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High nervous necrosis virus (NNV) diversity in wild wrasse (Labridae) in Norway and Sweden

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ABSTRACT: Wild goldsinny wrasse *Ctenolabrus rupestris*, corkwing wrasse *Symphodus melops* and ballan wrasse *Labrus bergylta* were collected at 8 sampling sites in Sweden and Norway during summer 2014. Brain tissue from 466 wrasses were analyzed for nervous necrosis virus (NNV) infections by real-time RT-PCR, and positive samples were subjected to sequencing and phylogenetic analysis of partial segments of the RNA2 and RNA1 genes. This study shows that NNV is present in wild ballan, corkwing and goldsinny wrasse along the coastline of Sweden and Norway. The overall prevalence in the sampled labrids was 6.7%. Prevalence was 6.4% in goldsinny, 6.3% in corkwing and 18% in ballan wrasse. The wrasse RNA2 NNV sequences revealed high genetic variability and were divided into 3 clusters within the cold water barfin flounder NNV (BFNNV) and warm water cluster red-spotted grouper NNV (RGNNV) genogroups. Within the BFNNV genogroup, wrasse NNVs clustered in 2 sub-genogroups, with grey mullet NNV (GMNNV) and with Atlantic halibut NNV (AHNNV). These groups were previously dominated by virus originating from Atlantic cod *Gadus morhua* and Atlantic halibut *Hippoglossus hippoglossus* from the northeast Atlantic. The presence of NNV in wild wrasse and the surprising high genetic variability observed in this study should be considered before moving wild-caught wrasse between geographically distant sites. The results show that use of wild-caught wrasse as brood fish in wrasse farming represents a risk of introducing NNV into aquaculture.

KEY WORDS: Betanodavirus · Wild marine fish · NNV · Genotypes · Wrasse

INTRODUCTION

Salmon louse *Lepeophtheirus salmonis* is the biggest threat towards environmentally sustainable salmon aquaculture. In Norway, wrasses are extensively used as cleaner fish to remove salmon lice from farmed Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*. The main species used are ballan wrasse *Labrus bergylta*, goldsinny wrasse *Ctenolabrus rupestris* and corkwing wrasse *Symphodus melops*. Wrasses are mixed with smolts at the start of the marine production cycles.

The demand for wrasse has increased dramatically due to the development of resistance against antiparasitic drugs. Fish are mainly supplied from wild populations, and in 2016, more than 22 million wrasses were caught and used as cleaner fish in Norway. Additionally, there was an import of at least 1 million wild-caught wrasses from Sweden, and a supply of around 1 million farmed ballan wrasse (Directorate of Fisheries 2017).

Wrasses live in temperate waters, and in Norway, there is a mismatch between the abundance of wrasses in the south and the extensive fish farming in

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the north. Millions of wrasses are therefore caught in the south and transported to fish farming sites further north. The longest transport distance is from the Swedish west coast near Gothenburg to the southern part of Nordland County in Norway (see Fig. 1). To supply fish farms with wrasses independent of the fishing season, and in sufficient numbers, commercial farming of ballan wrasse has been established. Goldsinny wrasse is a relatively robust species and has also been considered for commercial farming.

Use of wrasse to delouse salmonids has a long history in Norway (e.g. Bjordal 1988, 1990, Kvenseth & Kvenseth 1997). But there has been limited focus on the health of the wrasse species, despite an almost total loss of cleaner fish throughout the salmonid life cycle (see review in Mortensen & Karlsbakk 2012). The causes of mortalities are, however, not fully understood, and we believe that mortality events are influenced by sub-optimal conditions and the poor physiological status of the wrasses — particularly during the spawning period (Harkestad 2011, Skiftesvik et al. 2014) and by diseases. Several studies have revealed pathogenic and opportunistic bacteria that are involved in disease outbreaks and accumulated mortalities of wrasses (Jensen et al. 2003, Bergh & Samuelsen 2007, Harkestad 2011, Nilsen et al. 2014). There is, however, limited information on viral diseases of wrasses which may be involved in the mortality events, both in farmed and in wild-caught wrasse. Some fish pathogenic viruses are well known to have serious consequences for intensive farming of marine fish, and there is a report on viral haemorrhagic septicaemia (VHS) associated with mortality in several wrasse species (Munro et al. 2015). Nervous necrosis virus (NNV), belonging to the genus *Betanodavirus* (Family *Nodaviridae*) is known to infect a high number of marine fish species, including several wild fish species found in Norwegian waters (Nylund et al. 2008). In Norway, the disease viral nervous necrosis (VNN) has caused severe problems in the farming of cod (Patel et al. 2007) and halibut (Grotmol 1997). Similar problems with NNV in fish farming has been shown in other regions of the world. Hence, using wild-caught wrasses in the establishment of an intensive production may incur the risk of introducing NNV. Furthermore, there is a risk of introducing new virus genotypes into the marine environment when wild-caught fish are transported over long distances.

The aim of this study was to investigate the presence of NNV in wild populations of wrasse along the west coast of Sweden and along the Norwegian coast as far north as possible. The sampling was a part of a larger investigation of wild wrasse species along the Swedish and Norwegian coastline.

MATERIALS AND METHODS

Locations and sampling

Wrasses were sampled at 8 sites along the Swedish west coast and the Norwegian coast, from Gulmarfjorden (Lysekil) in Sweden in the south to the Lofoten islands (Tysfjord) in the north (Fig. 1, Table 1), during summer 2014. Fish were caught in traps consisting of pots baited with shrimp or in fyke nets. Traps were soaked for approximately 24 h, wrasse were collected, brought live to the laboratory and killed by a blow to the head. All sampling was performed according to the Norwegian Law on Animal Welfare, and all personnel handling fish held a FELASA-C certificate. Wrasse from Sites D and G were killed, immediately frozen, sent by over-night mail to the laboratory and sampled while thawing.

Species was determined for each fish collected, and the brain was removed by autopsy and kept in

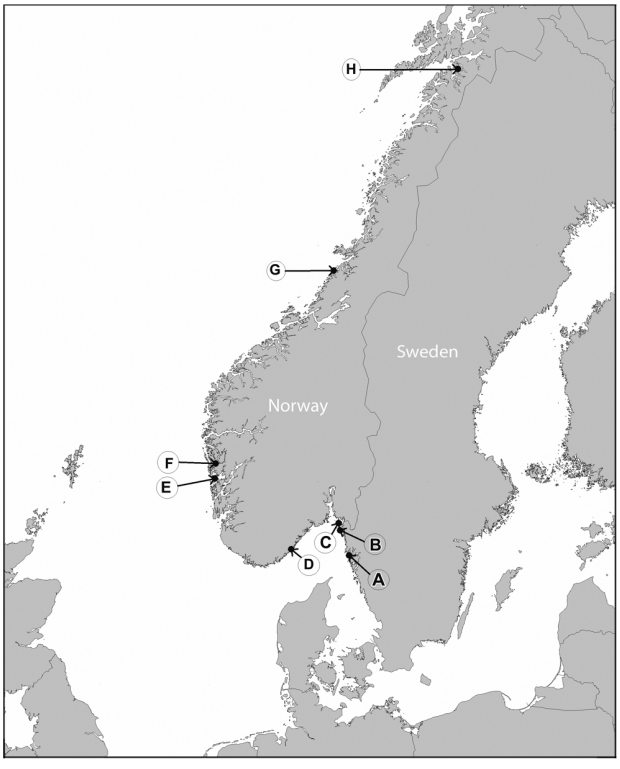

Fig. 1. Geographic distribution of sampling sites in this study. Sites A and B are in Sweden; Sites C to H are in Norway. GPS position of each sampling site is given in Table S1 in the Supplement at www.int-res.com/articles/suppl/d126p043_supp.pdf

Table 1. Sampling sites and number of wrasses of the different species included in the study, and the number of real-time RT-PCR positive fish (brains). The genogroups (clades), include all sequences obtained, consisting of both RNA2 and RNA1 segments. Geographical site: location of sampling; N: number of fish included for investigation in each species; NNV positives: number of fish which tested positive. Sites A and B are in Sweden; Sites C to H are in Norway as far north as Tysfjord close to the Lofoten islands. AHNNV: Atlantic halibut nervous necrosis virus (NNV); GMNNV: *Gadus morhua* (Atlantic cod) NNV; RGNNV: red grouper NNV; na: not available (not possible to determine genotype)

Site	Geographical location	Species	N	NNV positives	Genogroup			
					AHNNV	GMNNV	RGNNV	na
A	Lysekil	Goldsinny	55	5	5			
		Corkwing	45	1				1
		Ballan	11	2	1		1	
B	Koster	Goldsinny	53	4	2			2
		Corkwing	23	3	2			1
C	Hvaler	Goldsinny	54	2	2			
		Corkwing	6	2	1			1
D	Flødevigen	Goldsinny	58	3				3
E	Austevoll	Corkwing	15	0				
F	Bergen	Goldsinny	15	0				
		Corkwing	23	1			1	
G	Flatanger	Goldsinny	29	3		2		1
H	Tysfjord	Goldsinny	79	5		1		4

RNAlater® solution (Ambion) in a 1:10 ratio of tissue to fixative. Samples were stored at −20°C until further processing and analysis. The total sample size was 506 fish. The location and number of fish at each site are given in Table S1.

RNA extraction

RNA from brain samples stored in RNAlater was extracted using the RNAeasy kit (QiaGen) according to the manufacturer's instructions. In brief, samples were homogenized in kit lysis buffer for 30 s at 6000 rpm in a MagNA Lyser (Roche) instrument. Lysate was centrifuged at maximum speed for 60 s (Heraeus Fresco 21; Fisher Scientific) at 4°C and further processing performed as instructed in the kit. RNA was eluded in nuclease-free water in a total volume of 30 µl and stored at −80°C until further analysis.

Real-time RT-PCR

Screening for NNV in brain samples was performed by real-time RT-PCR, using an assay published by Korsnes et al. (2005). All samples were also analyzed for expression of the wrasse elongation factor to assess the quality of the RNA extracted. The primers and probe in use were: forward 5′-CCC CTC CAG GAT GTC TAC AAA and reverse 5′-AAC ACG RCC SAC GGG KAC W and TaqMan probe 5′6FAM-ATY GGY GGT ATT GGA AC-MGBNFQ. All samples with no real-time RT-PCR amplification of the elongation factor were removed from the study. The real-time RT-PCR reactions were performed using 2 µl template RNA in a final reaction volume of 12 µl, using the TaqMan® RNA-to-C_T™ master mix (Applied Biosystems; Fisher Scientific). Both assays utilized TaqMan probes, with reactions concentrations of primers of 900 nM and probes with 200 nM (NNV) and 250 nM (elongation factor). All real-time RT-PCR reactions were performed on a LightCycler 96 instrument (Roche). The reactions conditions consisted of a hold of 900 s at 48°C, followed by a hold at 95°C for 600 s and then 45 cycles at 60°C for 60 s and 95°C for 15 s (with a ramping time of 4.4 s).

Genotyping and phylogenetic analysis

Samples identified as positive for NNVs were carried forward for genotyping. RNA was transcribed into cDNA by using SuperScript®VILO™ (Invitrogen) according to the manufacturer's instructions. PCR amplification of a partial segment of RNA1 and RNA2 gene segments was done using primers described by Korsnes et al. (2005) and Bigarré et al. (2010). PCR amplification was performed with HotStartTaq® DNA polymerase (QiaGen) according to the manufacturer's instructions. Gel electrophoresis in 1% Seakem LE agarose (BioNordica) with GelRed

10.000X in water stain (VWR) was used to test the success of the amplification and served as an additional criterion for selecting samples for sequencing. PCR products selected for sequencing were purified with illustra™ ExoProStar™ kit (VWR) to remove excess nucleotides and unincorporated primers. Selected samples were sequenced bidirectionally by cycle sequencing technology according to the BigDye version 3.1 protocol and Automated Sanger DNA Sequencing using the capillary-based Applied Biosystem 3730XL Analyzer at the Sequencing Facility, MBI at University of Bergen. Resulting RNA1 and RNA2 sequences were assembled and aligned in Vector NTI software (ThermoFisher Scientific). Phylogenetic analysis of RNA2 (610 nt) were performed using MEGA 5.10 to produce a tree with maximum likelihood criteria. RNA2 sequences from different genotypes and genogroups of NNV were retrieved from GenBank and included in the phylogenetic analysis. A total of 50 RNA2 sequences were included, 15 of which were obtained from wrasse in the present study.

RESULTS

Prevalence of NNV

A total of 40 wrasses were removed from the study due to failure of amplification of the elongation factor gene used as quality control of RNA, reducing the number of fish from 506 to 466. NNV was detected in fish at 7 out of 8 sampling sites investigated, and in total, 31 individual fish tested positive for NNV in brain tissue. Overall prevalence of the virus was 6.7% (n = 466). Prevalence at the species level was 6.4% in goldsinny (n = 343), 6.3% in corkwing (n = 112) and 18% in ballan wrasse (n = 11). Prevalence at the various sites showed some variation, where Site A was 7.2% (n = 111), Site B was 9.2% (n = 76), Site C was 6.7% (n = 60), Site D was 5.2% (n = 58), Site F was 2.6% (n = 38), Site G was 10.3% (n = 29) and Site H was 6.3% (n = 79). At Site E no fish were positive for NNV (n = 15) (Table 1).

Genotypes and phylogenetic analysis

The RNA2 sequences grouping with Atlantic halibut NNV (AHNNV) showed the highest nucleotide (nt) and corresponding deduced amino acid (aa) identity to sequences from Atlantic halibut, 98.7 to 99.3% and 98.0 to 98.5%, respectively (615 bp and 205 aa compared). The RNA2 sequences grouping in grey mullet NNV (GMNNV) show the highest nt and aa identity to sequences from northeast Atlantic cod, 98.7 to 99.8% and 99.0 to 99.5%, respectively. The 2 sequences grouping with red-spotted grouper NNV (RGNNV) showed ≤96.8% nt and ≤97.6% deduced aa sequence identity with other genogroup members. The highest nt identity was seen with a RGNNV isolate from cultured seabass *Dicentrarchus labrax* from the Mediterranean Sea (Tunisia; HE796793) and cultured groupers (Serranidae) from Indonesia and Malaysia (e.g. HQ859945).

RNA1 was sequenced from 14 samples (GenBank accession numbers are given in Table S2 in the Supplement at www.int-res.com/articles/suppl/d126p043_supp.pdf), representing 11 from which RNA2 sequences had been obtained, and 3 additional. Sequence analyses identified these 3 as closest to AHNNV. The genogroups determined on the basis of RNA2 and RNA1 agreed (9 AHNNV, 2 GMNNV). The 12 wrasse NNV RNA1 with affinity to the AHNNV showed highest identity to sequences from halibut (98.9 to 99.6% nt and 98.3 to 99.6% aa identity; 702 bp and 234 aa compared). The 2 RNA1 sequences with affinity to GMNNV showed 99.3 to 100.0% nt and 99.6 to 100.0% aa identity to sequences from northeast Atlantic cod from Norway. In total, 18 wrasse NNV infections were genotyped (Tables 1 & S2). Assembled sequences of RNA1 (12) and RNA2 (15) from this study were submitted to GenBank.

The phylogenetic analysis included NNV RNA2 sequences from 15 fish (see Table S2 for GenBank accession numbers), originating from all sites except Site E. The phylogenetic tree is presented in Fig. 2, based on a partial segment (610 nt) of RNA2. The wrasse NNV sequences represented 3 clusters: 2 in sub-groups in barfin flounder NNV (BFNNV) and 1 as a sub-group in RGNNV. In BFNNV, 3 NNV sequences from Sites G and H clustered in the GMNNV sub-group, which is dominated by NNV from northeast Atlantic cod *Gadus morhua*. Ten wrasse NNV sequences clustered with the AHNNV sub-group of BFNNV. The AHNNV sub-group was dominated by NNV from Atlantic halibut *Hippoglossus hippoglossus*. The distribution of genotypes according to site and host species is shown in Table 1.

DISCUSSION

We analyzed wild wrasse species collected at 8 different sites along the coast of Sweden and Norway for NNV infections. The virus was detected in brain

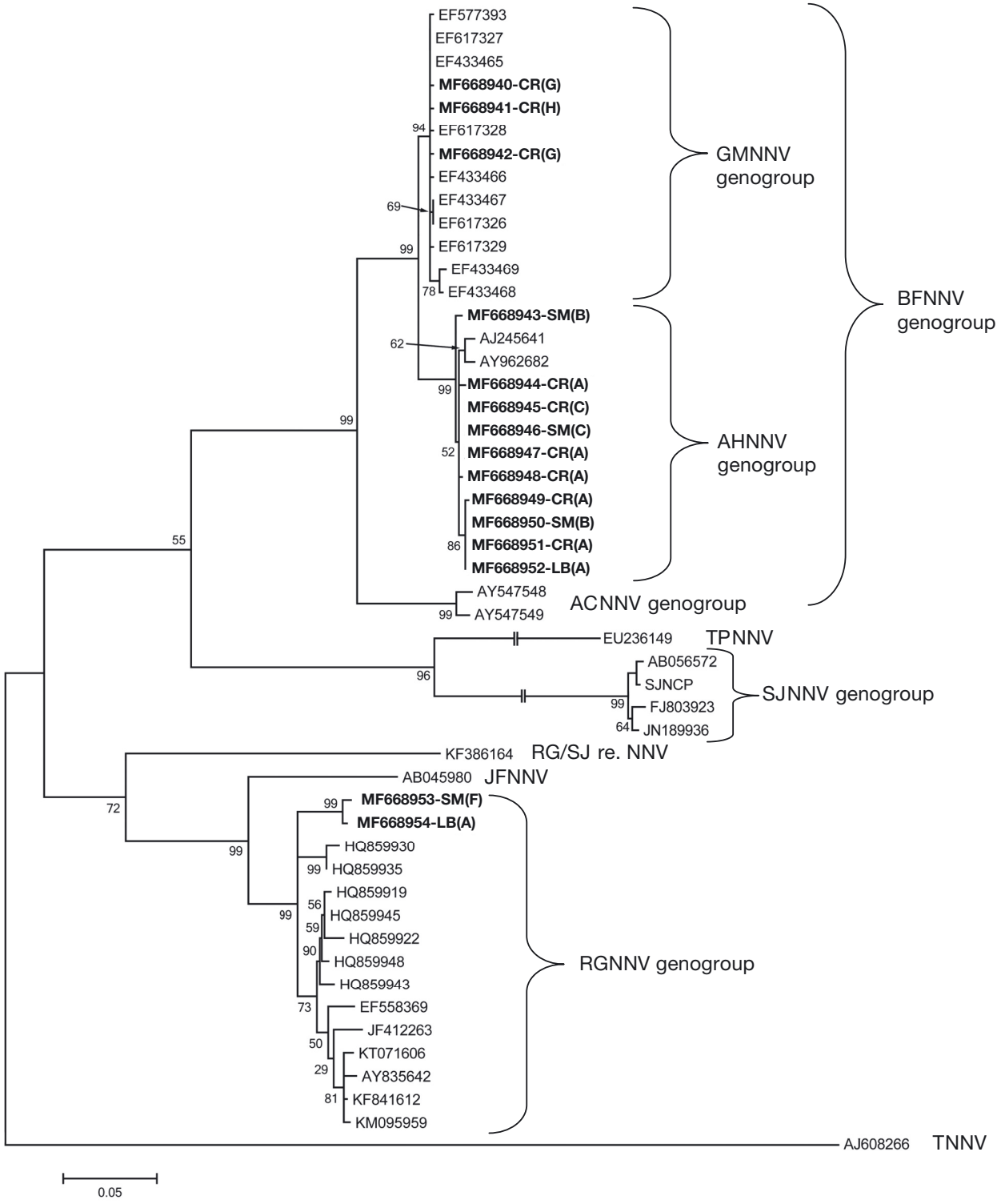

Fig. 2. Phylogenetic distribution of nervous necrosis virus (RNA2 sequences) from Scandinavian labrids. Novel sequences indicated in **bold**, with accession number followed by a host code (CR: goldsinny; SM: corkwing; LB: ballan wrasse) and a letter in brackets referring to the different geographic locations (A–F; Table 1). MEGA 5.10 maximum likelihood tree, model K2 I+G, based on 50 sequences (alignment 610 nt). Numbers at nodes represent bootstrap-support values based on 1000 replications. The branches to the tiger puffer (TPNNV) and the striped jack nervous necrosis virus (SJNNV) genogroups are shortened (50%). Rooted with turbot nervous necrosis virus (TNNV). Major genogroups recognized in previous studies indicated. Scale bar: substitutions per site

tissue of wrasses at 7 of these sites and in all the 3 wrasse species examined. To our knowledge this is the first study to report NNV in wild wrasse in Scandinavian waters. The results also showed that there are no geographic differences in the overall distribution of the virus, as NNV was detected at both the northern- and southernmost sampling sites. The prevalence of NNV was consistent within the various geographic populations investigated, ranging between 2.6 and 10.3%. No clinical signs of disease were observed in the fish sampled. There was variation in the viral load (data not shown) among the fish, and failures to obtain sequences to phylogenetic analysis in 16 samples were linked to low viral load (observed as high cycle threshold [C_T]-values in real-time RT-PCR analysis). The real-time RT-PCR analysis suggests a carrier state of NNV. Detection of NNV in wild wrasse is consistent with other reports of NNV in wild cold-water marine fish species, including Atlantic cod *Gadus morhua*, saithe *Pollachius virens*, pollack *Pollachius pollachius*, plaice *Pleuronectes platessa* and mackerel *Scomber scombrus* (Nylund et al. 2008).

The phylogenetic analysis included partial RNA2 sequences from 15 of the 31 NNV-positive fish. Most of the sampling sites were represented, except Sites D and E (see Fig. 1), and all 3 wrasse species. Although the number of wrasse NNV sequences is low, some interesting results arise.

Firstly, the phylogeny revealed that NNV sequences from wrasse clustered in 2 of the 4 major genogroups suggested by Nishizawa et al. (1997): the cold-water host fish species genogrup BFNNV, and — more surprisingly — the warm-water RGNNV. Two sequences, 1 from Site A from ballan wrasse and 1 at Site F from corkwing wrasse, belong to RGNNV. This might demonstrate a wide distribution of various genotypes of NNV within wrasse species. The 2 RGNNV sequences form a unique sub-genogroup within the phylogenetic tree generated (Fig. 2).

Second, in BFNNV, sequences from wrasse clustered in 2 different sub-genogroups which were assigned to Atlantic cod NNVs (GMNNV) and Atlantic halibut (AHNNV). Interestingly, the 3 sequences originating from the 2 northernmost sites (Sites G and H) all cluster in GMNNV, while sequences from the southernmost sites (Sites A, B, C and E) cluster in AHNNV. Although the GMNNV wrasse sequences are few, and are all from goldsinny, they may suggest a geographic and host species difference between the 15 NNV sequences analyzed. Actually, the 9 and 3 genotyped infections from the southernmost and northernmost goldsinny samples all represent AHNNV and GMNNV, respectively, a significant departure from random (Fisher's exact test, $p < 0.01$). The existence of host species-specific sub-genogroups within BFNNV has been suggested by Nylund et al. (2008), but in this case NNVs from goldsinny showed high genetic similarity to virus originating from Atlantic cod and did not form a distinct sub-genogroup of their own. Therefore, goldsinny may prove to be susceptible to NNV from cod. Thirteen of the infections showed similarity to AHNNV, mostly originating from halibut in Norway with VNN. However, Thiéry et al. (2004) found an infection with a AHNNV-like genotype (AJ698094) in seabass in France, a perciform fish, showing that this virus group is not host-specific. The present findings of related virus variants in wild wrasse seems to substantiate this, although none of the virus sequences obtained represented virus identical to those characterized from halibut.

Import or long-distance transfers of live fish always represents a risk of introducing new pathogens, alien species and as well as introgression. At present, high numbers of wild-caught wrasses with unknown health and carrier status are repeatedly being moved from the Swedish west coast and southern Norway to aquaculture areas further north. On delivery, the transport water is discarded without treatment, and fish are directly transferred into the net pens. It has been shown that wrasse can escape the net pens after release (Woll et al. 2013). It is, however, not known if they survive in the recipient environment and mix with local populations, but results obtained by Jansson et al. (2017) indicate that this may have been the case at Site G, the only 'import' area in these studies. As we have found different genotypes of NNV in the wrasse species examined (Table 1), this indicates that there may be a geographical variation of NNV genotypes in wrasse. Goldsinny wrasse has the widest geographical distribution, and the sampling covered most of the species' geographical range in Scandinavia. Site H (Tysfjord) is the northernmost known goldsinny wrasse population. A population genetic study of goldsinny wrasse including samples used in this study showed a clear isolation-by-distance pattern along the coastline and genetic distinctiveness of the northernmost Tysfjord samples (Jansson et al. 2017). This implies that not only the NNV virus populations but their hosts as well are genetically structured in a similar manner and might differ in their susceptibility to viral infections.

It is not possible to determine wheter the genotype variation observed in this study could be linked to movement of wild wrasse, but if wrasse from the southern part of Norway are moved to the north, it would seem likely that different NNV genotypes are

moved as well. The impact this might have on local marine fish species is not known.

The study included a low number of ballan wrasse (reflecting the composition of fish species normally caught by the traps). Still, 2 out of 11 were positive, representing 2 different genotypes (RGNNV and AHNNV). Recently, Norwegian fish farmers have established intensive production of ballan wrasse to secure a stable supply of cleaner fish. In light of the problems caused by NNV in Norwegian marine fish farming, such as in Atlantic cod (Patel et al. 2007) and Atlantic halibut (Grotmol 1997), wrasse farmers should pay attention to NNV in brood fish, in order to avoid future disease problems in juveniles. As vertical transmission of NNV has been demonstrated (Breuil et al. 2002), this indicates a potential risk of introducing the virus with wild-caught wrasse. Hence, as a precautionary action, surveillance for, and preventive measures against, NNV should be implemented in the aquaculture production of ballan wrasse.

Wrasses are normally used in only one salmonid production cycle. There is a high loss of wrasses from the net pens. The farmers are making efforts to improve the survival and welfare of these cleaner fish. Re-use of wrasses has been proposed in order to meet the demand for cleaner fish and reduce the impact on wild populations. However, this conflicts with the principle of fallowing the sites between production cycles and may represent a risk of transferring pathogens between different generations of salmonids.

In this study, we have shown that NNV is naturally present in wild ballan, corkwing and goldsinny wrasse along the coastline of Sweden and Norway. Three different genotypes of nodavirus were found, representing the BFNNV and RGNNV genogroups. A possible geographic genotype difference between the northern and southern sampling sites was seen. The presence of NNV in wild wrasse and the high genetic variation should be considered in the management and use of wild-caught wrasse, as well as in selection of brood fish and industrial production of cleaner fish.

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