Gazameda gunnii

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# Development of genetic probes for rapid assessment of the impacts of marine invasive species on native biodiversity – *Maoricolpus roseus*

Jawahar Patil, Rasanthi Gunasekera, Felicity McEnnulty and Nicholas Bax

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# EXECUTIVE SUMMARY

The New Zealand Screw shell *Maoricolpus roseus* unintentionally introduced to south-eastern Tasmania in the 1920s, has now spread out to the 80 metre depth contour off the eastern Victorian and New South Wales coasts and is found as far north as Botany Bay (Bax et al., 2003). This species has colonized more habitat than any other high-impact benthic marine pest in Australia and the risk of its further spread and establishment is very likely owing to its wide temperature and depth tolerance. This project was initiated to develop genetic probes for the rapid detection and assessment of this species as a prelude to determining its impacts on the native flora and fauna as well as to fill our knowledge gaps in its phenology and life history strategies. In particular it is important to know whether this species has a planktonic life history stage that could lead to its dispersal in ships' ballast water.

Obtaining suitable samples of the *M. roseus* and its closely related species from Australia and its native range in New Zealand, although trivial, were time consuming and samples were often not forthcoming. Specifically, difficulties were encountered in obtaining samples of the native screw shell *Gazameda gunnii*, which took special attempts involving dedicated divers and a scientific cruise. *Maoricolpus roseus* samples were obtained from 6 and 2 locations in Australia and New Zealand respectively with the majority of the shells collected being empty.

Three sets of gene probes were developed, each of which targeted a unique region in the mitochondrial COI locus. All the three sets of probes were tested against as many closely related species as could be obtained and the reaction conditions optimized for maximum sensitivity and specificity. Further sequence comparison of COI locus of *M. roseus* and members of Cerithioidea, including the native Australian turritellids, *Gazameda iredalei*, *G. gunnii* and *Turitella terebra* suggest significant differences which should permit the design of specific probes for *M. roseus* and perhaps for the others such as the *G. gunnii*.

In light of limited sequence variation within and between *M. roseus roseus* and *M. roseus manukauensis,* we attempted to amplify the entire mitochondrial genome of *M. roseus.* In all 13224bp of the expected ~15000 bp of the mtDNA was sequenced and characterized. It is hoped that this information will assist in fine scale genetic discrimination between populations of *M. roseus* as well as in identifying source population of introduction into Australian waters.

A nested polymerase chain reaction (PCR) was used to analyse environmental plankton samples collected in the Derwent Estuary between August 2003 and June 2004. The results suggest that the species does have a planktonic larval existence prior to settlement. Results also support earlier beliefs that the species is predominantly a spring-summer spawner, although *M. roseus* was detected in plankton samples on two occasions in winter months.

The presence of *M. roseus* in the plankton and water available for ships' ballast suggests that the risk of this species being spread by shipping may need to be

managed within the National System for Prevention and Management of Marine Pest Incursions.

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# 1 INTRODUCTION

## 1.1 Background

The economic cost of aquatic exotic species is significant (Pimental et al., 2000). The extent of the introduced marine species problem in Australia became apparent with the outbreaks of highly visible introduced species (eg. *Asterias amurensis* in Hobart and Port Phillip Bay; *Mytilopsis* sp. in Darwin) capable of adversely affecting and impacting industry, aquaculture and biodiversity (Morrice 1995; Goggin, 1998; Ferguson, 2000; Bax et al., 2002).

The majority of marine pest species in Australian waters is restricted to shallow waters usually close to port environments and/or disturbed or heavily impacted areas. The turritellid gastropod, *Maoricolpus roseus* is atypical in this respect. In its native region of New Zealand it is found on all substrata from soft sediments to exposed habitats, living in crevices on rock walls, and in sheltered pockets on more exposed reefs from low-water to approximately 200 metres depth on the continental shelf (Scott 1997). Initially only known from around south-eastern Tasmania, *M. roseus* has now spread out to the 80 metre depth contour off the eastern Victorian and New South Wales coasts and is found as far north as Botany Bay (Bax et al., 2003). Due to this habitat flexibility the potential exists for *M. roseus* to have greater ecological and environmental impacts over larger areas than introduced species restricted to specific inshore environments. Given its wide temperature, depth and substrate tolerances, *M. roseus* has the potential to spread further around the southern Australian coastline. It is the only pest recorded (to date) in marine areas under Commonwealth jurisdiction. The dense beds of this invasive species change the benthic structure of marine coastal and shelf ecosystems from southern Tasmania to central New South Wales with unknown (and unexamined) effects on ecosystem services from these environmentally and economically important ecosystems. Dense beds of this burrowing filter feeder may have adverse impacts on our native species such as scallops with which they occupy the same beds, or native turritellids such as *Gazameda gunnii*, which has been steadily declining in numbers concurrently with the increase in *M. roseus*. The Minister for Environment and Planning approved on 7th May 2004 the listing of *G. gunnii* onto Schedule 4 (Vulnerable) of the Threatened Species Protection Act under Schedule 3 (Threatened) in Tasmania.

## 1.2 Objectives

The primary aim of this project was to develop genetic tools to identify larval *Maoricolpus roseus* in planktonic and benthic samples, as a precursor to further environmental studies. Larval *M. roseus* are indistinguishable morphologically from many other gastropods. Reproduction in *M. roseus* is via direct development and fertilized embryos are held in egg capsules within the female's mantle cavity. These develop into trochophore larvae and then into actively swimming veliger larvae within the egg capsules (Allmon et al., 1994). We are currently unable to determine whether these veligers are released into the plankton for a period prior to settlement or whether they

immediately settle out as benthic juveniles. Anecdotal evidence tends to suggest they have a planktonic life (Bax et al., 2003). The duration of this putative planktonic life and, and thus how the plankton may be transported around and threaten Australia's coastline is currently the focus of a number of ecological studies (Professor Craig Johnson, University of Tasmania, personal communication).

A secondary aim of this project (although not a project deliverable) is to amplify and characterise the entire mitochondrial genome of *M. roseus* to assist in establishing the genetic diversity within the species and to identify the source population of introduction to Australia.

# 2. METHODS

## 2.1 Sample collection

Adult individuals of *Maoricolpus roseus,* and native turritellid species were either collected from the wild or obtained from preserved laboratory or museum collections. Fresh tissue was either immediately subjected to DNA extraction, or frozen and stored at –20 C or -80 C. The museum collected samples typically came preserved in 95 % ethanol.

## 2.2 DNA extraction and sequencing

All genomic DNA extractions from fresh or frozen samples were performed on 10-50 mg of tissue samples using the CTAB protocol or DNeasy tissue kit (QIAGEN) following supplier's instructions. In case of samples obtained from museum collections, the tissue was dissected and rinsed several times in distilled water and allowed to re-hydrate for 10 minutes two times before DNA extraction.

Amplification and sequencing of the mitochondrial cytochrome oxidase subunit I (COI) was carried out using the universal primers LCO1490 and HCO2198 (see Table1 for sequence and references). A separate PCR reaction was carried out on all samples using universal nuclear 18S ribosomal DNA primers (Table 1; NSF1179 and NSR 1642) to confirm suitability of each sample for PCR.

Standard PCR reactions were done in a 25 μl volume containing 0.2 μM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer, and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions were: 94 C for 9 minutes then 35 cycles (94 C, 30s / 54 C, 30s / 72 C, 1 minute) followed by 72 C for 5 minutes. Aerosol-resistant pipette tips were used with all PCR solutions and negative control reactions were performed with each PCR cocktail.

PCR products were purified using the QIAquick PCR purification kit (QIAGEN). Reactions for sequencing the COI region were carried out on both strands, using the original amplification primers, with the ABI Big Dye dideoxy terminator cycle sequencing kit (Applied Biosystems). Electrophoresis was carried out on an ABI-377 or ABI-3100 automated DNA sequencer and sequence data were edited with Sequence Navigator software (Applied Biosystems). Sequence data were aligned using CLUSTAL_X (Thompson et al., 1997). These sequences along with additional sequences from GenBank were used to assess the level of COI variation within *M. roseus* and between this species and other species of the family Turritellidae and the Superfamily Cerithoidea. Additional sequence analysis was carried out using the software DNASTAR and a phylogenetic tree of *M. roseus* from different geographical region and some closely related species was constructed using the Neighbourhood-joining method.

**Table 1. Sequences of primers used**

| Name | Gene | Sequence (5'-3') | Application | Reference |
|---|---|---|---|---|
| LCO 1490(F) | mt COI | GGTCAACAAATCATAAAGATATTGG | PCR -Universal | Folmer et al., 1994 |
| HCO 2198(R) | mt COI | TAAACTTCAGGGTGACCAAAAAATCA | PCR -Universal | Folmer et al., 1994 |
| NSF 1179 | 18S-rDNA | AATTTGACTCAACACGGG | PCR -Universal positive control | Wuyts et al., 2001 |
| NSR1642 | 18S-rDNA | GCGACGGGCGGTGTGTAC | PCR -Universal positive control | Wuyts et al., 2001 |
| CMRSF1 | mt COI | TTCTCTCTGCATTTAGCTGGTGTTTCTTCA | PCR and sequencing *M. roseus*-specific | This study |
| CMRSF4 | mt COI | GTGCTGAGCTTGGACAGCCAGGTGCGTTGC | PCR and sequencing *M. roseus*-specific | This study |
| CMRSR2 | mt COI | TGCTAGCACAGGAAGCGAAAGTAGTAACAA | PCR and sequencing *M. roseus*-specific | This study |
| CMRSR3 | mt COI | CACCCAGTCCCTACCCCTCTTTCTACAGCA | PCR and sequencing *M. roseus*-specific | This study |
| CMRSR4 | mt COI | ACAGCAGCTGAAGAAAGGAGAAGTAGAAGA | PCR and sequencing *M. roseus*-specific | This study |

## 2.3 "*Maoricolpus roseus*–specific" primer design

Regions of 30 bp that varied between species of Turritellidae, but were conserved and unique to *Maoricolpus roseus* were identified in the ~660 bp region of the mt COI locus. Following initial identification, the primer sites were analysed for secondary structures and self-complementarity using OLIGO (Rychlik, 1996). Each of the identified oligonucleotides was checked for uniqueness against the COI sequences in GenBank. Three primer pairs considered to be "*M. roseus*-specific" were synthesised for empirical validation (Table 1).

PCR reactions specific to *M. roseus* were carried out in a 25 μl volume containing 0.2 μM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions for the specific primers were as follows: 94 C for 9 minutes then 35 cycles (94 C, 30s / 61 C, 30s / 72 C, 30s) followed by 72 C for 5 minutes. All three primer combinations were tested. Aerosol-resistant pipette tips were used with all PCR solutions and negative control reactions were performed with each PCR cocktail. The products of the *M. roseus*-specific PCR and the 18S positive control PCR corresponding to each of the samples were either mixed or run separately on a 1.8% agarose gel. All gels were stained with ethidium bromide, exposed under UV light and documented with a Nikon Coolpix digital camera.

## 2.4 Detection of larval *Maoricolpus roseus*

To test the sensitivity of the developed *Maoricolpus roseus* probe, egg capsules containing veliger larvae of *M. roseus* were removed from the mantle cavity of brooding females under a compound microscope. The sizes of the egg capsules and veligers were measured and numbers per egg capsule estimated. These egg capsules were

then transferred directly to PCR tubes (1 or 2 or 5 egg capsules per tube) and 10µl sterile Milli-Q water was added. The samples were then twice snap frozen at -80 C and thawed at 37 C to disrupt the cells. Immediately the PCR cocktail (as above) was added directly to the tubes and subjected to PCR amplification using CMRSF1 and CMRSR2 primers

## 2.5 Detection of *Maoricolpus roseus* in plankton samples

To determine if the probes would effectively detect *Maoricolpus roseus* in environmental samples, the probes were tested against genomic DNA extracted from environmental samples that were collected from the Derwent River estuary between August 2003 to June 2004 as part of another NHT-funded project (Hayes et al., 2004). In brief, three five-minute samples were collected with an electric mono pump (CP 25) and sieved through a 100µm mesh plankton net. The mono pump was calibrated at 1-2 m head, delivering 31 litres per minute (range of 30 – 31.6 litres per minute). The total volume of water sampled for each plankton sample was approximately 155 litres. All samples were fixed in SET buffered 90 % ethanol. The plankton samples were collected from two locations: the Royal Yacht Club and the Domain slipways in the Derwent River estuary. At least one sample was taken every month except January 2004, when no samples were taken.

## 2.6 Amplification and sequencing of partial mitochondrial genome

Large fragments of the *Maoricolpus roseus* mitochondrial genome were amplified by designing appropriate primers (Table 2). Amplification of mtDNA was initially undertaken in an effort to obtain sequence of cytochrome oxidase (COI) gene for use in developing species-specific probes as well as for population sequencing studies using the universal COI primers (Folmer et al., 1994). Two new primers from this sequence were designed to be used in conjunction with primer designes based on the published (Accession No. AY010322) *M. roseus* mitochondrial 16S rDNA sequences and universal *cyt b* primers of Wilding et al., (1999). Because we had no prior information on gene order, each of the COI primers was used with *cyt b* and 16S rDNA primers. In turn the 16S rDNA and *cyt b* primers were paired with one another in two different combinations. The long range PCR ProofStart DNA polymerase system (QIAGEN) was employed to reduce PCR errors and amplify large fragments. This uses *Taq* DNA polymerase as the main polymerase with a low-concentration of proofreading ProofStart DNA polymerase. Initial amplification yielded three fragments of approximately 8 kb, 4.5 kb and 3.5 kb. The smaller 3.5 and 4.5 fragments were contained within the large 8 kb fragment. All three fragments were cloned into 2.1-Topo TA cloning vector. Sequencing was carried out using M13 forward and M13 reverse as well as gene specific primers. Where these primers did not adequately cover the sequence, internal sequencing primers were designed and sequencing was performed on an ABI 377 or ABI 3100 automatic sequencer. Subsequently new primers were designed to amplify the remaining part of the mitochondrial genome and an additional 2 and 3 kb fragments were amplified and sequenced as above.

**Table 2. Sequences of primers used to amplify large fragments of Mitochondrial genome**

| Name | Gene | Sequence (5'-3') | Application | Reference |
|---|---|---|---|---|
| MR.COI-F2 | mt COI | CGATTATTAACATACGATGACGAGGAATGC | PCR and sequencing | This study |
| MR.COI-R2 | mt COI | AGGAACAAGCCAGTTACCGAACCCGCCAAT | PCR and sequencing | This study |
| MR.16SrDNA-F1 | mt 16SrDNA | TAGAGATGACATGTTATTCGCGCTGGTGAT | PCR and sequencing | This study (based on AY010322) |
| MR.16SrDNA-R1 | mt 16SrDNA | ATCACCAGCGCGAATAACATGTCATCTCTA | PCR and sequencing | This study (based on AY010322) |
| cyt.b-R1 | mt *cyt b* | AGGGAACTTTTTCTCCATCTCTGT | PCR-Universal sequencing | Wilding et al., 1999 |
| cyt.b-F1 | mt *cyt b* | ACAGAGATGGAGAAAAAGTTCCCT | PCR-Universal sequencing | Reverse compliment of cyt b-R1 primer |

# 3. RESULTS

## 3.1 Sample collection

To determine if there is any genetic difference between different populations of *Maoricolpus roseus* and therefore if there were one or more introductions to Australian waters, we have obtained samples from various locations both in Australia and New Zealand including the subspecies *Maoricolpus roseus manukauensis* (Fig 1 and Table 3). Over 26 different research providers, museums and private consultants were contacted in an effort to obtain samples of *Maoricolpus roseus* and related native turritellid species in Australia. Six different research providers and museums were contacted in New Zealand (Appendix C). However, despite extensive attempts to obtain samples of these native turritellids we managed to obtain only five native species (Table 3). Samples of *Gazameda gunni* were obtained by CSIRO field teams from the Tasman Peninsula by remote grab off RV Explorer and off south-eastern Victoria using a benthic sled towed by the National Facility RV Southern Surveyor (Williams 2004). Two specimens of *Gazameda iredalei* were collected by a diver from South Australia, as were the three samples of *Turritella terebra* from the Northern Territory. *Gazameda tasmanica* and *Colpospira australis* were obtained from the Tasmanian Museum and Art Gallery collection.

Although it is relatively easy to collect *M. roseus* samples from the wild in southeastern Tasmanian waters, the majority of the shells collected were empty (and often harboured hermit crabs). Our limited observations suggest that between 65-98 % of the shells collected from Tasmania were either empty or harboured a hermit crab. Similarly, all the frozen samples collected from Eden by NSW Fisheries were empty. Samples of *M. roseus* from Tasmanian and New South Wales waters were collected by divers or by remote sampling devices on the larger research vessels owned by the Tasmanian Aquaculture and Fisheries Institute (TAFI).

**Table 3. Samples obtained for DNA extraction**

| Species | n | Sample Location | Sample Date | Preservation Method | Collector |
|---|---|---|---|---|---|
| *Maoricolpus roseus* | 16 | Tasmania - Pirates Bay | 03/08/2003 | Frozen | Anthony Reid, University of Tasmania |
| *Maoricolpus roseus* | 12 | Tasmania - Dennes Point | 28/06/2003 | Frozen | Anthony Reid, University of Tasmania |
| *Maoricolpus roseus* | 10 | Tasmania – Triabunna | 24/01/2001 | Frozen | Felicity McEnnulty, CMR |
| *Maoricolpus roseus* | 8 | Bass Strait #1 | 28/03/2003 | Frozen | Anthony Reid, University of Tasmania |
| *Maoricolpus roseus* | 5 | Bass Strait #2 | 28/03/2003 | Frozen | Anthony Reid, University of Tasmania |
| *Maoricolpus roseus* | 10 | Tasmania - Nubeena | 31/03/2004 | Frozen | Felicity McEnnulty, CMR |
| *Maoricolpus roseus* | 0 | New South Wales – Eden | October 2003 | Frozen | Craig Brand, New South Wales Fisheries |
| *Maoricolpus roseus* | 6 | New Zealand – Bucklands Beach | 25/10/2003 | 95% ethanol | Margaret Morley, Auckland Museum |
| *Maoricolpus roseus* | 7 | New Zealand - Tauranga | 15/09/2003 | 95% ethanol | Graeme Inglis, NIWA |
| *Maoricolpus roseus* | 2 | New Zealand - Tauranga | 17/09/2003 | 95% ethanol | Graeme Inglis, NIWA |
| *Maoricolpus roseus* | 3 | New Zealand – Whangarei #1 | 30/09/2003 | 95% ethanol | Graeme Inglis, NIWA |
| *Maoricolpus roseus* | 1 | New Zealand – Whangarei #2 | 30/09/2003 | 95% ethanol | Graeme Inglis, NIWA |
| *Maoricolpus roseus* | 2 | New Zealand – Whangarei #3 | 30/09/2003 | 95% ethanol | Graeme Inglis, NIWA |
| *Maoricolpus roseus* | 1 | New Zealand – Whangarei #4 | 02/10/2003 | 95% ethanol | Graeme Inglis, NIWA |
| *Maoricolpus roseus* | 5 | New Zealand – Otago Harbour | 13/02/2004 | 95% ethanol | Keith Probert, University of Otago |
| *Maoricolpus roseus manukauensis* | 6 | New Zealand - Manukau Harbour | October 2003 | 95% ethanol | Margaret Morley, Auckland Museum |
| *Turritella terebra* | 3 | Northern Territory –Darwin | 13/01/2003 | 95% ethanol | Richard Willan, Museum and Art Gallery of the Northern Territory |
| *Gazameda iredalei* | 2 | South Australia - Yorke Peninsula | December 2003 | 95% ethanol | Karen Gowlett-Holmes, CMR |
| *Gazameda gunnii* | 5 | Tasmania - Nubeena | 31/03/2004 | Frozen | Felicity McEnnulty, CMR |
| *Gazameda gunnii* | 13 | New South Wales - Disaster Bay | 28/04/2004 | 7 Frozen, 6 in 95% ethanol | Alan Williams, CMR |
| *Gazameda tasmanica* | 3 | Tasmania – Burnie | 17/02/1970 | 95% ethanol | Tasmanian Museum and Art Gallery specimen |
| *Colpospira australis* | 1 | New South Wales - 11nM SSE off Montagu Island | 05/09/1994 | 95% ethanol | Tasmanian Museum and Art Gallery specimen |

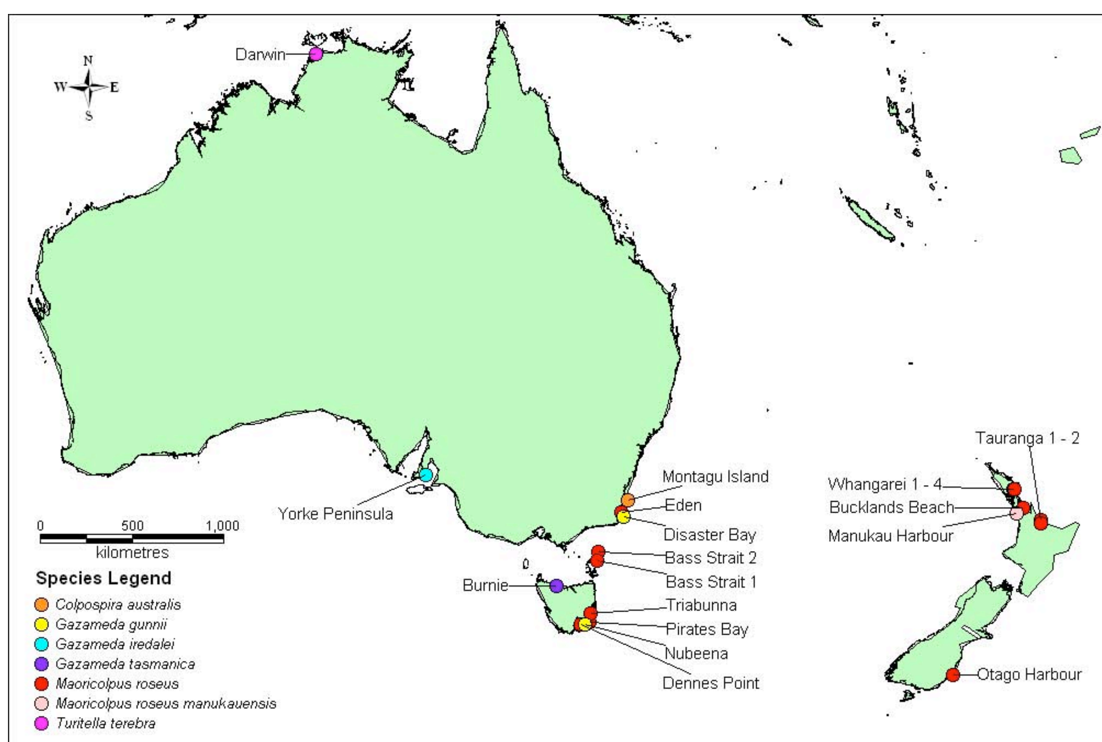


**Figure 1. Map of sample locations**

## 3.2 DNA Extraction and PCR amplification.

The samples from which DNA was extracted are listed in Table 4. It was possible to amplify regions of both the nuclear 18S rDNA and mitochondrial COI locus from most of the *M. roseus* samples. But no amplification was possible from some of *M. roseus* samples collected from New Zealand. It could be due to degradation of the DNA templates. We have successfully amplified both 18S rDNA and mt COI locus from *Maoricolpus roseus manukauensis*, *Gazameda iredalei, Gazameda gunnii* and *Turritella terebra* samples. It was possible to amplify only the 18S rDNA loci from *Gazameda tasmanica* and no amplification was possible from *Colpospira (Ctenocolpus) australis* (Table 4). There are two possible reasons for these results (a) the chosen primer pairs are inadequate to amplify the loci; or (b) the template DNA is degraded. The latter is the most probable cause as these samples had been preserved in ethanol for over nine years. Although the samples from New Zealand were obtained recently, some of them did not amplify, implying that the preservation of these samples was not carried out adequately.

**Table 4. Species of Turritellidae tested for amplification of the mitochondrial COI (universal primer pairs HCO-F / LCO-R), nuclear 18S rDNA internal control (universal primer pairs NSF1179 / NSR1642) and *M.roseus*-specific primers pairs (CMRSF1 / CMRSR2 ; CMRSF4 / CMRSR3 ; CMRSF4 / CMRSR4). All the *M.roseus*-specific primers pairs gave similar results.**

| Species | Collection Location | Sample code | n | 18SrDNA control PCR | COI locus | *M.roseus*-specific PCR results (61 C) |
|---|---|---|---|---|---|---|
| *Maoricolpus roseus* | Tasmania Pirates Bay | Mr_PB | 4 | +ve | +ve | +ve |
| *Maoricolpus roseus* | Tasmania Dennes Point | Mr_DP | 4 | +ve | +ve | +ve |
| *Maoricolpus roseus* | Tasmania Triabunna | Mr_TB | 4 | +ve | +ve | +ve |
| *Maoricolpus roseus* | Bass Strait #1 | Mr_BS1 | 4 | +ve | +ve | +ve |
| *Maoricolpus roseus* | Bass Strait #2 | Mr_BS2 | 4 | +ve | +ve | +ve |
| *Maoricolpus roseus* | Tasmania Nubeena | Mr_NB | 4 | +ve | +ve | +ve |
| *Maoricolpus roseus* | New Zealand Bucklands Beach | Mr_BB | 4 | +ve | +ve | +ve |
| *Maoricolpus roseus* | New Zealand Tauranga | Mr_TR1 | 7 | -ve | -ve | -- |
| *Maoricolpus roseus* | New Zealand Tauranga | Mr_TR2 | 2 | -ve | -ve | -- |
| *Maoricolpus roseus* | New Zealand Whangarei #1 | Mr_WR1 | 3 | -ve | -ve | -- |
| *Maoricolpus roseus* | New Zealand Whangarei #2 | Mr_WR2 | 1 | -ve | -ve | -- |
| *Maoricolpus roseus* | New Zealand Whangarei #3 | Mr_WR3 | 2 | -ve | -ve | -- |
| *Maoricolpus roseus* | New Zealand Whangarei #4 | Mr_WR4 | 1 | -ve | -ve | -- |
| *Maoricolpus roseus* | New Zealand Otago Harbour | Mr_OH | 5 | -ve | -ve | -- |
| *Maoricolpus roseus manukauensis* | New Zealand Manukau Harbour | Mrm_MH | 4 | +ve | +ve | +ve |
| *Turritella terebra* | Northern Territory Darwin | Tt_DR | 2 | +ve | +ve | -ve |
| *Gazameda iredalei* | South Australia Yorke Peninsula | Gi_YP | 2 | +ve | +ve | -ve |
| *Gazameda gunnii* | Tasmania Nubeena | Gg_NB | 3 | +ve | +ve | -ve |
| *Gazameda gunnii* | New South Wales Disaster Bay | Gg_DB | 3 | +ve | +ve | -ve |
| *Gazameda tasmanica* | Tasmania Burnie | Gt_BN | 3 | +ve | -ve | -- |
| *Colpospira australis* | Tasman Sea, 11nM SSE of Montague Island | Ca_MI | 1 | -ve | -ve | -- |

## 3.3 Sequence Analysis

Clustal alignment of the partial mt-COI sequence data (nucleotides) of *Maoricolpus roseus* obtained from several geographic regions revealed that the COI region is highly conserved within the species (Figure 2). All the individuals we sequenced from different locations within Australia were identical. However there were seven mismatches between the sequences of *M. roseus* from different locations of Australia and *M. roseus* from Bucklands Beach, New Zealand. Also, six mismatches were found between *Maoricolpus roseus manukauensis* and *M. roseus* from Australia. All the Australian samples clustered into a single group, with the only New Zealand sample of *M. roseus* (Mr_BB) and the subspecies *M. roseus manukauensis* (Mrm_MH) segregating into two close sister groups (Figure 3).

When sequences corresponding to the mt-COI region of *M. roseus* and other species of the family Turritellidae were aligned (Figure 4), it was possible to identify short sequences that were unique for *M. roseus* to serve as target specific PCR primers. Alignment of the three specific primer binding regions, CMRSF1 / CMRSR2; CMRSF4 / CMRSR3 and CMRSF4 / CMRSR4, from different species of Turritellidae especially native to Australia are given in Table 5a, 5b and 5c respectively. Each of the primer pairs constituted a total sequence region of 60 bp in length. Over this 60 bp primer region, the target sequence shows a minimum 10 nucleotide differences between *M. roseus* and the remaining species analysed. However there was only one nucleotide difference between *M. roseus* (Australia) and *Maoricolpus roseus manukauensis* in all three primer combinations.

The relatively high sequence difference (16%-31%) at the primer binding site between *Maoricolpus* and the other species of native Turritellidae, implied that there was a good possibility of developing species-specific PCR assays at all three primer loci.

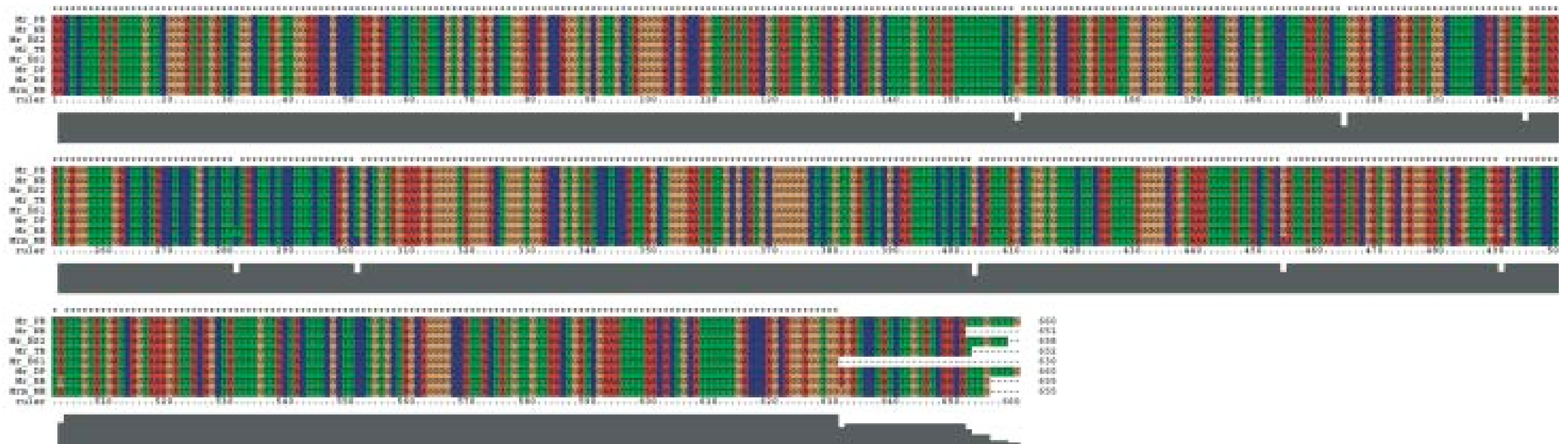

**Figure 2. Clustal alignment of the partial mt-COI sequences of *Maoricolpus roseus* obtained from several geographic regions. Details of the sample codes are given in Table 4.**

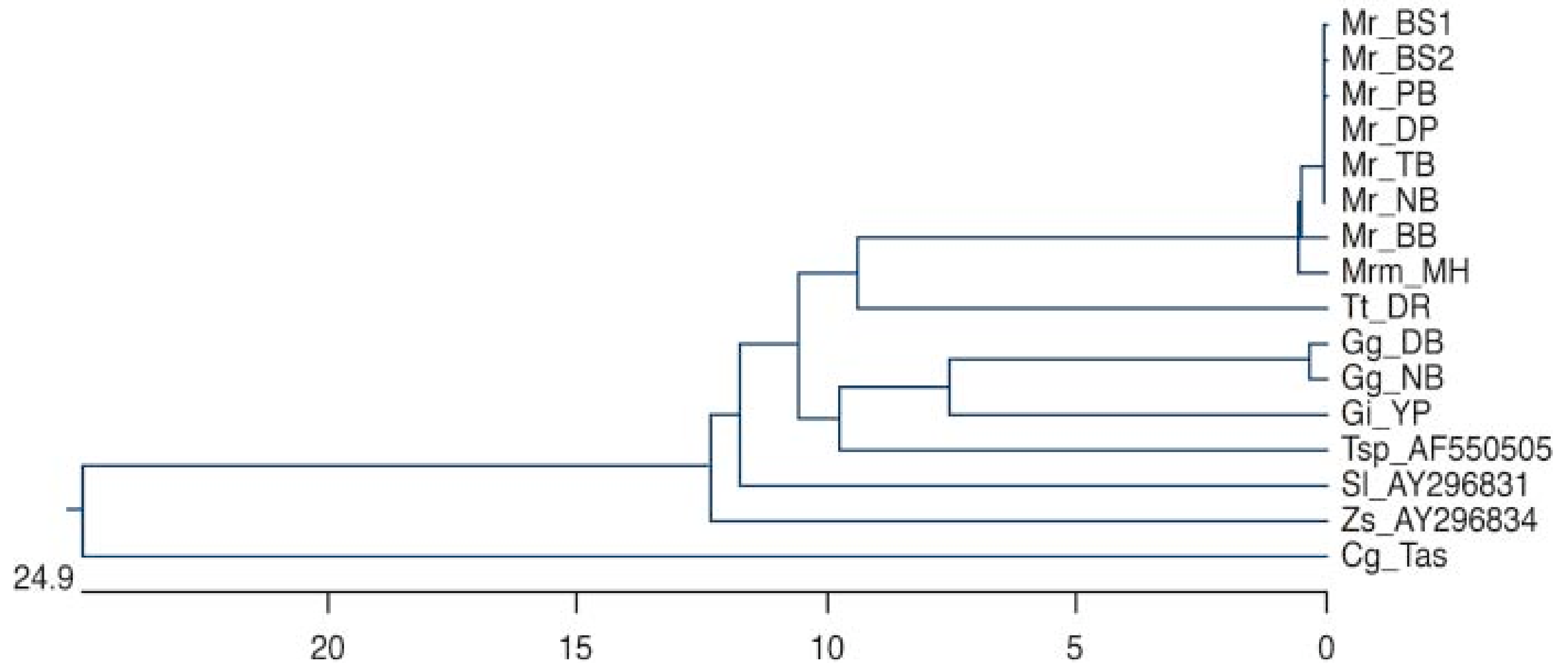


**Figure 3.** **A phylogenetic tree of *Maoricolpus roseus* from different geographical regions and some closely related species. Details of most of the sample codes are given in Table 4. Remaining sample codes are Tsp-*Turitella* sp; Sl-*Strombus luhuanus*; Zs-*Zeacumantus subcarinatus*; Cg- *Crassostrea gigas***

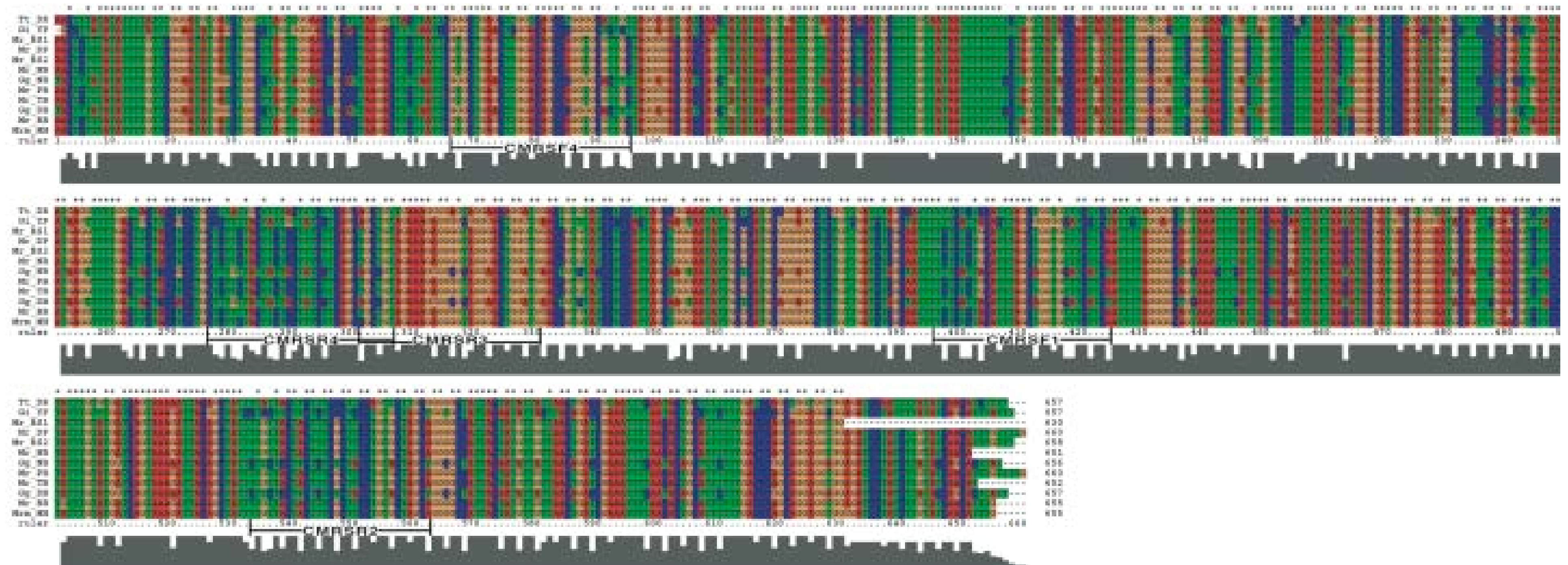

**Figure 4. Clustal alignment of the partial mt-COI sequences of *M. roseus* and other species of the family Turritellidae Details of the sample codes are given in Table 4. The positions of the respective primers (probes) are marked below the alignment.**

**Table 5a.** ***Maoricolpus roseus*-specific PCR primers, CMRSF1 and CMRSR2, aligned with corresponding sequence from other native species of Turritellidae (a dot indicates the nucleotide is the same as in the top sequence).**

| Species | n | CMRSF1 → | ← CMRSR2 |
|---|---|---|---|
| Mr_DP | 3 | **5'**TTCTCTCTGCATTTAGCTGGTGTTTCTTCA | TGCTAGCACAGGAAGCGAAAGTAGTAACAA **3'** |
| Mr_BS1 | 3 | .............................. | .............................. |
| Mr_BS2 | 3 | .............................. | .............................. |
| Mr_NB | 3 | .............................. | .............................. |
| Mr-PB | 3 | .............................. | .............................. |
| Mr_TB | 3 | .............................. | .............................. |
| Mr_BB | 3 | ........A..................... | .............................. |
| Mrm_MH | 3 | ........A..................... | .............................. |
| Gg_DB | 3 | ..T..AT.A........A....CA..A..T | A.....T.....T..A..G..A..G.GA.G |
| Gg_NB | 3 | ..T..AT.A........A....CA..A..T | A.....T.....T..A..G..A..G.GA.G |
| Gi_YP | 2 | ..T..AT.A..C.....G..G.CA..C..T | A...........G..G..G..A..A.GG.G |
| Tt_DR | 2 | ..T.....C....................T | C..A..A..T.....G.....A.....T.. |

**Table 5b.** ***Maoricolpus roseus*-specific PCR primers, CMRSF4 and CMRSR3, aligned with corresponding sequence from other native species of Turritellidae (a dot indicates the nucleotide is the same as in the top sequence).**

| Species | n | CMRSF4 → | ← CMRSR3 |
|---|---|---|---|
| Mr_DP | 3 | **5'**GTGCTGAGCTTGGACAGCCAGGTGCGTTGC | CACCCAGTCCCTACCCCTCTTTCTACAGCA **3'** |
| Mr_BS1 | 3 | .............................. | .............................. |
| Mr_BS2 | 3 | .............................. | .............................. |
| Mr_NB | 3 | .............................. | .............................. |
| Mr-PB | 3 | .............................. | .............................. |
| Mr_TB | 3 | .............................. | .............................. |
| Mr_BB | 3 | .............................. | .............................. |
| Mrm_MH | 3 | .............................. | .............................G |
| Gg_DB | 3 | .G.....A...........C..G..AC.TT | ..T.....T...G.G..C........G..T |
| Gg_NB | 3 | .G.....A...........C..G..AC.TT | ..T.....T...G.G..C........G..T |
| Gi_YP | 2 | .G.....A..C........C..G..TC.CT | ...........CG................T |
| Tt_DR | 2 | .G...........T.....T..G..A...T | ..T..T..T.....T..C.....A.....T |

**Table 5c. *Maoricolpus roseus*-specific PCR primers, CMRSF4 and CMRSR4, aligned with corresponding sequence from other native species of Turritelidae (a dot indicates the nucleotide is the same as in the top sequence).**

| Species | n | CMRSF4 → | ← CMRSR4 |
|---|---|---|---|
| Mr_DP | 3 | **5'**GTGCTGAGCTTGGACAGCCAGGTGCGTTGC | ACAGCAGCTGAAGAAAGGAGAAGTAGAAGA **3'** |
| Mr_BS1 | 3 | .............................. | .............................. |
| Mr_BS2 | 3 | .............................. | .............................. |
| Mr_NB | 3 | .............................. | .............................. |
| Mr-PB | 3 | .............................. | .............................. |
| Mr_TB | 3 | .............................. | .............................. |
| Mr_BB | 3 | .............................. | .........................A.... |
| Mrm_MH | 3 | .............................. | .....G........................ |
| Gg_DB | 3 | .G.....A...........C..G..AC.TT | ..G..T.....G..T..T.AT.A..AC.A. |
| Gg_NB | 3 | .G.....A...........C..G..AC.TT | ..G..T.....G..T..T.AT.A..AC.A. |
| Gi_YP | 2 | .G.....A..C........C..G..TC.CT | .....T.....G..T.AC.AT....A...G |
| Tt_DR | 2 | .G...........T.....T..G..A...T | .....T...........T.A...C.AT.AG |

## 3.4 Specificity of the PCR assay

The specificity of the predicted *Maoricolpus roseus*-specific primers was empirically tested by PCR amplification of genomic DNA of 42 samples representing 5 different species of the family Turritellidae. Amplifications were carried out on of the family Turritellidae (Table 4). Separate amplifications of all three potential *M. roseus*-specific primer pairs were carried out and all of them gave similar results. As summarised in Table 4., only the *M. roseus* samples amplified the target sequence. Representative PCR results following amplification of DNA from five different species of Turiitellidae using the three different primer pairs are presented in Figures 5a, 5b and 5c respectively. The expected 113bp (CMRSF1 / CMRSR2), 181 bp (CMRSF4 / CMRSR4) and 205 bp (CMRSF4 /CMRSR3) target DNA fragments were amplified only from *M. roseus* samples irrespective of their geographical origin (Figure 5; lanes 9-14 and Table 4) in all three amplifications. In contrast, none of the *G. iredalei* (Figure 5; lanes 1-2), *G. gunnii* - Nubena (Figure 5; lanes 3-4), *G. gunnii* – Disaster Bay (Figure 5; lanes 5-6), and *T. terebra* (Figure 5; lanes 7-8) returned PCR negative for the *M. roseus*-specific primers. In concurrent PCR amplification studies, a universal primer pair targeted at the 18S rDNA generated an expected fragment size of about 460 bp (Figure 5; lanes 1-14, top band and Table 4) from all the samples, indicating that an adequate quantity and quality of template DNA was supplied in each PCR reaction.

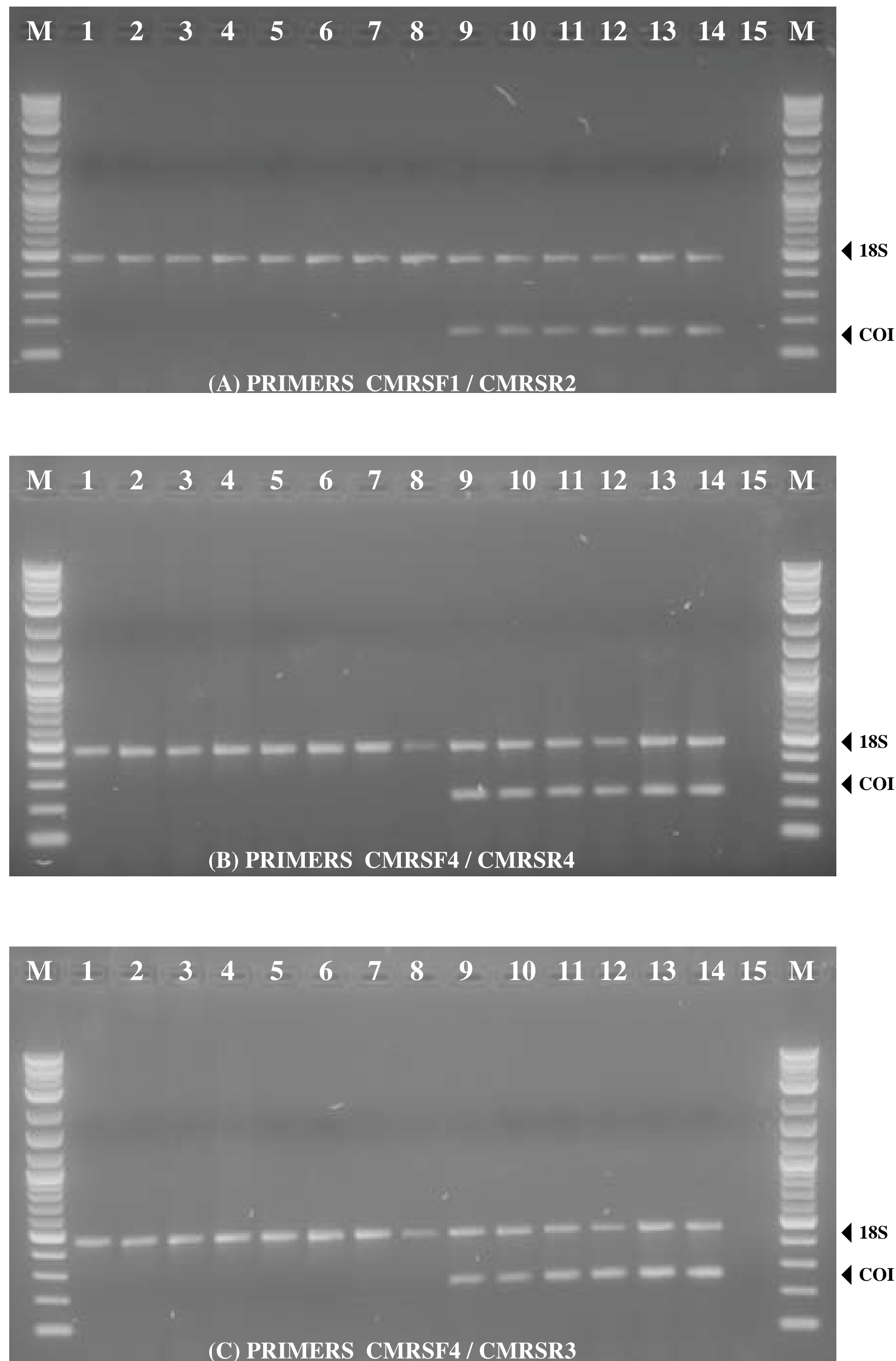


Figure 5. A representative gel photographs showing "*M.roseus*-specific" PCR products separated on a 1.8% agarose gel. Figure 5A (primers CMRSF1 & CMRSR2), 5B (primers CMRSF4 & CMRSR4), and 5C (primers CMRSF4 & CMRSR30) represents amplifications of three potential *M.roseus*-specific primer pairs. The upper band is the positive internal control reaction (18S, arrowhead) and the lower band is the diagnostic *M.roseus*-specific (COI, arrowhead). lane M, Standard size markers (2-log DNA ladder, New England Biolabs); lanes 1-2, Gi_YP; lanes 3-4, Gg_NB; lanes 5-6, Gg_DB; lanes 7-8, Tt_DR; lanes 9-10, Mrm_MH; Lanes 11-12, Mr_BB; Lanes 13, Mr_DP; Lane 14, Mr_NB; lane 15, negative control.

## 3.5 Detection of larval *Maoricolpus roseus*

Egg capsules containing veliger larvae of *Maoricolpus roseus* were removed from the mantle cavity of frozen samples collected from Lords Bluff, Triabunna on 24th January 2001. The veligers ranged from 0.1 - 0.2 mm in length and there was an average of 45 veligers per egg capsule, (Figures 6, 7 & 8).

**Figure 6. A bunch egg capsules of *Maoricolpus roseus* protruding from adult female's mantle cavity (first whorl of shell removed)**

**Figure 7. A magnified egg capsule of *Maoricolpus roseus* containing larvae, Capsule diameter = 1 mm**

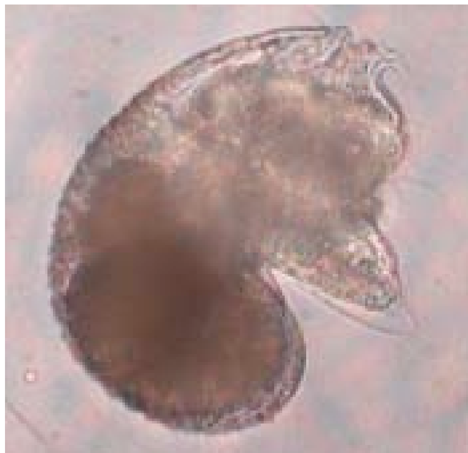

**Figure 8. Close up of veliger of Maoricolpus roseus removed from egg capsule, diameter = 0.1 mm**

It was possible to amplify *M roseus*-specific mitochondrial COI amplicon using the isolated egg capsules as templates, without the need to extract the DNA. The accuracy was 100% even when one egg capsule was used as template (about 45 larvae). Although attempted it was physically impossible to isolate and test the probes on individual larvae as most of them tended to rupture and burst open upon dissection of the egg capsules. This may not be a problem when dealing with live specimens rather than the frozen samples that were used in this study.

## 3.6 Detection of *Maoricolpus roseus* in environmental samples

A total of 90 (30 sampling points X 3 replicates) plankton samples were analysed using the primer pairs CMRSF1 / CMRSR2 and CMRSF4 / CMRSR3. The details of the samples processed are presented in Table 6. The results indicate that the *Maoricolpus roseus* larvae were detected sporadically throughout the year with higher frequency in the summer months. The results from the two sets of probes employed were not only identical, but also the subsequent sequencing of the products confirmed/verified the identity of the signals to be that of *M. roseus*. On the basis of this we can conclude that *M. roseus* does have a planktonic larval phase.

**Table 6. The results of plankton samples collected from Derwent River estuary, Hobart. Samples were collected at the Royal Yacht Club or the Domain slipways. All the positive samples were sequenced and confirmed positive for *Maoricolpus roseus*.**

| Sampling date | Location | CMRSF1 / CMRSR2 | CMRSF4 / CMRSR3 |
|---|---|---|---|
| 12/8/03 | Royal Yacht Club | -ve | -ve |
| 15/8/03 | Royal Yacht Club | +ve | +ve |
| 8/9/03 | Royal Yacht Club | -ve | -ve |
| 23/9/03 | Royal Yacht Club | -ve | -ve |
| 25/9/03 | Royal Yacht Club | -ve | -ve |
| 1/10/03 | Royal Yacht Club | -ve | -ve |
| 6/10/03 | Royal Yacht Club | -ve | -ve |
| 22/10/03 | Royal Yacht Club | -ve | -ve |
| 24/10/03 | Royal Yacht Club | +ve | +ve |
| 3/11/03 | Domain | -ve | -ve |
| 4/11/03 | Domain | -ve | -ve |
| 12/11/03 | Royal Yacht Club | +ve | +ve |
| 19/11/03 | Domain | +ve | +ve |
| 21/11/03 | Royal Yacht Club | -ve | -ve |
| 24/11/03 | Royal Yacht Club | -ve | -ve |
| 8/12/03 | Domain | -ve | -ve |
| 2/2/04 | Domain | +ve | +ve |
| 3/2/04 | Domain | -ve | -ve |
| 10/2/04 | Domain | +ve | +ve |
| 25/2/04 | Royal Yacht Club | -ve | -ve |
| 1/3/04 | Royal Yacht Club | -ve | -ve |
| 3/3/04 | Domain | +ve | +ve |
| 18/3/04 | Domain | +ve | +ve |
| 2/4/04 | Royal Yacht Club | +ve | +ve |
| 23/4/04 | Domain | +ve | +ve |
| 28/4/04 | Royal Yacht Club | -ve | -ve |
| 3/5/04 | Domain | +ve | +ve |
| 10/5/04 | Domain | -ve | -ve |
| 20/5/04 | Domain | -ve | -ve |
| 11/6/04 | Domain | +ve | +ve |

## 3.7 Sequence of partial mitochondrial genome

Although not an agreed project deliverable, we decided to amplify and sequence the entire mitochondrial genome of *Maoricolpus roseus* for two reasons. First, as mentioned above there was limited genetic variation within and between the COI locus of *M. roseus* and *M. roseus manukauensis*, precluding inferences on potential source of introduction. Secondly, our decision to defer empirical validation of probes in the absence of the *G. gunnii* sequence provided the opportunity to carry out amplification and sequencing of the mitochondrial genome. The exact length of the *M. roseus* mt-DNA genome has not been determined, however, based on the size of other known gastropods and molluscs we estimate it to be around 15 kb. We have successfully amplified and sequenced the 13224 bp fragment of *M. roseus* mitochondrial genome. We made several attempts to amplify the remaining roughly 1-2 kb of the

genome but were unsuccessful. It is hoped that the 13224bp sequence information will assist in future population studies of the species as well as for making inferences on the potential source/s of introduction into Australian waters.

# 4. DISCUSSION

## 4.1 Significance of *Maoricolpus roseus* in Australia

It is hard to compare the environmental impacts of invasive species. Species having the most impact are those which can change a system – system engineers. These system engineers typically operate as effective generalist predators (eg. *Asterias amurensis*); or through changing system structure by smothering (eg. *Mytilopsis* sp., *Caulerpa taxifolia*), adding vertical structure (eg. *Undaria pinnatifida*), or by altering sediment structure (eg. *M. roseus*). *M. roseus* consolidates mobile sediments creating empty reefs of shells many layers deep (Professor Craig Johnson, University of Tasmania, personal communication). The empty shells provide homes for hermit crabs (Bax et al., 2003) and can provide holdfasts for other introduced species (Bax, personal observation). It is currently not possible to say whether the primary sediment consolidation impact of *M. roseus* is more or less significant than secondary impacts though the provision of novel habitat for hermit crabs. However, with an area of impact equaling the landmass of Tasmania and including waters under State and Commonwealth jurisdiction, *M.roseus* may be the most damaging marine pest existing in Australia today.

Despite, a long history of invasion and spread in the coastal and continental shelf waters of south east Australia, there is very limited information on the life cycle and reproductive biology of *M. roseus* and nothing has been done to reduce or mitigate its spread to new areas. Anecdotal evidence suggests that the animal has a planktonic life history stage (Bax et al., 2003). However without definitive information, management measures to reduce the risk of this species being transported to Western Australia and the Great Australian Bight in ballast water will not be implemented. This work, developing a tool for the rapid identification of *M. roseus,* provides the opportunity to provide this information. It is a first step towards understanding how *M. roseus* is spread and how we can act to limit its future impacts.

*Maoricolpus roseus* is a member of the family Turritellidae, gastropods found in soft sediments from the shallow subtidal to the continental shelf and slope in tropical and temperate waters. Twenty two native turritellid species have a distribution that potentially overlaps with the currently known distribution of *M. roseus* in Australian waters (Figure 9 and Appendix D). Despite exhaustive efforts we managed to obtain samples of only four of these species. Turritellids have never been studied specifically in Australia and any collections held by museums, federal or state environmental and fisheries agencies and other private environmental consultants are the result of general benthic surveys. Such large scale surveys on the continental shelf are usually conducted by dredging and since turritellids are small (5 - 80 mm length) the majority of these species would not be retained by a normal dredge or benthic sled and so few are collected. In addition, samples taken from benthic sediments are almost universally preserved in formalin so the majority of turritellids present in existing collections were unsuitable for genetic analysis. The species we did manage to obtain were in the larger size range of the turritellids and were collected from shallow subtidal depths.

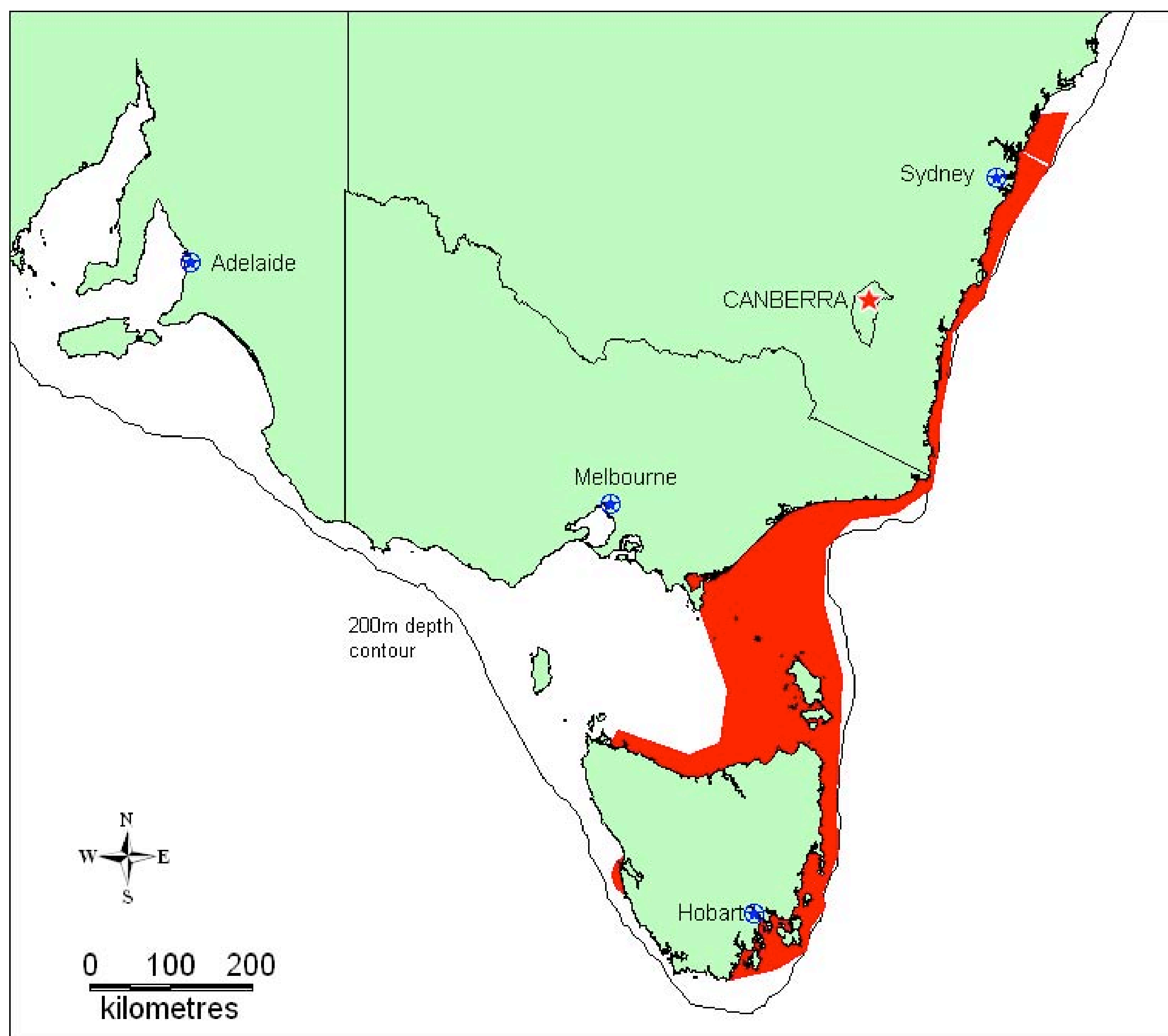


**Figure 9. Current known distribution of *Maoricolpus roseus* in Australian waters (based on Bax et al., 2003).**

## 4.2 Probe development, specificity and sensitivity

We used a combination of genetic sequence comparison and empirical testing to develop *M. roseus*-specific PCR primers targeted at the mt-COI locus. These primers can be used to detect and identify *M. roseus* larvae dissected from the egg sacs or in unsorted plankton samples. The feasibility of specific PCR amplification as a rapid means for detection and or quantification of larvae has been previously demonstrated for the bay scallop (Frischer et al., 2000) and the zebra mussel, *Dreissena polymorpha* (Frischer et al., 2002), based on 18S rDNA loci, and for the sea star, *Asterias amurensis* (Deagle et al., 2003) and the bivalve *Crassostrea gigas* (Patil et al., 2004), based on the mt-COI locus.

It is well known that bivalve molluscs (Patil et al., 2004, Hare et al., 2000 and references therein) exhibit high sequence variation at the mt-COI locus permitting the design of species-specific probes. Similarly in this study, the high sequence variation observed at the mt-COI locus between the members of the family Turritellidae allowed us to design PCR primers that

appear to be specific for *M. roseus.* This sequence variation was particularly evident in all the three primer pairs chosen (Table 5a-c), making it possible to design not one but three sets of probes for specific detection of *M. roseus*. This availability of multiple gene probes for specific detection assists in cross verification and validation of results. All three sets of probes consist of two species specific primers each. The use of two species-specific primers as probes provide better discrimination than one conserved primer with an opposing species-specific primer (Rocha-Olivares, 1998) as has been previously demonstrated in two other species of molluscs (Hare et al., 2000; Patil et al., 2004). The large interspecific sequence diversity at all six primer binding sites (Tables 5a-c) suggests that it might be possible to custom make species specific probes for other species of turritellids.

Including additional turritellids to those used in this study, especially those from the geographical region where it is intended to use the probes, would be advisable to ensure the specificity of the chosen primer pair for *M. roseus* in a new area. Of particular emphasis should be tiny turritellids commonly missed in large scale surveys.

The three PCR assays (probes) developed in this study, successfully amplified the target DNA in the 32 *M. roseus* samples, despite their geographic diversity (6 and 2 sampling sites in Australia and New Zealand respectively). However, it must be noted that these samples did not provide a broad coverage of New Zealand or mainland Australia (eastern Victoria and New South Wales). Therefore, we can not completely rule out the possibility of intraspecific polymorphism at the primer sites that might preclude amplification in some samples, producing a false negative result. Genetic characterisation of the mt-COI locus of *M. roseus* throughout its natural range in New Zealand as well as its *de novo* range, where it has been introduced, would be required to test the possibility of false negative results. However, such false negative results are unlikely as even the subspecies *M. roseus manukauensis* was successfully amplified by all the three probe sets, despite exhibiting a single base pair polymorphism at all the probe sites. Moreover, the COI locus seems to have low levels of intraspecific polymorphism in marine molluscs (Hare et al., 2000 and references therein and Patil et al., 2004).

No false positives were obtained for the probe pairs tested on several species of turritellids, including the native and threatened screw shell *G. gunnii* (Figure 5a-c, lanes 3-6), implying that the probes are species specific. Primer specificity in PCR is mostly conferred by the last few nucleotides at the 3' end of oligonucleotide, so even a single unique nucleotide can be used to direct species-specific PCR (Newton et al., 1989; Bottema et al., 1993). The *M. roseus* specific probes used here had in excess of 10 base pair mismatches with the corresponding sequence from other sequenced native turritellids, suggesting the probes should be specific to *M. roseus.*. We consider the primers to be species-specific, not because they have been tested on all potential congeners, but because they exhibit significant sequence divergence, particularly from those of native Australian turritellids. The primers may be species specific outside of this geographic region, but further study would be needed to verify this possibility. If the primers were used for screening environmental samples, it would be prudent to confirm the identity of a subset of positive results by sequencing.

Genetic identification of microscopic organisms in environmental samples is more difficult than identifying pure or isolated samples (Patil et al., 2004). At least two reasons have been proposed to explain this difference. First, dilution of target DNA in the background of environmental DNA samples might reduce the success of amplification. Second, PCR inhibitors such as humic material in the environmental samples could compromise the efficiency of PCR reactions. A common approach to enhance sensitivity of gene probes in analysing

environmental samples is the use of nested PCR. Our studies indicate that nested PCR increases sensitivity by at least 100 times over standard PCR when dealing with environmental samples; others have demonstrated 10 000 times higher sensitivity when compared with standard PCR (Miserez et al., 1997).

Detection levels achieved using purified target DNA are of little significance when dealing with mixed environmental samples, and therefore as previously described (Patil et al., 2004) we attempted to determine the number of larvae that can be consistently detected. Unfortunately we were unable to isolate individual *M. roseus* larvae because fresh larvae were unavailable at the time of the sensitivity trials and frozen larvae tended to disintegrate when removed from previously frozen egg capsules. Tests were therefore conducted on whole egg capsules, which on average contained about 45 veliger larvae per capsule. A follow up experiment will be conducted with larvae obtained from fresh specimens to determine lower limits of detection of the assay. Based on previous experience we expect that as few as 5 larvae spiked into environmental/ballast water samples could be routinely detected (Patil et al., 2004).

## 4.2 Mapping the mitochondrial genome

One of the key requirements to manage any pest populations is the source of introduction or infection. It is possible that the *M roseus* introduction into Australian coastal waters has occurred through multiple introductions from various geographic regions of New Zealand. It is also possible that one particular population might have led to the introduction and establishment of all of Australian populations. Based on the limited COI sequence information it appears that the latter may be the case as there is no genetic variation between the samples collected from different locations in Australia. This is the currently accepted view in the literature (Bax et al., 2003). However, this may also be due to limited sequence variation within and between *M. roseus* populations in Australia and New Zealand at the COI locus. To assist in fine scale genetic discrimination of different populations of *M. roseus* and to elucidate the potential source of introduction to Australian waters, we decided to characterise the entire mitochondrial genome of the *M. roseus.* A nearly complete mitochondrial genome sequence was generated (13324 bp of the estimated ~15 kb) during this study. Close analysis of the genome suggest that the gene order is more similar to the black chiton, *Kathrina tunicata* than to *Littorina saxtalis*, another gastropod mollusc for which the mtDNA genome sequence has been partially described. This perhaps implies that the two gastropods may have evolved independently from a common ancestor. However it will require further rigorous analysis against all the known mitochondrial genomes of molluscs to establish a true evolutionary relatedness.

The mitochondrial genome of the *M. roseus* is highly AT rich (63.3%), somewhat more than for the sequenced portions of *Mytilus* (62 %; Hoffman et al., 1992), however less than that of *Katharina tunicata* (69 %; Boore and Brown 1994). The sequenced genome reported here encompasses genes for 11 proteins (ATPase 6, ATPase 8, COI, COII, Cytochrome b, ND1, ND2, ND4, ND4L, ND5 and ND6) 2 Ribosomal RNAs (large and small subunits) and 17 tRNAs. Based on current information, the gene order closely resembles that of the black chiton *K. tunicata* (Boore and Brown 1994). A detailed description of the mtDNA genome will be published elsewhere.

## 4.3 Testing for a planktonic life history

Given the importance of the presence of a planktonic life history stage in the management of *Maoricolpus roseus* we decided to conduct a preliminary evaluation based on environmental plankton samples collected monthly from the Derwent Estuary as part of a separate NHT-funded project: "Empirical Validation stage 1" (Hayes et al., 2004). Nested PCR was used to enhance sensitivity (Deagle et al., 2003;Patil et al., 2004), and *M. roseus* was detected. The possibility of a contamination as well as non-specific amplification was ruled out since two independent amplifications using two sets of primers yielded identical results. The sequence identity was confirmed by sequencing, and the majority of DNA extractions occurred before any *M. roseus* DNA was worked on in the lab. These results support earlier suggestions that *M. roseus* has a planktonic larval stage prior to settlement (Bax et al., 2003). Although the data are sparse, they also confirm earlier observations that *M. roseus* is predominantly a spring-summer (Austral) spawner, generally between October and March. However, we also obtained positive samples in June and August suggesting that *M. roseus* may have a second spawning period in autumn/winter, or that small levels of spawning occur year-round in different populations. It is also possible that *M. roseus* may have a protracted larval existence in the absence of a suitable substratum for settlement.

These preliminary observations indicate that *M. roseus* has a planktonic life history, possibly a protracted one and therefore would be susceptible to movement in ships' ballast water from the Derwent estuary to other temperate Australian ports. The probe results do not indicate the abundance of the larvae in the water column; real time PCR is being developed to assist the rapid quantitative estimation of invasive species in a separate Natural Heritage Trust funded project. It is surprising, given the presence of *M. roseus* in the Derwent Estuary since at least the 1930s (Bax et al., 2003), that it has not already been spread to major ports such as the ports of Melbourne and Geelong in Port Phillip Bay. The seastar, *Asterias amurensis* moved from the Derwent Estuary to Port Phillip Bay in a much shorter time, although there is some discussion over whether it was transported by ballast water or as a fouling organism. Additional studies on the period and abundance of *M. roseus* larvae in the water column are recommended to evaluate the risk that ships' ballast poses in spreading this species. The probes developed here would greatly assist in such a study.

# 5. CONCLUSION

Three sets of genetic probes were developed to detect *M. roseus* in environmental samples. Regions of relatively high sequence diversity flanked by conserved regions at the COI locus enabled the development of this nested PCR assay for detection of *M. roseus* larvae in plankton samples. The relatively high efficiency of this assay stems from its ability to circumvent the need for sorting or isolating larvae prior to PCR analysis or post characterisation of PCR products. The 13224 bp mitochondrial genome sequence generated as part of this study will assist in fine scale genetic discrimination of *M. roseus* populations in Australia and New Zealand. Results from preliminary application of the probes indicate that the animal undergoes a planktonic larval stage and is predominantly a spring-summer spawner. This suggests that it can be transported in ships' ballast water and its presence needs to be considered in the management of this vector.

This is fourth in a list of high profile alien marine species in Australia for which we have developed a PCR detection assay, the others being *Asterias amurensis* (Deagle et al., 2003), *Gymnodinium catenatum* and *Crassostrea gigas* (Patil et al., 2004). Ultimately we aim to provide a rapid, high throughput and cost-effective method for routine simultaneous monitoring of marine pest larvae to establish their potential translocation vectors and routes and to facilitate management practices that will mitigate their spread to new environments.

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# APPENDIX A - ACKNOWLEDGEMENTS

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# APPENDIX B – DETAILED CONSULTANCY WORKPLAN

| Stage | Activity | Milestone | *Due Date* | *Performance against milestones* |
|---|---|---|---|---|
| 1. | Contract finalisation:<br>Finalise contract | Contract finalised | | Contract signed 2 April 2004 |
| 2. | Probe identified:<br>Sequence 18S rDNA and mtCOI locus of *Maoricolpus roseus*".<br>Design potential *M roseus* specific primers<br>Determine conditions that maximise positive discrimination between *M. roseus* and other native gastropods | Sequence for genetic probe identified<br>Progress Report 1 submitted to EA. | Aug 30, 2003 | Both the 18S rDNA and mitochondrial COI loci of *M. roseus* have been sequenced<br>Alignment of the sequences with those of supefamily Cerithioidea and a member of the family Turritellidae, *(Turritella terebra)* identified suitable regions for *M roseus* specific probes.<br>Fresh specimens of Australian and New Zealand members of the family Turritellidae are being sought to optimise probe specificity.<br>Progress report submitted in October 2003 |
| 3. | Probe tested:<br>Test in natural plankton samples, spiked with a specified number of *Maoricolpus roseus* larvae, or tissue.<br>If required re-validate the positive "signatures" by direct sequencing | Probe optimised and tested for natural plankton samples<br>Progress Report 2 submitted to EA. | Dec 31 2003 | *In silico* analysis of sequence data suggest significant sequence variation at COI locus and hence the potential for developing probes to specifically detect *M roseus.*<br>Empirical testing of probes against DNA of other species of Turitellids and plankton samples is deferred for the time being, until specimens of *G gunni* are obtained.<br>A survey involving 9 divers has been planned for Wednesday 24 Feb 2004 to obtain adult samples of *G gunni* and larvae of *M roseus*<br>A 8 kb fragment of the mitochondrial genome of *M. roseus* has been amplified and partially (5.2 kb) sequenced. |
| 4 | Submit Final report to Environment Australia | Final report submitted to Environment Australia | 31 March 2004 | |

# APPENDIX C – RESEARCHERS CONTACTED FOR SAMPLES

**Table x. List of people contacted in search of Turritellid samples in Australia (n=26)**

| Name | Institution | State | Response |
|---|---|---|---|
| **Museums** | | | |
| Brian Smith | QVM | Tasmania | None in collection |
| Fred Wells | WAM | Western Australia | Samples in formalin |
| Liz Turner | TMAG | Tasmania | *Gazameda subsquamosa, Colpospira (Ctenocolpus) australis* |
| Richard Willan | MAGNT | Northern Territory | *Turritella terebra* |
| Chris Rowley | Museum Victoria | Victoria | Samples in formalin |
| Karen Gowlett-Holmes | SAM | South Australia | none |
| **CSIRO Marine Research** | | | |
| Felicity McEnnulty | CMR Hobart | Tasmania | *Maoricolpus roseus, Gazameda gunnii* |
| Alan Butler | CMR Hobart | Tasmania | SEF samples in formalin |
| Karen Gowlett-Holmes | CMR Hobart | Tasmania | *Gazameda iredalei* |
| Mark Lewis | CMR Hobart | Tasmania | Contact for scallop fishers |
| Alan Williams | CMR Hobart | Tasmania | *Gazameda gunnii* |
| **State Government Agencies** | | | |
| Catriona Macleod | TAFI | Tasmania | Samples in formalin |
| Malcolm Haddon / Julian Harrington | TAFI | Tasmania | *Maoricolpus roseus* |
| Neville Barrett | TAFI | Tasmania | Sampling locations |
| Alan Jordon | TAFI | Tasmania | Sampling locations |
| Colin Shepherd | DPIWE Marine Environment | Tasmania | Sampling locations |
| Iona Mitchell | DPIWE Water Resources | Tasmania | Sampling locations |
| Duncan Worthington / Craig Brand | NSW Fisheries Abalone research | New South Wales | *Maoricolpus roseus* |
| Jason Tanner | PIRSA/SARDI | South Australia | None in collection |
| Greg Parry | MAFRI | Victoria | Samples in formalin |
| **Universities** | | | |
| Jeff Ross | Universisty of Melbourne | Victoria | None in collections for Victoria or Tasmania |
| Anthony Reid | Unversity of Tasmania | Tasmania | *Maoricolpus roseus* |
| **Private Environmental consultants** | | | |
| Adam Davey | Aquenal | Tasmania | Samples in formalin |
| Graeme Edgar | Aquenal | Tasmania | Sampling locations |
| Professor Tony Underwood | Centre for Research on Ecological Impacts of Coastal Cities | New South Wales | None in collection |
| Matt Edmunds | Australian Marine Ecology | Victoria | None in collection |

**Table Z. List of people contacted in New Zealand in search to obtain Turritellid samples(6)**

| Name | Institution | Response |
|---|---|---|
| **Museums** | | |
| Brian Marshall | Museum of New Zealand | Samples in formalin or too long in ethanol |
| Todd Landers | Auckland Museum | *Maoricolpus roseus* and *Maoricolpus roseus manukauensis* |
| **Government Agencies** | | |
| Dennis Gordon | NIWA Wellington | Samples in formalin |
| Graeme Inglis / Isla Fitridge | NIWA Christchurch | Samples from port survey *Maoricolpus roseus* |
| Simon Thrush | NIWA Hamilton | None collected |
| **Universities** | | |
| Keith Probert | University of Otago | *Maoricolpus roseus* |

# APPENDIX D – TURRITELLID SPECIES OVERLAPPING *MAORICOLPUS ROSEUS* IN AUSTRALIAN WATERS (WILSON 1993)

| Species | Distribution | Length (mm) | Depth (metres) |
|---|---|---|---|
| *Colpospira bundilla* | sQLD to sWA | 5 | 100 - 550 |
| *Colpospira mediolevis* | SA to sWA | 5 | 110 |
| *Colpospira wollumbi* | sQLD to sWA | 5 | 77 - 237 |
| *Colpospira yarramundi* | sQLD to eastern Bass Strait | 6 | 75 - 550 |
| *Turritellopsis kimberia* | SA | 8 | 35 - 170 |
| *Colpospira smithiana* | sQLD to sWA | 10 | to 1500 |
| *Colpospira cordismei* | NSW to Bass Strait | 12 | ? |
| *Colpospira guilleaumei* | NSW to Bass Strait | 15 | 75 - 100 |
| *Colpospira circumligata* | Bass Strait to SA | 17 | 58 - 200 |
| *Colpospira sinuata* | sQLD to Bass Strait | 18 | 75 -100 |
| ***Colpospira australis*** | **NSW to sTAS and SA** | **20** | **50 - 200** |
| *Colpospira decoramen* | sQLD to Bass Strait | 20 | 200 -1000 |
| *Colpospira quadrata* | NSW to SA | 20 | 82 - 860 |
| *Colpospira atkinsoni* | NSW to Bass Strait | 22 | 75 - 100 |
| *Colpospira accisa* | NSW to sWA | 30 | 75 - 100 |
| *Colpospira runcinata* | NSW to TAS and central WA | 30 | 70 |
| ***Gazameda iredalei*** | **Bass Strait to sWA** | **40** | **0 - 50** |
| ***Gazameda tasmanica*** | **sQLD to sWA** | **40** | **8 – 90** |
| ***Gazameda gunnii*** | **sQLD to VIC and TAS** | **56** | **8 - 140** |
| ***Maoricolpus roseus*** | **NSW to TAS** | **80** | **0 - 200** |
| *Turritellopsis neptunensis* | sQLD to SA | ? | 190 |
| *Gazameda declivis* | Northern Australia from Central WA to eastern Bass Strait | 60 | 75 - 100 |
| ***Turritella terebra*** | **nWA to central QLD** | **170** | **0 -** |

N.B. Species which have been subject to genetic analysis are shown in bold. *Turritella terebra* does not overlap with *Maoricolpus roseus*.