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Original Article

Cryptic *Sebastes norvegicus* species in Greenland waters revealed by microsatellites

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Identification of cryptic species can have profound implications in fishery management, conservation and biodiversity contexts. In the North Atlantic, the genus *Sebastes* is currently represented by four species, although additional cryptic species have been assumed. The connectivity of the gene-pools within the genus in Greenland waters, in particular, remains largely unexplored. Using a panel of 13 microsatellite markers for 720 fish, we explored the species complex of *Sebastes norvegicus* in Greenland waters. Genetic analyses provided evidence for three cryptic species in samples that were morphologically identified as *S. norvegicus*. They were termed *S. norvegicus*-A, *S. norvegicus*-B, and *S. norvegicus*-giants. A few phenotypic features exist to identify adult *S. norvegicus* giants, but no characteristics have been identified for the two other cryptic species. The proposed cryptic species should be recognized in the management regime to ensure sustainable exploitation and conservation of *Sebastes* species in Greenland waters.

Keywords: Bayesian population clustering, discriminant analysis of principal components, golden redfish, isolation-with-migration.

Introduction

Cryptic species are two or more species that are categorized as a single species as no apparent morphological distinction between them has been established (Bickford *et al.*, 2007). Since speciation may not always be accompanied by morphological change, "biological species" (Mayr, 1963) may evolve reproductive isolation despite identical or similar phenotypic traits (Bickford *et al.*, 2007). Marine habitats appear to support cryptic speciation due to their wide species diversity, although not much attention has been paid to them so far (Miglietta *et al.*, 2011). Special consideration is required for the identification and subsequent management of a cryptic species complex to ensure sustainability (Bickford *et al.*, 2007). Molecular markers are the best way to identify and describe cryptic species complexes (Miglietta *et al.*, 2011), thus providing a basis for their management and conservation.

Redfish (*Sebastes* spp.) in the North Atlantic are morphologically similar, leading to persistent difficulties in species identification (Johansen, 2003; Schmidt, 2005; Pampoulie and Daníelsdóttir, 2008). This morphological similarity has motivated hypotheses of recent speciation within the genus, which may be linked to separation by depth (Barsukov *et al.*, 1984). Contemporary redfish species are thought to have evolved from a North-east Pacific ancestor at the opening of the Bering Strait ~3 million years ago (Hyde and Vetter, 2007), followed by recent and rapid evolutionary divergence of North Atlantic *Sebastes* spp. (Sundt *et al.*, 1998). As many as 92

recognized species of *Sebastes* are found in the North Pacific (Love *et al.*, 2002), but only four species are known in the North Atlantic: *S. mentella* Travin, 1951 (beaked redfish), *S. norvegicus* Ascanius, 1772 (golden redfish, previously called *S. marinus*), *S. fasciatus* Storer, 1854 (acadian redfish), and *S. viviparus* Krøyer, 1845 (Norway redfish). Redfish are long-lived species, and the maximum recorded lifespan for *S. norvegicus* and *S. fasciatus* are 60 and 75 years, respectively. They are late maturing (e.g. 10–14 years) with generation lengths between 16 and 26 years (Drevetnyak *et al.*, 2011; HELCOM, 2013). All *Sebastes* species are ovoviviparous (live-bearer) with internal fertilization and display mate recognition and courtship behaviour, which may provide an additional mechanism for reproductive isolation (Johns and Avise, 1998). Cryptic *Sebastes* species have been detected in the southern hemisphere (Rocha-Olivares *et al.*, 1999), Northwest Pacific (Kai *et al.*, 2002), Gulf of Alaska (Gharrett *et al.*, 2005), south of Point Conception (Hyde *et al.*, 2008), and Pacific coast of southern Japan (Kai and Nakabo, 2004). Furthermore, cryptic *S. norvegicus* species have been suspected in the North Atlantic (Johansen *et al.*, 2000; Schmidt, 2005; Pampoulie and Daníelsdóttir, 2008). Within the *S. mentella* complex in the North Atlantic, two genetically distinctive morphs ("shallow" and "deep") have been described (Stefánsson *et al.*, 2009; Shum *et al.*, 2015; Saha *et al.*, 2017). Stefánsson *et al.*, (2009) proposed the "shallow" and "deep" morphs are incipient species. A deep evolutionary divergence between these two morphs has recently been supported by Saha *et al.*, (2017).

The genus *Sebastes* is widely distributed across the North Atlantic. *S. mentella* and *S. norvegicus* have trans-Atlantic distributions. *S. fasciatus* is restricted to the Northwest Atlantic, whereas *S. viviparus* represents its "ecological counter species" in the Northeast Atlantic. Geographical boundaries for the latter two species are not well defined (Whitehead, 1986). The four species exhibit overlapping depth distributions. *S. fasciatus* is typically found at 150–300 m water depth (Roques *et al.*, 2001), *S. norvegicus* at 100–300 m, *S. mentella* at 50–800 m and *S. viviparus* largely above 120 m depth (Barsukov *et al.*, 1984). However, these species can frequently be caught in the same trawl haul as a consequence of overlapping distributions (Barsukov *et al.*, 1984). Morphological characters such as eye diameter, size of lower jaw beak, and the direction of spines on the pre-operculum have been used for species identification of adults (>25 cm), but morphological species identification of juveniles remains difficult and controversial (Barsukov *et al.*, 1984), as for many rockfish species (Kendall, 1991; Pearse *et al.*, 2007). Morphological identification tends to be particularly problematic in Greenland waters (Johansen, 2003), which are the assumed main nursery ground for *S. mentella* and *S. norvegicus* extruded along the Reykjanes Ridge (Anderson, 1984; Magnusson and Johannesson, 1995). This region undoubtedly serves as a significant habitat for *Sebastes* throughout their life-cycle; yet, species structure in these waters remains largely unexplored.

Fisheries and management of *S. norvegicus* in the central North Atlantic are limited to a single management unit constituting Greenland, Iceland and the Faroe Islands waters (ICES 2016). Catches in the past decades have been around 40 thousand tons and are thus an important fishing resource. However, the management advice does not take account of the wide distribution of the stock (ICES 2016). While there has been considerable research interest for *S. mentella* (for review see Cadrin *et al.*, 2010; Saha *et al.*, 2017), much less attention has been paid to *S. norvegicus*. Analysing panels of mtDNA, microsatellites and AFLP markers, Schmidt (2005) suggested the occurrence of two cryptic *S. norvegicus* species in the North Atlantic. Pampoulie *et al.*, (2009), in accordance with Schmidt (2005), reported two different gene pools of *S. norvegicus* in the North Atlantic. A so-called "giant" morph has been described in the Irminger Sea (Reykjanes Ridge, Figure 1) within the *S. norvegicus* complex with a larger size, fewer gill rakers, distinct otolith morphology, and faster growth rate before maturation compared to "ordinary" *S. norvegicus* (Kotthaus, 1961). Allozyme frequencies of "giant" *S. norvegicus* appeared to be similar to those of *S. mentella* according to Altukhov and Nefyodov (1968), whereas mtDNA analyses indicated a close relationship to *S. norvegicus* (Schmidt, 2005). Furthermore, based on haemoglobin, allozyme (Johansen *et al.*, 2000) and microsatellite data (Pampoulie and Daníelsdóttir, 2008), "giants" were claimed to represent a distinct species. Artamonova *et al.* (2013), on the other hand, suggested that the "giants" were hybrids between *S. mentella* and *S. norvegicus*. Thus, the taxonomic status of the "giants" remains unresolved and further investigations are required to clarify its connectivity with the co-existing gene pools.

With the many ambiguities associated with *Sebastes* in Greenland waters, the main objective of the present study has been to investigate the species structure of *Sebastes* in these waters, paying special attention to the possible existence of cryptic species within the *S. norvegicus* complex. Here, we used an unprecedented number of microsatellite loci to analyse a large number of *S. norvegicus* specimen collected from Greenland and nearby waters to investigate the taxonomic status of the "giants" and to clarify its connectivity with the co-existing gene pools. We define "species" as separately evolving metapopulation lineages (De Queiroz, 2007). For the species delimitation, reproductive isolation of the metapopulations has been considered necessary (Mayr, 1963). We define "genetic clusters/metapopulations" as possible "cryptic species" if the extent of reproductive isolation, quantified through gene flow and genetic distances, is larger than or comparable with that between the established *Sebastes* species (see De Queiroz, 2007).

Material and methods

Sampling

In total, 12 samples consisting of 720 fish from *Sebastes* spp. were studied (Table 1). *S. norvegicus* ($N = 411$) and *S. mentella* ($N = 185$) were sampled from Greenland waters in spring and fall 2011–2012. The sample sets of adult and juvenile *Sebastes* were caught with trawls from research and commercial vessels. Species were identified on-board based on body size, beak size, eye diameter, and direction of spines in the pre-operculum, as suggested by Barsukov *et al.*, (1984). For comparison, reference samples of *S. viviparus* (Icelandic and Norwegian, $N = 79$) and *S. fasciatus* (Flemish Cap, $N = 45$) were included from the EU REDFISH project (QLK5-CT1999-01222) (Figure 1 and Table 1). Except for two samples, as shown in Table 1, all other samples were previously examined by Saha *et al.*, (2017) for the population genetic investigation in *S. mentella*.

Microsatellite genotyping

DNA was isolated from ethanol preserved gill tissue using E-Z 96 Tissue DNA kit (Omega Bio-Tek, Inc, USA). We analysed 13 microsatellite loci: Seb09, Seb25, Seb31, Seb33, Seb45, Smen05

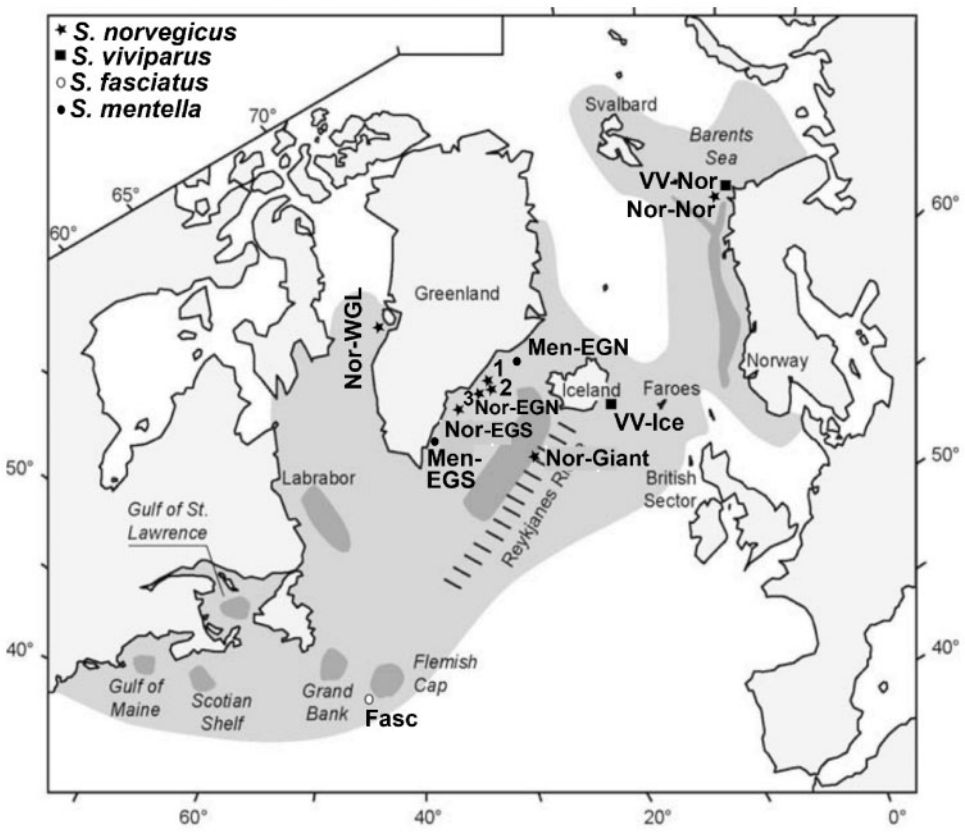

Figure 1. Sampling of *Sebastes* spp. for the present investigation. Distribution (light grey shading) and larval extrusion areas (dark grey) of the species across the North Atlantic are shown (source: Johansen, 2003). *S. norvegicus* and *S. mentella* samples were collected from Greenland waters. Reference samples of *S. norvegicus* (Norway), *S. norvegicus* giants (Reykjanes ridge), *S. viviparus* (Norway and Iceland) and *S. fasciatus* were included for comparison (see Table 1).

Table 1. Details of the *Sebastes* spp. samples analysed.

Species	Sample code	Location	Lat/Long (Mean)	Time	*N*	Depth (m)	Length (cm)	F	Ad
S. norvegicus	Nor-Nor	Noway	69.19/15.08	Oct 2001	41	195–417 (258)	29–54 (36)	15	100
	Nor-EGN1[a]	East Greenland	64.42/−35.18	April 2012	55	375	28–54 (37)	55	99
	Nor-EGN2[a]		64.38/−35.3	Feb 2012	71	375	28–51 (38)	46	99
	Nor-EGN3		64.26/−35.15	Feb 2011	108	365–370 (367)	26–57 (38)	58	87
	Nor-EGS		62.2/−40.65	Aug 2011	70	188–332 (239)	17–49 (28)	44	29
	Nor-WGL	West Greenland	69.28/−53.1	Jun 2012	49	521	43–62 (54)	65	100
S. norvegicus (giants)	Nor-Giant	Reykjanes Ridge	61/−29	Aug 1996	17	594–786 (692)	71–84 (79)	82	100
S. mentella	Men-EGN	East Greenland	64.24/−35.14	Mar 2011	137	367–377 (372)	28–63 (36)	56	64
	Men-EGS		61.15/−41.66	Aug 2011	48	423–512 (453)	20–40 (30)	42	56
S. viviparus	VV-Ice	Iceland	64.1/−13.47	Mar 2001	53	155–305 (175)	11–27 (18)	47	45
	VV-Nor	Norway	70.5/20.52	1992, 2001[b]	26	75–170 (137)	14–24 (20)	NA	100
S. fasciatus	Fasc	Flemish Cap	45.79/−47.16	Oct 2001	45	240–322 (295)	15–31 (21)	62	36

[a]Samples not included in Saha *et al.* (2017). Mean depth and length shown in brackets.

[b]Samples collected in two years. No temporal genetic differences between samples observed.

N = sample size, F = % females, Ad = % adults (≥ 29 cm) (Barsukov *et al.*, 1984), NA = data not available. For sampling depth and fish length, mean values are given within brackets. For sample codes, Nor = *S. norvegicus*, Men = *S. mentella*, VV = *S. viviparus*, Fasc = *S. fasciatus*, Nor = Norway, EGN = Northeast Greenland, EGS = Southeast Greenland, WGL = West Greenland, and Ice = Iceland.

(Roques *et al.*, 1999a), Sal1, Sal3, Sal4 (Miller *et al.*, 2000), Smen10 (Stefánsson *et al.*, 2009), and Spi4, Spi6, Spi10 (Gomez-Uchida *et al.*, 2003), arranged in three multiplexes (Supplementary Table S1). These 13 microsatellites were also used by Saha *et al.* (2017) to study genetic population structure of *S. mentella*. Nine of these microsatellites were previously analysed by Pampoulie and Daníelsdóttir (2008) to resolve species identification in North Atlantic *Sebastes*. PCR was performed in 2 µl volume comprising 1× Qiagen Multiplex Master Mix, 0.1–1.0 µM primer and 15–25 ng DNA. The PCR products were labelled with fluorescent dye at the 5' end of the forward primer (Applied Biosystems, Foster City, CA). Amplifications were

performed in a GeneAmp 9700 (Applied Biosystems) with a PCR profile consisting of an initial denaturation step of 95 °C for 15 min followed by 25 cycles of 95 °C for 30 s, 56 °C for 90 s, and 72 °C for 60 s, ending with 60 °C for 45 min. The PCR products were separated with an ABI 3130 XL automated sequencer (Applied Biosystems) and their sizes were determined using GeneMapper 4.0 (Applied Biosystems). To detect possible scoring errors or null alleles, we analysed the data with Micro-Checker (Van Oosterhout *et al.*, 2004). The locus Spi4 was not successfully genotyped for the samples of *S. viviparus*, likely due to poor DNA quality. Eventually, this locus was not used in the analyses where the samples of *S. viviparus* were included.

Descriptive statistics

We used FSTAT (Goudet, 1995) to calculate number of alleles, gene diversities, allelic richness ($N = 13$), and inbreeding coefficient (F_{IS}). Pairwise F_{ST} (Weir and Cockerham, 1984), observed and expected heterozygosities (H_O and H_E) were estimated in Arlequin 3.5 (Excoffier and Lischer, 2010). Deviations from linkage and Hardy-Weinberg equilibria (HWE) were tested in Genepop 4.2 (Rousset, 2008) with unbiased estimates of Fisher's exact test with the implemented Markov chain Monte Carlo (MCMC) method (dememorization 10 000, batches 1000, and iterations 10 000). A false discovery rate control (FDR, Benjamini and Yekutieli, 2001) was applied to avoid type- I error, while preserving power, whenever multiple comparisons were made. To identify the cause of departures from HWE, we followed Waples (2015) to determine whether (1) deviations from HWE were locus and/or sample specific, (2) the departures represented heterozygote deficiencies or excesses, (3) there was a positive correlation between F_{ST} and F_{IS} values at the loci. Finally, we re-tested deviations from HWE in the identified clusters (described below).

Population cluster analyses

Since the accuracy of clustering can vary on the basis of data and statistical features of the methods used (Bohling *et al.*, 2013), we applied two approaches to identify genetic clusters in the samples: Bayesian Analysis of Population Structure (BAPS) (Corander *et al.*, 2006) and Discriminant Analysis of Principal Components, DAPC (Jombart *et al.*, 2010). BAPS 6.0 was used for the model "clustering of individuals" as sampling was not possible on mating grounds and seasons. The model-based Bayesian method of the program assigns individuals to clusters by minimizing deviations from HWE and gametic phase disequilibrium within groups. The program is based on a stochastic optimization algorithm, which finds the posterior mode of the genetic structure. It provides faster execution than MCMC methods (Corander *et al.*, 2006). BAPS was run several times to ensure proper replication for the given number of clusters (K). K was treated as a random variable. For the analysis of 12 samples, $K = 15$ was the upper bound limit. $K = 10$ was used as the upper bound limit for the BAPS analysis of the seven *S. norvegicus* samples. Finally, the number of clusters (K) in the data were selected on the basis of the highest posterior probability values observed. The admixture analyses were performed subsequently. The data for 12 microsatellites were used to analyse the total sample set, and 13 microsatellites were used for the analyses of the *S. norvegicus* samples (see rationale above).

As a non-model approach to detect the number of genetic clusters and assignment of individuals in the *S. norvegicus* samples, we applied DAPC (Jombart *et al.*, 2010) in *adegenet* 1.4–2 (Jombart, 2008) in R 3.1.1 (R Core Team, 2014). *S. norvegicus* genotypes ($N = 411$) for the 13 microsatellite loci were used for this analysis. Unlike BAPS, DAPC does not rely on the assumptions of HWE or linkage equilibrium. This multivariate analysis summarizes the variation in the original variables (alleles) in a PCA and then maximizes differences between clusters while minimizing variation within clusters (Jombart *et al.*, 2010). It provides a graphical representation of between-cluster structures to reveal complex population structures. DAPC uses the Bayesian Information Criterion (BIC) to assess the best supported model and the number and component of clusters. The function *find.clusters* was applied to determine the number of clusters in the data. The function *optima.a.score* was used to estimate the optimal number of principal components to retain and avoid overfitting the discriminant functions.

The extent of divergence among the cluster identified by BAPS analysis was quantified by the chord distance (D_{CE}, Cavalli-Sforza and Edwards, 1967) using 12 microsatellites. Pair-wise distances were used to construct an unrooted-phenogram using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) available in Populations (Langella, 2002). To estimate confidence of nodes in the tree, 1000 bootstraps were performed on loci. The phylip format tree generated by Populations was viewed in Fig Tree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/ Fig Tree v1.4.2).

Isolation-with-migration

The isolation-with-migration (IM) model implemented in IMa2 (Hey, 2009) was applied to estimate historical gene flow and population demographic parameters for the predicted three *S. norvegicus* clusters ($N = 44$ for each, based on BAPS and DAPC results). Additionally, a comparative analysis was carried out on the cluster pair "viviparus-fasciatus" ($N = 44$ for each, based on BAPS result). We selected this species pair as a reference because they seldom occur in sympatry and, therefore, should provide the best examples of "good" species for comparison with cryptic species. We could not run all six putative species because of excessive computational time (see Beerli, 2004). The IM model assumes random mating, free recombination among loci and no recombination within loci. This MCMC based method uses sampling of gene genealogies to estimate posterior probability densities of demographic parameters scaled by mutation rate per generation per year (μ). After several test runs with uniform and non-uniform priors of various ranges, we used uniform priors for the parameters ($q = 30$, $m = 2$, $t = 20$) and a step-wise mutation model for the 12 microsatellite loci.

To achieve reliable estimates, we optimized estimated effective sample sizes of parameter t (time since divergence), swapping rates, autocorrelation, trend-line plots of the parameter and the coherence of estimates in the first and second halves of a run. We used a burn-in of 500 000 steps, after which 2 000 000 more steps were run to save 20 000 genealogies for each analysis. To ensure good convergence for the estimation, 80 chains were used for each run with medium heating scheme (−ha0.975–hb0.75). Finally, we estimated historical rates of bidirectional migration (*m1* and *m2*) as effective number of migrants per generation ($2NM = (4N_e\mu) * (M/\mu/2)$, independent of mutation rate), population size parameters ($\Theta = 4N_e\mu$, N_e = effective population size), and time since divergence in generations ($t\mu$) without converting for a given mutation rate and generation time.

Table 2. Estimates of pairwise F_{ST} differentiation, based on 12 microsatellite markers, between *Sebastes* samples (cf. Table 1).

	Nor-Nor	Nor-EGN1	Nor-EGN2	Nor-EGN3	Nor-EGS	Nor-WGL	Nor-Giant	Men-EGN	Men-EGS	VV-Ice	VV-Nor
Nor-EGN1	0.040										
Nor-EGN2	0.041	0.000									
Nor-EGN3	0.049	0.000	0.000								
Nor-EGS	0.010	0.015	0.016	0.019							
Nor-WGL	0.048	0.040	0.043	0.044	0.022						
Nor-Giant	0.083	0.097	0.096	0.102	0.057	**0.010**					
Men-EGN	0.082	0.073	0.071	0.086	0.078	0.094	0.125				
Men-EGS	0.066	0.056	0.053	0.068	0.059	0.079	0.111	**0.003**			
VV-Ice	0.134	0.111	0.108	0.118	0.115	0.136	0.181	0.120	0.100		
VV-Nor	0.142	0.122	0.117	0.127	0.119	0.139	0.195	0.132	0.109	0.018	
Fasc	0.154	0.164	0.161	0.162	0.142	0.162	0.201	0.179	0.154	0.120	0.131

All values, except two in bold, are significant at $p = 0.000001$.

Results

Descriptive statistics

The number of alleles per locus ranged from 3 to 41, whereas mean allelic richness corrected for the smallest sample size ($N = 13$) varied between 2.81 and 18.18 (Supplementary Table S2). Observed gene diversities per locus and sample were between 0.22 and 0.97 (mean = 0.78). Mean expected heterozygosity was 0.76. Before FDR control, 50 of the total 154 tests deviated significantly from HWE (40 significant deviations in *S. norvegicus* samples, Supplementary Table S2). Only 12 tests remained significant after FDR control (ten in *S. norvegicus* samples). All but one *S. norvegicus* sample deviated from HWE, even after FDR control (Supplementary Table S2). All of the significant deviations from HWE showed heterozygote deficits. Although a positive correlation ($r = 0.34$) was observed between F_{ST} and F_{IS} values at the 13 loci, the correlation was statistically not significant (Supplementary Figure S1). However, a statistically significant positive correlation was supported when eight of the loci showing a greater number of deviations were considered. The correlation between F_{ST} and F_{IS} was the strongest for six of the microsatellite loci ($r = 0.92$, $P = 0.005$). Micro-Checker detected no null alleles or large allele drop-outs for any of the loci used. Of 792 tests for pairwise linkage disequilibrium in the data with 12 samples genotyped in 12 microsatellite loci (see rationale above), 67 (8.5%) were significant. Only three tests, however, remained significant after application of the FDR control. In the data with ten samples (excluding *S. viviparus*) genotyped for 13 microsatellite loci, 74 (9.5%) tests deviated from linkage equilibrium. Only four of these tests remained significant after FDR control. None of the tests for deviation from HWE after FDR control of the identified *S. norvegicus* clusters was significant (Supplementary Table S3).

Pattern of genetic differentiation

Almost all pairwise F_{ST} values between samples were highly significant (64 of 66; Table 2). Differentiation between the two *S. mentella* samples (Men-EGN and Men-EGS) and two of the *S. norvegicus* samples (Nor-WGL and Nor-Giant) was not supported after FDR control. The *S. fasciatus* (Fasc) sample stood out as the most divergent of all samples.

Bayesian population cluster analysis (BAPS) identified seven clusters (posterior probability = 1) in the data (Figure 2). One of these clusters included only a single individual. For the *S. norvegicus* samples, three highly differentiated genetic clusters were displayed by the population mixture analysis (Figures 2 and 3); these clusters were designated "Norvegicus-A/*S. norvegicus*-A", "Norvegicus-B/*S. norvegicus*-B", and "Giants/*S. norvegicus* giants." In contrast, only one cluster was found for each of the *S. viviparus*, *S. mentella*, and *S. fasciatus* species. One individual, however, morphologically identified as *S. fasciatus*, clustered with *S. norvegicus*-B (Figure 3). The cluster "Giants" included fish from the Reykjanes Ridge (Nor-Giant, $N = 17$), West Greenland (Nor-WGL, $N = 30$), and East Greenland ($N = 7$). The cluster "Norvegicus-A" consisted of fish mainly from Greenland (both east and west) plus four fish from Norwegian waters, whereas the "Norvegicus-B" cluster included fish from both Greenland and Norwegian waters (plus one specimen from Flemish Cap). Nine fish morphologically identified as *S. norvegicus* clustered with *S. mentella*. When only seven *S. norvegicus* samples genotyped in 13 microsatellites were analysed with BAPS, three *S. norvegicus* clusters were supported by the data (not shown).

The DAPC using data from 13 microsatellites suggested five clusters in the seven *S. norvegicus* samples (Figure 4). Seven principal components and four discriminant functions were retained for this analysis. The graphical representation of between-cluster structures, first, showed three distinct clusters which were consistent with *S. norvegicus*-A, *S. norvegicus*-B, *S. norvegicus*-giants. Second, the DAPC indicated sub-structuring within both the *S. norvegicus*-A and *S. norvegicus*-B clusters. When the DAPC was forced to show three clusters, the assignments of the individuals into clusters were congruent with those obtained with BAPS. No significant deviation from the HWE was observed in the clusters identified by the DAPC (Supplementary Table S3).

Pairwise F_{ST} comparisons among the six genetic clusters obtained from the BAPS result (Table 3) showed that the genetic differentiation among the three clusters of *S. norvegicus* was comparable to that among the established species, *S. mentella*, *S. fasciatus*, and *S. viviparus*. The distinction of the clusters in the NJ tree of chord distances was supported by bootstrap values between 55 and 84% (Supplementary Figure S2).

Estimates of demographic parameters

Evidence of low, but significant, historical gene flow (i.e. hybridization) was observed in all cluster-pairs included in the IM analyses (Table 4). The extents of hybridization among the *S. norvegicus* clusters were comparable to hybridization between the two established species, *S. viviparus* and *S. fasciatus*. The smallest estimate of population size parameter was found for the "Giants" cluster.

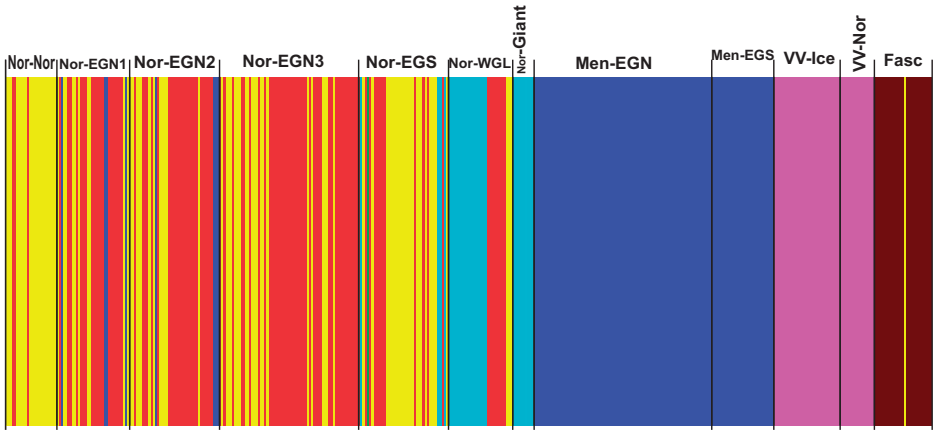

Figure 2. Population mixture analysis of the 12 samples of *Sebastes* spp. by BAPS 6 (Corander *et al.*, 2006). For a colour representation of the figure, it is referred to the online version. Seven genetic clusters, as indicated by seven different colours, were predicted (sample names at the top, cf. Table 1). Red, yellow, and cyan colours represent *S. norvegicus*-A, *S. norvegicus*-B and *S. norvegicus*-giants, respectively. One of the clusters composed of only one individual as shown by the green bar within the Nor-EGS sample. This figure is available in black and white in print and in colour at *ICESJMS* online.

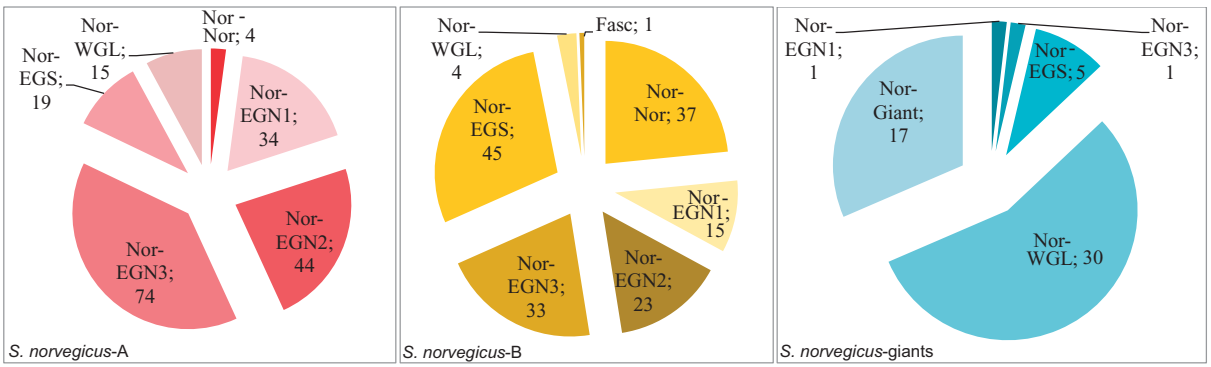

Figure 3. Pie-diagram showing components of three clusters obtained from the *S. norvegicus* complex by Bayesian population mixture analysis. Numbers of fish clustered from specific samples are presented (cf. Table 1). This figure is available in colour at *ICESJMS* online version of the article, while in black and white in print.

Discussion

The present study provides evidence for the existence of previously unrecognized cryptic *Sebastes* species in Greenland waters. Given the genetic distances and isolation-with-migration pattern, we propose three cryptic species within the *S. norvegicus* complex: *S. norvegicus*-A, *S. norvegicus*-B, and *S. norvegicus* giants. Neither *S. fasciatus* nor *S. viviparus* were present in samples from Greenland, and both seemed to be distinct species in their respective areas. The results indicate that *S. norvegicus*-B may be distributed across the North Atlantic whereas *S. norvegicus*-A is restricted to the Northeast Atlantic. The *S. norvegicus* giants were found in the Irminger Sea and Greenland waters, predominantly around West Greenland.

The tests for deviation from HWE revealed significant heterozygote deficits at all loci surveyed in the *S. norvegicus* samples. Two likely reasons for these deficits are the presence of null alleles and/or different reproductively isolated groups in the samples (Wahlund effect). Although the data were carefully analysed in Micro-Checker, the existence of "cryptic" null alleles cannot be ruled out. The incidences of heterozygote deficits were clearly *S. norvegicus* sample specific. Most importantly, a positive correlation was observed between F_{ST} and F_{IS} at these loci, supporting a Wahlund effect (see Waples, 2015). This interpretation is in line with other studies of *Sebastes* using most of the microsatellites analysed herein (Roques *et al.*, 2001; Schmidt, 2005; Pampoulie and Daníelsdóttir, 2008; Saha *et al.*, 2017). Support for a Wahlund effect in our data also appeared in the Bayesian clustering output (Figures 2 and 3). Most notable is the clear segregation of the three distinct genetic clusters within the *S. norvegicus* samples.

Genetic distinctiveness of the clusters was supported by the F_{ST} estimates (Table 3), which again were consistent with previous studies using the same morphological and microsatellite markers to distinguish species (Roques *et al.*, 1999b; Schmidt, 2005). The DAPC also suggested the existence of genetic clusters in the *S. norvegicus* samples (Figure 4) that were also identified with BAPS. Sub-structuring within two of the *S. norvegicus* clusters in the DAPC result may indicate the existence of different populations of the species in these waters.

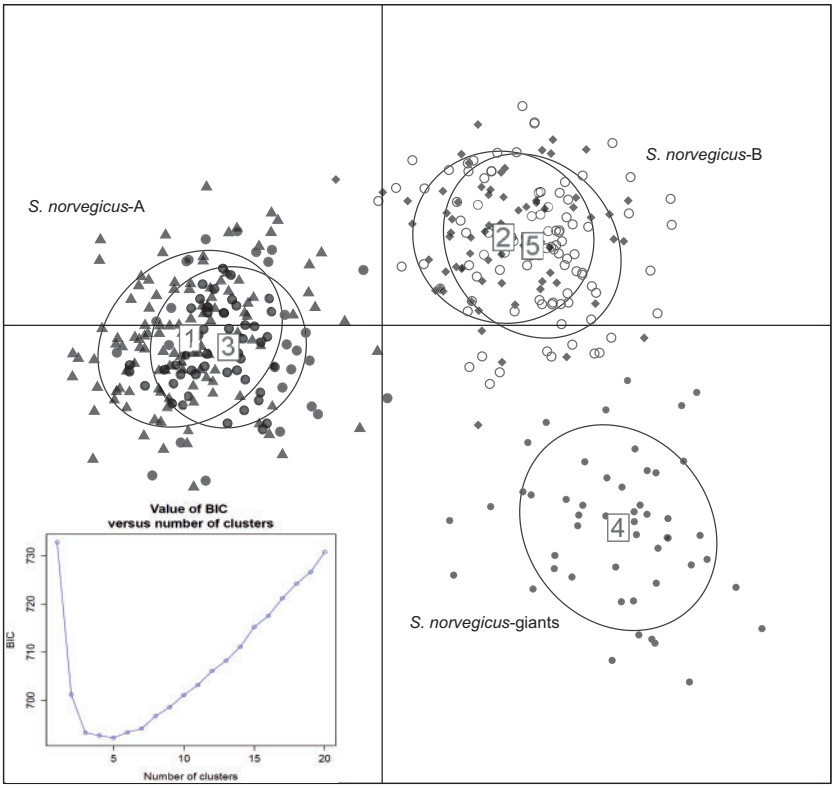

Figure 4. DAPC analyses of the seven *S. norvegicus* samples using 13 microsatellite loci. BIC value suggested a total of five genetic clusters in the data. Although no sub-structuring was observed within *S. norvegicus*-giants, sub-structuring was supported within both *S. norvegicus*-A and *S. norvegicus*-B.

Table 3. Estimates of pairwise F_{ST}'s between six genetic clusters obtained by BAPS.

	Norvegicus-A	Norvegicus-B	Giants	Mentella	Viviparus
Norvegicus-B	0.099				
Giants	0.160	0.074			
Mentella	0.106	0.085	0.132		
Viviparus	0.141	0.131	0.184	0.115	
Fasciatus	0.208	0.154	0.207	0.178	0.124

All values are significant at $p = 0.000001$.

Observation of gene flow between the established species and the observed three *S. norvegicus* clusters may reveal the extent of divergence among these *Sebastes* clusters, and therefore clarifies their taxonomic status. In this respect, the IM model provides a coalescent-based framework to infer evolutionary divergence (Hey, 2009). Our results show low but significant gene exchange among *S. norvegicus* clusters (Table 4), which implies that they are emerging *Sebastes* species such as the "shallow" and "deep" morphs of *S. mentella* (Stefánsson *et al.*, 2009; Saha *et al.*, 2017). However, a key feature of our results is that isolation observed by IM analyses among the three clusters within *S. norvegicus* was much greater than that between the "shallow" and "deep" morphs of *S. mentella* (Saha *et al.*, 2017) and similar to isolation between two established species, *S. viviparus* and *S. fasciatus*. The phylogenetic tree of chord distances also showed genetic divergence among the three *S. norvegicus* clusters that was similar to that among the established species. These observations support the hypothesis of reproductive isolation among the three genetic clusters within the *S. norvegicus* complex. The extent of the divergence is consistently similar to or larger than that among the three established species, *S. mentella*, *S. fasciatus*, and *S. viviparus*. Hence, the existence of novel cryptic *Sebastes* species in the North Atlantic is evident from our data.

The first mention of a "giant" form of *S. norvegicus* dates to 1961 (Kotthaus, 1961), and the "giants" have later been suggested as a valid species in several studies (e.g. Johansen *et al.*, 2000; Johansen, 2003; Pampoulie and Daníelsdóttir, 2008). Interestingly, Pampoulie and Daníelsdóttir (2008) used nine microsatellites that were also used in the present study. The failure to recognize "giants" as a valid species in other studies (Schmidt, 2005; Pampoulie *et al.*, 2009) may be due to the small numbers of fish encountered. Schmidt (2005), using mtDNA, microsatellites and AFLP markers, suggested the occurrence of two cryptic species roughly corresponding to the present "Norvegicus-A", and "Norvegicus-B." But, the author found no genetic differentiation between the "giants" ($N = 12$) and the most widely distributed cryptic species ("Norvegicus-B"). The hypothesis that "giants" were hybrids between *S. mentella* and *S. norvegicus*, as indicated by Artamonova *et al.* (2013), is unlikely given the observed Q-values (genome ancestry fraction, almost 100% pure) in Bayesian clustering. However, *S. norvegicus* individuals in the present study appear to be less admixed (S2) than those in the investigation by Pampoulie and Daníelsdóttir (2008). The discrepancy may be linked to both the resolution power and applied methods of the two different studies. Compared to the investigation by Pampoulie and Daníelsdóttir (2008), we analysed a larger panel of

Table 4. Results from the IM analysis of *Sebastes* clusters.

	Results from the analysis of Norvegicus-A (A), Norvegicus-B (B), and Giants (G) clusters												
	$2NM_{(A\rightarrow B)}$	$2NM_{(B\rightarrow A)}$	$2NM_{(A\rightarrow G)}$	$2NM_{(G\rightarrow A)}$	$2NM_{(B\rightarrow G)}$	$2NM_{(G\rightarrow B)}$	$t\mu_1$	$t\mu_2$	Θ_1	Θ_2	Θ_A	Θ_B	Θ_G
Peak value	1.51	0.01	0.03	0.01	1.03	1.26	5.03	0.25	29.98	29.98	7.25	8.05	1.73
Lower 95% HPD	0.57	0.00	0.00	0.00	0.39	0.21	3.23	0.09	21.70	11.98	3.26	6.10	1.07
Upper 95% HPD	2.29	5.57	0.33	0.63	1.89	2.19	19.99	1.06	29.98	29.98	16.07	14.21	2.93
	Results from the analysis of *S. viviparus* (V) and *S. fasciatus* (F) clusters												
	$2NM_{(V\rightarrow F)}$	$2NM_{(F\rightarrow V)}$	$t\mu_3$	Θ_3	Θ_V	Θ_F							
Peak value	0.41	0.33	2.89	29.98	17.86	11.98							
Lower 95% HPD	0.01	0.00	1.23	20.09	12.40	8.72							
Upper 95% HPD	0.95	0.91	19.99	29.98	24.73	16.13							

The estimates of gene flow are expressed as effective number of migrants per generation (2*NM*) and the direction of gene flow is indicated by arrows. Estimates of time since divergence ($t\mu$) are presented in generations without converting for a given mutation rate, and generation time. And the estimates of population size parameters (Θ) are shown for the studied clusters. Both peak values and values within 95% highest posterior density (HPD) are shown.
Here, $t\mu_1$ = estimate of time since divergence between Norvegicus-A and Norvegicus-B (plus Giants) clusters, $t\mu_2$ = estimate of time since divergence between Norvegicus-B and Giants clusters, and $t\mu_3$ = estimate of time since divergence between *S. viviparus* and *S. fasciatus* clusters. Θ_1 = estimate of ancestral population size parameter of Norvegicus-A and Norvegicus-B (plus Giants), Θ_2 = estimate of ancestral population size parameter of Norvegicus-B and Giants, and Θ_3 = estimate of ancestral population size parameter of *S. viviparus* and *S. fasciatus* clusters.

microsatellites in a larger number of fish using a different method as implemented in BAPS (Corander *et al.*, 2006). The program STRUCTURE, used by Pampoulie and Daníelsdóttir (2008), has a tendency to classify "pure" individuals as "hybrids" (Bohling *et al.*, 2013). Pampoulie and Daníelsdóttir (2008) analysed a different set of samples than that in the present study. It is also possible that the extent of hybridization varies in different region as was observed by Saha *et al.* (2017).

The existence of cryptic species in the North Atlantic within the genus *Sebastes* should be expected, given that marine habitats are believed to support numerous cryptic species (Miglietta *et al.*, 2011), and speciation within this genus is assumed to be relatively recent (Hyde and Vetter, 2007), thereby allowing less time for morphological divergence to evolve. Several studies have reported cryptic species within the genus *Sebastes* in the Pacific. Based on mitochondrial DNA and AFLP analyses, Kai *et al.* (2002) suggested that three morphotypes of *Sebastes inermis* may actually represent different species. A new species, *S. kiyomatsui*, was described by Kai and Nakabo (2004) in the Pacific coast of southern Japan. Gharrett *et al.* (2005), using mitochondrial and microsatellite DNA, described two cryptic species within *S. aleutianus* samples, for which no phenotypic characteristics have been unequivocally recognized.

Estimates of the demographic history of the suggested three cryptic *S. norvegicus* species were congruent with the interpretation of their genetic connectivity based on both F_{ST} estimates and DAPC analyses. The observed genetic divergence is the outcome of an interaction between gene flow, effective population sizes, and time since divergence; the principle that provides the basis of the IM model (Hey, 2009). The disjunct distributions of *S. fasciatus* (found in the Northwest Atlantic) and *S. viviparus* (found in the Northeast Atlantic) support the lowest gene flow between them, as predicted by the IM model (Table 4). The observed lowest genetic diversity for the *S. norvegicus* giants is also consistent with a recently evolved species with a small population size. Divergence between the cryptic species was estimated to be more ancient than between two established species, but the gene flow between cryptic *S. norvegicus* species was greater than between established species. This is possibly linked with the observation that cryptic *S. norvegicus* species exist in sympatry but not *S. fasciatus* and *S. viviparus*.

The present results suggest the existence of three novel cryptic species, but the data cannot explain the underlying mechanisms causing speciation. Given that the North Atlantic was colonized by ancestral populations after the last ice age ~12 k years ago (Alley, 2000), one can speculate that the formation of these species may have occurred by allopatric speciation in separate refugia. The occurrence of *S. norvegicus* giants in deeper water provides an ecological basis for this speculation. Hence, the observed low gene flow between the species implies a by-product on their secondary contact. Stefánsson *et al.* (2009) presented a similar explanation for the proposed two incipient *S. mentella* species in the North Atlantic. On the other hand, ovoviviparity and internal fertilization in *Sebastes* spp. suggest assortative mating (Helvey, 1982; Kendall, 1991), which can facilitate sympatric speciation (e.g. Barluenga *et al.*, 2006). Disentangling sympatric from allopatric speciation may require a thorough examination since many features of sympatric speciation are compatible with both models (Barluenga *et al.*, 2006). In the Northeast Pacific, speciation within the genus *Sebastes* is associated with divergence in water depth implying models of parapatric speciation (Ingram, 2011). However, the observed specific morphological traits in the *S. norvegicus* giants may point to selection as a driver of speciation. Conservatively, one can assume that the three cryptic species have evolved through the combined effects of assortative mating, selection and ecological divergence.

As for the pattern of distribution of the three cryptic species, "Norvegicus-B" was found in both Greenland and Norwegian waters. One individual caught in the Northwest Atlantic and morphologically identified as *S. fasciatus* also appeared to be "Norvegicus-B", implying that this species is distributed throughout the North Atlantic. In contrast, "Norvegicus-A" was found only around Greenland and Norwegian waters, and the "giants" were found only around Greenland, mostly off the west coast. Schmidt (2005) reported a similar distribution for the observed two *S. norvegicus* clusters, but did not acknowledge the "giants." Our sample set did not include *S. norvegicus* from Canadian waters, so the occurrence of the three cryptic species in this region remains unresolved.

Fisheries for *S. norvegicus* in Greenland and Iceland waters (ICES subareas 5 and 14) are managed on the basis of a single population unit (ICES, 2016), and the possible presence of cryptic species is not considered. The current management practice does not ensure sustainable exploitation in a fishery for both target and cryptic species, since cryptic species with smaller stock sizes may be more vulnerable to exploitation. Recognition of cryptic species and subsequent estimation of population size are, therefore, critical. For example, a sustainable fishing pressure on the entire *S. norvegicus* stock complex according to ICES advice (ICES 2016), might not ensure a sustainable harvest on the *S. norvegicus* giants in such a "mixed" *S. norvegicus* fishery. Efforts should be made to identify morphological traits so that the fisheries would be able to distinguish among the species to improve species specific catches and initiate separate assessment and advice. Reassessments of the stock dynamics for the separate species would enable a management designed to ensure sustainable exploitation of all species constitutive of the *S. norvegicus* complex, even if they are caught in mixed fisheries.

In some cases, as evident with the wide confidence intervals (Table 4), our results were insufficient to achieve precise estimates of the demographic history of the species. An additional inadequacy with the application of the IM method on our data might be that not all the clusters were included in a single run and, therefore, may have missed other contemporary clusters (i.e. "ghost populations"). However, given that no severe bias has been observed in comparable studies (e.g. Chan *et al.*, 2013; Saha *et al.*, 2017), bias in the IM results of the present study are likely to be minor and should not affect our main conclusions. Although the present genetic data provide good evidence for the existence of "reproductively isolated/cryptic species", other evidences (e.g. diagnosability, different ecological niches) will be required to provide a more consistent hypothesis as suggested by De Queiroz (2007). It will also be important to assess the temporal stability of the observed species structure.

Conclusions

The finding of the three novel cryptic *Sebastes* species in Greenland waters implies a rich species diversity for the genus in this region. A prevalent difficulty in the identification of *Sebastes* species may be linked with these cryptic species. Effort should be taken to detect phenotypic traits to identify these species. *Sebastes* spp. exhibit late maturation and slow growth rates, making them vulnerable to high exploitation. In this regard, findings of the new cryptic species should be considered in the management of *Sebastes* fisheries, to avoid the loss of important gene pools and biodiversity due to indiscriminate exploitation.

Data accessibility

Electronic supplementary data are available at the *ICESJMS* online version of the manuscript. Genotypes and biological data of the fish are available at the PANGAEA® data archive (https://doi.pangaea.de/10.1594/PANGAEA.873897).

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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