Utility of 18S rDNA and ITS sequences as population markers for *Lepeophtheirus salmonis* (Copepoda: Caligidae) parasitising Atlantic salmon (*Salmo salar*) in Scotland

[1]A.P. Shinn, [1]B.A. Banks, [2]N. Tange, [1]J.E. Bron, [1]C. Sommerville, [2]T. Aoki & [1]R.Wootten

[1]*Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland;* [2]*Laboratory of Genetics and Biochemistry, Department of Aquatic Bioscience, Tokyo University of Fisheries, Konan 4-5-7, Minato-ku, Tokyo 108, Japan*

Abstract

Genetic differentiation within the salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837), was investigated by the sequencing of specific nucleotide regions. Partial sequences of the 18S ribosomal RNA gene and the ribosomal internal transcribed spacer (ITS-1) region from single sea lice were amplified by the polymerase chain reaction (PCR). Lice were collected from wild and farmed Atlantic salmon (*Salmo salar* L., 1758) from nine selected localities around the Scottish coastline. A 0.9kb fragment of the 18S ribosomal RNA gene was amplified and compared for several samples of lice which showed no observable differences between the lice from different collection sites confirming the absence of cryptic species. The 454 nucleotide ITS-1 sequence showed differences between derived sequences from 13 sea lice samples from 4 collection sites which included 2 farm sites and 2 sites where lice were taken from wild fish. Across all samples, there was a 92.14% similarity in the ITS-1 sequence. The percentage similarity in the ITS-1 sequence in samples of lice from two fish farms were 99.71% (site A) and 95.72% (site D) but only 86.90% (site B) and 86.03% (site C) similarity was shown in lice samples taken from sites where wild salmonids were caught. The greater similarity between the ITS-1 sequence within farm sites may be attributed to a restricted gene flow within lice populations in Atlantic salmon cage sites.

Contents

Introduction

The characterization of salmon louse (*Lepeophtheirus salmonis* (Krøyer, 1837)) populations has recently become the subject of considerable interest as a tool to investigate louse population origins and interactions. Nordhagen, Heuch & Schram (1998) investigated experimentally the usefulness of morphometric parameters to identify the origins of the salmon lice populations in Norway. They concluded that it was not possible to discriminate populations of lice on the basis of size, as lice of different origins raised at the same temperature on a common host showed no significant differences in dimensions and similar life-cycle features such as egg production and growth rate. Similarly, in an unpublished experiment, the present authors applying multivariate analyses to morphometric data of several populations of lice taken from naturally infected wild and farmed salmonids sampled throughout a year, concluded that the discrimination of lice on the basis of size was not sufficiently clear to be useful.

Other studies have investigated the allelic frequencies of polymorphic enzymes (allozymes) of populations of the genus *Lepeophtheirus*. For example, Shinn et al. (1998) found that, using nine

polymorphic enzymes, it was possible to obtain some discrimination of lice from wild fish on the east coast of Scotland from both wild and farmed fish sampled on the west coast of Scotland. Isdal, Nylund & Nævdal's (1997) allozyme study of Norwegian populations of lice similarly found differences between northern and southern populations of farmed lice. De Meeüs et al. (1992) used allozymes to demonstrate an adaptive polymorphism to salinity in *L. europaensis* Zeddam, Berrebi, Renaud, Raibaut & Gabrion, 1988 parasitic on *Scophthalmus rhombus* L., 1758 and *Platichthys flesus* L., 1758 which they suggested, when coupled with a restricted movement of hosts between environments of differing salinity, could in time give rise to genetically isolated sub-populations.

Other approaches in the investigation of the population characteristics of *L. salmonis* include the use of inductively coupled plasma mass spectrometry (ICPMS) to ascertain the elemental signature of adult female lice (Shinn et al., this volume). In this study it was demonstrated that adult female lice from seven localities could be discriminated from each other on the basis of 16 elements and lice from farmed fish could be discriminated from lice on wild salmonids within the same locality.

Molecular techniques permit finer resolution of genetic differences between populations and individuals that cannot be detected using techniques such as morphometrics and allozyme analysis. Todd et al. (1997) using RAPDs, stated that genetic differentiation between populations of lice on farmed salmon and wild salmonids and between individual farm sites was evident to the extent that the presence of putative "farm markers" within certain individual wild lice suggested they originated from farm sites. While RAPDs allow for the comparison of amplified DNA bands of similar molecular weight, without direct sampling it is unknown whether these similar sized bands correspond to the same portion of the genome. This study describes an investigation which characterizes populations through determination of the nucleotide sequence of selected areas of the *L. salmonis* genome, namely the 18S ribosomal RNA gene and the internal transcribed spacer (ITS).

Information resulting from analysis of the 18S

rRNA gene sequence has previously been used to determine whether or not the species under investigation contains cryptic species or genetically isolated populations. Similarly, determination of the nucleotide sequence of internal transcribed spacers (ITS) have been successfully implemented as a taxonomic tool to discriminate closely related or morphologically indistinguishable species of parasite e.g apicomplexans (Homan et al., 1997); gyrodactylids (Monogenea) (Cunningham, McGillivray & MacKenzie, 1995; Cunningham, 1997); schistosomes (Digenea) (Littlewood & Johnston, 1995; Van Herwerden, Blair & Agatsuma, 1998); and nematodes (Chilton, Gasser & Beveridge, 1995). Nucleotide variations in ITS regions have also been used to characterise strains or isolates of *Cyptocaryon irritans* (Diggles & Adlard, 1997); *Enterocytozoon bieneusi* (Rinder, Katzwinkel-Wladarsch & Loscher, 1997a); *Eimeria maxima* (Barta et al., 1998); *Echinostoma caproni* (Morgan & Blair, 1998) and *Hymenolepis diminuta* (Okamoto et al., 1997). Sequence similarity in the ITS region has also provided evidence for synonomy eg. *Teladorsagia* spp. (Trichostrongylidae) (Stevenson, Gasser & Chilton, 1996) and *Fasciola* spp. (Hashimoto et al., 1997); sub-speciation e.g. *Echinococcus multilocularis* (Rinder et al., 1997b) and *Paragonimus westermani* (Blair et al., 1997) or the identification of cryptic species as has been suggested for geographically distanced isolates of *Necator americanus* (Romstad et al., 1998). Overall, these studies suggest that the 18S and ITS regions may be used to provide a means of population characterization and it is with this in mind that this study has been undertaken.

Materials and methods

Sample collection and preparation

Sea lice were collected from eight sites in Scotland. Samples were collected from wild Atlantic salmon (*Salmo salar* L., 1758) at three sites and from farmed Atlantic salmon at five sites (Fig. 1). After collection sea lice were maintained in sea water (10°C) for 48 hrs to remove host products from the gut before use and then homogenised in

buffer (1mM Tris-HCl pH7.0, EDTA 1mM, 2-mercaptoethanol 0.1mM). Samples were then frozen in liquid nitrogen and stored at –70°C.

Genomic DNA extraction

Frozen lice samples were kept on ice, homogenised in a cell lysis buffer (10mM Tris-HCl pH 8.0; 150mM NaCl; 10mM EDTA-NaOH pH 8.0; and 1% SDS) and then treated with proteinase K (1mg/ml) overnight at 55°C and RNaseA (0.5mg/ml) for one hour at 37°C to digest cellular proteins and RNA. Genomic DNA was then precipitated using 0.3M NaOH-Ac and isolated using a standard phenolic-chloroform extraction (Sambrook et al., 1989) and precipitated in ice-cold 75% ethanol. The purified DNA pellets were dried at room temperature overnight and resuspended in TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA).

PCR amplification

Universal and species specific primers (given below) were selected for each gene and amplified using PCR (Saiki et al., 1988).

18S ribosomal DNA

The nematode *Caenorhabditis elegans* rRNA primers complementary to the annealing positions 2074-2098 5'-GGGCAAGTCTGGTGCC-3' (18S-1) and 2931-2946 5'-GGTCTGTGATGCCCTT-3' (18S-2) were used (Ellis, Sulston & Coulson, 1986). A 25µl reaction mix was used, containing 25pM of each primer, 50-100ng DNA and a PCR bead (Amersham Pharmacia-Biotech)(1.5 units of Taq, 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl$_2$, 200uM of each dNTP). The thermocycle programme was 5 min at 95°C, 30 cycles of 30s at 95°C, 30s at 55°C, 90s at 72°C, then 5min at 72°C.

Internal Transcribed Spacer (ITS)

The *Gyrodactylus salaris* 18S rDNA forward primer ITS-1 5'-TTTCCGTAGGTGAACCT-3' correspond-
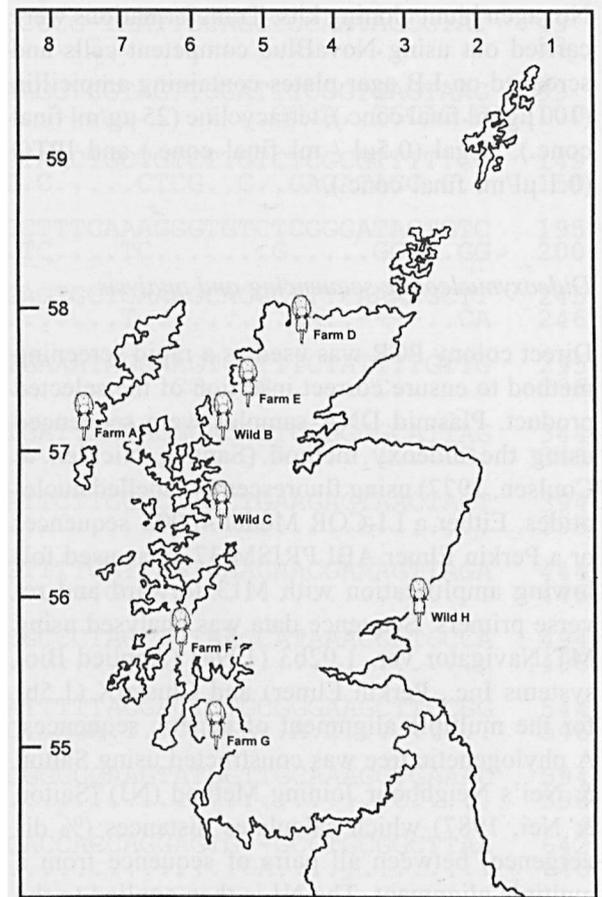

Fig. 1. Map of *Lepeophtheirus salmonis* collection sites in Scotland. The sequence of ITS-1 was determined and compared for individual lice collected from sites A-D. Lice from these sites and from sites E-H were used to determine a partial sequence of the 18S rRNA gene.

ing to the annealing position 1,938-1955 (Cunningham, McGillivray & MacKenzie, 1995) and the *Schistosoma* 28S rDNA reverse primer ITS-2 5'-TCCTCCGCTTAGTGATA-3' (Littlewood & Johnston, 1995) were used as primers in the PCR. The PCR reaction mix was as for the 18S rDNA amplification. The thermocycling programme was 5min at 95°C, 30 cycles of 60s at 95°C, 90s at 52°C, 120s at 72°C, then 5min at 72°C.

Cloning into pMOS Blue T-vector

PCR products were purified and ligated into pMOS *Blue* T-vector (pT7Blue) (Amersham, UK) using

Novagen blunt cloning kits. Transformations were carried out using NovaBlue competent cells and screened on LB agar plates containing ampicillin (100 μg/ml final conc.), tetracycline (25 μg/ml final conc.), X-gal (0.5μl / ml final conc.) and IPTG (0.1 μl/ml final conc.).

Dideoxynucleotide sequencing and analysis

Direct colony PCR was used as a rapid screening method to ensure correct insertion of the selected product. Plasmid DNA samples were sequenced using the dideoxy method (Sanger, Nicklen & Coulsen, 1977) using fluorescently labelled nucleotides. Either a LI-COR Model 4000L sequencer or a Perkin Elmer ABI PRISM 377 was used following amplification with M13 forward and reverse primers. Sequence data was analysed using MT Navigator ver. 1.02b3 (1994) (Applied Biosystems Inc., Perkin Elmer) and ClustalX (1.5b) for the multiple alignment of genetic sequences. A phylogenetic tree was constructed using Saitou & Nei's Neighbour Joining Method (NJ) (Saitou & Nei, 1987) which calculates distances (% divergence) between all pairs of sequence from a multiple alignment. The NJ is then applied to the distance matrix and the bootstrap tree which provides confidence estimates for the groupings was drawn using Treeview 1.5 (Page, 1996).

Results

a) 18S ribosomal DNA

The precise sequence for a 0.9kb fragment of the 18S ribosomal RNA gene was determined from eight sea lice (2 from each site – 2 wild and 2 farmed sites), using two clones per specimen (i.e. a total of 16 clones) and from both forward and reverse sequences for each clone. Further, the DNA for this region was extracted, purified, cloned and sequenced for lice taken from the same samples on two separate occasions placing increased confidence in the sequence identity. A 904 nucleotide (nt) sequence was determined (Fig. 2) for each clone with no differences between clones, between indi-

vidual lice or between sites. The most similar crustacean 18S previously recorded is that of the branchiuran parasite *Argulus nobilis* with a similarity of 83.92% (914 nt) (Abele, Kim & Felgenhauer, 1989). The nucleotide sequence for this partial sequence of the 18S ribosomal RNA gene has been submitted to the GenBank Data Library under the acquisition number AF043979.

b) Internal Transcribed Spacer (ITS-1)

A total of twenty sea lice from six sites (3 wild and 3 farm) were prepared for cloning. Two or three clones from each louse were prepared for sequencing. Forward and reverse sequences for each clone were obtained, aligned and the sequences compared. Where differences occurred in the forward and reverse sequence and / or between clones from each louse, the sequences were rejected. A total of thirteen lice from four sites (2 wild and 2 farm) were selected and compared and a 454 nucleotide sequence was derived (Fig. 3). Between all samples (n = 13) there was an overall 92.14% similarity in nucleotide sequences with 28 transitions, 28 transversions, 17 indels and 3 inversions (between 6 nucleotide positions). Within the ITS-1 sequence, there are three highly conserved regions between nucleotides 1–32, 91/94–183/189 (*x/y* denotes the range across all specimens), and 410/411–446/456 and one hypervariable region between base pairs 289/299–401/411. The similarity between all sequences is given in Table 1 and a phylogenetic tree produced from determined bootstrap values (1000 iterations) is given in Fig. 4. To summarize, the percentage similarity between lice within farm sites was 99.71% and 95.72% for sites A and D respectively and 86.90% and 86.03% between lice from within wild sites B and C. This is clearly shown in Fig. 4 where lice from salmon held in farm cages cluster together in the dendrogram, whilst sea lice from wild salmonids are distributed throughout the dendrogram.

A sequence has been submitted to GenBank (acquisition no. AF043980 for a louse collected from a farmed salmonid (site D)). The most similar crustacean sequence in GenBank was the ITS sequence for the ostracod *Darwinula brasiliensis*

```
L. salmonis   GGGCAAGTCTGGTGCCAGCAGCCGCG-TAATTCCAGCTCCAATAGCGTAT   49
A. nobilis    ........................G.........................   50

L. salmonis   ATTAAAGTTGTTGCGGTCAAAAAGCTCGTAGTTGGATTTCGGTGAGTAAG   99
A. nobilis    ..........C......T....................CC.A..TGCAG.C   100

L. salmonis   GAGGGGTTCATCTTGTTTGATGTAACTGCTGTCTTGTCTCGCGTTTTTCG   149
A. nobilis    .G.C...C.GC..CA.GGCGGT.C.....CTCG..C..GACATACC.C..   150

L. salmonis   ACAGAGGTTT---CGATGGTGCCTTTCAAAGGGTGTCTCGGGATACTGTC   195
A. nobilis    GTT..TC...GTC.CGC.AT..TC....TC.......G.....GG.C.GG   200

L. salmonis   GGGTTTACTTTGAAAAAATTAGAGTGCTCAAAGCAGGCTTTTAAAAGCTT   245
A. nobilis    AC....................................T.........GA..-----..CA   246

L. salmonis   GAATATTAGTGCATGGAATAATGAAATAGGAAGTCGTTTCTATTTTGTTG   295
A. nobilis    .....A.G.........................CC...G......C......   296

L. salmonis   GTT-TTCGGAAATCGACTTAATGATTAATAGGGACAGTCGGGGGCATTAG   344
A. nobilis    ...C.......CA...GG.................G.A..........C.   346

L. salmonis   TATTCAGACGACAGAGGTGAAATTCTTGGACCGTCTGAAGACTAACTACT   394
A. nobilis    ....GC....TT.....................T....GC.....G........   396

L. salmonis   GCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTTAGA   444
A. nobilis    ......................................G...................   446

L. salmonis   GGTTCGAAGGCGATCAGATACCGCCCTAGTTCTAACCATAAACGATGCCA   494
A. nobilis    ...................................................   496

L. salmonis   GCTAGCGATCCGCAGTTGTTTATTTTTACGACTCTGCGGGAAGCTTCCGG   544
A. nobilis    A.C.........TC.GC...AC..AGA.T...C.GA..A.C.........   546

L. salmonis   GAAACCAAAGCTTTTGGGTTCCGGGGGAAGTATGGTTGCAAAGCTGAAAC   594
A. nobilis    ...........T..C.....................................C.....   596

L. salmonis   TTAAAGGAATTGACGGAAGGGCACCACCAGGAGTG--GCCTGCGGCTTAA   642
A. nobilis    ...................................GA.............   ·646

L. salmonis   TTTGACTCAACACGGGAAATCTCACCAGGCCCGGACACTGGAAGGATTGA   692
A. nobilis    ...................C...................T.G........   696

L. salmonis   CAGATTGAGAGCTCTTTCTCGATTCGGTGGGTGGTGGTGCATGGCCGTTC   742
A. nobilis    ...................T.....A.........................   746

L. salmonis   TTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAACGAGACTC   792
A. nobilis    ............C.......................................C.   796

L. salmonis   TGTCCTGCTAAATAGAGTTAACGTCATTTTTTTTGCGTTAACTTTTCTTC   842
A. nobilis    .T..............GCGG..GA..C.CA.GCAGTGTC....G..-....   845

L. salmonis   TTAGAGGGACTGGTGGCGTCTAGCCACACGAGATTGAGCAATAACAGGTC   892
A. nobilis    ..........A........T...................G..........   895

L. salmonis   TGTGATGCCCTT                                        904
A. nobilis    ............                                        907
```

Fig. 2. The determined partial 18S ribosomal RNA gene sequence from *Lepeophtheirus salmonis* is given and compared to *Argulus nobilis* (Branchiura: Crustacea). Letters in bold and underlined represent the primers used for PCR amplification.

Pinto & Kotzian (Schon, Butlin, Griffiths & Martens, 1998) with a similarity of 47.94% (315 nt).

Discussion

The ability to identify different sea lice populations will make a major contribution to understanding characteristics including the dispersion and epidemiology of sea lice infections and contribute information to the ongoing dispute between salmon farming and wild salmonid fishery interests. To begin investigating the population characteristics of *L. salmonis*, two regions of the sea louse genome, namely 18S rDNA and ITS-1 were targeted. No variation in a partial fragment (904 base pairs)

```
Farm A₁    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGCCAATTATACTACTAGCGTCCTC--    58
Farm A₂    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGCCAATTATACTACTAGCGTCCTC--    58
Farm A₃    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGCCAATTATACTACTAGCGTCCTC--    58
Farm A₄    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGCCAATTATACTACTAGCGTCCTC--    58
Wild B₁    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGCCAATTATACTACTAGCGTCCTC--    58
Wild B₂    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGCCAATTATTCTACTAGCGTCGTTTC    60
Wild C₁    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGCCAATTATACTACTAGCGTCCTC--    58
Wild C₂    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGCCAATTATTCTACTAGCGTCGTTTC    60
Farm D₁    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATGCCAATTATACTACTAGCGTCCTC--    58
Farm D₂    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGCCAATTATTCTACTAGCGTCGTTTC    60
Farm D₃    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGCCAATTATTCTACTAGCGTCGTTTC    60
Farm D₄    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGCCAATTATTCTACTAGCGTCGTTTC    60
Farm D₅    TTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGCCAATTATTCTACTAGCGTCGTTTC    60
           ****************************** ********* ********** *


Farm A₁    ----ACGGGCGCTGTAAACTTTAAAAAAAAAAAAAAAGAAAATTTTCAACGGTGGATATCT    114
Farm A₂    ----ACGGGCGCTGTAAACTTTAAAAAAAAAAAAAAAGAAAATTTTCAACGGTGGATATCT    114
Farm A₃    ----ACGGGCGCTGTAAACTTTAAAAAAAAAAAAAAAGAAAATTTTCAACGGTGGATATCT    114
Farm A₄    ----ACGGGCGCTGTAAACTTTAAAAAAAAAAAAAAAGAAAATTTTCAACGGTGGATATCT    114
Wild B₁    ----ACGGGCGCTGTAAACTTTAAAAAAAAAAAAAAAGAAAATTTTCAACGGTGGATATCT    114
Wild B₂    ACG-ACGG-CGCTGTAAACTTTAAAACAAAAAAAAAGAAAATTTTCAACGGTGGATATCT     118
Wild C₁    ----ACGGGCGCTGTAAACTTTAAAAAAAAAA----GAAAATTTTCAACGGTGGATATCT     110
Wild C₂    ACG-ACGG-CGCTGTAAACTTTAAAACAAAAAAAAAGAAAATTTTCAACGGTGGATATCT     118
Farm D₁    ----ACGGGCGCTGTAAACTTTAAAAAAAAAA----GAAAATTTTCAACGGTGGATATCT     110
Farm D₂    ACGGACGG-CGCTGTAAACTTTAAAACAAAAAAAAAGAAAATTTTCAACGGTGGATATCT     119
Farm D₃    ACGGACGGGCGCTGTAAACTTTAAAACAAAAAAAAAGAAAATTTTCAACGGTGGATATCT     120
Farm D₄    ACG-ACGG-CGCTGTAAACTTTAAAACAAAAAAAAAGAAAATTTTCAACGGTGGATATCT     118
Farm D₅    ACG-ACGG-CGCTGTAAACTTTAAAACAAAAAAAAAGAAAATTTTCAACGGTGGATATCT     118
           ****  ***************** ****         *********************


Farm A₁    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    174
Farm A₂    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    174
Farm A₃    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    174
Farm A₄    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    174
Wild B₁    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    174
Wild B₂    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    178
Wild C₁    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    170
Wild C₂    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    178
Farm D₁    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    170
Farm D₂    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    179
Farm D₃    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    180
Farm D₄    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    178
Farm D₅    AGGTTCTCGTACCGAGGAAGAACGCAGCGAACTGCGATAAGCAATGCGAATTGCAGAACG    178
           ************************************************************
```

Fig. 3. The determined 454 nucleotide sequence for the internal transcribed spacer (ITS1) from different populations of *Lepeophtheirus salmonis*. Letters in bold and underlined represent the primers used for PCR amplification. Stars represent areas of identity between sequences.

of the 18S rRNA gene was found between samples of lice collected from four different sites. The 18S rRNA gene was found to be highly conserved between samples demonstrating that the "*L. salmonis*" population from farmed and wild salmonids collected in Scottish waters represents a single species i.e. without cryptic species present.

Sequences of the intergenic spacer ITS-1 how-

ever, were variable between sites with greater similarity demonstrated between individual lice taken from farmed salmonids than those taken from wild salmonids. Variation within the ITS-1 region is not uncommon and has been reported from *Schistosoma* where both size and sequence variation among copies of the ITS-1 repeat within individuals occurs (Van Herwerden et al., 1998). Similarly,

```
Farm A₁      CGAGTCATTGGATTTTCGAACGCAACTGGCGGTCTGACGCAACAATCAGACCATGTGTGT     234
Farm A₂      CGAGTCATTGGATTTTCGAACGCAACTGGCGGTCTGACGCAACAATCAGACCATGTGTGT     234
Farm A₃      CGAGTCATTGGATTTTCGAACGCAACTGGCGGTCTGACGCAACAATCAGACCATGTGTGT     234
Farm A₄      CGAGTCATTGGATTTTCGAACGCAACTGGCGGTCTGACGCAACAATCAGACCATGTGTGT     234
Wild B₁      CGAGTCATTGGATTTTCGAACGCAACTGGCGGTCTGACGCAACAATCAGACCATGTGTGT     234
Wild B₂      CGAGTCATTTGATTTTCGAACGCATTCGGCGGTCCGACGTAGCAATCGGACCATGTGTGT     238
Wild C₁      CGAGTCATTGGATTTTCGAACGCAACTGGCGGTCTGACGCAACAATCAGACCATGTGTGT     230
Wild C₂      CGAGTCATTTGATTTTCGAACGCATTCGGCGGTCCGACGTAGCAATCGGACCATGTGTGT     238
Farm D₁      CGAGTCATTTGATTTTCGAACGCAACCGGCGGTCCGACGTAGCAATCGGACCATGTGTGT     230
Farm D₂      CGAGTCATTTGATTTTCGAACGCATTCGGCGGTCCGACGTAGCAATCGGACCATGTGTGT     239
Farm D₃      CGAGTCATTTGATTTTCGAACGCATTCGGCGGTCCGACGTAGCAATCGGACCATGTGTGT     240
Farm D₄      CGAGTCATTTGATTTTCGAACGCATTCGGCGGTCCGACGTAGCAATCGGACCATGTGTGT     238
Farm D₅      CGAGTCATTTGATTTTCGAACGCATTCGGCGGTCCGACGTAGCAATCGGACCATGTGTGT     238
             ********  **************    ******* ****  *  ***** ***********


Farm A₁      TTCAGTGTCAACAAAAATACCAATCTTAATGTGATTGAACTTCAGTTCTCTCATGAAATA     294
Farm A₂      TTCAGTGTCAACAAAAATACCAATCTTAATGTGATTGAACTTCAGTTCTCTCATGAAATA     294
Farm A₃      TTCAGTGTCAACAAAAATACCAATCTTAATGTGATTGAACTTCAGTTCTCTCATGAAATA     294
Farm A₄      TTCAGTGTCAACAAAAATACCAATCTTAATGTGATTGAACTTCAGTTCTCTCATGAAATA     294
Wild B₁      TTCAGTGTCAACAAAAATACCAATCTTAATGTGATTGAACTTCAGTTCTCTCATGAAATA     294
Wild B₂      TTCAGTGTCAACAAAACTCCCAATCTTAATGTGATTGAATTTCGATTCTCTCATGAAACA     298
Wild C₁      TTCAGTGTCAACAAAAATACCAATCTTAATGTGATTGAACTTCAGTTCTCTCATGAAATA     290
Wild C₂      TTCAGTGTCAACAAAACTCCCAATCTTAATGTGATTGAATTTCGATTCTCTCATGAAACA     298
Farm D₁      TTCAGTGTCAACAAAACTCCCAATCTTAATGTGATTGAATTTCGATTCTCTCATGAAACA     290
Farm D₂      TTCAGTGTCAACAAAACTCCCAATCTTAATGTGATTGAATTTCGATTCTCTCATGAAACA     299
Farm D₃      TTCAGTGTCAACAAAACTCCCAATCTTAATGTGATTGAATTTCGATTCTCTCATGAAACA     300
Farm D₄      TTCAGTGTCAACAAAACTCCCAATCTTAATGTGATTGAATTTCGATTCTCTCATGAAACA     298
Farm D₅      TTCAGTGTCAACAAAACTCCCAATCTTAATGTGATTGAATTTCGATTCTCTCATGAAACA     298
             *************** *  ******************* ***   ************* *


Farm A₁      ACTGTGC-TTGCGGCTCGGACTGACGAAGTAGTCACCCGTCTCTGACGGCAGAGATTTCG     353
Farm A₂      ACTGTGC-TTGCGGCTCGGACTGACGAAGTAGTGACCCGTCTCTGACGGCAGTGATTTCG     353
Farm A₃      ACTGTGC-TTGCGGCTCGGACTGACGAAGTAGTCACCCGTCTCTGACGGCAGTGATTTCG     353
Farm A₄      ACTGTGC-TTGCGGCTCGGACTGACGAAGTAGTCACCCGTCTCTGACGGCAGTGATTTCG     353
Wild B₁      ACTGTGCCTTGCGGCTCGGACTGACGAAGTAGTCACCCGTCTCTGACGGCAGTGATTTCG     354
Wild B₂      AGAGGGGCTTGCGGCCTTCTCTGACGAAGTAGTCACCCGCTTGCGGA-----TGATTTCG     353
Wild C₁      ACTGTGCCTTGCGGCTCGGACTGACGAATTAGTCACCCGTCTCTGACGGCAGTGATTTCG     350
Wild C₂      AGAAGGGCTTGCGGCCTTCTCTGACGAATTAGTCACCCGCTTGCGGA-----TGATTTCG     353
Farm D₁      AGAGGGGCTTGCGGCCTTCTCTGACGAAGTAGTCACCCGTCTCTGAA-----TGATTTCG     345
Farm D₂      AGAGGGGCTTGCGGCCTTCTCTGACGAAGTAGTCACCCGCTTGCGGA-----TGATTTCG     354
Farm D₃      AGAGGGGCTTGCGGCCTTCTCTGACGAAGTAGTCACCCGCTTGCGGA-----TGATTTCG     355
Farm D₄      AGAGGGGCTTGCGGCCTTCTCTGACGAAGTAGTCACCCGCTTGCGGA-----TGATTTCG     353
Farm D₅      AGAAGGGCTTGCGGCCTTCTCTGACGAATTAGTCACCCGCTTGCGGA-----TGATTTCG     353
             *     *  ******   *******  ****** **** ****   *    *  ******


Farm A₁      CCGGAAAGAGTTGAGAAGGTTCAGAGAAAACCTCTTTTGAGATCAAGACCTGAAATAGGC     413
Farm A₂      CCGGAAAGAGTTGAGAAGGTTCAGAGAAAACCTCTTTTGAGATCAAGACCTGAAATAGGC     413
Farm A₃      CCGGAAAGAGTTGAGAAGGTTCAGAGAAAACCTCTTTTGAGATCAAGACCTGAAATAGGC     413
Farm A₄      CCGGAAAGAGTTGAGAAGGTTCAGAGAAAACCTCTTTTGAGATCAAGACCTGAAATAGGC     413
Wild B₁      CCGGAAAGAGTTGAGAAGGTTCAGAGAAAACCTCTTTTGAGATCAAGACCTGAAATAGGC     414
Wild B₂      GCAGAGCTGGCTGAGAGGCTCCGGAGCAAACTTCTTTAACAATAACGACCTGAGACAGGC     413
Wild C₁      CCGGAAAGAATTGAGAAG-TTCAGAGAAAACCTCTTTTGAGATCAAGACCTGAAATAGGC     409
Wild C₂      GCAGAACTGGCTGAGAAGCTCCGGAGCAAACTTCTTTAACAATAACGACCTGAGACAGGC     413
Farm D₁      CCGGAAAGAGTTGAGAAGGTTCAGAGAAAACCTCTTTTGAGATCAAGACCTGAAATAGGC     405
Farm D₂      GCAGAGCTGGCTGAGAGGCTCCGGAGCAAACTTCTTTAACAATAACGACCTGAGACAGGC     414
Farm D₃      GCAGAGCTGGCTGAGAGGCTCCGGAGCAAACTTCTTTAACAATAACGACCTGAGACAGGC     415
Farm D₄      GCAGAGCTGGCTGAGAAGCTCCGGAGCAAACTTCTTTAACAATAACGACCTGAGACAGGC     413
Farm D₅      GCAGAACTGGCTGAGAAGCTCCGGAACAAACTTCTTTAACAATAACGACCTGAGACAGGC     413
             *  **     ***** * *|* **  **** *****       ** * ******* * ****
```

Fig. 3. Continued.

```
Farm A₁        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      454
Farm A₂        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      454
Farm A₃        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      454
Farm A₄        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      454
Wild B₁        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      455
Wild B₂        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      454
Wild C₁        AAGATAACCCGCCCAACTTAACCATATCACTAAGCGGAAGA                      450
Wild C₂        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAAGA                      454
Farm D₁        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      446
Farm D₂        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      455
Farm D₃        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAGGA                      456
Farm D₄        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAAGA                      454
Farm D₅        AAGATAACCCGCCCAACTTAAGCATATCACTAAGCGGAAGA                      454
               *********************************** **
```
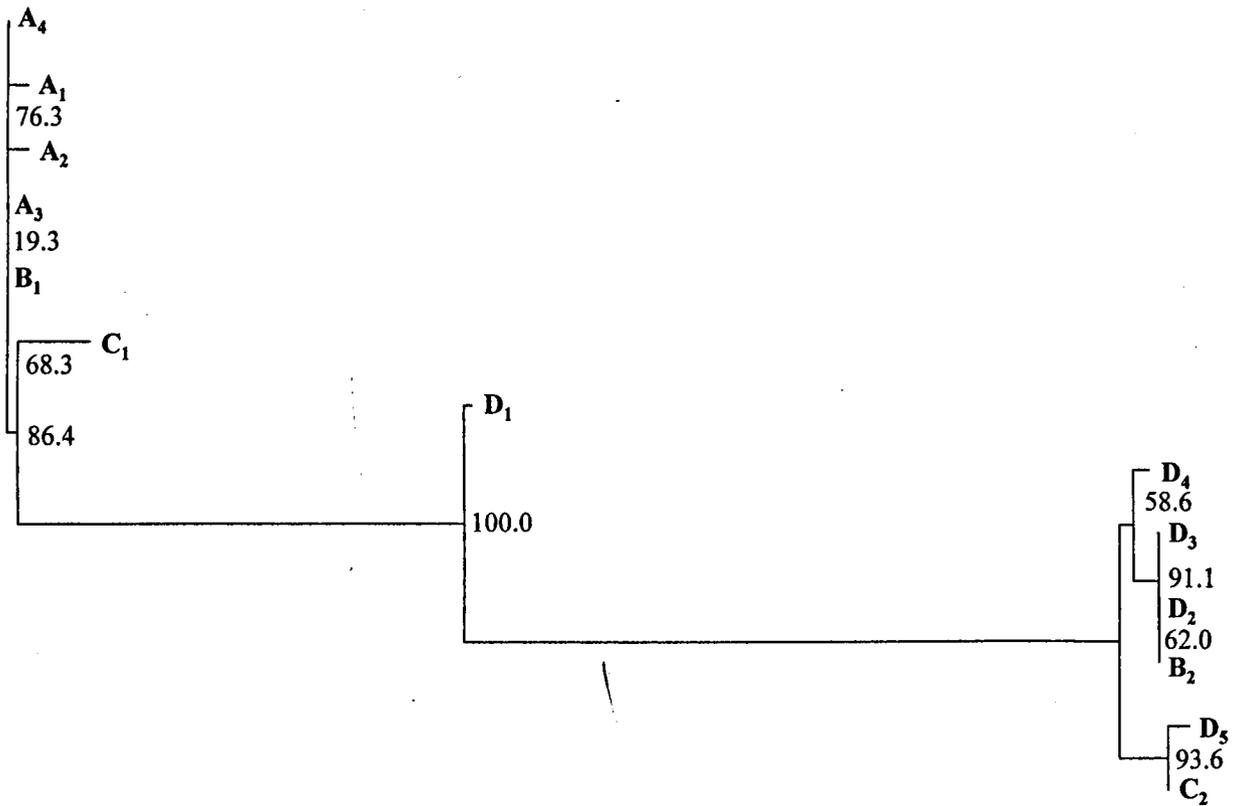
Fig. 3. Continued.

Fig. 4. Unrooted phylogenetic tree of *Lepeophtheirus salmonis* showing the relationship between the samples based on the ITS1 sequence constructed using Saitou & Nei's Neighbour Joining Method (NJ). Samples A_1-A_4 and D_1-D_5 represent lice taken from two Atlantic salmon farms and samples B_1-B_2 and C_1-C_2 represent lice taken from two wild salmon collection sites. Nodes show bootstrap determined confidence estimates for each grouping expressed as percentages.

Diggles & Adlard (1997) found 11 variable nucleotides in the 169-170 sequence representing ITS-1 between 16 isolates of *Cryptocaryon*. Further, they reported genetic drift amounting to 2.9-3.5% divergence when the original samples of 3 isolates were compared to those maintained in a laboratory over a 36 month period. The 0.29-4.28% divergence between samples of lice collected from farmed salmonids when compared to background levels (i.e. wild lice) of 13.10-13.97% divergence between samples, may serve to illustrate that cage sites represent a bottleneck to genetic flow. A reduc-

Table 1. Percentage similarity between nucleotide sequences for the internal transcribed spacer (ITS-1) obtained from four populations of *Lepeophtheirus salmonis*. Lice collected at sites A and D were from farmed salmonids whilst those lice collected from sites B and C were from wild salmonids.

Sample	A_1	A_2	A_3	A_4	B_1	B_2	C_1	C_2	D_1	D_2	D_3	D_4	D_5
A_1	-												
A_2	99.34	-											
A_3	99.78	99.78	-										
A_4	99.56	99.78	100.00	-									
B_1	99.56	99.56	99.78	99.78	-								
B_2	86.49	86.68	86.90	86.90	86.90	-							
C_1	97.58	97.80	98.02	98.02	98.24	85.37	-						
C_2	86.49	86.68	86.90	86.90	86.68	99.12	86.03	-					
D_1	92.75	92.95	93.17	93.17	93.17	91.39	93.30	91.17	-				
D_2	86.74	86.49	86.71	86.71	86.71	99.78	85.19	98.90	91.19	-			
D_3	85.90	86.07	86.30	86.52	86.30	99.12	84.78	98.02	90.77	99.82	-		
D_4	86.49	86.68	86.90	86.90	86.90	99.56	85.81	99.56	91.61	99.34	98.68	-	
D_5	86.06	86.24	86.46	86.46	86.24	98.45	85.59	99.56	90.73	98.53	97.58	98.90	-

Summary statistics for lice within sites: within A (n = 6) 99.71 ± 0.23% similarity; within B (n = 1) 86.90% similarity; within C (n = 1) 86.03% similarity; and within D (n = 10) 95.72 ± 4.04% similarity. Across all lice within farm sites (n = 36) there was 91.97 ± 5.70% similarity; within wild sites (n = 6) there was 90.39 ± 6.45% similarity: and across all lice and all sites (n = 78) there was 92.14 ± 5.88% similarity.

tion in the genetic variability or population isolation within farm sites, may stem from a combination of environmental and anthropogenic induced factors including farming intensity, loch flush rates, fallowing history, the retention of lice larval stages and the frequency of chemotherapeutant application. While fallowing is anticipated to eliminate the resident lice population, it is not known to what extent the gene pool is propagated across the fallowing period to the next stock of fish by either the interaction of the planktonic larvae with neighbouring farm sites or the activity of local salmonids, both of which may serve as a reservoir of genetic identity. Using the features of the hypervariable region identified within the ITS-1 region in this study, it may be possible to design population specific tags or primers that could subsequently be used to ascertain the origin of nauplii and copepodites on farmed and wild salmonids. The acquisition of such population markers could make significant inroads into our ability to monitor the dispersal, fate and impact of planktonic larvae not only on wild salmonids, but between farm sites and across fallow periods.

Acknowledgements

References

Abele LG, Kim W, Felgenhauer BE. 1989. Molecular evidence for the inclusion of the phylum Pentastomida in the Crustacea. *Mol. Biol. Evol.* 66: 685-691.

Barta JR, Coles BA, Schito ML, Fernando M.A, Martin A, Danforth HD. 1998. Analysis of infraspecific variation among five strains of *Eimeria maxima* from North America. *Int. J. Parasitol.* 28: 485-492.

Blair D, Agatsuma T, Watonobe T, Okomoto M, Ito A. 1997. Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. *Parasitology* 115: 411-417.

Chilton NB, Gasser RB, Beveridge I. 1995. Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the *Hypodontus macropi* complex (Nematoda: Strongyloidea). *Int. J. Parasitol.* 25: 647-651.

Cunningham CO. 1997. Species variation within the internal transcribed spacer (ITS) region of *Gyrodactylus* (Monogenea: Gyrodactylidae) ribosomal RNA genes. *J. Parasitol.* 83: 215-219.

Cunningham CO, McGillivray DM. MacKenzie K. 1995. Phylogenetic analysis of *Gyrodactylus salaris* Malmberg, 1957 based on the small subunit (18S) ribosomal RNA gene. *Mol. Biochem. Parasitol.* 71: 139-142.

De Meeüs T, Marin R, Renaud F. 1992. Genetic heterogeneity within populations of *Lepeophtheirus europaensis* (Copepoda: Caligidae) parasitic on two host species. *Int. J. Parasitol.* 22: 1179-1181.

Diggles BK, Adlard RD. 1997. Intraspecific variation in *Cryptocaryon irritans. J. Eukaryotic Microbiol.* 44: 25-32.

Ellis RE, Sulston JE, Coulson AR. 1986. The rDNA of *C. elegans*: sequence and structure. *Nucl. Acids Res.* 14: 2345-2364.

Hashimoto K, Watanobe T, Liu CX, Init I, Blair D, Ohnishi S, Agatsuma T. 1997. Mitochondrial DNA and nuclear DNA indicate that the Japanese *Fasciola* species is *F. gigantica*. *Parasitol. Res.* **83**: 220-225.

Homan WL, Limper L, Verlaan M, Borst A, Vercammen M, Knapen F van. 1997. Comparison of the internal transcribed spacer, ITS-1, from *Toxoplasma gondii* isolates and *Neospora caninum*. *Parasitol. Res.* **83**: 285-289.

Isdal E, Nylund A, Nævdal G. 1997. Genetic differences among salmon lice (*Lepeophtheirus salmonis*) from six Norwegian coastal sites: evidence from allozymes. *Bull. Eur. Assoc. Fish Pathol.* **17**: 17-22.

Littlewood DTJ, Johnston DA. 1995. Molecular phylogenetics of the four *Schistosoma* groups determined with partial 28S ribosomal RNA gene sequences. *Parasitology* **111**: 167-175.

Morgan JAT, Blair D. 1998. Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial CO1 and ND1 genes for distinguishing among *Echinostoma* species (Trematoda). *Parasitology* **116**: 289-297.

Nordhagen JR, Heuch PA, Schram TA. 1998. Size as an indicator of the origin of salmon lice *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Proceedings and Abstracts of the Fourth International Crustacean Congress*. Amsterdam 20-24 July, p.102.

Okamoto M, Agatsuma T, Kurosawa T, Ito A. 1997. Phylogenetic relationships of three hymenolepidid species inferred from nuclear ribosomal and mitochondrial DNA sequences. *Parasitology* **115**: 661-666.

Page RDM. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Comp. Applic. Biosciences* **12**: 357-358.

Rinder H, Katzwinkel-Wladarsch S, Loscher T. 1997a. Evidence for the existence of genetically distinct strains of *Enterocytozoon bieneusi*. *Parasitol. Res.* **83**: 670-672.

Rinder H, Rausch RL, Takahashi K, Kopp H, Thomschke A, Loscher T. 1997b. Limited range of genetic variation in *Echinococcus multilocularis*. *J. Parasitol.* **83**: 1045-1050.

Romstad A, Gasser RB, Nansen P, Polerman AM, Chilton NB. 1998. *Necator americanus* (Nematoda: Ancylostomatidae) from Africa and Malaysia have different ITS-2 rDNA sequences. *Int. J. Parasitol.* **28**: 611-615.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*. 2nd edition. New York: Cold Harbor Laboratory Press.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Nat. Ac. Sciences*, USA **72**: 5463-5467.

Schon I, Butlin RKB, Griffiths HI, Martens K. 1998. Slow molecular evolution in an ancient asexual ostracod. *Proc. Roy. Soc.* London, Ser. B **265**: 235-242.

Shinn AP, Bron JE, Gray DJ, Sommerville C. (this volume) Elemental analysis of Scottish populations of the ectoparasitic copepod *Lepeophtheirus salmonis* (Krøyer, 1837). *Contrib. Zool.*

Shinn AP, Banks BA, Bron JE, Powell SF, Sommerville C. 1998. Differentiation of populations of the ectoparasitic copepod *Lepeophtheirus salmonis* (Krøyer, 1837) (Crustacea: Caligidae) in Scottish waters. *British Soc. Parasitol*, Spring Meeting, Abstracts, University of Exeter 5th-8th April, 102.

Stevenson LA, Gasser RB, Chilton NB. 1996. The ITS-2 rDNA of *Teladorsagia circumcincta*, *T. trifurcata* and *T. davtiani* (Nematoda: Trichostrongylidae) indicates that these taxa are one species. *Int. J. Parasitol.* **26**: 1123-1126.

Todd CD, Walker AM, Wolff K, Northcott SJ, Walker AF, Ritchie MG, Hoskins R, Abbott RJ, Hazon N. 1997. Genetic differentiation of populations of the copepod sea louse *Lepeophtheirus salmonis* (Krøyer) ectoparasitic on wild and farmed salmonids around the coasts of Scotland: Evidence from RAPD markers. *J. Exp. Mar. Biol. Ecol.* **210**: 251-274.

Van Herwerden L, Blair D, Agatsuma T. 1998. Intra- and inter-specific variation in nuclear ribosomal internal transcribed spacer 1 of the *Schistosoma japonicum* species complex. *Parasitology* **116**: 311-317.