Electrophoretic identification of whitebait species

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Abstract

The shortjawed (SJ) kokopu (*Galaxias postvectis*) is rare and listed as category A conservation status by DOC and as indeterminate (status not resolved) by IUCN. It may be in decline given that there is substantial habitat loss and modification. If existence of SJ kokopu is to be maintained, information regarding its presence within whitebait runs both in terms of timing and geography, is essential. Both SJ kokopu and koaro (*Galaxias brevipinnis*) are involved in the whitebait fishery and commonly occur together with three other *Galaxias* spp. Unfortunately, morphological data do not allow the discrimination between koaro and SJ kokopu whitebait, making it impossible to study the ecology of SJ kokopu whitebait independently.

Using information encoded within the DNA we developed genetic markers from the easily identified adult fish for *G. brevipinnis, G. postvectis*, *G. maculatus* and *G. fasciatus*. Using these markers we screened 48 whitebait morphologically identified as being either *G. brevipinnis* or *G. postvectis*. Of these 48 individuals, 42 were shown to be *G. brevipinnis,* two were shown to be *G. fasciatus* and four individuals produced no genetic markers (i.e. the DNA was degraded). None were shown to be *G. postvectis*. Because none of the whitebait screened were SJ kokopu, we were unable to proceed to identification of distinguishing morphological features. Results have also shown that the traditional method of identification of species using morphological features can be inaccurate.

Introduction

GENERAL BACKGROUND

The primary aim of this study was to develop morphological markers that will allow successful and consistent differentiation between juveniles (whitebait) of shortjawed kokopu (SJ kokopu, *Galaxias postvectis*) and koaro (*G. brevipinnis*).

The SJ kokopu may be in decline given that there is thought to be substantial habitat loss and modification (R. M. McDowall, pers. comm.). The SJ kokopu is quite widely distributed: it has been reported from Northland, Waikato, Bay of Plenty-East Cape, Taranaki, Wellington, Wairarapa, Marlborough Sounds, West Coast, South Westland and Fiordland's south coast (McDowall, 1990). It is thought that it prefers small streams in unmodified native forest, usually mixed broad leaf/podocarp, with pools which have extensive cover such as large bouldery areas (providing deep cavities), overhanging banks, or submerged log jams.

Both SJ kokopu and koaro are involved in the whitebait fishery and since whitebait migrate in mixed species shoals, they commonly occur together

with three other species of *Galaxias: G. maculatus*, *G. fasciatus* and *G. argenteus*. The composition of these shoals can differ consistently between rivers indicating that entry to a river may be closely controlled by environmental factors. Knowledge regarding the presence of SJ kokopu in whitebait runs both in terms of timing and geography is scarce since their whitebait can not be distinguished from that of the koaro, particularly since normally it is probably a matter of distinguishing a few SJ kokopu from a mass of koaro (e.g. McDowall and Eldon, 1980). This has led to the idea that genetic markers within the DNA (DNA being a molecule which does not change in response to environmental factors or aging) should be identified from the easily distinguishable adults. These markers can than be used to distinguish the juveniles which is an essential task if the SJ kokopu is to be conserved successfully.

The objectives of this study were:

1. Develop a DNA-based genetic marker that distinguishes between juvenile koaro and SJ kokopu.

2. Genetic sorting of 100-150 'koaro type' whitebait individuals into either koaro or SJ kokopu.

3. Renewing research into morphological methods which differentiate between koaro and SJ kokopu juveniles that have been clearly distinguished by 'genetic sorting'.

Method

SAMPLES

Controls

Tissue from koaro and SJ kokopu adults was collected from each of four individuals caught in the Kaniere catchment near Hokitika (Striplands Creek, NZMS 2660, 23502:58266). *G. fasciatus* and *G. maculatus* controls (three and four individuals respectively) were isolated from West Coast whitebait specimens and positively identified at species level before DNA extraction.

Test samples

Whitebait samples collection was greatly facilitated by DoC staff who obtained samples personally (DoC Motueka) or collected them from whitebait fishermen (DoC Hokitika). NIWA (Greymouth) also obtained samples from fishermen fishing the Grey and Buller Rivers. Samples were preserved in absolute ethanol prior to sending them to NIWA by courier. Suitable sample sites were chosen on the basis of likely/known presence of adult SJ kokopu. However, within that constraint, samples were collected on the basis of convenience and availability given the limited funds available.

DNA EXTRACTION

The extraction procedure was optimised for the use of relatively harmless reagents and speed. DNA was extracted from fin tissue (adults) or a piece of tissue (5 x 3 mm) resulting in a notch in the back just in front of the dorsal fin (whitebait). This left the specimen virtually intact for future morphological analysis.The block of tissue was placed in 500 μl of extraction buffer (1% SDS, 1 x SET (150mM NaCl, 20 mM Tris, 1mM EDTA pH 8.0) 0.25 mg/ml Proteinase K (Boehringer Mannheim)) in a 1.5 ml Eppendorf tube before incubation at 50°C for three hours. Tubes were cooled to room temperature before the addition of 5 M NaCl to a final concentration of 1.6 M. After centrifugation at 8400 rpm in a microfuge (IEC MicroMax, SciTech, Christchurch, New Zealand) for 10 minutes at room temperature, the supernatant was transferred to a fresh tube. Total nucleic acids were precipitated using an equal volume of isopropyl (propan-2-ol) alcohol (BDH) and incubation at -20°C for one hour. Nucleic acids were collected by centrifugation at 13400 rpm for 10 minutes. The pellet was washed using a 1 ml volume of 70% ethanol (BDH) and recentrifuged at 13400 rpm for five minutes. After air drying for 30 minutes the pellet was suspended in 50 μl sterile deionized water. DNA yield was assessed by UV spectrophotometry at 260 nm. DNA working stocks were prepared in sterile deionized water to a final concentration of 25 ng total nucleic acids/μl.

PCR AMPLIFICATION

Details of the PCR reactions and the background to ribosomal DNA profiles are given in Appendix I. Primers for DNA amplification (unprotected) were synthesised by Custom Primers, BRL Life Technologies, PO Box 12-502, Auckland 6, New Zealand. NS1 was of the sequence ACC CTG GTT GAT CCT GCC AGT and NS8 had the sequence TGA TCC TTC TGC AGG TTC ACC TAC. Reactions (25 μl) consisted of 25 ng total nucleic acids, 100 μM dNTPs, 1 μM each primer, 1 x Boehringer Mannheim Taq reaction buffer and 1 U Boehringer MannheimTaq. The PCR machine used was a Perkin Elmer 2400. The temperature regime used consisted of 94°C 5 min; 94°C 15 sec, 50°C 20 sec, 72°C 1 min (38 cycles); 72°C 5 min.

AGAROSE GEL ELECTROPHORESIS

PCR products were separated and visualised using agarose gel electrophoresis and ethidium bromide staining. Samples (5 μl) were mixed with 1.5 μl loading dye (0.25% bromophenol blue, 40% w/v sucrose), before loading onto a 1% agarose submarine gel and running the samples in 0.5 x TBE (44.5 mM Tris, 44,5 mM boric acid, 1 mM EDTA) containing 0.08 μg/ml ethidium bromide. After electrophoresis bands were visualised using 300-312 nm UV illumination before photographing on 667 Polaroid film using a DS34 Polaroid camera. Results were compared with known controls to identify samples at a species level.

Results

The mixed species whitebait samples were sorted by R. M. McDowall or L. H. Dijkstra into *G. maculatus, G.fasciatus* and '*G. brevipinnis* type' fraction. No *G. argenteus* individuals were found. All *G. brevipinnis* type samples were analysed resulting in data for 13 Motueka River, 12 Buller River, 20 Grey River and three Totara River individuals.

DNA was extracted from a total of 48 fish and the 28S rDNA region was amplified using universal primers and the polymerase chain reaction. While it was intended to study more individuals, they were not supplied. Results were compared to known species standards and are presented in tables 1-4. Known species standards were specifically developed as part of this study and consisted of DNA markers amplified from adult fish for *G. postvectis, G. brevipinnis, G. maculatus* and *G. fasciatus* (see Figures 1 and 2). No *G. postvectis* whitebait were found and consequently we could not attempt to attribute specific morphological features to *G. postvectis* whitebait. On two occasions fish had been incorrectly identified by L. H. Dijkstra as *G. brevipinnis* type when they were in fact *G.fasciatus* as determined by genetic analysis. Subsequent visual re-examination also identified them as *G. fasciatus.* This illustrates the potential for erroneous identification using morphological data and highlights the benefits of using genetic markers where accurate identification is essential.

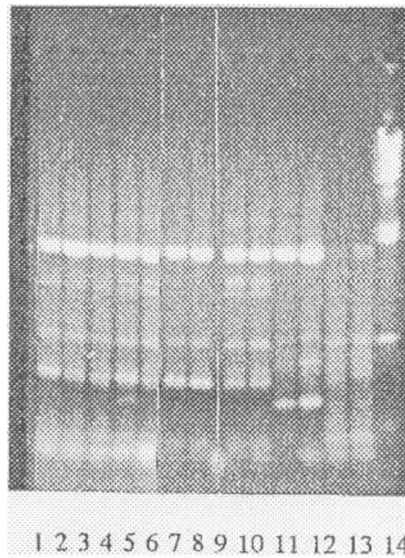
Table 1: Motueka River, Motueka

	Species		Species		Species
nwb1	*G. brevipinnis*	nwb6	*G. brevipinnis*	nwb11	*G. brevipinnis*
nwb2	*G. brevipinnis*	nwb7	*G. brevipinnis*	nwb12	*G. brevipinnis*
nwb3	*G. brevipinnis*	nwb8	*G. brevipinnis*	nwb13	*G.fasciatus*
nwb4	*G. brevipinnis*	nwb9	*G. brevipinnis*		
nwb5	*G. brevipinnis*	nwb10	*G. brevipinnis*		

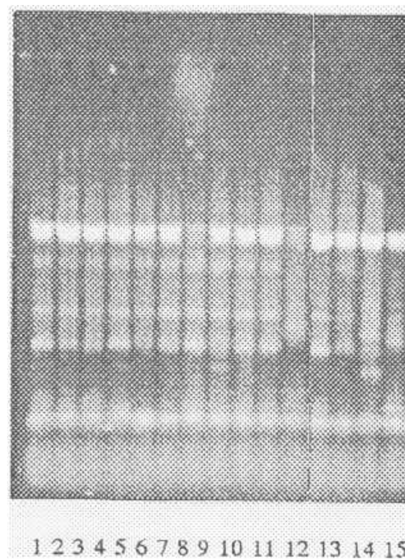
Table 2: Buller River, Westport

	Species		Species		Species
bwb1	*G. brevipinnis*	bwb5	*G. brevipinnis*	bwb9	*G. brevipinnis*
bwb2	*G. brevipinnis*	bwb6	*G. brevipinnis*	bwb10	*G. brevipinnis*
bwb3	*G. brevipinnis*	bwb7	*G. brevipinnis*	bwb11	*G. brevipinnis*
bwb4	*G. brevipinnis*	bwb8	*G. brevipinnis*	bwb12	*G. brevipinnis*

Figures 1 and 2: Typical PCR products identifying whitebait samples as *Galaxias brevipinnis* or *Galaxias fasciatus*



Lane
1 *G. brevipinnis*
2 *G. brevipinnis*
3 *G. brevipinnis*
4 *G. brevipinnis*
5 *G. brevipinnis*
6 Adult *G. postvectis*
7 Adult *G postvectis*
8 Adult *G. brevipinnis*
9 Adult *G. brevipinnis*
10 Adult *G. maculatus*
11 Adult *G. maculatus*
12 Adult *G. fasciatus*
13 Adult *G. fasciatus*
14 Lambda/Hind III marker



Lane
1 *G. brevipinnis*
2 *G. brevipinnis*
3 *G. brevipinnis*
4 *G. brevipinnis*
5 *G. brevipinnis*
6 *G. brevipinnis*
7 *G. brevipinnis*
8 *G. brevipinnis*
9 *G. brevipinnis*
10 *G. fasciatus*
11 Adult *G. postvectis*
12 Adult *G. brevipinnis*
13 Adult *G. maculatus*
14 Adult *G. fasciatus*

Table 3: Grey River, Greymouth

	Species		Species		Species
gwb1	*G. brevipinnis*	gwb8	*G. brevipinnis*	gwb15	*DNA degraded*
gwb2	*G. brevipinnis*	gwb9	*G. brevipinnis*	gwb16	*G. fasciatus*
gwb3	*G. brevipinnis*	gwb10	*G. brevipinnis*	gwb17	*DNA degraded*
gwb4	*G. brevipinnis*	gwb11	*DNA degraded*	gwb18	*G. brevipinnis*
gwb5	*G. brevipinnis*	gwb12	*DNA degraded*	gwb19	*G. brevipinnis*
gwb6	*G. brevipinnis*	gwb13	*G. brevipinnis*	gwb20	*G. brevipinnis*
gwb7	*G. brevipinnis*	gwb14	*G. brevipinnis*		

Table 4: Totara River, Hokitika

	Species
twb1	*G. brevipinnis*
twb2	*G. brevipinnis*
twb3	*G. brevipinnis*

Conclusions

This study has resulted in the development of reliable genetic markers which are able to distinguish koaro and SJ kokopu whitebait individuals. The ability of these markers to resolve these species at an early age serves as a useful management tool. However, since genetic sorting of whitebait into SJ kokopu and koaro can be expensive, it would still be advantageous to find morphological markers which are equally capable of sorting the two species. Unfortunately, the limited number of early age individuals supplied, resulted in no genetic identification of SJ kokopu whitebait, and consequently no unique morphological features characteristic for juvenile SJ kokopu were found. Given that this study has shown that a large number of whitebait need to be screened to identify SJ kokopu individuals, we believe that morphological markers are essential to obtain the detailed information regarding presence and timing of SJ kokopu whitebait within runs.

Recommendation

That the Department of Conservation consider an extension to this program for a further year to allow for a wider range of whitebait to be screened thereby ensuring that *Galaxias postvectis* juveniles are encountered.

Acknowledgements

Considerable effort was made by DoC and NIWA field staff to collect samples with no specific funding allocated for this purpose. In particular we would like to thank Craig Miller and David Rees (DoC Hokitika and Motueka respectively), as well as Paul Lambert (NIWA, Greymouth).

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APPENDIX I

THE PCR REACTION

We have used the polymerase chain reaction (PCR) to produce appropriate genetic markers. PCR has revolutionised techniques in molecular biology because it allows amplification of the DNA at specific regions of interest. This target DNA would otherwise be present in amounts that are so small relative to the rest of the DNA that its study would be prohibited. The quantity of amplified DNA produced is extremely large relative to the quantity of original template, and separation of product from template prior to analysis is not normally required.

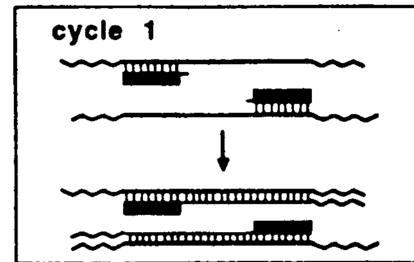
The basis of the reaction is enzymatic amplification of DNA up to 3000 nucleotides long (although the use of new PCR enzymes enable amplification much larger regions, e.g. 30 kb). The region(s) amplified are defined by primers (typically 10-30 nucleotide of synthetic DNA) which have been chosen to flank a specific or random (i.e. unknown) piece of DNA. The basis of the reaction is shown in Figure 3. The enzyme used is heat stable and usually Taq polymerase. It is able to catalyse duplication of the DNA by extending the primers across the region they delineate by joining bases complementary to the target sequence. The reaction is arranged to go through a series of temperatures in a cyclic manner. The first temperature in the cycle melts the template DNA to expose the target 'template' region in preparation for of the primer hybridisation. The next temperature step allows primer hybridisation to occur. Following this is a temperature stage which promotes the extension of the primers along the template to produce a copy of the target DNA. The cycle is repeated by melting the DNA duplexes so that the copy produced in the first cycle becomes a template in the following cycle. The DNA is amplified geometrically so that theoretically, over 20 cycles the original DNA template is increased a million fold.

PCR primers are short 20-30 base long sequences which complement the areas which flank the study region. This requires that some prior knowledge regarding the sequence of the target gene is necessary so that primers can be designed. However this requirement has been negated to some degree with the design of universal primers (i.e. primers which will amplify the same locus in many species) which were created based on consensus sequences from mammals, frogs and birds. For example, three pairs of primers which correspond to the flanking regions of either the 12s rRNA or cytb genes can amplify over a hundred different species including mammals, birds amphibians, fish and invertebrates (Kocher et al., 1989). Universal primers have therefore provided an opportunity for obtaining sizeable pure quantities of the DNA of interest for RFLP/sequencing analysis without the difficult extraction/cloning procedures required for such loci as rDNA and the mtDNA genome which were necessary in the past.

Figure 3: Schematic representation of the PCR reaction (after Oste, 1988)

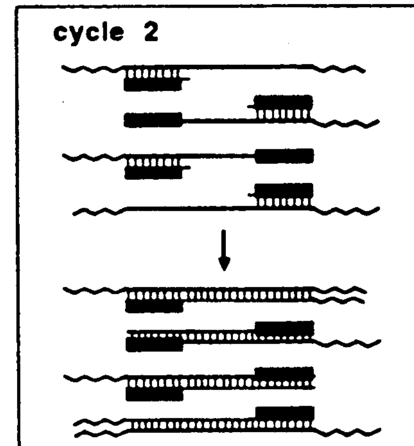
1) Isolate DNA

2) Denature and anneal primers



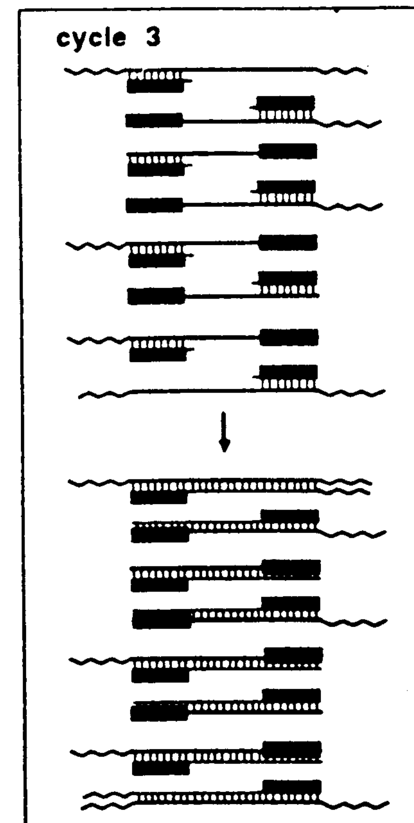
3) Primer extension

4) Denature and anneal primers



5) Primer extension

6) Denature and anneal primers



7) Primer extension

8) Repeat cycles

RIBOSOMAL DNA PROFILES AND THEIR USE IN SPECIES IDENTIFICATION

In this work we have used universal primers for the 28S ribosomal RNA gene as described by Stiller and Waaland (1993), White et al. (1990) and Sogin (1990). Ribosomes make the proteins and therefore are an integral and absolutely essential component of the cell. Part of the ribosome consists of ribosomal RNA (rRNA). The exact function of rRNA within the ribosome is unknown but it is thought to have both structural and more active functional roles. Because there may be millions of ribosomes in a cell, the genes which code for rRNA are quite numerous and may amount to up to 5000 identical copies of the gene per genome.These rRNA genes are arranged in tandem into large blocks situated on one or more chromosomes.

Ribosomal RNA genes are highly conserved meaning that their sequences are very similar between individuals, populations and even species. They are consequently much more useful for distinguishing differences at the genus and family level. The reason that they have been useful at distinguishing *Galaxias* species lies with our chance finding that a number of secondary products are produced in addition to the basic 1.8 kb 18S/5.8S/28S repeat unit typical of a eukaryotic ribosomal gene. These secondary products of unknown identity we have found to be highly diagnostic at a species level.

Figure 4: Structural representation of ribosomal DNA genes (after Avise, 1994)

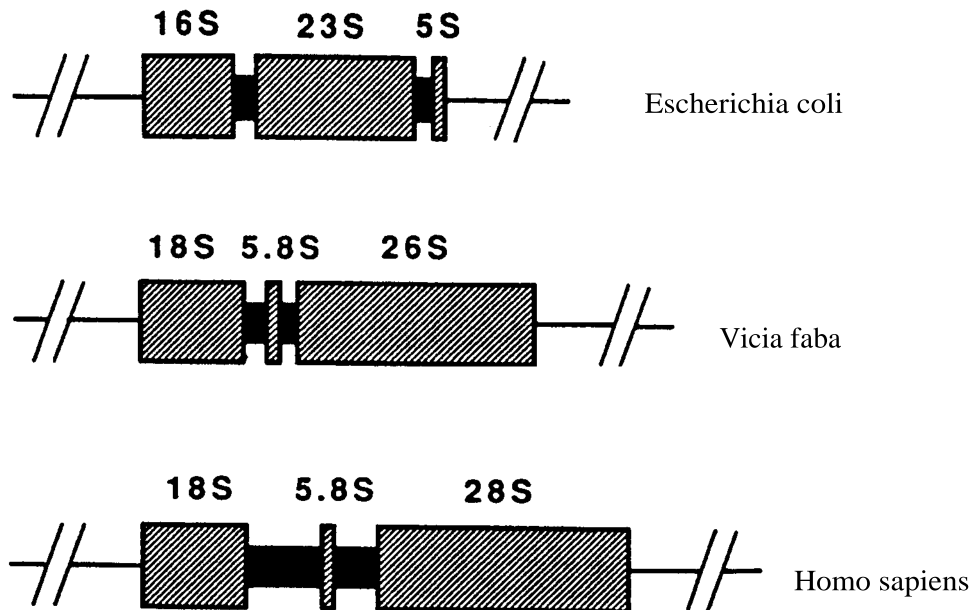


Figure 3.15. Structural features of the rDNA repeat module (drawn to approximate scale) in *E. coli*, and in a representative plant and animal (after Appels and Honeycutt, 1986). Hatched regions indicate loci encoding the "small" (16S and 18S) and "large" (23S, 26S, 28S) subunits of ribosomal RNA, as well as the "5S" rRNA elements. Black regions indicate internal transcribed spacers, which often differ in length.

APPENDIX 2: Interim report

EXECUTIVE SUMMARY (INTERIM)

DOC Ref No. 94/4

Investigation Title:	Electrophoretic identification of whitebait species
Study Venue:	West Coast, New Zealand
Investigation Leader:	L H Dijkstra, NIWA, Christchurch
Associated Researcher:	R M McDowall, NIWA, Christchurch
Investigation Status:	Current
Client:	DOC, Science and Research Division
Finish Date:	June 1995

I INVESTIGATION OVERVIEW

The primary aim of this study is to develop a morphological marker which will allow successful and consistent differentiation between juveniles (white-bait) of shortjawed kokopu (SJ kokopu, *Galaxias postvectis*) and koaro (*Galaxias brevipinnis*). The SJ kokopu is rare and listed as category A conser-vation status by DOC and as indeterminate by IUCN. It may be in decline given that there is thought to be substantial habitat loss and modification.

Both SJ kokopu and koaro are involved in the whitebait fishery and commonly occur together with 3 other species of *Galaxias*. Knowledge regarding the presence of shortjawed kokopu in whitebait runs both in terms of timing and geography is scarce. However if existence of SJ kokopu is to be maintained this information is essential for successful management. Unfortunately, mor-phological data do not allow the discrimination between koaro and shortjawed kokopu whitebait which has become a serious problem, particularly since normally it is a matter of distinguishing a few shortjawed kokopu from a mass of koaro. This has led to the idea that genetic markers within the DNA (DNA being a molecule which does not change in response to environmental fac-tors or aging) should be identified from the easily distinguishable adults. These markers can then be used to distinguish the juveniles.

OBJECTIVES

1. Develop a DNA-based genetic marker that distinguishes between juve-nile koaro and shortjawed kokopu.

2. Genetic sorting of 100-150 'koaro type' whitebait individuals into either koaro or shortjawed kokopu.

3. Renewing research into morphological methods which differentiate between koaro and shortjawed kokopu juveniles that have been clearly distinguished by 'genetic sorting'.

METHODS

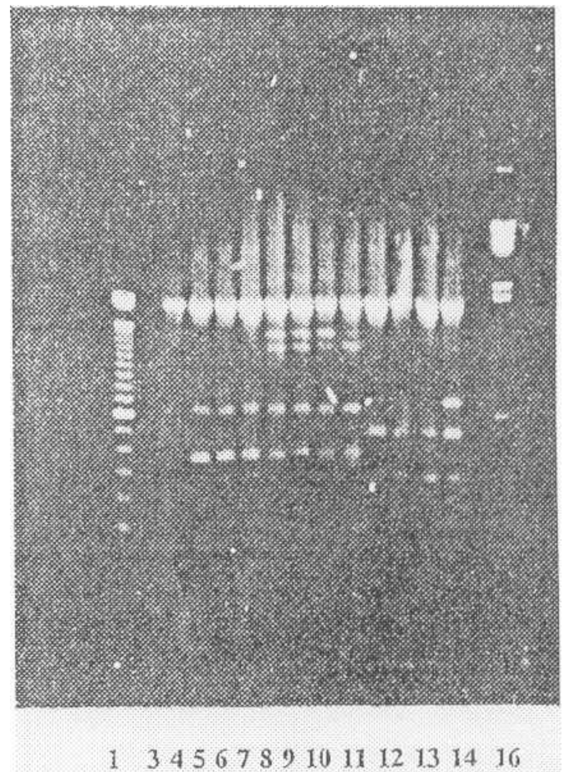
Genetic markers were developed from adult fish. DNA was extracted from fin tissue collected from 4 individuals for each species caught in the Kaniere catchment near Hokitika (Striplands Ck, J33 23502: 58266). To increase our certainty of the ability of the technique to resolve the different whitebait species we also included 4 whitebait specimens identified as *Galaxias maculatus* which were bought locally and were collected from the East Coast. Marker loci were obtained by exploiting variation among 18S nuclear rRNA genes using the polymerase chain reaction (PCR). This technique involves enzymatic amplification producing millions of copies of target DNA (in this case 18S rRNA genes) so that it may be studied independently from the rest of the genome.

Differences between individuals arise because of the number and the size of the products produced. Products were visualised using 1% agarose gel electrophoresis followed by ethidium bromide.

INTERIM RESULTS

DNA was successfully extracted from 4 SJ kokopu and 4 koaro adults as well as 4 inanga juveniles. Only a small amount of tissue was required to provide adequate amounts of DNA. For a juvenile (whitebait) specimen this translates to a centimetre long portion taken from the back on one side just in front of the dorsal fin. This left the specimen virtually intact for future morphological analysis. The extraction protocol used was a high salt protein precipitation procedure which circumvents the need for the usual lengthy phenol extractions. Genomic DNA was amplified using universal primers developed by Sogin (1990) to produce a profile unique to each species consisting of several products each in the 0.2 to 1.6 kilobase range (a base constitutes one link in the DNA chain). The ethidium bromide stained agarose gel illustrating these profiles is presented below. Unfortunately logistical problems encountered by DOC with regard to obtaining a suitable ethanol preserved sample for genetic screening meant that the work could not be completed with regard to linking genetic markers to morphological characteristics.

Lane 1: 100 by ladder (marker)
Lanes 3-6: SJ kokopu adults
Lanes 7-10: koaro adults
Lanes 11-14: inanga whitebait
Lane 16: Lambda/Hind III digest (marker)



INTERIM CONCLUSIONS

We have found a genetic marker which we believe to be suitable for distinguishing juvenile individuals of koaro and SJ kokopu. This marker allows up to 80 'koaro-like' individuals to be genetically sorted into either koaro or SJ kokopu per day.

RECOMMENDATIONS

During the next (1995) whitebait season we hope that DOC will fund this work to completion and provide a sample of 100-200 whitebait which is known to consist of either koaro or SJ kokopu individuals. We will then sort them into the two species using our genetic marker and endeavour to find a morphological characteristic which can distinguish the two species in the future.

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