INTRODUCTION

Sea lice *Lepeophtheirus salmonis* (Krøyer) are ectoparasitic copepods that feed on several salmonid species. These parasites are a major cause of economic loss in the aquaculture industries of Northern Europe, the USA, Canada, and the Pacific coast of Japan (Denholm et al. 2002, Grant 2002). Since the mid 1990s, the synthetic pyrethroid insecticides cypermethrin (‘Excis’ and ‘Beta-max’, Novartis) (Hart et al. 1997) and deltamethrin (‘AlphaMax’, Alpharma) have almost completely replaced organophosphates for lice treatments and were used in more than 90% of the treatments in Norway for sea lice in 1999 (Denholm et al. 2002). Several clinical treatment failures with deltamethrin and cypermethrin have been reported from Norway (Sevatdal & Horsberg 2000), and reduced sensitivity has been documented using bioassays (Sevatdal & Horsberg 2003).

Pyrethroid insecticides have been used widely to control many arthropod pests since their introduction in the 1970s. However, because of their intensive use, many pest species have developed resistance to these compounds. This has stimulated extensive research into the incidence of resistance and the nature of the underlying mechanisms. Knowledge of resistance mechanisms is a valuable tool for developing strategies to detect and manage the spread of resistant individuals (Horowitz & Denholm 2001).

The primary target site for pyrethroids is the voltage-gated sodium channel, a large membrane protein that mediates the increase in sodium ion permeability during the rising phase of action potentials in neurons and other excitable tissues (Catterall 2000). Pyrethroids modify the normal operation of the channel by slowing its activation and inactivation kinetics, which results in uncontrolled bursts of action potentials leading to nerve exhaustion and death (Narahashi 1992). Pyrethroids are excellent insecticides because they selectively target the sodium channels of arthropods with little cross-toxicity to mammals. In insects, the
main voltage-gated sodium channel is encoded by the \textit{para} gene that was first cloned from \textit{Drosophila melanogaster} (Loughney et al. 1989). Subsequent studies have generated full or partial sequences of \textit{para} gene homologues from a range of insect species (reviewed in Soderlund & Knipple 2003). The insect channel protein is similar to that in vertebrates, consisting of 4 internally repeating homology domains (I–IV) with a minimum of 6 membrane-spanning segments (S1–S6) in each domain (Catterall 2000).

An important mechanism of pyrethroid resistance, termed knockdown resistance (or kdr), is characterised by a reduced sensitivity of the insect nervous system to these compounds and is caused by alterations to the \textit{para}-type sodium channel protein that render it less sensitive to the toxic effects of these insecticides. Mutations leading to resistance are most commonly located in the domain II region of the channel protein and have now been identified at 5 different residues (see Vais et al. 2001, Soderlund & Knipple 2003 and references therein). The most common is leucine 1014, where mutations to phenylalanine (L1014F), serine (L1014S) and histidine (L1014H) generally confer moderate levels of resistance (10 to 30 fold) to pyrethroids. Other secondary mutations in this domain that give enhanced (super-kdr) resistance include methionine 918 to threonine (M918T) in housefly \textit{Musca domestica} (L.) and horn fly \textit{Haematobia irritans} (L.) (Williamson et al. 1996, Guerrero et al. 1997), threonine 929 to isoleucine (T929I) in diamondback moth \textit{Plutella xylostella} (L.) and head louse \textit{Pediculus humanis} (L.) (Schuler et al. 1998, Lee et al. 2000) and leucine 925 to isoleucine (L925I) in tobacco whitefly \textit{Bemisia tabaci} (Genn.) (Morin et al. 2002). These secondary mutations are usually found in combination with the L1014F kdr mutation and give enhanced resistance levels of up to 1000 fold for certain pyrethroids. Mutations have also been reported outside domain II, although there is evidence that these may incur fitness penalties and none of these have yet been found in more than one species (Park et al. 1997, Pittendrigh et al. 1997, Head et al. 1998, He et al. 1999, Liu et al. 2000). All mutation numbering in this paper is based on the housefly \textit{para} sequence (EMBL accession CAA65448) where kdr mutations were first identified.

Little is known about the mechanism(s) of pyrethroid resistance in sea lice. The current study was conducted to investigate whether one or more of the mutations described above also cause pyrethroid resistance in sea lice. We have PCR-amplified and sequenced regions in domain II of the \textit{Lepeophtheirus salmonis} \textit{para}-type sodium channel gene that contain 5 of the mutation sites conferring kdr-type resistance to pyrethroids in insects, including the common L1014F mutation site. The predicted amino acid sequences from pyrethroid sensitive and resistant \textit{L. salmonis} individuals have been compared. We report a single amino acid substitution, glutamine 945 to arginine (Q945R) that was only found in pyrethroid resistant lice samples and may therefore represent a novel mutation site in the domain II region of the sodium channel.

**MATERIALS AND METHODS**

**Experimental sea lice strains.** Sea lice were collected from salmon farms in selected regions in Norway (Fig. 1). The selection was based partially on geography, but also on reports of treatment failure or reduced pyrethroid sensitivity documented by bioassays (Sevatdal & Horsberg 2003). Atlantic salmon were netted from cages at the fish farm, and anaesthetized lightly with benzocaine (50 mg l$^{-1}$). The parasites were gently removed using forceps, snap frozen in liquid nitrogen and subsequently stored at $-80^\circ$C. In cases of treatment failures with pyrethroids, only individuals surviving the treatment were collected to maximise the chances of selecting resistant individuals.

**Resistance history.** There were no reports of resistance from farms in Brekke, Tysnes and Sveio and bioassays confirmed normal sensitivity. In Hitra, Bømlo and Hjelmeland the fish health service reported clinical treatment failures with pyrethroids, which were confirmed by bioassays (Sevatdal & Horsberg 2003). From Dirdal, there were only anecdotal reports of treatment failures.

**RT-PCR amplification and cloning of sea lice sodium channel fragments.** Frozen adult sea lice were ground to
a fine powder in liquid nitrogen in 1.5 ml microfuge tubes using tight-fitting pestles. Total RNA was then extracted using the SV Total RNA Isolation kit from Promega (Madison, WI). For first-strand synthesis, 5 µg RNA was incubated with 0.5 µg Oligo (dT) at 70°C for 10 min and placed on ice for 1 min. Synthesis buffer (2.5 mM MgCl$_2$, 0.5 mM dNTP and 10 mM DTT) and 200 units Superscript II RT (Life Technologies) were added and the reactions incubated at 42°C for 50 min. The reactions were terminated at 70°C for 15 min and these products used as templates for PCR. Degenerate oligonucleotide primers were designed for conserved motifs in previously published sodium channel sequences (Williamson et al. 1996, Martinez-Torres et al. 1997) available in the EMBL/GenBank databases. Primer sequences used in this study are shown in Table 1, and their location on the sodium channel are shown in Fig. 2A & B. PCR amplifications using primers SCLS-1F and SCLS-2R for the primary reaction, followed by primers SCLS-3F and SCLS-2R for secondary, nested reactions produced a fragment of approximately 250 bp which included the known domain II mutation sites. Separate reactions using primers SCLS-12F and SCLS-27F for the primary reaction followed by primers SCLS-6F and SCLS-27F for the secondary reaction were used to amplify a fragment of approximately 1.9 kb that spanned segments IIS4 through to domain IIIS3. All PCR amplifications were done in 50 µl reaction volumes with 100 ng genomic DNA, 1× buffer containing 1.5 mM MgCl$_2$, 200 µM dNTPs, 1µM of each primer and 1 Unit Taq polymerase (Qiagen). The reactions were hot-started by denaturation at 94°C for 4 min and run through 35 cycles of 30 s at 94°C, 1 min at 50°C and 1 min at 72°C with a final elongation for 10 min at 72°C. The products were cloned into the pMOSBlue vector (Amer sham Pharmacia Biotech) and sequenced using Big Dye terminator v1.1 cycle sequencing kit (Applied Biosystems) on Applied Biosystems 377 or 310 sequencers. Sea lice-specific para gene primers (Table 1) were designed from the sequences obtained and these improved the efficiency of the PCR reactions. These primers also allowed the use of genomic DNA as template for amplification in subsequent reactions.

**Amplification from genomic DNA.** Genomic DNA was extracted using DNeasy Tissue kit from Qiagen. Between 4 and 10 lice were analysed from each sampling area. Gene fragments spanning the major mutation sites (L1014, T929, M918, etc.) were amplified in 2 separate PCR amplifications. Primers SCLS-6F and SCLS-7R amplified a product of 1388 bp that included an intron of 1202 bp. Primers SCLS-14F and SCLS-25R amplified a product of 166 bp (Fig. 2A, Table 1). PCR conditions were as described above, except that 50 ng genomic DNA was used as template. PCR gene fragments were directly sequenced as described above using the internal primers, SCLS-15F and SCLS-16R, designed from the cDNA sequence (Table 1). All sequences were analysed using the Vector NTI software package (Informax).

The *Lepeophtheirus salmonis* para sodium channel sequence was deposited in EMBL nucleotide sequence database with accession number AJ812299.

**RESULTS**

Using degenerate primers for the domain II region of the known sodium channel sequences, we amplified and sequenced a 318 bp fragment of the *Lepeophtheirus salmonis* para-type sodium channel gene. This region contains 5 of the mutation sites previously associated with kdr-type resistance in a range of insect species including the common L1014F mutation site. The nucleotide and encoded amino acid sequences of this fragment, including the nucleotide sequence of an accompanying intron, are shown in Fig. 3. This is the first sodium channel sequence reported for a crustacean. An alignment of a 106 amino acid conserved sequence, encompassing the M918 and L1014 mutation sites, against a range of other vertebrate and invertebrate sequences is shown in Fig. 4. The *L. salmonis* sequence showed 75% similarity to the bee pathogen *Varroa destructor* Anderson (AY259834) and 73% similarity with the cattle tick *Boophilus microplus* Canestrini (AF134216) within this region of the para-type sodium channel (Fig. 5). Fig. 5 shows a phylogenetic tree, generated in Vector NTIP, depicting similarity between different sodium channel sequences.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Degeneracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLS-1F</td>
<td>5’- AARYTNGCNAARTCNGTGCC -3’</td>
<td>128</td>
</tr>
<tr>
<td>SCLS-2R</td>
<td>5’- ARRAANARRTNARNACCAC -3’</td>
<td>2048</td>
</tr>
<tr>
<td>SCLS-3F</td>
<td>5’- GCNAARTCNTGGCCNAC -3’</td>
<td>128</td>
</tr>
<tr>
<td>SCLS-27F</td>
<td>5’- ACITTYMGIGTNIMTNMGNC -3’</td>
<td>256</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genomic DNA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLS-6F</td>
<td>5’- CCACACTGTAACCTGCTATATC -3’</td>
<td></td>
</tr>
<tr>
<td>SCLS-7R</td>
<td>5’- AGTCCCAAAATGTTCAACCGG -3’</td>
<td></td>
</tr>
<tr>
<td>SCLS-8R</td>
<td>5’- GGATTGTGAGGAATCGGTC -3’</td>
<td></td>
</tr>
<tr>
<td>SCLS-12F</td>
<td>5’- CATGCCCCACACTGGAACCTG -3’</td>
<td></td>
</tr>
<tr>
<td>SCLS-14F</td>
<td>5’- CTTTTATGACTGTTGATGCGT -3’</td>
<td></td>
</tr>
<tr>
<td>SCLS-15F</td>
<td>5’- TCCATGTTGGGACTGTATGTA -3’</td>
<td></td>
</tr>
<tr>
<td>SCLS-25R</td>
<td>5’- TGCACCCCGAGAGATTTGTA -3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primer sequences used in the study. Degenerate bases are represented using standard IUB codes: R = A + G, Y = C + T, M = A + C, N = A + C + G + T. The degeneracy of each primer is shown.

To look for possible mutations in pyrethroid resistant samples, it was necessary to switch from RT-PCR (using RNA as the template) to PCR using genomic DNA templates. This assay was more robust and enabled testing of individual lice samples. We therefore re-amplified these fragments from genomic DNA of single adults using lice-specific *para* primers designed from the cDNA sequence. This analysis allowed us to accurately determine the genotype of multiple individuals from various locations and also revealed the presence of a large intron (1202 bp) in this region of the gene (Fig. 3).

PCR products from a total of 47 adults from the 7 locations were sequenced (Table 2). None of the 5 previously identified mutations in this region of channel protein were detected, but a novel change from glutamine to arginine at the end of IIIS5 at residue corresponding to Q945 of the housefly *para* sequence was found. The mutation was detected in samples from Hitra, Bømlo, Hjelmeland 2000 and Dirdal. The mutation was only detected in samples from areas with reported treatment failure with pyrethroids. The pyrethroid sensitivity was confirmed with bioassays in all samples except from Dirdal (Sevatdal & Horsberg 2003).

**DISCUSSION**

New insecticide/pesticide/anti-parasitic compounds are expensive to develop, and as a consequence the management of organisms such as sea lice depends on maintaining the efficacy of the few chemicals avail-
Fig. 3. Nucleotide and amino acid sequences of the *Lepeophtheirus salmonis* para-type sodium channel fragments (IIS5, IIS6) cloned in this study. Coding nucleotides are in uppercase and the intron sequence is in lower case. Transmembrane protein domains are shown by lines above the sequence. The Q945R mutation site is indicated with a box.

![Nucleotide and amino acid sequences of the *Lepeophtheirus salmonis* para-type sodium channel fragments (IIS5, IIS6) cloned in this study. Coding nucleotides are in uppercase and the intron sequence is in lower case. Transmembrane protein domains are shown by lines above the sequence. The Q945R mutation site is indicated with a box.](image)

Fig. 4. Alignment of the *Lepeophtheirus salmonis* sodium channel sequences with corresponding sequences from a range of other organisms. ▼: positions of the Q945R, M918T (super-kdr) and L1014F (kdr) mutation sites.

![Alignment of the *Lepeophtheirus salmonis* sodium channel sequences with corresponding sequences from a range of other organisms. ▼: positions of the Q945R, M918T (super-kdr) and L1014F (kdr) mutation sites.](image)
able. The cloning and sequencing of sea lice sodium channel gene sequences described in this paper and the identification of a novel mutation potentially responsible for target site resistance to pyrethroids offers the opportunity of developing a molecular diagnostic tool that could quickly detect the mutation in individual sea lice and thereby facilitate the early detection of resistance in different populations.

Mutations in the sodium channel conferring kdr resistance to pyrethroids have been characterised in many pest species. There are potentially many resistance-associated mutation sites in the 4 homologous domains of the sodium channel (Soderlund & Knipple 2003). However, most of the mutations found so far occur in the S4-S5, S5 and S6 segments of the domain II region of the channel protein. To date, 5 mutation sites that correlate with resistance in a range of insect species have been identified within this region. We therefore focused our work to look for similar mutation(s) in this region of the sea lice channel protein. Comparisons of the sequences of sensitive sea lice with lice from areas with documented reduced sensitivity to pyrethroids failed to reveal mutations at any of these known sites. We did, however, identify a single point mutation at a novel position in the IIIS5 segment of the Lepeophtheirus salmonis channel. This mutation, Q945R (numbering according to the Musca domestica para sequence [CAA65448]), was located within a highly conserved region of the channel and involved a non-conservative replacement of an uncharged glutamine side-chain by a positively charged arginine residue. The occurrence of the mutation correlated with resistance, as it was only found in samples from areas with reports of treatment failures. These samples had also been confirmed through bioassays to show significantly reduced sensitivity to pyrethroids (Table 2) (Sevatdal & Horsberg 2003).

In samples collected in 1999 from Sveio and Dirdal, the Q945R mutation was found only in Dirdal. These 2 areas are in different fjord systems and approximately 130 km apart. Analysis of samples from 2000 revealed the Q945R mutation in samples from Bømlo, Hjelmeland and Hitra. Samples from Bømlo had a high frequency of the mutation. This area is located approximately 20 km from Sveio and Tysnes. In spite of this, no mutations were detected in the Sveio and Tysnes samples. In Hitra, which is about 450 km north of Bømlo, the mutation was detected. However, in Brekke, which is between Bømlo and Hitra (about 180 km north of Bømlo), the mutation was not detected. The samples collected from Hjelmeland in 2002 showed normal sensitivity towards pyrethroids and the Q945R mutation, which was evident in the 2000 samples from this region, was not found. The areas in which the mutation has been detected thereby conform well with sensitivity data from bioassays and anecdotal reports of reduced efficacy of pyrethroids in delousing procedures.

**Table 2. Detection rate of mutation Q945R and reduced pyrethroid sensitivity for each sampling site. Nt: not tested**

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>Year</th>
<th>No. of samples with mutation/ Total sample size</th>
<th>Reported clinical treatment failure with pyrethroids</th>
<th>Sensitivity confirmed with bioassays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hitra</td>
<td>2000</td>
<td>3/6</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Brekke</td>
<td>2000</td>
<td>0/6</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Tysnes</td>
<td>2000</td>
<td>0/4</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Bømlo</td>
<td>2000</td>
<td>3/5</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Sveio</td>
<td>1999</td>
<td>0/4</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Hjelmeland</td>
<td>2000</td>
<td>1/10</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hjelmeland</td>
<td>2002</td>
<td>0/10</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Dirdal</td>
<td>1999</td>
<td>4/6</td>
<td>Yes</td>
<td>Nt</td>
</tr>
</tbody>
</table>

Alignments of known vertebrate and invertebrate sodium channel sequences show a high degree of sequence conservation across
this region of the channel protein, suggesting that this sequence is important for the normal function of the channel. It is therefore unlikely to tolerate polymorphisms, unless they exert an alternative selective advantage (e.g. conferring protection against insecticides). Indeed, all of the available sodium channel sequences in the EMBL/GenBank databases contain the glutamine residue at this position except for one of the mammalian channel isoforms (Scn11a, also known as Nav1.9) from mouse, Mus musculus and rat, Rattus norvegicus (AF118044 and AF059030), which also contains an arginine residue. Interestingly, this channel isoform is thought to be insensitive to tetrodotoxin (TTX), a highly potent sodium-channel blocking agent, although to our knowledge this residue has not been implicated as forming part of the TTX binding site.

To date there are reports of more than 20 different resistance-associated mutations (Soderlund & Knipple 2003) (Fig. 2B). This implies that there could be resistance-associated mutations elsewhere in the sodium channel of Lepeophtheirus salmonis. Cloning of the full sodium channel gene is necessary to investigate other possible mutation sites. However, the detection of this new mutation in L. salmonis and the correlation with bioassay results and anecdotal reports of pyrethroid resistance is an intriguing finding. Because of the difficulty in cultivating several generations of sea lice in the laboratory to select for a highly resistant population, physiological expression studies will be necessary to study the importance of this new mutation in terms of its in vivo effect on pyrethroid sensitivity and other functional properties on the sodium channel. At present, there is little structural information regarding the binding site for pyrethroids at the voltage-gated sodium channel. The sodium channel is a large, complex transmembrane protein and the difficulties in purifying and crystallising such proteins are well known. Hence, the information derived from the various mutations that have been shown to affect channel sensitivity are providing the first real clues as to which regions of the channel and which residues are involved in channel-pyrethroid interactions (Vais et al. 2001, Soderlund & Knipple 2003). Several recent studies have successfully confirmed the mechanism of other mutations (L1014F, T929I and M918T) by expressing the wild-type and mutated sodium channel genes in Xenopus oocytes and assaying their sensitivity to pyrethroids using electrophysiological techniques (Smith et al. 1997, Lee et al. 1999, Vais et al. 2000, 2001). These studies have shown that the resistance mutations are able to affect channel sensitivity to pyrethroids in 2 ways: (1) by altering the physiological properties of the channel to make it a poorer template for pyrethroid binding, and (2) by reducing overall affinity of the channel for pyrethroids so that pyrethroid-modified channels are able to recover more quickly. Studies are now in progress to explore the functionality of the Q945R by similar methods.

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