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ORIGINAL PAPER

Population connectivity of the European squid *Loligo vulgaris* along the West Iberian Peninsula coast: comparing mtDNA and SNPs

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Abstract

Understanding genetic diversity and population connectivity in marine organisms is essential for fisheries management. In the present study, we examined the population genetics of the European squid, *Loligo vulgaris*, along the western Iberian Peninsula at two genetic resolutions using the mitochondrial cytochrome oxidase subunit I gene (COI) and genomic markers obtained via double digest restriction-site associated DNA sequencing (ddRADseq). The results obtained revealed 79 haplotypes out of 160 COI sequences, while the SNP data set included 86,431 loci after filtering for 38 individuals with 86,319 neutral data. Mitochondrial COI analyses revealed high haplotype (0.961) and nucleotide (0.010) diversities, and the haplotype network reveals complex sub-structure in Turkish waters within a panmictic population. Both Tajima's D and Fu's Fs tests suggest that the population of *L. vulgaris* analysed is evolving neutrally. Pairwise F_{st} for neutral SNPs were low ($0 < F_{st} < 0.002$) and not significant showing high homogeneity among populations, while pairwise comparations for candidate adaptive SNPs (112 loci) showed F_{st} values ranging from 0.026 to 0.234. While neutral SNPs showed admixture, the candidate adaptive SNPs showed a moderate significant structure with a latitudinal discrimination. Overall, both genetic approaches showed homogeneity and strong genetic flux identifying a unique population along the Western Iberian Peninsula.

Keywords DdRADseq · Fisheries management · Iberian Peninsula · Molecular analyses · Panmixia · Squids

Introduction

Squids comprised more than 82% (30 Mt) of the world cephalopod catch from 2009 to 2018 (FAO 2020), with catches fluctuating considerably from year to year. *Loligo vulgaris*, commonly known as European squid, holds significant socioeconomic importance, particularly in the east Atlantic. Its fishery, dominated by small-scale hand-jig fisheries in Portugal and Spain (Moreno et al. 2002), also includes catches as by-catch in bottom trawl fisheries elsewhere. The species spans the Eastern Atlantic from the North Sea to northern Namibia, including the Mediterranean Sea (González et al. 2010; Vidal and Boletzky 2014). With a lifespan of 12 to 15 months and a planktonic phase lasting two to three months (Moreno et al. 2015), spawning seasonality varies by region. In the north and east Atlantic, peak spawning occurs in winter (Moreno et al. 2015). Along the north-western Spanish coast, spawning peaks between December and April (Guerra and Rocha 1994), whereas in Portuguese waters, it occurs in late autumn/early winter and

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late spring (Moreno et al. 2002). Due to increased fisheries pressure on squid populations in recent years it is crucial to understand population structure and connectivity to adopt data-based fisheries management strategies (Hanlon 1998; Cheng et al. 2021).

Delineation of fisheries stocks is often determined according to geographical or political concerns and without adequate knowledge of biological information. This can lead to the overexploitation of vulnerable populations even when catch rates fall within quotas (Ward 2000; Bonanomi et al. 2015; Casey et al. 2016; Garcia-Mayoral et al. 2016). The apparent lack of geographical barriers in marine environments has historically led to the conclusion of widespread panmixia across areas within the same species and so stock management was undertaken depending on the area without considering that the species harvested might be formed by more than one stock (Hauser and Carvalho 2008; Waples and Naish 2009). However, recent studies on genetic fisheries realised that population subdivision in marine species exist, although genetic differentiation is much lower than that detected in freshwater or terrestrial species. It has been demonstrated that the biological significance of low levels of genetic structure is correlated with environmental factors or by temporal and spatial replication (Shaw et al. 2010).

Furthermore, ignoring the importance of genetic diversity of a determined exploited species could result in a decrease of its capability of adaptation to environmental changes (Okumuú and Çiftci 2003). Therefore, genetic analyses are necessary to detect the existence of subpopulations and gene flow between them to know if more than one population is contributing to a local fishery (Chauhan and Rajiv 2010; Garcia-Mayoral et al. 2016).

A wide variety of molecular markers are now available to understand spatial and temporal genetic structure of species of commercial interest (Chauhan and Rajiv 2010; Goodall-Copestake et al. 2012; Nowland et al. 2019). While traditional markers such as allozymes, fragment length, mitochondrial DNA, microsatellites, and tandem repeats have been employed in exploring inquiries concerning stock structure, they possess limitations in discerning intricate patterns of genetic variation (Waples and Naish 2009; Chauhan and Rajiv 2010).

Reduced representation next generation sequencing approaches, such as restriction-site-associated DNA (RADseq), enable the cost-effective acquisition of thousands of single-nucleotide polymorphisms (SNPs) to be obtained across individuals from non-model organism at low cost. This method generates a large number of loci, enhancing the capability to detect fine-scale structure and identify genomic regions under selection using restriction enzymes (Mastretta-Yanes et al. 2015; Rochette and Catchen 2017; Nowland et al. 2019; Sherman et al. 2020).

Generally, squids are characterised by having a great dispersal potential due to its high capability of swimming long distances and for having a planktonic stage that last several weeks (Shaw et al. 2010). In line with this, most population genetic studies on species within the family Loliginidae have utilized conventional molecular technologies. For instance, allozymes were applied in research on *Loligo forbesii* (Brierley et al. 1995), mtDNA was employed in studies involving to *Sepioteuthis lessoniana* and *Alloteuthis spp.* (Aoki et al. 2008; Roura et al. 2019; respectively) and microsatellites were utilized in investigations concerning *Doryteuthis gahi* (McKeown et al. 2019). These studies have reported little to no difference in population structure across different ranges of spatial and temporal scales. In contrast, a study which employed next-generation sequencing-based methods (restriction site-associated DNA sequencing; RADseq) on *Doryteuthis opalescens* along the Californian coast (Cheng et al. 2021) revealed low, but significant levels of genetic differentiation between samples caught in different months.

Only two studies have previously explored the connectivity of the *L. vulgaris* population. Garoia et al. (2004) analyzed six microsatellites from four populations across the Adriatic Sea, revealing a genetically homogeneous single population. Similarly, García-Mayoral et al. (2020) assessed the genetic diversity between paralarvae collected north and south of Galicia, NW Spain, approximately 300 km apart, using mtDNA COI, but found no genetic structure among them. Both approaches were carried out with a very limited number of loci (six and one respectively) and with spatial scales < 500 km, which could potentially bias the genetic structure recorded. Accordingly, the aim of this study was to explore the population genetic structure of *L. vulgaris* along the western side of the Iberian Peninsula, from southern Portugal to northwest Spain (~ 1000 km distance) at two genetic resolutions: (I) single gene, amplifying the mitochondrial cytochrome oxidase subunit I gene (COI) and genome-wide, (II) applying the double digest restriction-site associated DNA sequencing (ddRADseq) targeting thousands of nuclear loci.

Materials and methods

Sample collection

Paralarvae squids were collected in two areas of NW Spain (south-western and northern waters off Galicia) and Portugal (Fig. SI 1). The samples captured in southern Galicia were obtained on board of R/V Mytilus from June to November 2017 and May to October 2018 within the project CALECO. A Multinet Hydrobios Mammoth net of 250 µm mesh size was employed to collect 200m^3 at seven

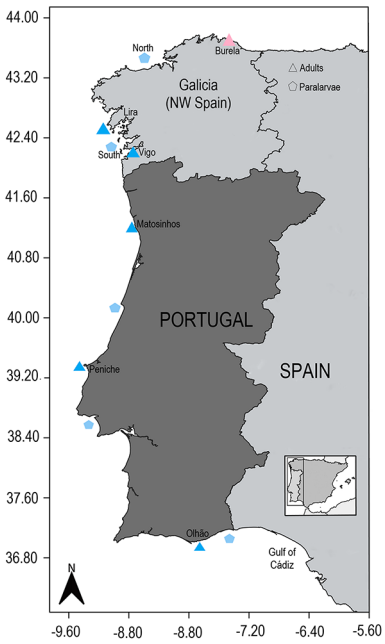

Fig. 1 Sampling sites of *Loligo vulgaris* through the western coast of the Iberian Peninsula. Paralarvae from North and South Galicia were caught using a Multinet and adults were collected by fishing vessels. Adults from Burela were identified as *L. forbesii* (pink triangle); thus, they were not used for this study. Triangles represent locations with adult samples and pentagons represent locations where paralarvae were caught

different strata: 105 – 85, 85 – 55, 55 – 35, 35 – 20, 20 – 10, 10 – 5 and 5 –0 m. Two types of surveys were undertaken in the area: six surveys at night in the Ría de Vigo along four transects (Fig. SI 1d), and four 24 h surveys on six transects surrounding Cíes and Ons islands. The samples collected in the northern Galician waters (Fig. SI 1b) were obtained on board R/V Sarmiento de Gamboa on June 2017 within the project DREAMER with a bongo net of 500 µm mesh size. Samples from Portugal were collected on board the R/V Noruega in April and May 2017, 2018, and 2019 within the project SARDINHA2020 using oblique bongo net tows of 200 and 500 µm mesh size. All zooplankton samples were fixed on board in 96% ethanol. In the laboratory, samples were stored at -20ºC and loliginid paralarvae were sorted and stored in 70% ethanol. In total, 354 loliginid paralarvae were obtained in the different surveys: 57 in North Galicia, 255 in South Galicia and 42 in Portugal. For molecular analyses, the individual mantle from each loliginid paralarvae was dissected as the rest of the body was used for other analyses.

During 2019 and 2020, 120 adults of *Loligo* spp. (20 individuals per location) were obtained in fishing vessels from six different locations situated along the Western Iberian Coast: Burela, Lira and Vigo (NWSpain), Matosinhos, Peniche and Olhão (Portugal) (Fig. 1). Mantle and branchial tissue samples were collected, preserved in 70% ethanol and stored at -20ºC.

DNA extraction

To extract the genomic DNA from the mantle tissue of paralarvae the QIAmp DNA Micro Kit (QIAGEN) was used and for adults the NucleoSpin Tissue Kit (Macherey-Nagel) was employed. The manufacturer's protocol for the respective kits was followed. DNA quality and quantity were checked in a Nanodrop® 2000c UV-Vis spectrophotometer and Qubit™ 3.0 fluorometer (Thermo Fisher Scientific, Nagel) following the manufacturer's protocol.

Mitochondrial COI data

Molecular techniques

A 604 bp fragment of cytochrome c oxidase subunit I (COI) was amplified using the universal primers HCO2198 and LCO1490 (Folmer et al. 1994). PCR reactions were completed in 25-µl of volume containing 1-µl of genomic DNA, PCR buffer at 1x concentration, 0.3-µM each primer, 0.2-mM nucleotides and 0.025-U. μl^{-1}DreamTaq DNA Polymerase (Thermo Scientific). The PCR tests were conducted

Table 1 Molecular diversity indices and population demographic statistics for *L. vulgaris*. N, number of individuals; H, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; S, number of segregating sites; k, average number of nucleotide differences; D, Tajima's D (Tajima 1984); Fs, Fu's Fs (Fu 1997).*$0.05 \geq P \geq 0.01$; **$0.01 > P \geq 0.001$; NS, not significant.

Code	Location	Phase	n	H	h (± standard deviation)	π	S	k	D	Fs
NG_GAL	North Galicia	paralarvae	8	6	0.929 ± 0.084	0.01218	15	7.36	1.390^{ns}	0.305^{ns}
SG_GAL	South Galicia	paralarvae	85	46	0.948 ± 0.014	0.00961	53	5.81	−1.46*	−25.27**
Pt	Portugal	paralarvae	5	5	1.00 ± 0.126	0.01325	14	8	1.38^{ns}	$1'-0.64^{ns}$
Li	Lira	Adult	15	13	0.981 ± 0.031	0.00949	18	5.73	0.15^{ns}	−5.85*
Vi	Vigo	Adult	10	9	0.978 ± 0.054	0.01067	16	6.44	0.64^{ns}	-2.79^{ns}
Ma	Matosinhos	Adult	13	10	0.949 ± 0.051	0.0093	23	5.61	-1.04^{ns}	-2.59^{ns}
Pe	Peniche	Adult	9	7	0.917 ± 0.092	0.01205	15	7.28	1.53^{ns}	-0.39^{ns}
Ol	Olhão	Adult	15	14	0.990 ± 0.028	0.01214	24	7.33	-0.03^{ns}	−6.57**
Overall		–	160	79	0.961 ± 0.006	0.01019	70	6.15	0.33^{ns}	-3.85^{ns}

in a Tgradient thermocycler (Biometra) following the conditions: 3 min at 95ºC, 40 cycles of 30 s at 95ºC, 45 s at 48ºC and 1 min at 72ºC, followed by 7 min at 72ºC. A negative control (no DNA) was included in all PCR amplifications.

PCR products were separated on a 2% agarose gel in Tris acetate EDTA buffer, stained with Red Safe and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories). The amplified fragments were cleaned for sequencing using ExoSAP-IT™ PCR Product Cleanup (Applied Biosystems) for 15 min at 37ºC, followed by inactivation for 15 min at 80ºC. Sequencing was performed in StabVida (Portugal) and the chromatograms were analysed using ChromasPro 2.1.8. (Technelysium Pty Ltd). All generated sequences were identified using BLAST (Basic Local Alignment Search Tool) through web servers of the National Center for Biotechnology Information (NCBI).

Data analyses

To assess the genetic diversity, the software DnaSP v.6 (Rozas et al. 2017) was used to calculate the number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), number of segregating sites (S), and the average number of nucleotide differences (k). The software ARLEQUIN v3.5.2 (Excoffier et al. 2005) was used to perform the neutrality tests of Tajima's D (Tajima 1984) and Fu's Fs (Fu 1997). Pairwise F_{ST} was calculated to examine the population differentiation using ARLEQUIN v3.5.2. The mitochondrial COI sequence data was used to generate a median joining haplotype network using PopART (Leigh and Bryant 2015). Additionally, in order to get a better prospective of the genetic relationships, three sequences from North Sea, six from Portugal, seven from North-east Atlantic, five from central east Atlantic, six from West-central Atlantic, 13 from Italy and Israel and 18 from Turkey were obtained from GenBank and BOLD databases (Table SI 1).

Single nucleotide polymorphism (SNP) data

Molecular techniques

For the obtention of SNPs, tissue from 47 individuals corresponding to adults and paralarvae of *L. vulgaris* were collected from six locations across the western Iberian Peninsula coast (Fig. 1): North Galicia ($n=7$), Lira ($n=8$), Vigo ($n=8$) (three locations from NW Spain) and Matosinhos ($n=8$), Peniche ($n=8$) and Olhão ($n=8$) (three locations from Portugal).

Double-digest restriction site associated DNA (ddRAD) libraries were constructed following the protocol prepared by Peterson et al. (2012). The genomic DNA was digested using the restriction enzymes *Pst*I and *Nla*III. Libraries were amplified using PCR and single-end sequencing with a target length of 150 bp. Sequencing was conducted on Illumina Next Seq500 platform with 150 cycles in high-output mode at the Australia Genome Research Facility (AGRF) (Melbourne, Australia).

Bioinformatics and genotyping

Library reads were demultiplexed and cleaned using the program *process_radtags* in STACKS (Catchen et al. 2013), followed by *de novo* assembled and genotyped using the STACKS *de novo* pipeline.

Subsequently, to determine the optimised values for the parameters m, M and n, a subset of 12 samples across sites were used to find the best fitted values to use in the STACKS pipeline as described by Paris et al. (2017) and Rochette and Catchen (2017). Therefore, in the *ustacks* module, the minimum number of raw reads allowed to initiate a new stack (m) and the number of mismatches allowed between stacks to merge them into putative locus (M) were three. Then, the *cstack* module was used to match the loci across all samples to build a catalogue of putative RAD tags. After the catalogue construction, the putative loci were matched against the catalogue with *sstacks* module followed by the corresponding filtering by the *population* module in STACKS. The *population* module was used to retain SNPs present in at least 75% of the samples and in all the locations, with a minimum minor allele count of three as suggested by Linck and Battey (2019) and O'Leary et al. (2018), and a maximum observed heterozygosity > 0.5 required to process a nucleotide site at a locus. Analyses were restricted to one random SNP per locus (using the *---write_random_snp* option in the *population* module). Later, vcftools 0.1.16 was used to remove low-confidence genotypes by filtering the minimum read depth per locus > 15. The final VCF file obtained was converted to genepop format using PGDSpider 2.1.1.5 (Lischer and Excoffier 2012).

Data analyses

To identify loci under selection, outliers were identified and analysed with two different approaches. The first was BAYESCAN 2.1 and prior odds for the neutral model were set to 100 with 20 pilot runs, followed by 5,000 iterations (5,000 samples, a thinning interval of 10, and a burn-in length of 50,000) (Foll and Gaggiotti 2008). After probabilities were calculated, the function *plot_bayescan* was employed to identify outliers at a False Discovery Rate (FDR) of 0.05. The second method was based on Principal Component Analysis using PCAdapt R package (Luu et al. 2017) to

perform genome scans for local adaptation employing a Monte Carlo approach based on a Bayesian factor model. The loci identified were selected as outlier loci and the following analyses were applied to neutral and outlier datasets, separately.

Genetic diversity, including observed and expected heterozygosity within and among populations was analysed together with inbreeding coefficient (F_{is}), pairwise F_{st} and its respective *p*-values using GenoDive 3.0 (Meirmans 2020). The number of alleles (A) and allelic richness (A_R) were calculated with the R package *PopGenReport* (Adamack and Gruber 2014). Moreover, departure from Hardy-Weinberg Equilibrium (HWE) through all populations was calculated using the function hw.test() from the R package *pegas* version 1.01 (Guo and Thompson 1992). The R package *adegenet* was used for discriminant analysis of principal component (DAPC) to study the population structure and to estimate the membership probabilities using the function compoplot() (Jombart 2008; Jombart et al. 2010). Mantel test was used to identify patterns of isolation by distance (IBD) by comparing a genetic distance of F_{st} matrix vs. a matrix of geographic distance (Km) employing GenoDive 3.0 (Meirmans 2020). Google Earth was used to calculate geographical distances between sampling locations taking the shortest distance by sea.

Results

Mitochondrial DNA data

High-quality genomic DNA was successfully extracted and sequenced from 303 out of 354 loliginid paralarvae. Among these, 143 paralarvae corresponded to *Alloteuthis media*, 56 to *A. subulata*, and 104 to *L. vulgaris.* Additionally, 69 squid adults were identified as *L. vulgaris* (Table 1), with individuals from Burela being identified as *L. forbesii* and thus excluded from the study. Six sequences of *L. vulgaris* paralarvae and seven sequences of adults were removed due to low quality. A total of 160 COI sequences from the western Iberian Peninsula coast of *L. vulgaris* were utilized to calculate molecular diversity indices and population demographic statistics. In total, 79 haplotypes were obtained. Haplotype diversity among paralarvae from each location ranged from 0.948 to 1.00, while for adults, it ranged from 0.917 to 0.990. Nucleotide diversity ranged from 0.0096 to 0.0133 for paralarvae and from 0.0093 to 0.1214 for adults (Table 1).

Overall, neither Tajima's D nor Fu's Fs test could not reject the null hypothesis of neutral evolution, which implies that population frequency of a neutral mutation fluctuates randomly through genetic drift. On the other hand, paralarvae from south Galicia showed negative significant values suggesting a recent population expansion. Meanwhile, adults from Lira and Olhão presented significant negative values for Fu's Fs test, but results of Tajima's D showed non-significant values and therefore, could not reject the null hypothesis of neutral evolution.

A complex haplotype network comprising 100 haplotypes resulted from analysis of 228 sequences of *L. vulgaris* (Fig. 2). Five main haplotypes (H2, H4, H5, H7, H9) were shared among the populations. The paralarvae of south Galicia shared the five main haplotypes, of which haplotype H5 was the most common ($n = 15$). Haplotypes H2, H7 and H9 were present within six populations from the Iberian Peninsula, where H7 contained most of the sequences (17) from all the Spanish locations, Matosinhos and Olhão. The paralarvae from Portugal only shared two haplotypes (H2 and H9), which are separated by up to of 11 mutations from the main population network. Squids from North-eastern Atlantic and Mediterranean shared the five main haplotypes mentioned before and 80 unique haplotypes belonging to the different locations were found (Fig. 2). Haplotype H5 contained the highest number of sequences shared ($n = 26$; 11.04% of the total), followed by H7 ($n = 22$; 9.65%) and H2 ($n = 19$; 8.33%), while haplotypes H4 and H9 shared the same number of sequences each ($n = 14$; 6.14%). Additionally, the haplotype network presented a complex population structure, with three different subgroups separated by high number of mutations from the main population: (i) adults collected exclusively in Turkey; (ii) adults collected at the North Sea ; (iii) and a group constituted by haplotypes H9 and H2 from different locations.

Pairwise Φ_{ST} comparison between locations throughout the Iberian Peninsula were not significant. The exception were samples from Peniche that showed significant pairwise F_{st} values with Lira and Olhão. Moreover, samples from Lira, Matosinhos and Olhão showed marginally significant differences with individuals from the Northeast Atlantic (NEA) and Mediterranean Sea. Most of the Φ_{ST} values between locations from the North-eastern Atlantic showed no significant values. Turkey exhibited significant values comparing to the remaining populations. Additionally, samples from the central east Atlantic (CEA), west central Atlantic (WCA) and NEA showed slight significant differences between them (Table 2).

SNP data

Genotyping

A total of 421,487,725 sequence reads of 150 bp were obtained from four lanes of Illumina NextSeq500 sequencing. The number of reads retained ranged from 40,811 to

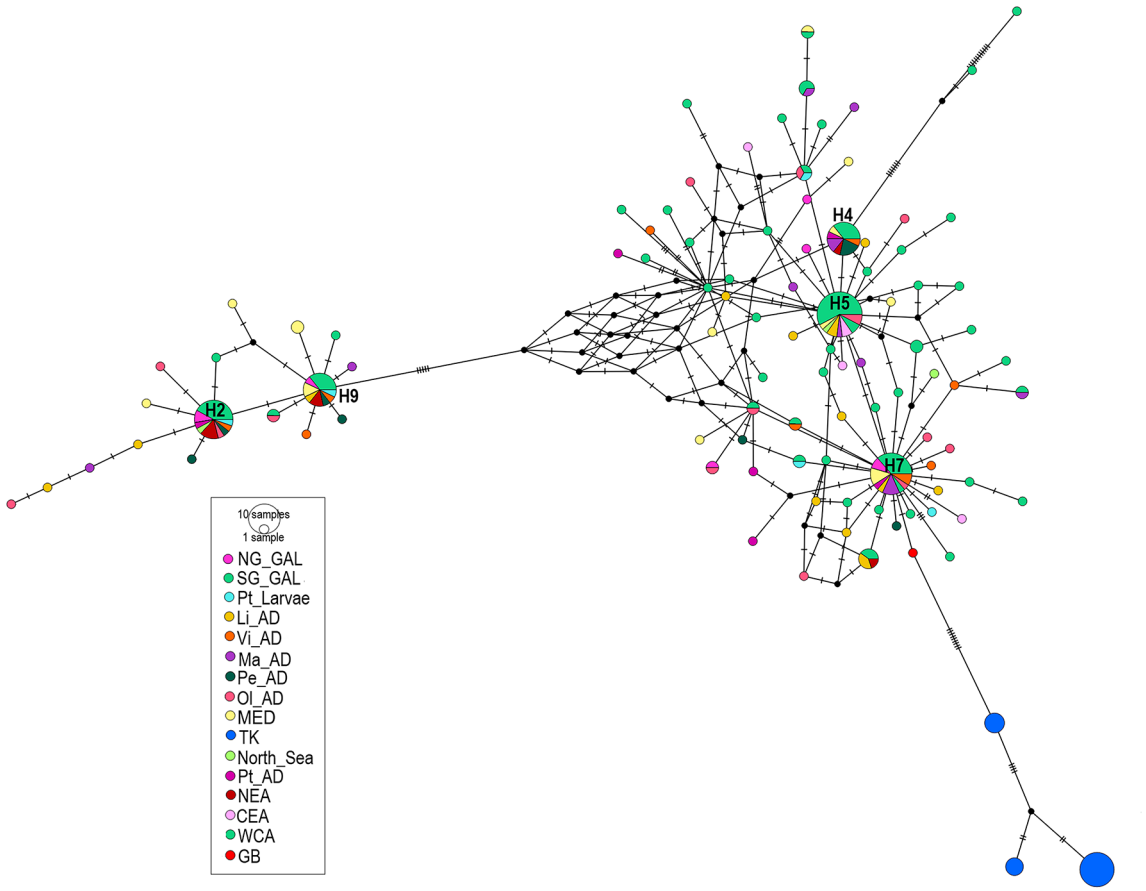

Fig. 2 Haplotype network for *L. vulgaris* obtained in this study including 98 paralarvae and 130 adults. NG_GAL: paralarvae from North Galicia; SG_GAL: paralarvae from South Galicia; Pt_larvae: paralarvae from Portugal; Li_AD; adults from Lira; Vi_AD: adults from Vigo; Ma_AD: adults from Matosinhos; Pe_AD: adults from Peniche; Ol_AD: adults from Olhao; MED: adults from Mediterranean Sea; TK: adults from Turkey; adults from North Sea; Pt_AD: adults from Portugal; NEA: adults from North-east Atlantic; CEA: adults from Central east Atlantic; WCA: adults from West-east Atlantic; GB: adults from Great Britain

24,443,428 with an average of 8,967,823 reads per individual. After removing individuals with a low number of reads (with < 1,450,346 sequence reads) and a mean coverage below 10x, the final data set included 38 individuals from five populations located throughout the western Iberian Peninsula coast with eight individuals each, except those from North Galicia and Olhão with seven individuals (Table 3). The catalogue initially contained 6,220,673 loci and 121,755 loci were retained after the filtering steps, of which 83.23% were polymorphic (101,339 loci). Then, loci with read depths below 15 were removed, and the final number of SNPs retained was 86,431.

No outlier SNPs were identified using BayeScan, while PCAdapt identified 112 outliers. These 112 loci were considered as candidate adaptive loci and analyses were undertaken for the putatively neutral data set of 86,319 loci and the putatively adaptive data set of 112 loci.

Genetic diversity and population structure

The number of alleles and allelic richness were similar across all populations. The population with higher number of alleles and allelic richness was Lira (141.471 and 1.639, respectively), while Olhão had the lowest values (139.494 and 1.616). Observed heterozygosity was lower than expected heterozygosity for all populations for neutral SNPs, while Matosinhos was the only population that showed lower observed heterozygosity than expected heterozygosity for candidate adaptive SNPs (Table 3). Mean expected and observed heterozygosity were higher for candidate adaptive SNPs than neutral SNPs. The inbreeding coefficient was similar and positive among localities for the neutral dataset, whereas the candidate adaptive SNPs had negative values except Matosinhos (F_{is}=0.183, p-value = 0.000) and Lira (F_{is}= 0.037, p-value = 0.139). The mean F_{is} was 0.129 (p-value = 0.000) for neutral SNPs and 0.028 (p-value = 0.088) for candidate adaptive SNPs.

Table 2 COI-based pairwise Φ_{ST}-values between locations of *Loligo vulgaris* in the Atlantic Ocean and Mediterranean Sea. NG_GAL: paralarvae from North Galicia; SG_GAL: paralarvae from South Galicia; Pt_larvae: paralarvae from Portugal; Li_AD: adults from Lira; Vi_AD: adults from Vigo; Ma_AD: adults from Matosinhos; Pe_AD: adults from Peniche; Ol_AD: adults from Olhao; MED: adults from Mediterranean Sea, TK: adults from Turkey; adults from North Sea; pt: adults form Portugal; NEA: adults from North East Atlantic; CEA: adults from Central East Atlantic; WCA: adults from West Centra Atlantic. *$0.05 \geq P \geq 0.01$; **$0.01 > P \geq 0.001$

Pop.	NG_GAL	SG_GAL	Pt larvae	Li_AD	Vi_AD	Ma_AD	Pe_AD	Ol_AD	MED	TK	North_Sea	Pt_AD	NEA	CEA	WCA	GB
NG_GAL	0															
SG_GAL	0.00623	0														
Pt larvae	−0.0396	−0.00488	0													
Li_AD	0.01898	−0.0007	−0.00251	0												
Vi_AD	−0.04517	−0.00315	−0.02894	0.00059	0											
Ma_AD	0.00334	0.00458	0.02927	0.00959	-0.02609	0										
Pe_AD	0.03737	0.02999	0.00131	0.0423*	-0.00332	0.01624	0									
Ol_AD	−0.00319	−0.00711	−0.02186	-0.00812	-0.00443	0.00465	0.03727*	0								
MED	−0.00735	0.00747	−0.00119	0.00128	−0.02541	−0.01198	0.02753	0.00494	0							
TK	0.29492**	0.21652**	0.28942**	0.2453**	−0.02015	0.26619**	0.2952**	0.2407**	0.2538**	0						
North_sea	−0.04348	−0.06271	−0.07143	−0.03388	0.26352**	0.00731	0.01563	−0.06504	0.00616	0.32378**	0					
Pt_AD	−0.05043	−0.0121	−0.03448	−0.00059	-0.05882	−0.03883	−0.03256	−0.01738	−0.01953	0.27898**	−0.05882	0				
NEA	−0.01516	0.04368	−0.04932	0.06045*	0.01811	0.0944*	0.00921	0.06417*	0.06643*	0.3482**	−0.03279	0.00275	0			
CEA	0.08432	0.0006	0.05	0.00051	0.05632	0.04261	0.09069	−0.00512	0.04213	0.32682**	−0.08808	0.04797	0.14901*	0		
WCA	0.02869	-0.01782	0.03465	−0.01595	0.0098	−0.00624	0.07561	−0.02143	−0.01029	0.30578**	−0.08108	0.00571	0.1307*	−0.05832	0	
GB	0.07143	0.05154	0	0.01905	0.02222	0.05128	0.08333	0.00952	0.04167	0.43841**	0	0	0.19048	0.1000	0.06667	0

Moreover, the populations follow the Hardy-Weinberg equilibrium for both datasets (Table 4).

*$0.05 \geq P \geq 0.01$; **$0.01 > P \geq 0.001$; NS, not significant.

Pairwise F_{st} for neutral SNPs were low ($0 < F_{st} < 0.002$) and not significant showing high homogeneity among populations, while pairwise comparations for candidate adaptive SNPs dataset showed F_{st} values ranging from 0.026 to 0.234. Values between Lira and Matosinhos / Olhao had the highest values (p-value = 0.04 and 0.063, respectively). Neutral SNPs data set obtained a mean F_{st} of 0.008 (p-value = 0.000) and candidate adaptive SNPs had F_{st} of 0.102 (p-value = 0.000). While neutral SNPs showed admixture, the candidate adaptive SNPs showed a moderate significant structure (Table 5). Discriminant analysis of principal components (DAPC) of all locations did not detect any differentiation between clusters using all SNPs and neutral SNPs (Fig. 3). On the other hand, the first principal component of the DAPC showed a slight gradient of differentiation from north to south of the west Iberian Peninsula (Fig. 3). On the contrary, results with candidate adaptive SNPs showed a slight differentiation from Matosinhos against the rest of populations. This pattern was also seen with the membership probability. Mantel tests showed negative and non-significant values with both datasets, indicating that it does not exist any correlation between genetic differentiation and geographic distances (p-value = 0.340).

Significance (Bonferroni corrected p-value < 0.05) indicated in bold.

Discussion

This is the first study undertaken to investigate the population structure of *Loligo vulgaris* in the western coast of Iberian Peninsula using mitochondrial and nuclear DNA markers.

The results obtained with both markers (mtDNA and SNP) indicated that the different Atlantic sites sampled along the western Iberian Peninsula presented a high genetic diversity. Overall, *L. vulgaris* presented high values of haplotype and nucleotide diversity and expected heterozygosity.

Comparing with other species of loliginids from the NW Iberian Peninsula, *L. vulgaris* had the highest genetic diversity followed by *Alloteuthis media* (as per COI analysis). Meanwhile, *A. subulata* demonstrated the lowest values of haplotype and nucleotide diversity based on COI (Roura et al. 2019; García-Mayoral et al. 2020). Comparing these values with other squids distributed in other areas, *L. vulgaris* is still the species with the highest values followed by *Sepioteuthis lessoniana* from Taiwan, based on the control region (Aoki et al. 2008) (Table 6). Surprisingly, *Loligo forbesi* had the lowest values based in mtDNA COIII, while microsatellites confirmed the high genetic variability (H_E=0.79) (Shaw et al. 1999). Studies by Cheng et al. (2021) with SNPs revealed that other species of loliginids such as *Doryteuthis opalescens* had lower H_E (0.07–0.14).

The strong genetic flow along the western Iberian Peninsula coast (WIP) is confirmed by pairwise F_{st} obtained with SNPs (Table 5) that showed no population structure. This is supported by the DAPC analyses, which detected admixture among populations (Fig. 3), and the lack of isolation by distance throughout the WIP revealed by the Mantel test. All evidence, both using conventional and modern genetic analysis, suggest that *L. vulgaris* can be considered a panmictic population along the WIP, with a small variation in the North-South axis (Fig. 3), though not significant. This fact could mean that it has a high potential of adaptation to new or changing environmental conditions (Timm et al. 2020). Furthermore, similar patterns of genetic homogeneity were also observed by Garoia et al. (2004), who revealed that populations of *L. vulgaris* in the Adriatic Sea have high gene flow showing panmixia (based on microsatellites). Other species of squids such as *Loligo forbesii* showed to be geographically homogeneous through the North East Atlantic shelf (Shaw et al. 1999).

Studies applying mitochondrial cytochrome b region and COI markers in Humboldt squid, *Dosidicus gigas* along the eastern Pacific coast discovered that populations found in the same hemisphere did not show population structure, but the genetic differences between hemispheres became more obvious (Sandoval-Castellanos et al. 2010; Ibánez et al. 2011). Moreover, research applying microsatellites and mtDNA in the longfin squid, *Doriteuthis gahi*, revealed two different genetic populations between the north and south of South America in the south-eastern Pacific Ocean (McKeown et al. 2019). Some of these differences are due to the hydro-geographic changes such as the marine currents in the area and depth water changes which can affect the migrations of the squids. A similar pattern is seen in the veined squid, *L. forbesii* that showed genetic homogeneity through the continental shelf, but when populations from deeper waters in Azores and from the coastal waters in the NE Atlantic are compared, it was observed that populations from Azores were an isolated population (Shaw et al. 1999). The investigation made by Aoki et al. (2008) with *Sepioteuthis lessoniana*, using nucleotide sequences of the mitochondrial DNA non-coding region 2, did not present differences within the Japanese populations, nor between Japan and Pacific Ocean, but a weak structure with those found in the East and South China Seas was detected caused by the distance and the different oceanographic conditions limiting the gene flow. Those results demonstrated that squid species display genetic homogeneity up to a scale of hundreds of kilometres between populations. This fact

Table 3 Genetic diversity indices and statistics for *L. Vulgaris* from five populations based on 86,319 neutral and 112 candidate adaptive SNPs. N, sample size; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{is}, inbreeding coefficient (fixation index); a, number of alleles; A_R, mean allelic richness

			He		Ho		F_{is}		A		AR	
Pop.	N	Phase	Neutral	Adaptive	Neutral	Adaptive	Neutral	Adaptive	Neutral	Adaptive	Neutral	Adaptive
NG	7	Paralarva	0.194	0.149	0.183	0.170	0.139	-0.067	1,40,163.8	160.518	1.624	1.433
Li	8	Adult	0.197	0.118	0.183	0.121	0.139	0.037	1,41,471.0	163.428	1.639	1.459
Ma	8	Adult	0.194	0.377	0.182	0.334	0.137	0.183*	1,40,572.0	220.067	1.628	1.965
Pe	8	Adult	0.196	0.172	0.181	0.199	0.145	-0.088	1,41,059.0	164.165	1.634	1.466
Ol	7	Adult	0.191	0.274	0.181	0.315	0.134	-0.066	1,39,494.4	192.650	1.616	1.720

Table 4 Chi-square (X^2) values to test hardy-Weinberg equilibrium

SNPs	X^2	df	*p*-value
Neutral	2.850	1	0.452
Adaptive	2.584	1	0.498

Table 5 Pairwise F_{ST}-values between populations of *Loligo vulgaris* in the western Iberian Peninsula coast. Above diagonal F_{st} from neutral SNPs and below diagonal F_{st} from candidate adaptive SNPs dataset

	NG	Li	Ma	Pe	Ol
NG	–	0.001	0.002	0.001	0.002
Li	0.033	-	0.001	0.000	0.001
Ma	0.129	**0.234**	–	0.001	0.000
Pe	0.036	0.074	0.094	–	0.001
Ol	0.087	0.211	0.026	0.040	–

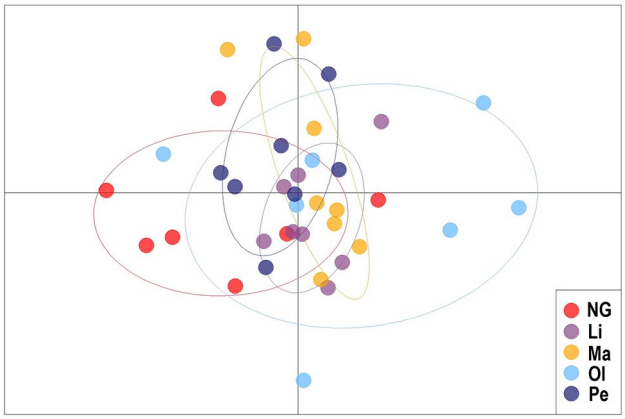

Fig. 3 Discriminant analysis of principal components (DAPC) of *L. vulgaris* using all 86,431 SNPs and 86,319 neutral SNPs. NG: North Galicia; Li: Lira; Ma: Matosinhos; Ol: Olhao; Pe: Peniche

could be observed in *Loligo vulgaris* when pairwise was calculated between samples from NEA, CEA and WCA, showing slight significant differences between these three areas (Table 2).

Other factors that may result in the high genetic diversity obtained for *L. vulgaris* could be its population size, a constant evolution of the mtDNA reflected by the high number of segregating sites, and their swimming capability during the paralarval and juvenile phases. If environmental conditions are optimal, they could start schooling within two months or earlier (Turk et al. 1986), as observed in other loliginid paralarvae – *Alloteuthis media* – off the coast of Morocco (Roura et al. 2019). Furthermore, adults are capable to migrate long distances, up to 500 km and have a planktonic phase that last for approximately two/three months (Moreno et al. 2015) ensuring that a high number of haplotypes will expand to other locations (Allcock and Strugnell 2012; Leone et al. 2017). Other explanation to the high genetic diversity could be the multiple paternity that can be found, not only among the egg strings laid by females but also within the same egg strings (Hanlon 1998).

Table 6 Nucleotide (π) and haplotype (h) diversity of the mitochondrial control region, cytochrome c oxidase I and III

Order	Species	π	h	Region	Location	Source
Myopsida	*Loligo vulgaris*	0.0101	0.948	COI	Galicia (NW Spain)	García-Mayoral et al. 2020
	Alloteuthis media	0.0027	0.857	COI	Galicia (NW Spain)	García-Mayoral et al. 2020
	Alloteuthis subulata	0.0023	0.473	COI	Galicia (NW Spain)	García-Mayoral et al. 2020
	Doryteuthis gahi	0.0020	0.770	COI	Chile	McKeown et al. 2019
	Sepioteuthis lessoniana	0.0124	0.897	Control region	Taiwan	Aoki et al. 2008
	Loligo forbesii	NA	0.160	COIII	North East Atlantic	Shaw et al. 1999
Oegopsida	*Illex argentinus*	0.0008	0.310	COI	Argentina	Roldán et al. 2014
	Architeuthis dux	0.0017	0.613	Control region	Global	Winkelmann et al. 2013
	Dosidicus gigas	0.0008	0.370	COI	Western South America	Ibañez et al. 2011
	Onkya robusta	0.0044	NA	COI	North Pacific	Kurita 2018

Both sexes, males and females, mate with multiple partners during migrations and also during their time in the spawning area contributing the gene mixing within populations (Hanlon 1998). Thus, the inbreeding coefficient could result in high values, while Hardy-Weinberg equilibrium still exists which means random mating.

All individuals from the Atlantic Ocean and North Sea populations share some of the main haplotypes, except those haplotypes belonging to Turkey in the Mediterranean Sea. Overall, pairwise F_{ST} values showed homogeneity between the samples in the Atlantic Ocean, and North Sea, whereas the population from Turkey reflected genetic structure compared with the other localities, as it was also showed in the haplotype network (Table 2; Fig. 1). This genetic structure pattern (Mediterranean vs. Atlantic) has also been observed in other marine species such as the Blue shark and European hake (Cimmaruta et al. 2005; Leone et al. 2017). These genetic differences across the Gibraltar strait could be due to changes in the sea level during the Quaternary glaciation that resulted in isolation of the Mediterranean populations because of the closure of the Gibraltar strait. Modern physical factors, such as the differences of salinity and temperature along the Almerian-Alboran front, also affect the genetic connectivity between hake populations from NE Atlantic and Mediterranean Sea (Cimmatura et al. 2005).

Nonetheless, we must be cautious with the interpretation of the genetic signal observed in the samples from Turkey that were collected from Yumurtalik (Keskin and Atar 2013), because other samples collected in the Mediterranean Sea (FAO 37 and Israel, shown in yellow Fig. 2), showed homogeneity with those from the Atlantic Ocean. It is important to note that the samples from Turkey and Israel were collected less than 600 km apart from each other, but they are completely different without a single haplotype in common (Fig. 2). Unfortunately, we cannot give a scientific answer to this genetic variability, but to suggest the possibility of an incorrect assignment of those sequences, the existence of a sibling species in that particular area, editing errors in the sequences uploaded, or the existence of a nuclear copy of COI that is evolving independently of the mitochondrial counterpart (Buhay 2009). It will be necessary to expand the sample locations along the Mediterranean Sea, to unravel this question.

It is also important to note the shape of the haplotype network obtained, with two main groups of haplotypes (H2 & H9 vs. H4, H5 and H7, Fig. 2) separated by unsampled haplotypes. One of the possibilities for the high number of mutations between subgroups could be due to the elevated rates of evolution in COI (Strugnell and Lindgren 2007). Also, the presence of a copy of the mitochondrial COI gen into the nuclear genome (known as numts or pseudogene) could explain such variation in the group containing H2 and H9, because the mitochondrial DNA transferred to the nucleus lost its function and accumulated mutations owed to the lack of evolutionary constrains (Benesh et al. 2006; Song et al. 2008). Numts have been observed in cephalopods (Strugnell and Lindgren 2007), however, if this was the case, the insertion of the numts into the nuclear genome should be relatively recent because there are no modifications in the length, the open reading frame, or presence of stop codons in the sequences that appear in that divergent group (Buhay 2009).

Results in this study suggest that *L. vulgaris* from the West Iberian Peninsula coast form a unique population with high genetic diversity, maintaining this pattern through the east Atlantic coast. Further exploration on genetic connectivity and diversity through the East Atlantic waters and Mediterranean Sea applying ddRADseq would be interesting to find subtle differences among different oceanographic areas. As well as, to study the differences in population structure or diversity during the different phases of the life cycle of *L. vulgaris.*

Applying RADseq to study paralarvae can provide insights into ecological changes occurring over shorter timescales, which are crucial for fisheries management. Previous studies have suggested the presence of potentially distinct cohorts depending on the season when paralarvae hatch (García-Mayoral et al., 2024). This information could be valuable for understanding population dynamics and implementing effective fisheries management strategies.

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Data availability The authors declare that any raw data files needed in any format are available from the corresponding author upon request.

Declarations

Conflict of interest All authors included in this study declare that they have no conflict of interest.

Ethical approval All applicable national and institutional guidelines for the care and use of animals were followed.

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