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Halioticida noduliformans infection in eggs of lobster (*Homarus gammarus*) reveals its generalist parasitic strategy in marine invertebrates

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ABSTRACT

A parasite exhibiting Oomycete-like morphology and pathogenesis was isolated from discoloured eggs of the European lobster (*Homarus gammarus)* and later found in gill tissues of adults. Group-specific Oomycete primers were designed to amplify the 18S ribosomal small subunit (SSU), which initially identified the organism as the same as the '*Haliphthoros*' sp. NJM 0034 strain (AB178865.1) previously isolated from abalone (imported from South Australia to Japan). However, in accordance with other published SSU-based phylogenies, the NJM 0034 isolate did not group with other known *Haliphthoros* species in our Maximum Likelihood and Bayesian phylogenies. Instead, the strain formed an orphan lineage, diverging before the separation of the Saprolegniales and Pythiales. Based upon 28S large subunit (LSU) phylogeny, our own isolate and the previously unidentified 0034 strain are both identical to the abalone pathogen *Halioticida noduliformans.* The genus shares morphological similarities with *Haliphthoros* and *Halocrusticida* and forms a clade with these in LSU phylogenies. Here, we confirm the first recorded occurrence of *H. noduliformans* in European lobsters and associate its presence with pathology of the egg mass, likely leading to reduced fecundity.

1. Introduction

The Oomycetes are parasitic or saprotrophic eukaryotes that group within the Stramenopile clade (Phillips et al., 2008). They include numerous taxa which infect and cause disease in aquatic invertebrates (Noga, 1990). Several Oomycete genera are known pathogens of lobsters and Crustacea in general. *Lagenidium*, has been identified as a mortality driver in larval American lobster (*Homarus americanus*) (Nilson et al., 1976) and other members of the genus have been detected in several commercially significant shrimp and crab species (Armstrong et al., 1976; Bian et al., 1979; Bland and Amerson, 1973; Lightner and Fontaine, 1973). Species belonging to the genera *Saprolegnia* and *Aphanomyces* are also notable pathogens of freshwater crayfish (Alderman et al., 1984; Diéguez-Uribeondo et al., 1994); often associated with catastrophic mortalities in natural stocks in Europe (Holdich et al., 2009).

The genus *Haliphthoros* comprises three species; *H. milfordensis, H. philippinensis and H. sabahensis.* These typically infect eggs and early life stage marine invertebrates. Infection has been described in spiny rock lobster (*Jasus edwardsii*) (Diggles, 2001), blue crab (*Portunus pelagicus*) (Nakamura and Hatai, 1995a, 1995b), mud crab (*Scylla serrata, S. tranquebarica*) (Leano, 2002; Lee et al., 2017), American lobster (*Homarus americanus*) (Fisher et al., 1975), white shrimp (*Penaeus setiferus*) (Tharp and Bland, 1977), black tiger prawn larvae (*Penaeus monodon*) (Chukanhom et al., 2003), and abalone (*Haliotis* spp.) (Hatai, 1982; Sekimoto et al., 2007). Experimental challenges have also demonstrated the susceptibility of pea crab eggs (*Pinnotheres* sp.) (Ganaros, 1957; Vishniac, 1958) the European lobster (*Homarus gammarus*) (Fisher et al., 1975), ova of the blue crab (*Callinectes sapidus*) (Tharp and Bland, 1977), adult pink shrimp (*Penaeus duoraram*) (Tharp and Bland, 1977) and, the ova and larvae of brine shrimp (*Artemia salina*) (Tharp and Bland, 1977). Furthermore, *Haliphthoros* has also been isolated from the surfaces of several algae which may give an indication of its lifecycle outside of an invertebrate host infection (Fuller et al., 1964). With the exception of *H. sabahensis* in mud crab (Lee et al., 2017), all of these descriptions were solely based on the morphological characteristics of cultures isolated from the site of infection. The infection occurring in black tiger prawn (Chukanhom et al., 2003), however, was later

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sequenced and analysed phylogenetically (Sekimoto et al., 2007). It is therefore possible that, based on morphological descriptions alone, several of these infections could have been misdiagnosed as '*Haliphthoros*' and more accurate diagnostics are required (Stentiford et al., 2014).

Halocrusticida (*syn. Halodaphnea*) is a closely related genus isolated from marine Crustacea, erected to contain 6 taxa belonging to the genus *Atkinsiella* (Nakamura and Hatai, 1995a). All six infect invertebrates with *A. hamanaensis, A. okinawaensis* and *A. panulirata* originally isolated from decapods (*Scylla serrata, Portunus pelagicus* and *Panulirus japonica*, respectively) (Bian and Egusa, 1980; Kitancharoen and Hatai, 1995; Nakamura and Hatai, 1995b). *Atkinsiella dubia*, a crab parasite, was the only species not to be reclassified as a member of the *Halocrusticida* (Atkins, 1954; Nakamura and Hatai, 1995a; Sparrow, 1973).

Sekimoto et al. (2007) isolated an unidentified Oomycete (NJM 0034) from white nodules in the mantle of an abalone (*Haliotis rubra*) imported to Japan from southern Australia. The pathogen most closely resembled a species of *Haliphthoros* based on characteristic morphological features such as hyphal fragmentation by cytoplasmic restriction. However, zoosporogenesis, which has traditionally served as the principle method of species identification to discern between *Haliphthoros* and its close relatives, was not observed. Upon discovery of the unidentified NJM 0034 isolate (herein referred to as 0034), Sekimoto et al. (2007) analysed three different gene loci; the ribosomal small subunit (SSU), the ribosomal large subunit (LSU) and the cytochrome *c* oxidase subunit II (cox2). In the SSU and cox2 phylogenies, 0034 branched just prior to the divergence of Peronosporales and Saprolegniales, separately from the other members of *Haliphthoros*. In the LSU phylogeny, 0034 formed a clade with '*Haliphthoros* sp. *NJM 0131*', originally isolated from black tiger prawn (Chukanhom et al., 2003; Sekimoto et al., 2007). Muraosa et al. (2009) later described a second abalone parasite sharing morphological characteristics with *Haliphthoros* and erected a new genus to describe the species as *Halioticida noduliformans*. *H. noduliformans* was later isolated in wild Japanese mantis shrimp (*Oratosquilla oratoria*) from Japan and cultured abalone (*Haliotis midae*) from South Africa (Atami et al., 2009; Macey et al., 2011) and found to share 100% sequence identity to the previously sequenced 0034 in the LSU gene region (Macey et al., 2011).

As part of an ongoing programme considering novel and emerging pathogens of the European lobster (*Homarus gammarus*) in the United Kingdom, we carried out a histopathology and molecular diagnostic survey of lobsters displaying cloudy/discoloured eggs. We designed and applied new Oomycete-specific SSU PCR primers to reveal the presence of 0034 associated with the egg pathology, and generated LSU sequences from the lobster pathogen to determine whether it was the same as that in *Haliotis rubra* in Japan (Sekimoto et al., 2007). We also designed and tested 0034-specific SSU primer sets for use as molecular diagnostic tools. Our SSU analysis confirmed that 0034 cannot belong to the genus *Haliphthoros* and has no directly related SSU sequence types.

2. Material and methods

2.1. Sample collection

2.1.1. Animal sampling

From July 2015 to October 2016, 323 egg bearing female lobsters were obtained from various fishermen and wholesale facilities around Cornwall and the Isles of Scilly, United Kingdom, originally recruited to take part in a larval rearing program at the National Lobster Hatchery, Padstow (UK). The landing of egg bearing females was carried out under authorisation granted by the Cornwall Inshore Fisheries and Conservation Authority (IFCA). During this period, a total of 21 animals developed abnormal egg colouration (Fig. 1) (6.5% of the total number of animals that entered the hatchery). Eighteen of the suspect 21 animals were maintained in wholesaler tanks for up to 7 days prior to transport to the hatchery. The remaining three were chilled and immediately transported. Animals that developed pathological signs of infection (n = 21) had spent between 24 and 106 days within the hatchery tank system. In order to understand the nature of the disease, animals were anaesthetised under ice for up to one hour, depending size. Heart, hepatopancreas (HP), gonad, gut, muscle, gill and eggs were removed using sterile dissecting equipment and fixed for DNA extraction, histopathology, and transmission electron microscopy. Six eggs from a subset of animals were cut in half so that histological and molecular analysis could be applied to the same individual egg. From 4th to 9th July 2016, an additional 17 egg bearing lobsters were collected on landing, from wholesalers in the south of Cornwall and processed in the same manner as above. These animals did not enter any holding tanks and are herein referred to as 'wild'. Wild lobsters were chilled on landing and sampled that same day.

Fig. 1. Gross pathology of infected eggs of *Homarus gammarus*. Pale, discoloured eggs observed in brood clutch of berried hen. Black eggs indicate healthy, uninfected eggs.

2.1.2. Environmental sampling

Littoral marine, brackish water and sediment samples were collected from Newton's Cove and the Fleet Lagoon (Weymouth, SW England) by Hartikainen et al. (2014), together with agricultural soil samples (Gosling et al., 2014), and freshwater samples from the River Avon (Bickton) and California Lake (Berkshire) (Hartikainen et al., 2016).

2.2. Histology

Lobster egg samples were fixed in Davidson's Seawater Fixative for 24–48 h before transferring tissues to 70% industrial denatured alcohol (IDA). Cassettes were processed using a Leica Peloris Rapid Tissue Processor and subsequently embedded in paraffin wax. Sections were cut using a rotary microtome set at 3 µm thickness, adhered to glass slides and stained using a standard haematoxylin and eosin protocol.