high background, or results were confounded when reducing the stringency and consequent lack of specificity. Reducing the hybridisation temperature rendered probes non-specific. Raising the hybridisation temperature appeared to increase the background and it was considered that the higher temperatures may have affected the protein component of the blocking solutions. Further modifications such as addition of poly-adenine to the hybridisation process had no effect. Increasing the hybridisation time resulted only in increasing the level of background further. Changing the tail from DIG-labelled adenine to DIG labelled cytosine served to reduce overall signal but did not affect contrast between target and background. Hence, the technique was abandoned in favour of using the capture probes and streptavidin coated plates as this more closely represented the project aims.

The results of the initial testing of the streptavidin plate process, following optimisation of the process, are shown in Table 7. Further results of empirical testing of universal and specific probes are shown in Table 8. To test and compare the universal probes, equal concentrations of 16S-1 and 16S-2 were applied to replicate streptavidin coated wells and tested as described above using specific probes shown to successfully bind to target PCR amplicons during the initial testing. 12S probes were not tested as the dot-blot technique showed these to result in a weaker hybridisation for reasons unknown. Table 8a shows that 16S-2 appeared consistently to bind more target DNA than 16S-1 producing a markedly stronger reaction when all other factors (amplicon concentration, specific probe concentration and all test conditions) were equal. 16S-2 was used as a

[2] SSC with 0.1% Tween 20

capture probe in subsequent tests. Background/non-specificity of the capture reaction was not apparent as using 12S-derived specific probes did not react with the 16S amplicons captured by the 16S universal probes. The remainder of Table 8 shows that some probes appeared more specific than others, with the following probes performing as expected: vario16 for *Variola* spp; lutjan2 for *Lutjanus* spp.; lethrin16 for *Lethrinus* spp; plectro16 for *Plectropomus* spp; plaevis3 for *Pl. laevis*; and caltiv1 for *C. altivelis*. Some probes, however, showed some mild cross reactivity, but this was always weaker than hybridisation to the target amplicon and might be eliminated with DNA extracted from tissue rather than amplified 16S region. Further testing with alternate detection techniques was designed to investigate this.

Using a similar technique, universal probes 12S-1 and 12S-2 were used as capture probes with the specific probes lminia12, cundul12 and lethrin12. Assayed with target 12S amplicons from target species and non-target species, the 12S complex was excluded from further evaluation in the project as neither the target species nor other species reacted.

Table 3a. Theoretical examination of potential species-specific probe sequences

Target Species	Target region (specific region)	Probe sequence 5′ to 3′ within target region	Conserved in replicate samples of species	Specificity to target species/ genus	Virtual melting temperature (°C)	Hairpins/ stuttering potential identified
Plectropomus spp.	12S (90-134)	CAAGATGCATATTTCAGTGCGATTTTTCGTATTYTCTTC	Yes	Yes	61	No
Plectropomus spp.	16S-1 (267-324)	TAGATCATACCCTCTGTAAYTAAACAAAAACAATRCAAGCCTAATGATACCTC	5/53 mis-match with *Pl. laevis;* 9/53 with *Pl. areolatus*	Yes	63	No
Plectropomus spp.	16S-2 (370-402)	ACATGGAGTGGGCACACAGTTCCTATAAAC	Yes	Yes	62	No
Pl. laevis	16S (1)	CCATACCCTCTGTATCTATACAAAAACAACACAAGCCTAATGG	Yes	only 7/43 mismatch with *Pl. leopardus* and *Pl. maculates*	64	No
Pl. laevis	16S (2)	GTTATCAGTATTTTTGACCAAAAGAGATCCGGCAAC	Yes	Yes	62	No
Pl. laevis	16S (3)	CTGTATCTATACAAAAACAACAC	Yes	Yes	41	No
Variola spp.	12S-1 (105-131)	CAATATCATCCGTATTCACTGTAACAGGGAAT	Yes	only 4/28 mismatch with *C. altivelis*	59	No
Variola spp.	12S-2 (214-230)	GTGTGTTCCCTCTGCAG	Yes	only 4/17 mismatch with many species	52	No
Variola spp.	16S-1 (271-319)	CACACC**M**CCTAC**Y**CCTATAACAA**Y**AGGC	Yes	Yes	60	No
Variola spp.	16S-2 (373-402)	ACGGAA**Y**GGGAG**Y**ACACGCTCCCG**R**AACC	Yes	Yes	70	No
Lethrinus miniatus	12S (108-122)	CCGTGGTGCACCGTTGAAGG	Yes	Yes	61	No
L. miniatus	16S (279-324)	TAAATAGCAAGAGAATAAACCAAATAAACCCCTATCCG	Yes	Yes	60	No
Lethrinus spp.	12S (88-136)	GAATGTTTCAATT**R**CATCTT**Y**CGT**DR**T**R**CACCGTTAA	Yes	Yes	62	No
Lethrinus spp.	16S-1 (271-322)	ATTTATGTTTAACCTTCTCTAACAAGAGAAGAAAAAC**Y**AAATAAACCCCTA	Would not hybridise *L. miniatus*			
Lethrinus spp.	16S-2 (378-413)	AGGAGCACAA**Y**TACTCCCACAGCT	Yes	Yes	61	No
Lutjanus spp.	12S (105-136)	TGTGATCATTCGTAATTCCTTA**W**TRTTA**K**GG	Would not hybridise *L. argentimaculatus*			
Lutjanus spp.	16S-1 (268-317)	AAACAAGGAC**Y**GAACCGAATGAGCCC	Yes	Yes	63	No
Lutjanus spp.	16S-2 (383-402)	GAGAGCACCCCCTCTCACA**RY**C	Yes	Yes	60	No
Scomberomorus commerson	16S-1 (276-321)	CCTGAACAAAGGACTAAACCAAATGAACCATG	Yes	Yes	61	No

Target Species	Target region (specific region)	Probe sequence 5′ to 3′ within target region	Conserved in replicate samples of species	Specificity to target species/ genus	Virtual melting temperature (ºC)	Hairpins/ stuttering potential identified
Scomberomorus commerson	16S-2 (370-401)	GAGCGGACCGGGAGTACTATCTCCTAA	Yes	Yes	62	No
Cromoleptes altivelis	16S-1 (270-317)	CTTCAACATTAATATCCTGAACATAGGACACGAATAGCTT	Yes	Yes	61	No
Cromoleptes altivelis	16S-2 (377-402)	AAGGACCGAATGTACTACATTTATAACCAAGAGT	Yes	Yes	60	No
Cheilinus undulates	12S (100-134)	AATCTACCGTCCGTATGCCATGTGG	Yes	Yes	61	No
Cheilinus undulates	16S-1 (271-318)	CGTAAACATCTTCAAAACAACGAACCGAACA	Yes	Yes	61	No
Cheilinus undulates	16S-2 (372-400)	GCGGAATGGGCTTACATTCCCTAAG	Yes	Yes	60	No

Table 3b. Further theoretical examination of potential species-specific probe sequences

Target Species	Target region (specific region)	BLAST (comparison with mtDNA sequences published in Genbank*) Matches to expected target species not listed*	Comment	Suitability for practical determination of probe suitability
Plectropomus spp.	12S (90-134)	No other matches		YES
Plectropomus spp.	16S-1 (267-324)	No other matches	Mismatches to some plectropomads cannot be alleviated with shortening as situated mid-sequence	NO
Plectropomus spp.	16S-2 (370-402)	Partial alignment to human, mouse, some fungi, some terrestrial bacteria – low percentages		YES
Pl. laevis	16S (1)	No other matches		YES
Pl. laevis	16S (2)	No other matches		YES
Pl. laevis	16S (3)	No other matches		YES
Variola spp.	12S-1 (105-131)	May crossreact with goby (*Boleophthalmus pectinirostris*)found in Japan/Korea (5/32 mismatches)		YES
Variola spp.	12S-2 (214-230)	No other matches	Tm too low but cannot extend without increasing potential for cross reactivity with multiple other coral reef finfish	NO
Variola spp.	16S-1 (271-319)	No other matches		YES
Variola spp.	16S-2 (373-402)	No other matches	Tm too high but cannot reduce length and retain specificity	NO
Lethrinus miniatus	12S (108-122)	No other matches		YES
L. miniatus	16S (279-324)	No other matches		YES
Lethrinus spp.	12S (88-136)	No other matches		YES
Lethrinus spp.	16S-2 (378-413)	No other matches	Only 4/24 matches with 1 published sequence for *S. commerson*. This was not noted from the samples tested in QLD waters	YES
Lutjanus spp.	16S-1 (268-317)	No other matches	May need to reduce annealing temperature to detect *L. argentimaculatus*. As Tm is 63°C this should not be a problem	YES
Lutjanus spp.	16S-2 (383-402)	No other matches		YES
Scomberomorus commerson	16S-1 (276-321)	May hybridise to *Thunnus thynnus thynnus* and some other tuna-like fishes		YES
Scomberomorus commerson	16S-2 (370-401)	May hybridise *Scomberomorus tritor* (West African Spanish Mackerel	Can assume the West African species would not be found in QLD waters	YES
Cromoleptes altivelis	16S-1 (270-317)	No other matches		YES
Cromoleptes altivelis	16S-2 (377-402)	Cross reaction with some grouper		NO
Cheilinus undulates	12S (100-134)	No other matches		YES
Cheilinus undulates	16S-1 (271-318)	No other matches		YES
Cheilinus undulates	16S-2 (372-400)	Possible weak cross reaction with some other wrasse	Keep hybridisation temperature optimum to prevent cross reaction	YES

Table 4. Theoretical examination of potential universal probe sequences

Target region	Probe sequence 5' to 3' within target region	Conserved in QPIF database and Genbank (BLAST)	Virtual melting temperature (°C)	Hairpins/ stuttering potential identified	Comment	Suitability for practical determination of probe suitability
12S-1 (66-86)	CTTACTGCTAAATCCTCCTTC	YES	50	NO	Low-temp option	YES
12S-2 (166-191)	CTACACCTCGACCTGACGTTTTGG	YES	60	NO		YES
12s-3 (230-260)	GGGTAAGCTGACGACGGCGGTATATAGGCGG	YES	68	NO		YES
12S-4 (328-360)	TCTAAAGCACCGCCAAGTCCTTTGGGTTTTAAG	YES		Possible (underlined)	Eliminate option with possible hairpin	NO
12Sa-L (Palumbi)	AAACTGGGATTAGATACCCCACTAT	YES	55	NO		YES
12Sb-H (Palumbi)	GAGGGTGACGGGCGGTGTGT	YES	64	NO		YES
16S-1: (98-132)	GTGCGAAGGTAGCGCAATCACTTGTC	YES	62	NO		YES
16S-2 (228-259)	ATAAGACGAGAAGACCCTAT	YES	49	NO	Low-temp option	YES
16S-3 (472-498)	CATAAGACGAGAAGACCCTATGGAGCTTTAGAC	YES	63	NO		YES
16Sar-L (Palumbi)	CGCCTGTTTATCAAAAACAT	YES	50	NO	Low-temp option	YES
16Sbr-H (Palumbi)	ACGTGATCTGAGTTCAGACCGG	YES	59	NO		YES

Table 5. Oligonucleotide probes selected from Tables 3 and 4 for further testing

OLIGO ID	TARGET SPECIES/GENUS	TARGET REGION AND PROBE SEQUENCE 5' to 3'	ACTUAL MELTING TEMPERATURE DETERMINED EMPIRICALLY BY MANUFACTURER
PLECTRO-12	*Plectropomus* spp.	12S CAAGATGCATATTTCAGTGCGATTTTTCGTATTYTCTTC	68
PLECTRO-16	*Plectropomus* spp.	16S ACATGGAGTGGGCACACAGTTCCTATAAAC	63
PLAEVIS-1	*Pl. laevis*	16S CCATACCCTCTGTATCTATACAAAAACAACACAAGCCTAATGG	62
PLAEVIS-2	*Pl. laevis*	16S GTTATCAGTATTTTTGACCAAAAGAGATCCGGCAAC	67
PLAEVIS-3	*Pl. laevis*	16S CTGTATCTATACAAAAACAACAC	41
VARIO-12	*Variola* spp.	12S CAATATCATCCGTATTCACTGTAACAGGGAAT	61
VARIO-16	*Variola* spp.	16S CACACC**M**CCTAC**Y**CCTATAACAA**Y**AGGC	56
LMINIA-12	*Lethrinus miniatus*	12S CCGTGGTGCACCGTTGAAGG	66
LMINIA-16	*Lethrinus miniatus*	16S TAAATAGCAAGAGAATAAACCAAATAAACCCCTATCCG	66
LETHRIN-12	*Lethrinus* spp.	12S GAATGTTTCAATT**R**CATCTT**Y**CGT**DR**T**R**CACCGTTAA	67
LETHRIN-16	*Lethrinus* spp.	16S AGGAGCACAA**Y**TACTCCCACAGCT	57
LUTJAN-1	*Lutjanus* spp.	16S AAACAAGGAC**Y**GAACCGAATGAGCCC	63
LUTJAN-2	*Lutjanus* spp.	16S GAGAGCACCCCCTCTCACA**RY**C	56
SCOMMER-1	*Scomberomorus commerson*	16S CCTGAACAAAGGACTAAACCAAATGAACCATG	65
SCOMMER-2	*Scomberomorus commerson*	16S GAGCGGACCGGGAGTACTATCTCCTAA	62
CALTIV-1	*Cromoleptes altivelis*	16S CTTCAACATTAATATCCTGAACATAGGACACGAATAGCTT	66
CALTIV-2	*Cromoleptes altivelis*	16S AAGGACCGAATGTACTACATTTATAACCAAGAGT	66
CUNDUL-1	*Cheilinus undulates*	12S AATCTACCGTCCGTATGCCATGTGG	62
CUNDUL-2	*Cheilinus undulates*	16S CGTAAACATCTTCAAAACAACGAACCGAACA	65
UNI-12S-1-LO	All species	12S CTTACTGCTAAATCCTCCTTC	45
UNI-12S-2	All species	12S CTACACCTCGACCTGACGTTTTGG	59
UNI-16S-1	All species	16S GTGCGAAGGTAGCGCAATCACTTGTC	64
UNI-16S-2-LO	All species	16S ATAAGACGAGAAGACCCTAT	49

Table 6. Results of dot-blot hybridisation to universal probes

	Melting temp	Hybridisation conditions	*Plectropomus leopardus*	*Pl. maculates*	*Variola louti*	*Variola albimarginata*	*Lutjanus sebae*	*Lut. malibaricus*	*Lethrinus nebulosus*	*Leth. olivaceus*	*Cromileptes altivelis 1*	*Cr. altivelis 2*	*Scomberomorus commerson 1*	*Sc. commerson 2*	*Cheilinus umdulates 1*	*Ch. undulates 2*
16S-1	64	58°C/1hr	+	+	+	+	+	+	+	+	+	+	weak	+	+	+
		64°C/1hr	+	+	+	+	+	+	+	weak	+	+	+	+	+	weak
16S-2	63	58°C/1hr	+	+	+	weak	weak	+	+	+	+	+	+	+	+	+
		54°C/1hr	ND[1]	ND[1]	+	+	ND[1]	+	+	+	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]	+
		63°C/1hr	+	+	weak	weak	weak	weak	weak	+	+	+	+	+	weak	weak
12S-1	45	40°C/1hr	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
12S-2	59	54°C/1hr	ND[1]	ND[1]	ND[1]	ND[1]	+	+	ND[1]	+	ND[1]	ND[1]	weak	weak	ND[1]	+

ND = hybridisation not detected

1 = high background may be masking any chromogenic signal

Table 7. Initial screen of genus/species probes with the capture probe 16S-2 using the streptavidin plate method described in the text, following optimisation, to test amplified 16S region from some coral reef finfish

Expected positive reactions shaded grey; unexpected results shaded yellow; NT = not tested.

0.5 pmol capture probe; hyb temp 40°C	**Probe (0.5 pmol)**			
	vario16	lutjan2	plectro16	lethrin16
V. louti	0.754	NT	0.063	NT
Pl. laevis	NT	NT	1.489	0.092
Leth. nebulosus	NT	0.244*	NT	0.244
Lut. sebae	0.077	0.244*	NT	NT

*Amplicons were of lower concentration as seen by agarose gel electrophoresis

0.5 pmol capture probe; hyb temp 37°C	**Probe (0.5 pmol)**									
	Lethrin16	Plectro16	Caltiv1	Caltiv3	Lminia16	Lutjan1	Lutjan2	Vario16	Plaevis2	Plaevis3
Leth. nebulosus	1.009	0.068	NT	NT	0.069	NT	NT	NT	NT	NT
Cr. altivelis	0.080	0.171	2.565	1.610	NT	NT	NT	NT	NT	NT
Lut. sebae	NT	NT	NT	NT	NT	0.963	0.787	0.083	NT	NT
V. louti	NT	NT	NT	NT	NT	0.070	0.065	1.033	NT	NT
Pl. laevis	NT	NT	NT	NT	NT	NT	NT	NT	1.518	1.897
Pl. maculatus	NT	NT	NT	NT	NT	NT	NT	NT	1.068	0.055

0.5 pmol capture probe; hyb temp 37°C	**Probe (0.5 pmol)**								
	scommer2	scommer1	Lminia16	Plaevis1	Plaevis2	Cundul16	Plectro16	Caltiv1	Caltiv3
Sc. commerson	1.588	1.314	NT	NT	NT	NT	NT	NT	NT
Leth. miniatus	NT	NT	0.359	NT	NT	NT	NT	NT	NT
Pl. maculatus	NT	NT	NT	0.667/ 0.950	0.561/ 0.699	NT	NT	NT	NT
Ch. undultatus	NT	NT	NT	NT	NT	1.081/ 1.348	NT	NT	NT
V. louti	NT	NT	NT	NT	NT	NT	NT	NT	NT
Pl. laevis	0.066	0.084	NT	NT	NT	NT	1.324	0.062	0.072
Cr. altivelis	NT	NT	NT	NT	NT	NT	NT	1.528/ 1.203	1.024/ 1.139

Table 8a. Comparison of universal capture probes 16S-1 and 16S-2 at hybridisation temperature 37°C

Key: NT = not tested; - = negative, no reaction; + = positive reaction where number of + is relative to intensity of reaction. Unexpected results shaded yellow

Specific probe	16S amplicon source DNA and capture probe							
	V. albimarginata		*Pl. laevis*		*C. altivelis*		*V. louti*	
	16S-1	16S-2	16S-1	16S-2	16S-1	16S-2	16S-1	16S-2
Vario16	+	++	NT	NT	NT	NT	++	++++
Plectro16	NT	NT	++	++++	NT	NT	NT	NT
Plaevis3	NT	NT	++	++++	NT	NT	NT	NT
Caltiv3	NT	NT	NT	NT	++	++++	NT	NT
Plectro12	NT	NT	-	-	NT	NT	NT	NT
Vario12	-	-	NT	NT	NT	NT	-	-

Table 8b. Probe for detection of *Variola* spp. (Vario16) tested at 37°C

16S amplicon source species	Amplicon target concentration	Hybridisation time	Reaction (+/- or OD 620nm)
Pl. maculatus	40 ng / µL	1 hour	-
Pl. leopardus	40 ng / µL	1 hour	-
Pl. areolatus	40 ng / µL	1 hour	-
Leth. miniatus	40 ng / µL	1 hour	-
Lut. erythropterus	40 ng / µL	1 hour	-
Lut. malabaricus	40 ng / µL	1 hour	-
Leth. olivaceus	40 ng / µL	1 hour	-
Leth. nebulosus	40 ng / µL	1 hour	-
Lut. argentimaculatus	40 ng / µL	1 hour	-
Ch. undulatus	40 ng / µL	1 hour	-
***V. louti* #26**	40 ng / µL	1 hour	+
***V. louti* #27**	40 ng / µL	1 hour	+
***V. louti* #28**	40 ng / µL	1 hour	+
***V. louti* #30**	40 ng / µL	1 hour	+
***V. albimarginata* #29**	40 ng / µL	1 hour	+
***V. albimarginata* #31**	40 ng / µL	1 hour	+
***V. albimarginata* #29**	40 ng / µL	30 min	2.818
***V. albimarginata* #29**	20 ng / µL	30 min	2.38
***V. albimarginata* #29**	10 ng / µL	30 min	1.812
***V. albimarginata* #29**	5 ng / µL	30 min	1.235
***V. albimarginata* #29**	2.5 ng / µL	30 min	0.699

Table 8c. Probe for detection of *Lutjanus* spp. (lutjan2) tested at 37°C for 1 hour

POSITIVE REACTIONS	NEGATIVE REACTIONS
Lut sebae #41	*Pl. maculatus #2*
Lut sebae #42	*Pl. laevis #8*
Lut sebae #43	*Pl. leopardus #15*
Lut sebae #45	*Pl. areolatus #21*
Lut sebae #81	*V. louti #30*
Lut. erythropterus #46	*V. albimarginata #31*
Lut. erythropterus #47	*Leth. miniatus #40*
Lut. erythropterus #48	*Leth. olivaceus #57*
Lut. erythropterus #49	*C. altivelis #66*
Lut. erythropterus #50	*Sc. commerson #75*
Lut. malabaricus #51	*Ch. undulatus #85*
Lut. malabaricus #52	
Lut. malabaricus #53	
Lut. malabaricus #54	
Lut. malabaricus #55	
Lut. argentimaculatus #76	

Table 8d. Probe for detection of *Lutjanus* spp. (lutjan1) tested at 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
Lut sebae #45	*Pl. maculatus #2*
Lut. erythropterus #48	*Pl. leopardus #15*
Lut. malabaricus #52	*V. louti #30*
Lut. malabaricus #53	*V. albimarginata #31*
Lut. malabaricus #54	*Leth. miniatus #38*
Lut. malabaricus #55	*Leth. olivaceus #56*
Lut. argentimaculatus #77	*Leth. nebulosus #61*
Sc. commerson #75 (weak)	*C. altivelis #68*

Table 8e. Probe for detection of *Lethrinus* spp. (Lethrin16) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
Leth miniatus #36	*Pl. maculatus #2*
Leth miniatus #37	*Pl. laevis #8*
Leth miniatus #38	*Pl. leopardus #15*
Leth miniatus #39	*V. louti #30*
Leth miniatus #40	*V. albimarginata #31*
Leth. olivaceus #56	*Lut sebae #45*
Leth. olivaceus #57	*Lut. erythropterus #48*
Leth. olivaceus #58	*Lut. malabaricus #55*
Leth. olivaceus #59	*C. altivelis #66*
Leth. olivaceus #60	*Sc. commerson #75*
Leth nebulosus #61	*Lut. argentimaculatus #77*
Leth nebulosus #62	*Ch. undulatus #84*
Leth nebulosus #63	
Leth nebulosus #64	
Leth nebulosus #65	

Table 8f. Probe for detection of *Plectropomus* spp. (Plectro16) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
Pl. maculatus #1	*V. louti #30*
Pl. maculatus #2	*V. albimarginata #31*
Pl. maculatus #3	*Leth miniatus #36*
Pl. laevis #8	*Leth. olivaceus #56*
Pl. laevis #9	*Leth nebulosus #61*
Pl. laevis #10	*Lut sebae #45*
Pl. leopardus #11	*Lut. erythropterus #50*
Pl. leopardus #12	*Lut. malabaricus #51*
Pl. leopardus #14	*C. altivelis #66*
Pl. leopardus #15	*Sc. commerson #75*
Pl. leopardus #23	*Lut. argentimaculatus #77*
Pl. leopardus #24	*Ch. undulatus #84*
Pl. areolatus #22	
Pl. areolatus #82	

Table 8g. Probe for detection of *Pl. laevis* (Plaevis3) tested 37°C for 1 hr

POSITIVE REACTIONS	NEGATIVE REACTIONS
Pl. laevis #6	*Pl. maculatus #1*
Pl. laevis #7	*Pl. maculatus #2*
Pl. laevis #8	*Pl. maculatus #3*
Pl. laevis #9	*Pl. maculatus #4*
Pl. laevis #10	*Pl. maculatus #5*
	Pl. leopardus #11
	Pl. leopardus #14
	V. louti #30
	V. albimarginata #31
	Pl. areolatus #22
	C. altivelis #68

Table 8h. Probe for detection of *Leth. miniatus* (lminia16) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
Leth. miniatus #36	*Pl. maculatus #1*
Leth. miniatus #37	*Pl. leopardus #14*
Leth. miniatus #38	*V. louti #30*
Leth. miniatus #39	*V. albimarginata #31*
Leth. miniatus #40	*Pl. areolatus #22*
Leth. olivaceus #57	*C. altivelis #68*
Leth nebulosus #61	

Table 8i. Probe for detection of *Sc. commerson* (scommer1) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
Sc. commerson #71	*Pl. maculatus #1*
Sc. commerson #72	*Pl. areolatus #82*
Sc. commerson #73	*V. louti #30*
Sc. commerson #75	*V. albimarginata #31*
Lut. erythropterus #50 (weak)	*Leth miniatus #40*
Lut. malabaricus #53 (weak)	*Lut sebae #42*
Lut. argentimaculatus #77 (very weak)	*Leth. olivaceus #58*
	Leth nebulosus #63
	C. altivelis #66
	Ch. undulatus #84

Table 8j. Probe for detection of *Sc. commerson* (scommer2) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
Sc. commerson #71	*Pl. maculatus #1*
Sc. commerson #72	*Pl. areolatus #21*
Sc. commerson #73	*Leth miniatus #40*
Sc. commerson #75	*Lut sebae #42*
V. louti #30 (very weak)	*Lut. erythropterus #50*
V. albimarginata #31 (weak)	*Lut. malabaricus #51*
	Leth. olivaceus #57
	Leth nebulosus #63
	C. altivelis #66
	Lut. argentimaculatus #77
	Ch. undulatus #84

Table 8k. Probe for detection of *C. altivelis* (caltiv1) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
C. altivelis #66	*Pl. maculatus #1*
C. altivelis #67	*Pl. laevis #8*
C. altivelis #68	*Pl. leopardus #14*
	V. louti #30
	V. albimarginata #31
	Leth miniatus #40
	Lut sebae #42
	Lut. erythropterus #50
	Lut. malabaricus #51
	Leth. olivaceus #58
	Leth nebulosus #63
	Sc. commerson #72
	Lut. argentimaculatus #77
	Ch. undulatus #84

Table 8l. Probe for detection of *C. altivelis* (caltiv2) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
C. altivelis #66 *C. altivelis #67* *C. altivelis #68* *V. louti #30* (very weak) *V. albimarginata #31* (weak) *Lut sebae #42* (weak) *Lut. erythropterus #50* *Lut. malabaricus #51* *Sc. commerson #72*(weak) *Lut. argentimaculatus #77*(very weak)	*Pl. maculatus #1* *Pl. laevis #8* *Pl. leopardus #14* *Leth miniatus #40* *Leth. olivaceus #58* *Leth nebulosus #63*

Table 8m. Probe for detection of *Ch. undulatus* (cundul-16) tested 37°C for 30 mins

POSITIVE REACTIONS	NEGATIVE REACTIONS
Ch. undulatus #84 *Ch. undulatus #85* *Pl. areolatus #82* (weak) *V. albimarginata #31* (weak)	*Pl. maculatus #1* *Pl. laevis #8* *V. louti #30* *Lut sebae #42* *Lut. erythropterus #50* *Lut. malabaricus #51* *Leth nebulosus #63* *Sc. commerson #75* *Lut. argentimaculatus #76* *C. altivelis #66*

Discussion

These results demonstrate that the 16S rDNA region of the mitochondrial DNA can be used to design universal and species-specific probes. The 12S region did not react so well. The results of the universal probes alone showed that the 12S universal probes did not react as strongly as the 16S and this is likely the reason why the capture probe and specific probes in tandem did not produce a visible chromogenic reaction. Hence, the 12S complex was excluded from further testing in this project as the tests described above used amplified DNA so that the DNA extraction methods would not influence the determination of probe specificity. As the 12S did not work sufficiently well to give a reaction with amplified material, it can be assumed that the native DNA would not react either.

It was interesting to note that dot-blot hybridisation worked to demonstrate the universal binding of the generic probes but was not successful for assaying the specific probes. This may be explained by the detection methods, as the chromogenic agents NBT and BCIB are less sensitive than other methods (Roche Biosciences DIG Application Manual for Filter Hybridisation), however, it was expected that sensitivity would be sufficient to detect the DIG-labelled probes binding to the target numbers in amplified material. Nevertheless, the failure of this technique provided the opportunity to test the capture probe mechanism intended for the next stage of the project. Here, biotinylated universal probes served to bind mitochondrial DNA (in this case the 16S amplicon) and immobilise it onto a streptavidin coated surface. After washing away unbound material, the bound complex was exposed to specific probes labelled with digoxygenin. After washing away unbound probe, hybridisation was detected with anti-digoxygenin fragments and the chromogenic agent TMB. This first application of the capture probe system proved to be successful in determining the specificity of probes designed to hybridise within the 16S amplicon. Surprisingly, the probes were seen to be mostly specific even at 37°C, when the dot-blot method was non-specific even at temperatures within 5°C below the melting temperature. Some of the probes showed some weak cross reaction with non-target species but in most cases this was a markedly and visibly less intense signal indicating that optimisation of the final methodology could undoubtedly eliminate this. This will be investigated further in the next section of the project.

As previously stated, it was determined by QBFP officers that the required set of detection probes would include:

a) Fish subject to quota/bag size:

- *Plectropomus* spp. (coral trouts) targeting *Pl. maculates*, Pl. leopardus*, Pl. laevis* and Pl. areolatus**
- Species specific *Pl. laevis* (chinese footballer)*
- *Variola louti** and *V. albimarginata** (coronation trout and lyretail)
- Species specific *Lethrinus miniatus* (Red throat emperor)*
- *Lethrinus* spp. to target the emperors including red throat, longnosed, spangled*
- *Lutjanus* spp. to target red emperor*, smallmouth nannygai*, largemouth nannygai*, mangrove jack* (snappers)*
- Species specific *Scomberomorus commerson* (Spanish mackerel)*

b) Protected species / no-take

- Species specific *Cromoleptes altivelis* (Barramundi cod)*
- Species specific *Cheilinus undulates* (Humphead maori wrasse)*
- Species specific *Lutjanus gibbus* (Paddletail / red snapper)
- Species specific *Epinephelus tukala* (Potato cod)
- Species specific *Epinephelus lanceolatus* (Queensland grouper)
- Species specific *Lutjanus boha* (Red bass)
- Species specific *Symphorus nematophorus* (Chinaman fish)

Those marked with * were genera or species for which the partial 16S rDNA sequence had been determined prior to this project. As a result of this section of the project, it can be concluded that the following probes are likely to be effective:

Plectropomus spp.:	plectro16
Pl. laevis:	plaevis3
Variola spp.:	vario16
Lethrinus spp.:	lethrin16
Lutjanus spp.:	lutjan2
Sc. commerson:	scommer2 (needs further optimisation of hybridisation conditions)
C. altivelis:	caltiv1
Ch. undulates:	cundul2 (needs further optimisation of hybridisation conditions)

Of the species/genera on the list for which sequence data is known, only *L. miniatus* remains yet to be determined as the 16S probe was not specific and the 12S probe was not detected.

1.3. Application and comparison of hybridisation protocols suitable for field testing

Summary

Rationale: Conventional membrane dot blots or Southern blots are not suited to field use because of the specialist equipment, reagents and expertise required. Much of the published methodology related to ELISA-like DNA techniques involve an amplification step that is not appropriate to a field test. This phase investigated the use of various probe capture methods, capture surfaces and capture apparatus to compare sensitivity, ease of use and cost. It was anticipated that the use of mtDNA targets in a test with increased sensitivity would result in amplification being unnecessary as each cell has many thousands of target copies compared with nuclear DNA targets.

Experimental design: The probes identified above were used to examine and compare a number of alternative methods. To reduce costs and time, comparisons initially involved the conserved probe and only one or two of the specific probes, and those methods that appear the better ones were compared using all probes. These include:

a) The use of commercially prepared streptavidin coated microwells. The conserved probe was biotin-labelled and therefore bound very strongly to the streptavidin,

thus immobilising the probe and providing a specific capture platform for denatured mtDNA (or mtDNA fragments if degradation has occurred in a sample). Specific probes were labelled with DIG and the presence of DIG labels detected colorimetrically. The presence of a positive signal should indicate the presence of that species in that sample. A variation of this compared where the probes are both hybridised to denatured target DNA in solution simultaneously and this solution added to the streptavidin coated well.

b) The use of Star-wells®, with increased surface area, and commercial dipstick apparatus, coated in-house with streptavidin and subsequent capture probe mechanisms described above. In-house immobilisation of streptavidin on these surfaces intended to investigate the use of passive binding and also two reported methods of protein-avidin-biotin-complex (PABC) binding (Suter et al., 1989; and a BK-101 biotinylation kit supplied by Sigma). This method was removed form the project following advice from the manufacturer that other consumers had observed significantly high background.

c) The use of commercially available DNA-binding plastic microwells to which the capture probe (unlabelled) was bound and used to capture denatured target mtDNA. DIG-labelled specific probes were applied to bind specific targets. The omission of the capture probe was investigated, where the denatured target DNA binds directly to the coated surface and specific probes were used to determine the presence of target species.

d) The use of commercial DNA-binding solutions to coat microwells, Star-wells® with increased surface areas, and plastic commercial dipstick apparatus. Coated plastics were examined as in (c) above.

e) The use of streptavidin coated magnetic beads with largely increased surface area, and could be immobilised against side of tube for washing purposes throughout the procedure. These beads were tested as platforms for both capture probe mechanisms described in (a) above.

All described methods were compared with respect to accuracy, sensitivity, reproducibility, ease of use and cost.

Performance indicators: Identification of optimal methodology for FIFI development. Preliminary discussion regarding potential commercialisation or publication of FIFI with decisions influenced by methods/commercial products used in optimal procedures.

Methods

For consideration as suitable for a boat-side test, an optimal technique will meet certain criteria. Firstly, it should be portable and not require large, heavy or precision laboratory equipment. Such equipment would be a safety hazard and may not perform on a boat moving in the marine environment. Moreover, the working environment will be subject to salt and dampness in the air that may compromise some precision apparatus. So, while QBFP vessels are equipped with electrical power, apparatus such as thermal cyclers or hybridisation ovens are not considered suitable for a boat-side test. Secondly, the technique should not include noxious, toxic or corrosive chemicals that even with the utmost care may be a safety hazard in such an unstable environment. Thirdly, the technique should be sufficiently simple that experienced laboratory personnel are not required, and the test can (with minimal training) be performed by QBFP officers. And lastly, the test should be sufficiently robust to provide confidence in its results, so that QBFP officers may determine the necessity for confiscation and demonstrate the reasons to the fisherperson suspected of contravening the regulations. With these considerations, a number of potential techniques to employ the DNA probes were investigated, as described below.

a) *Commercially prepared streptavidin coated microwell plates*

This method was used to assess the specific probes as described above, using amplified target material. Here, the method was assessed for adaptation to DNA extracted from

tissue using the tissue boiling method. After boiling in a heating block the tissue extract was snap-chilled using a cold block (Promega).

i) The first experiment used a modified version of the method described by the commercial company Pierce[3]. Biotinylated universal probe 16S-1 was diluted to 20µM in double strength (2X) hybridisation buffer. Specific probes were diluted to approximately 50 nM in 2X hybridisation buffer. Fifty µL universal probe were mixed with an equal volume of specific probe and heated to 60°C using a shaking heating block. One hundred uL denatured DNA was heated also to 60°C, then the DNA was quickly added to the probe mixture. The mixture was incubated at 60°C with shaking for 1.5 hours.

   Meanwhile, commercially prepared high-binding capacity streptavidin coated plates (Reacti-Bind™ High Binding Capacity (HBC) plates, Pierce, via Quantum Scientific, QLD) were washed three times with 5X SSCT. Hybridised DNA was added to the microwells and incubated for a further one hour at 60°C. Microwells were washed three times with 0.5X SSCT, soaked for 15 mins and washed a further three times. Hybridisation was detected with anti-DIG Fab fragments conjugated to horse radish peroxidase (Roche Biosciences) in a 1/1000 solution with 100 mM Tris-HCl, 150 mM sodium chloride and 1% blocking agent. The antibody solution was added to the plate, incubated for one hour and washed three times in 0.5 X SSCT. The chromogenic agent 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was added (100 uL per well) and a chromogenic reaction was detected using a photospectrometer at a wavelength of 405 nm.

ii) In the second experiment the biotinylated probe and DNA were added to the strepatavidin coated microwells. Biotinylated probe was serially diluted from 20 µM to 0.5 µM to determine if capture probe concentration was a determining factor. Capture probe and DNA was incubated for one hour at 60°C and plates were washed three times with 0.5 X SSCT. Specific probe was similarly serially diluted and added to the washed wells in 100 µL aliquots and incubated for one hour at 60°C. Plates were washed four times in 2X SSCT. Hybridisation was detected as described above.

iii) To determine between lack of specificity and failure of technique, two probes demonstrated to work using amplified material were re-assessed using the first method of adding both probes to DNA rather than pre-capture. Here, 20 µM both probe types were used as otherwise described in (i) above. The chromogenic reagent was changed to TMB.

iv) In attempt to overcome non-specific binding, an alternative commercial blocking solution of 5X SSC, 0.1% Tween 20, 1% blocking agent, 1 µg/mL poly (A) and 5 µg/mL poly (dA) was assayed (Superblock, Pierce via Quantum Scientific). This was used to dilute capture probe to 20 µM and added to streptavidin coated plates for 30 mins. After washing equal volumes of blocking solution and DNA extract containing 20 µM specific probe were added to the microwells and incubated at 60 °C for one hour. Hybridisation detection was performed as above using TMB.

v) To investigate the non-specific binding of the Anti-digoxygenin fragments to the microwells bound to biotinylated probe, and possible reaction between the biotinylated capture probe and the chromogen, a serial dilution (100 nM to 16 pM) of probe was bound to the microwells as described above. No DNA and no specific probe were used, but anti-DIG and TMB were applied. Anti-DIG and TMB were also applied to microwells to which no biotinylated probe had been added, and TMB was added to microwells that had been coated with the probe.

vi) Having determined that the antibody conjugate was binding to the wells, different blocking solutions were assayed: 1% blocking solution with 1% and 5% foetal bovine serum (FBS), 1% and 5% FBS with no blocking solution, 1% blocking solution with 10% bovine serum albumen (BSA), and 10% BSA with no blocking solution. Streptavidin coated plates were washed in 5X SSCT, blocked for 30 mins, washed three times with 0.5 X SSCT and conjugate was added at concentrations of 1/800,

[3] Pierce: http://www.piercenet.com

1/1000 and 1/1500 dilutions of the commercial product where dilutions were made in te corresponding blocking solutions. Conjugate was incubated in wells for one hour at 60 °C and plates were washed five times in 0.5 X SSCT.

vii) Having optimised blocking agent as commercial blocking solution containing 5% FBS, the optimum concentration of the commercial blocking agent and the duration for blocking was investigated. Blocking agent at 1%, 2% and 5% with 5% FBS were used as described above (vi) with blocking durations of one minute to one hour and tested with 1/1000 dilution of antibody conjugate. In addition, low-binding capacity streptavidin plates were compared.

vii) Having determined that low-binding capacity streptavidin coated wells and a blocking solution of 2-5% with 5% FBS was optimum to eliminate non-specific binding of antibody conjugate, this combination was assayed using the capture probe/specific probe method of detecting specific DNA. Biotinylated capture probe was diluted in blocking solution to 50 µM and microwells were coated for 30 mins. DNA was heat denatured, mixed with 20 µM specific probe pre-heated to 60 °C, added to washed coated wells and incubated at 60 °C for one hour. Washing and detection were as described above.

b) *Streptavidin coated magnetic beads*

This method was used following the promise of streptavidin binding of the capture probe and providing a much-increased surface area that may increase the sensitivity lacking in the coated microwells.

Approximately 1 µg biotinylated probe 16S-2 was mixed with 100 µL BioMag Streptavidin beads (QIAgen, VIC) and incubated at room temperature for 30 mins to bind. Approximately 500 µL DNA prepared from *P. laevis* #7 and *L. miniatus* #37 *and* by tissue boiling was mixed with an equal volume of 2X hybridisation buffer, added to the beads and incubated for 1 hour at 37°C. The tube of beads was placed on a magnetic stand (QIAgen, VIC) and the liquid removed with a pipette. Specific probe plectro16 was diluted to 20 µM in 1 X hybridisation buffer and 100 µL was added to the beads and incubated for 1 hour at 37°C. Liquid was removed by binding the beads to the magnetic stand and beads were washed twice in 2 X SSCT. Anti-digoxygenin conjugate (1/1000 commercial preparation, Roche Biosciences) was added, incubated at 37°C for 30 mins and beads were washed a further two times before addition of TMB and development of a chromogenic signal. Hybridisation time, stringency of wash solution and number of washes were adjusted to optimise the technique.

Following the manufacturers recommendation, attempts to reduce non-specific binding included pre-washing the beads with different solutions and comparison of different hybridisation buffers. Using probes 16S-2 and vario16, these assays were conducted without adding any DNA so any non-specific binding to the beads could be detected. Wash solutions assayed were:

i) Maleic acid buffer: 0.1M maleic acid, 0.15M sodium chloride, pH 7.5, containing 1% blocking agent;

ii) Maleic acid buffer containing 5% FBS;

iii) 5% FBS.

Hybridisation buffers assayed were:

i) hybridisation buffer from previous experiments,

ii) hybridisation buffer containing poly-adenine.

Finally, to mimic standard filter hybridisation techniques, an extra blocking step (maleic acid buffer containing 1% blocking agent) was added to the procedure prior to addition of antibody conjugate to reduce the non-specific binding.

c) Commercially available DNA binding solutions

DNA binding solutions were examined for binding the capture probe directly to a plastic surface.

i) Polystyrene microwells were washed three times with phosphate buffered saline, and air dried before adding 100 µL Reacti-Bind™ (Pierce, via Quantum Scientific, QLD) and incubating at room temperature for 2 hours. Liquid was removed by aspirating with a pipette. Probes 16S-1 and 16S-2 (unlabelled) were diluted to 5 µM in hybridisation buffer, 100 µL was added to the wells and plates were incubated at room temperature overnight. Liquid was removed by aspiration. To determine if the technique was viable, 50 µL 16S amplicons from *Pl. laevis* and *L. miniatus* were purified (QIAquick columns, QIAgen) were mixed with 100 µL 1.5X hybridisation buffer containing 5 µM specific probes plectro16 and lethrin16 respectively, and added to the microwells. Wells were incubated at 37°C for two hours, washed three times with 0.5X SSCT, soaked in 0.5X SSCT for 15 mins and washed a further three times. Hybridisation detection was conducted using anti-DIG conjugate as described above.

   This method was repeated with modification. Equal volumes of Reacti-Bind™ and water containing the capture probe were premixed in a glass vial (to prevent binding to the mixing vessel) as recommended by the manufacturer, and this was applied to the microwells and incubated overnight at room temperature. This method was applied also to DNA extracted from tissue from the same target species using the tissue boiling method.

ii) To increase the sensitivity of the technique the latter method was modified by using the capture probe/Reacti-Bind™ mixture to coat plastic "dipsticks" (Immunosticks, Nunc) with an increased surface area. This was applied to 16S amplicons from *Pl. laevis* and tissue extracts from the same sample.

d) "In-house" designed platforms

The results of the commercially available immobilisation, capture and detection techniques indicated that a more sensitive and robust method was required. Hence, some novel applications were assayed.

i) It was determined from the techniques above that streptavidin was an effective immobiliser of the capture probe but that increased sensitivity was required. The dipsticks provided greater surface area but were not available with streptavidin coating. Hence, the first in-house method aimed to coat the dipsticks with streptavidin and determine if this would provide the desired sensitivity. Streptavidin does not effectively bind to polystyrene but other proteins such as albumin will do so. The protein-avidin-biotin-capture (PABC) method (Suter e*t al,* 1989) is a technique of immobilising streptavidin onto plastic surfaces. Essentially, this method conjugates biotin to a protein and the biotin is used as a binding surface for a layer of streptavidin.

   EZ-Link™ Biotin-LC-ASA (Pierce, via Quantum Scientific, QLD) was used to prepare biotinylated ovalbumin (Sigma Aldrich) as recommended by the manufacturer for biotinylation of proteins. The ovalbumin/biotin conjugate was diluted to 50 µg/mL in a coating buffer (20 mM Boric acid, 50 mM sodium chloride, pH 8.6) and added to clean cryotubes (Nunc) in 600 µL volumes. Dipsticks (Immunosticks™ with MaxiSorb™ surface, Nunc) were removed from their vials and placed in the cryotubes so that the paddle-like structure was immersed in the conjugate, and sticks were incubated at 37°C for one hour. Dipstick vials were retained. Following incubation, the sticks were washed four times in sterile double-distilled water, air dried and replaced into the original storage vials. Cryotubes used for coating were excluded as they would also have reacted also with the coating solution. Streptavidin (Sigma Aldrich) was diluted in PBS containing 0.1% BSA to a concentration of 5 µg/mL and added to each dipstick vial in 600 µL volumes to cover the coated portions of the sticks, and then

incubated at room temperature for one hour. Sticks and tubes were washed in PBS three times, air dried and stored at 4°C.

The streptavidin coated sticks were used as a capture and detection platform as described above using 16S-2 as a capture probe, plectro16 as the specific probe, 16S amplicons from *Pl. laevis #7*, and DNA extracted from *Pl. laevis #7* with the tissue boiling method.

ii) An alternative to immobilising streptavidin simply so that biotinylated probes could be immobilised was to simplify the process and investigate the direct binding of the capture probe to the plastic surface of the dipstick using cross-linking agents (other than the proprietary React-Bind™ used above). Chemical binding of DNA to a protein surface has limitations as crosslinking chemical agents favour protein:protein crosslinking. Similarly, when binding DNA to plastics. However, the Immunosticks (Nunc) used as dipsticks above, have a proprietary coating that binds proteins (Maxisorb) as these consumables are intended for binding antibodies. In preparation for binding the oligonucleotide capture probes to the Maxisorb surface of the dipstick, two strategies were investigated. Firstly, the probe 16S-2 was resynthesised by a commercial provider (Proligo, NSW) with a 5' amine group for direct binding, and secondly, 16S-2 with a 5' phosphate group was bonded with a diamine spacer. The spacer was to examine if the formation of a spatial "brush-like" layer of capture probe may work more effectively to capture the surrounding target mitochondrial DNA than the rigid tight structure where probe is bound directly to the surface.

To synthesise the diamine spacer, 5' phosphorylated 16S-2 was diluted to 15 nmol/10 µL in a reaction buffer (10 mM sodium phosphate, 0.15M sodium chloride, 10 mM EDTA, pH 7.2), and 7.5 µL was combined with 1.25 mg 1-ethy-3-[3-dimethylaminopropyl]carbodiiamide hydrochloride (Pierce, via Quantum Scientific) and 25 µL ethylenediiamine dihydrochloride (EDC) (0.25M in 0.1 imidazole) (Sigma Aldrich). After complete mixing, this was incubated at 37°C overnight. Unreacted EDC and byproducts were removed using a desalting column (Zeba columns, Pierce, via Quantum Scientific). This synthesised 1.25 µMol ethylene diamine content bound to 15 nmol probe in 32.5 µL, assuming reaction reached its maximum capacity. This concentrated probe was stored at -18 °C.

Each dipstick is reported by the manufacturer to bind approximately 500 ng IgG per cm^2, so each stick has the capacity to bind approximately 15 pmol protein molecules. The probe with the spacer was diluted 1/1000 in 0.05M carbonate buffer[4] to provide a working stock and this was further diluted to provide test solutions containing up to 10 pmol, 10^2 pmol, 10^3 pmol and 10^4 pmol. These different concentrations were used because there was no confirmation that the addition of the spacer had worked to maximum capacity.

The 5'-aminated 16S-2 probe was diluted in carbonate buffer to a master stock of 1.5 µM, and this was diluted to a working stock of 1/100. The working stock was further diluted to test solutions containing approximately 10 pmol, 10^2 pmol, 10^3 pmol and 10^4 pmol.

Volumes of 900 µL each test solution from both capture probes were transferred into the dipstick vials, the dipsticks were immersed, and vials were incubated at room temperature in the dark overnight. Sticks were transferred into cryotubes containing PBS with 0.5% blocking agent for 15 mins, then into cryotubes containing 1% sucrose and incubated at room temperature for one hour. Finally the sticks were washed three times in PBS containing 0.05% Tween 20, air-dried, placed in clean vials and stored at 4 °C.

For the initial assessment of this technique, double strength hybridisation buffer was prepared in 450 µL volumes in clean cryotubes. This was made up to 900 µL using 450 µL DNA extract using the tissue boiling method from *Pl. leopardus#12* (tissue positive), 50 µL purified 16S amplicon from *Pl. leopardus #12* and 400 µL

[4] 1.59 g sodium carbonate, 2.93 g sodium hydrogen carbonate, 1L water, pH 9.6

sterile nuclease-free water (amplified positive), or 450 µL sterile, nuclease-free water (negative control). Coated dipsticks were placed into the hybridisation mix and incubated at 37 °C for two hours in a shaking incubator. Sticks were washed three times with a squirt bottle containing 2 X SSCT and placed into vials containing 450 µL double strength hybridisation buffer and 450 µL 50 mM sodium hydroxide (0.4M final concentration) to denature captured mitochondrial DNA. Specific probe plectro16 was added to give a concentration of 5 µM and the denatured DNA was neutralised with 72 µL 1M Tris hydrochloride (pH 7.0) to allow hybridisation to occur between the DNA and the specific probe. Tubes were incubated at 37°C for one hour with gentle shaking and washed three times with 2X SSCT. Detection of hybridisation was made using anti-DIG-POD conjugate as above, and TMB chromogenic agent.

In a brief test for specificity, the diaminated spacer test was repeated using the specific probes Lutjan2 and Lethrin16 at 10^3 pmol with DNA extracted from *Lut. erythropterus#47, Leth olivaceus #56* and *Cr. altivelis #68.* Hybridisation times of 30 mins, one hour and two hours were compared.

Results

a) Commercially prepared streptavidin coated microwells

The series of experiments to optimise the use of commercially prepared streptavidin coated microwells demonstrated that although the method performed, it had low sensitivity. Tables 9a to 9h show the final results where the specific probes consistently produced a stronger chromogenic reaction with the target tissue than non-target tissue when the capture probe 16S-2 was used, however the reaction was so weak that it would be unreliable.

The method recommended by the manufacturers of the high-binding capacity plates (Pierce) was assayed and modified significantly. Table 9a demonstrated that the technique had potential but was non-specific. The non-specificity occurred also when the capture probe was incubated with the DNA prior to addition of specific probe (Table 9b), and the concentration of both probes did not alleviate the problem (Table 9c), however it was noted that the chromogenic reaction decreased in intensity with the lower concentrations of capture probe. The manufacturers recommended commercial blocking agent did not alter the non-specificity (Table 9d). Tables 9e and 9f shows that the non-specific reactions were caused, at least in part, by the direct binding of the anti-digoxygenin conjugate to the HBC microwells, and investigation into a number of blocking agents showed addition of 5% foetal bovine serum (FBS) to a 1% blocking solution (Roche Biosciences) to be most successful in preventing the conjugate binding (Table 9g), although it was not eliminated completely. Finally, the HBC microwells were exchanged for standard streptavidin coated plates (Pierce, via Quantum Scientific) and a marked reduction in non-specific binding of the anti-DIG conjugate was demonstrated in the presence of 2-5% blocking solution (Table 9h).

Following these assays it was determined that the use of HBC streptavidin coated microwells was non-specific and the use of LBC was not sufficiently sensitive for reliable detection of specific binding of probes.

b) Streptavidin coated magnetic beads

Initial assays showed that, as with the streptavidin coated microwells, this method showed a lack of specificity with previously demonstrated specific probe Plectro16 reacting with *P. laevis, L. miniatus* and beads to which DNA has not been added (Table 10a). Experimentation with pre-washing the beads in different wash solutions failed to eliminate the non-specific binding of antibody conjugate, although it was noted that the lowest background was achieved from pre-washing the beads in 1% blocking agent with 5% FBS and using the hybridisation buffer described for previous techniques. The most background was noted from beads that did not contain blocking agent in the pre-wash but did contain FBS. The addition of an additional blocking step dramatically reduced the non-specific binding of the antibody conjugate and false-positive reactions, however the

positive results were weak even with amplified 16S amplicon from the target species (Table 10b and Figure 1).

c) Commercially available DNA binding solutions

Initial assays where microwells were precoated with Reacti-Bind™ showed no reaction. When the binding reagent was mixed with the capture probe prior to coating the microwells, the capture using 16S-2 and hybridisation to specific probes worked with amplified target but the reaction was very weak when applied to DNA extracted from tissue (Table 11). When the method was applied to plastic dipsticks with a larger surface area, the amplified material gave a clearly visible chromogenic reaction, but the extracted DNA did not. No background reaction was noted in the negative control using either the microwells or the dipsticks.

d) "In-house" designed platforms

i) Streptavidin-coated dipsticks using the PABC technique resulted in an intense reaction with the amplified target material, but no reaction with the whole-cell extract or the negative control with no DNA.

ii) Binding capture probe directly to dipsticks with formation of a chemical bond using 5' terminal phosphate molecules of the probe was thought to increase number of capture probe molecules available for DNA binding. This was tested with PCR amplified 16S mitochondrial DNA as a positive control, whole cell DNA as a test and no DNA as a negative control. With capture probe concentrations of 1/10 and 1/100 (10^3 and 10^2 pmols) strong chromogenic reactions were seen with the PCR amplified material, and acceptable obvious signals were seen from the whole cell DNA. However the background signals from the negative control tests were considered unacceptable although obviously lower than the whole cell DNA (Figure 2). Moreover, the expected increase/decrease resulting from capture probe titration was not apparent.

iii) Binding capture probe to dipsticks with formation of dual chemical (amine) bonds and insertion of inert spacer molecule was thought to facilitate washing and elimination of background signals. This was tested with PCR amplified mtDNA as a positive control, whole cell DNA as a test and no DNA as a negative control. This technique showed great promise and sensitivity, with clearly visible signals from amplified and unamplified material. Background was minimal. Results showed the expected increase in chromogenic intensity as probe concentration increased (Figure 3). If left to develop >20 mins the negative reactions start to show a colour change and if left too long could mimic the positive colour reaction. Using a stop-solution of weak hydrochloric acid at 20 mins after development arrested further colour change seen in the tubes, including the negative control.

In a brief test for specificity using the specific probes Lutjan2 and Lethrin16 with DNA extracted from *Lut. erythropterus#47, Leth olivaceus #56* and *Cr. altivelis #68,* the negative controls and non-target species reacted to give very weak colour change while the target species reacted strongly, even when hybridisation time was reduced to 30 mins.

Table 9. Progressive optimisation for using streptavidin coated microwells

a) Initial assessment of method using 16S-1 capture probe and specific probe Plaevis1 (at this time, Plaevis3 had not been determined as the better specific probe for this species). Unexpected results are shaded yellow.

	OD (405 nm)
Sc. commerson #71	0.114
C. altivelis #68	0.098
L. sebae#45	0.137
L. miniatus #40	0.141
V. lout #28	0.120
Pl. laevis #7	0.239
no DNA	0.046

b) Titration of capture probe and specific probe concentration

Capture probe concn	*P.laevis #7* Plaevis1 concn						*C. altivelis #68* Plaevis1 concn					
	20 µM	10 µM	5 µM	2.5 µM	1.2 µM	0.6 µM	20 µM	10 µM	5 µM	2.5 µM	1.2 µM	0.6 µM
20 µM	0.428	0.366	0.474	0.446	0.442	0.543	0.531	0.362	0.459	0.369	0.337	0.450
10 µM	0.363	0.286	0.343	0.374	0.380	0.355	0.342	0.287	0.249	0.293	0.305	0.327
5 µM	0.362	0.305	0.291	0.339	0.337	0.329	0.299	0.287	0.290	0.285	0.380	0.295
2.5 µM	0.337	0.272	0.263	0.281	0.273	0.250	0.267	0.236	0.262	0.255	0.320	0.284
1.2 µM	0.238	0.198	0.179	0.189	0.162	0.156	0.209	0.174	0.186	0.195	0.106	0.190
0.6 µM	0.096	0.081	0.067	0.071	0.065	0.139	0.062	0.066	0.066	0.070	0.083	0.203
0	0.042	0.044	0.045	0.043	0.047	0.044	0.043	0.042	0.044	0.044	0.044	0.044

c) Consecutive use of capture and specific probes shown to work previously with amplified target material

	Pl. laevis #7	*V. louti #28*	*Leth. miniatus #40*	*Lut. sebae #41*	*Cr. altivelis #66*	*Sc. commerson #71*
plectro16	0.640	0.627	0.675	0.708	0.597	0.438
scommer2	0.554	0.529	0.625	0.653	0.494	0.557

d) Change of blocking reagent to commercial preparation *Superblock*

Capture probe	Specific probe	DNA source	OD (620nm)
16S-2	plaevis3	*Pl. laevis #7*	0.516
16S-2	plaevis3	*L. sebae #45*	0.496
16S-2	plaevis3	*C. altivelis #66*	0.583
16S-2	plaevis3	*Sc. commerson #71*	0.574
16S-2	plaevis3	none used	0.491
16S-2	plaevis3	none used	0.589
16S-2	none used	*Pl. laevis #7*	0.507
none used	plaevis3	*Pl. laevis #7*	0.557

e) Chromogenic detection of anti-DIG conjugate binding to biotinylated probe complexed with streptavidin and directly with HBC streptavidin coated microwells.

Biotinylated probe concn	Anti-DIG concn	OD (620 nm)
100 nMol	1/800	0.526
1 nMol	1/800	0.390
500 pMol	1/800	0.410
250 pMol	1/800	0.433
125 pMol	1/800	0.235
62.5 pMol	1/800	0.480
31.25 pMol	1/800	0.517
15.625 pMol	1/800	0.412
none	1/800	0.263
none	1/800	0.289
none	1/800	0.414
none	1/800	0.392
200 pmol	none	0.041
200 pmol	none	0.040
200 pmol	none	0.064
200 pmol	none	0.042

f) The effect of different blocking reagents on the ability of anti-DIG conjugate to bind HBC streptavidin coated microwells

	Conjugate dilution		
	1/800	1/1000	1/1500
1% blocking solution	0.170	0.150	0.126
1% blocking solution + 1% FBS	0.133	0.145	0.111
1% blocking solution +5% FBS	0.127	0.116	0.103
1% blocking solution + 10% BSA	0.209	0.140	0.188
1% FBS	0.424	0.358	0.225
5% FBS	1.488	0.988	0.408
10% BSA	0.507	0.409	0.236
control (no blocking and no conjugate)	0.045		

g) The ability of anti-DIG conjugate to bind to HBC and LBC streptavidin coated microwells with different concentrations of blocking solution (OD620)

Type of microwells	Blocker concn.	blocking duration						
		60 mins	30 min	10 mins	5 mins	2 mins	1 min	none
High binding capacity	1%	0.207	0.186	0.274	0.207	0.171	0.173	0.913
	2%	0.198	0.227	0.228	0.269	0.155	0.124	0.634
	5%	0.216	0.203	0.221	0.185	0.178	0.171	0.610
Low binding capacity	1%	0.191	0.218	0.210	0.164	0.168	0.116	0.828
	2%	0.086	0.093	0.103	0.081	0.078	0.073	0.724
	5%	0.073	0.071	0.085	0.072	0.067	0.065	0.502

h) Performance of optimised technique using streptavidin coated microwell plates (OD620)

capture probe	vario16		lutjan2		lethrin 16	
	V. louti #29	*Sc. commerson #71*	*L. sebae #45*	*Sc. commerson #71*	*Leth, nebulosus #61*	*Sc. commerson #71*
16S-1	0.107	0.099	0.095	0.096	0.121	0.111
16S-2	0.113	0.096	0.104	0.075	0.118	0.092

Table 10. Optimisation of streptavidin coated magnetic beads

Key: intensity of chromogenic reaction given as + = a definite but weak colour change and +++ = a mid/dark blue. Weak = pale blue. Results highlighted yellow are unexpected/undesirable results.

i) Effect of adjusting hybridisation duration, wash stringency and wash number

Hybridisation duration		1:00	1:30	2:00	2:00
Stringency of wash		2X SSCT	0.5X SSCT	0.5X SSCT	0.5X SSCT
Number of washes		wash X2	wash X2	wash X6	wash X12
Target DNA	*Pl. laevis #8*	+++	+++	+++	+++
	L. miniatus #38	++	+	+	+
	No DNA	++	+	weak	weak

ii) Results of a trial involving an additional blocking step prior to adding antibody conjugate

	Relative intensity of chromogenic reaction
P. laevis #7	+
P. laevis #8	+
P. laevis #9	+
L. miniatus #38	-
16S amplicon from *P. laevis #7*	+
No DNA	-/very weak

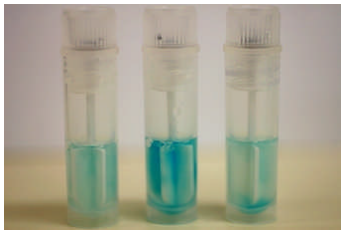
Table 11. Results of assay using Reacti-BindTM to immobilise capture probes

Initial assessment of Reacti-Bind™ using polystyrene microwells (OD_{620})

	16S-1 (capture)		16S-2 (capture)	
	lethrin16	plectro16	lethrin16	plectro16
L. minatus#38 amplicon	0.060	NT	0.199	NT
L. minatus#38 tissue extract	0.051	NT	0.068	NT
Pl. laevis #8 amplicon	NT	0.089	NT	0.277
Pl. laevis #8 tissue extract	Nt	0.061	NT	0.088
no DNA	0.041	0.035	0.040	0.037

Tubes 1-3 were whole cell DNA from *Pl. laevis* #7, #8 and #9 tested with 16S-2 capture probe and specific probe plectro16.
Tube 4 was whole cell DNA from *Leth. miniatus #38* with probes 16S-2 and plectro16.
Tube 5 was PCR amplified DNA from *Pl. laevis #7* with probes 16S-2 and plectro16 (positive control). Tube 6 had no DNA added and was tested for background with probes 16S-2 and plectro16 (negative control)

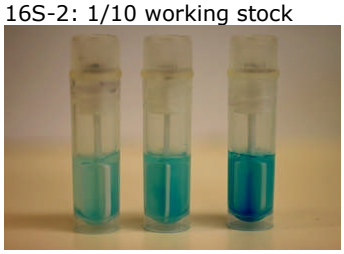
Figure 1. Streptavidin coated beads with capture probe 16S-2 treated with DNA from two species and the specific probe plectro16



Development: 12 mins
Tubes: DNA; PCR; negative
16S-2: neat working stock

Development: 13 mins
Tubes: DNA; PCR; negative
16S-2: 1/10 working stock

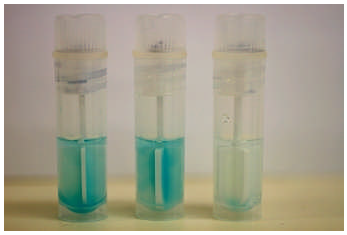
Development: 14 mins
Tubes: DNA; PCR; negative
16S-2: 1/100 working stock



Development: 14 mins
Tubes: DNA; PCR; negative
16S-2: 1/1000 working stock

Figure 2. Results of dipsticks coated with aminated probe 16S-2 bound directly to Maxisorb coated polystyrene (specific probe = plectro16; DNA tubes are tested with *Pl. leopardus* DNA; PCR tubes are tested with 16S amplicon from *Pl. leopardus*; negative tubes contain no target DNA)

Development of 9 mins
16S-2: neat working stock

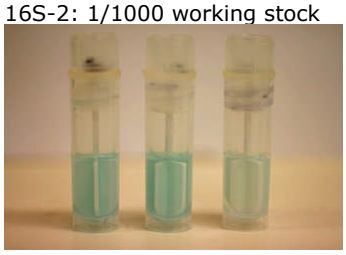


Development of 9 mins
16S-2: 1/10 working stock

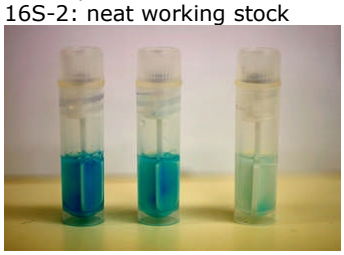
Development of 9 mins
16S-2: 1/1000 working stock

Development of 15 mins
16S-2: neat working stock

Development of 15 mins
16S-2: 1/10 working stock



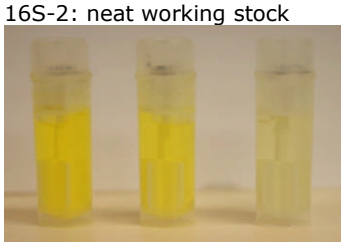
Development of 15 mins
16S-2: 1/1000 working stock



Development of 20 mins
16S-2: neat working stock

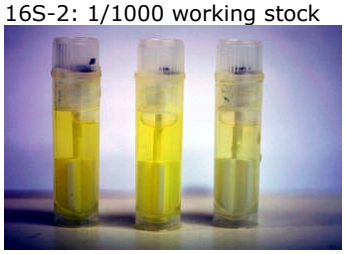
Development of 20 mins
16S-2: 1/10 working stock

Development of 21 mins
16S-2: 1/1000 working stock



Development stop at 20 min
16S-2: neat working stock

Development stop at 20 min
16S-2: 1/10 working stock



Development stop at 20 min
16S-2: 1/1000 working stock

Figure 3. Results of dipsticks coated with diaminated spacer probe 16S-2, specific probe = plectro16. Tubes for each image are (left to right) DNA tubes (*Pl. leopardus* DNA); PCR tubes (16S amplicon from *Pl. leopardus);* negative tubes contain no target DNA

Discussion

The original project proposed the inclusion of Star-wells® coated in-house with streptavidin and commercial DNA-binding plastics in the potential methods of capture probe immobilisation. Subsequent to the proposal, the manufacturers of Star-wells® (Nalge Nunc International) recommended that this method be withdrawn from the project as other consumers had observed significantly high background, presumably from the topology of the microwells. Likewise, the manufacturers of the commercial DNA-binding plastic microwells advised that consumers of the product had reported a lack of sensitivity and they were recommending detection of amplified material only (NucleoLink Microwell plates, Nalge Nunc International). None of the other commercially available methods that were tested here were acceptable for a boat-side test with respect to sensitivity and specificity, although appeared to give desired results when using pre-amplified target material as opposed to DNA extracted directly from test tissue. Extensive experimentation to optimise components of the procedure using streptavidin coated surfaces did not rectify the sensitivity problem but appeared to overcome background signal and hence the initial issues with specificity. Increasing the surface area using

dipsticks coated with streptavidin in-house or commercially coated beads also did not enhance the reaction with DNA extracted direct from tissue. However increasing the surface area and using a biochemical cross-linking process to immobilise the capture probe appeared to provide a platform for sufficiently sensitive detection of mitochondrial DNA from tissue that had undergone a crude heat-induced cell lysis procedure (boiling). The preliminary experiments described indicated that this technique could provide the specificity demonstrated by the probes in the previous section of the project. The potential for this technique for a boat-side test is positive as the dipsticks can be coated with the capture probe in the laboratory and stored. The subsequent technique is viable on board a boat with electric power with the minimum of equipment – only a heating block, a small portable shaking incubator and a few pipettors are required in addition to space in a small on-board freezer.

It is not known why chemical crosslinking gave a more sensitive reaction than the more commonly used streptavidin, although it may be hypothesised that more capture probe became immobilised using this method. Regarding the specificity and lack of background, the direct aminated capture probe binding to the dipstick surface showed an initial background problem similar to the first assays of the other methods, hence the success is possibly a direct result of the spatial access to the probes provided by the addition of a spacer between the probe and the solid surface. The polystyrene surface of the dipstick was pre-coated by the manufacturer with MaxiSorb^{TM} that has a high affinity for polar groups and is designed for adsorbtion of high molecular weight glycoproteins, particularly antibodies. This suggests that binding a smaller amine molecule at the end of a spacer at the 5' terminus of an oligonucleotide would be highly efficient. Certainly, it was noted in the experiments above (Figure 3) that maximum chromogenic intensity was obtained using 1/10 dilution of the probe conjugate, equivalent to 10^3 pmols which is far in excess of the 15 pmols antibody molecules stated by the manufacturer as the binding capacity.

The formation of the "micro-brush" by virtue of the spacer arm may have simplified the washing process thus preventing non-specific binding, trapping or 'clogging up' of or with the detection components such as the anti-DIG conjugate that would subsequently result in a non-specific chromogenic reaction. The manufacturer's technical handbook states that spacer arms are often required because steric effects can limit the distance between potential reaction sites[5]. In other words, it is recommended to include a spacer to maximise potential binding of the conjugate to the substrate (ie, in this case the dipstick), however it follows that the spacer may provide easier access by the tissue DNA and downstream detection reagents including the washing solutions.

Using carbodimide reactions to form conjugates of DNA via a 5' phosphate is not a novel concept, being first described by Ralph *et al* in 1962 and later modified to use EDC as described here (Ghosh *et al.* 1989). However, these early reports describe modification of DNA molecules for downstream processing and further binding to hybrid molecules, and this may be the first report of using such modification to immobilise active DNA probes for hybridisation on a solid surface. Therefore, at this stage of the project a provisional patent of the process was sought and a draft is attached as Appendix to this report.

As only one technique provided the required sensitivity and specificity, a comparison of costs, ease of use etc as stated in the objectives was not conducted. However it was noted that at the time of the project the cost for a single test, including a test tissue, a positive control of suspected tissue source and a negative control would cost approximately AUD20.

[5] Pierce Biotechnology: Crosslinking Reagents Technical Handbook. *www.piercenet.com/xlink95d*

OBJECTIVE 2: To optimise and validate this method as a field test to detect the presence of, and to identify, restricted coral reef fin fish species from samples that have had phenotypic markers removed (such as fillets), and to confirm that cross-reactivity with unrestricted species will not occur

Summary

Rationale: As the method has the intended use of compliance and possible legal proceedings, a full validation of the system is required for integrity and scientific/legal acceptance. Validation should include a study of the limits of detection and examine the ability to detect restricted species in a mixed-species sample, and the ratio of targeted species to non-target species that can be detected.

Experimental design: Following further optimisation, reproducibility and robustness testing, validation should be carried out using the optimised method(s) from (3) above on fresh samples from target species (3 of each of 16 species), other fish included in the Plan (3 each of ~30 species), and other fish found on the Great Barrier Reef that are not included in the Plan (3 each of ~20 species), a total of 250 analyses.

It was intended that positive reactions would be confirmed with mtDNA sequencing and compared to the mtDNA database. This will determine/confirm the specificity of the described test (FIFI). Subsequent methodology adjustments will be investigated as necessary. The sensitivity of the optimised method(s) will be examined firstly through testing FIFI with known concentrations of DNA extractions and tissue masses, for each specific probe. This will be followed by creating samples with known ratios of target species and non-target species, and subsequent testing using FIFI. This will determine the minimum ratio of target species FIFI will detect in a mixed sample (such as one fillet in 10). Subsequent methodology adjustments will be investigated, if necessary, to ensure that FIFI is not so sensitive that it will detect trace levels that result from the use of the same filleting knife, or one erroneous fillet placed in a container of 100 others.

Performance Indicators: Determination and validation of specificity and sensitivity of FIFI, determination of performance of FIFI when applied to mixed samples.

Method

Testing the repetition/reproducibility of the dipstick method showed occasional background of varying degree of intensity, and varying intensity of target tissue when no factors of the test were altered. The following optimisation processes were conducted in an attempt to increase the robustness. Unless otherwise stated, these optimisations were performed at least in duplicate to determine repeatability of the results.

a) confirmation of required capture probe concentration and confirmation of target denaturation

Replicate tests were conducted with capture probe concentrations of 10^2 and 10^3 pmols, specific probe plectro16, and DNA extracted from *Pl. areolatus* #22 and *Cr. altivelis* #67 prepared using the boiling method and the alkaline lysis method.

b) confirmation of required capture probe concentration mitochondrial DNA capture time

Replicate tests were conducted with capture probe concentrations of 10^2 , 5X 10^2 and 10^3 pmols, specific probe plectro16, and DNA extracted from *Pl. leopardus* #23 and *Cr. altivelis* #67 prepared using the boiling method. Capture of mtDNA was ceased after 30 mins, 60 mins and two hours.

c) assessment of washing procedures

It was noted from previous experiments that the intensity of the signal in repeat tests varied and the presence of weak reactions in the negative controls were intermittent. It was observed during the procedure that different operators interpreted the washing steps differently, with washing consisting of either vertical: dipping the stick in and out of the wash solutions or horizontal: with a "swizzle stick"-like action. A series of tests were conducted to determine if the washing method had an effect on signal intensity and

presence of weak background reactions. The tests were conducted with DNA extracted from *Pl. leopardus* #23. Washing variations were:

i) ten vertical dips

ii) 2X ten vertical dips (in two vials of wash solution)

iii) 3X ten vertical dips (in three vials of wash solution)

iv) 1X "swizzle" with five forward and reverse actions and 2X ten vertical dips (in two vials of wash solution)

v) 2X "swizzle" with five forward and reverse actions (in two vials of wash solution) and 1X ten vertical dips

vi) 3X "swizzle" with five forward and reverse actions (in three vials of wash solution)

d) confirmation and optimisation of denaturation and neutralisation of DNA

The dipstick method depends upon the sample being denatured when adding to the capture probe. The inconsistent results may be caused by the re-naturation of the DNA prior to binding to the capture probe. Experiment (a) above noted that the alkaline lysis method produced expected signals when applied to the dipstick, albeit weak. This technique of DNA extraction may be more suited to the boat-side application as temperature may not be as easily controlled as in the laboratory. Effective denaturation was determined by addition of thymol blue which turns blue at pH >10. Neutralisation/renaturation was determined by further addition of bromocresol purple. While thymol blue turns yellow at pH <8, the bromocresol purple will confirm neutral pH >6.8.

i) Finfish tissue (50 mg) was homogenised in 400 µL lysis buffer. Alkaline denaturants at concentrations 50 mM, 1M and 2M were titrated to determined optimum volume to obtain pH>10. Neutralising agents were 1M Tris (pH7) for the 50 mM NaOH, and 4M Tris (pH7) for the higher concentrations.

b) To confirm the denaturation/neutralisation in the present of hybridisation buffer, 450 µL of each denatured reaction, including pH indicator) was added to an equal volume of 2X hybridisation buffer.

c) Excess volumes of alkaline were added to the lysate, mixed with equal volumes of 2X hybridisation buffer, and where this remained alkaline, the neutralising Tris was titrated to obtain neutral pH.

d) The dipstick test was repeated with a modified denaturation protocol of adding 45 µL 2M NaOH to the lysate. After adding 500 µL 2X hybridisation buffer to 400 µL denatured lysate, the mixture was applied to the capture probe coated sticks with simultaneous addition of 100 µL 4M Tris to neutralise and let hybridisation to the capture probe occur. The remainder of the dipstick tests was as described above. The test was conducted using plectro16 specific probe and *Pl. leopardus* #23. A comparison between 2X vertical dips washes (ten dips each) and 2 X swizzle washes was conducted.

e) Optimisation of washing stringency

i) Using the optimised denaturation procedure, triplicate dipstick tests were conducted with plectro16 specific probe and *Pl. leopardus* #23. In one, the wash solutions were SSCT as described above. In the other two the wash solutions were 2X SSCT with 0.1% sodium dodecyl sulphate (SDS) and 5X SSCT with 0.1% SDS.

ii) Using the same sample, a comparison of 2X SSCT and 5X SSCT was made, and one, two and three washes were used at each wash step of the procedure.

f) Increased concentration of blocking agent.

Replicate experiments were conducted using 1%, 2% and 5% blocking solution.

g) Rationalising the number of steps to reduce non-specific binding potential.

The amended DNA denaturation procedure was used with *Pl. leopardus* #23 and bound to the capture probe as previously described. While hybridising, a specific probe-anti-DIG complex was formed from 1µL anti-DIG POD Fab fragments, 2 µL specific probe plectro16 and 10 µL conjugate buffer. This mixture was incubated at 37^{o}C for 30 minutes. After washing the captured DNA, the probe/antibody complex was added to the sticks with 100 µL 4M Tris (pH7) and incubated at 37^{o}C for one hour. Sticks were washed three times with 2X SSCT and hybridisation was detected with TMB.

h) Optimisation of anti-DIG concentration.

It was apparent from the previous experiments that antibody was binding non-specifically to the dipstick. A series of replicate tests were conducted using the probe/antibody complex method with anti-DIG concentrations of 150 mU, 100 mU and 75 mU. The DNA in the positive control tubes was derived from *Pl. leopardus* #23 and the specific probe was plectro16.

As none of the amendments and re-optimisation procedures conducted above improved the robustness and repeatability of the method, the primary theories of the methods were reinvestigated. Firstly, the binding of the capture probe to the plastic dipstick surface was examined. The 5' diaminated probe 16S-2 was modified to have a 3' digoxygenin tail using the same procedure described above for labelling the specific probes. The binding of the probes to the plastic was conducted using titrated dilutions of the probe (1/10 to 1/2500 of working stock, or 10^{3} pmols to 10 pmol), binding was conducted with and without shaking during the process and blocking was compared using four blocking solutions: milk powder (10% solution), bovine serum albumin (10% solution), Startblock (Pierce, via Quantum Scientific) and Superblock (Pierce, via Quantum Scientific).

Binding of 16S mitochondrial DNA to the immobilised capture probe was re-examined using partial 16S amplicons prepared using the Palumbi *et al* (1991) primers as described during the comparison of DNA extractions above. The amplicons were labelled with digoxgenin during the amplification using a PCR DIG probe synthesis kit (Roche Molecular Biochemicals, Cat. no. 1636 090). Concentrations of labelled amplicons were estimated according to the Roche Molecular Biochemicals DIG Application Manual, and standard solutions containing 30 pmol, 60 pmol and 120 pmol were prepared. Hybridisation to dipsticks coated with variable concentrations of capture probe 16S-2, and detection of hybridisation were conducted as described above.

Having determined the steps of the dipstick method worked in principle, it was hypothesised that the lack of robustness may be a result of variable yields of DNA as the confirmation of binding was shown to be reproducibly successful when using amplified target material, yet intermittent when applied to cell lysates. Hence a number of variations were examined that were reported to effectively concentrate DNA. Firstly, an investigative test was examined that concentrated the DNA during the extraction process, meaning a larger tissue sample could be used to obtain the same volume of extractant with a higher concentration of DNA. Muscle tissue from *Pl. leopardus* #23 was cut into approximately 50 mg, 100 mg, and 250 mg pieces. Each slice was homogenised in sufficient lysis buffer to form a loose slurry, boiled for ten minutes, centrifuged at 2500 X g for five mins and the supernatant was removed into a clean tube. One tenth volume of 3M potassium acetate (pH5.5) and two volumes of absolute ethanol were added and mixed by inverting the tube a three to four times. Tubes were incubated on ice for 15 mins and centrifuged at 12,000 X g for ten minutes. The supernatant was discarded, and the pellet was air-dried for 15 minutes before resuspension in 50 µL sterile distilled water. The concentrated extracts were boiled and snap chilled prior to use on the dipstick with the plectro16 specific probe.

A second modification intended to effectively concentrate the DNA extract within the hybridisation solution. This modification was one that could be applied in the field test. In one tube, equal volumes (250 µL each) of 4X hybridisation buffer and 40% polyethylene glycol were prewarmed to 37^{o}C. In a second tube 375 µL 3X hybridisation buffer was mixed with 125 µL 2.7M MOPS and prewarmed. Each was mixed with 500 µL

lysate prepared from 100 mg tissue from *Pl. leopardus* #23 using tissue boiling that had been denatured with snap chilling and addition of 15 µL 2M sodium hydroxide, specific probe plectro16, 4 µL Poly(A) from a 100 µg / mL stock. When mixed and equilibriated to 37 ^{o}C, the mixture was neutralised with 60 µL 4M Tris (pH7) to allow hybridisation to occur while incubating for two hours. Hybridisation was detected as described above using 50 mU anti-DIG.

From the promising results of the latter method, the test was repeated using DNA extracted from twelve non-target species and at different hybridisation temperatures.

Finally, the modifications and optimisations described above were combined and performed in duplicate on target and non-target species that were most likely to cross react using the probe sequence similarities. Each coated dipstick was incubated at 60 ^{o}C with 500 µL 4X hybridisation buffer mixed with 500 µL 40% PEG to prehybridise and block any uncoated plastic. Meanwhile, 100-150 mg test tissue was macerated in tissue boiling solution, boiled for ten mins, mixed with 15 µL 2M sodium hydroxide and snap-chilled in a slurry of ice. Half of the prehybridisation solution was discarded from the dipstick tube and the reminder was rapidly mixed with 60 µL 4M Tris (pH7), 5 µL specific probe and 100 µg poly(A). Finally, 400 µL denatured DNA was added and the stick rapidly replaced and returned to 60^{o}C with gentle shaking for two hours. Each stick was washed twice with 0.2X SSCT preheated to 60^{o}C, using a swizzle action. For each stick 900 µL conjugate buffer with 1% blocking agent was mixed with 50 mU anti-DIG POD and added to clean tubes, the washed dipsticks were added and incubated at room temperature for ten mins. Each dipstick was washed twice with 1X SSCT at room temperature using a swizzle action. Washed dipsticks were placed into 900 µL freshly aliquoted TMB and observed for up to 20 mins or until a blue chromogen formed in the target species (irrespective of the observations in non-target species).

Based upon the results of the previous experiments, no further modification or optimisation was carried out.

Results

For ease of interpretation, all results summaries below refer to results that were seen in all replicates. Where the replicates did not perform similarly, then the more unfavourable results are stated. This is to ensure that the test is not described from its best performance that may not be reproduced in each and every event.

a) confirmation of required capture probe concentration and confirmation of target denaturation

Both concentrations of capture probe produced similar results.

Boiling lysis method produced stronger results than the alkaline lysis method with clearly stronger signal in the target species DNA compared with the non-target species. However, a weak signal was observed in the non-target species that was not present using DNA prepared with the alkaline lysis method.

b) confirmation of required capture probe concentration mitochondrial DNA capture time

Strongest signals were noted from 10^3 pmols and 5 X 10^2 pmols incubated at two hours and one hour respectively. No cross reaction was noted at any time period. No reaction was noted in the negative controls at any time period.

c) assessment of washing procedures

Using one or two dipping steps gave a weak background signal. Using a "swizzle"-like action eliminated the background signal and also reduced the target signal somewhat.

	target DNA	**no DNA**
1X 10 vertical dips	**+++**	**++**
2X 10 vertical dips	**++**	**+**
3X 10 vertical dips	**++**	**-**
1X swizzle, 2X 10 dips	**+**	**-**
2X swizzle, 1 X 10 dips	**+**	**-**
3X swizzle	**+** (weak)	**-**

d) confirmation and optimisation of denaturation and neutralisation of DNA

i) Volumes of alkaline reagents added to 400 µL lysate to obtain pH>10 (as seen by thymol blue as yellow to blue reaction):

50 mM NaOH: 800 µL
1 M NaOH: 20 µL
2 M NaOH: 10 µL

Volume 4M Tris to neutralise (as seen by thymol blue, as blue to yellow reaction):

400 µL lysate + 800 µL 50 mM NaOH: 80 µL
400 µL lysate + 20 µL 1M NaOH: 30 µL
400 µL lysate + 10 µL 2M NaOH: 25 µL

Bromocresol purple confirmed pH >6.8 in all reactions (as seen as yellow to purple reaction).

ii) When alkaline denatured lysates were added to equal volumes of 2X hybridisation buffer, all three immediately turned acidic as seen by thymol blue turning yellow.

iii)

Lysis solution volume	**Alkaline conc. and volume**	**thymol blue reaction on adding 2X hyb.**	**neutralising 4M Tris volume**
300 µL	800 µL, 50 mM	acid	N/A
300 µL	300 µL, 1M	alkaline	200 µL
400 µL	200 µL, 1M	alkaline	150 µL
600 µL	60 µL, 2M	alkaline	150 µL
600 µL	45 µL, 2M	alkaline	100 µL

Overall results show that DNA denatured with 50 mM will renature immediately upon addition of 2X hybridisation buffer and will likely affect the efficiency and sensitivity of the test.

iv) Testing a stronger alkaline denaturant and comparing the dipping washes with the "swizzle" washes showed a very weak background signal with the dipping washes, but no background signal with the "swizzle" washes. In both cases, the target tissue gave a strong signal.

e) Optimisation of washing stringency

i) Dipsticks washed with SSCT showed weak background signal in the negative control. Dipsticks washed in either concentration of SSC with SDS showed no background but a marked reduction in target tissue signal, to a degree that would be unacceptable in an objective field test.

ii) Comparison of 2X SSCT and 5X SSCT showed no apparent effect. The number of washes in a wash step decreased the intensity of the signal. However, the stringency was not sufficient to prevent background signal.

	5X SSCT		2X SSCT	
	#23	**no DNA**	**#23**	**no DNA**
1 wash	+++	+++	+++	+++
2 washes	++	++	++	++
3 washes	+	+	+	+

f) Increased concentration of blocking agent

No difference was observed between the differing concentrations of blocker, and the background signal was of similar intensity to the target tissue.

	1% blocker	**2% blocker**	**3% blocker**
#23	++	++	++
no DNA	++	++	++

g) Rationalising the number of steps to reduce non-specific binding potential

The amended procedure did not alleviate the background signal in negative controls, although it was reduced in some replicates. The intensity of the positive signal was not affected by the shortened procedure.

h) Optimisation of anti-DIG concentration

	Anti-DIG Fab fragments		
	150 mU	**100 mU**	**75 mU**
#23	+++	+++	+++
no DNA	++++	++(+)	+ (weak)

It was markedly apparent that the higher the amount of Anti-DIG added to the tubes, the higher the visible background signal.

Confirmation of the binding of capture probe to plastic dipstick:

BLOCKER	**Static binding (probe dilutions)**					
	1/10	**1/50**	**1/100**	**1/500**	**1/1000**	**1/2500**
MILK	+++++	++++	++++	+++	++++	++
BSA	+++++	++++	++++	++++	++++	++++
STARTBLOCK	+++++	++++	++++	++++	++++	+++
SUPERBLOCK	+++++	++++	++++	++++	++++	++++
	Shaking during binding (probe dilutions)					
	1/10	**1/100**	**1/1000**	**1/2500**	**no probe**	
MILK	+++++	++++	++++	+++	-	
BSA	+++++	++++	++++	++++	+	
STARTBLOCK	+++++	++++	++++	+++	-	
SUPERBLOCK	+++++	++++	+++	++	+	

These results show that the probe was indeed binding to the plastic. A weak non-specific binding was occurring if BSA or Superblock is used, but not when using a milk-based blocker or Startblock. Shaking during the binding process did not appear to have any effect on the efficiency.

Confirmation of capture of 16S mitochondrial DNA:

Capture probe concn.	**Concentration of DIG-labelled amplicon**			
	0	**30 pmol**	**60 pmol**	**120 pmol**
1/50	+ (w)	++++	++++	++++
1/100	+ (w)	++++	++++	++++
1/500	+	++++	++++	++++
1/1000	+	++++	++++	++++
1/2500	+	++++	++++	++++

These results show that the 16S DNA did bind to the immobilised capture probe on the dipstick. It is likely that the lower 30 pmol amount of DNA was sufficient to saturate the captured probes even with the higher probe concentrations, as no variation was observed between probe or amplicon variations. Background was not completely eliminated, but was very weak with the higher probe amounts. This suggested the lower probe amounts did not mask all the plastic and there was some residual non-specific binding of the antibody. This can be interpreted to infer that although the milk based blocker was effective in the previous experiment, the blocker was not completely effective in blocking the naked plastic in a reproducible manner.

Concentration of extracted DNA:

Tissue boiling, no modification		**Physical concn of DNA extract**		**Effective concn in hybridisation**			
				MOPS		**PEG**	
#23	**no DNA**	**#23**	**no DNA**	**#23**	**no DNA**	**#23**	**no DNA**
++	+	++++	+	++	-	+++	-

These results show that, as previously, tissue boiling with no modification showed a relatively weak signal and there was little differentiation between the positive and the weak background where no DNA was added. Concentrating the DNA prior to the test improved the signal and increased the differentiation, which gave promise to the theory that concentration was needed. Superior results were observed when the DNA and hybridisation reaction is effectively concentrated with the addition of PEG to the hybridisation solution. Although no background was observed when using MOPS< the positive signal was markedly weaker than observed using PEG.

Optimisation of hybridisation temperature when using PEG in the hybridisation solution:

	37°C	**42 °C**	**45 °C**	**48 °C**	**55 °C**
Pl. leopardus #23	+++	+++	+++	+++	++
Lut. sebae #45	++	++	+	-	-
Lut. argentimaculatus #76	+	+	weak	-	-
Lut. malabaricus #54	-	-	-	-	-
Leth. olivaceus #59	++	+	+	+	-
Sc. commerson #71	+++	++	++	++	-
Leth. nebulosus #61	++	++	++	weak	-
V. louti #28	-	-	-	-	-
Leth. miniatus #38	++	++	++	+	-
Cr. altivelis #66	+	+	+	+	-
Ch. undulatus #85	+	-	-	-	-
Lut. erythropterus #47	+	-	-	-	-
No DNA	-	-	-	-	-

These results showed that for optimum specificity of the Plectro16 probe, an hybridisation temperature of 55°C is needed. This is representative of the expected temperature early in the project (ie, 8°C below the melting temperature of the probe), that was shown at that time to not support hybridisation using the methods at that time.

Combined method incorporating optimisations and modifications:

Specific probe	**Tissue sample**	**Expected result**	**Observed result 1**	**Observed result 2**
Lutjan	*L. sebae #45*	+	+	w
	L. erythropterus#48	+	+	+
	L. marabaricus #54	+	+	+
	L. argentimaculatus #76	+	+	w
	V. albimarginata #31	-	-	-
	Leth. nebulosus #61	-	-	-
	Pl. areolatus #21	-	-	-
	Leth. miniatus #39	-	-	-
	Cr. altivelis #66	-	-	-
	no DNA	-	-	-
Vario	*V. albimarginata #31*	+	+	+
	V. louti #28	+	+	+

Specific probe	Tissue sample	Expected result	Observed result 1	Observed result 2
	Pl. maculatus #1	-	weak	-
	Pl. laevis #6	-	weak	-
	Leth. olivaceus #59	-	-	-
	L. sebae #45	-	-	-
	L. marabaricus #54	-	-	-
	L. argentimaculatus #76	-	-	-
	No DNA	-	-	-
Lethrin	*Leth. miniatus #39*	+	+	+
	Leth. olivaceus #59	+	weak	weak
	Leth. nebulosus #61	+	+	+
	Ch. undulatus #85	-	-	-
	L. erythropterus#48	-	-	-
	Sc. commerson #71	-	-	-
	Pl. leopardus #23	-	-	-
	V. albimarginata #31	-	-	-
	No DNA	-	-	-
Plectro	*Pl. leopardus #23*	+	+	+
	Pl. maculatus #1	+	+	+
	Pl. laevis #6	+	+	+
	Pl. areolatus #21	+	-	-
	L. argentimaculatus #76	-	-	-
	V. albimarginata #31	-	-	-
	Leth. olivaceus #59	-	-	-
	V. louti #28	-	-	-
	No DNA	-	-	-
Caltiv	*Cr. altivelis #66*	+	+	+
	Pl. laevis #6	-	-	-
	Leth. olivaceus #59	-	-	-
	L. sebae #45	-	+	+
	Sc. commerson #71	-	-	-
	Ch. undulatus #85	-	-	-
	L. marabaricus #54	-	weak	+
	No DNA	-	weak	-
Scommer	*Sc. commerson #71*	+	+	+
	Pl. leopardus #23	-	-	-
	Leth. nebulosus #61	-	-	-
	L. argentimaculatus #76	-	-	-
	V. louti #28	-	-	-
	Cr. altivelis #66	-	-	-
	Ch. undulatus #85	-	-	-
	L. erythropterus#48	-	+	-
	No DNA	-	-	-
Plaevis	*Pl. laevis #6*	+	+	+
	Pl. leopardus #23	-	+	+
	Pl. maculatus #1	-	+	+
	Pl. areolatus #21	-	+	-
	V. albimarginata #31	-	-	-
	V. louti #28	-	-	-
	L. sebae #45	-	-	-
	No DNA	-	-	-
	L. erythropterus#48	-	-	-
Lminia	*Leth. miniatus #39*	+	+	weak
	Leth. olivaceus #59	-	weak	-
	Leth. nebulosus #61	-	-	-
	L. marabaricus #54	-	-	-
	Ch. undulatus #85	-	-	-
	Pl. maculatus #1	-	weak	+
	Sc. commerson #71	-	-	-
	Cr. altivelis #66	-	-	-
	No DNA	-	weak	weak

These results show that despite the rigorous re-optimisation and modification processes, the method is not fully robust and reproducible. On occasions the negative control gives a weak chromogenic signal. Similarly, although all the specific probes react with the target species as expected, there are some that show non-specificity in either one or both of the replicates.

Discussion and conclusions

Despite significant modification and re-optimisation attempts, the dipstick method did not achieve sufficient robustness to demonstrate consistently blank negative controls, or consistent reactions with target DNA. Inconsistent signals in the negative control is not acceptable for a field test where it will not be known if this relates to a weak contamination rendering the test invalid, or if it is a weak non-specific reaction in which case it could be ignored if the positive control shows a strong signal. Similarly false positive results were occasionally seen from cross reaction with non-target DNA. Such results may weaken any future legal investigation or enquiry into the legality of confiscation based upon the result.

It is not known why the inconsistencies occurred. The experiments above demonstrated that each phase of the technique was successful, yet in combination the method was not reproducible. One reason for this may be inconsistencies between batches of reagents, although reagents were mostly purchased from Roche an internationally recognised supplier of biochemicals with a reported high level of quality assurance. Another reason may be inconsistencies between operators, indeed the project laboratory staff changed between the design of the dipstick and the optimisation/modification phase. However, rather than serve as an explanation, if this is the case it serves to further demonstrate the lack of robustness of the method. A boat-side field test would be expected to be sufficiently robust for multiple operators in below-optimum conditions, yet it appears that different operators do not show consistency even in the laboratory and the test is susceptible to even minor changes in operator variations. One such operator variation is washing action (dips or swizzles) which was investigated and demonstrated to have significant effects on the result.

Given the inconsistencies, the practical component of the project (including further evaluation of specificity and sensitivity) was discontinued at this point. While there were sufficient positive results to write a convincing report, the costly continuation into the field use would have illustrated the method's deficiencies.

BENEFITS, FURTHER DEVELOPMENT AND PLANNED OUTCOMES

The work described herein showed a technique with great promise, yet lacked the robustness required for a boat-side test that could be used as a preliminary test for legal proceedings. The reasons for the inconsistencies are not known. Each phase of the technique was shown to perform in isolation, yet in combination the seemingly random occurrences of non-specificity and false positive reactions could not be eliminated, despite rigorous modification and re-optimisation attempts. In legal cases involving high market value seizures, such an unreliable tool would be of more detriment to a prosecution than using no pre-screen at all. Given the lack of robustness, it is considered therefore that QBFP Officers continue to use discretion for determining necessity for confiscation of suspected non-compliances and that the legal cases continue to use mitochondrial DNA sequence determined in the laboratory as proof of species identity.

However, given the partial success of the work (albeit lacking consistency) there are components of the work that will be invaluable for future investigation and application. Since the drafting of this project in 2004, molecular biotechnology has progressed rapidly. Included within this progress are the advances in "point-of-care" testing. Point-of-care testing applies previously laboratory-bound technology to small, often disposable, devices that can be operated either in a practitioners surgery by a medical practitioner, or pen-side by a veterinarian, for a rapid diagnosis or detection of a disease agent. Many of those point-of-care devices currently in use are based upon immunological methods, detecting antibodies or proteins associated with a target agent and would not serve the

purpose of species detection for coral-reef finfish as discussed in the introduction (above) with reference to the protein work of Yearsley *et al* (2001). However, there are many recent publications describing the potential for combining state of the art electronics, chemistry and biology to produce hand-held devices that rapidly detect target DNA sequences. These are so numerous that there a number of dedicated journals to this subject, such as Biomedical Microdevices (published by Springer) or Lab on a Chip (published by the Royal Society of Chemistry). Kumar and Kumar (2008) review recent advances in these biosensors and differentiate the basal technologies. One common theme in all DNA biosensors is the immobilisation of single stranded DNA probes and a measurement of hybridisation between the probes and complementary DNA strands in the target, similar to the basic principles described in the FIFI dipstick. Kumar and Kumar describe immobilisation by adsorption, cross-linking or covalent binding (as with our dipstick). The key advances, however, concentrate on the measurement and detection of hybridisation and may involve changes in electrical conductance or capacitance, fine changes in mass, fine changes in temperature or changes in light transmission. Hence, in simplified terms, a biosensor may be summarised simply as a portable device supporting DNA hybridisation using one of these mechanisms and translating the changes into biologically relevant results

One technique that is commonly referred to in the development of these potential point-of-care test devices is isothermal DNA amplification. Where conventional DNA amplification by the polymerase chain reaction (PCR) requires accurate thermal cycling apparatus and agarose gel electrophoresis resolution methods to produce exponential amplification of low copies of target DNA to easily detectable levels in a few hours, the isothermal methods require only a single temperature after an initial heat denaturation. More recently, the incorporation on DNA helicase to unwind double stranded DNA has eliminated the requirement for the initial heat denaturation step. This helicase-dependent isothermal amplification (HDA) amplifies DNA in a linear manner and can produce detectable concentrations of target nuclear DNA in an hour. HDA is comprehensively reviewed by Jeong *et al* (2009). A recent modification of HDA is EXPAR (Tan *et al*., 2007) where a similar technique reportedly amplifies exponentially thus greatly reducing the required time to detect only 100 fg of target DNA per litre in less than 10 minutes. EXPAR is detected using fluorescence detection of incorporated SYBR-green (changes in light transmission at particular wavelength). However, an even faster mechanism is reported by these authors that couples EXPAR with colourimetric detection of aggregation of gold nanospheres coated with DNA oligonucleotide probes. The colourimetics may be as simple as biotin/streptavidin complexes as assayed in our dipstick method, although the nanoparticles provide a vastly increased surface area than any dipstick or magnetic bead. Preliminary testing of this technique reportedly detects as low as 10 fg per litre in 4 minutes.

Obviously, these techniques are far advanced from the dipstick method described in this project. However, HDA and its derivatives still necessitate species specific primer probes to differentiate target species. In this regard, the work described in this report could, at the very least, provide this basic input data. The described species specific probes in combination with one of the universal probes or primers could be used as species specific primer sets for such devices. These techniques involving amplification may resolve the inconsistencies observed from the described dipstick, as examination of the individual phases of the dipstick test suggests the DNA extraction is still the most likely step to result in variable yields. Amplification would ensure that the number of target molecules increased to detectable concentrations regardless of the initial load, particularly as there are many multiple copies of mitochondrial DNA molecules in each cell of the tissue.

Another method currently receiving interest in the field of point-of-care testing is the PCR array chip. Currently in development, this technique combines polymerase chain reaction (and its associated high sensitivity) with microarray that has reduced sensitivity but the ability to combine multiple assays in a single reaction. Most reports of these solid-phase PCRs have necessitated manual PCR mixing, multiple step pipetting or expensive high precision liquid handling robots.

It must be noted, however, that any amplification methods including HDA, EXPAR or PCR arrays are not always conducive to a field test where contamination risks are high, skilled experienced laboratory staff are not available and the facilities and power supply may be basic. PCR, isothermal amplification or arrays require a thermal cycler or static temperature chamber, and either gel electrophoresis apparatus or laser driven fluorescence detectors. The sub-optimal conditions are exacerbated further when the test needs to be conducted on a boat on the ocean. In addition to common sources of contamination, the marine environment is particularly unfavourable for high-precision apparatus where salt precipitation may cause extreme damage.

Ramalingam *et al* (2009) report a revolutionary experimental device applying microfluid technologies to the PCR array chip whereby small volumes of cell lysate fluids and generic PCR mixtures are applied to a small chip, which is then sealed to prevent evaporation and contamination. Microchannels or microcapillaries in the chip distribute the fluid to multiple independent reactors in the chip. Each reactor is populated with different immobilised primer pairs. The addition of template and amplification mix completes the requirements for a closed system of microvolume PCR. Amplification is measured in real time by the incorporation of SYBR-green and detected by a single wavelength purpose built laser in a miniature thermal cycler constructed specifically for the chips. This method is described more fully in the publication by Ramalingam *et al* (2009), although details of the miniaturised thermal cycler are not provided in the publication, and are presumably patented. With point-of-care testing as the primary goal, these authors have reportedly minimised the risk of cross-contamination by the innovative sealing mechanism of the chip and the application of capillary action to prevent bubble formation and enhance fluid access to the bioreactors. Moreover, this technique has the advantage that multiple groups of primer pairs can be grouped on a single chip. The benefit of this to our coral reef finfish application is that all target species can be tested for in a single reaction, and can be tested using different primer pairs for the same species in different reactor cells for multiple confirmatory tests. According to Ramalingam *et al* (2009) the most difficult part of the development will be identifying the specific primers for any potential application target. This identification of specific probers/probes has been achieved in this project for the coral reef finfish of interest to QNFP officers in Queensland, and would be a valuable precursor to the application of this technology.

CONCLUSION

While the simple approach to a dipstick development described in this report was not entirely successful, recent advances suggest that such a device has great potential using more sophisticated technology based on the probe sequences described herein. Biosensors are a rapidly growing area and the combination of electrochemistry, microfluid engineering and biology are highly likely to produce cost effective usable applications from the prototypes described above in the near future. Indeed, Kumar and Kumar (2008) cite likely potential applications in food and environmental analysis (eg, detection of pathogens or genetically modified organisms), bioterrorism (eg. detection of biological agents), health monitoring (eg. detection of mutations) and diagnostics.

It is suggested therefore, that QPIF and FRDC retain the probe sequences intellectual property as commercial-in-confidence for this eventuality. Large array chips such as that described by Ramalingam *et al* (2009) could include other probes for many species of coral reef and other marine fin fish, extending the client base from QBFP Officers to include, for example, food substitution detection by statutory bodies or large food trade entities such as supermarket chains. The application of the probe sequences to such a device would probably entail a necessary third party with the expertise in biosensor engineering.

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APPENDIX 1: Intellectual property

Provisional patent attached.

APPENDIX 2: Staff engaged on the project

Principal investigator:	Dr Jane Oakey, Senior Scientist, Biosecurity Queensland, Queensland Primary Industries and Fisheries
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