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Assessing the genetic connectivity of two octocoral species in the Northeast Atlantic

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Foreword

Natural England commission a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Background

Understanding patterns of connectivity for species of conservation concern is crucial in the design of networks of ecologically coherent Marine Protected Areas (MPAs) and connectivity is one of a number of principles considered in the design of such a network currently being enacted by the UK Governments. However, data concerning connectivity are deficient for many invertebrate sessile taxa. This study was commissioned to assess the population genetic structure and genetic connectivity of two temperate octocoral species around southwest Britain and the North East Atlantic *Eunicella verrucosa* and *Alcyonium digitatum*.

Eunicella verrucosa is a threatened, IUCN red-listed sea fan and is recognised as a species of principal importance in English waters.

It has been specifically identified for protection within a UK MPA network.

Alcyonium digitatum is a soft coral and a common on rocky reefs and on a broad range of subtidal rock habitats. As such it will be represented in the UK MPA network.

The findings of this work have been, and will continue to be, used to help design and deliver the UK marine conservation policy and to inform the implementation of the MPA networks around the UK.

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Further information

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Eunicella verrucosa genetic library 1 was constructed at the Evolutionary Genetics Core Facility, Cornell University, USA. The isolation of a second library for *E. verrucosa* and a library for *Alcyonium digitatum*, together with marker development and genotyping, was undertaken at the NERC Biomolecular Analysis Facility (NBAF) at the University of Sheffield (Grant No. NBAF-362); Deborah Dawson, Gavin Horsburgh, Andy Krupa, Maria-Elena Mannarelli and Terry Burke (Sheffield) and Jill Lovell and Andy Gillies (Edinburgh) provided technical assistance and advice.

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Summary

Elucidating patterns of connectivity for species of conservation concern is crucial in the design of networks of ecologically coherent marine protected areas (MPAs); as such, connectivity is one of a number of key factors considered in the design of such a network currently being enacted by the UK Governments. However, data concerning connectivity are deficient for many invertebrate sessile taxa. This study assessed the population genetic structure and genetic connectivity of two temperate octocoral species, *Eunicella verrucosa* and *Alcyonium digitatum*, around southwest Britain and the North East Atlantic. To achieve this objective, two novel panels of microsatellite loci were developed and screened in multiple populations of each species from across the study area. Microsatellite panels for both species showed evidence of cross-species amplification, and should prove useful as monitoring tools for the target species and some congeneric species.

Eunicella verrucosa, a threatened and IUCN red-listed sea fan, is recognised as a species of principal importance in English waters and has been specifically identified for protection within a UK MPA network. In this study, *E. verrucosa* was sampled from the northerly extremes of its eastern Atlantic range, from southwest Britain (England and Wales), western Ireland, northwest France and southern Portugal. Our findings suggest that within this area connectivity appears to be defined at regional scales, and localised cases of inbreeding and differentiation suggest that the population structure of this species may best be described as a meta-population. *Alcyonium digitatum*, a soft coral, was sampled in the central portion of its range from southwest Britain and the North Sea, western Ireland and northwest France; *A. digitatum* is a common species of rocky reefs and a broad range of subtidal rock habitats, and as such will be represented in the UK MPA network. *A. digitatum* exhibited very little population structure and showed apparent panmixia across the sampled range. However, high levels of heterozygote deficiencies and inbreeding in the majority of populations implies that the genetic structure of some populations of this species are defined by self-seeding and rarer dispersal events that nonetheless occur sufficiently often to counteract divergence due to genetic drift. Coalescent analyses indicated that in both species, migration between regions occurs asymmetrically. The presence of few duplicate genotypes in both data sets suggests that sexual reproduction predominates in both species across the sampled area.

These findings have implications for marine conservation policy. Within UK waters the genetic data identified no significant barriers to gene flow in *A. digitatum*. For *E. verrucosa*, the situation appears more complex: connectivity within the UK range of this species appears robust and no significant genetic variants between *E. verrucosa* populations in English and Welsh waters were observed. However, UK populations were distinct from Irish, French and Portuguese populations, suggesting limited connectivity between regions and a need to safeguard that portion of the overall genetic diversity of the species present in UK waters.

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1. Introduction

1.1 Defining marine connectivity

Assessing population divergence and inferring how diversity is governed by import and export of individuals between populations is achieved by assessing 'connectivity'.

Connectivity has been broadly defined as "the extent to which populations in different parts of a species' range are linked by exchange of larvae, recruits, juveniles or adults" (Palumbi 2003). Understanding connectivity is a priority for the sustainable management of resources, the tracking of invasive species pathways, the determination of the impact of climate change and the design of protected areas (Levin 2006). Connectivity may also be defined as being either demographic or genetic (reviewed in Lowe and Allendorf 2010). Demographic connectivity refers to how population growth, or survival and birth rates, are affected by immigration, emigration, and recruitment (Thomas and Bell 2013), whereas genetic connectivity refers to how the extent of gene flow from migration mitigates divergence through genetic drift within subpopulations (Lowe and Allendorf 2010). The work reported here represents a study of genetic connectivity.

In marine populations, the number of individuals maintaining each type of connectivity is often plotted as a function of dispersal distance from source populations in a so called 'dispersal kernel' (eg, Steneck 2006, Steneck *et al.* 2009). In such diagrams, sufficient genetic connectivity to prevent local extinctions can be achieved by very few individuals relative to the large proportion of individuals that maintain demographic connectivity. Gene flow resulting from as little as one immigrant per generation may spread advantageous alleles, may mitigate local inbreeding effects or may act to maintain similar allele frequencies (termed 'adaptive', 'inbreeding' and 'drift' genetic connectivity respectively; Lowe and Allendorf 2010). Demographic connectivity is thought to stabilise populations, in cases where immigration compensates for low recruitment in the resident population, or at larger geographic scales where colonization of new unoccupied patches compensates for high extinction rates in occupied patches (Lowe and Allendorf 2010). Marine populations are often described as 'open' or 'closed'; these concepts are also related to connectivity and describe the relative rates of recruitment from distant vs local sources, respectively. An open population implies that the extent of larval dispersal is great given the potentially large distance propagules may travel on oceanic currents, coupled with observed genetic homogeneity over large spatial scales (Cowen *et al.* 2000). Thus, the term 'open' can relate to both demographic and genetic connectivity, and distinguishing between them is important to prevent false assumptions that the extent in demographic openness will result in a corresponding change in genetic openness (Johnson 2005).

1.2. Using population genetics to infer connectivity

Inferring the extent of gene flow (and thus genetic connectivity) within and between populations based upon variations in allele frequencies (ie, heterozygosity) is usually calculated using Wright's F-statistic, which describes the correlation between two randomly chosen alleles within subpopulations relative to two alleles randomly sampled from the total population. As such, the extent of inbreeding can also be determined due to the correlation between alleles if they occur in the same subpopulation (Wright 1951, Balloux and Lugon-Moulin 2002).

Molecular techniques are currently the only means through which genetic connectivity and recruitment can be directly measured and they can be used to determine gene flow indirectly by estimating the extent and pattern of genetic divergence between populations, or more directly by assignment of individuals to a particular subpopulation or parental combination (Lowe and Allendorf 2010). A wide selection of molecular techniques is available for detecting allelic variants with genetic markers (=genotyping). Methods vary in cost and resolution, but all are based upon statistical validation of differences between either DNA sequences or the frequency and composition of alleles (copies of a gene of interest) within and between individuals from the populations of interest (see Ryman et al. 2006). As genes recombine and exhibit inter-generational variation (leading to genetic drift), assignment of marine larvae to a source adult population or individual can be inferred from population genetic models derived from evolutionary theory (Hellberg et al. 2002).

Laboratory techniques to assess genetic connectivity take either a multi-locus or single-locus approach, ie, genetic information is derived from alleles found in several genomic positions or in just one. In addition, genetic markers may be dominant (eg, RAPDs or AFLPs) or codominant (eg, microsatellites or SNPs). Dominant markers generally identify variation in anonymous genes from unidentified regions within the genome simultaneously; the methodology is relatively quick and affordable, and high levels of variation between individuals can be demonstrated. Critically, however, dominant markers allow only presence and/or absence scoring (in which dominance alone is detectable and can be non-comparable between studies; see Sunnucks 2000), meaning that individual alleles are not recognised, with a resulting loss in information content. Use of codominant markers –such as microsatellite loci– permit both allele variants from co-dominant inheritance to be scored and, therefore, evolutionary models based upon Mendelian inheritance can be applied (Hellberg et al. 2002). Microsatellites are used in the research outlined in this report.

Microsatellites, or simple sequence repeats (SSRs), are short tandem repeats of DNA motifs

typically of 2-10 base pairs (Hellberg et al. 2002). Microsatellites are thought to have some functional roles –chromatin organization, regulation of DNA metabolic processes and gene regulation– although, as they constitute a high proportion of non-coding DNA relative to protein-coding regions, they are generally considered to be evolutionary neutral markers (a contestable concept, see Li 2002). As genetic markers, microsatellites have several favourable properties for inferring population structure: they have relatively high rates of mutation and are highly polymorphic, multi-allelic and co-dominant (Andreakis et al. 2009). As neutral markers, the extent of polymorphism is proportional to their underlying rate of mutation, and evolutionary models can be readily applied to microsatellite size and frequency data to infer population divergence (Ellegren 2004). As such, microsatellites are extremely popular in assessing connectivity in marine populations. This is particularly evident, for example, from both fisheries and marine invertebrate research, where microsatellites have been used to infer source populations, to examine migratory routes and to examine the effects of over-fishing on genetic diversity (eg, Miller et al. 2009).

In summary, molecular techniques have been employed with increasing frequency for conservation purposes, for example, in fisheries management and in marine reserve design (Hellberg et al. 2002; Hedgecock et al. 2007). Importantly, molecular markers can give an empirical measure of realized gene flow (ie, a measure of inter-generational genetic input mediated by migration), as opposed to theoretical values (eg, inferred from but not measured directly from ocean currents); nonetheless, estimates based on population genetic statistics are only as good as the underlying resolution and accuracy of the molecular markers used, and should always be interpreted with caution.

In the current study, two novel panels of microsatellite loci were developed (Appendices 1 and 2) and screened in multiple populations of two octocoral species, *Eunicella verrucosa* and *Alcyonium digitatum*, from Britain, Ireland, France and Portugal. These new markers allowed gene flow and connectivity to be explored both within English and Welsh waters, and also with populations in neighbouring countries, allowing wider source-sink dynamics to be explored.

1.3. Marine protected areas

In Europe, commitments to marine environmental protection are being implemented under several policies, including the OSPAR convention (1992) - an amalgamation of the 1972 Oslo and 1974 Paris conventions that were developed initially to address pollution risk in the NE Atlantic, which now has fifteen signatories (<http://www.ospar.org>). More recently, the EU Marine Strategy Framework Directive (MSFD 2008) has been developed which requires that

each member state establish 'good environmental status' and 'coherent and representative networks' of MPAs by 2020 (eg, <http://www.defra.gov.uk>).

Commitments to develop an MPA network in the UK are being met by the wider OSPAR network (Evidence Review MCZ Final Report, Defra 2013) as well as the Marine (Scotland) Act, the Northern Ireland Bill and the Marine and Coastal Access Bill of 2009, which implements initiatives in marine planning, licensing, marine conservation and coastal access. The new MPA network will include and develop upon areas previously protected under European legislation, including European Marine Sites: Special Areas of Conservation (SAC) and Special Protection Areas (SPA); marine areas of Ramsar sites (for protection of wetlands); Sites of Scientific Special Interest (SSSIs); Marine Conservation Zones (MCZs) in English, Welsh seas and Northern Irish waters and Nature Conservation MPAs which include Scottish inshore and (Evidence Review MCZ Final Report: Defra 2013).

In England and adjacent offshore waters (the primary region under consideration in this study) development of proposals for national MCZ designations was conducted between 2008-2010 by four regional stakeholder groups, in line with national guidance from the UK government statutory nature conservation bodies, Natural England and the Joint Nature Conservation Committee (JNCC) and Defra (2008). The four English regional groups were Finding Sanctuary (southwest England), Balanced Seas (southeast England) Net Gain (the North Sea) and Irish Sea Conservation Zones (the Irish Sea excluding Welsh and Irish territorial waters) (Defra 2013). In addition, an independent scientific advisory panel of primarily academic marine experts was appointed to support the work of the four regional groups, and each regional group worked with the scientists and stakeholders to decide upon potential areas to be designated as MCZs following the requirements of the Ecological Network Guidance (Natural England and JNCC 2010). The groups' final recommendations were submitted to Natural England and JNCC who provided formal advice alongside the recommendations in summer 2012 (Natural England & JNCC 2012; Defra 2013). Thirty-one of the 127 proposed sites were accepted by the UK government for further consideration in December 2012; final consultation on these sites continued until March, 2013 (Natural England 2013a) and 27 sites in English waters were designated as MPAs in November 2013 (see: <https://www.gov.uk/government/collections/marine-conservation-zone-2013-designations>). Two additional tranches of sites for consideration as MPAs have subsequently been announced.

To date, however, there appear to be relatively few cases where genetic assessment of connectivity has been incorporated into MPA network design. An explicit requirement to

consider genetic data was not included in the original ecological network guidance (Natural England & JNCC 2010) for UK MPAs, though connectivity was identified as one of the seven key principles (Principle 5) for network design (Defra 2008; Natural England & JNCC 2010), a principle restated by the Joint Administrations statement of 2012. Thus, the need for incorporating molecular population data into MPA network design is widely recognised (eg, von der Heyden 2009). This study goes some way towards addressing this deficit for invertebrate taxa in English and European waters.

1.4. Study organisms

This study focused upon two important octocoral species in the UK and North East Atlantic, the 'pink sea fan' *Eunicella verrucosa* and 'dead man's fingers' *Alcyonium digitatum*.

Eunicella verrucosa is an IUCN red-listed species; it is recognised as a species of principal importance in English waters and has been specifically identified for protection within a UK MPA network (see: <http://www.naturalengland.org.uk/ourwork/conservation/biodiversity/protectandmanage/habsandspeciesimportance.aspx>). It is subject to damage from bottom trawling activity, it is protected in the UK under the Wildlife and Countryside Act of 1981 and is also a UK Biodiversity Action Plan (BAP) species (Hall-Spencer et al. 2007). Despite an extensive range in the North East Atlantic, from Angola to western Ireland, its range in the British Isles is limited to the South West, Pembrokeshire and southern and western Ireland (eg, Grasshoff 1992). *Alcyonium digitatum* is a common species of rocky reefs and a broad range of subtidal rock habitats, and as such will be represented in the UK MPA network. It has an extensive trans-Atlantic range that spans Portugal to Norway, Iceland and the North Sea in the NE Atlantic, and eastern Canada to Cape Hatteras in the USA in the NW Atlantic (Hartnoll 1975, Watling and Auster 2005). *Alcyonium digitatum* is not protected, but it is locally depleted in some areas by benthic trawling (Hinz et al. 2011). Both species often co-exist in the same habitat and they are often presented in publicity materials to promote UK-based marine conservation efforts. Colonies representative of specimens found in UK waters are shown in Figure 1.

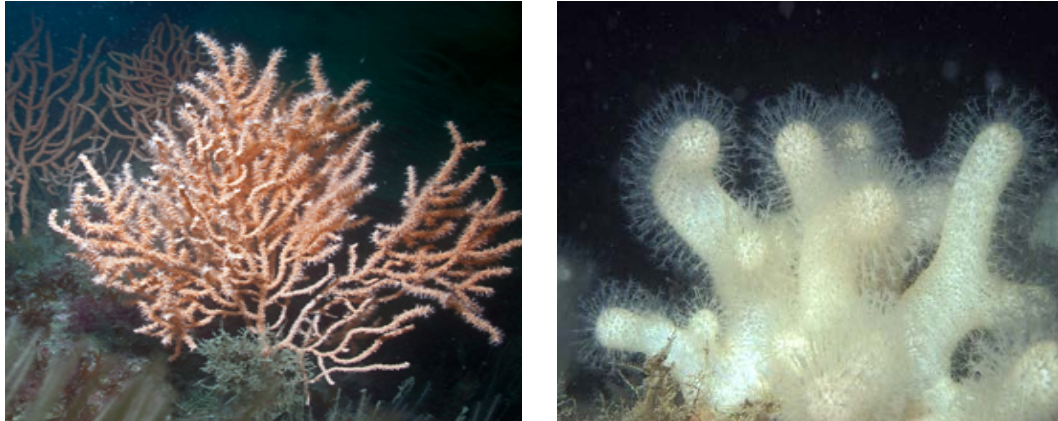


Figure 1. Examples of octocorals of the UK and NE Atlantic: *Eunicella verrucosa* (left) pink morph (© Paul Kay, from naturalengland.org.uk); *Alcyonium digitatum* (right) white morph (© Keith Hiscock, marlin.ac.uk).

1.4.1. *Alcyonium digitatum* (Linnaeus, 1758)

Alcyonium digitatum (O. Alcyonacea: S. O. Alcyoniina: F. Alcyoniidae)

Members of the order Alcyoniina, of which Alcyoniidae is the eponymous family, are commonly termed ‘true soft corals’ and lack an internal proteinaceous axis such as those found in holaxonians (ie sea fans and sea rods). As such, they are usually lobate or digitate, and fleshy or encrusting and rely upon hydrostatic pressure for support (Fabricius and Alderslade 2001). *Alcyonium* is a highly speciose genus with a circum-global distribution spanning both polar and tropical regions, and including the Mediterranean, and Pacific, and Atlantic Oceans (van Ofwegen *et al.*2001). There are between 75 – 135 species, all of which are thought to be heterotrophic passive suspension feeders, typically found on rocky overhangs and ledges up to forty metres deep, although some species may occur in soft sediment (McFadden *et al.*2001). Taxonomic relationships within this genus are often poorly resolved (eg, McFadden 1999) and, as is the case for most coral and octocoral taxa, species descriptions have typically been based upon morphological characterisation of their spicules. It is clear that the full extent of molecular and morphological diversity within this genus is still unknown and a large depth distribution of some species of >1000m has meant that some species have only recently been discovered and described, eg, *Alcyonium megasclerum* at 1000-1350m and *A. profundum* at 2200 – 2600m in Cape Verde (Stokvis and Ofwegen 2006).

1.4.2. *Eunicella verrucosa* (Pallas, 1766)

Eunicella verrucosa (O. Alcyonacea: S. O. Holaxonia: F. Gorgoniidae),

Eunicella Verrill 1869 is an octocoral genus within the suborder Holaxonia (an unspiculated, proteinaceous axis with a hollow core, Fabricius and Alderslade 2001) comprising approximately 36 species. At least nine of these are found in the eastern Atlantic, primarily in western Africa, the Atlantic coasts of Europe and the Mediterranean. Based on sclerite analysis, the genus has been ascribed to the family Gorgoniidae (eg, Grasshof 1992); Gorgoniidae are characterised by small sclerites (Fabricius and Alderslade 2001), often reticulating flabellate forms and includes the charismatic sea fans and sea plumes, well known from tropical coral reefs. In the northeast Atlantic and Mediterranean, three species of *Eunicella* are well represented, *E. verrucosa*, *E. cavolini* and *E. singularis*, the latter two being among the most prevalent octocorals (along with *Paramuricea clavata* and *Corallium rubrum*) in rocky sublittoral communities of the western Mediterranean (Gori et al. 2007; Sartoretto and Francour 2012). *Eunicella singularis* is the only known species of the genus to harbour symbiotic dinoflagellates of the genus *Symbiodinium* more commonly associated with tropical Anthozoa, although mostly at shallow depths as deeper colonies are typically azooxanthellate (Gori et al 2011). *Eunicella cavolini* and *E. verrucosa* are obligate heterotrophs and the latter has been shown to switch its diet seasonally from zooplankton in the winter months to sedimentary organic matter in the summer (Cocito et al 2013). *Eunicella verrucosa* is also the only species of the three to occur in the UK, where it has a limited distribution and is threatened by anthropogenic activity and is therefore a protected species. As for many anthozoans, morphological variants are recognised in *E. verrucosa* which has two colour morphs - colonies are either pink or white, although they can also be pale orange or in some cases exhibit patches of several colours from pink to pale brown on the same colony (personal observations). *Eunicella singularis* is also known to display two distinct morphotypes where colonies vary in branching pattern with depth (Gori et al 2012). Besides its limited UK distribution, *E. verrucosa* has an extensive range in the eastern Atlantic, which extends from Angola to western Ireland and includes Cape Verde, the Canary Islands and Madeira (Grasshof 1992, Stiasny 1936). It is also prevalent in the western Mediterranean basin, but has a more sporadic distribution in Spain, France and the Tyrrhenian Sea (Sartoretto and Francour 2012). Its distribution therefore spans the marine provinces of the Gulf of Guinea, West African Transition, Lusitanian, Mediterranean and the southern part of the Northern European Seas (or Boreal) (as defined by Spalding et al 2007). *Eunicella verrucosa* typically inhabits rocky substrates in areas of high turbidity and moderate to high water flow at upper and lower circa-littoral depths between 10-155m, although colonies occasionally extend deeper. In the UK, the distribution of *E. verrucosa* is restricted to the

southwest of England, southern Wales and the south and west coasts of Ireland where, despite high abundance and density in some areas, it is considered nationally rare but locally common (Hiscock et al 2010). Moreover, some colonies are found as shallow as 4m, therefore the UK likely constitutes the most northerly and shallowest portion of the species' range with large peripheral populations are located in Donegal Bay, Ireland, Pembrokeshire, Wales and Worbarrow Bay, Dorset, England (Tinsley 2005, MarLIN.co.uk).

1.5. Aims and objectives

The aim of this research is to provide the first assessment of genetic connectivity and population structure in *Eunicella verrucosa* and *Alcyonium digitatum* in southwest Britain, and to determine the extent of connectivity between populations in this area and beyond at a variety of spatial scales. This research was commissioned by Natural England in 2008 (Contract no. SAE 03-02-146; project No. RP0286) to fill an evidence gap identified by other Natural England commissioned research and to coincide with the design and development of the UK's first Marine Protected Area network; the project ran from 2008 – 2012. Few molecular markers are available for temperate Anthozoa, and none are available to determine population-level relationships within these species. Therefore, this research comprised two parts:

- 1) Development of novel microsatellite markers for each species.

The objective of this part of the project was to identify robust molecular markers to allow accurate assessment of genetic connectivity and population structure in each study species. These microsatellite loci represent (to our knowledge) the first developed from octocorals in the northeast Atlantic, including the UK, and are the first developed for each species.

- 2) Assessment of genetic connectivity of *Eunicella verrucosa* and *Alcyonium digitatum* in UK waters and the proximal areas of the northeast Atlantic.

Using the microsatellites (Objective 1), population structure and genetic connectivity was assessed in *Eunicella verrucosa* and *Alcyonium digitatum* in UK waters (with particular focus on southwest England and Wales), northwest France, western Ireland, and Portugal (in the case of *E. verrucosa*) and the North Sea (in the case of *A. digitatum*). The objective of this research was to gain an overview into the genetic diversity within and between populations of each species, and, subsequently, to make inferences about the degree of genetic connectivity at various spatial scales, (from

adjacent sites to regionally disparate populations) and to identify the potential vulnerability of each species.

Despite the reproductive biology of each species being relatively poorly understood, we hypothesized that genetic structure would be less pronounced in *A. digitatum*. Anecdotal evidence suggests that this species is likely to produce lecithotrophic larvae, as does *E. verrucosa*, although *A. digitatum* is unusual in that it spawns hibernally and as such may be more exposed to wind-driven surface currents.

2. Molecular methods and microsatellite isolation

2.1. Site selection

Samples of *Eunicella verrucosa* were collected at thirty sites ranging from southern Portugal to western Ireland, including sites in northwest France and southwest England and Wales (Figure 2). Samples from Pembrokeshire, Wales and Lyme Bay, Dorset represent the most northerly and easterly known locations of *E. verrucosa*, respectively, within the UK; samples from St. John's Point, Donegal Bay, Ireland and Lyme Bay, Dorset represent the most northerly and easterly known locations of *E. verrucosa*, respectively, across the species range. Samples of *Alcyonium digitatum* were collected from twenty-one sites across the same region, together with additional samples from two sites in the North Sea; these sites cover the more southerly end of this species' range in Europe (Tables 1 and 2).



Figure 2. Map showing the 30 sampling locations of *Eunicella verrucosa* analysed (see also Table 1) in the NE Atlantic and Mediterranean. Exact coordinates were not available at all sites and some points are approximate.

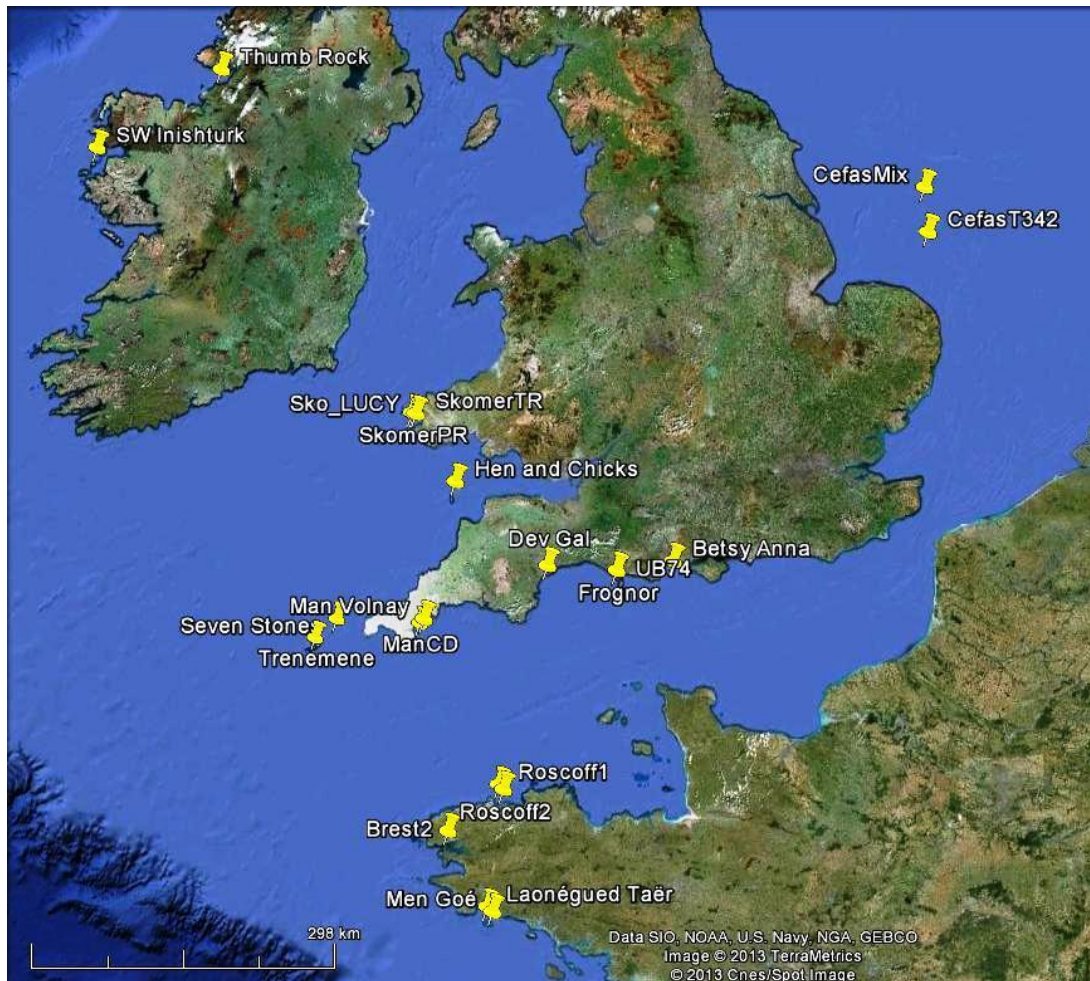


Figure 3. Map showing the 21 sampling locations of *Alcyonium digitatum* analysed (see also Table 2) from Britain, northwest France and Ireland. Exact coordinates were not available at all sites and some points are approximate.

2.2. Sample collection

All octocoral samples used in this study were collected between September 2008 and May 2012, with the exception of several *Eunicella verrucosa* colonies that were collected from Skomer Marine Reserve in Pembrokeshire, Wales in 2007. Virtually all samples were collected by SCUBA diving by J. R. Stevens, J. Kent, L. Holland and many volunteer divers (see acknowledgements); additionally, two sets of *A. digitatum* were collected from the east coast of England during bottom trawls conducted during CEFAS research cruises ('CEFAS MIX' and 'T342 CEFAS', Table 2). Due to depth and time restrictions imposed by SCUBA, typical depths of populations sampled were forty metres or shallower. All samples of *Alcyonium digitatum* were collected specifically for this research. Similarly, the majority of *Eunicella verrucosa* samples were collected specifically for the project, though some additional samples were acquired from previously collected populations in the Marseille area of France, southern Portugal ('EvARM', Table 1), and several aquaria-maintained colonies

originating from near Padstow on the north coast of Cornwall were provided by London Zoo ('nr Padstow', Table 1).

At each site, *E. verrucosa* colonies were sampled by removing a 3 cm terminal branch using sea-snips; as this species is protected in the UK, all specimens were collected according to UK wildlife licensing laws as per the terms and conditions of Natural England licences granted to J. R. Stevens (Natural England licences 20080861 and 20090943) and subsequently by the Marine Management Organisation (license number MMO-001). Wherever possible in sites with high sea fan abundance, we avoided small (juvenile) colonies and tried to sample from larger, apparently healthy individual sea fans approximately 1m apart, although as we attempted to sample a minimum of forty individuals per site (in order to maximise statistical validity of genotypic data), it was not always feasible to sample only from larger fans. No colonies were tagged or tracked *in situ* during this study, although most individuals collected at Skomer Marine Reserve are well mapped and known individually to staff of the Countryside Council for Wales (now Natural Resources Wales [NRW]); therefore, genotypes of these individual colonies could theoretically be assigned to a known living individual.

A. digitatum colonies were sampled by removing a 1 cm³ section of tissue from the end of a thumb-like 'branch' with sea-snips. As with *E. verrucosa*, where possible samples were taken from individual colonies spaced at least one metre apart, to avoid sampling clonal individuals (duplicate genotypes); other studies of hard corals have identified potentially clonal individuals at spatial scales from 5m apart (eg, Goffredo *et al.* 2009; Foster *et al.* 2012). For both species, however, distribution of samples was highly site-specific and dive time limitations meant that collecting samples over a wide bottom area was not always possible. Additionally, given ambiguity over reproductive strategies employed by the two species, and their unknown pelagic larval durations and potential distances of larval dispersal, this strategy may be a somewhat crude means by which to avoid collecting duplicates. Thus, following genotyping, duplicate haplotypes were detected and removed prior to statistical analysis.

During sample collection, individuals were placed into mesh dive bags and immediately after collection (either on the boat, on the dock or in the laboratory), were divided into batches of up to ten individuals (depending upon the size of the sample taken) and placed into 50ml falcon tubes containing 100% ethanol, roughly corresponding to a 1:5 – 1:10 volume of sample tissue to alcohol. With larger *A. digitatum* fragments, incisions were made into the tissue (taking care not to cut through the sample), to maximize entry of ethanol into the internal tissues. Several sets of donated samples appeared to be degraded and thus when

conducting our own sampling this step was carried out to minimise endonuclease activity prior to DNA extraction. Ethanol was changed within twenty-four hours of collection, and was usually replaced twice in order to reduce levels of degraded and mucous material. Samples were then catalogued, recorded in the project database and placed individually into glass vials containing 100% ethanol, which were then stored in cold room facilities at the University of Exeter at 4°C pending analysis; in some instances, a small amount of tissue was removed straight after processing and placed in 1.5ml microcentrifuge tubes containing ethanol during cataloguing of samples for immediate DNA extraction. Following initial processing, timing of subsequent analysis steps did not appear to affect the success of molecular analysis, suggesting that preservation and storage protocol used is robust.

Table 1. List of sampling sites for *Eunicella verrucosa*. Bold type (Code) indicates sites or areas in which *Alcyonium digitatum* was also sampled. * The number of white colour morph colonies sampled at some sites is indicated; all other sampled colonies were pink. Some samples were donated to the project; however, when preserved in ethanol, pigment leached out of the colonies and they appeared white. Therefore, the original colour morph of donated these colonies cannot be confirmed (shown as n/a). N represents samples included in the final dataset and not the number collected or extracted; these N individuals amplified in at least 11/14 loci. Samples from EvMai were donated and exact coordinates were not supplied for this sample. Samples are colour coded here and in subsequent results into the following groups; **BLUE** = Britain, **PINK** = Ireland, **YELLOW** = Portugal, **RED** = Mediterranean (one site) and **GREEN** = northwest France.

Country	Code	N	Colour Morph*	Date Collected	GPS	Site
U.K.	DevBF	40	pink	5.6.09	50°20'4.73"N 4° 8'52.09"W	Bovisand, Plymouth Sound, Devon
U.K.	PlyMew	44	pink	3.7.09	50°18'38.00"N 4° 6'30.55"W	Mewstone Ledges, Plymouth Sound, Devon
U.K.	DevHD	36	pink	8.08.09	50°12'30.60"N 4°20'33.60"W	Hand Deeps, Plymouth Sound, Devon
U.K.	loSHath	30	pink	4.8.10	49°52'57.12"N 6°20'59.91"W	Hathor, Isles of Scilly, Cornwall
U.K.	loSLR	22	pink	11.06.09	49°58'60.00"N 6°18'48.00"W	Lion Rock, Isles of Scilly, Cornwall
U.K.	loSnnw	23	20 pink, 3 white	11.06.09	49°58'7.20"N 6°15'19.20"W	NNW Flat Ledge, Isles of Scilly, Cornwall
U.K.	JTEten	7	pink	07/2009	50°39'11.27"N 2°53'10.65"W	East Tennents Reef, Lyme Bay, Dorset
U.K.	Lundy	22	pink	9.8.09 / 20.9.09	51°10'19.80"N 4°41'15.60"W	Lundy Island, Devon (Battery N=19, Jenny's Cove N=3)
U.K.	LymeHW	9	pink	22.4.09	50°40'31.80"N 2°56'7.50"W	Heroine (shipwreck), Lyme Bay, Dorset
U.K.	ManMo	30	pink	21.03.10	50° 2'45.66"N 5° 2'40.02"W	SS Mohegan (shipwreck) Manacles rocks, Cornwall
U.K.	ManRR	43	pink	23.3.09	50° 2'40.02"N 5° 2'32.22"W	Manacles, Raglan Rocks, Cornwall
U.K.	ManV	24	pink	23.3.09	50° 4'22.32"N 4°59'48.12"W	Volnay (shipwreck), Manacles, Cornwall
U.K.	Sawtooth	12	pink	07/2009	50°41'6.65"N 2°48'7.34"W	Sawtooth, Lyme Bay, Dorset
U.K.	Skomer	39	n/a	3.06.06, 2007	51°44'40.14"N 5°17'42.30"W	Skomer Island, Pembrokeshire
U.K.	WestTen	43	pink	18.08.09	50°38'52.80"N 2°57'46.80"W	West Tennents Reef, Lyme Bay, Devon
U.K.	nrPad	7	pink	6.10.05	50°35'40.98"N 4°56'54.12"W	Camel Estuary, nr Padstow, Cornwall
Ireland	Ire_BR	29	pink	16.5.12	54°34'37.20"N 8°25'44.58"W	Black Rock, St John's Head, co. Donegal
Ireland	Ire_TR	48	pink	16.5.12	54°28'17.88"N 8°26'41.40"W	Thumb Rock, Mullaghmore, co. Sligo
Portugal	EvARM	27	check	n/a	37° 5'25.34"N 8°20'45.06"W	Jardim de Veira, Armação de Pêra
Portugal	Faro1	41	pink	26.5.10	37° 2'15.84"N 8°21'21.90"W	Amazonia das Gorgónias, Portimão, Armação de Pêra
Portugal	Faro2	43	pink	26.5.10	37° 3'5.16"N 8°21'10.68"W	Poço, Portimão, Armação de Pêra,
Portugal	Faro3	42	5 brown, 37 white/brown	27.5.10	37° 6'5.94"N 8°34'35.70"W	Portimão, nameless site
Portugal	Faro4	35	pink	27.5.10	37° 6'15.48"N 8°33'33.96"W	Portimão, nameless site
Portugal	Faro5	44	pink	28.5.10	36°58'48.78"N 7°59'27.24"W	Pedra da Greta, Portimão
France	EvMai	13	n/a	n/a	n/a	Maire-Pharillons Fromages (N=10), Liban (N=3), Provence
France	Brest3	43	7 pink, 36 white	19.5.10	48°18'40.62"N 4°25'21.78"W	Pointe de Rozegat , Rade de Brest, Brittany
France	LTGlen	40	pink	12.5.11	47°43'38.39"N 4° 3'35.75"W	Laonégued Taër, Glenan Archipelago, Brittany
France	MenGlen	43	pink	12.5.11	47°41'19.86"N 3°59'31.70"W	Men Goé, Glenan Archipelago, Brittany
France	Ros2	36	29 pink, 6 white	21.5.10	48°42'33.71"N 3°54'11.78"W	La Vieille, Baie de Morlaix, Brittany
France	Ros1	40	33 pink, 7 white	20.5.10	48°44'49.50"N 3°57'42.24"W	Astan, Baie de Morlaix, Brittany

Table 2. List of sample sites for *Alcyonium digitatum*. Bold type indicates sites or areas in which *Eunicella verrucosa* was also sampled. ¹ The number of orange colour morph colonies sampled at sites is indicated; all other colonies were white. ² CEFAS samples were obtained from benthic trawls; 'CefMix' was a combination of four adjacent trawls of 300m each, starting with T86, where N = 25, 11, 8, 4 respectively; 'CefT342' was collected from one trawl of 600m (the coordinates of the start of the trawl for T342 and T86 are given). N represents the number of individual samples included in the final data set (not the total numbers collected or extracted); these N individuals amplified at at least 9/11 loci.

Country	Code	N	Orange? ¹	Collected	GPS	Site Name
U.K.	Dgal	7		7.5.09	50°33'18.72"N 3°26'21.43"	The Galicia (shipwreck), Devon
U.K.	DorBA	24		10.5.09	50°36'58.74"N 1°49'57.84"	The Betsy Anna (shipwreck), Dorset
U.K.	Frog	18		25.9.10	50°32'02.00"N 2°33'6.00"	Frognor 1 (shipwreck), Lyme Bay, Dorset
U.K.	HC	36	N=1	27.7.10	51°12'12.51"N 4°40'50.29"	Hen and Chickens, Lundy, Devon
U.K.	Tren	42		1.8.10	49°51'54.00"N 6°23'9.00"V	Trenemene reef, Isles of Scilly, Cornwall
U.K.	Stone	40		1.8.10	50° 1'58.80"N 6° 7'7.20"W	Seven Stones reef, Isles of Scilly
U.K.	ManCD	33		22.3.09	50° 2'43.39"N 5° 2'44.99"	Carn-du rocks, The Manacles, Cornwall
U.K.	ManV2	28		23.3.09	50° 4'22.32"N 4°59'48.12"	Volnay (shipwreck) Manacles, Cornwall
U.K.	Lucy	22		13.6.09	51°44'28.08"N 5°16'36.54"	The Lucy (shipwreck), Skomer, Pembrokeshire
U.K.	PR	51		13.6.09	51°44'40.25"N 5°18'27.18"	Payne's Rock, Skomer, Pembrokeshire
U.K.	TR	21		15.6.09	51°44'17.52"N 5°15'21.54"	Tusker Rock, Skomer, Pembrokeshire
U.K.	UB74	19		25.9.10	50°31'50.21"N 2°33'19.26"	UB74 (shipwreck, WW1 German U-boat), Lyme Bay, Dorset
U.K.	CefMiX	27	N=6	5/2009	53°38'40.55"N 1°32'49.96"	² CEFAS trawls (4 sites, beginning T86), Humberside, North Sea
U.K.	CefT342	33	N=19	05/2009	53°16'33.86"N 1°34'9.29"E	² CEFAS trawl (T342), nr Norfolk, North Sea
France	Bre2	43	N=43	19.5.10	48°20'20.94"N 4°34'32.52"	"Mengam", Rade de Brest, Brittany
France	LTGlen	29	N=29	12.5.11	47°43'38.39"N 4° 3'35.75"	Laonégued Taër, Glenan Archipelago, Brittany
France	Mglen	34	N=33	12.5.11	47°41'19.86"N 3°59'31.70"	Men Goé, Glenan Archipelago, Brittany
France	Ros1	41		20.5.10	48°44'49.50"N 3°57'42.24"	"Astan", Baie de Morlaix, Brittany
France	Ros2	41		21.5.10	48°42'33.71"N 3°54'11.78"	"La Vieille", Baie de Morlaix, Brittany
Ireland	IreIT	48	N=48	12.5.12	53°43'08.50"N 10°7'19.14"	SW Inisturk island, co. Sligo, Ireland
Ireland	IreTR	18	N=48	15.5.12	54°28'17.88"N 8°26'41.40"	Thumb Rock, Mullaghmore, co. Donegal,

2.3. DNA extraction

Wherever possible, DNA was extracted from approximately 10-20 whole octocoral polyps that were plucked manually from each colony using forceps. If polyps had retracted following collection and their removal from calyces was impossible, scalpels were used to shave a slice of either 1cm² (approximately) of surface tissue from *Alcyonium digitatum*, or coenenchymal tissue (excluding the gorgonin axis) of 1-2cm in length from *Eunicella verrucosa*. Shavings were inspected visually to verify presence of at least 10-20 polyp cross-sections or calyces per slice to ensure animal tissue was present for digestion (in addition to the proteinaceous skeletal matter).

Genomic DNA was extracted from both octocoral species using the Wizard® SV Genomic DNA Purification System (Promega Corporation, Madison, Wisconsin) according to the manufacturer's protocol for animal tissues using a microcentrifuge. Full details of the DNA extraction methods used are given in Appendices 1 and 2 (Holland *et al.* 2013a, b), the two technical papers published from this research.

2.4. DNA quantification

Following extractions, 1.5µl of genomic DNA was quantified using a FLUOstar OPTIMA plate-reading fluorometer; full details of the DNA quantification method used are given in Appendices 1 and 2 (Holland *et al.* 2013a, b), the two technical papers published from this research. DNA concentrations varied widely; poor DNA yields were more common for *Alcyonium digitatum* (less than 10ng/µl) than for *Eunicella verrucosa*, although for both species, concentrations above 50ng/µl were considered to be high. Ranges were typically between 20-40ng/ µl (data not shown). Following DNA quantification, aliquots were taken from stock solutions, diluted to working concentrations of 10ng/ µl and transferred by population into deep-well plates ready for downstream PCR pipetting.

2.5. Isolation of microsatellites

Microsatellites were developed and tested for both species at the NERC Biomolecular Analysis Facility (NBAF), Department of Animal and Plant Sciences, University of Sheffield between 2009 and 2012. Additionally, a further microsatellite library was developed for *Eunicella verrucosa* at the Evolutionary Genetics Core Facility, Cornell University (October 2008 and March 2009); loci from this library were also tested in Sheffield and added to microsatellites developed at NBAF to complete the final multiplex panels. All libraries were constructed from DNA obtained from one individual. For *E. verrucosa*, the individual was collected from East Tenants Reef,

Lyme Bay, Devon, in September 2008 (50 39.143 N, 02 52.728 W). For *A. digitatum*, the individual in question was collected from the Volnay Wreck in the Manacles area off the Lizard Peninsula, Cornwall, in March 2008 (N50 04.372 W04 59.802). A small (2cm) branch clipping was used to extract DNA from *E. verrucosa* with a Qiagen Plant Mini kit according to manufacturer's instructions (QIAGEN), while a Wizard Kit (Promega) was used to extract DNA from *A. digitatum* following the manufacturer's animal tissue protocol (Promega). Presence of high-molecular weight genomic DNA was verified on 2% agarose gels in TBE (tris base, boric acid and EDTA) and quantified with a Nanodrop 2000 (ThermoScientific), prior to library construction (*E. verrucosa*: 16.5ng/ul, A260:A280 = 1.77, *A. digitatum* 14.5ng/ul, A260:A280 = 1.71). Full details of the protocols used for microsatellite isolation and testing are described in the two technical papers published from this research (Holland et al. 2013a, b) in *Conservation Genetics Resources*; see Appendices 1 and 2.

2.6. Data preparation: *Eunicella verrucosa*

All genotyping for *Eunicella verrucosa* was done using three multiplexes comprising 17 loci (Table 3). From these, three loci were impossible to score reliably due to practical problems (incompatible bleed-through/flashover or consistently bad amplification), resulting in a final data set of 14 loci that were scorable and suitable for further analysis. To avoid spurious results from missing data, individuals for which more than six out of 14 loci had failed to amplify (five failures allowed, 36%) were removed from the final data matrix. This resulted in 1055 individuals from 30 different sites around southwest England and Wales, western Ireland, northwest France, Portugal and the Mediterranean (Figure 2, Table 3). Some analyses were subsequently conducted with more stringent screening, in which an individual with a failure rate of four or more loci out of 14 was excluded from the data matrix, ie individuals with three failed loci (no amplification) or fewer (21%) were kept in the data matrix – this resulted in a revised data set comprising 979 individuals.

After removal of individuals with four or six missing loci, the occurrence of duplicate genotypes was tested for using the 'Identity Analysis' option in the program Cervus v3.0.3 (Kalinowski *et al.* 2007), allowing for no mismatching loci. This equates to a measure of clonality and occurrence of individuals potentially resulting from asexual reproduction within each population. In the most stringent data set (four or more failed amplifications), from 478,731 pairwise comparisons of 979 individuals, 28 exact-match genotypes were detected – in some cases this included individuals with unequal amplification success (eg, data from 12 loci from one individual that exactly

matched data from 10 loci that amplified successfully in the second individual would count as a 100% match). If duplicate genotypes occurred within the same population, all but one were removed, resulting in a final data matrix comprising 955 individuals, of which two were an exact match ('EvArm_22' and 'Faro2_10', from separate but adjacent sites in southern Portugal approximately 4 km distant).

Using this data matrix, evidence for the presence of null alleles, stuttering and large allele dropout was checked for in all populations using the program Microchecker v2.2.3 (van Oosterhout *et al.* 2004), with a maximum allele size set at 400bp, a 95% confidence interval and 1000 iterations. Input GENEPOP files, suitable for import into Microchecker, were generated in Cervus v3.0.3 (Kalinowski *et al.* 2007) and were thus automatically Bonferroni corrected; therefore, this correction was not re-applied in Microchecker. This program omits missing data from analyses and, as allele sizes in this dataset did not conform to expected sizes for perfect motif repeats, analyses were conducted including suspect data. Results from this analysis are presented in Table 3.

2.7. Data preparation: *Alcyonium digitatum*

Using a stringent dataset, where the failure allowance threshold was set at two out of eleven loci, the occurrence of duplicate genotypes was tested using the 'Identity Analysis' option in Cervus v3.0.3 (Kalinowski *et al.* 2007). Of 666 genotypes, eleven were identical to another haplotype; in this case all identical pairs happened to be from the same populations as each other and therefore one of each pair was removed from the data, resulting in a final data matrix of 655 individuals. Using this data matrix, evidence for the presence of null alleles, stuttering and large allele dropout was checked using Microchecker v2.2.3 (van Oosterhout *et al.* 2004) as above.

2.8. Final selection of usable microsatellite loci

2.8.1. *Eunicella verrucosa*

For *E. verrucosa*, 18 polymorphic microsatellite loci were identified and tested in all populations in three multiplexes. However, similar allele sizes and technical problems with some loci resulted in four loci being dropped from further analyses; thus, a final panel of fourteen usable microsatellite loci (amplified in three PCR reactions per individual) was optimized for use in all further analyses of *E. verrucosa* populations (Table 3).

2.8.2. *Alcyonium digitatum*

For *A. digitatum*, eleven loci were identified. Initially, these loci were also multiplexed 3 or 4 loci per multiplex; however, significant failures in amplification with primers labelled this way, large allele sizes and potential problematic primers (eg, potential cross annealing) resulted in poor returns and unreliable reads; therefore, all *A. digitatum* loci were run as duplexes for all populations (ie, 6 PCR reactions per individual). Eleven loci were selected for final analysis (Table 3).

2.9. Final panels in comparison with other octocoral microsatellites

In order to compare microsatellites generated in this study with other research, properties of all microsatellites isolated from octocorals in studies published to date were summarized; in total, this comprises twelve panels in ten species including those from this study (Table 2.5). Panels have been described from three Mediterranean species, two from the Caribbean and three from the Pacific; *Eunicella verrucosa* and *Alcyonium digitatum* represent the first temperate NE Atlantic octocorals and the only anthozoans in the British Isles to have microsatellite markers developed for them (besides the anemone *Nematostella vectensis*, an introduced species only found in estuarine environments in the south and south east of England, Darling *et al.* 2006). At the time of writing, no polar octocorals appear to have microsatellites or other genetic markers published yet.

Numbers of loci isolated from octocorals vary between two and fourteen per species with an average of eight usable loci retained; this is a relatively low number compared to the initial numbers of positive clones isolated or potential sequences identified during the development and screening process. For example, where data are available, from the number of primers tested as few as 5% may be usable (Liu *et al.* 2005a, b) to a maximum of 54% (Mokhtar-Jamai *et al.* 2010). In several cases, octocoral microsatellites are initially so few in numbers that several libraries may be combined to obtain sufficient numbers of polymorphic loci (LeDoux *et al.* 2010, Liu *et al.* 2005b, Mokhtar-Jamai *et al.* 2010, this study for *Eunicella verrucosa*).

Furthermore, where papers proceed the publication of a primer note, some loci prove unusable when tested in a larger dataset, therefore numbers of loci in population genetic assessments of some species are even lower than in the corresponding primer note (eg, Andras *et al.* 2013, Costantini *et al.* 2007 and Costantini and Abbiati 2007, Yasuda *et al.* 2008). Overall, there does not appear to be a clear relationship between numbers of primers tested and numbers of final usable loci; in this study,

the return rate for *E. verrucosa* was approximately 18% (between both libraries) and was 20% for *A. digitatum*, whereas in the blue coral *Heliopora coerulea*, a 191 primer set yielded a 6% return of eleven loci. There is not enough data at this stage to determine if success at isolating microsatellites in octocorals is taxonomically correlated; a similar return (26%) was obtained for *Eunicella singularis* (Cataneo *et al.* 2010) to *E. verrucosa*, although reasons for this may be coincidental and with different laboratories using different enrichment approaches or probes, it is difficult to determine if similarity is based upon methodology or genomic characteristics.

In terms of motifs, it is clear that octocorals may contain some complicated, long, and imperfect repeats, for example, *Corallium lauuense* (Baco *et al.* 2006) and *Heliopora coerulea* (Yasuda *et al.* 2008). The *Alcyonium digitatum* panel includes three penta-nucleotide repeats, whereas *Eunicella verrucosa* did not contain any and had a high proportion of tetra-nucleotide repeats (7/14). A penta-nucleotide was also reported in *Gorgonia ventalina* (Andras and Rypien 2009). Numbers of alleles per locus also varied between species (although as the data in Table 2.5 comes from primer notes, it is worth pointing out that in some cases only a few individuals were tested and therefore the entire range of allelic diversity may be under-represented). In the Plymouth Mewstone Ledges population presented in the *E. verrucosa* primer note, between 2-10 alleles were found per locus (N=44), a similar number to *E. singularis* (2-9, Cataneo *et al.* 2010; in the current study between 1-8 alleles/locus in *E. singularis* were found (N=12); see Table 2 in the primer note for *E. verrucosa*, Appendix 1). For *A. digitatum*, 3-27 alleles / locus were found among the Isles of Scilly Trenemene population (N= 42). It appears that *A. digitatum* has a higher allelic richness than *E. verrucosa*, and that (from limited data), *Eunicella spp.* are on the lower end of allelic richness in octocorals genotyped with microsatellites so far.

Table 3. Eighteen loci multiplexed and genotyped in all *Eunicella verrucosa* samples
Eunicella verrucosa multiplexes

Multiplex 1				
Locus	Dye	MOTIF	Size Range	No. Alleles
01C02	HEX	(AATG)17	233-274	15
Exe15	HEX	(TTAC) 6	105-121	5
Exe21	HEX	(TTGA) 8	166-182	5
Exe33	6FAM	(CAA)12	148-183	13
Exe41	6FAM	(GATT)6	230-239	3
Exe49	6FAM	(GTT) 22, imperfect	337-394	18
Exe24*	HEX	(GTT) imperfect	317-364	9
Multiplex 2				
Locus	Dye	MOTIF	Size Range	No. Alleles
06E10	6FAM	(AATC)8	92-176	18
Exe17	6FAM	(TATC) 13, imperfect	237-266	9
Exe34	VIC	(TCAA) 7	140-157	5
Exe47	6FAM	(GAT) 6	204-217	5
Exe50	PET	(CAA) 14	127-170	14
06F03	NED	(GA)8	147-153	2
Exe24*	6FAM	(GTT)imperfect	317-364	9
Multiplex 3				
Locus	Dye	MOTIF	Size Range	No. Alleles
Exe10	6FAM	(GA) 10	223-260	11
Exe48	6FAM	(GAT)6	151-154	2
Exe29	NED	(GAT) 26, imperfect	125-167	9
Exe44	VIC	(TGA), 6 imperfect	139-146	2
Exe35	PET	(GTAT) 7	141-151	2

*Exe24 was attempted in two multiplexes – best results were obtained with Multiplex 2 and therefore data from Multiplex 1 were discarded for this locus. Unreliable reads, problems with bleed-through and flashover resulted in a loss of data from four loci: 06F03, Exe29, Exe44 and Exe35. Final analyses were thus conducted using the remaining fourteen loci (though the discarded loci were selected from initial screens and are likely to be informative if optimised). Size ranges and allele numbers are from the entire *E. verrucosa* dataset (N=955); data for the four discarded loci originate from initial screens and probably do not represent the full extent of allelic polymorphism at these loci.

3. Population genetic analysis and statistics

3.1 Data preparation

To reduce confounding effects of asexually produced clones on connectivity estimates between populations, duplicate genotypes were identified and removed if found within the same population using the software Cervus (Kalinowski 2007), as outlined in sections 2.6 and 2.7 above. The quality and reliability of genetic data was tested and corrected as appropriate following tests for null alleles (Microchecker, van Oosterhout *et al.* 2004), for neutrality and outlier loci (LOSITAN, Antao *et al.* 2008), and for linkage disequilibrium (Genepop v4.2 (Raymond and Rousset 1995, Rousset 2008)).

3.2 Genetic diversity and population structure

Metrics of genetic diversity between populations were obtained using several methods. Allelic richness (A_r) was calculated by rarefaction algorithms implemented in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003) to account for variation in sample size. Next, measures of differentiation based upon Wright's fixation indices (pairwise F_{ST}) were calculated in Arlequin v3.5.1.2 (Excoffier 2005); these were used to correct P-values for multiple tests using the false discovery rate method (FDR, Benjamini and Hochberg, 1995). In addition, principal component analyses (PCA) were performed on a covariance matrix of Nei's unbiased genetic distances, which is a suitable measure of genetic diversity if sample and/or loci numbers used are small (Nei 1978). In the case of *Eunicella verrucosa*, initial PCA runs successfully distinguished three regional clusters; however, a Lyme Bay population (JT East Tennents) was also isolated. As this population consisted of only seven sampled individuals, it was removed from the analysis along with two other populations of less than ten individuals (nr Padstow and Heroine Wreck, Lyme Bay) to avoid spurious results due to insufficient sampling (Figure 2). For a more detailed description of statistical analyses and parameterization, see Holland (2013).

4. Results

4.1. Overview

Approximately 20% of the primers tested yielded informative microsatellites – a typically low return for anthozoan taxa. The occurrence and evolution of microsatellites in cnidarian genomes remains poorly understood; they are thought to be much lower in abundance than in vertebrates (Marquez et al. 2000; Meglecz et al. 2012) and, as more available genomic resources become available and yields of microsatellite loci remain low, it appears that this assertion is valid (Davies et al. 2012; Meglecz et al. 2012). Recent research suggests that in Cnidaria, di-nucleotide repeats may be less common than in other metazoans and that tri- and tetra-nucleotides may be the most common in non-chordate taxa; this is also the case with the microsatellite locus panels resulting from the research presented here (Holland et al. 2013a, b). *Alcyonium digitatum* contained penta-nucleotide motifs and, in general, its microsatellites had larger size ranges and more alleles than those of *Eunicalla verrucosa*; these differences remain unexplained. Regardless, as this work demonstrates, microsatellites offer a useful tool for inferring population structure and gene flow among octocorals in temperate and deep-sea conservation contexts (Baco et al. 2006). We applied the loci identified to relatively large sets of specimens of *A. digitatum* and *E. verrucosa* collected from across the northeast Atlantic and demonstrated that connectivity of these two octocoral species with a partly overlapping range is markedly different. One species, *E. verrucosa*, shows a regional-scale differentiation in line with previous research on corals in this region and the other, *A. digitatum*, exhibits panmixia across a large spatial scale. Whether these different patterns correspond to variation in life history traits or the evolutionary history of each species remains unclear, but our study validates the utility of using microsatellites as a measure of connectivity in temperate sessile taxa whose assumed dispersal potential is being used to infer Marine Protected Areas.

4.2. Allelic richness

For *Alcyonium digitatum*, the number of alleles combined across all loci for each population ranged between 54 and 144, and the average number of alleles per locus ranged between 5 and 31 in a given population (with an average total allele number of 118 per population and 10.8 alleles per locus). There is a linear relationship between the number of samples included from each population and the number of alleles observed, with the lowest numbers of alleles corresponding to the lower

sample sizes (eg, 54 alleles in the seven individuals sampled from the Galicia wreck site in Devon). Sample sizes of at least 30 individuals are required to attain the average total number of alleles observed per population, although as for *Eunicella verrucosa*, (see below) there is evidence that genetic diversity was under-represented in my samples, as allelic numbers correlated with sample size failed to reach a plateau (data not shown). After correction for sample size, allelic richness measures varied from 4.9 (the Devon Galicia wreck) to 6.1 (Roscoff 2) with a mean of 5.7 across all populations. There was no striking pattern of geographic variation in the distribution of allelic richness as sites from different regions were mixed when ranked according to A_r (data not shown). Nonetheless, it is interesting to note that both Irish sites had amongst the lowest A_r values (5.5 and 5.6) for *A. digitatum* as they did for *E. verrucosa*. The lowest value found in the Devon Galicia sample is highly likely to be an artefact of a small sample ($N=7$). The highest allelic richness was observed in samples from Brittany (Brest 2 and Roscoff 2, 6.0 and 6.1 respectively).

For *Eunicella verrucosa*, the total number of alleles across all loci ranged between 39 and 83. The average total number of alleles in a population was 67; it appears that approximately 30 individuals need to be sampled per population to attain the average total number of alleles. However, there is some indication that representative genetic diversity was not sampled in its entirety as correlated allelic numbers and samples size failed to plateau and lower allele numbers tended to coincide with lower sample numbers (data not shown). Allelic richness varied between 2.5 and 3.48 (mean 3.1), with the highest and lowest values found in populations from Marseilles and Lyme Bay respectively, When A_r values were ordered in ascending size in the context of sampling location (data not shown), both Irish samples had the lowest A_r (2.78 and 2.92 for Black Rock and Thumb Rock respectively). These sites are at the northerly extreme of my sampling scheme and represent the most northerly populations of *Eunicella verrucosa* in its range. On the contrary, two of the highest values were found in the Lyme Bay area in Devon, UK, the area thought to contain the most easterly sea fans in their range (West Tennents reef, 3.32 and the Lyme Bay Heroine Wreck, 3.48). Otherwise there were no clear patterns of allelic richness variation, and the overall variation was small (Table 4).

Populations	N	Ar
Ev Marseilles	13	2.50
Ev Armacao	27	3.16
Faro1	41	3.13
Faro3	42	3.26
Faro2	43	3.06
Faro4	35	3.21
Faro5	44	3.17
Brest3	43	3.15
LTGlen	40	3.01
MenGlen	43	2.99
Roscoff2	40	3.09
Roscoff1	36	3.25
Ire_BlackRock	29	2.78
Ire_ThumbRoc	48	2.92
DevonBF	40	3.09
PlymouthMew	44	3.20
HandsDeep	36	3.35
IoScilly Hath	30	3.02
IoScilly LR	22	3.10
IoScilly nnw	23	2.96
JT East Ten	7	3.00
Lundy	22	3.17
Lyme Bay HW	9	3.48
Manacles Mo	30	3.08
Manacles RR	43	3.27
Manacles V	24	3.07
Sawtooth	12	2.94
Skomer	39	3.16
West Ten	43	3.32
nr Padstow	7	2.99

Populations	N	Ar
Brest2	43	5.96
LT_Glen	29	5.73
MenGlen	34	5.84
Ros1	41	5.94
Ros2	41	6.07
IreITWest	48	5.56
IreTR	18	5.52
DevGal	7	4.91
DorBA	24	5.61
Frog	18	5.85
HenChicks	36	5.55
IoSTren	42	5.93
IoS_Stones	40	5.83
ManCD	33	5.87
ManV	28	5.91
SkoLUCY	22	5.77
SkoPR	51	5.70
SkoTR	21	5.80
UB74	19	5.61
CefasMix	27	5.84
CefasT342	33	5.93

Table 4. Allelic richness estimates (Ar) for each population sampled for *Eunicella verrucosa* (left) and *Alcyonium digitatum* (right). For detailed site descriptions, please refer to Tables 1 and 2.

4.3. Population differentiation

Tables 5 and 6 present the results of pair-wise F_{ST} s calculated between populations of *Alcyonium digitatum* (Table 5) and *Eunicella verrucosa* (Table 6) based on allele frequencies in the microsatellite data, based on 30 and 20 populations, respectively. Each matrix presents pair-wise fixation indices (F_{ST}); yellow cells indicate significant differentiation and, by extension, reduced connectivity. Axes are collection sites

colour coded by region: green = Brittany, pink = Ireland, yellow = Portugal, red = Mediterranean (*E. verrucosa*); green = Brittany, pink = Ireland, blue = UK, dark blue = east coast UK (*A. digitatum*).

Figures 4 and 5 present principle components analysis of samples of the two study species, *Alcyonium digitatum* (Figure 4) and *Eunicella verrucosa* (Figure 5) based on microsatellite data; spatial clustering of data suggest connectivity within regional scales for *E. verrucosa*, but no obvious pattern (ie, high connectivity) across the sampled range of *A. digitatum*.

Table 5. *Alcyonium digitatum* – pair-wise F_{ST} between 20 populations

	Brest2	LT_Glen	MenGlen	Ros1	Ros2	IreITWest	IreTR	DorBA	Frog	Hen Chicks	IoSTren	IoS_stones	ManCD	ManVtake2	SkoLUCY	SkoPR	SkoTR	UB74	CefasMix	CefasT342	
Brest2	-	.	.	.	+
LT_Glen	.	-	.	.	+	+	+
MenGlen	.	.	-	+
Ros1	.	.	.	-	+
Ros2	+	+	.	.	-	+	.	.	.	+	.	+	+	+	.	+
IreITWest	+	-	+
IreTR	-	+
DorBA	-
Frog	-	+
HenChicks	.	.	+	.	+	-	.	.	+	+	+
IoSTren	-	+
IoS_Sstones	+	-	+
ManCD	.	+	.	.	+	.	+	.	.	+	.	.	-	+	.	+	.	.	+	+	
ManVtake2	+	+	-
SkoLUCY	-
SkoPR	.	.	.	+	+	+	.	.	-
SkoTR	-
UB74	-	.	.	.
CefasMix	+	.	.	+
CefasT342	.	+	.	.	.	+	.	.	+	+	+	+	+

Table 6. *Eunicella verrucosa* – pair-wise F_{ST} between 27 populations

	EvMAI	Ev ARM	Faro1	Faro3	Faro2	Faro4	Faro5	Brest3	LTGlen	MenGlen	Ros2	Ros1	IreBR	IreTR	DevBF	PlyMew	HandsDeep	IoSHath	IoSLR	IoSnnw	LundynBATT	ManMo	ManRR	ManV	Sawtooth	Skomer	WestTen
EvMAI		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
EvARM	+		-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Faro1	+			-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Faro3	+				-	-	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Faro2	+					-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Faro4	+						-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Faro5	+							+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Brest3	+	+	+	+	+	+	+		-	-	-	-	+	+	-	-	+	-	+	-	+	-	+	-	-	-	+
LTGlen	+	+	+	+	+	+	+			-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-
MenGlen	+	+	+	+	+	+	+				+	+	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-
Ros2	+	+	+	-	+	-	+						+	+	-	+	+	-	+	-	-	-	-	-	+	-	+
Ros1	+	+	+	+	+	+	+						+	+	-	+	+	-	+	-	-	-	-	-	-	-	+
IreBR	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	-	+	+	+	-	+	+	+	+	+
IreTR	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	-	+	+	+	+	+	+	+	+	+
DevBF	+	+	+	+	+	+	+						+	+				-	+	+	+	-	+	+	+	+	+
PlyMew	+	+	+	+	+	+	+				+	+	+	+				-	+	+	+	-	+	+	+	+	+
HandsDeep	+	+	+	+	+	+	+	+		+	+	+	+	+				-	+	+	+	-	+	+	+	+	+
IoSHath	-	-	-	-	-	-	-											-	+	+	+	-	+	+	+	+	+
IoSLR	+	+	+	+	+	+	+	+			+	+	+	+				-	+	+	+	+	+	+	+	+	+
IoSnnw	+	+	+	+	+	+	+						+	+				-	+	+	+	-	+	+	+	+	+
LundynBATT	+	+	+	+	+	+	+			+	-	-	+	+				-	+	+	+	-	+	+	+	+	+
ManMo	-	-	+	-	-	-	-							+				-	+	+	+	-	+	+	+	+	+
ManRR	+	+	+	+	+	+	+	+		+	+	+	+	+				-	+	+	+	-	+	+	+	+	+
ManV	+	+	+	+	+	+	+		+	+	-	-	+	+				-	+	+	+	-	+	+	+	+	+
Sawtooth	+	+	+	+	+	+	+				+	+	+	+				-	+	+	+	-	+	+	+	+	+
Skomer	+	+	+	+	+	+	+						+	+				-	+	+	+	-	+	+	+	+	+
WestTen	+	+	+	+	+	+	+	+			+	+	+	+				-	+	+	+	-	+	+	+	+	+

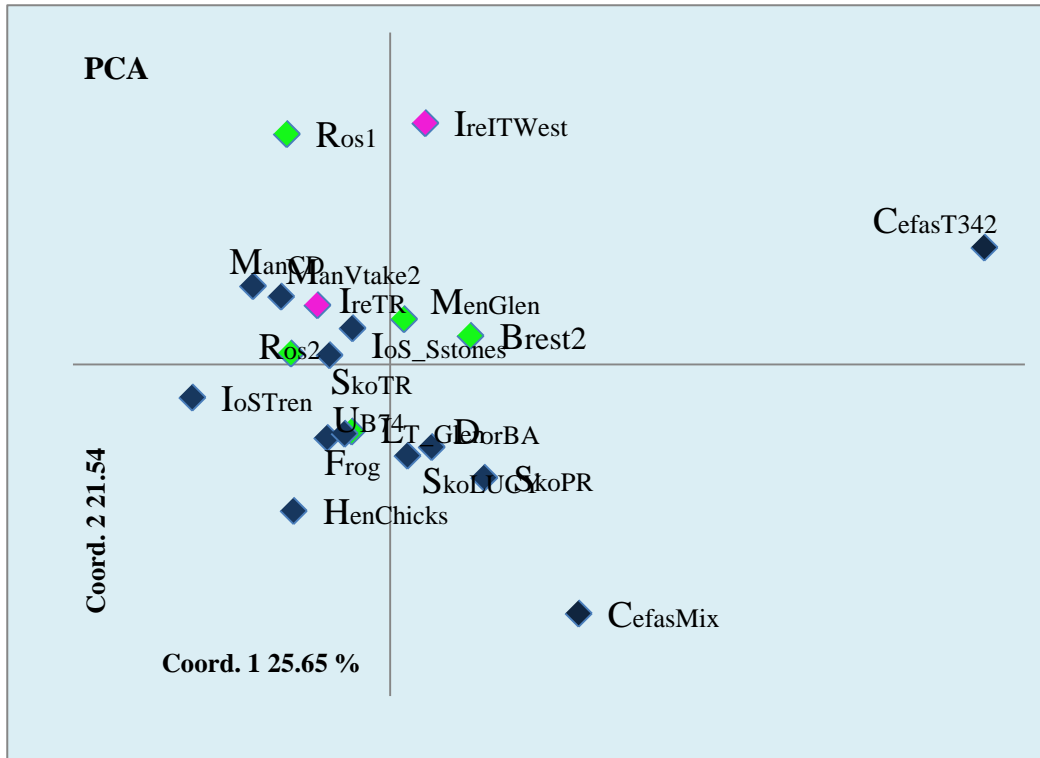


Figure 4. PCA plot of 20 *Alcyonium digitatum* samples based on analysis of 11 loci

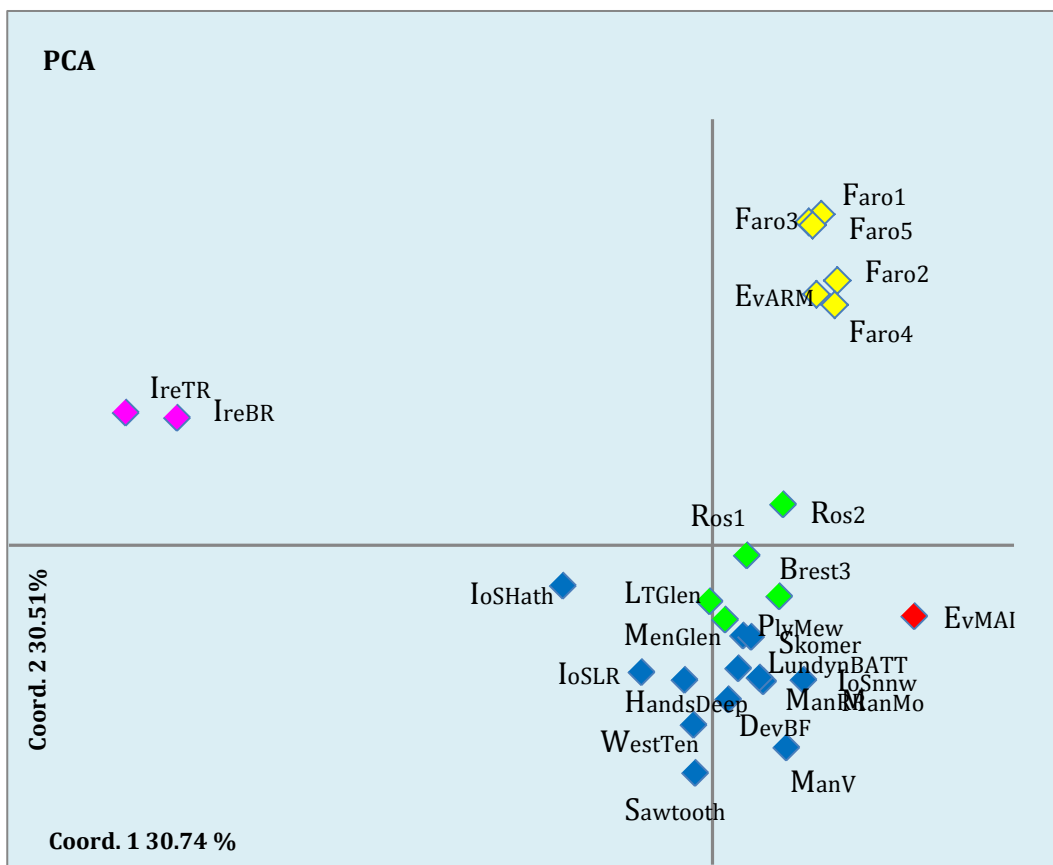


Figure 5. PCA plot of 27 *Eunicella verrucosa* samples based on analysis of 14 loci

5. Discussion

5.1. Using molecular markers to infer connectivity

Molecular methods are invaluable in understanding marine ecosystem function as they can be used to infer interactions at several levels including measurement of gene flow and connectivity, assessment of population structure and parentage, phylogenetic relationships and biogeography (Feral 2002). The panels of novel microsatellite loci developed during this study are comparable to panels developed from other species in terms of locus numbers, and have proven to be a reliable method to identify population structure in the target study species and other congeneric species. A need for high resolution markers such as microsatellites for the study of octocoral population genetic structure has recently been recognized, eg, Gori *et al.* (2012), who were unable to discriminate between depth-related morphotypes in *Eunicella singularis* or between different *Eunicella* species. It is anticipated that the microsatellite panels developed as part of this research project will be useful across their respective genera, *Eunicella* and *Alcyonium*, due to their cross-species amplification.

During the microsatellite development stage, a high rate of attrition resulted in low yields of useable loci from the total number tested (approximately 20%; see thesis Chapter 2). Reasons for low success in octocoral (and anthozoan) microsatellite isolation remain unclear, although microsatellites are thought to be rare in cnidarian genomes (eg, Liu *et al.* 2005a, b; see Holland 2013 for further details). Development of alternate markers is challenging in octocorals; anthozoan mitochondria are very stable evolutionarily, exhibit little variation and evolve approximately 10-20 times more slowly than rates inferred for vertebrate mitochondria (Shearer *et al.* 2002). Reasons for this may include elements such as homing endonuclease genes ('selfish DNA') that have been found in some actinarians (anemones) and which are thought to stabilise the mitochondrial genome (Goddard *et al.* 2006). In octocorals, mitochondria are atypical with reference to other Anthozoa, with anomalies including slow evolution (McFadden *et al.* 2010), alternate mitochondrial gene orders between families (Brugler and France 2008), and the presence of rare mismatch repair homologs that may suppress mitochondrial mutation rates (Bilewitch and Degnan 2011). Therefore mtDNA markers are of limited use for population-level analyses in octocorals, as has been demonstrated by several studies including Calderon *et al.*'s (2006) research on genetic structure of four Mediterranean gorgonian species, where

COI variation was so low, relationships between even geographically distant populations could not be deduced. In *Eunicella* spp., COI, internal spacer ITS regions and mutation suppression homolog msh1 genes have failed to resolve species level relationships (Calderon *et al.* 2006, Gori *et al.* 2012). However, RFLPs generated from COI PCR amplicons may have use in the species-level identification of scleractinian coral larvae (Shearer and Coffroth 2006).

Nonetheless, previous research on octocorals has combined DNA sequence data with microsatellite data, or used an assortment of mitochondrial and nuclear markers to infer population structure or phylogeography, which may offer better resolution when combined. For example, Concepcion *et al.* (2010) used nuclear signal recognition particle subunit 54kDa (SRP54) and mitochondrial NADH dehydrogenase subunits 2 and 6 (ND2 and ND6) to track the spread of *Carijoa riisei* between the Atlantic/Caribbean and Pacific, with SRP54 being considered the most promising marker for resolving closely related lineages (see also Concepcion *et al.* 2008). Herrera *et al.* (2012) used a combination of mitochondrial genes (including NADH subunits 2, 3 and 6, CO1 and msh) and nuclear (ITS) markers to examine phylogeography of the deep-sea bubblegum coral *Paragorgia arborea*. In threatened Mediterranean *Corallium rubrum* populations, sequences from the nuclear elongation factor 1a gene (EF1a) have been combined with microsatellite data to infer population structure (Aurelle *et al.* 2011). EF1a corroborated microsatellite data but with less resolution (although sample sizes were different between the two datasets). In the work presented here, a lack of suitable population-level DNA sequence markers resulted in reliance purely on microsatellites to examine *E. verrucosa* and *A. digitatum* genetic connectivity. A lack of congruence and lowered resolution between sequence data and microsatellites in other taxa (eg, *Corallium rubrum*, Costantini and Abbiati, 2007) and the lower resolution associated with sequence data in octocorals suggests microsatellites represent a relatively high resolution marker for exploring octocoral population structure. And, while promising population level nuclear markers are emerging for scleractinians (e.g, β -tubulin, Nunes *et al.* 2009), microsatellites are likely to remain a viable choice of population marker for octocorals in the immediate future.

In this study polymorphism and hence allelic richness was higher in *A. digitatum* than *E. verrucosa* (interestingly, the low polymorphism reported for *E. verrucosa* here is the lowest of any octocoral, with the exception of congeneric *Eunicella singularis*; see Holland 2013). A negative correlation has previously been demonstrated between

F_{ST} values and locus polymorphism (here measured by allelic richness and heterozygosity) in walleye pollock (O'Reilly *et al.* 2005) and sockeye salmon (Olsen *et al.* 2004). The latter authors found significantly lower F_{ST} estimates for highly polymorphic microsatellites (defined as $H_s > 0.84$) compared to moderately polymorphic microsatellites and isoenzymes ($H_s < 0.6$); therefore in this study confidence in F_{ST} values may be drawn from the relatively low average heterozygosity value found across the markers used.

In summary, microsatellites are the marker of choice for conservation genetic studies of many organisms and, as this study demonstrates, they have also proven to be robust markers for the elucidation of population genetic structure in octocorals. Nonetheless, while at present genomic data are relatively scarce in octocorals, recent advances in technology may soon lead to other classes of genetic marker, eg, SNPs, offering even broader coverage of octocoral genomes in the near future.

5.2. Connectivity patterns in the northeast Atlantic

The North East Atlantic region can be divided into several provinces, e.g. JNCC (2004), Spalding *et al.* (2007); regions recognised comprise: Lusitanian (including the South European Atlantic Shelf, Saharan Upwelling, and the Azores, Canaries and Madeira Islands ecoregions), the Temperate North Atlantic (ie Boreal, including the south and west Iceland, Faroe Plateau, southern Norway, northern Norway and Finnmark, Baltic Sea, North Sea and Celtic Sea ecoregions) and the Mediterranean, West African Transition, and Gulf of Guinea. The ranges of *Eunicella verrucosa* and *Alcyonium digitatum* therefore each span several provinces. It has been suggested that the general pattern for genetic subdivision in marine taxa in the North East Atlantic is delineated by the Mediterranean, western and northern European areas (Roman and Palumbi 2004). Although this is an oversimplified view, evidenced by affinity between Irish and Spanish samples in some studies (Sotelo *et al.* 2008), admixture between western and northern Europe (Luttikhuizen *et al.* 2008) or genetic breaks observed between the Mediterranean and NE Atlantic (Lowe *et al.* 2011), it seems that *E. verrucosa* supports this theory. In this study, strong divergence between southern Europe and the British Isles, some differentiation between England and Brittany, and strong divergence between western Ireland and everywhere else in the sampled area was observed, highlighting regional scale variation.

Alcyonium digitatum was sampled from a more northerly area and appears to be

highly admixed in this region; therefore, the pattern identified by Palumbi and Roman (2004) for this species cannot be supported or refuted without the inclusion of samples from further afield. In this study, little divergence was seen in *A. digitatum* between Brittany, the UK and the North Sea, such a pattern has also been reported for cuttlefish (Wolfram *et al.* 2006). It is clear that the two octocorals studied here differ in their connectivity patterns around Britain and Ireland; samples from the North Sea and western Ireland showed little divergence in *A. digitatum*, whereas samples of *E. verrucosa* from Ireland were more distinct. McFadden (1999) found no genetic difference in *Alcyonium hibernicum* in Ireland and the Isle of Man. In summary, little divergence around Britain is seen here for the two octocorals species studied, a finding that accords with a number of previous studies on marine invertebrates (eg, Muths *et al.* 2009).

Genetic patterns in many species may be explained by historical range expansions from southern refugia. During the Pleistocene, (1.8 million–12,000 year ago), Europe was subject to a series of ice ages, the most severe of which was 18,000 years ago and is known as the last glacial maximum (LGM; Luttikhuizen *et al.* 2008). Glaciers and sea ice extended as far south as southern Britain and France and essentially restricted the range of terrestrial and marine fauna to mid/southern Europe, from where it expanded and retracted to coincide with glacial and interglacial periods. The Mediterranean and Atlantic-Iberian coasts were not under ice and therefore have a continuous marine history, since the opening of the Gibraltar Strait five million years ago (Duran *et al.* 2004), although the Mediterranean and Atlantic basins were separated during glacial periods, which may explain genetic divergence in some taxa between the two (Baus *et al.* 2005). Northern Atlantic areas were thus colonised more recently and boreal-temperate communities around the British Isles are characterized by an assemblage of species that returned from southern temperate regions or that survived in northern glacial refugia, such as those in southwest Ireland and northwest Scotland (Luttikhuizen *et al.* 2008, Jolly *et al.* 2006). Genetic signatures reflecting range expansions and retractions are detectable and have been utilized to suggest migration patterns or locations of refugia for several Lusitanian and Boreal marine species. For example, in a study of the seaweed *Fucus serratus* Coyer *et al.* (2003) proposed that high microsatellite allelic diversity in Brittany compared to elsewhere in the NE Atlantic and Nova Scotia implies that this area was a refuge during the last LGM or has been recolonized since. Although there is some uncertainty over the extent of glacier and permafrost coverage in Britain and Ireland, it is likely that most of the northern part of the current range of *E. verrucosa* was

close to the southerly limit of the ice sheet (Hoarau *et al.* 2007). Therefore the current range of *E. verrucosa* might not have expanded substantially as the ice retreated.

However, a lack of sampling at range limits for *A. digitatum* and limited genetic structure within either species make it difficult to infer range expansion pathways in this study. *E. verrucosa* is clearly divergent in Ireland, and in common with *A. digitatum* has an apparently reduced effective population size and allelic richness in this region. Sampling at closer intervals between Ireland and Britain and northwest France may elucidate potential source areas for these Irish populations. The range of *A. digitatum* extends much further north and re-colonization in the UK may have followed a northerly or southerly route (eg, if it persisted in potential northern refugia, Luttikhuisen *et al.* 2008).

5.3. Inferring connectivity from life-history strategies

This research has demonstrated that estimating connectivity patterns based upon reproductive traits, when they are unknown (eg, *E. verrucosa*) or even relatively well understood (eg, *A. digitatum*) is highly unreliable. *E. verrucosa* and *A. digitatum* are thought to disperse less than 1km and more than 10km, respectively, from a parent colony (marlin.ac.uk). However, our findings suggest that dispersal is vast in both species and at scales of hundreds of kilometres, evidenced by lack of structure at this scale in the southwest Britain. In summary, these data suggest that dispersal estimates being used as a proxy for connectivity in the UK MPA network design guidelines are highly unlikely to be accurate (Jones and Carpenter 2009; Roberts *et al.* 2009) and further highlight the need to incorporate genetic connectivity data into reserve design and management.

5.4. Conservation implications

Globally, unprecedented rates of biodiversity loss, declining fish stocks, habitat degradation and detrimental impacts of climate change (eg, Hall-Spencer and Moore 2000, Martin *et al.* 2008, Robinson *et al.* 2008) have led to international efforts to protect marine ecosystems. Marine reserves have proven their value and efficiency globally. Benefits of them usually manifest in the augmentation of biomass and abundance of target fish (Tetreault and Ambrose 2007; PISCO 2011), protection of coral reefs (Mumby *et al.* 2007, Harborne *et al.* 2008), and coincident indirect benefits such as improved ecosystem services and economical value (Roncin *et al.* 2008; PISCO 2011) or increased ecosystem resilience led by a reduction of disease within protected areas (Raymundo *et al.* 2009). Spillover effects and enhancement of

adjacent populations is sometimes a beneficial 'side-effect' of an MPA (Goni *et al.* 2008), although this benefit is uncertain and requires, for example, suitable habitat (Forcada *et al.* 2009). The science behind MPA design is complicated and a lack of data concerning, for example, availability of suitable habitats, local hydrodynamics and connectivity of species of interest may impede their success (reviewed in Sale *et al.* 2005). As such, connectivity is recognised as a key ecological criterion in the design of MPAs (eg, Foley *et al.* 2010), although distinctions between genetic and demographic connectivity are rarely made.

In Europe, each member state is required to implement 'coherent and representative networks' of Marine Protected Areas by 2020 as a requirement of the Marine Strategy Framework Directive (MSFD), and the sixteen signatories of the OSPAR Commission have pledged to halt further degradation and biodiversity loss in the OSPAR maritime area by 2020 and to develop an ecologically coherent network of well-managed MPAs (Jones and Carpenter 2009, ospar.com). At a national level, legislation to protect the marine environment and moves to develop a network of marine protected areas began under the 2009 Marine and Coastal Access Act, which in England was directed by four regional groups that suggested candidate sites for protection to the UK Government through the Marine Conservation Zone (MCZ) project (jncc.defra.gov.uk). This culminated in 127 sites around Great Britain being put forward to the UK Government for consideration; of these, 27 were eventually designated as MPAs in November 2013 (see: <https://www.gov.uk/government/collections/marine-conservation-zone-2013-designations>). Two additional tranches of sites for consideration as MPAs have subsequently been announced by the UK government <https://www.gov.uk/government/publications/marine-conservation-zones-february-2014-update>. *Eunicella verrucosa* is one of seven Cnidaria targeted by the network, *A. digitatum* is not. From the data presented here, it appears that genetic connectivity, at least for some sessile benthic invertebrates, needs consideration at European levels and would likely fall within the remit of OSPAR.

In this study, although not within the scope of England's MCZ project, the marginality seen in Irish *Eunicella verrucosa* populations could be levied as a case for their protection at large spatial scales. Marginal populations usually contain rare alleles (three private alleles were found here), they may recruit slowly, and may be genetically divergent due to isolation, all of which imply vulnerability and reduced resilience (Sanderson 1996). Compared to the overall range of *E. verrucosa* (Angola to western Ireland), the extent of it in the UK is very small, and divergence from

Portugal and Brittany also highlights the genetic uniqueness of British populations. Coupled with its status as an IUCN red-listed octocoral, a UK BAP Priority species, and with its unofficial role as a 'poster child' for UK marine biodiversity and conservation efforts, an argument could be made for the protection of *E. verrucosa* across its range as a connected meta-population. This approach may be strengthened by the high rates of local inbreeding noted in the UK, including populations in Plymouth Sound, the Manacles, the Isles of Scilly and Lyme Bay, but not at Lundy, currently the only designated MCZ where populations are not apparently genetically isolated. When our findings concerning the range over which connectivity is being maintained in *E. verrucosa* and *A. digitatum* are viewed in the light of current guidance on the spacing of MPAs, ie at 40 – 80 km (Guideline no. 12; Natural England & JNCC 2010), it would appear that, in general, the spacing of designated and candidate MPAs in UK waters may be adequate to maintain connectivity in these two octocoral species; this assumes, of course, that – irrespective of distance – local marine currents are suitable to facilitate the movement of sufficient viable larvae of each species.

Alcyonium digitatum is ubiquitous, has no protective status, is not peripheral to its global range in the UK and appears to have large-scale genetic homogeneity and high genetic diversity. Although widespread and abundant around UK coasts, *A. digitatum* is, however, locally vulnerable in some areas, primarily due to fishing activity. For example, some populations of *A. digitatum* have been damaged by benthic scallop dredging for *Pecten maximus* and *Aequipecten opercularis* (the king and queen scallop respectively), such as in Lyme Bay, Devon (Hinz *et al.* 2011), whereas others are likely to be detrimentally affected by trawling for benthic fish including *Solea solea* (sole), such as in Anglesey, Wales (Kaiser *et al.* 1998). The overall extent of anthropogenic disturbance and its long-term effect upon *A. digitatum* has not been adequately studied, as data concerning its recovery potential are scarce or inconclusive; Kaiser *et al.* (1998) were unable to quantify changes in the biomass of *A. digitatum* pre- and immediately post-trawl. However, they assert that it is likely to be affected in the long term by intense and repeated fishing activity given the significant proportion of the biomass that they represent. Hinz *et al.* (2011) recorded a 67% reduction in abundance after trawling activity compared to control sites and also observed that surviving colonies were reduced in size. Although this research is limited in terms of geographical scope, it is highly unlikely that benthic trawling using heavy mobile fishing gear will be anything but detrimental for *A. digitatum* populations (and indeed most other epibenthic and sessile marine

invertebrate fauna). Furthermore, the reduced colony size noted by Hinz *et al.* (2011) also implies that remaining colonies may not be sexually mature, as maturity is not reached until 2 - 3 years of age (Hartnoll 1975). In this study, inbreeding depression seen at almost all sites from the North Sea to western Ireland highlights the possibility that this species is not freely able to exchange genetic material between populations, and that these areas may be isolated with high rates of self-seeding (as suggested for this pattern in another octocoral, *Corallium lauuense*, Baco and Shank 2005). Reduced heterozygosity and impaired sexual reproduction is known to result from trawling damage (eg, Henry and Kenchington 2004). Reduced numbers of colonies and smaller sizes have already been observed in areas of Lyme Bay subject to trawling activity (Hinz *et al.* 2011); significant inbreeding coefficients detected in all three of my samples collected from there and across the region further highlight vulnerability of this species despite its prevalence. Therefore, genetic patterns observed here in *A. digitatum* may also provide a hypothetical proxy to highlight the occurrence of inbred and damaged sessile populations in areas in need of protection for other targeted species.

To date, 27 MPAs have been designated in English waters by the UK Government's Department for Food, the Environment and Rural Affairs (Defra); these comprise 22 inshore sites and 5 offshore sites: <https://www.gov.uk/government/collections/marine-conservation-zone-2013-designations>. Of these MPAs, two were sampled for both species in this study, the Manacles (Lizard Point, Cornwall) and the Isles of Scilly (Natural England 2013b). High levels of inbreeding in both *E. verrucosa* and also in *A. digitatum* in these areas highlight the vulnerability of populations there and therefore support designation of these areas as MCZs, should a goal of the network be to conserve genetic diversity (as it is of the IUCN). However, highly significant inbreeding coefficients were also detected at other populations in areas not put forward for protection, indicating that many populations of *E. verrucosa* remain vulnerable, at least in terms of fitness loss (data not shown here, see Holland 2013). The first UK MCZ to be designated was the island of Lundy in the Bristol Channel. However, *Eunicella verrucosa* from Lundy does not appear to be subject to inbreeding and a lack of distinction of this population in PCA analyses suggests it is not an isolated population, despite the apparent geographic isolation of the site. Significantly, this study sampled *E. verrucosa* from seven of the eight SACs (Appendix 3) identified as containing Grade A/B reefs in southwest England and Wales (JNCC 2014), the only grade A/B reef site not sampled being Lands End and Cape Bank (UK0030375). Consequently, we anticipate that our findings will be of

particular relevance to legislation aimed at protecting these reef SACs in these regions.

Designation of an MCZ network in the UK has been primarily stakeholder driven (Defra 2013). The UK Marine and Coastal Access Act of 2009 obliges Defra to review achievements of MCZs individually and as part of the network every six years. Flexibility towards the network design regarding addition, alteration in sizing or indeed addition or removal of sites to and from the network is unclear, but at present, from the genetic connectivity data generated in this study, it appears that localized, unconnected reserves are of limited relevance to *Eunicella verrucosa*, which, given the disparate spread of inbred populations, requires conservation of the whole UK meta-population if the full range of genetic diversity of the species in English and Welsh waters is to be conserved; we anticipate that the recently designated MPAs in these waters should go some way towards addressing this issue. In Ireland, divergent and inbred populations could be used to advocate protection of *E. verrucosa* in this part of its range, and the same could be applied to southern Portugal. During the MCZ network design process, *E. verrucosa* was recorded in only four MPAs nationally (designated under existing European legislation, Jackson *et al.* 2008), and its habitat ('wave-exposed circalittoral bedrock', Jackson *et al.* 2008) in less than five MPAs. This suggests that, as well as connectivity, the criteria of representivity and replication are also not currently met for *E. verrucosa*. As far as is discernible, new MCZs are extensions of existing MPAs and no new sites have been designated specifically to protect *E. verrucosa*. In summary, the designation of 22 inshore sites in the recently designated MCZ network is likely to fall short of its conservation objectives with regard to *E. verrucosa* (and, by extension, possibly other sessile invertebrates). Empirical data concerning connectivity of *E. verrucosa* were not included in the draft guidelines (Natural England & JNCC 2010) (as this study is the first to obtain such data), although connectivity based upon potential dispersal distances inferred from its status as a 'low disperser' may have been (Jones and Carpenter 2009, Roberts *et al.* 2009). Whitsand and Looe Bay in Cornwall has been designated as an MCZ and is highlighted as an important site for *E. verrucosa* (Natural England 2013a). The current study did not include samples from this site, although samples from just east of the MCZ boundary were analysed; all three samples (Hand Deepes, Plymouth Mewstone Ledges and Plymouth Breakwater Fort) showed significant evidence of inbreeding. These findings highlight the importance of protecting *E. verrucosa* in this area.

5.5. Implications for policy

These findings have important implications for policy. Within UK waters the genetic data identified no significant barriers to gene flow in *A. digitatum*, although a high prevalence of inbreeding at almost all sampled sites suggests that the dispersal biology of this species, perhaps coupled with site-specific current patterns, may be acting to limit gene flow. Nonetheless, the molecular analysis suggests that, despite high levels of inbreeding, contemporary gene flow is sufficient to maintain genetic connectivity.

For *E. verrucosa*, the situation appears more complex: again, connectivity within the UK range of this species appears robust and no significant genetic variants between *E. verrucosa* populations in English and Welsh waters were observed. However, English and Welsh populations are genetically distinct from populations from Ireland, France and Portugal, suggesting limited connectivity between regions and a need to safeguard the distinct portion of the overall genetic diversity of the species present in UK populations of *E. verrucosa*.

Conservation of marine species with meta-population characteristics and no clear geographically defined connectivity patterns is challenging due to the confounding effects of ecological connectivity (ie, contemporary gene flow from on-going larval import and export) and evolutionary processes (ie, rare migration events that homogenize populations coupled with mutation and drift, Marko and Hart 2011). This can be particularly troublesome in long-lived species with overlapping generations and high levels of clonality, such as sponges and corals, as distinct genotypes may persist for decades to centuries even after gene flow has ceased; thus, traditional *F* statistics may not always be sufficiently sensitive to be indicative of present-day patterns of connectivity (Botsford et al. 2009). These factors should be borne in mind when implementing ecological networks such as MPZ networks.

5.6. Summary

This study highlights the utility of using molecular data to explore patterns of genetic connectivity in two important benthic invertebrate species, *Eunicella verrucosa* and *Alcyonium digitatum*, in coastal waters in the northeast Atlantic. Significantly, both species demonstrate a high degree of contemporary genetic connectivity. However, while *A. digitatum* shows very little population sub-structure, *E. verrucosa* does exhibit patterns of regional differentiation across the northeast Atlantic. Specifically, populations from Britain, Ireland, France and Portugal are to a greater or lesser

degree distinct from each other. Moreover, it is apparent that the *E. verrucosa* populations in English and Welsh waters contain a distinct portion of the overall genetic diversity of the species, including several unique combinations of microsatellite genotypes.

In terms of demographic patterns, higher connectivity in *A. digitatum* may be driven by its habit of spawning in winter, and thus having larvae that disperse further than those of *E. verrucosa*; thus, the hypothesis that the former species would exhibit less population subdivision appears to be correct. However, the extent of demographic vs genetic connectivity in both species remains elusive. For both species, high rates of inbreeding were found which are challenging to explain without the application of hydrodynamic models; furthermore, the effects of inbreeding on fitness have not yet been demonstrated for either species – both of which would be interesting topics for future study.

6. References

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7. Appendices

Appendix 1: Publications arising from this research

Holland, L.P., D.A. Dawson, G.J. Horsburgh and J.R. Stevens (2013)
Isolation and characterization of eleven microsatellite loci from the ubiquitous temperate octocoral *Alcyonium digitatum* (Linnaeus, 1758). *Conservation Genetics Resources*, 5: 767–770.

Holland, L.P., Dawson, D.A., Horsburgh, G.J., Krupa, A.P. and Stevens, J.R. (2013) Isolation and characterization of fourteen microsatellite loci from the endangered octocoral *Eunicella verrucosa* (Pallas, 1766). *Conservation Genetics Resources*, 5: 825–829.

Appendix 2: Grade A/B reef SACs in southwest England and Wales

The eight SACs currently identified as containing Grade A/B reefs in southwest England and Wales (JNCC 2014):

- UK0013694 Isles of Scilly Complex
- UK0030374 Lizard point
- UK0030375 Lands End and Cape Bank
- UK0013111 Plymouth Sound and Estuaries
- UK0030373 Start Point to Plymouth Sound & Eddystone
- UK0030372 Lyme Bay and Torbay
- UK0013114 Lundy
- UK0013116 Pembrokeshire Marine

This study included samples of *E. verrucosa* from seven of the eight; the only grade A/B reef site not sampled being UK0030375 Lands End and Cape Bank.

Appendix 3: Outputs from the research project

Papers:

- Holland et al 2013. *Alcyonium* primer note. *Conservation Genetics Resources*
- Holland et al 2013. *Eunicella* primer note. *Conservation Genetics Resources*
- Holland & Stevens – Connectivity papers (in prep.)

- This report
- A fully catalogued sample collection (tissues, DNAs)

Advances in knowledge:

- New panels of microsatellite loci for octocorals
- Information on gene flow and connectivity in *Alcyonium digitatum* and *Eunicella verrucosa*.
- Insights into connectivity in octocoral species in southern England and Wales of relevance to marine protected area planning and designation in English waters.

Appendix 4: Misc. Natural England policy documents used as background

Habitats and species of principal importance in England

<http://www.naturalengland.org.uk/ourwork/conservation/biodiversity/protectandmanage/habsandspeciesimportance.aspx>

<http://www.naturalengland.org.uk/ourwork/marine/mpa/mcz/features/species/pinksea-fan.aspx>