Molecular markers reveal spatially segregated cryptic species in a critically endangered fish, the common skate (*Dipturus batis*)

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Many sharks and skates are particularly vulnerable to overfishing because of their large size, slow growth, late maturity and low fecundity. In Europe dramatic population declines have taken place in common skate (*Dipturus batis* L.), one of the largest demersal fish in regional shelf seas, leading to extirpations from substantial parts of its former range. Here we report the discovery of cryptic species in common skate collected from the northeast Atlantic continental shelf. Data from nuclear microsatellite markers indicated two clearly distinct clades and phylogenetic analysis of mitochondrial DNA sequences demonstrated monophyly of each one of them. Capture locations showed evidence of strong spatial segregation, with one taxon occurring mainly in waters off the southern British Isles and around Rockall, while the other was restricted to more northerly shelf waters. These apparently cryptic species showed overlapping substrate and depth preferences, but distributional limits were closely related to temperature gradients, potentially indicating thermal limits to their distributions. This discovery of hidden diversity within a large, critically endangered marine vertebrate demonstrates how marine biodiversity can be underestimated, even in such a relatively well-studied and heavily exploited region.

Keywords: elasmobranch; marine fishes; biodiversity; fisheries; genetic differences; extinction

1. INTRODUCTION

Many large marine fish species have undergone striking declines in abundance during the twentieth century (Casey & Myers 1998). Worldwide assessments suggest 52 per cent of the world's fish stocks are now fully exploited, with an additional 28 per cent considered overexploited (FAO 2009). Conventional fisheries management is based on the stock unit concept which can essentially be defined as a fish population that is assumed

to be spatially and temporally homogeneous for management purposes owing to some common characteristic (Begg & Waldman 1999). However, in recent years evidence has accumulated suggesting that many stocks show significant temporal and spatial segregation operating at finer scales than the unit as a whole. This is not merely of biological interest, but could have serious implications for management and conservation of exploited species. Knowledge of patterns and drivers of fish movements may help to prevent overexploitation and extirpation of local stock components (Mucientes *et al.* 2009).

Direct tracking of the movements of individuals has been used successfully to reveal spatial dynamics for many species, however there are limitations with

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Figure 1. Common skate capture locations. Individuals identified genetically from the 'northern' clade are green; individuals from 'southern' clade are coloured red. Circles indicate locations where multiple samples were collected, triangles where single samples were collected. Depth information derived from the General Bathymetric Chart of the Oceans (<http://www.gebco.net>). Inset, a 'northern' *D. batis*.

retrieving data from large numbers of individuals and over multiple generations (Sims *in press*). Overcoming these constraints on our ability to infer long-term patterns of population connectivity would require considerable investment within a long-term tracking programme. A complementary approach is to quantify gene flow and stock boundaries using molecular markers (Hansen & Hemmer-Hansen 2007). Importantly, such population genetic studies have occasionally identified 'cryptic' or superficially morphologically indistinguishable species in marine systems (Bickford *et al.* 2007). Such studies have not only helped to improve our estimates of biodiversity, but have also informed conservation and management.

In this study we used a combination of population genetic and phylogenetic methods to explore spatial genetic structure of one of Europe's largest demersal fish, the common skate (*Dipturus batis* L.), with the aim of providing information on connectivity between remaining populations. The species is widely distributed from the Mediterranean Sea to Norway and Iceland, and is the largest of all known skates; it can grow to a total length of 2.85 m, 2 m disc width and weight of up to 113 kg (Wheeler 1978). It has a wide depth range (30–600 m), and is caught in a variety of habitats, from sea lochs, to shelf sand plains and deep offshore seamounts. Historically, the species was widespread and abundant, but its highly *K*-selected life history has made it particularly vulnerable to overfishing (Reynolds *et al.* 2005). Fisheries pressures have been implicated in driving declines in population abundance, and the species is now extirpated from large parts of this range, including habitats within the Mediterranean Sea, North Sea, Irish Sea and English Channel (Dulvy & Reynolds 2002). The last remaining strongholds of common skate include continental shelf-edge habitat off the western coast of Scotland, the western

waters of the Celtic Sea (Ellis *et al.* 2005), and along the Norwegian coast (Dolgov *et al.* 2005). The species is listed as 'Critically Endangered' on the IUCN Red List of Threatened Species, and recently, there was a ban placed on landing common skate within the European Union while species management plans are considered (Clarke 2009).

2. MATERIAL AND METHODS

(a) Sampling

Fin-clips from 123 individuals were collected for phylogeographic study from northern European waters between October 2007 and September 2008 (figure 1; electronic supplementary material, appendix A). Samples were obtained opportunistically from research cruises, by-catch assessments, quantitative surveys and an electronic tagging study. Tissue was immediately preserved in absolute ethanol prior to storage at -20°C . Extraction of genomic DNA was undertaken using the Promega (Madison, WI, USA) Wizard extraction kit.

(b) Phylogeographic and population genetic analyses

The mitochondrial control region was chosen for phylogeographic analysis owing to high nucleotide polymorphism in skate species (Valsecchi *et al.* 2005). DNA sequences for a partial section of this region were generated for all sampled individuals (GenBank accessions GQ392065 to GQ392081, electronic supplementary material, appendix A and for detailed methods, appendix B). To reconstruct genealogical relationships among haplotypes, a maximum parsimony haplotype network was constructed using TCS 1.2.1 (Clement *et al.* 2000).

Individual genotypes were generated for six micro-satellite loci (LER1 21, 33, 34, 40, 44 and 50) following

El Nagar *et al.* (in press). Data were checked for deviations from Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium with GENEPOP 4 (Raymond & Rousset 1995). MICROCHECKER (Van Oosterhout *et al.* 2004) was also used to screen data for genotyping errors. Ordination of data was completed by multi-dimensional scaling in PRIMER 6 (Clarke & Warwick 2001). To test for population structure among samples, Bayesian clustering of samples was conducted with STRUCTURE 2.2 (Pritchard *et al.* 2000) and gene flow between clusters was examined by estimating individual admixture coefficients. For detailed methods see electronic supplementary material, appendix B.

(c) Environmental data

Spatial distributions of phylogenetic clades were explored in relation to environmental variables. Depth data were sourced from the General Bathymetric Chart of the Oceans 30s dataset (<http://www.gebco.net>). Sea surface temperatures at 4 km spatial resolution were sourced from the PO.DAAC Ocean ESIP Tool (POET) dataset (<http://poet.jpl.nasa.gov/>). Substrate data were obtained from UKSeaMap (Connor *et al.* 2006).

(d) Phylogenetics of Rajiformes

To place results in a broader phylogenetic context, samples from an additional 13 skate species were collected (electronic supplementary material, appendix C), and sequenced for partial fragments of the mitochondrial DNA control region and cytochrome *b* gene (GenBank accessions GQ392082 to GQ392133; electronic supplementary material, appendix A). The cytochrome *b* gene was chosen as it can successfully distinguish skate species (Smith *et al.* 2008). Sequences were aligned alongside those of outgroups *Mitsukurina owstoni* (GenBank accession EU528659) and *Scyliorhinus canicula* (GenBank accession Y16067). After confirming homogeneity of phylogenetic signal of the two sequence sets (partition homogeneity test, $p = 0.30$; Swofford 2000), alignments were concatenated and phylogenetic analyses were undertaken using maximum likelihood and Bayesian inference in PHYML (Guindon & Gascuel 2003) and MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001). To estimate divergence times a Bayesian dating approach was also employed (Rutschmann 2004). For detailed methods see electronic supplementary material, appendix B.

3. RESULTS

(a) Population phylogeography

Analysis of partial control region sequences produced a 729 bp alignment with 34 polymorphic sites (electronic supplementary material, appendix D). Two distinct clades were identified (figure 2a). The largest clade included 85 individuals and 15 distinct haplotypes. The second clade was 27 steps removed, and included 38 individuals, but only two haplotypes. The clades had only partially overlapping spatial distributions. The larger 'southern' clade occurred in the Western Approaches and Celtic Sea, extending out to Rockall, while the second 'northern' clade was present off Northern Ireland and Scotland, including the North Sea (figure 1). Only four of the 123 individuals showed deviation from a pattern of complete allopatry; one individual from the 'southern' clade present in inshore waters off the Isle of Lewis, Scotland, one individual from the 'northern'

Figure 2. (a) Network of control region haplotypes. (b) Multi-dimensional scaling plot of genetic similarities of sampled individuals derived from microsatellite DNA allele frequencies. Three individuals genotyped at less than half the loci were removed from the analysis. Red circles, 'southern' clade; green circles, 'northern' clade. (c) Estimated admixture coefficients based on Bayesian clustering ($K = 2$). Each column corresponds to a single individual and is coloured in proportion to its estimated admixture coefficient.

clade present off County Kerry, Ireland, and two individuals caught off County Mayo, Ireland.

Multi-dimensional scaling of microsatellite DNA data clearly delineated into two groups (figure 2b), with membership matching clades identified with mitochondrial DNA. Significant linkage disequilibrium ($p < 0.001$) was identified between loci LERI 40 and 50; therefore, LERI 40 was removed from subsequent analyses. Bayesian clustering in STRUCTURE also identified the most likely number of clusters represented in the data as two (electronic supplementary material, appendix E), allocating individuals accurately to mitochondrial DNA clade. Admixture analysis, using the 'southern' and 'northern' clusters as ancestral sources, identified little evidence of contemporary gene flow, only two individuals had admixture coefficients below 0.99 (figure 2c) with both having

Figure 3. Maximum likelihood phylogeny based on combined control region and cytochrome *b* sequences. Numbers above and below branches indicate maximum likelihood bootstrap percentages and Bayesian inference posterior probabilities, respectively. Bootstrap values less than 50 per cent and posterior probabilities less than 70 per cent not shown. Statistical support within species not shown.

90 per cent probability intervals that encompassed coefficients of 1.0; suggesting no admixture. No microsatellite loci deviated significantly from HWE when samples were split into 'northern' and 'southern' clusters, however all loci deviated significantly when samples were pooled together, consistent with a Wahlund effect and significant population structure within the pooled sample, but not within the two clusters (electronic supplementary material, appendix F).

(b) *Phylogenetic analysis*

The cytochrome *b* alignment was 414 bp in length with 164 sites polymorphic and 143 parsimony informative. The control region alignment was 811 bp in length with 564 sites polymorphic and 461 parsimony informative (electronic supplementary material, appendix G). Phylogenetic reconstructions of northern European skates showed strong support for division of sampled Rajidae into two sub-families (Rajinae and Arhynchobatinae), and three described tribes (Arhynchobatini, Rajini and Amblyrajini; figure 3). Generic divisions (*Bathyrhaja*, *Rajella*, *Amblyraja*, *Leucoraja*, *Raja* and *Dipturus*) also received strong statistical support, with all reciprocally monophyletic. Critically, the reconstruction demonstrated strong support for the monophyly of the 'northern' and 'southern' clades of *D. batis* with respect to other *Dipturus* species, but the position of the two clades relative to longnosed skate (*Dipturus oxyrinchus*) remained unresolved. Assuming a 31 Ma divergence between Rajini and Amblyrajini (Valsecchi *et al.* 2005), divergence between 'northern' *D. batis*, 'southern' *D. batis* and *D. oxyrinchus* took place 4.1 Ma (95% credibility interval, 2.1–7.0 Myr ago; electronic supplementary material, appendix H).

(c) *Environmental correlates of clade distribution*

Both *D. batis* clades were sampled across a range of substrates, including deep ocean rises, shelf sand plains, shelf coarse sediment plains and shelf mounds. The 'northern' clade was also present in sea lochs and shelf mud plains. There was no clear distinction between clades in water depth at capture location (figure 1), with the 'southern' clade found between 54 and 311 m (mean: 129 m), and the 'northern' clade sampled between 44 and 176 m (mean: 109 m). The 'southern' clade was present in waters with surface temperatures ranging from 8°C in winter to 19.7°C summer, while the 'northern' clade was present in temperatures ranging from 6.4°C in winter to 16.9°C in summer (figure 4). Despite this overlap, the data indicate potential thermal limits to apparent distributions of these clades. Specifically, the 'southern' clade was found in waters with surface summer temperatures at least 2.8°C warmer in summer, while the 'northern' clade occurred in waters with surface winter temperatures 1.6°C colder in winter (figure 4).

4. DISCUSSION

We tested for evidence of population genetic differences among samples of common skate from northern European waters. Our data revealed two evolutionarily distinct lineages, with largely allopatric distributions. The consistency of genetic differences between mitochondrial and nuclear markers, remarkably low level of admixture and strong evidence of monophyly of the clades, suggests the occurrence of two species. The alternative hypothesis of strong spatial genetic structuring

Figure 4. Distribution of 'northern' and 'southern' clades relative to sea surface temperature in (a) September and (b) March. Data are averaged over a 5-year period (2004–2008, both inclusive).

among individuals that exhibit no intrinsic reproductive barrier is unlikely for several reasons. First, while the two clades appear largely allopatric, overlap was present yet reproductive isolation has been maintained. Second, studies of other European skates across much larger spatial scales have revealed only relatively weak genetic differences (Chevolot *et al.* 2006, 2007). By contrast, the 'northern' and 'southern' clades of common skate could only be joined in a statistical parsimony network if the connection limit was dropped well below the recently proposed standard for designating operational species (Hart & Sunday 2007). Finally, the most likely estimate of divergence time between the clades was relatively ancient (4.1 Ma; 95% CI 2.1–7.0), and older than the estimated divergence times of *Amblyraja hyperborea* and *A. radiata* (2.3 Ma; 95% CI 1.0–4.2), and the divergence of *Raja microocellata* and *R. brachyura* (3.0 Ma; 95% CI 1.3–5.3).

Estimates of time from divergence in skate correspond to a period of cooling in the Arctic and Atlantic Oceans approximately 4 Ma, which has been hypothesized as contributing to divergence between species of *Macoma* bivalves (Nikula *et al.* 2007). In the common skate, divergence could have been related to onset of cooler conditions emerging in the Arctic at that time, similar to suggestions that divergence in wobbegong sharks in Australia was ecologically driven and associated with the occupation of novel habitats (Corrigan & Beheregaray 2009). An alternative explanation is that divergence was related to isolation within glacial refugia following the onset of Pleistocene (approx. 2.6 Ma) ice sheet expansion (Hewitt 1996), as has been suggested for cryptic bryozoan species in the northeast Atlantic (Gomez *et al.* 2007). The comparatively low mitochondrial DNA diversity of the 'northern' clade of common skate relative to the 'southern' clade is also consistent with reductions of genetic diversity during glacial advances, but distinguishing between these scenarios will require sampling other *Dipturus* species, and resolution of the phylogeny of the sampled *Dipturus*.

The conserved body morphology of skates, and their restriction to benthic habitats, belies a group with a high degree of biodiversity and endemism (Ebert & Compagno 2007). Many skate species are spatially segregated because of distinct patterns of habitat use

(Arkhipkin *et al.* 2008), and our results show some evidence for potential thermal limits to northerly and southerly distributions of the *D. batis* clades (figure 4). These analyses are based on surface water temperatures, which may not be representative of sea bottom temperature during summer months when the shelf seas become seasonally stratified. However, electronic tagging of common skate off the west coast of Scotland has revealed extensive vertical movements in summer, including near surface occurrence (Wearmouth & Sims 2009), suggesting our analyses capture at least part of the thermal regime to which fish are exposed. If correct, the identification of thermal limits has important implications for species distributions, given past and projected climate change.

The phylogeny of northern European species in this study extends that of previous work demonstrating monophyly of tribes and genera within European Rajiformes (Tinti *et al.* 2003; Turan 2008). The broad taxonomic sampling was also useful in excluding the possibility that samples were misidentifications, as the majority of skate species commonly encountered in the region were included. In light of our results, formal taxonomic interpretation of *D. batis* now requires investigation. Historically, several large species of skate have been listed from the northeastern Atlantic, but have been subsequently synonymized with *D. batis*. Therefore, whether our results represent the rediscovery of a formerly described species, or identification of a new taxon is at present unclear. Detailed morphological study of samples collected over wider spatial scales may help to uncover diagnostic features that separate these genetically segregated species. Until this taxonomic uncertainty is resolved, we regard these two taxa as representing 'cryptic' species, following Bickford *et al.* (2007) who considers 'two or more species to be cryptic if they are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable'.

The results of this study have important conservation implications for the common skate. They have identified a new taxon for IUCN Red List assessment, and current estimates of population distributions and abundances of *D. batis* will require revision. In this case, we consider it possible that major declines have actually taken place in

two endangered species with only partially overlapping distributions. Given our study only covered a section of the reported range of common skate, there is a need for spatial surveys of the distribution of both taxa. The use of the molecular markers from this study may help to distinguish species, and identify breeding grounds and nursery areas if required for conservation measures. Importantly, our finding of cryptic species within *D. batis* adds to a body of evidence identifying evolutionarily significant units within vulnerable elasmobranch species. Using similar methods, cryptic species have also been identified in hound sharks (*Mustelus* sp.; Gardner & Ward 2002), scalloped hammerhead sharks (*Sphyrna* sp.; Quattro *et al.* 2006) and wobbegong sharks (*Orectolobus* sp.; Corrigan *et al.* 2008), and these methods have helped to confirm the taxonomic distinctness of Australian river sharks (Wynen *et al.* 2009). They have also helped to identify strong spatial genetic differences within coastal elasmobranchs, for example grey nurse sharks (Ahonen *et al.* 2009). Overall, these studies strongly indicate that significant marine diversity remains to be discovered. Our results from common skate demonstrate that this is applicable to even apparently well-known ecosystems, such as the Western European shelf margins.

All sampling procedures were approved by the Marine Biological Association (MBA) Ethical Review Committee and were licensed by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

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