WP 4. Integrated Ecosystem Assessment: DNA Barcoding

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Abstract

This report provides a summary of the morphological and genetic identification of cephalopod species caught in the North East Atlantic. Cephalopod samples were obtained through a combination of research surveys, port sampling and fish market sampling. Each sample was initially examined using morphological characteristics with 14 species identified. Tissue samples were also taken from each specimen and subsequent DNA analysis was performed. DNA Barcoding using the COI region was successfully performed on a total of 1155 samples with the number of species identified increasing to 30 species. All of these species have either been previously recorded in the NE Atlantic waters or their range is expected to extend into these waters. This study revealed which cephalopod species are commonly misidentified on morphological assessment alone, and hence, which are the species that require further development of identification guides to contribute to more accurate fisheries landings data records.

Keywords: DNA Barcoding, species identification, cephalopods, NE Atlantic

Introduction and Methods

Cephalopods are a non-quota species under EU legislation. This is one of the contributing factors which has led to inaccuracies in species identification across many cephalopod groups, with landings usually only reported at family level [ICES, 2020]. In order to assess the species diversity present and verify the species caught and landed in the Atlantic Area, we combined morphological and genetic techniques on samples collected throughout 2018 – 2019. Samples were acquired via combination of fish markets, supermarkets, port landings and scientific research cruises and were collected by both junior and senior Cephs and Chefs researchers with varying levels of experience. Morphological assessment was made by the researcher collecting the data. Samples collected aboard Marine Institute (MI) / Centre for Environment Fisheries and Aquaculture Science (UK) (CEFAS) / Marine Scotland vessels were identified with the aid of a cephalopod identification key (Laptikhovsky and Ourens, 2017). Tissue samples were taken from the mantle of each specimen and stored in 96-100% ethanol and DNA analysis was performed in molecular biology laboratories at either National University of Ireland Galway (NUIG) or Portuguese Institute for Sea & Atmosphere (IPMA).
DNA was extracted using the Invitrogen ™ Purelink ™ Genomic DNA Extraction Kit, as per manufacturer's instructions. A 650bp region of the COI gene was amplified using universal invertebrate primers LCO1490 5'-ggtcaacaaatcataaagatattgg-3' and HCO2198 5'-taaacttcagggtgaccaaaaaatca-3' (Folmer et al., 1994) under PCR conditions outlined in Allcock et al. (2007). Each PCR contained 12.5 µL of Thermo Scientific™ DreamTaq Green PCR Master Mix (2X), 0.5 µM of each primer, 2.5 µL of DNA and 9 µL H_20 resulting in final reaction volume of 25 µL. A negative control was also included to ensure cross-contamination did not occur. PCR products were checked on a 1% (w/v) agarose gel and sized using DNA HyperLadder™ 1kb. PCR products were purified using Invitrogen™ Purelink™ PCR Purification Kit, according to manufacturer's instructions. Purified PCR products were then standardized to 12 ng/µL in accordance with the DNA sequencing facility specifications. Samples were prepared for sequencing by adding 5 µL of each purified PCR product to 5 µM forward primer LCO1490 resulting in a 10 µL reaction volume. A total of 1155/1169 sequences were successfully obtained; DNA of sufficient quality could not be retrieved from the remaining 14 samples. DNA sequences were imported into MEGA Software v.10 (Molecular Evolutionary Genetics Analysis), trimmed (to remove poor quality sequence at the ends), and the species identified using BLAST Local Alignment tool on NCBI Genbank database and the Barcode of Life (BOLD database). Where specimens could not be confidently identified using these tools, RAxML (Randomized Axelerated Maximum Likelihood) phylogenetic software was used to compute evolutionary trees and species identified based on their relationship to comparator sequences. A summary of the analyses performed and results obtained is presented below. Implications and future recommendations are discussed.

Market sampling

Initial market research in Galway yielded little in terms of locally caught cephalopod products. Cephalopod consumption in the home is much less common in Northern European countries than our southern European counterparts, which explains the low demand for fresh cephalopod produce. A total of six supermarkets and five fish mongers in Galway were visited on a monthly basis from August 2018 – December 2018 (except November 2018). Supermarkets stocked 100% frozen imported produce, for example, species such as *Loligo duvauceli, "Loligo edulis", Todarodes pacificus* and *Loligo opalescens*. Fish mongers also had frozen imported products, but two also sold locally caught cephalopods, when available. When fresh cephalopods were in stock, these samples were taken for DNA analysis. See Table 1 for market sampling summary and results.

Table 1: Market sampling summary, Galway Ireland

Market Name	Date	Species marketed as	DNA barcode ID	Count
Gannet Fishmonger	Aug-18	*Sepia officinalis*	*Sepia officinalis*	1
		Loligo sp.	*Loligo forbesii*	2
Galway Bay Seafoods	Sep-18	*Loligo forbesii*	*Loligo forbesii*	1
Gannet Fishmonger		*Loligo* sp.	*Loligo forbesii*	2
Galway Bay Seafoods	Oct-18	*Loligo forbesii*	*Loligo forbesii*	4
Gannet Fishmonger		*Loligo* sp.	*Loligo forbesii*	4
Gannet Fishmonger	Dec-18	*Loligo* sp.	*Loligo vulgaris*	1
			Loligo forbesii	2

Port Landings Castletownbere

A subset of cephalopod landings from ICES area 6b2 were provided by the Fisheries Co-operative at Castletownbere port, Ireland in August 2018. Fishers record their landings using appropriate standardised codes which are documented in logbooks and submitted to the Sea-fisheries Protection Authority (SFPA). These *Loligo forbesii* samples were recorded as 'SQC' or Long fin squid. Samples were transported to NUIG for analysis. Biological information was recorded from a total of 156 individuals over a wide range of sizes (ML range 43mm – 221mm, average ML 82.5mm), with DNA analysis performed on 29 of these individuals. All samples were identified as *Loligo forbesii* using DNA barcoding (Table 2).

Research survey samples

Samples were collected by members of the Cephs and Chefs team aboard various research surveys led by the Marine Institute, CEFAS, Marine Scotland, IEO and IFREMER. They were analysed by either NUIG or IPMA.

A genetic sampling protocol was circulated to all partners in May 2019, with a list of priority species *Loligo forbesii, Loligo vulgaris, Alloteuthis subulata, Alloteuthis media, Illex coindetii, Todarodes sagittatus, Todaropsis eblanae and Sepia officinalis.* As *Loligo forbesii* is one of the most commercially important species, there was a special emphasis on taking tissue samples from this species for a separate population genetics study.

Selected samples were also sent to us at the request of senior researchers in CEFAS and IEO to confirm species identity, emphasizing the need for DNA barcoding to support morphological identification. Table 2 displays an overview of all samples analysed throughout the project.

Table 2: Overview of all samples collected and analysed from research surveys, fishmarkets and port landings (NUIG)

Survey samples (tissue and eggs)	Total samples analysed	Total samples collected	No. of failed sequences	Total no. of successful barcodes
Marine Institute IAMS 2018	77	171	0	77
Marine Institute IGFS 2018	391	779	12	379
Marine Institute IAMS 2019	130	530	1	129
CEFAS April 2019	0	28	0	0
CEFAS - egg samples	8	8	1	7
IFREMER EVHOE 2019	24	65	0	24
Marine Scotland Rockall 2019	38	150	0	38
Marine Scotland WSIBTS 2019	116	261	0	116
IEO DESCARSEL N Spain 2019	76	201	0	76
IEO ARSA Gulf of Cadiz 2019	94	196	0	94
IEO ARSA Gulf of Cadiz 2020	0	20	0	0
IEO Guinea Bissau 2019	25	74	0	25
IPMA surveys and Uni. of Caen	84	84	0	84
Market samples				
Port en Bessin Market 2019	60	60	0	60
Galway Market September - December 2018	17	17	0	17
Port samples				
Port landing Castletownbere, Ireland 2018	29	29	0	29
				1155

Samples analysed at IPMA:

Analysis of 84 research survey samples at IPMA between 2018 and 2019 yielded 11 cephalopod species:

- *Octopus vulgaris*
- *Octopus salutii*
- *Eledone cirrhosa*
- *Eledone moschata*
- *Sepia elegans*
- *Sepia orbignyana*
- *Sepia officinalis*
- *Rossia macrosoma*
- *Illex coindetii*
- *Todaropsis eblanae*
- *Loligo forbesii*

These samples were collected aboard research vessels from IPMA and Université de Caen Normandie. 27 samples are from Portuguese Waters (FAO Division 27.9.a) and 57 are from the Bay of Biscay (FAO Division 27.8.a).

Identification via DNA Barcoding versus Morphological Identification

Figure 1 displays a summary of all species identified via DNA Barcoding at NUIG. The graph shows the proportion of each species that was correctly identified, the proportion that was misidentified and the proportion that could not be assigned a species level ID using morphological characteristics (but was later confirmed to be that species via DNA barcoding). *Loligo forbesii* samples were excluded from this as the dataset was so large it skewed the graph. DNA barcoding confirmed that 580 *Loligo forbesii* samples were correctly identified with just one sample misidentified as *Alloteuthis subulata*.

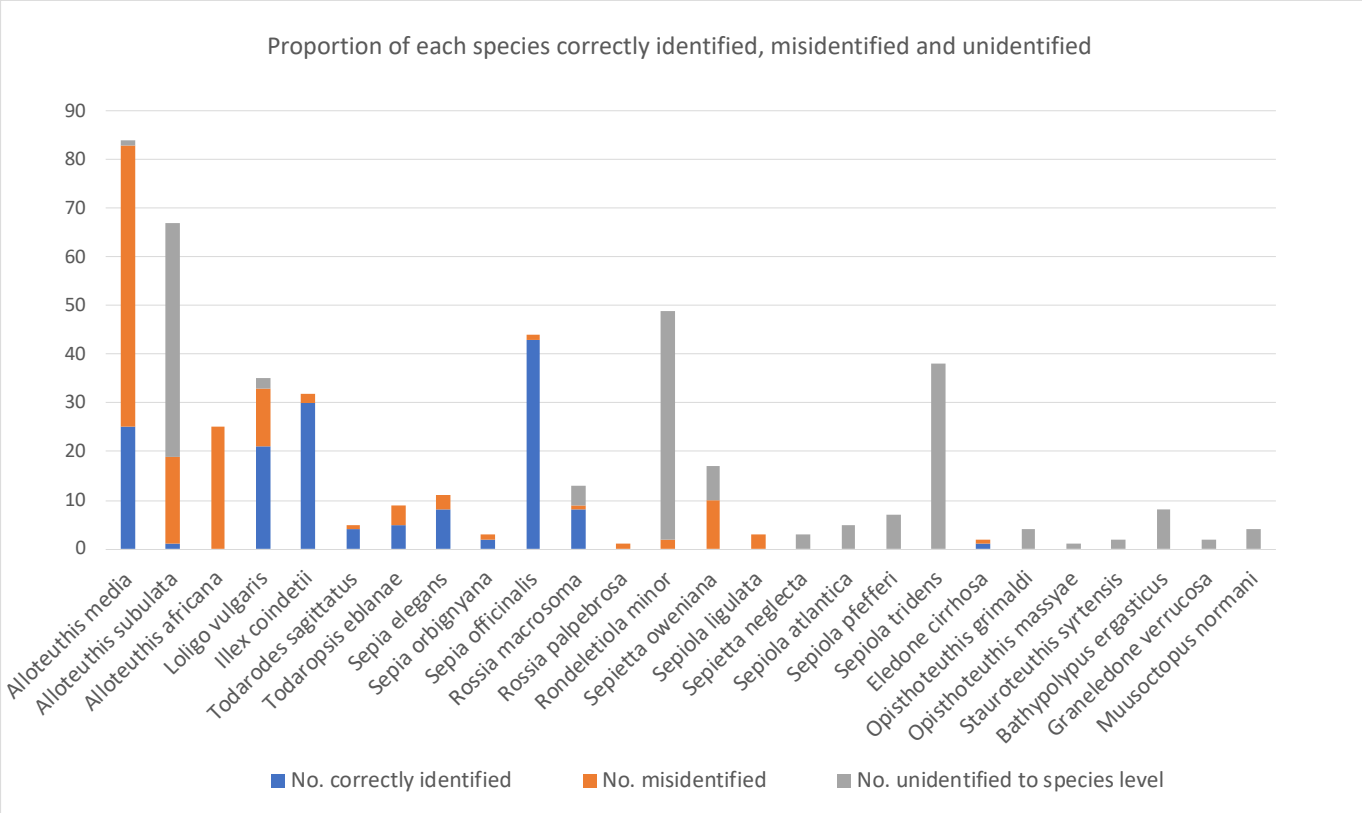

Figure 1 Summary of all species identified via DNA Barcoding at NUIG, excluding *L. forbesii,* collected in the Atlantic Area during the Cephs and Chefs project between 2018 - 2019.

In the majority of cases, specimens were assigned a species-level ID based on morphological inspection (Table 3), however, some specimens could only be identified to a higher taxonomic rank (Table 4). As expected, there were some misidentifications between morphological identification and identification of samples via DNA barcoding. Both tables below display the assigned morphological ID, the barcode species ID and relevant quantities of each.

Table 3: Barcode ID versus Morphological ID – Samples with species level ID

Barcoded Species ID	Count	No. Correctly Identified	No. Misidentified	Misidentified as
Alloteuthis media	83	25	46	*Alloteuthis subulata*
	-	-	1	*Alloteuthis africana*
	-	-	11	*Loligo forbesii*
Alloteuthis subulata	19	1	18	*Alloteuthis africana*
Alloteuthis africana	25	0	25	*Alloteuthis media*

Loligo forbesii	581	580	1	*Alloteuthis subulata*
Loligo vulgaris	33	21	12	*Loligo forbesii*
Illex coindetii	32	30	1	*Alloteuthis subulata*
	-	-	1	*Loligo forbesii*
Todarodes sagittatus	5	4	1	*Illex coindetii*
Todaropsis eblanae	8	5	2	*Illex coindetii*
	-	-	1	*Rossia macrosoma*
Sepia elegans	11	8	3	*Sepia orbignyana*
Sepia orbignyana	3	2	1	*Sepia elegans*
Sepia officinalis	44	43	1	*Sepia orbignyana*
Rossia macrosoma	9	8	1	*Sepiola atlantica*
Rossia palpebrosa	1	0	1	*Rossia macrosoma*
Rondeletiola minor	2	0	2	*Sepiola atlantica*
Sepietta oweniana	10	0	10	*Sepiola atlantica*
Sepiola ligulata	3	0	3	*Sepiola atlantica*
Eledone cirrhosa	2	1	1	*Sepia orbignyana*
Failed sequences	2	-	-	-

Table 4: Barcode ID versus Morphological ID – Samples which could not be identified to species based on morphology.

Barcoded Species ID	Count	Morphologically identified as:
Alloteuthis subulata	48	*Alloteuthis* sp.
Alloteuthis media	1	
Opisthoteuthis grimaldi	4	Cirrate octopus
Opisthoteuthis massyae	1	
Stauroteuthis syrtensis	2	
Bathypolypus ergasticus	8	Deep-sea octopus
Graneledone verrucosa	2	
Muusoctopus normani	4	
Histioteuthis reversa	1	Deep Sea squid
Loligo vulgaris	9	*Loligo* sp. (incl. eggs)
Loligo forbesii	8	
Fail	1	
Rondeletiola minor	47	Sepiolidae sp.
Rossia macrosoma	4	
Sepietta neglecta	3	

Sepietta oweniana	7
Sepiola atlantica	5
Sepiola pfefferi	7
Sepiola tridens	38
Todaropsis eblanae	1
Fail	11

Species with high morphological identification accuracy were as follows: *Loligo forbesii, Sepia officinalis, Todarodes sagittatus,* and *Illex coindetii.* Species with low morphological identification accuracy were *Alloteuthis subulata, Alloteuthis media, Alloteuthis africana*, and *Sepietta oweniana*. These results show that *Alloteuthis* and Sepiolidae species are the most frequently misidentified groups, or groups for which identification is difficult solely using morphological characteristics.

For Irish waters specifically, only two cephalopod identification guides exist (Lordan, 1995a; Lordan, 1995b). These were originally designed as a compilation of the species likely to be present, rather than a comprehensive guide. Lordan 1995a consists of only six squid species: *Alloteuthis subulata*, *Loligo forbesii, Todaropsis eblanae, Illex coindetii, Todarodes sagittatus Ommastrephes bartrami* and Lordan 1995b only three sepiolid species: *Sepiola atlantica, Sepiola oweniana* and *Rossia macrosoma* commonly caught in ground fish surveys in Irish waters. Therefore, the guide by Laptikhovsky and Ourens (2017) which contains 22 species was heavily relied-upon for morphological identification of specimens in the Marine Institute, CEFAS and Marine Scotland research cruises.

Genetic data supports the existence of three species of *Alloteuthis* (*A. subulata*, *A. media* and *A. africana*) however each species exhibits a variable morphology across its distributional range, and it is likely that many misidentifications exist throughout the literature (Jereb et al., 2015). Despite several recent efforts to discriminate between these species effectively, much confusion remains, and this is reflected in the proposed identifications based on morphology herein. Genetic data confirms the presences of three clearly defined species. Molecularly we identify *A. subulata* and *A. media* as those individuals matching sequences of *A. subulata* and *A. media* (respectively) in Anderson et al. (2008). Original type specimens for both *Alloteuthis subulata* and *Alloteuthis media* have been lost so Anderson et al. (2008) applied the name *subulata* to an *Alloteuthis* clade with narrow tentacular clubs and *media* to a clade whose members had central tentacular club suckers more than 9% head width, essentially following Naef (1923). Naef (1923) considered *A. media* to be the species with larger tentacular clubs, following the illustration by Rondelet (Rondeletius, 1554), which Linnaeus used to illustrate *A. media* in his Systema Naturae (Bello, 2019). *Alloteuthis* specimens were collected onboard

IAMS 18, IGFS 18, DESCARSEL 19, ARSA 19 and Guinea Bissau 19 surveys (see Figures 2 and 3). 70% of *Alloteuthis media* specimens were misidentified, together with 95% of *Alloteuthis subulata* and 100% of *Alloteuthis africana* specimens collected. *Alloteuthis* specimens also comprised up to 23% of the samples which were unidentified to species level.

Anderson et al. (2008) and Lefkaditou et al. (2012) both combined morphological and molecular data to discriminate between these species, evaluating the usefulness of taxonomic characters. Anderson et al. (2008) found that a commonly used morphological character – relative fin length, is not effective in discriminating between the species. They did however find that central club sucker size could be used to distinguish between *A. media* and *A. subulata,* and head width distinguished *A. africana* from the other two. However, it is clear that our results indicate that these taxonomic characters were either not incorporated into the morphological assessment undertaken by the researchers, or alternatively, were not successful in delineating between these morphotypes, possibly because of variation in morphology throughout the species ranges. Both *A. media* and *A. subulata* are caught as bycatch throughout European waters but are mainly of fishery interest in Spain and Portugal. In this region, they are reported at species level or as *Alloteuthis sp*., but doubt remains as to the accuracy of this identification. Designation of neotypes which have been sequenced could help stabilize the nomenclature, but descriptions of how *Alloteuthis* morphology varies throughout species ranges are badly needed to support fisheries data.

Sepiolidae species are difficult to identify using external morphological characteristics and the results show that all specimens of *Rondeletiola minor*, *Rossia palpebrosa*, *Sepietta oweniana*, and *Sepiola ligulata* specimens collected were misidentified as *Sepiola atlantica* (Table 3). *Rossia macrosoma* identification however was much more accurate, with only 11% being misidentified, although some specimens were only identified as Sepiolidae sp. Members of the Sepiolidae group comprised 58% of the individuals unidentified to species level shown in Table 4. The distribution of all nine Sepiolidae species verified by DNA barcoding is displayed in Figure 4 below. Whilst these are not a targeted fishery in Europe, species of Sepiolidae are frequently caught as bycatch and most European countries do not record these at species level in the landings data (ICES, 2020).

All species barcoded during this study (n = 30) have either been previously recorded in the Atlantic Area or their range is expected to extend into these waters (Jereb and Roper, 2005; 2010; Jereb et al., 2014; Jereb et al., 2015).

Accurate identification of species is necessary to produce a well-informed integrated ecosystem assessment. This report highlights where the inaccuracies occur most frequently and which fisheries are most at risk as a result of underreporting.

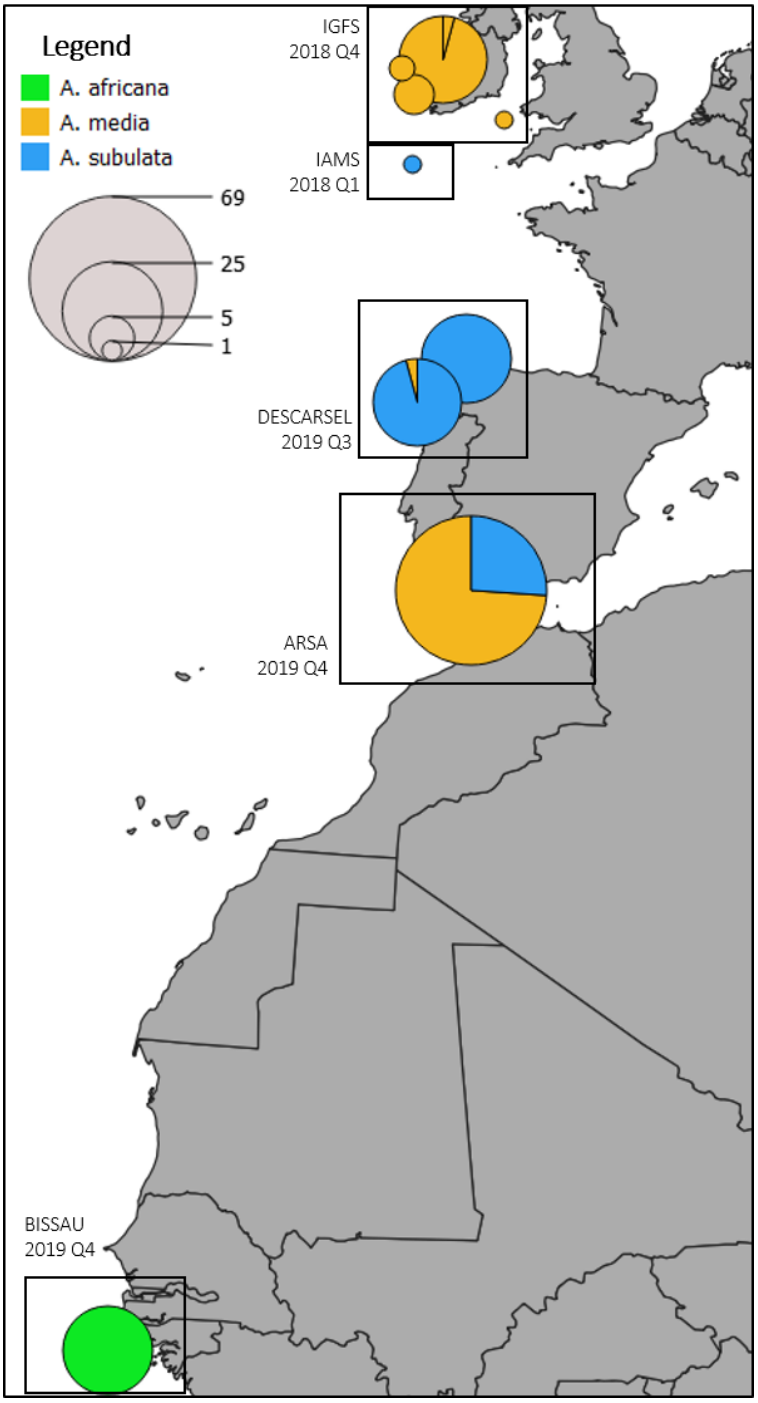

Figure 2 Distribution of all genetically verified *A. subulata*, *A. media* and *A. africana* samples collected in the Atlantic Area during the Cephs and Chefs project between 2018 - 2019. Size of circles refers to number of individuals.

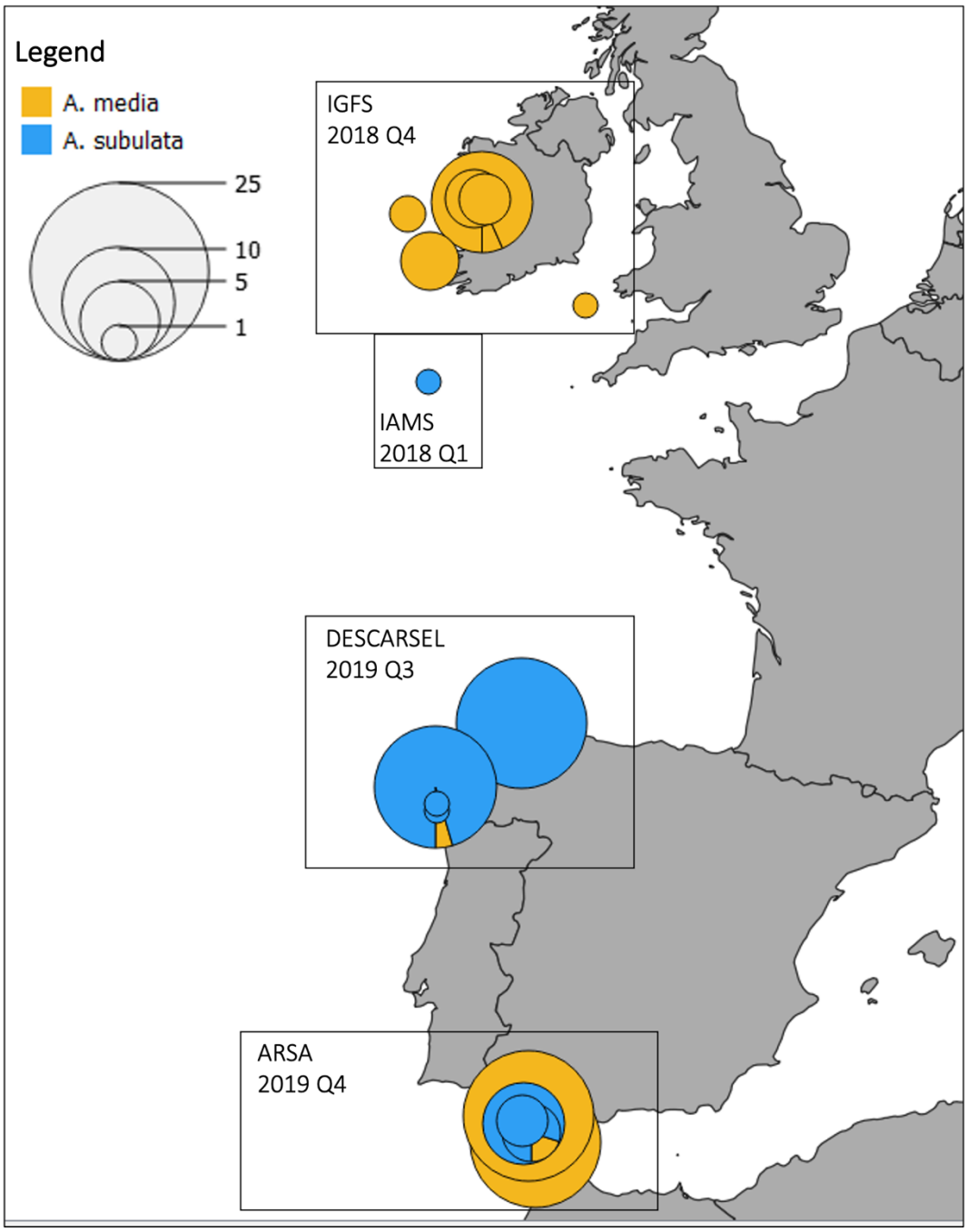

Figure 3 Distribution of all genetically verified *A. subulata* and *A. media* collected in the Atlantic Area during the Cephs and Chefs project between 2018 - 2019. Size of circles refers to number of individuals.

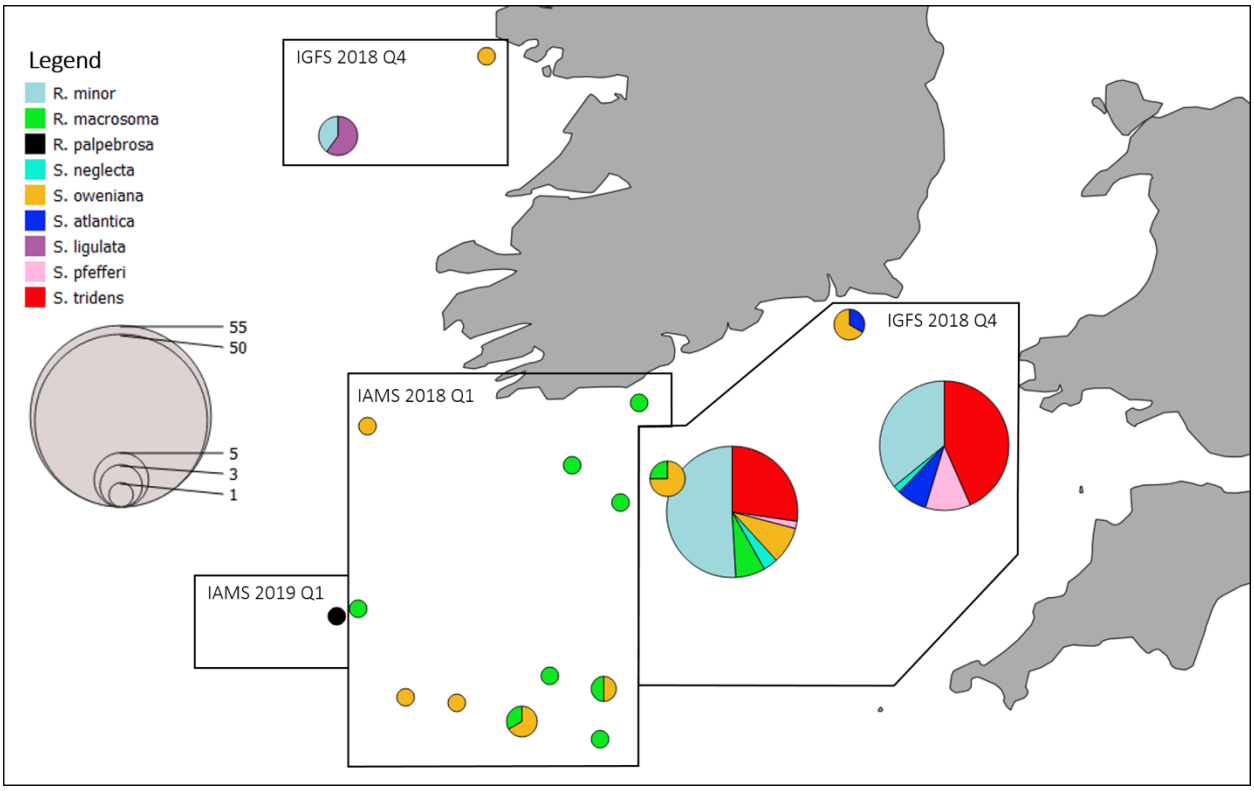

Figure 4 Distribution of all nine genetically verified Sepiolidae species collected in the Atlantic Area during the Cephs and Chefs project between 2018 - 2019. Size of circles refers to number of individuals.

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