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Research Article

Molecular identification of *Didemnum vexillum* Kott, 1982 from sites around the UK coastline

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Abstract

Didemnum vexillum Kott, 1982 is a tunicate known as the carpet sea squirt that is non-native to the UK. It has been reported from sites in the UK and Ireland based on morphological characteristics. Here we present the results of Cytochrome Oxidase I mitochondrial gene analyses to confirm species identification of specimens taken from several of the sites around the UK mainland coast where non-native tunicate colonies have been reported. The results confirm the identity of the UK carpet sea squirt samples as *Didemnum vexillum* and provide additional information on COI haplotypes present in the UK.

Key words: aquatic invasive tunicates, COI gene, haplotypes, carpet sea squirt

Introduction

Didemnum vexillum Kott, 2002, commonly known as the carpet sea squirt, is a colonial ascidian which has rapidly become an invasive species in several locations throughout the world including North America, northern Europe and New Zealand (Griffith et al. 2009). It has been identified based on morphological characteristics at a number of coastal sites in the UK and Ireland, where it is thought to have been introduced through a variety of pathways including hull fouling, ship ballast water and contaminated aquaculture equipment (Beveridge et al. 2011). Comparative morphology of samples from around the world by Lambert (2009) and molecular evidence gathered by Stefaniak et al. (2012) suggest that *D. vexillum* may be native to Japan, though its range may extend to continental Asia. This was based on the number of unique COI haplotypes found in Japan. Of the 23 haplotypes from around the world described by Stefaniak et al. (2012), Japan has 22 and 17 of these were unique.

Bullard et al. (2007) suggested that *D. vexillum* has the potential to alter marine communities and affect economically important fishing and aquaculture activities. Problems can arise due to the bio fouling of structures and equipment, overgrowing and smothering of commercially important species, and by the covering of sensitive habitats (e.g. fish spawning grounds, scallop settlement grounds). In 2002, *D. vexillum* was discovered growing on Georges Bank, an important commercial fishing ground off the east coast of the USA, where it had formed large mats over the seabed, negatively impacting on the benthic fauna and the associated fisheries (Valentine et al. 2007; Lengyel et al. 2009). In New Zealand, due to the failure of eradication attempts in 2003, *D. vexillum* has become a serious problem affecting the aquaculture industry by overgrowing and smothering mussels resulting in significant commercial losses (Coutts and Forrest 2007).

The first report in the UK was from Holyhead Harbour, North Wales, where *D. vexillum* was observed overgrowing other marine organisms on structures in the harbour in 2008. Due to the low levels found, it was assumed to be a recent arrival (Griffith et al. 2009). It is thought to have been introduced either on the hull of a

recreational vessel originating from eastern Ireland where *D. vexillum* was reported in 2005, or from a visiting vessel from The Netherlands or France where *D. vexillum* was reported in the 1990s (Griffith et al. 2009). Other marinas around the Welsh coast were surveyed but *D. vexillum* was not found anywhere else (Holt et al. 2009). A pilot eradication programme was undertaken in Holyhead Harbour in 2009, and by May 2010 no trace of colonies were found on the treated structures. However, later that year small colonies were found, so a repeat eradication was carried out in 2011–2012 (Holt and Cordingley 2011).

D. vexillum was confirmed at Darthaven Marina (Dartmouth, Devon) in 2008 although there was evidence it could have been there since 2005 (Griffith et al. 2009). A survey of selected marinas in England carried out in late 2009 also detected colonies in the Solent, specifically Gosport, Lymington, and Cowes (Bishop et al. 2010a, b; Laing et al. 2010). As there is limited shellfish aquaculture in these areas, the industry would not be significantly affected (Laing et al. 2010).

The first record of *D. vexillum* in Scotland was in November 2009 from Largs Yacht Haven on the west coast; found during a routine non-native species survey by the Scottish Association of Marine Science (SAMS). In response to this discovery, Scottish Natural Heritage commissioned a dedicated survey of 12 west coast marinas, which was carried out in February 2010. Results indicated that *D. vexillum* had remained within the Largs marina, where it was found to be growing preferentially on artificial structures such as pilings, ropes, and tyres as well as on other marine organisms attached to these structures, including: mussels, barnacles, and other tunicates. A follow-up survey two months later of sites within the vicinity of Largs marina revealed colonies at three additional locations, including Fairlie Quay where it was found to be well established (Beveridge et al. 2011). Prior to this discovery, the marina had been surveyed in 2006 as part of a rapid assessment of Scottish marinas for non-native species, and no *D. vexillum* had been found (Ashton et al. 2006). As *D. vexillum* overgrows a range of artificial structures and other marine organisms, there is the possibility of serious consequences for the aquaculture industry on the west coast of Scotland. In the same way as witnessed in New Zealand, there is the potential for *D. vexillum* to overgrow and smother rope-grown mussels, scallop cages, and oyster trestles, or cause bio-fouling problems on fin-fish cages.

In 2011, during a shore survey of the Whitstable Flats, North Kent organised by the Kent Wildlife Trust (KWT) and the Museum of Wales, a colonial sea squirt was discovered and confirmed as being *D. vexillum* based on larval morphology. Further surveys of other areas of the Kent coastline were then carried out by the KWT and Natural England and colonies were identified at other locations, including an oyster hatchery (Hitchin 2011).

The discovery of *D. vexillum* during non-native species surveys highlights the importance of carrying out regular monitoring by individuals who are trained to identify or recognise suspect non-native species. Molecular methods are widely used as a fast and inexpensive way of identifying species that may appear to be morphologically very similar or identical. In this study, we used the Cytochrome Oxidase I mitochondrial gene (COI) analyses as a DNA barcode method for quick identification (Stefaniak et al. 2009; Smith et al. 2012; Stefaniak et al. 2012). We also examined two tissue homogenisation techniques (glass versus steel beads) for use with whole colony tissue samples to establish which technique gives the higher concentration of extracted DNA for direct sequencing. Samples of colonial ascidians, identified as *D. vexillum* using morphological characteristics, were collected from several sites around the coast of the UK. Of particular interest were the colonies found in Kent which did not fit with the usual ecological and habitat preferences of the species (Hitchin 2011). Previous studies using the COI gene method only included samples from a single location in the UK: Holyhead, North Wales (Stefaniak et al. 2012).

Material and methods

Sample collection and preparation

Fourteen samples of colonial ascidians morphologically identified as *Didemnum* sp. were collected from seven sites around the UK (Table 1) and immediately preserved in 95% ethanol. Two tissue sub-samples (10 mg) from each sample were weighed into 2 ml Safelock tubes (Eppendorf) and placed in a freezer at – 20ºC overnight. The tissue sub-samples were removed from the freezer and finely chopped using a scalpel then stored in the freezer until DNA extraction. In order to further breakdown the tissue to make DNA extraction more efficient, we tested two homogenisation methods that are currently used for the breakdown of biological tissues. One method

Table 1. Location of *Didemnum* samples collected for this study and sequencing results.

UK Sites	GPS or location	Collection Date	Colony description	Site description	Collector	Sequences (n) F|:forward, R|; reverse	GenBank Number
Largs Marina	55.774 -4.859	23/04/2012	pale, cream	covering mussels	Dr E. Cook	3F, 3R	KR612213
Fairlie Quay Pier 11	Pile 2 no. 4	26/07/2011			Dr J. Bishop	1F, 1R	KR612209
Fairlie Quay Pier 15	Pile 1 no. 2	26/07/2011			Dr J. Bishop	1F, 1R	KR612210
Fairlie Quay Pier 21	Pile 1 no. 6	26/07/2011			Dr J. Bishop	1F, 1R	KR612211
Hunterston terminal 23		26/07/2011			Dr J. Bishop	1F, 1R	KR612212
Darthaven Marina D1	50.35083 -3.57167	11/06/2012	small colonies on mussel	Marina Pontoons	Dr J. Bishop	2F, 2R	KR612214
Darthaven Marina D2	50.35083 -3.57167	11/06/2012	small colonies on mussel	Marina Pontoons	Dr J. Bishop	1F, 1R	KR612215
Darthaven Marina D3	50.35083 -3.57167	11/06/2012	small colonies on brown algae	Marina Pontoons	Dr J. Bishop	2F, 2R	KR612216
Gosport Marina G1	50.80667 -1.12056	18/06/2012	Small colonies (few cm)	Marina Pontoons	Dr J. Bishop	1F, 1R	KR612217
Gosport Marina G2	50.80667 -1.12056	18/06/2012	Small colonies (few cm)	Marina Pontoons	Dr J. Bishop	3F, 3R	KR612218
Gosport Marina G3	50.80667 -1.12056	18/06/2012	Small colonies (few cm)	Marina Pontoons	Dr J. Bishop	1F, 1R	KR612219
Holyhead	unknown	unknown			unknown	1F, 1R	KR612220
Kent A	unknown	2012	large colonies	Aquaculture site	Kent Wildlife Trust	1F, 1R	KR612221
Kent B	unknown	2012	large colonies	Aquaculture site	Kent Wildlife Trust	1F, 1R	KR612222

used glass beads (710–1180 µm) (Sigma-Aldrich, Gillingham, UK) while the other used a single steel bead (5 mm) (Qiagen Ltd., Crawley, UK). This resulted in a total of 28 samples for DNA extraction and PCR analysis.

DNA extraction: DNA extraction was carried out by adding 500 µl of 1% CTAB to each sample tube. For each sub-sample either glass beads (125 mg) or a single steel bead was added. All tubes were then vortexed three times, each time for 10 seconds, and then placed in a TissueLyser II (Qiagen Ltd.) for 2 × 3 min at 25 Hz. Homogenised samples were left for 30 min at room temperature to allow foam generated during the procedure to dissipate. The homogenates were briefly vortexed and the extracts were pipetted into clean 2 ml microcentrifuge tubes. DNA extraction was carried out using a QIAsymphony DNA Extraction kit and the QIAsymphony SP instrument (Qiagen Ltd.) according to the manufacturer's instructions. Extracted DNA concentration was measured using the Nanodrop 1000 (Thermo Scientific) for all 28 samples. Extracted DNA was stored at -80ºC.

PCR and Sequencing: A pair of tunicate specific PCR primers Tun_forward 5'-TCGACTAATCAT AAAGATATTA-3' and Tun_reverse2 5'-AAC TTGTATTTAAATTACGATC-3' (Stefaniak et al. 2009) were used to amplify an approximately 600 bp section of the COI mitochondrial gene. PCR amplification was carried out in a volume of 25 µl consisting of 1× NH_4 buffer (Bioline, London, UK), 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 µM forward and reverse primer, 0.5 U BioTaq enzyme (Bioline), 2 µl of extracted DNA, and molecular grade water (Sigma). Thermocycling was carried out using the following conditions: an initial denaturation step at 94ºC for 1 min followed by 60 cycles of 94ºC for 10 sec, 50ºC for 30 sec and 72ºC for 50 sec, and a final extension step at 72ºC for 10 min. The PCR products were visualized on 1.5% TAE agarose gel containing ethidium bromide, under UV illumination. The product was excised from the gels and purified using MinElute gel purification kit (Qiagen Ltd.) according to the manufacturer's instructions and eluted in 12 µl elution buffer. Concentrations of the purified PCR products were

estimated following agarose gel electrophoresis alongside mass markers (Low DNA Mass Ladder, Invitrogen Ltd., Paisley, UK). All fourteen sub-samples purified PCR products from the steel bead beating homogenisation method were analysed by direct sequencing. However, at a later date, to resolve the 5' end sequences for two sub-samples, the purified PCR products from the glass bead homogenisation method were analysed by direct sequencing.

Purified PCR product (60–120 ng) was used as template in sequencing reactions using the GenomeLab DTCS – Quick Start Kit (Beckman Coulter UK Ltd., High Wycombe, Buckinghamshire, UK), and using 4 µL of Quick Start Mix and 1.5 µL of GenomeLab Sequencing Reaction Buffer (Beckman Coulter Ltd.), according to the manufacturer's protocol. The same primers as used for PCR were used in sequencing reactions at a concentration of 0.5 µM. Sequence reactions were analysed on a Beckman Coulter CEQ 8800 DNA analysis system (Beckman Coulter Ltd.). In addition, to resolve ambiguous nucleotide bases, PCR products from four of the steel bead homogenised sub-samples and two of the glass bead homogenised sub-samples were later sent for sequencing to DNA Sequencing and Services, University of Dundee. Forward and reverse sequencing reactions were performed for each PCR product, and sequences were manually checked using Sequencher software (Intelligenetics/Gene Codes Corp., Ann Arbor, MI, USA).

Sequences generated were compared to sequences in the NCBI database using the BLAST tool (Zhang et al. 2000).

Results

All tissue samples yielded DNA, with tissue lysing using the steel bead resulting in a higher concentration of DNA (12.1–44.2 ng/µl) compared to using glass beads (8.4–16.5 ng/µl).

BLAST analyses of the sequence data showed that all the sequences corresponded with *D. vexillum* COI sequences in GenBank. Primer sites and/or poor quality sequence data at 5'-prime and 3'-prime ends of sequences were removed. The resulting sequences were 519–587 bases in length. COI haplotype sequences for *D. vexillum* were downloaded from the GenBank public database (COI haplotypes were those as described in Smith et al. 2012 and Stefaniak et al. 2012) and aligned with sequences generated during the current study using the Clustal multiple alignment function in Bioedit (Hall 1999). As a consequence of data loss following the trimming of poor quality 3' and 5' sequence, six of the samples from the current study were missing the first variable nucleotide position and one sample missed the second variable nucleotide position. There was 98.8–100% similarity between individual samples. An overview of the variable nucleotide positions found between COI haplotypes in this study, and compared with the most similar haplotypes from the GenBank public database is presented in Figure 1.

From the fourteen colonial ascidian sample sequences obtained in this study, the samples from Largs and Hunterston, one from Fairlie, three from Darthaven and one from Gosport Marina were identical to Haplotype 3 sequence. Two samples from Fairlie and one from Gosport were identical to Haplotype 5. One of the Gosport samples and the sample from Holyhead were identical to the Haplotype 2 sequence. The two samples from Kent were identical to the Haplotype 1 sequence. (Figure 2). All the samples fall within the *D. vexillum* Clade A, which are found globally (Stefaniak et al. 2012). Sequences were submitted to GenBank and given Accession numbers (Table 1).

Discussion

Of the fourteen *D. vexillum* samples identified during this study, six were identical to the Haplotype 3, which is the most frequent haplotype identified worldwide, including France, Ireland, The Netherlands, and Wales (Stefaniak et al. 2009, 2012). Haplotype 2 was also found in samples previously taken from Wales (Stefaniak et al. 2012), which has been confirmed in this study from Holyhead. Interestingly, the three Gosport samples each represented a different haplotype. However, based on the haplotype data presented in Stefaniak et al. (2012), it is not unusual for sites to have up to three haplotypes present; therefore, a single transfer may be responsible for all. Concerning the three samples from Fairlie, one was identical to the widespread Haplotype 3 and two matched Haplotype 5, found previously in the USA, France, The Netherlands, Japan, and New Zealand. To investigate possible routes of introduction to the UK, or between sites within the UK, it would be important to take more samples from each of these sites. These could then be analysed using the DNA COI barcoding method to look at haplotype diversity and relative frequency, similar to work done by Stefaniak et al. (2012).

Nucleotide Position	10	22	85	214	247	385	421	433	536	556
Haplotype 3 JF738058	C	G	A	C	C	T	T	A	C	T
Fairlie Pier 11	?	.	.	.	.	.	.	.	.	.
Hunterston Terminal 23	.	.	.	.	.	.	.	.	.	.
Darthaven Marina D1	.	.	.	.	.	.	.	.	.	.
Darthaven Marina D2	.	.	.	.	.	.	.	.	.	.
Darthaven Marina D3	.	.	.	.	.	.	.	.	.	.
Gosport Marina G1	.	.	.	.	.	.	.	.	.	.
Largs	?	.	.	.	.	.	.	.	.	.
Haplotype 6 JF738060	T	A	.	.	.	.	.	T	.	.
Haplotype 5 JF738059	T	A	.	.	.	.	.	T	.	C
Fairlie Pier 15	?	A	.	.	.	.	.	T	.	C
Fairlie Pier 21	?	A	.	.	.	.	.	T	.	C
Gosport Marina G2	T	A	.	.	.	.	.	T	.	C
Haplotype 2 JF738057	T	A	T	T	T	C	A	G	.	T
Gosport Marina G3	?	?	T	T	T	C	A	G	.	.
Holyhead	?	A	T	T	T	C	A	G	.	.
Haplotype 1 JQ663509	T	A	T	T	T	C	A	G	T	.
Kent A	T	A	T	T	T	C	A	G	T	.
Kent B	T	A	T	T	T	C	A	G	T	.

Figure 1. Aligned sequences showing variable sites of *D. vexillum* samples from the UK and related Haplotypes. Dots represent where bases were the same as Haplotype 3 (JF738058), "?" indicates no sequence was obtained at 5' end.

Despite their different morphology, all the colonial ascidians samples tested from Kent were molecularly identified as *D. vexillum*. However, the Kent samples did show the most nucleotide differences, being identical to Haplotype 1, which had so far only been identified in the USA and Japan (Stefaniak et al. 2012). Whereas the majority of *D. vexillum* samples from around the UK were found to colonise artificial structures permanently covered by seawater, in Kent some colonies were found covering sandstone boulders in the lower and mid shore regions that are exposed during low tide. A range of colour forms were also present in the Kent colonies, from pale yellow to a bright orange form found at an oyster hatchery where it covered artificial and natural substrates. The Kent colonies also showed varying surface features, with some showing no sign of the common cloacal channels that can be used as an identifying feature (Hitchin 2011). Minchin and Sides (2006) also describe various growth forms and colours of what was identified at the time as *Didemnum* sp. colonies, from the east coast of Ireland. Observations of *Didemnum* sp. A colonies from North America described in Bullard et al. (2007) exhibited a wide range of morphology, with colour ranging from pinkish to tan to pale orange, and a range of forms from long and rope-like to encrusting mats, and covering a range of artificial and natural substrates. As with the Kent

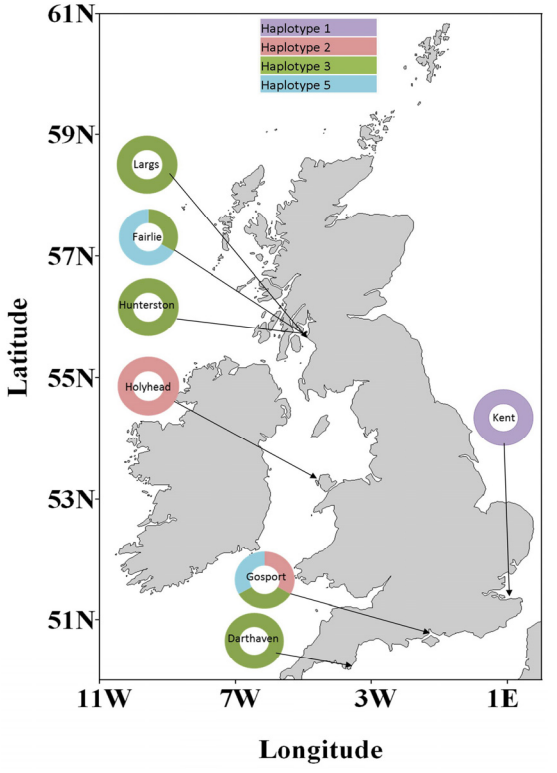

Figure 2. Location and haplotype frequency of samples of *Didemnum vexillum* analysed in this study from around the UK. The charts represent the number of haplotypes present at each site. The legend at the top of the map gives the colour code for each of the haplotypes found.

colonies, some of the rock-encrusting mats were also found growing in the lower intertidal zone, which is exposed at low tide. Stefaniak et al. (2009) indicated from molecular data that, despite the differences in external morphology, *Didemnum* sp. A was a single species, and that it should be named *Didemnum vexillum*. It has been suggested that some of these morphological differences could be due to environmental adaptations with growth form related to habitat type (Bullard et al. 2007), and it could be that morphological variation is common throughout populations of *D. vexillum*.

Within the genus *Didemnum*, identification to species level is difficult because their external morphological features can be very similar. It is only by looking at their microscopic internal structures that differences can become apparent. However, this requires extensive expertise, and it is very time consuming. The molecular method used in this study has been widely used to confirm the non-native ascidian species, *D. vexillum*. This provides biologists and ecologists with a tool to confirm the identity of suspect samples more rapidly, while providing information on the diversity of the species from haplotype identification, which could help to localise the possible origin and routes of spread of the invasive species. As previously shown by Smith et al. (2012), the work carried out in this study demonstrates that DNA can be extracted without having to dissect any particular parts of the tunicate tissue, which is an onerous task, and that the extracted DNA is suitable to identify the sample to species level with high confidence. As no specific tissue dissection is required, this method can be carried out by individuals with no expertise in the morphological identification of *Didemnum*. This can speed up the process of identifying any suspicious samples collected from around the coastline.

It is acknowledged that eradication of an invasive species is time consuming, costly, and rarely completely effective (Eno et al. 1997; Manchester and Bullock 2000). The only eradication attempt of *D. vexillum* in the UK has been carried out in Holyhead Harbour. Following a feasibility study (Kleeman 2009), eradication using a combination of plastic wrappings and accelerant (acetic acid or chlorine) was tested. By carrying out the second attempt as one single large treatment as opposed to a succession of smaller treatments, the efforts have so far been partially successful. As of September 2013, small colonies were detected on the breakwaters and additional treatment was planned. However, in the longer term, containment appears to be the way forward for management of the species (Sambrook et al. 2014). No further spread has been observed from the other locations throughout the UK. Shortly after the detection in Largs, a cost-benefit analysis was commissioned by the Scottish Government to investigate various methods of response (Nimmo et al. 2011). The report provided three recommendations: do nothing, attempt eradication, and containment and management. The third option, containment and management, was considered to be the most cost effective response. This highlights the need for continued biosecurity, good practice, and awareness-raising among industry, stakeholders, marina and harbour operators, and the general public. A Biosecurity Plan for the Firth of Clyde has since been developed that provides guidance on reducing the risk of introducing invasive non-native species and how to manage existing species in the most appropriate ways. While eradication of invasive non-native species may be possible in certain locations, it is not always feasible, and the most effective way forward for management is a combination of regular monitoring, rapid identification techniques, and good biosecurity practices.

Molecular methods are ideally suited to addressing important management issues related to invasive species such as clarifying taxonomic identification and pathways of introduction (Hess et al. 2009). As the requirement for non-native species surveys and monitoring increases through regulations such as the Marine Strategy Framework Directive and the newly adopted European Union Regulations on Invasive Alien Species (Regulation 1143/2014), there will likely be higher demand for quick, reliable tools to aid in the identification of suspect species so that appropriate responses can be made, and the potential risks posed by invasive species can be reduced.

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