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# Species identification of marine invertebrate early stages by whole-larvae *in situ* hybridisation of 18S ribosomal RNA

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ABSTRACT: The ability to identify early life-history stages of organisms is essential for a better understanding of population dynamics and for attempts to inventory biodiversity. The morphological identification of larvae is time consuming and often not possible in those species with early life-history stages that are radically different from their adult counterparts. Molecular methods have been successful in identifying marine larvae; however, to date these methods have been destructive. We describe here an *in situ* hybridisation (ISH) technique that uses oligonucleotide probes specific for the 18S ribosomal RNA gene to identify marine larvae. Our technique leaves the larvae intact, thus allowing the description of larvae whose morphology was not previously known. Only 1 mismatch between the rRNA sequences of target and non-target species is sufficient to discriminate species, with nearly 100% efficiency. We developed a colourimetric assay that can be detected with a dissecting microscope, and is thus suitable for autofluorescent or large eggs and larvae that cannot be sorted under a microscope. Probe binding is revealed by an enzymatic reaction catalysed by either a horseradish peroxidase or an alkaline phosphatase. ISH was broadly applicable: it was effective in identifying eggs, larvae and adult tissues, soft-bodied larvae (polychaetes) and larvae with hard shells (bivalves), larvae belonging to different phyla and from different environments. Further advantages of this method are its relatively low cost, that only a minimal amount of equipment is needed, and that 100s of specimens can be processed quickly and simultaneously.

KEY WORDS: Whole-larvae *in situ* hybridisation · Oligonucleotide probes · Species identification · Molecular ecology · Ribosomal RNA · Polychaetes · Bivalves



## INTRODUCTION

Our understanding of the mechanisms governing population biology and dynamics, and our ability to predict changes in these populations and to manage marine invertebrate and fish species are complicated by their life cycles. Marine larvae are radically different from their adult counterparts, in morphology, habitat and mode of nutrition. During development, they undergo very rapid and extensive developmental changes. Moreover, larvae of a number of species are planktonic and have the potential to travel 100s to 1000s of kilometres transported by currents, to mix in the water masses with other species, before undergoing metamorphosis and beginning their adult life (Kinlan et al. 2005). Both adult benthic ecology and larval ecology need to be considered to develop a fuller understanding of the relative importance of larval and benthic dynamics to a species' spatial and temporal distributions, abundances and population structure (Eckman 1996). In many marine species, the first step towards larval ecology is merely to be able to identify early life stages in the environment.

Many marine invertebrate and fish larvae cannot be identified to the species level, either because closely related species are essentially identical morphologi-

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cally, or because the larval forms of a species are unknown (Levin 1990). In species that have yet to be reared in the laboratory, which applies to the vast majority of all species, how does one match an unknown larva to a described species? In addition, there is an increasing realisation that the forms of many invertebrate larvae are very plastic and are determined by a number of environmental variables such as food (Sewell et al. 2004) and the physico-chemical properties of water (e.g. temperature in Shirley et al. 1987).

As an alternative to morphological methods, a number of biochemical and molecular methods have been developed (for review see Garland & Zimmer 2002). Immunological (Demers et al. 1993) and polymorphic allozyme electrophoresis techniques (Hu et al. 1992) have been used to discriminate larvae at the family or even the species level. However, environmental conditions, ontogenic changes in the larvae and sample preservation may alter protein concentration or conformation of the protein's epitope (Demers et al. 1993, Anderson et al. 1999). Larval proteins may also be highly conserved and may not differ sufficiently among species to be used as species-specific markers. In addition, general antibody cross-reaction problems limit the resolution of immunological techniques (Garland & Zimmer 2002). Molecular methods using diagnostic DNA sequences isolated from adult specimens have been used successfully to identify larvae (Coffroth & Mulawka 1995, Morgan & Rogers 2001, Larsen et al. 2005), a number of which were based on ribosomal RNA sequences (Olson et al. 1991, Medeiros-Bergen et al. 1995, Comtet et al. 2000, Frischer 2000). Ribosomal RNAs (rRNA) are such excellent phylogenetic markers because they are extremely conserved in overall structure, allowing identification at higher taxonomic levels, and yet also highly variable in certain regions, allowing identification at the species level. To date, molecular identification methods have required DNA or RNA extraction and were therefore destructive.

Whole-cell *in situ* hybridisation methods using oligonucleotide probes targeting rRNA have successfully identified bacteria and archea (DeLong et al. 1989, Amann et al. 1990, Pernthaler et al. 2002), diatoms (Scholin et al. 1997), nanoflagellates (Lim et al. 1996), Microsporidia (Hester et al. 2000), ciliates (Petroni et al. 2002) and picophytoplankton (Simon et al. 2000). Despite the clear advantage of this technique for identifying larval stages, surprisingly, there has been only 1 attempt to extend it to marine larvae (Goffredi et al. 2006). These authors used fluorescent oligonucleotide probes to identify barnacle larvae by *in situ* hybridisation (FISH), but this method is limited by the strong autofluorescence of many marine eggs and larvae (Pradillon 2002), making it difficult to consider a general application of FISH for marine larvae.

We describe here a non-fluorescent method that uses horseradish peroxidase- or digoxygenin-labelled probes to which binding is revealed by a colour reaction. Coloured larvae can then easily be seen under a standard dissecting microscope while sorting plankton. This approach is similar to the one first described for bacterial cells (Amann et al. 1992). Here, we developed species-specific oligonucleotide probes targeting the 18S rRNA for invertebrate species in 2 different contexts where larval identification was needed for understanding dispersal processes. We targeted 4 polychaete species from a hydrothermal vent of the East Pacific Rise (*Alvinella pompejana*, *Alvinella caudata*, *Riftia pachyptila* and *Tevnia jerichonana*) and an introduced alien oyster species currently invading the southern North Sea (*Crassostrea gigas*). In both cases, the aim was to identify a defined target species, among a mix of non-target species. By developing our method for use on such different larvae, i.e. polychaete larvae with a cuticle and bivalve larvae with a shell, we were able to show the broad applicability of our technique.

## MATERIALS AND METHODS

**Sample collection.** To design species-specific probes, sequences of all closely related species that occur in the same biogeographic range as the target species should be compared. At the time we designed probes for this study, only some of these sequences were available in GenBank. For this reason, in addition to specimens of the target species, closely related species were collected to obtain 18S rRNA gene sequences. Adult specimens of 9 polychaete species, including our 4 target species, were collected from hydrothermal vents between 9° N and 21° S on the East Pacific Rise (EPR) during cruises from 1994 to 2004 (Table 1). Upon reaching the surface after the submersible ascent, individuals were directly stored in liquid nitrogen or in 96% ethanol. *Crassostrea gigas* and 3 additional bivalve species were collected around the island of Sylt (Germany) in 2001 and stored in 70% ethanol (Table 1).

*In situ* hybridisation (ISH) assays were performed on larvae obtained from cultures, on eggs collected from adult specimens, or on adult tissues. *Crassostrea gigas* larvae were obtained from Guernsey Sea Farms. They were preserved in 70% ethanol in seawater and had been kept for up to 4 yr when used in ISH. *C. gigas*, *Ostrea edulis* and *Mytilus edulis* adult specimens were collected from an intertidal mussel bed south of the island of Juist (Germany). Tissues were preserved in 70% ethanol in seawater and kept up to 1 yr before ISH was performed. For deep-sea polychaete species, larvae were not available, so we used eggs collected from

Table 1. Polychaete and mollusc specimens from which 18S rRNA sequences were obtained and used for probe design. EMBL: European Molecular Biology Laboratory; EPR: East Pacific Rise

| Species | No. of individuals | Tissue | Origin | EMBL accession number |
|---|---|---|---|---|
| *Alvinella pompejana* | 2 | Gills | 9° N / EPR, 2000 | AM159573 |
| | 1 | Gills | 17° S / EPR, 2004 | |
| | 1 | Gills | 21° S / EPR, 2004 | |
| *Alvinella caudata* | 1 | Gills | EPR, 1994 | AM159574 |
| | 1 | Gills | 17° S / EPR, 2004 | |
| | 1 | Gills | 21° S / EPR, 2004 | |
| *Paralvinella grasslei* | 2 | Body wall | 13° N / EPR, 2002 | AM159575 |
| *Paralvinella pandorae* | 2 | Entire body | 17° S / EPR, 2004 | AM159576 |
| *Hesiolyra bergi* | 1 | Body wall | 13° N / EPR, 2002 | AM159577 |
| *Amphisamytha galapagensis* | 1 | Entire body | 14° S / EPR, 2004 | AM159578 |
| | 1 | Entire body | 17° S / EPR, 2004 | |
| *Nereis sandersi* | 1 | Body wall | 21° S / EPR, 2004 | AM159579 |
| *Riftia pachyptila* | 1 | Eggs | 17° S / EPR, 2004 | AM159580 |
| *Tevnia jerichonana* | 1 | Eggs | 17° S / EPR, 2004 | AM159581 |
| *Crassostrea gigas* | 1 | Adductor muscle | Sylt Island | AM182263 |
| *Macoma balthica* | 1 | Foot muscle | Sylt Island | AM182265 |
| *Ensis americanus* | 1 | Foot muscle | Sylt Island | AM182264 |
| *Cerastoderma edule* | 1 | Foot muscle | Sylt Island | AM182262 |

fresh specimens. They were preserved in several different ways: stored in 96% ethanol, fixed for 4 h to 2 d in 4% paraformaldehyde (PFA) in seawater and stored in phosphate-buffered saline (PBS: 145 mM NaCl, 1.4 mM $NaH_2PO_4$, 8 mM $Na_2HPO_4$, pH 7.5):ethanol (50:50), or fixed for 4 h to 2 d in 3% formalin in seawater and stored in 70% ethanol in seawater. Eggs had been preserved for up to 3 yr when used for ISH.

In order to assess the influence of the developmental stage on *in situ* hybridisation in polychaetes, we used embryos and larvae of a shallow-water species, *Platynereis dumerilii*, since vent larvae were not available. *P. dumerilii* larvae were obtained from laboratory cultures (Prof. A. Dorresteijn, Giessen University) at different developmental stages from 4 h old embryos to 6 d old juveniles. They were either stored in 96% ethanol or fixed for 4 h in 4% PFA in PBS and washed in ethanol:PBS (50:50). These larvae had been preserved for up to 1 yr when used for ISH.

**18S rRNA sequences.** DNA samples from polychaete and bivalve adult specimens were obtained using the method described by Zhou et al. (1996). Tissues were digested with Proteinase K, and DNA was recovered after a standard chloroform-isoamyl alcohol extraction procedure, precipitation in isopropanol, washing in ethanol, and resuspension in sterile-filtered water. As genetic differentiation was shown using the COI mitochondrial gene in several vent polychaete species across their geographic distribution range (Hurtado et al. 2004), specimens originating from distant sites were selected, when available, to check for 18S rRNA intraspecific variability (Table 1). PCR amplification of the 18S rRNA genes was performed using 4 sets of primers (Table 2). A >1600 bp fragment of the 18S rRNA gene was amplified from DNA of *Alvinella pompejana*, *A. caudata*, *Paralvinella grasslei*, *P. pandorae*, *Amphisamytha galapagensis*, *Nereis sandersi*, *Riftia pachyptila* and *Tevnia jerichonana* using the primer combination 1f/2023r, from DNA of *Crassostrea gigas*, *Ensis americanus* and *Macoma balthica* using the primer combination Univ15f/Univ1765r, and from *Cerastoderma edule* using the universal primers developed by Sogin (1990). A ≈1400 bp fragment was amplified from DNA of *Hesiolyra bergi* by using the primer combination 1f/1486r. Each PCR contained 10 µl of 10× Eppendorf *Taq* buffer, 71 µl of $H_2O$, 25 µM of each dNTP, 150 mg BSA $l^{-1}$, 1 U of Eppendorf *Taq* polymerase, each primer at 0.5 µM, and 1 µl DNA template. PCR amplification was initiated by a 5 min denaturation step at 96°C, followed by 30 cycles of 94°C for 1 min, 51°C (for primer pairs 1f/2023r or 1f/1486r), 53°C (for primer pair Univ15f/Univ1765r), or 64°C (for universal primer pair from Sogin 1990) for 1 min and 72°C for 2 to 3 min; a final elongation step was performed at 72°C for 10 min. Amplified DNA was purified with a QIAquick PCR purification kit (Quiagen). Additional internal primers were designed for sequencing reactions (Table 2). Sequencing reactions were carried out on both strands, using the ABI BigDye prism dideoxy sequencing dye terminator kit and an

Table 2. Primers used for the PCR amplification and sequencing reaction of the 18S rRNA genes

| Primer | Sequence 5′–3′ | Treatment | Source |
|---|---|---|---|
| 1f | CTG GTT GAT YCT GCC AGT | PCR amplification and sequencing | Winnepenninckx et al. (1995) |
| Univ 15f | CTG CCA GTA GTC ATA TGC | PCR amplification and sequencing | Frischer (2000) |
| Univ f | CAA CCT GGT TGA TCC TGC CAG T | PCR amplification and sequencing | Sogin (1990) |
| 1486r | ACC AAC TAA GAA CGG CC | PCR amplification and sequencing | Present study |
| 2023r | GGT TCA CCT ACG GAA ACC | PCR amplification and sequencing | Modified from Winnepenninckx et al. (1995) |
| Univ 1765r | ACC TTG TTA CGA CTT TTA | PCR amplification and sequencing | Frischer (2000) |
| Univ r | CTG ATC CTT CTG CAG GTT CAC CTA C | PCR amplification and sequencing | Sogin (1990) |
| 429f | AGG GTT CGA YTC CGG AG | Sequencing (polychaetes) | Present study |
| 915f | TTT GAA AAA ATT AGT GTG YTC | Sequencing (polychaetes) | Present study |
| 1373f | TAA TTT GAC TCA ACA CGG G | Sequencing (polychaetes) | Present study |
| 1854f | CAC ACC GCC CGT C | Sequencing (polychaetes) | Modified from Winnepenninckx et al. (1995) |
| 505r | GTG GGT AAT TTG CGC G | Sequencing (polychaetes) | Present study |
| 987r | RAR GTC CTI TTC YAT TAT TCC | Sequencing (polychaetes) | Present study |
| 361f | ATC AGG GTT CGA TTC CGG | Sequencing (*Macoma balthica*) | Present study |
| 570f | GCC AGC AGC CGC GGT | Sequencing (bivalves) | Frischer (2000) |
| 919f | GAT TAA GAG AGA CTG CCG | Sequencing (*Crassostrea edule*) | Present study |
| 1138f | GAA ACT TAA AGG AAT | Sequencing (bivalves) | Frischer (2000) |
| 570r | ACC GCG GCT GCT GGC | Sequencing (bivalves) | Frischer (2000) |
| 1138r | ATT CCT TTA AGT TTC | Sequencing (bivalves) | Frischer (2000) |
| 1145f | AAT TGA CGG AAG GGC ACC | Sequencing (*Ensis americanus*) | Present study |
| 1216r | ACC GGG TGA GGT TTC CCG | Sequencing (*M. balthica*) | Present study |

ABI PRISM 3100 generic analyser (Applied Biosystems). Sequence data were edited with Sequencing Analysis software (Version 3.7, Applied Biosystems) and Sequencher 4.5 (Gene Codes Corporation). Sequences were submitted to GenBank, and accession numbers are given in Table 1.

**Probe design.** Probes were designed using the package software ARB (Ludwig et al. 2004). All 18S rRNA gene sequences of polychaetes and bivalves available in online databases at the time of the study, as well as the 13 sequences obtained in this study, were imported and aligned in the ARB database. Alignments were manually corrected. For species for which we obtained 18S rRNA sequences from several individuals originating from distant populations, these were always 100% identical over the total length analysed. This indicates that our species-specific 18S rRNA probes could not have produced false negative identification caused by intra-specific variation.

Species-specific probes were designed using the PROBE-DESIGN function of the ARB software. They were named after the first letter of the genus and species name and the position targeted on the 18S rRNA gene. Probes were chosen to have at least 1 mismatch with any non-target species, and they were assigned so that the species used as a reference for the specificity test were available. For example, *Alvinella pompejana* probes were chosen so that the species presenting the most similar sequence at the target site was *A. caudata*. In some other regions, the 18S rRNA sequence was more similar to *Paralvinella grasslei* or *P. pandorae*, 2 other alvinellid species present at EPR vent sites. Since eggs of these 2 species were not available for the specificity test, probes in such regions were discarded. Probes were also designed to minimise self-complementarity and loop formation, which was checked using the OLIGO Primer Analysis software (Molecular Biology Insights). Potential complimentarity with non-target organisms whose sequences were not available at the time the probes were designed was checked again in June 2006 using the BLAST function of online databases against all published sequences.

**Whole larvae *in situ* hybridisation.** For ISH, probes were labelled with 2 different haptens: (1) horseradish peroxidase (HRP) (Biomers); and (2) digoxygenine (DIG) (Thermo Electron).

ISH was usually conducted in 1.5 ml tubes. However, with particularly fragile, rare, or tiny larvae, for which accidental pipetting while changing buffers had to be strictly avoided, all steps were conducted under a binocular dissecting microscope in 4-well Nunclon plates. Eggs, larvae, or tissues were first rehydrated in a graded series of ethanol in PBS. Then, different permeabilisation procedures were evaluated: 0.02, 0.05, 0.1, or 0.2 M HCl for 10 min at room temperature (RT); 0.1, 0.25, or 0.5% sodium dodecyl sulphate (SDS) for 15 min at RT; 1, 10, or 100 µg Proteinase K $ml^{-1}$ with Tween 0.1% for 1 to 30 min at 37°C or 10 min to 3 h at RT; 1 mg collagenase $ml^{-1}$ for 10 min at 37°C with Tween 0.1%; 0.05, 0.1, 0.2, or 0.5% acetic acid for 15 min at RT with Tween 0.1% (detailed protocols are available upon request). After

washing in PBS, hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.02% [w/v] SDS, 10% [w/v] dextran sulphate, 1% [w/v] blocking reagent [Boehringer], 0 to 60% [v/v] formamide [Fluka]) and 125 pg $\mu l^{-1}$ HRP-labelled probe or 250 pg $\mu l^{-1}$ DIG-labelled probe were pipetted onto the larvae. Overnight incubation (12 to 16 h) at 46°C was then carried out. Unspecific binding was removed by stringent washing in buffer with 14 to 900 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8) and 0.01% (w/v) SDS, at 48°C. Stringency in the washing buffer was modulated through NaCl concentration, according to the formamide concentration in the hybridisation buffer (Pernthaler et al. 2001).

For hybridisation with HRP-labelled probes, a final wash in PBS was performed at RT. Probe binding was revealed by the addition of a solution of 1.25 mM TMB (3,3,5,5 tetramethylbenzidine; Research Diagnostics), which is a substrate oxidised by the HRP. In positive hybridisations a blue colour developed within 20 min at RT. Colour intensity was evaluated by eye. It varied from very light blue to very dark, and results were recorded according to an arbitrary scale of 8 levels (very light, light, medium-light, medium, medium-strong, strong, dark and very dark).

For hybridisation with DIG-labelled probes, after washing the unbound probe, larvae were blocked in PBS-0.5% (w/v) blocking reagent for 30 min at RT, and then incubated with an anti-DIG–AP (alkaline phosphatase) antibody (1.5 U $ml^{-1}$, Fab fragment, Roche) in 100 mM Tris-HCl, 150 mM NaCl and 1% (w/v) blocking reagent overnight at 4°C. Unbound antibody was removed in a 30 min wash in PBS, and 2 × 5 min washes in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) at RT. The antibody was detected by incubation in NBT/BCIP staining solution (Roch) diluted 1:50 in 100 mM Tris-HCl and 100 mM NaCl. Red-purple colour developed after 10 to 180 min, and the colour reaction was stopped with TE-buffer (10 mM Tris-HCl, 1 mM EDTA). Colour intensity was evaluated by eye, and arbitrarily scaled from light red to dark purple.

Successive ISH was conducted with different HRP probes on eggs. After the first ISH, eggs were washed in PBS for 30 min and in a high-stringency (without NaCl) washing buffer for 1 h. TMB incubations were performed to check that no HRP probe remained, and eggs were washed again in PBS before incubation in hybridisation buffer with the new probe.

Images of ISH were recorded with a Nikon Coolpix 995 digital camera mounted on the binocular microscope, using the same exposure settings for each picture.

**DNA extraction and PCR amplification from eggs and larvae.** In order to assess the number of false positive and false negative results in ISH, DNA was extracted from a single egg or larva after ISH using the procedure described by Schizas et al. (1997), and the 18S rRNA gene was amplified and partially sequenced using the procedure described above. After ISH, eggs were washed in PBS before DNA extraction. This procedure was performed on 18 *Riftia pachyptila* eggs and on 6 *Platynereis dumerilii* larvae.

## RESULTS

### Probe design

In Alvinellidae, the 18S rRNA sequences of the 2 *Alvinella* species examined in this study differed by at least 3% over 1792 bp, and by at least 4 and 5% over >1650 bp from all other alvinellid 18S rRNA sequences available (*Paralvinella grasslei*, *P. pandorae*, *P. palmiformis*). In addition, *Alvinella* sequences have several large insertions in their 18S rRNA, unique to each species. These insertions provided highly specific sequences, with no or very low identity to sequences of non-target species, and were therefore chosen for the species-specific *Alvinella* probes. These probes differed by at least 3 base pairs from the target sequences in other alvinellid species (Table 3).

In Siboglinidae, despite the overall high identity (between 98 and 99%) between their 18S rRNA genes, we identified short sequence stretches that were sufficiently unique to *Riftia pachyptila* and *Tevnia jerichonana* to serve as target sites for species-specific probes. Both species are present at EPR vent sites, and their 18S rRNA genes are more similar to each other than to all other siboglinids, with 99% identity over 1783 bp. The chosen target sequences had at least 1 unique base pair when compared with representatives of other siboglinid species (e.g. RP158; Table 3) and in most cases at least 2 base pair differences.

For *Crassostrea gigas*, the most closely related co-occurring bivalve species in the North Sea are *Ostrea edulis* and *Mytilus edulis*. Their 18S rRNA sequences are, respectively, 97 and 92% identical to the *C. gigas* 18S rRNA over >1750 bp. *C. gigas*-specific probes were designed by targeting sequences exhibiting at least 2 base pair differences to *O. edulis* and *M. edulis* (Table 3).

### Specificity tests

Designed probes were evaluated using eggs collected on adult specimens for polychaete vent species and using larvae obtained from culture and adult tissues for *Crassostrea gigas*. Specificity was determined in a series of hybridisations with increasing formamide

Table 3. Oligonucleotide probe sequences specific for 4 vent polychaete species (*Alvinella pompejana*, *A. caudata*, *Riftia pachyptila*, *Tevnia jerichonana*) and for the oyster *Crassostrea gigas*

| Probe | Target organism | Probe sequence 5′–3′ | Temp. (°C) | Target sequence 5′–3′ in target species and closest non-target species | Source |
|---|---|---|---|---|---|
| AP176 | *Alvinella pompejana* | ACCAACGACAACTACCACG | 58 | CGUGGUAGUUGUCGUUGGU (*A. pompejana*)<br>.U....CC.GCCTAC...G (*A. caudata*) | Present study |
| AP1420 | *Alvinella pompejana* | AGGACCACGGGCACACTG | 60 | CAGUGUGCCCGUGGUCCU (*A. pompejana*)<br>U...U.......U..... (*A. caudata*) | Present study |
| AC175 | *Alvinella caudata* | AGTAGGCAGGACCAAGGC | 58 | GCCUUGGUCCUGCCUACU (*A. caudata*)<br>C..G.........GCGA. (*A. pompejana*) | Present study |
| AC1455 | *Alvinella caudata* | GCCTGCCCTCCCACCTG | 60 | CAGGUGGGAGGGCAGGC (*A. caudata*)<br>GC...........C... (*A. pompejana*) | Present study |
| RP158 | *Riftia pachyptila* | GCTCACGCGGTCGGAAC | 58 | GUUCCGACCGCGUGAGC (*R. pachyptila*)<br>............C.... (*T. jerichonana*) | Present study |
| RP1752 | *Riftia pachyptila* | CGACCTCTAAGCCGTCAA | 56 | UUGACGGCUUAGAGGUCG (*R. pachyptila*)<br>.....C.U.......... (*T. jerichonana*) | Present study |
| TJ202 | *Tevnia jerichonana* | CGAACGACGCACCGATTG | 58 | CAAUCGGUGCGUCGUUCG (*T. jerichonana*)<br>........C........C (*R. pachyptila*) | Present study |
| CG773 | *Crassostrea gigas* | CATTGTACAGGCGAAGCG | 56 | CGCUUCGCCUGUACAAUG (*C. gigas*)<br>..AA.......C...... (*Ostrea edulis*) | Present study |
| CG1543 | *Crassostrea gigas* | AGAATTACACACCCCAAT | 50 | AUUGGGGUGUGUAAUUCU (*C. gigas*)<br>.........C......A. (*O. edulis*) | Present study |
| CG1546 | *Crassostrea gigas* | GGGAGAATTACACACCCC | 56 | GGGGUGUGUAAUUCUCCC (*C. gigas*)<br>....C........A.... (*O. edulis*) | Present study |
| EUK516 | *Eukarya* | ACCAGACTTGCCCTCC | 52 | GGAGGGCAAGUCUGGU | Amann et al. (1990) |
| NonEUB338 | Negative control | ACTCCTACGGGAGGCAGC | 60 | | Wallner et al. (1993) |

concentrations, causing an increase in stringency. These series were performed with HRP-labelled probes for each target species and with DIG-labelled probes for *C. gigas*. Specificity of any given probe did not differ between HRP- and DIG-labelled probes. Examples of such series are given in Fig. 1. With the HRP-labelled probe RP158, which is specific for *Riftia pachyptila*, we showed that even only 1 base pair difference is sufficient to discriminate the target species from other closely related species (Fig. 1a, see also Fig. 3c,d).

Stringent hybridisation conditions were evaluated for each designed probe. Each probe allowed the discrimination of the target species without producing false positives among the non-target organisms tested (Figs. 2 to 4, Table 4). For each assay, nearly 100% of the eggs of the target species were positively identified. The rare unstained individuals were damaged, and, in those cases, we could expect a loss of target ribosomes.

All probes did not give equally intense signals under stringent conditions (see Figs. 2 & 3). Probes AP1420, AC1455, RP1752, CG1543 and CG1546 showed stronger signals than probes AP176, AC175, RP158, TJ202 and CG773.

### Effect of fixation

We tested whether the use of different fixation methods would influence the ISH reaction using eggs of the vent polychaetes. Ethanol-fixed eggs always showed a strong hybridisation signal, as well as eggs fixed with formalin or paraformaldehyde for a few hours. However, paraformaldehyde fixation times of >24 h resulted in low or undetectable hybridisation signals. Increasing permeabilisation time, or the use of more concentrated permeabilisation solutions did not improve ISH in specimens fixed in paraformaldehyde for >24 h.

### Effect of permeabilisation

Permeabilisation procedures were adapted to the type of structure surrounding the larvae. *Crassostrea gigas* larvae are protected by their calcite shell. Efficient permeabilisation was achieved by using HCl at relatively high concentrations (0.1 M) (Fig. 4d). In polychaetes, the cuticle develops in early larval stages (Hausen 2005). In oocytes of vent species and in early embryos of *Platynereis dumerilii* (4 h embryos) no

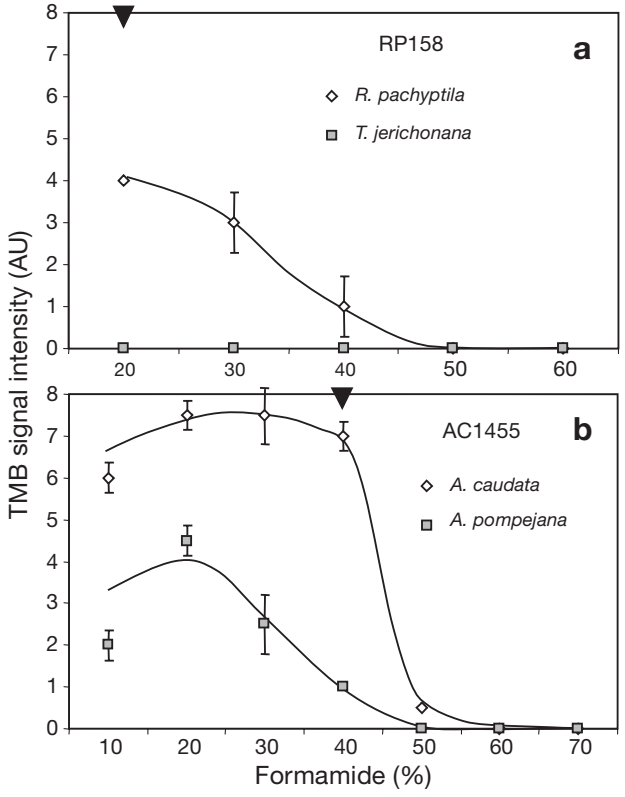


Fig. 1. Comparison of the melting curves derived from the whole-egg hybridisation for the duplex between a specific horseradish peroxidase (HRP) probe and the 100% complementary target sequence and mismatched target sequence, with increasing formamide concentration as measured by the tetramethylbenzidine (TMB) signal intensity (AU: arbitrary units). (a) Duplex between RP158 and the *Riftia pachyptila* sequence (complementary) and the *Tevnia jerichonana* (1 mismatch). (b) Duplex between AC1455 and the *Alvinella caudata* sequence (complementary) and the *A. pompejana* sequence (3 mismatches). Optimised formamide concentrations are indicated by the black arrowheads. Error bars = ± SD

permeabilisation was required (Fig. 5a), although a short incubation in 0.02 M HCl resulted in a more homogeneous colouration. For larvae older than 1 d (trochophore stage), HCl treatment was not efficient (Fig. 5b). Increasing HCl concentration or incubation times resulted in a heavy loss of morphology. Proteinase K is commonly used in embryo and larva permeabilisation protocols. We used it in concentrations varying from 1 to 100 µg $ml^{-1}$, with incubation times varying between 5 min and 3 h. Signal intensity obtained with this permeabilisation treatment was always weak (Fig. 5c), and the highest Proteinase K concentrations resulted in a complete loss of signal and ultimately a loss of morphology. Permeabilisation with collagenase, acetic acid, or a combination of both did not increase the signal intensity (data not shown). Finally, best results for polychaete larvae were achieved using 0.5% SDS (Fig. 5d). Optimal permeabilisation and hybridisation procedures are summarised in Table 5.

### Post-ISH analysis

Environmental samples usually include a mix of larvae from different species, and we therefore examined if it is possible to identify >1 species by hybridising eggs or larvae several times successively with different species-specific HRP-labelled probes. Using eggs of vent species and *Platynereis dumerilii* larvae, we found that early larval stages can go through ISH procedures at least 2 times successively without loss of morphology. Between hybridisations, eggs or larvae have to be washed with a high-stringency washing buffer (without NaCl) in order to remove the attached probe from the first ISH. Probe signal intensity did not vary significantly whether a species-specific probe was applied at the first or at the second hybridisation (Fig. 3g).

In some cases it may be desirable to analyse larval genes using PCR, for example to validate probe identification of larvae, or to examine genes besides 18S rRNA for additional phylogenetic information. We extracted DNA from single *Riftia pachyptila* eggs and single *Platynereis dumerilii* larvae after they had been hybridised. The 18S rRNA gene could be amplified by PCR in 16 (89%) of the *R. pachyptila* eggs and all *P. dumerilii* larvae. Sequencing of the first 700 base pairs confirmed that there were no differences in the 18S rRNA sequences of specimens examined with and without ISH treatment. This method thus allows further examination of ISH-treated specimens using PCR=based methods.

### HRP or DIG probes?

In order to evaluate the ISH procedure on natural plankton samples that may include considerable amounts of sand, algae and other debris, *Crassostrea gigas* larvae were mixed with plankton samples collected around the island of Juist. Unspecific blue background labelling of debris was observed, sometimes making it difficult to pick out larvae in the sample. We therefore developed an alternative protocol using a DIG probe combined with an AP-labelled anti-DIG antibody instead of the HRP probe. Since the kinetics of the reaction catalysed by AP are much slower than those catalysed by HRP, background labelling did not develop, or only after several hours. This time lapse is then sufficient to sort the larvae. DIG-labelled probes were also used successfully with polychaete eggs (example in Fig. 2h).

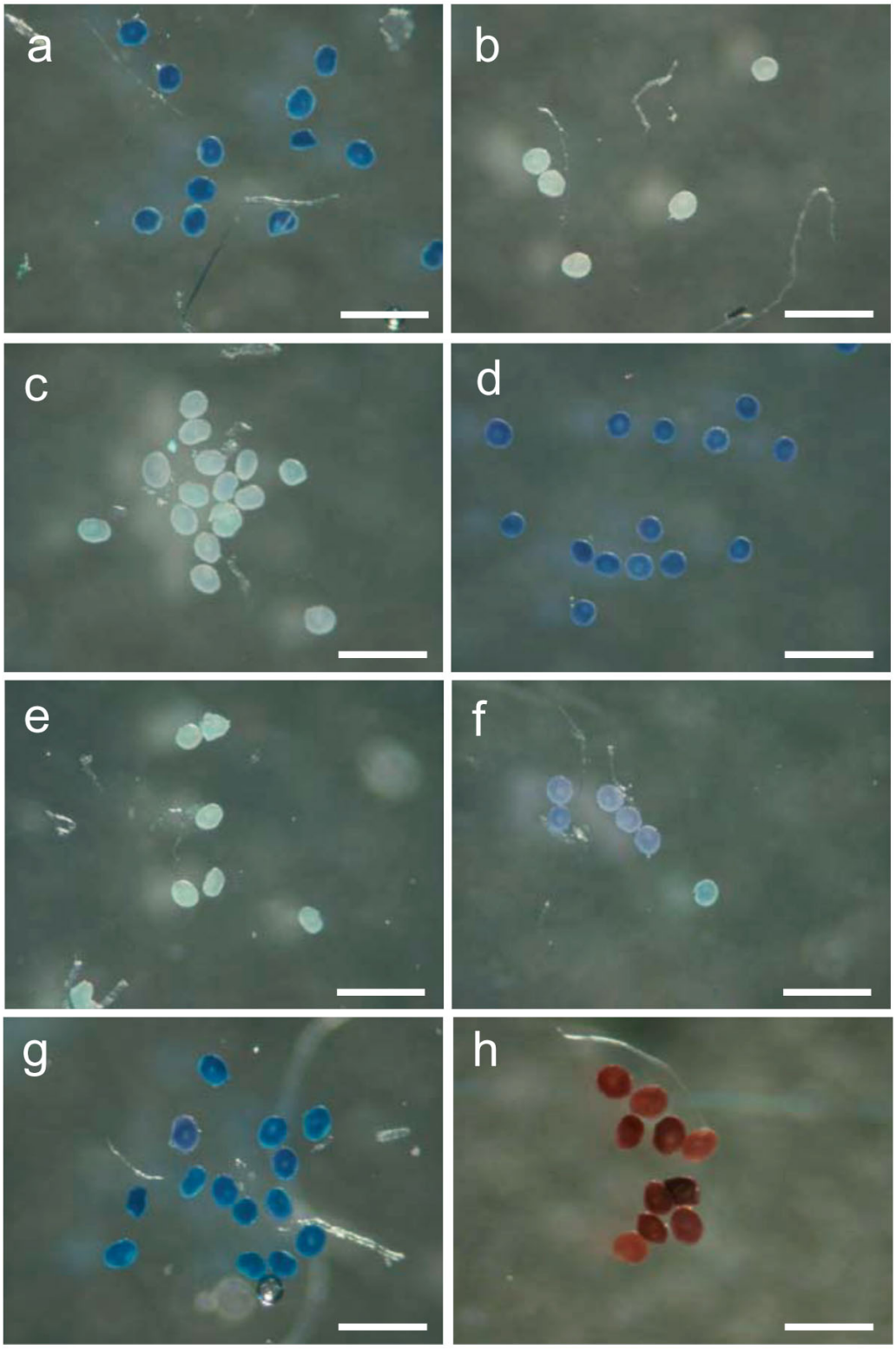


Fig. 2. *Alvinella pompejana* and *A. caudata.* Evaluation of specific probes. Blue colour indicates hybridisation with HRP probe. Reactions were performed under stringent conditions, as defined in Table 4, with *A. caudata* (a,c,e,g,h) and *A. pompejana* (b,d,f) eggs using specific probes: AC1455 (a,b), AP1420 (c,d) and AP176 (e,f); AC1455 after a first *in situ* hybridisation (ISH) with AP1420 (g). Panel (h) shows hybridisation with the general eukaryote probe EUK516 labelled with digoxygenin as indicated by the red-purple colour. Scale bars = 500 µm

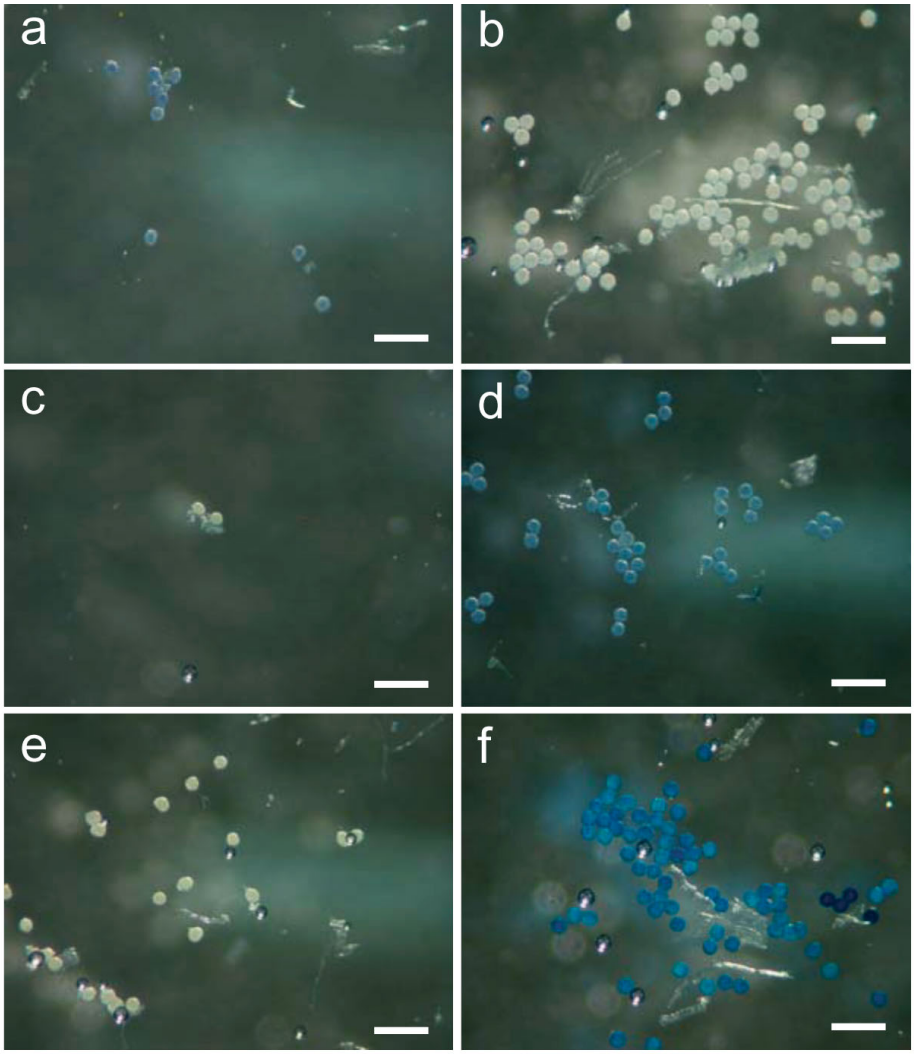


Fig. 3. *Riftia pachyptila* and *Tevnia jerichonana*. Evaluation of specific probes. Blue colour indicates hybridisation with HRP probes. Hybridisation reactions were performed under stringent conditions, as defined in Table 4, with *T. jerichonana* (a,c,e) and *R. pachyptila* (b,d,f) eggs using specific probes TJ202 (a,b), RP158 (c,d) and RP1752 (e,f). Scale bars = 500 µm

Table 4. ISH experiments demonstrating specificity of the probes, with formamide (FA) concentration in the hybridisation buffer required for specific ISH. For non-target species, the number of mismatches is indicated in parentheses

| Probe | Expected specificity | %FA | Signal demonstrated with: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Alvinella pompejana* oocytes | | *Alvinella caudata* oocytes | | *Riftia pachyptila* oocytes | | *Tevnia jerichonana* oocytes | |
| AP176 | *A. pompejana* | 20 | + | | – | (10) | – | (no match) | | |
| AP1420 | *A. pompejana* | 30 | ++ | | – | (3) | – | (no match) | | |
| AC175 | *A. caudata* | 20 | – | (6) | + | | – | (no match) | | |
| AC1455 | *A. caudata* | 40 | – | (3) | ++ | | – | (no match) | | |
| RP158 | *R. pachyptila* | 20 | – | (8) | | | + | | – | (1) |
| RP1752 | *R. pachyptila* | 40 | – | (7) | | | ++ | | – | (2) |
| TJ202 | *T. jerichonana* | 20 | – | (11) | | | – | (2) | + | |
| | | | *Crassostrea gigas* larvae & tissue | | *Ostrea edulis* tissue | | *Mytilus edulis* tissue | | | |
| CG773 | *C. gigas* | 10 | + | | – | (3) | – | (4) | | |
| CG1543 | *C. gigas* | 10 | ++ | | – | (2) | – | (2) | | |
| CG1546 | *C. gigas* | 10 | ++ | | – | (2) | – | (3) | | |

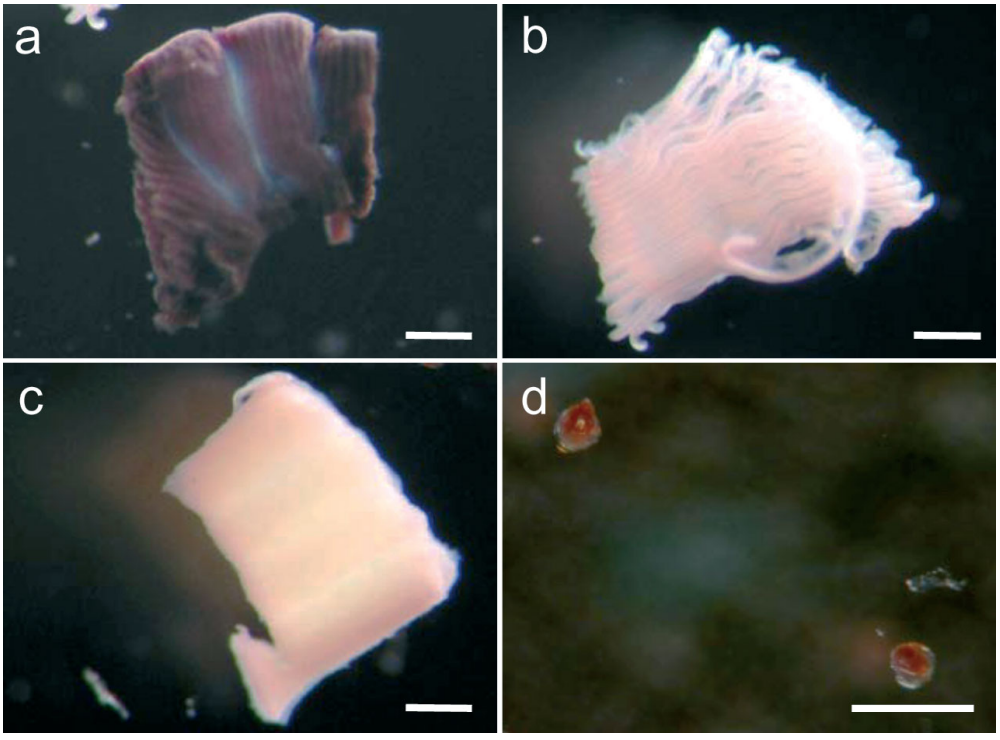


Fig. 4. *Crassostrea gigas*. Evaluation of specific probe CG1546 using DIG probes. The deep-red staining indicates hybridisation with the probe: (a) *Crassostrea gigas* gill tissue; (b) *Mytilus edulis* gill tissue; (c) *Ostrea edulis* gill tissue; and (d) *C. gigas* larvae. Scale bars = 500 µm

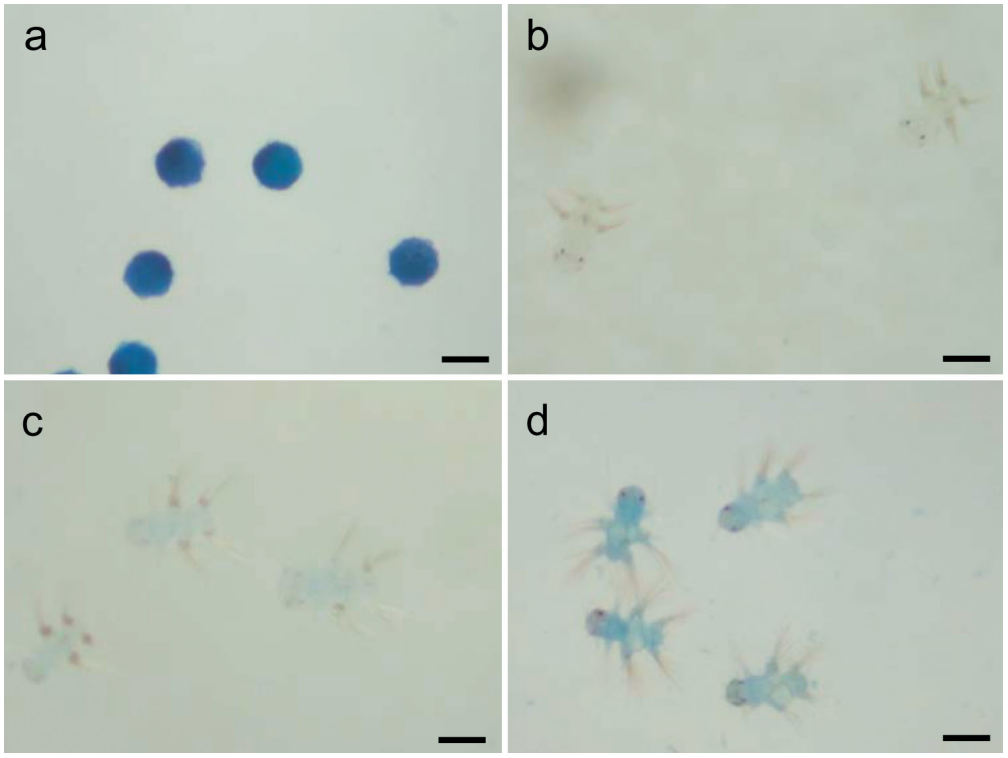


Fig. 5. *Platynereis dumerilii*. *In situ* hybridisations at different developmental stages: (a) HCl (0.02 M)-treated 4 h old embryos, EUK516; (b) HCl (0.02 M)-treated 6 d old larvae, EUK516; (c) Proteinase K (10 µg ml$^{-1}$, 20 min, with Tween)-treated 3 d old larvae, EUK516; and (d) SDS (0.5%)-treated 6 d old larvae, EUK516. Scale bars = 150 µm

Table 5. Summary of steps for ISH with marine eggs and larvae with HRP- or DIG-labelled oligonucleotide probes. All steps are conducted at room temperature except when specific temperature is mentioned (see 'Materials and methods' for details). ON: over night

| Stage | Eggs and early embryos | Soft-bodied polychaete larvae | Hard-shell bivalve larvae |
|---|---|---|---|
| Permeabilisation | • Rehydrate in a graded series of ethanol in PBS | | |
| | • Incubate 10 min in 0.02 M HCl (facultative)<br>• Wash in PBS (facultative) | • Incubate 20 min in 0.5% SDS<br>• Wash in PBS | • Incubate 10 min in 0.1 M HCl<br>• Wash in MilliQ water<br>• (1) Wash in 70% ethanol and let dry at RT, or (2) wash in PBS |
| Hybridisation | • Add 30 µl (1.5 ml tube procedure) or 300 µl (plate procedure) hybridisation buffer containing the probe at 125–250 pg $\mu l^{-1}$<br>• Incubate ON (12–16 h) at 46°C<br>• Wash 3 × 40 min in washing buffer at 48°C<br>• Wash in PBS 10 min | | |
| Antibody reaction (only for DIG-labelled probes) | • Incubate in 0.5% blocking reagent in PBS 30 min<br>• Incubate with antibody solution ON at 4°C<br>• Wash in PBS 30 min<br>• Wash in TBS 2 × 5 min | | |
| Probe binding visualisation | HRP-labelled probes:<br>• Incubate in TMB staining solution maximum 45 min<br>• Observation<br>DIG-labelled probes:<br>• Incubate in NBT/BCIP staining solution maximum 3 h<br>• Stop colour reaction with TE buffer<br>• Observation | | |
| Post-hybridisation (tested only for HRP-labelled probes) | • Wash in PBS 30 min<br>• Wash in high stringency washing buffer and proceed with new ISH | | • Not tested |

## DISCUSSION

All molecular methods developed so far for species identification in larval stages have been destructive, preventing further analysis of the larvae, which would be valuable for those that have not yet been described (Garland & Zimmer 2002), such as hydrothermal vent larvae. The whole-larvae colourimetric ISH method presented here allows the identification of larvae to the species level, without damaging morphology (however, ultrastructural details of the larval shell in bivalves that are examined by scanning electron microscopy and used for species identification might be lost after HCl treatments). For each species, we were able to develop probes that bound specifically to their target with nearly 100% efficiency, and without producing false positives with closely related species, even when target and non-target sequences differed by only 1 mismatch. By making slight changes in the permeabilisation steps, we showed that the ISH method is effective with eggs, as well as with larvae and with adult tissues. The colour-based assay produced a bright blue or red signal, according to the labelling system used. Although not tested here, the simultaneous use of 2 probes labelled with each of the 2 haptens would allow 2-colour ISH assays in which 2 species could be simultaneously identified. Compared to fluorescent methods, such colour methods are better suited to be used with a standard dissecting microscope, where the bright signal produced by the probe hybridisation can easily be distinguished, and large amounts of plankton can be efficiently sorted.

ISH identification assays must meet the challenge of designing probes to discriminate among sequence differences at the species level, while retaining insensitivity to polymorphism within the target species. The 18S rRNA gene evolves slowly and has been used to resolve deep branching orders among different orders and families of organisms including invertebrates (Winnepenninckx et al. 1995, Bleidorn et al. 2003). It usually does not vary at the species level, and in some cases does not differ between closely related species. Here, even in families where 18S rRNA sequences are highly similar such as the siboglinid tubeworms, we showed that it is still possible to design species-specific probes based on single mismatch discrimination between target and non-target species (Fig. 1a). Since the 18S rRNA gene has both regions that are highly conserved and highly variable, probes can be targeted

to signature sites characteristic for species, genera, families, or orders (Amann et al. 1990). Within mixed environmental samples where one has no precise idea of the potential species present in the sample, nested approaches can be carried out by successively applying probes specific to the lower and to the higher taxonomic level. In addition, the conserved nature of the 18S rRNA gene at the species level makes it suitable for identifying individuals over a broad geographical range. Another advantage of the 18S rRNA gene is the fairly large database of sequences available, allowing the design of probes for a wide range of species and comparison with a maximum of non-target sequences.

In groups where the 18S rRNA gene evolves so slowly that not even 1 base pair difference can be used to discriminate the target species, other ribosomal genes could be used. The 28S rRNA gene, which is longer than the 18S rRNA gene, may potentially provide a higher number of probe binding sites (Peplies et al. 2004). The mitochondrial 16S rRNA gene could also be used, since mitochondrial genes are known to evolve more rapidly than nuclear ones. Finally, genes such as the mitochondrial cytochrome *c* oxidase subunit I (COI) have been proposed as good candidates for species identification, because this gene has a high inter-specific variability together with low intra-specific variability (Hebert et al. 2003). However, when using non-rRNA sequences to design probes for ISH methods, further methodological developments are required, since mRNA is much less abundant and stable than rRNA.

The design of a good probe also depends on its binding efficiency, which is influenced by its target site in the rRNA gene. It was previously shown that the 16S rRNA of Bacteria and Archaea, and the 18S rRNA of Eukarya (*Saccharomyces cerevisiae*) are not equally accessible to probe binding (Behrens et al. 2003). Certain domains, such as the sequence stretch at Positions 585 to 656 (*Escherichia coli* numbering), are consistently inaccessible to probe binding in prokaryotic 16S rRNA and in eukaryotic 18S rRNA. Similarly, the probe CG773 targeting the corresponding area in *Crassostrea gigas* 18S rRNA gave a weak signal, adding evidence that this region of the 18S rRNA gene should be avoided when designing new probes. Our ISH experiments also showed that all probes targeting the 5'-end of the 18S rRNA gene (AP176, AC 175, RP158, TJ202) gave relatively low signals in the target species. Behrens at al. (2003) predicted a rather weak accessibility in the corresponding region in *S. cerevisiae*. On the other hand, we also found that the probes targeting the 3'-end of the gene (AP1420, AC1455, RP1752, CG1543, CG1546) gave a rather strong signal. In this case, our pattern does not completely fit data from Behrens et al. (2003), since AP1420 and AC1455 target areas with rather low predicted accessibility; whereas RP1752, CG1543 and CG1546 target areas with medium to high predicted accessibility. However, data from Behrens et al. (2003) also showed that even a slight shift along the rRNA sequence can produce a very strong increase in the probe signal.

ISH assay efficiency and sensitivity also strongly depend on the preservation and permeabilisation treatments. Preservation with cross-linking fixatives such as formalin or PFA should never exceed a few hours, because they tend to reduce considerably the probe penetration to the target molecules. A negative effect of formalin fixative has also been reported for ISH on diatoms (Miller & Scholin 2000).

Successful ISH depends strongly on the initial permeabilisation steps, in particular when HRP-labelled probes are used. Depending on the type of structure surrounding the larvae, permeabilisation has to be adapted. In eggs and very early embryos, cell membrane and fertilisation envelope might be relatively easy to permeabilise, whereas in older stages, which have developed cuticles or shells, much stronger permeabilisation procedures might be required.

The optimal permeabilisation depends on the species and also on the life stage of the larvae. Treating a mixed sample of larvae from the environment with a single permeabilisation method might leave some larvae impermeable and result in false negative results. With strong permeabilisation, softer larvae might lose their integrity and target rRNA, again producing false negatives. Prior to the use of an ISH assay, minimum sorting based on general morphology is helpful but does not require specific taxonomic expertise. Once this initial step is performed, larvae can be rapidly processed using ISH, and identified. We showed that ISH assays do not prevent the subsequent use of other methods. If necessary, post-hybridisation checks for false positives or negatives might be performed using methods based on DNA extraction and amplification.

Despite the relatively elevated cost of HRP probes compared to mono-labelled fluorescent probes, the total cost of 1 hybridisation assay was 0.94 Euros when performed in plates, and 0.12 Euros when performed in 1.5 ml tubes. DIG-labelled probes are cheaper than HRP probes, but higher concentrations are required for a sensitive result, and subsequent antibody detection increases the total cost of the assay. The cost of a DIG assay performed in plates is 2.1 Euros, and 0.23 Euros when performed in 1.5 ml tubes. Overall, considering that a large number of individuals (several 10s or even 100s in plate assay) can be processed in 1 single assay, ISH methods can be performed with minimum expense. Besides, very little equipment is required: only a standard dissecting microscope and a hybridisation oven are necessary to perform the assay. This method is thus well suited to be used on board during survey field trips.

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